

The Molecular Switching Mechanism at the Conserved D(E)RY Motif in Class-A GPCRs

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ABSTRACT The disruption of ionic and H-bond interactions between the cytosolic ends of transmembrane helices TM3 and TM6 of class-A (rhodopsin-like) G protein-coupled receptors (GPCRs) is a hallmark for their activation by chemical or physical stimuli. In the bovine photoreceptor rhodopsin, this is accompanied by proton uptake at Glu¹³⁴ in the class-conserved D(E)RY motif. Studies on TM3 model peptides proposed a crucial role of the lipid bilayer in linking protonation to stabilization of an active state-like conformation. However, the molecular details of this linkage could not be resolved and have been addressed in this study by molecular dynamics (MD) simulations on TM3 model peptides in a bilayer of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). We show that protonation of the conserved glutamic acid alters the peptide insertion depth in the membrane, its side-chain rotamer preferences, and stabilizes the C-terminal helical structure. These factors contribute to the rise of the side-chain pK_a (> 6) and to reduced polarity around the TM3 C terminus as confirmed by fluorescence spectroscopy. Helix stabilization requires the protonated carboxyl group; unexpectedly, this stabilization could not be evoked with an amide in MD simulations. Additionally, time-resolved Fourier transform infrared (FTIR) spectroscopy of TM3 model peptides revealed a different kinetics for lipid ester carbonyl hydration, suggesting that the carboxyl is linked to more extended H-bond clusters than an amide. Remarkably, this was seen as well in DOPC-reconstituted Glu¹³⁴- and Gln¹³⁴-containing bovine opsin mutants and demonstrates that the D(E)RY motif is a hydrated microdomain. The function of the D(E)RY motif as a proton switch is suggested to be based on the reorganization of the H-bond network at the membrane interface.

INTRODUCTION

G protein-coupled receptors (GPCRs) are a superfamily of membrane proteins that undergo conformational changes in response to extracellular chemical or physical stimuli. The ensuing conformational changes of their seven transmembrane (TM) helical structure lead to an activated receptor state that catalyzes GDP/GTP exchange in cytosolic G proteins (guanosine nucleotide-binding proteins). More than 600 GPCRs in humans belong to the class-A (1) that is characterized by structural homology with the visual photoreceptor rhodopsin. In contrast to ligandactivated GPCRs, rhodopsin is activated by 11-*cis* to all-*trans* photoisomerization of the retinal, which is covalently bound via a protonated Schiff base to the side-chain nitrogen of Lys²⁹⁶ of the apoprotein opsin (see Fig. 1).

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Functional studies of bovine rhodopsin have contributed to the identification of critical molecular activation steps that are thought to be shared by class-A GPCRs as has been reviewed in detail (2,3). It has been shown for the β_2 -adrenergic receptor that the breakage of ionic and H-bond interactions that link the cytosolic ends of TM3 and TM6 in the inactive receptor state is crucial for GPCR activation (4-6). Studies on bovine rhodopsin revealed that the corresponding distance increase between TM3 and TM6 (7) is followed by a proton uptake reaction in the side chain of Glu^{134} within the class-conserved D(E) RY motif at the C-terminal end of TM3 (8). This cytosolic "proton switch" (9,10) involves the proton exchange with the environment and is thus pH-dependent. It is evoked by preceding light-induced structural changes (11) and internal proton transfer reactions (12) among which the pH-insensitive internal "proton switch I," i.e., the transfer of the Schiff base proton to its counterion Glu¹¹³ (13), is the key step that leads to the active metarhodopsin II (MIIa) conformation (14).





FIGURE 1 (*A*) Two proton switches may explain the mechanism of activation of bovine rhodopsin. (*A.1*) The retinal isomerization produces a transfer of the Schiff base proton to Glu^{113} . (*A.2*) Glu^{134} positioned in the conserved D(E)RY motif at the C-terminal of TM3 (helix H3) takes up a proton, leading to the MIIbH⁺ intermediate. (*B*) The hallmark of the transition to active rhodopsin is the movement of helices 6 and 5 (H6, H5) away from H3. See Hofmann et al. (2) for details. To see this figure in color, go online.

The following movement of TM6 and TM5 (helices H6 and H5, MIIb state, see Fig. 1, *A.1* and *B*) precedes the protonation at Glu¹³⁴ in the conserved D(E)RY motif at the C-terminal end of TM3 (H3), leading to the MIIbH⁺ intermediate (7,10,15,16) (Fig. 1 *A.2*).

The latter step occurs with an unusually high $pK_a > 6$ (17), indicative of the energetic stabilization of the protonated state. Receptor activation thus follows a sequence of thermally activated structural transitions in multiple microdomains that in rhodopsin are spatially and temporally (from ps to ms) separated. The description of the activation process by a hierarchy of structural "on-off" transitions has originally been based on the spectroscopic identification of inactive rhodopsin states with partial active-like structural features (18) that may cause enhanced thermal receptor activation related to disease (19). Neutralization of the Glu134 side chain has been identified as one of the crucial activitypromoting factors (20,21).

