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Sequential Rearrangement of Interhelical Networks Upon Rhodopsin Activation in Membranes: The Meta II_a Conformational Substate

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Abstract: Photon absorption by rhodopsin is proposed to lead to an activation pathway that is described by the extended reaction scheme Meta I ⇌ Meta II_a ⇌ Meta II_b ⇌ Meta II_bH^+, where Meta II_bH^+ is thought to be the conformational substate that activates the G protein transducin. Here we test this extended scheme for rhodopsin in a membrane bilayer environment by investigating lipid perturbation of the activation mechanism. We found that symmetric membrane lipids having two unsaturated acyl chains, such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), selectively stabilize the Meta II_a substate in the above mechanism. By combining FTIR and UV−visible difference spectroscopy, we characterized the structural and functional changes involved in the transition to the Meta II_a intermediate, which links the inactive Meta I intermediate with the Meta II_b states formed by helix rearrangement. Besides the opening of the Schiff base ionic lock, the Meta II_a substate is characterized by an activation switch in a conserved water-mediated hydrogen-bonded network involving transmembrane helices H1/H2/H7, which is sensed by its key residue Asp83. On the other hand, movement of retinal toward H5 and its interaction with another interhelical H3/H5 network mediated by His211 and Glu122 is absent in Meta II_a. The latter rearrangement takes place only in the subsequent transition to Meta II_b, which has been previously associated with movement of H6. Our results imply that activating structural changes in the H1/H2/H7 network are triggered by disruption of the Schiff base salt bridge and occur prior to other chromophore-induced changes in the H3/H5 network and the outward tilt of H6 in the activation process.

Introduction

The dim light photoreceptor rhodopsin is widely recognized as perhaps the most thoroughly understood paradigm for G protein-coupled receptors (GPCRs) in cellular membranes.[1−3] Yet, despite the profound biological and pharmacological significance of GPCRs, the activation mechanism of rhodopsin and other class A GPCRs is still not well understood in structural terms. Elucidating the activation mechanisms of GPCRs and how they orchestrate the amazing diversity of cellular signals due to light, diffusible hormones, odorants, or neurotransmitters through their interactions with G proteins is currently among the most significant challenges in biochemistry, biophysics, and pharmacology. Activation of rhodopsin is triggered by the light-induced 11-*cis* to all-*trans* isomerization of its retinal chromophore, which is linked to the protein via a protonated Schiff base (PSB) in the transmembrane domain of the light receptor (Figure 1A). This initial event is propagated via distinct intermediates to the cytoplasmic receptor domain, allowing coupling to the visual G protein transducin which is activated by the signaling state Meta II.[4] The early spectral intermediates, Batho, BSI, and Lumi,[5] mainly involve gradual adjustments to the isomerized chromophore in the vicinity of the ligand binding pocket within the transmembrane core.[6,7] The conformational equilibrium between Meta I and several distinct Meta II states, on the other hand, involves major functional and structural changes which have been characterized by proton uptake measurements,[8,9] as well as UV−visible,[10] spin label electron paramagnetic resonance (EPR),[11−13] NMR,[14−20] and Fourier-transform infrared (FTIR) spectroscopy.[21−23]

For rhodopsin, a current picture is that isomerization of the 11-*cis* chromophore by visible light initiates concerted displacements of its transmembrane helices.[13,22] In the activation

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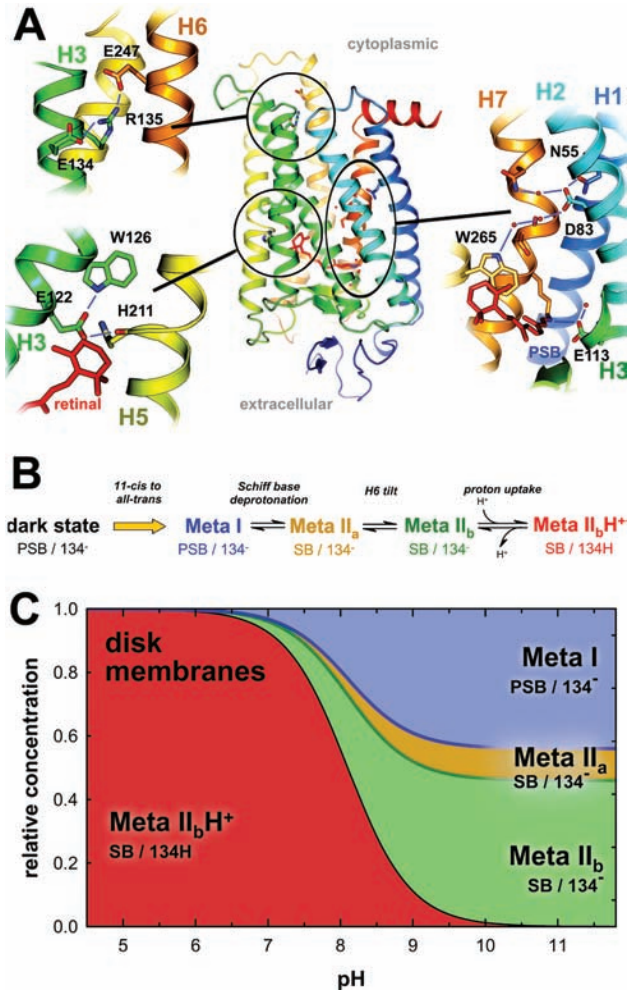

