The molecular switching mechanism at the conserved E(D)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 photoreceptor rhodopsin, this is accompanied by proton uptake at Glu-134 in the class-conserved E(D)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 here by MD calculations on TM3 model peptides in a DOPC bilayer. We show that protonation of the conserved glutamic acid alters its side chain rotamer preferences and stabilizes C-terminal helical structure. Both 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 but is unexpectedly not seen in MD calculations with an amide. Time-resolved FTIR spectroscopy of TM3 model peptides reveals different kinetics for lipid ester carbonyl hydration, suggesting that the carboxyl is linked to more extended H-bond clusters than an amide. Remarkably, this is also seen in DOPC-reconstituted E134- and Q134-containing opsin mutants and demonstrates that the E(D)RY motif is a hydrated microdomain where its functions as a proton switch is based on the reorganization of the H-bond network in the membrane surface.

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 which catalyzes GDP / GTP exchange in cytosolic G proteins (guanosine nucleotide-binding proteins). More than 600 GPCRs in humans belong to the class-A (1) which is characterized by structural homology with the visual photoreceptor rhodopsin. In contrast to ligand-activated GPCRs, rhodopsin is activated by 11-cis to all-trans photoisomerization of retinal, which is covalently bound via a protonated Schiff base to the side chain nitrogen of Lys-296 of the apoprotein opsin. 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 which link the cytosolic ends of TM3 end TM6 in the inactive receptor state is crucial for GPCR activation {Ballesteros, 2001 #1612; (4, 5). Studies on bovine rhodopsin revealed that the corresponding distance increase between TM3 and TM6 (6) is followed by a proton uptake reaction in the side chain of Glu-134 within the class-conserved E(D)RY motif at the C-terminal end of TM3 (7). This cytosolic “proton switch” (8, 9) involves the proton exchange with the environment and is thus pH-dependent. It is evoked by preceding light-induced structural changes (10) and internal proton transfer reactions (11) among which the pH-insensitive internal “proton switch I”, i.e., the transfer of the Schiff base proton to its counterion Glu-113 (12), is the key step that leads to the active metarhodopsin II (MIIa) conformation (13). The following movement of TM6 (MIIb) precedes protonation at Glu-134 in ($MIIbH^+$) (14). The latter occurs with an unusually high $pK_a > 6$ (15) indicative of the

energetic stabilization of the protonated state. Receptor activation thus follows a sequence of thermally activated structural transitions in multiple microdomains which 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 been originally based on the spectroscopic identification of inactive rhodopsin states with partial active-like structural features (16) which may cause enhanced thermal receptor activation related to disease (17) . Neutralization of the Glu134 side chain has been identified as one of the crucial activity-promoting factors (18, 19). Although the concept of concerted microdomain switches explains receptor activation (20), 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 Glu-134, 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 (21). 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 Glu-134 side chain with the sub-headgroup region are not known. In order to reveal these molecular details, we have performed MD calculations, fluorescence and time-resolved infrared-spectroscopic experiments on lipid-inserted TM3 model peptides. Our data give a detailed description of proton-induced secondary structure and topological changes in TM3. We demonstrate that the pK_a of the Glu-134 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 sub-headgroup hydration is addressed by infrared spectroscopy using both model peptides and E134- and Q134-containing opsin mutants.