Although the concept of concerted microdomain switches explains receptor activation (22), the underlying structures are not individually folding units in the strict sense of a protein domain. This raises the question whether short specific sequence motifs of an individually folding TM domain can exhibit local switching processes at all. In the case of the "proton switch II" at Glu¹³⁴, it has been argued that the C-terminal end of the isolated TM3 provides an "autonomous" structural switch that couples protonation to secondary structure formation by side-chain partitioning across the lipid water phase boundary (23). Lipid exposure has been suggested to be crucial for this coupling as the protonated side chain would be stabilized by the low dielectric of a bilayer. Whereas the predicted high pK_a of the side chain could be confirmed experimentally, neither the transmembrane topology of the TM3 domain nor the location of secondary structure formation could be determined unequivocally. Furthermore, structural details of the interaction of the Glu¹³⁴ side chain with the subheadgroup region are not known.

To reveal these molecular details, we have performed atomistic molecular dynamics (MD) simulations, fluorescence, and time-resolved infrared-spectroscopic experiments on lipid-inserted TM3 model peptides derived from the amino acid sequence of bovine rhodopsin. Our data provide a detailed description of proton-induced changes in the secondary structure and topology of TM3. We demonstrate that the pK_a of the Glu¹³⁴ carboxyl is not only tuned by the local dielectric environment at the phase boundary but also by the side-chain rotamer state. Finally, the role of the conserved carboxyl group for lipid subheadgroup hydration is addressed by infrared spectroscopy using both model peptides and Glu¹³⁴- and Gln¹³⁴-containing bovine opsin mutants.

MATERIALS AND METHODS

Peptide sequences used in MD calculations

The initial structure of the wild-type peptide P1 was derived from the TM3 of rhodopsin (PDB: 1F88, residues 108-138) and comprised the amino acids: TGCNLEGFFATLGGEIALWSLVVLAIERYVV. The numbering of all residues is based on the peptide sequence running from T1 to V31. The amino acid Glu¹³⁴ of the native rhodopsin sequence in the conserved D(E)RY motif of class-1 GPCRs corresponds to residue 27 in the peptide models and is designated Glu²⁷. To achieve comparability with previous spectroscopic studies, glutamic acids that do not belong to the conserved D(E)RY motif were replaced by alanine in all TM3 peptides investigated here (Table 1). In addition to fixing the ionized or protonated state of Glu^{27} in the calculations (systems P1 and P2), peptides with the $Glu^{27}Gln$ replacement were studied (system P3). However, spectroscopic studies of these peptides showed that it adopted a mixture of nonhelical and helical states that could be avoided by the additional Trp¹⁹Phe/Val³¹Trp double replacement. To validate corresponding experimental results, MD simulations were performed for peptides carrying this additional double replacement and a Glu or Gln residue at position 27, respectively (systems P4 and P5). Finally, peptides P6 and P7 included the replacement Leu²⁴Trp,

TABLE 1 Sequences of TM3 Model Peptides Used in This Study

Peptide Names	Sequence
P1	TGCNLEGFFATLGGEIALWSLVVLAIERYVV
P2	TGCNLAGFFATLGGAIALWSLVVLAIERYVV
P3	TGCNLAGFFATLGGAIALWSLVVLAIQRYVV
P4	TGCNLAGFFATLGGAIALFSLVVLAIERYVW
P5	TGCNLAGFFATLGGAIALFSLVVLAIQRYVW
P6	TGCNLAGFFATLGGAIALFSLVVWAIERYVV
P7	TGCNLAGFFATLGGAIALFSLVVWAIQRYVV
ICL2	AIERYVWVCKPMSNFRFG

All peptides were derived from the native TM3 sequence of bovine rhodopsin as represented by P1. Bold: position 27 carrying the glutamic acid of the conserved D(E)RY motif of class-A GPCRs. Underlined: additional amino acid replacements that increase helix stability or neutralize the side chain at position 27.

which was used as a fluorescence monitor for the repositioning of the peptide (see below). For comparison, MD simulations were also performed with the ICL2 peptide (AIERYVWVCKPMSNFRFG) derived from the second intracellular loop that extends from helix 3. It still carries the conserved D(E)RY motif but lacks the preceding transmembrane segment and was not inserted into a lipidic phase.