Figure 1. Extended Meta I-Meta II scheme for rhodopsin activation explains visual function by an ensemble of conformational substates. (A) Activation of rhodopsin involves several conformational switches, salt bridges, and interhelical networks, of which several are highlighted here in the dark state of rhodopsin.[49] (B) Triggering of these switches in the photoproducts of rhodopsin leads to a sequential progression of intermediates from inactive Meta I to the signaling state, involving formation of Meta II_a after disruption of the PSB salt bridge, Meta II_b after movement of H6, and Meta II_bH^+ after proton uptake by Glu134, which were initially characterized only in a detergent environment.[12] (C) In native disk membranes, the pH-independent thermodynamic equilibria between the Meta I, Meta II_a, and Meta II_b photoproduct states and the pH-dependent transition to Meta II_bH^+ gives rise to complex pH-dependent population curves that can be determined by combination of FTIR and UV−visible spectrometries.[21]

process, an outward tilt of transmembrane helix 6 (H6) from the H1−H4 helical core and a smaller rotation of H5 opens a site for binding and activation of the G protein transducin.[24] Nonetheless, the exact temporal sequence and mechanism whereby these helix rearrangements occur remains to be elucidated.[25] A reaction scheme has been proposed by Hofmann and Hubbell and colleagues[8,12] involving transitions from Meta I to a sequence of conformational substates designated as Meta II_a, Meta II_b, and Meta II_bH^+ (Figure 1B). In this extended reaction scheme, the transition from Meta I to Meta II_a is defined by the deprotonation of the PSB and hence disruption of the PSB salt bridge to its primary counterion, Glu113, on H3 (Figure 1A). The transition from Meta II_a to Meta II_b is defined by the EPR spectral signature associated with a rigid body tilt of H6 out the helix bundle,[12,13] which is followed by cytoplasmic proton uptake by Glu134 of the ERY motif in a H3/H6 interhelical network (Figure 1A) in the transition to Meta II_bH^+. Alternative kinetic models do not assume a strictly linear reaction scheme, but a modified square scheme,[26] in which a deprotonated early intermediate (Meta I_{380}) is observed already during the decay of Lumi and prior to the transition to the Meta II substates of the extended sequential scheme.[9]

However, the situation in a native-like membrane environment is only beginning to be fully elucidated.[21,22,27,28] Much of the information about the extended sequential reaction scheme has been derived from studies of rhodopsin and site-directed rhodopsin mutants in detergents such as dodecyl maltoside. Solubilization of rhodopsin in detergent micelles leads to substantial perturbations of the equilibria between the individual intermediates.[29] We have recently examined the thermodynamics of this reaction scheme using rhodopsin in native disk membranes, which leads to a complex, pH- and temperature-dependent population scheme for the conformational states and substates (Figure 1C).[21] In this scheme, Meta I, Meta II_a, and Meta II_b form coupled equilibria with the fraction of each intermediate being dependent on the particular value of ΔG^o of the associated transition, but not on the proton concentration. Only at lower pH, these intrinsically pH-independent equilibria are shifted to the Meta II_bH^+ state by the enthalpically favorable protonation of Glu134 from the aqueous solvent.[21,23] A reduced equilibrium involving only three of the four states, Meta I, Meta II_a, and Meta II_b, can hence be examined at high pH, at the alkaline end point of the population curves, where this last protonation step is absent. In native disk membranes, the Meta I and Meta II_bH^+ substates can be isolated under appropriate pH and temperature conditions (0 °C, pH 9, and 30 °C, pH 6, respectively) and are easily characterized by spectroscopic methods. In the alkaline range of the population curves (Figure

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1C), the ratio between Meta I and Meta II_b depends strongly on temperature, with higher temperature favoring Meta II_b due to a strong entropy increase compensating a positive and hence unfavorable enthalpy change. As there is only a very low population of the Meta II_a substate in disk membranes (Figure 1C), the Meta II_b state can be examined in its temperature-dependent equilibrium with Meta I, revealing a similar receptor conformation as Meta II_bH^+ [21]. In contrast to the deprotonation of the PSB, the protonation of Glu134 from the cytoplasm was concluded to release energetic rather than structural constraints.