METHODS

Peptide sequences used in MD calculations. The initial structure of the wild type peptide P1 was derived from transmembrane helix-3 (TM3) of rhodopsin (pdb entry 1F88, residues 108 to 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 E(D)RY motif of class-1 GPCRs corresponds to residue 27 in the peptide models and is designated E27. In order to achieve comparability with previous spectroscopic studies, glutamic acids that do not belong to the conserved E(D)RY motif were replaced by alanine in all TM3 peptides investigated here (Table I). In addition to fixing the ionized or protonated state of E27 in the calculations, peptides with the E27Q replacement were studied (P3). However, spectroscopic studies showed that it adopted a mixture of non-helical and helical states which could be avoided by the additional W19F / V31W double replacement. In order to validate corresponding experimental results, MD calculations were extended to P5 and P6 carrying the additional double replacement and a Glu or Gln residue at position 27, respectively. Finally, MD simulations were also performed with the ICL2 peptide (AIERYVWVCKPMSNFRFG) derived from the second intracellular loop which extends from helix 3. It still carries the conserved E(D)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 (22) into a system with 128 lipids of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and hydrated with 40 molecules of water (TIP3p, (23)) per lipid. Peptides with aspartic acid or glutamic acid in the D(E)RY motif, were generated in both protonated and unprotonated states. The Gromacs 4.5.5 package19 (24) was used and the leap-frog algorithm applied as integrator with a time step of 2 fs. An isothermal-isobaric scheme (NPT) was chosen (25, 26) 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 X 10^5 bar^{-1}(29). The barostat was coupled 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 (26) and SETTLE (30) algorithms. The long-range electrostatic interactions were treated using the particle mesh Ewald method, with a cut-off in real space of 1.0 nm, and a Fourier spacing of 0.12. The cut-off 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 (31) and AMBER99 (32) were used as force fields for lipids and peptides, respectively. The systems were minimized using the steepest descent algorithm with an energy step size of 0.01nm and 50,000 steps, equilibrated for 50 ns with position restraints on the heavy atoms of the peptide. The production simulations were run for 200 ns each.

Infrared spectroscopy of lipid-reconstituted model peptides and opsin mutants. Peptides P7 and P8 were synthesized with C- and N-terminus amidated and acetylated, respectively, HPLC-purified and trifluoroacetate was removed (ThermoFisher, Ulm, Germany). The peptides were reconstituted into vesicles of 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC; Avanti Polar Lipids, Inc. Alabaster, USA) by dissolving 10 mg DOPC and 1.5 mg of the peptides in 100-200 µL ethanol followed by solvent evaporation, resolvation in 40 µL ethanol and finally rapid dilution in 1960 µL H_2O (33). Vesicles were formed by at least 10 freeze-thaw cycles of the suspension (34). Opsin mutants carrying the stabilizing N2C/D282C amino acid replacements (35) where expressed in HEK-239S cells and detergent-solubilized as described (36), except for using 1% octylglucoside (OG) instead of dodecylmaltoside. Protein concentration was determined according to Bradford. The solubilized mutant opsins N2C/D282C and N2C/D282C/E134Q were mixed with DOPC in 1% OG in a 1:100 protein to lipid ratio in a total volume of 200-250 µL. They were simultaneously dialyzed in mini dialysis cups in the identical buffer (1 L of 5 mM phosphate buffer, pH 7.4) with one complete buffer exchange overnight.

The lipid-reconstituted peptide and 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 solutions, respectively (37) 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 (38, 39). Briefly, the r.h. above the sample is increased within 2-4 seconds by a heating current in the salt solution. 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 seconds. An additional waiting time of 5 min was allowed before repeating the experiment in an automated fashion for signal averaging.

RESULTS

MD-Calculations on the coupling of E27 protonation to transmembrane helical structure. Previous infrared spectroscopic data on a detergent-solubilized TM3 model peptides of bovine rhodopsin supported a protonation-dependent secondary structural transition near the E(D)RY motif in the visual photoreceptor and possibly other GPCRs but the extent of the helix formation along the sequence and its location relative to the membrane water interface could not be determined. In order to test the proposed pH-regulation of TM3 conformation on a more detailed molecular level, we have studied the influence of protonation on the secondary structure of TM3 of rhodopsin using MD simulations of peptides in a relaxed lipid bilayer for a family of related sequences that allow for the comparison with earlier peptide studies using FTIR, CD and fluorescence spectroscopy (21). The peptide P2 served as a single transmembrane helical model for the native TM3 sequence and the single carboxyl group in the side chain of E27 was chosen to be either protonated or unprotonated in the calculations. Figure 1 A shows the sequence-dependent probability of