Details of MD simulations

Each studied peptide was inserted using the g_membed tool (24) into a system with 128 lipids of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and hydrated with 40 water molecules (TIP3p (25)) per lipid. Peptides with aspartic acid or glutamic acid in the D(E)RY motif were simulated in both protonated and unprotonated states. The Gromacs 4.5.5 package (26) was used and the leap-frog algorithm applied as integrator with a time step of 2 fs. An isothermal-isobaric scheme (NPT) was chosen with the temperature coupled to a heat bath at 303 K, using the Nose-Hoover thermostat with a time coupling constant of 0.5 ps (27,28). The peptide, the lipid bilayer, and the solvent were coupled separately to the thermostat. The Parrinello-Rahman barostat was used to keep the pressure constant at 1.013 bar, with a time coupling constant of 10.0 ps and an isothermal compressibility of 4 \times 10⁵ bar⁻¹ (29). The barostat was used with a semi-isotropic scheme, where the pressure in the x-y plane (bilayer plane) and z direction (bilayer normal) were coupled separately. The covalent bonds were constrained with the LINCS (30) and SETTLE (31) algorithms. The long-range electrostatic interactions were treated using the particle mesh Ewald method, with a cutoff in real space of 1.0 nm, and a Fourier spacing of 0.12. The cutoff for van der Waals interactions was chosen to 1.5 nm, using a switch function starting from 1.4 nm. All the systems were simulated using periodic boundary conditions. SLIPIDS (32) and AMBER99 (33) were used as force fields for lipids and peptides, respectively. The systems were minimized using the steepest descent algorithm (< 50,000 steps) and equilibrated for 50 ns with harmonic position restraints in x, y, and z direction, with a force constant of 1000 kJ/mol/nm² on the heavy atoms of the peptide. Three production simulations for each studied system were run for 200 ns, the initial velocities of each system were randomly selected.

To test for possible force field artifacts, additional simulations of systems P2 and P3, with their respective replicas, were performed using the Lipid14/ Amber14sb force field (34,35). Here, the van der Waals cutoff was set to 1.0 nm.

Tools of the Gromacs suite (26) were used for the analysis of the simulation trajectories. More specifically, the helical content was calculated with the tool *g_helix*, and the dihedral angle χ (formed by atoms C α , C β , C δ , and C γ) was analyzed with the help of *g_sangle*. The average *z*-component of the center of mass (COM) of each residue was averaged over the last 50 ns of three independent simulations for each peptide.

Determination of Glu²⁷ pK_a on helical peptides and unstructured peptides

The pK_a of Glu²⁷ of peptides 2, 4, and ICL2 was computed using the multiconformer continuum electrostatics program (MCCE2) (36–38). To mimic the different dielectric environments of the peptides depending on their insertion depths, the pK_a values were analyzed for relative solvent dielectric constants between 2 and 80 (with $\Delta \epsilon = 1$). 15 titration points starting at pH 0 (intervals of 1 pH unit) were calculated for each peptide. The protein dielectric constant was set to 8 (suggested for small proteins).

Infrared spectroscopy of lipid-reconstituted model peptides and bovine opsin mutants

Peptides P6 and P7 were synthesized with the C- and N-terminus amidated and acetylated, respectively, the HPLC purified, and the trifluoroacetate removed (ThermoFisher, Ulm, Germany). The peptides were reconstituted into DOPC vesicles (Avanti Polar Lipids, Alabaster, AL) by dissolving 10 mg DOPC and 1.5 mg of the peptides in 100–200 μ L ethanol followed by solvent evaporation, resolvation in 40 µL ethanol, and finally rapid dilution in 1960 μ L H₂O (39). Vesicles were formed by at least 10 freeze-thaw cycles of the suspension (40). Bovine opsin mutants carrying the stabilizing Asn²Cys/Asp²⁸²Cys amino acid replacements (41) were expressed in HEK-239S cells and detergent-solubilized as described (42), except for using 1% (weight/volume) octylglucoside (OG) instead of dodecylmaltoside. These mutants were subsequently purified by using rho-1D4 immunoaffinity chromatography (42). The protein concentration was determined according to Bradford. The solubilized mutant bovine opsins Asn²Cys/Asp²⁸²Cys and Asn²Cys/Asn²⁸²Cys/Glu¹³⁴Gln were mixed with DOPC in 1% (w/v) OG in a 1:100 protein to lipid ratio in a total volume of 200–250 μ L. They were simultaneously dialyzed in minidialysis cups in the identical buffer (1 L 5 mM phosphate buffer, pH 7.4) with one complete buffer exchange overnight.

The lipid-reconstituted peptide and purified opsin samples were dried on an attenuated total reflectance (ATR) crystal under a gentle stream of nitrogen and hydrated overnight to 85% and 75% relative humidity (r.h.) using a reservoir of a saturated KCl or NaCl solution, respectively (43), separated from the DOPC film by a dialysis membrane and a 1 mm gap of air above the sample. The acquisition of time-resolved Fourier transform infrared (FTIR) difference spectra induced by hydration has been described in detail (44,45). Briefly, the r.h. above the sample is increased within 2–4 s by a heating current in the salt solution. Infrared (IR) absorption difference spectra are generated from the transmission at defined time intervals after the hydration pulse and the sample transmission at the initial equilibrium hydration. Positive absorption changes are caused by the more hydrated state, negative bands by the initially less hydrated sample. Relaxation of the different samples to the equilibrium hydration took 60 to 180 s. An additional waiting time of 5 min was allowed before repeating the experiment in an automated fashion for signal averaging.