Associated structural and functional changes within rhodopsin can be monitored by FTIR difference spectroscopy by following changes of band patterns in the amide I region due to the protein backbone, or by following band shifts of protonated Glu122 and Asp83. These latter residues serve as reporter groups in the H3/H5 and H1/H2/H7 interhelical networks (Figure 1A)—they respond to local changes in hydrogen bonding. The FTIR structural markers of conformational changes follow quite closely and in a concerted manner the deprotonation of the Schiff base as detected with UV−visible spectroscopy, demonstrating only a marginal population of Meta II_a in disk membranes.[21] Remarkably, by varying the lipid composition of the membrane environment, we found that the Meta II_a conformational substate is selectively stabilized in recombinant membranes with synthetic lipids having unsaturated acyl chains at both the glycerol *sn*-1 and *sn*-2 positions, such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). This selective stabilization allows a structural characterization of the Meta II_a substate that is of particular relevance in the context of the activation mechanism of rhodopsin. It provides the missing link between release of a major structural constraint on the activation pathway, namely the opening of the PSB salt bridge in the transition from Meta I, and the subsequent tilt of H6 detected by EPR spectroscopy defining the transition to Meta II_b.[11−13] We conclude that an ensemble of activated conformational substates is implicated in rhodopsin activation. Their populations are modulated by altering the lipid acyl chain composition as shown here, or the lipid polar head groups.[30,31] Our work illustrates in a particularly striking fashion how membrane lipid perturbation can illuminate and test membrane protein function analogously to site-directed mutagenesis.

Experimental Section

Rhodopsin Preparation. Rhodopsin in disk membranes was prepared from bovine retinae (Lawson, Lincoln, NE, USA),[32] purified on hydroxyapatite HTP gel (BioRad, Hercules CA, USA) in dodecyltrimethylammonium bromide (DTAB, Sigma, St. Louis, MO, USA) and reconstituted into 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids (Avanti, Alabaster, AL, USA) at a molar ratio of 1:100 according to established methods.[33] Proteoliposomes were characterized by isopycnic density gradient centrifugation, indicating a single lipid/rhodopsin band, and stored in 1 mM phosphate buffer, pH 6.5, at −86 °C.

FTIR and UV−visible Spectroscopy. Both FTIR and UV−visible spectroscopies were performed at 30 °C using previously described and characterized sandwich samples.[21,34] In short, membrane films were prepared by drying approximately 0.4 nmol of rhodopsin in disk or synthetic membranes under nitrogen from the low ionic strength membrane stocks. These films were covered with 80 μL of 200 mM bistrispropane (BTP) buffer in the pH range from 6.0 to 9.0 and equilibrated for 3 min to ensure sufficient hydration of the membrane stacks and complete adjustment of salt concentration and pH. In control experiments, membranes were washed with a large volume of 1 mM BTP buffer adjusted to the respective pH values prior to preparation of the sandwich samples, verifying the reliability of the pH adjustment in the samples. Water content in the samples was estimated to be approximately 90% (v/v) using the water OH stretch as an internal marker in FTIR absorption spectra. The nominal pH values were further corrected for the temperature dependence of the buffer pK_A. Additional experiments were performed at 0 and 10 °C. Photolysis was achieved by a 100-ms flash of an array of six ultrabright green (λ_{max} 520 nm) 5 mm-LEDs (Nichia, Tokushima, Japan, nominal 16 Candela at 20 mA current, 15° emission half angle) operated at 100 mA, by which approximately 70% of rhodopsin was photolyzed. FTIR difference spectroscopy was performed with a Bruker Vertex 70 spectrometer with a mercury−cadmium-telluride detector. IR spectra were recorded using time-resolved rapid-scan FTIR spectroscopy, with a spectral resolution of 4 cm^{-1} and an acquisition time of 6 s for the preillumination spectrum, and 120 ms for single postillumination spectra. UV−visible spectroscopy was performed with a Hewlett-Packard 8453 diode array spectrometer, using a neutral density filter with 10% transmission to minimize sample bleaching by the measuring beam; the spectral acquisition time was 200 ms. In all experiments, the thermal stability of the photoproduct states was confirmed in a series of postillumination spectra. The postillumination spectra were averaged to increase *S*/*N*.[21] Meta II_bH^+ was stabilized at 30 °C at pH 6.0, and Meta I at 0 °C at pH 9.0. The amount of isorhodopsin in the photoproduct mixture is approximately 10% of the amount of Meta I (as the sole all-*trans* photoproduct species with protonated Schiff), as shown previously under identical illumination conditions.[21] This would restrict the maximal amount of isorhodopsin to approximately 4 and 2.5% of the photoproducts in the case of disk and DOPC membranes. Therefore, the spectra were not corrected for isorhodopsin contribution to keep data manipulation on a clear level.