finding α-helical secondary structure during the entire simulation time of P2. The probability of α-helix formation in the C-terminal end of TM3 between residues 20 and 26 was about 10% higher for the protonated form. For comparison, a simulation is shown for a peptide with the additional E27Q replacement (P3). Such a replacement is generally considered a mimic of the protonated form of a glutamate side chain. However, the helicity in P3 barely exceeded that of P2 in the ionized form. Thus, the specific H-bonding properties of a carboxyl function rather than merely its charge state appear to be also critically involved in protonation-dependent secondary structure formation. The data agree with previous infrared absorption measurements of the protonation-induced C-terminal helix-formation when P2 was incorporated in a detergent micelle, whereas P3 had not been investigated spectroscopically, because it formed a large fraction of non-helical structure in detergent. This could be overcome by the additional Trp126Phe/Val138Trp replacement which stabilized the α-helical structure. Therefore, the corresponding peptides P5 and P6 (which carried the additional E27Q replacement) were simulated as well. The protonation-induced secondary structure formation in P5 was reproduced and slightly larger than in the wild type sequence (Fig. 1 B). Again, the replacement of the titratable amino acid E27 by the structurally homologous glutamine residue (P6) was not equivalent with protonation of the E27 side chain. The data agree with the proposed coupling of protonation and conformation at the C-terminus of TM3, but allow locating the helix stabilization to about five amino acids within the lipidic phase preceding the actual titration site. Furthermore, the data demonstrate the unexpected but apparently crucial importance of the detailed H-bond geometry and dynamics of the protonated carboxyl function as compared to the amide group despite their common neutral state.

Dynamics of E27 side chain conformation. A key assumption of the previously proposed mechanism that couples side chain protonation to conformation is the fluctuation of the Glu-134 side chain between an “exposed” and a “buried” geometry at the membrane surface once the ionic lock is disrupted upon photoactivation of rhodopsin. Therefore, the local structure was further assessed by monitoring fluctuations of the dihedral angle of E27 as a function of protonation. Both, the native P2 sequence and the P5 sequence with the C-terminal valine replaced by tryptophan exhibited fluctuations between the gauche and trans conformation of their ionized E27 side chain (Fig. 1 C). These fluctuations were strongly reduced upon protonation, leading to the prevalence of the gauche rotamer in P2, whereas the trans rotamer was stabilized in P5. Thus, the coupling of protonation to secondary structure formation is a robust feature of the ERY motif, whereas the side chain conformation appears to be further regulated by the sequence context, such that the sequence with the higher helical propensity (P5) favors the trans rotamer in the protonated state of the side chain. Irrespective of this context, however, protonation strongly reduced structural fluctuations. Figure 1 D visualizes the protonation-dependent rotamer preference in the transmembrane topology of P5, showing the position of the gauche rotamer of the ionized carboxyl at the level of the lipid phosphates, whereas in the protonated state, the preferred trans isomer locates the carboxyl to the sub-headgroup region.

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Protonation-dependent transmembrane positioning of the helical backbone. In addition to the local side chain rotamer preferences, we have studied the consequences of the protonation-induced α-helix stabilization for the general transmembrane topology of the peptides. This is visualized in Fig. 2 by following over time the center of mass (COM) position of the amino acids with respect to the lipid bilayer surface (represented by the COM of the

choline headgroup). In the protonated form, the COM of E27 of P2 exhibited a rather stable average distance from the lipid headgroup. In contrast, the ionized form showed a gradual movement towards the phase boundary during the simulations such that its burial depth was reduced by with respect to the protonated form. This behavior encompassed the entire E(D)RY motif and extended N-terminally into the bilayer, but did not include residues C-terminal of the E(D)RY motif. Also the replacement of the C-terminal valine by the more hydrophilic tryptophan in P5 did not change either the average COM of the C-terminus or the protonation-dependent shift of the back bone carbons. Thus, neither the secondary structure formation nor the shift of the E(D)RY motif toward the bilayer was controlled by the hydrophobicity of the extra-membraneous C-terminus but exclusively by the protonation state of E27.