RESULTS

Coupling of Glu²⁷ protonation to transmembrane helicity

Previous infrared spectroscopic data on detergent-solubilized TM3 model peptides of bovine rhodopsin supported a protonation-dependent secondary structural transition near the D(E)RY motif in the visual photoreceptor and possibly other GPCRs. However, the extent of helix formation along the sequence and its location relative to the membrane water interface could not be determined. To test the proposed pH-regulation of the TM3 conformation on a more detailed molecular level, we studied the influence of protonation on the secondary structure of TM3 of rhodopsin using atomistic MD simulations of peptides in a phospholipid bilayer.

For comparison with earlier experimental peptide studies using FTIR, CD, and fluorescence spectroscopy (23), a family of related sequences was investigated as well. The peptide P2 (compare Table 1) served as a single transmembrane helical model for the native TM3 sequence and the single carboxyl group in the side chain of Glu²⁷ was chosen to be either protonated or unprotonated in the calculations. Fig. 2 *A* shows the probability for observing the residues of peptide P2 in α -helical conformation. The propensity for α -helix formation was $\approx 10\%$ higher for the C-terminal end of P2 (residues 21–25) in its protonated form as compared with the unprotonated P2 peptide. A similar behavior was observed for the Lipid14/Amber14sb force field (see Fig. S1 in the Supporting Material).

For comparison, a simulation was performed for a peptide with the additional Glu²⁷Gln replacement (P3). Such a replacement is generally considered a mimic of the proton-

ated form of a glutamate side chain. However, the helicity in P3 barely exceeded that of P2 in its ionized form.

This result suggests that the specific H-bonding properties of a carboxyl group rather than merely its charge state are involved in (protonation-dependent) secondary structure formation. The data agree with previous infrared absorption measurements of the protonation-induced C-terminal helixformation: P2 was incorporated in a detergent micelle, whereas P3 had not been investigated spectroscopically because it formed a large fraction of the nonhelical structure in detergent. This problem was overcome by the additional Trp¹²⁶Phe/Val¹³⁸Trp replacement (residues 24 and 31 in peptides P4 and P5) that stabilized the α -helical structure in the experiments (23). However, the simulations of the corresponding peptides are not conclusive: the P4 and P5 peptides showed only a slightly enhanced α -helical content with respect to the wild-type sequence (Fig. 2 B). Also for the P4 peptide, a protonation-induced increase of helicity at the C-terminal part was observed (by 11% for residues 21-25). Again, the replacement of the titratable amino acid Glu²⁷ by the structurally homologous glutamine residue (P5) did not yield results equivalent to the peptide with protonated Glu²⁷ side chain. Such a difference in



FIGURE 2 Percentage of helicity per amino acid averaged over the last 150 ns of three independent simulations, the standard error per residue was calculated and plotted as error bars (using windows of 10 ns). Peptides P2 (*A*), P4 (*B*), and P6 (*C*) were studied in their protonated and unprotonated states and compared with the respective sequences carrying the additional Glu²⁷Gln replacement (P3, P5, and P7, respectively). (*D*) Probability distribution of the dihedral angle χ^2 of Glu²⁷, analyzed over the last 50 ns of each simulation, is shown. The colors red, blue, and green represent independent simulations of the same system. (*E*) Upper panel: transmembrane topology of P4 with Glu²⁷ in the protonated (*right*) and ionized (*left*) state is shown. Lower panel: close-up view of the favored protonation-dependent rotamer states of Glu²⁷ is shown. To see this figure in color, go online.

peptide helicity could not be observed between the protonated P2 peptide and the P3 peptide studied in the Lipid14/ Amber14sb force field combination (Fig. S1).

A stabilization of the α -helical structure was as well observed for the peptides P6 and P7. Protonation of Glu²⁷ of P6 led to a 23% increased α -helicity for the C-terminal end (residues 21–25) as compared with the unprotonated P6. However, opposite to the P2/P3 and P4/P5 pairs, the Glu²⁷Gln replacement stabilized the α -helical conformation comparable with the protonated P6.

Overall, the data agree with the proposed coupling of protonation and conformation at the C terminus of TM3 but allow to pinpoint the helix stabilization to about five amino acids within the lipidic phase preceding the actual titration site. Furthermore, the data suggest that, depending on the peptide sequence, the protonated carboxyl group and the amide group may differ in their influence on the peptide structure, despite their common neutral state.