Data Analysis. FTIR difference spectra were normalized using chromophore fingerprint bands of the dark state around 1237 cm^{-1}. Intensities of FTIR difference bands were determined for bands centered at 1768 cm^{-1} for Asp83[35] and at 1644 cm^{-1} for amide I. For Glu122, the difference intensity at 1745 cm^{-1} (+) and 1727 cm^{-1} (−) was evaluated correspondingly.[35] The Θ values are defined as $\Theta = (\Delta A - \Delta A_{\text{Meta I}})/(\Delta A_{\text{Meta IIbH+}} - \Delta A_{\text{Meta I}})$, where ΔA is the absorption for a respective functional group in a *photoproduct minus dark state* difference spectrum and $\Delta A_{\text{Meta I}}$ and $\Delta A_{\text{Meta IIbH+}}$ are the respective absorption changes in pure *Meta I minus dark state* and *Meta II_bH^+ minus dark state* difference spectra obtained at 0 °C, pH 9.0, and 30 °C, pH 6.0, respectively (Figure 2A). The protonation status of the Schiff base was calculated correspondingly as a Θ value by extending the above definition over the absorption range from 300 to 650 nm in UV−visible spectrophotometric experiments. Values of Θ were fitted to a modified Henderson−Hasselbalch function $\Theta = (\Theta^{alk} + 10^{pK_a\text{-pH}})/(1 + 10^{pK_a\text{-pH}})$, with Θ^{alk} being the alkaline end point of the curve.[21] In general, the alkaline end point values obtained with disk membranes and POPC membranes were in this study somewhat higher (by approximately 0.15 and 0.10, respectively) than in a previous study.[21] This is partly accounted for by using a Meta I reference state stabilized at 0 °C (instead of 10 °C in the previous study) and, in the case of disk membranes, presumably also by different batches of retinae being used in the preparations (all

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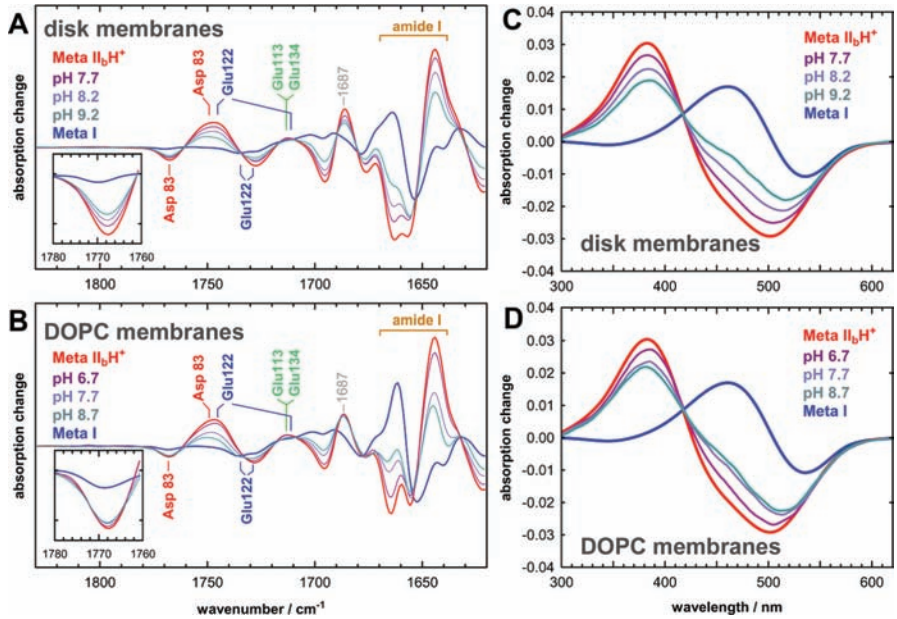