Coupling of the side chain pK_a of E27 to the dielectric environment. The results show that the protonation-dependent regulation of membrane insertion may provide a dielectric mechanism that stabilizes the peptide structure with the protonated side chain. We have tested the hypothesis that lowering of the dielectric constant with increased lipid exposure causes the unusually high $pK_a > 6$ of both Glu134 in the proton-dependent equilibrium between MII_a and MII_bH^+ states of light-activated rhodopsin (15) and of TM3 model peptides (21). The pK_a was computed in dependence of two factors: (a) the dielectric constants was varied between 2 and 80, corresponding to the transition from the hydrophobic membrane interior to the aqueous phase; (b) the effect of the secondary structure was addressed by computing the pK_a of the E27 side chain in peptides P2, P5, and ICL2. Peptides P2 and P5 have a defined helical structure in lipids, whereas the peptide ICL2 is water soluble and corresponds to the N-terminal part of the second intracellular loop of

rhodopsin. The pK_a of Glu134 in peptides P2 and P5 strongly depended on both the dielectric environment and the secondary structure: it ranged from 5.2 (ε = 80) to more than 10 at ε > 10 (Figure 3A). In contrast, the pk_a of the same side chain in the ICL2 sequence depended exclusively on the dielectric environment and ranged approximately one unit below the pK_a values of E27 in P2 and P5. Although the increased membrane insertion of the protonated E27 and the corresponding decrease in dielectric constant can partly explain the elevated pK_a, we found that the rotamer states contribute to pK_a regulation as well. In P2 and P5, the protonated form of E27 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 discussed below.

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E27 protonation exerts long range dielectric effects within the bilayer. The MD calculations demonstrate the interdependence of transmembrane topology, local hydrophobicity, side chain protonation and secondary structure. In order to verify experimentally the predicted change in the dielectric environment of the C-terminal end of TM3 as a consequence of protonation-induced repositioning relative to the bilayer, we have studied peptide P7, where L24 was replaced by tryptophan. This residue provides a fluorescence monitor by responding with a blue-shift of its emission upon lowering of the local dielectric constant in the majority of proteins (40). Figure 3B shows that the tryptophan emission was affected by pH, despite the fact that it is located by a helical turn deeper in the membrane than E27. In agreement with the predicted model, the emission of W24 became blue-shifted upon protonation of E27, demonstrating the relocation further 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,

confirming the unusually high pka of the E27 side chain carboxyl derived from MD calculations and seen with similar TM3 model peptides. The pH effect was abolished when E27 was replaced by Gln in P8, which proves the purely E27-mediated molecular mechanism. Emission from W24 in P8 was not only pH-insensitive but also observed at shorter wavelength than in P7 (Fig. 3B) which also agrees with the expected stabilization of lipid-inserted states of the C-terminal helical end by the presence of 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 E27 side chain provides a structural switch that implements physical interactions which can act largely independently of the context of the full length structure of the receptor. These interactions provide a structural metastability that will contribute to conformation and energetics of the full length receptor conformation by stabilizing substates in a protonation-dependent manner. Remarkably, this switch is tailored to the physical constraints of the lipid water phase boundary, such that protonation leads to the repositioning of the TM3 helical end, to a reduced flexibility of both the peptide backbone and the side chain of E27 and to altered side chain rotamer preferences.