Dynamics of Glu²⁷ side-chain conformation

A key assumption of the previously proposed mechanism that couples side-chain protonation to conformation is the fluctuation of the Glu¹³⁴ side chain between an "exposed" and a "buried" geometry at the membrane interface once the ionic lock is disrupted upon photoactivation of rhodopsin. The Glu²⁷ side-chain dihedral distribution ($\chi 2$, analyzed over the last 50 ns of each simulation) shows a slight shift of the preferred dihedral from *gauche* (60°) to *trans* (180°) configuration upon protonation. The shift toward a larger dihedral angle was more pronounced for peptide P4 (Fig. 2 *D*).

Thus, although the coupling of protonation to secondary structure formation was identified as a general feature of the ERY motif for the peptides studied here (see above), the side-chain conformation appears to be further regulated by the sequence context or the peptide insertion depth (see below). Fig. 2 E visualizes the protonation-dependent rotamer preference in the transmembrane topology of P4, showing the position of the gauche rotamer of the ionized carboxyl at the level of the lipid phosphates (*left*), whereas in the protonated state (*right*), the preferred *trans* isomer locates the carboxyl to the subheadgroup region.

Protonation-dependent transmembrane positioning of the helical backbone

In addition to helicity and local side-chain rotamer preferences, also the insertion depths of the peptides were influenced by the protonation state. The analysis of the membrane insertion depth as a function of sequence position shows that peptide P2 is shifted within the membrane by ≈ 2.2 Å toward the N-terminal membrane leaflet upon protonation of Glu²⁷ (Fig. 3, peptide shift averaged over all amino acids). Simulations of the same system using the Lipid14/Amber14sb force fields for the lipids and the peptide reflect an even larger peptide shift upon protonation by >4 Å (see Fig. S3).

For peptide P4 with Trp¹⁹Phe and Val³¹Trp replacements an overall shift upon protonation of Glu²⁷ could not be seen. In contrast, the shift is even reversed for the N-terminal part of the peptide (i.e., the interfacial Trp³¹ firmly anchors the C terminus of the peptide to the membrane interfacial region). This result is in agreement with the observed more pronounced shift of the Glu²⁷ side-chain dihedral for this peptide (Fig. 2 *D*).

Peptide P6 with the replacement Leu²⁴Trp shows a similar inward-shift of the C-terminal residues ($\approx 2-3$ Å) upon protonation; however, the anchoring of the N-terminal part remained unchanged. In summary, depending on the



FIGURE 3 Center of mass (COM) location of amino acids across the lipid bilayer for peptides P2, P4, and P6 for both protonated and unprotonated forms of Glu^{27} . The values were averaged over the final 50 ns of three independent simulations; the COM of the lipid head groups of the upper mono-layer was set to zero. To see this figure in color, go online.

position of interface-anchored tryptophans, the protonation state of the D(E)RY motif not only controls the local peptide helicity but as well the (local) membrane insertion depth and thus hydrophobicity.

Coupling of Glu²⁷ side-chain pK_a to dielectric environment

The results of a protonation-induced increased membrane insertion depth and increased helicity suggests a dielectric mechanism in the stabilization of the peptide structure and location: the increased hydrophobic immersion of Glu¹³⁴ upon protonation and the coupled lowering of the dielectric constant of its environment may contribute to the high $pK_a > 6$ of Glu¹³⁴ in the proton-dependent equilibrium between MIIa and MIIbH⁺ states of light-activated rhodopsin (17) and of TM3 model peptides (23). This hypothesis of an interplay between peptide protonation and localization was addressed by in silico analysis of the Glu²⁷ pK_a dependency on the dielectric environment.

The pK_a was computed in dependence of two factors: 1) the dielectric constant was varied between 2 and 80, corresponding to the transition between the hydrophobic membrane core and the aqueous phase; and 2) the effect of the secondary structure was addressed by computing the pK_a of the Glu²⁷ side chain in peptides P2, P4, and for comparison in ICL2. Peptides P2 and P4 have a defined helical structure in a lipid environment. In contrast, the peptide ICL2 is water soluble and corresponds to the N-terminal part of the second intracellular loop of rhodopsin. The pKa of Glu²⁷ in peptides P2 and P4 was observed to strongly depend on both the dielectric environment and the secondary structure: it ranged from 5.2 ($\epsilon = 80$) to more than 10 at $\epsilon < 10$ (Fig. 4 A). In contrast, the pK_a of the same side chain in the ICL2 peptide in coiled conformation showed a similar dependency on the dielectric constant; however, it ranged approximately one unit below the pK_a values of Glu²⁷ in P2 and P4.

Thus the increased membrane insertion of the protonated Glu^{27} and the corresponding decrease in dielectric constant can partly explain the elevated pK_a . Additionally, the rotamer states may contribute to pK_a regulation as well: in P2 and P4, the protonated form of Glu^{27} adopts preferentially the *gauche* and *trans* rotamer, respectively, and this correlates with an almost constant pK_a difference between the carboxyl in the two peptides over the tested range of dielectric constants. The effect of the rotamer on side-chain pK_a is further discussed below.

Glu²⁷ protonation exerts long-range dielectric effects within the bilayer

The MD analysis demonstrated the interdependence of transmembrane topology, local hydrophobicity, side-chain protonation, and secondary structure. The predicted change



FIGURE 4 Theoretical and experimental pK_a estimates for Glu^{27} . (*A*) pK_a calculations for Glu^{27} in P2, P4, and ILC2 as a function of the dielectric constant in a homogenous dielectric medium are shown. (*B*) Tryptophan fluorescence of lipid-reconstituted peptides is shown. The displayed pH-dependent spectral shifts were obtained by subtracting the emission spectra taken at the indicated pH values from an emission spectrum recorded at pH 8.8. Thereby, the emission in the ionized state of Glu^{27} in P6 is positive, and that of the protonated state is negative (*color-coded*). The pH sensitivity is abolished in P7 (*black*). Inset: emission spectra of P6 (*red*) and P7 (*blue*) at pH 8.8. To see this figure in color, go online.

in the dielectric environment of the C-terminal end of TM3 as a consequence of protonation-induced repositioning relative to the bilayer was experimentally validated using a peptide with the replacement Leu²⁴Trp (peptide P6). This residue provides a fluorescence monitor by a blue-shift of its emission upon lowering of the local dielectric constant in the majority of proteins (46).

Fig. 4 *B* shows that the tryptophan emission is affected by pH, despite the fact that Trp^{24} is located by a helical turn deeper in the membrane than Glu^{27} . In agreement with the predicted model, the emission of Trp^{24} became blue-shifted

upon protonation of Glu²⁷, demonstrating an increased insertion depth into the lipid bilayer. Whereas little pH sensitivity was observed between pH 3 and 6, the blue-shift was mainly induced between pH 6 and 7. This confirms the unusually high pK_a of the Glu²⁷ side-chain carboxyl derived from the theoretical pK_a analysis and also seen for similar TM3 model peptides (23). The pH effect was abolished when Glu²⁷ was replaced by Gln (P7 peptide), which further proves the Glu²⁷-mediated molecular mechanism. Emission from Trp²⁴ of the P7 peptide was not only pH-insensitive but also observed at shorter wavelength than for P6 (Fig. 4 *B*, *inset*). This result indicates a conformational stabilization of the C-terminal part by the neutral Gln side chain as opposed to a side chain that is in equilibrium with an ionized form.

In summary, the MD calculations and tryptophan fluorescence measurements show that the protonation of the Glu^{27} side chain provides a structural switch: the protonation of this site results in a shift of the peptide parallel to the membrane normal that is more pronounced for the non-Trp anchored peptide P2. This protonation-dependent switch observed in the model peptides is expected to contribute to the conformation and energetics of the fulllength receptor. Remarkably, this switch is tailored to the physical constraints of the lipid water phase boundary, such that protonation leads to repositioning of TM3, a reduced flexibility of both the peptide backbone and the side chain of Glu^{27} , and possibly to altered side-chain rotamer preferences.

Side chain-dependent dynamics of the subheadgroup H-bond network

The analysis of the MD simulations suggested an unexpected difference between the neutral protonated carboxyl group and the structurally similar amide group of Gln at position 27 in regulating local secondary structure. The protonated carboxyl group stabilized the C-terminal α -helical structure in P2, P4, and P6, whereas Gln²⁷ showed this effect only on P6/P7.

The only partial mimicking of the protonated state of Glu^{27} by Gln^{27} indicates that in addition to charge, the specific H-bonding geometries of the carboxyl function are required for peptide structure formation. Secondary structure depends on intramolecular backbone H-bonds that compete with intermolecular water H-bonds. Therefore, the interaction of the D(E)RY motif with water in the lipid ester carbonyl region could be crucial for the unique structure induction by a protonated carboxyl rather than an amide. This has motivated us to study the hydration of the DOPC carbonyl region in the presence of P6 and P7.

The infrared absorption of the ester carbonyl stretching mode and the OH-stretching vibration in corresponding lipid films was observed by time-resolved FTIR spectroscopy as a function of hydration. The technique employs a short hydration pulse that increases the relative humidity of air above the lipid film (from 85% to 90%–95% r.h.) within seconds. The experimental setup has been described in detail for the hydration of DNA and lipids (44,45) and allows to follow the relaxation of the sample to its initial r.h. with time resolution in seconds.