Side chain-dependent dynamics of the sub-headgroup H-bond network. The MD calculations have revealed the unexpected difference between the neutral protonated carboxyl group and the structurally similar amide group of Gln in the side chain at position 27 in regulating local secondary structure. Only the protonated carboxyl function stabilized the C-terminal α-helical structure in P2 and P5, whereas Q27 showed the effect in neither peptide. On the other hand, Q27 did reduce the dielectric constant at W24, as did protonation of E27. The only partial mimicking of the protonated state of E27 by Q27

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 which compete with intermolecular water H-bonds. Therefore, the interaction of the E(D)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 P7 and P8. The infrared absorption of the ester carbonyl stretching mode and the OH-stretching vibration in corresponding lipid films was observed by time-resolved Fourier-transform infrared (FTIR) spectroscopy as a function of hydration. The technique employs a short hydration pulse which increases the relative humidity of air above the lipid film (from 85% RH to 90-95%) within seconds. The experimental setup has been described in detail for the hydration of DNA and lipids (38, 39) and allows following the relaxation of the sample to its initial r.h. with seconds time-resolution. Figure 4 (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 stayed equilibrated during the entire time-course. This synchronicity was preserved in the presence of P7 (Fig. 4B), where an additional absorption change at 1663/1650 cm^{-1} revealed changes in peptide bond geometry / H-bonding. Figure 5 (A) shows the corresponding IR raw data for P8 and compares the C=O hydration response for P7 and P8. Whereas the H-bond-dependent change of the C=O stretching absorption scaled again strictly with hydration for both peptides, the relaxation time for P8 carrying the neutral Gln side chain was faster than for

P7. The data show that the presence of the carboxyl function slows down the re-equilibration of lipid-bound water with the gas phase above the lipid film.
This supports the critical role of carboxyl-specific H-bonds which need to be broken upon removal of the excess water taken up during transient hydration.

The data raise the question whether the different sub-headgroup hydration properties seen with a carboxyl or amide group at the membrane water interface of DOPC also persist in full length opsins. Opsin mutants carrying the stabilizing N2C/D282C double replacement were expressed, reconstituted in DOPC (Methods) and hydrated lipid films adsorbed on an ATR crystal in the same manner used for peptide-containing films. Figure 5 (B) shows the time-resolved IR raw data for stabilized opsin and a mutant that contains the additional E134Q 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 cm^{-1} difference band. In contrast to the peptide-containing films, the opsins exhibited a slower re-equilibration 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 re-equilibration for opsin with the amide-containing side chain at position 134 than with the carboxyl of the native E134 (Fig. 5 C). The different linkage of membrane hydration to sub-headgroup H-bonding is thus a site-specific feature that prevails in both the full length receptor structure and the TM3 model peptides.

DISCUSSION

We have studied molecular details of the structural and dynamical consequences of carboxyl protonation in the conserved E(D)RY motif at the C-terminal end of TM3 in class A GPCRs. Using MD calculations on DOPC-inserted peptides derived from TM3 of bovine rhodopsin

and spectroscopy on opsin mutants we have identified lipid protein interactions that couple carboxyl protonation to structural transitions that are of functional relevance in the full length receptor. In active bovine rhodopsin structures (41-43), the side chain of the protonated Glu134 in the class-conserved E(D)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 (44) 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. The MD calculations show that the E(D)RY motif forms a microdomain switch in the true sense: it is part of an independently folding transmembrane domain but adopts protonation-dependent structural sub-states that have counterparts in the crystal structures of inactive and active conformations of rhodopsin. This correspondence concerns the protonation-induced increase in C-terminal helicity and the transition from a *gauche* to a *trans* side chain rotamer preference as seen most prominently in the simulations with P5. The latter were addressed by MD calculations under the dielectric and geometric constraints of a membrane water interface which cannot be derived from the available GPCR structures. In the W31-terminated P5, the protonated side chain of E27 is located more than 0.43 A below the lipid headgroups but approaches the phase boundary to a distance smaller than 0.28 A in the protonated state. This change in relative COM distances can be largely attributable to the demonstrated E27 rotamer transition (Fig. 1), rather than to a pronounced general movement of the traverse helix position, because the COM displacements of the next three C- and N-terminal amino acids towards the membrane surface are all smaller than the E27 displacement (Table 2). Only the C-terminal W31 shows a larger, but opposite, displacement upon E27 protonation as it moves from a headgroup to sub-headgroup position (Fig. 2 and

Table 2). The high variability of tryptophan geometries at membrane surfaces has been studied in detail (45) and does not contradict the strong helix anchoring function of this residue (46). In fact, the data show that the helix anchoring role of W31 “forces” the rotamer switching in order to place the charged or neutral E27 side chain at the best matching region within the dielectric gradient provided by the lipid sub-headgroup layer. In agreement with this interpretation, the absence of the W31 anchor in P2 allows for a more substantial shift of 0.3 A of the TM helix toward the C-terminal end when the E27 side chain becomes ionized. Again the COM displacement is largest for E27 itself.