Fig. 5 *A* exemplifies this for a pure DOPC film for which the time-dependent water content was monitored by the absorption change of the OH stretching mode at 3370 cm^{-1} . It is plotted together with the amplitudes of the absorption change of the lipid C = O stretching mode at 1739/ 1712 cm^{-1} . The curves are averages of 10 such experiments and their perfect superposition demonstrates that the water content and the H-bond strength at the ester carbonyls equilibrated during the entire time course. This synchronicity was preserved in the presence of P6 (Fig. 5 *B*), where an additional absorption change at $1663/1650 \text{ cm}^{-1}$ revealed changes in peptide bond geometry/H-bonding. Fig. 6 *A* shows the corresponding IR raw data for P7 and compares the C = O hydration response for P6 and P7.

Whereas the H-bond-dependent change of the C = O stretching absorption scaled again strictly with hydration for both peptides, the relaxation time for P7 carrying the neutral Gln side chain was faster than for P6. The data show that the presence of the carboxyl function slows down the reequilibration of lipid-bound water with the gas phase above the lipid film. We speculate that carboxyl-specific H-bonds need to be broken upon removal of the excess water taken up during transient hydration.

The data raise the question whether the different subheadgroup hydration properties seen with a carboxyl or amide group at the membrane water interface of DOPC also persist in full-length opsins. Bovine opsin mutants carrying the stabilizing $Asn^2Cys/Asp^{282}Cys$ double replacement were expressed, reconstituted in DOPC (see Materials and Methods), and the hydrated lipid films adsorbed on an ATR crystal in the same manner used for peptide-containing films. Fig. 6 *B* shows the time-resolved IR raw data for stabilized opsin and a mutant that contains the additional Glu¹³⁴Gln mutation.

Both opsins reproduced the strong synchronicity between water content and ester carbonyl H-bonding, evident from the traces derived from the absorption changes at 3400 cm^{-1} and the $1740/1709 \text{ cm}^{-1}$ difference band. In contrast to the peptide-containing films, the opsins exhibited a slower reequilibration of hydration water with the gas phase, reaching the 90% recovery after one minute, rather than 30 s. However, the time courses reveal again a faster water reequilibration for opsin with the amide-containing side chain at position 134 than with the carboxyl of the native Glu¹³⁴ (Fig. 6 *C*). The different linkage of membrane hydration to subheadgroup H-bonding is thus a site-specific feature that prevails in both the full-length receptor structure and the TM3 model peptides.



FIGURE 5 Time-dependent hydration-induced IR absorption changes in DOPC films. (A) Decrease of the OH-stretching absorption of water and reduction of the lipid ester C = O stretching absorption difference band over time after the initial absorption changes were induced by a 4 s hydration pulse that increased the r.h. from 75% to 82% in a pure DOPC film. The increased H-bonding to the ester carbonyl led to its frequency downshift that caused the reduction of absorption at 1739 cm^{-1} (disappearance of the less-hydrated state) and the increase at 1712 cm^{-1} (appearance of the more-hydrated state). With the return to 75% r.h., both the water absorption and the C = O difference band vanish. Inset: reduction of the IR amplitudes at the water OH and lipid ester C = O stretching frequencies over time are shown. (B) Equivalent data is shown for a DOPC film containing the peptide P6 (in addition to the absorption changes of water and DOPC, the amide I mode of the peptide also responds to the hydration pulse with a frequency shift in the 1660 to 1640 cm⁻¹ range). Color codes are $\nu(C = O)$ (*orange*) and $\nu(OH)$ (blue). To see this figure in color, go online.

DISCUSSION

Proton-uptake of the conserved D(E)RY motif of the TM3 domain of class A GPCRs is a crucial step in receptor activation. In this study, we investigated the influence of protonation on the structure, dynamics, and membrane-insertion of



FIGURE 6 Hydration-induced IR absorption changes in lipid-reconstituted TM3 model peptides and full-length bovine opsin mutants. (*A*) Comparison is shown of the relaxation of water OH and lipid ester C = O stretching absorption bands of Glu²⁷- and Gln²⁷-containing peptides P6 and P7, respectively, in DOPC. Inset: the original time-dependent hydration-induced IR difference spectra of P7 is shown. (*B*) IR absorption changes induced by hydration of two opsin mutants in DOPC is shown. (*C*) Comparison is shown of the relaxation time course of the water OH (3400 cm⁻¹) and lipid ester C = O stretching absorption changes (~1741/1709 cm⁻¹) of the opsin mutants reconstituted in DOPC carrying the natural Glu¹³⁴ (opsin) or the Gln¹³⁴ mutation. Color codes are the same as in Fig. 5. To see this figure in color, go online.

corresponding model peptides in DOPC bilayers using both atomistic MD simulations and fluorescence spectroscopy on peptides and full-length bovine opsin mutants. A coupling of carboxyl protonation to structural transitions was observed that is suggested to be of functional relevance in the full-length receptor.

In active bovine rhodopsin structures (47-49), the side chain of the protonated Glu¹³⁴ in the class-conserved D(E)RY motif does not undergo specific intramolecular interactions. Instead, it resides in a hydrophobic region at

the TM3-TM4 interface close to the lipid-facing protein surface. This contrasts the inactive state (50) where its ionized form participates in H-bonding and ionic interactions between Arg¹³⁵ and Glu²⁴⁷ on TM6, i.e., the ionic lock that stabilizes the inactive state of the receptor (compare Fig. 7). The MD calculations show that the D(E)RY motif forms a microdomain switch in the true sense: it is part of an independently folding transmembrane domain but adopts protonation-dependent structural substates that have counterparts in the crystal structures of inactive and active conformations of rhodopsin. This correspondence concerns in particular the protonation-induced shift of the peptide P2 (H3 in rhodopsin) and transition of the Glu²⁷ rotamer states that are reflected in the transition from the rhodopsin dark state structure to the all-trans metarhodopsin II conformation (see repositioning of corresponding Glu^{134} in Fig. 7).

No shift was seen for the P4 peptide upon protonation, it is suppressed here by the helix-anchoring role of Trp^{31} that results in a more pronounced transition from a *gauche* to a *trans* side-chain rotamer. In agreement with this interpretation, the absence of the Trp^{31} anchor in P2 allows for a more substantial shift of the TM helix toward the C-terminal end when the Glu²⁷ side chain becomes ionized (see Fig. 3).

Membrane anchoring functions have also been described for tyrosines at TM helical ends (51). Remarkably, the potential of the charged side chain of Glu^{27} to promote the TM3 peptide shift is not impeded by the tyrosine of the D(E)RY motif: peptides anchored by Tyr²⁹ only (P2, P6) allow for significantly larger displacements upon protonation of Glu^{27} as compared with the tryptophananchored P4 peptide. This agrees with the strong effect of charged residues on TM helical end positioning (52–54). Nevertheless, for both peptides the ionized state of Glu^{27} favors a shift to the more hydrophilic membrane surface, whereas the protonated glutamic acid is preferentially located in the more hydrophobic subheadgroup region of the bilayer.



FIGURE 7 Comparison between the inactive conformation of bovine rhodopsin (1F88) (50) (*shown in light colors*) and the active all-*trans* rhodopsin conformation (47). The putative membrane interfacial region is indicated by a black line. The structures were fitted on the TM helices H1, H2, H4, and H7 (*gray*). To see this figure in color, go online.

Our data thus show a protonation-dependent partitioning of Glu²⁷ in regions of different hydrophobicity. This finding provides a molecular basis for the elevated pK_a of Glu¹³⁴ in the MIIb to MIIbH⁺ transition of light-activated rhodopsin. The results reveal further mechanistic details that have previously not been considered for the conformational switching process. First, the Glu²⁷ pK_a responded differently to the dielectric environment in P2 and P4, revealing a crucial role of the different side-chain rotamers for pK_a regulation. Second, a glutamine is generally considered a mimic of a protonated glutamic acid. However, the corresponding replacements made in P3 and P5 did not reproduce the effect of the protonated glutamic acid on stabilizing the C-terminal helical structure. For P3, this difference could not be reproduced in simulations using the Lipid14/Amber14sb force field combination, hinting to subtle albeit crucial differences between the studied force fields. Likewise to the former result, the rate of subheadgroup hydration was shown using FTIR spectroscopy to depend on a single amino acid replacement in the TM3 model peptide. Together with the MD simulations, this unexpected result hints at a crucial role of H-bond networks in addition to a side-chain positioning within the dielectric gradient at the membrane interface.

The significant difference in carbonyl dehydration kinetics seen also with the opsin mutants leads us to suggest that the ionized carboxyl is a hydration site at the opsin lipid interface, where proton uptake can lead to more extended remodeling of H-bond networks in the membrane interface. This may explain why in full-length bovine rhodopsin in membranes, the Glu¹³⁴Gln replacement leads to the loss of phosphodiester H-bond interactions normally seen in FTIR difference spectra of the formation of the MII G-protein complex (55). The described importance of the membrane interfacial region in these structural transitions provides a mechanistic rational why Glu¹³⁴ protonation is required for full receptor activation in membranes but not in detergent (56).

In summary, the proton switch mechanism of the D(E)RY motif can be understood on the basis of the dielectric properties of the membrane interface and the membraneanchoring capability of the sequence context. Correspondingly, the proton-induced structural changes occur in both the isolated microdomain and the full-length receptor. In both cases, the side-chain carboxyl is additionally connected to the H-bond network in the membrane surface, which is required for secondary structure stabilization.

SUPPORTING MATERIAL

Three figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)30407-6.

AUTHOR CONTRIBUTIONS

R.A.B. and K.F. designed the research; A.S. performed simulations as well as the simulation analysis; R.A.B. supervised the simulation analysis; P.J.R.

and S.M. prepared the opsin mutants; S.E. carried out the spectroscopic measurements; and A.S., K.F., and R.A.B. wrote the manuscript.

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