Membrane anchoring functions have also been described for tyrosines at TM helical ends (47). Remarkably, the potential of the charged side chain of E27 to promote the C-terminal transverse movement of TM3 is not impeded by the tyrosine of the E(D)RY motif: Y29 exhibits a three-fold larger displacement in P2 than in the tryptophan-anchored P5 (Table 2). This agrees with the strong effect of charged residues on TM helical end positioning (48, 49). Nevertheless, both peptides demonstrate that the ionized state of E27 favors the expected approach to the more hydrophilic membrane surface, whereas the protonated side chain is preferentially located in the more hydrophobic sub-headgroup region of the bilayer. The data show that the degree to which side chain rotamer switching or transverse TM helix movements accomplish this relocation depends on the sequence context. In combination with the electrostatic calculations (Fig.3 A), the data agree with the “partitioning” of the E27 side chain between regions of different hydrophobicity which can explain the elevated pK_a of Glu134 in the MII_b to MII_bH^+ transition of light-activated rhodopsin.

Our data reveal further mechanistic details that have previously not been considered for the conformational switching process. 1) The E27 pK_a responded differently to the dielectric environment in P2 and P5, revealing a crucial role of the different side chain rotamers for the pK regulation.

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2) A glutamine is generally considered a mimic of a protonated glutamic acid. However, the corresponding replacements made in P3 and P6 did not reproduce the effect of the protonated glutamic acid on stabilizing C-terminal helical structure. This unexpected result hints at a crucial role of H-bond networks in addition to the described side chain positioning within the dielectric gradient at the membrane surface. Likewise, it was not anticipated from the analyses that the rate of sub-headgroup hydration would respond to a single amino acid replacement in the TM3 model peptide. 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 surface. This may explain why in full length rhodopsin in membranes, the replacement of E134Q 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 (50). The described importance of the phase boundary in these structural transistions provides a detailed mechanistic rational why Glu134 protonation is required for full receptor activation in membranes but not in detergent (51).

In summary, the “proton switch” mechanism of the E(D)RY motif can be understood on the basis of the dielectric properties of the membrane-interface and the membrane-anchoring 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.

Peptide	(1) N-term C-term (31)
P1	TGCNLEGFFATLGGEIALWSLVVLAI **E** RYVV
P2	TGCNL<u>A</u>GFFATLGG<u>A</u>IALWSLVVLAI **E** RYVV
P3	TGCNL<u>A</u>GFFATLGG<u>A</u>IALWSLVVLAI **<u>Q</u>** RYVV
P5	TGCNL<u>A</u>GFFATLGG<u>A</u>IAL<u>F</u>SLVVLAI **E** RYV<u>W</u>
P6	TGCNL<u>A</u>GFFATLGG<u>A</u>IAL<u>F</u>SLVVLAI **<u>Q</u>** RYV<u>W</u>
P7	TGCNL<u>A</u>GFFATLGG<u>A</u>IAL<u>F</u>SLVV<u>W</u>AI **E** RYVV
P8	TGCNL<u>A</u>GFFATLGG<u>A</u>IAL<u>F</u>SLVV<u>W</u>AI **<u>Q</u>** RYVV
ICL2	AIERYVVCKPMSNFRFG

Table I: Sequences of TM3 model peptides used in this study. Bold: position 27 carrying the glutamic acid of the conserved E(D)RY motif of class-A GPCRs. Underlined: additional amino acid replacements which increase helix stability or neutralize the side chain at position 27.

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