Figure 2. FTIR and UV−visible marker bands in pH-dependent experiments monitor conformation of the receptor and protonation state of the retinal chromophore. Light-induced FTIR difference spectra *photoproduct minus dark state* were obtained at a series of pH values at 30 °C with rhodopsin in disk membranes (A). The pH-dependent transition from pure Meta II_bH^+ at acidic pH to a mixture of mostly Meta I and Meta II_b states at alkaline pH is shown (see Figure 1C). In disk membranes, this transition affects all spectral features in a mostly concerted way, including carboxylic acid and amide I bands. In DOPC membranes (B), the pH-dependence is selectively lagging for the band of Asp83 at 1768 cm^{-1} (shown enlarged in the inset) and an unassigned band at 1687 cm^{-1}. Corresponding UV−visible difference spectra (C and D) allow one to monitor the protonation status of the retinal Schiff base under identical conditions as in the FTIR experiments.

experiments here were performed with rhodopsin from a single batch of retinae).

Results

Fourier-Transform Infrared Spectroscopy Characterizes Intermediates of the Extended Reaction Scheme for Rhodopsin in Native Disk Membranes. Figure 2A shows pH-dependent, light-induced FTIR difference spectra obtained from rhodopsin in disk membranes at 30 °C in the pH range from 6 to 9. The intensities of the difference bands ΔA in the conformationally sensitive range between 1800 and 1600 cm^{-1} follow a curve with $\Theta = (\Delta A - \Delta A_{\text{Meta I}})/(\Delta A_{\text{Meta IIbH+}} - \Delta A_{\text{Meta I}})$ (see Experimental Section), which is similar for all bands in this spectral range. This implies that each difference spectrum can be represented to good approximation by a linear combination of *Meta I minus dark state* and *Meta II_bH^+ minus dark state* basis spectra with $(1-\Theta)$ and Θ as the respective coefficients, as reported previously.[21] It further implies that the structural changes underlying the single FTIR difference bands occur in a concerted manner. Such a simple linear relationship holds as long as the spectral signatures of Meta II_b and Meta II_bH^+ are similar, which is the case, and the population of the additional Meta II_a state in the equilibrium mixture is small.[21]

Membrane Lipids Perturb the Populations of Rhodopsin Conformational Substates. However, with rhodopsin reconstituted into synthetic DOPC lipid membranes, we found a strikingly different behavior. Here, the pH-dependent intensities of most of the difference bands in the spectral range between 1800 and 1600 cm^{-1} follow a similar pH-dependence, while the pH-dependence of the band at 1768 cm^{-1} of Asp 83 and of an unassigned band at 1687 cm^{-1} is much less pronounced (Figure 2B). This implies that the pH-dependent FTIR difference spectra cannot be represented by a linear combination of only two reference spectra, in contrast to the above experiments with disk membranes. Instead, there is a substantial contribution from another conformationally distinct state, which is Meta II_a, as shown further below.

Next, to quantify the pH-dependent intensities of individual marker bands, we calculated Θ for the difference band of Asp83 at 1768 cm^{-1}, of Glu122 at 1745 cm^{-1} (+) and 1727 cm^{-1} (−), and of the amide I marker band of Meta II at 1644 cm^{-1}. These values were then fitted to a modified Henderson−Hasselbalch function with a nonzero alkaline end point.[21] As evident from Figure 3B, the resulting curves have a similar apparent pK_a value of approximately 7.2, but differing alkaline end points. The alkaline end point of the Asp83 difference band is approximately 0.71, while those of Glu122 and of the amide I difference bands are approximately 0.36 and 0.38, respectively. To correlate this behavior with the protonation status of the Schiff base, we conducted a similar pH series of experiments using UV−visible spectroscopy (Figure 2D). Here the equilibrium was monitored between Meta I (λ_{max} 480 nm) with PSB and the Meta II substates (λ_{max} 380 nm) with a deprotonated Schiff base, with Θ being the contribution of Meta II species. As evident from Figure 3C, the resulting pH dependence of the retinal Schiff base deprotonation curve follows closely that of the structural changes sensed by the Asp83 FTIR difference band, but not those of Glu122 or the amide difference bands, which have a substantially lower alkaline end point. It is striking that a corresponding analysis of experiments with native disk membranes and POPC lipids membranes reveals a much smaller difference between the alkaline end point values for the Asp83 marker band and Schiff base deprotonation on one hand and Glu122 and the amide I marker band on the other hand (Figure 3A). Notably the lipid environment also affects the apparent pK_a values of the titration curves, similarly as described previously.[21] In particular for the POPC membranes, the apparent pK_a is downshifted as compared with disk membranes, which might be an effect of the varying effective thicknesses

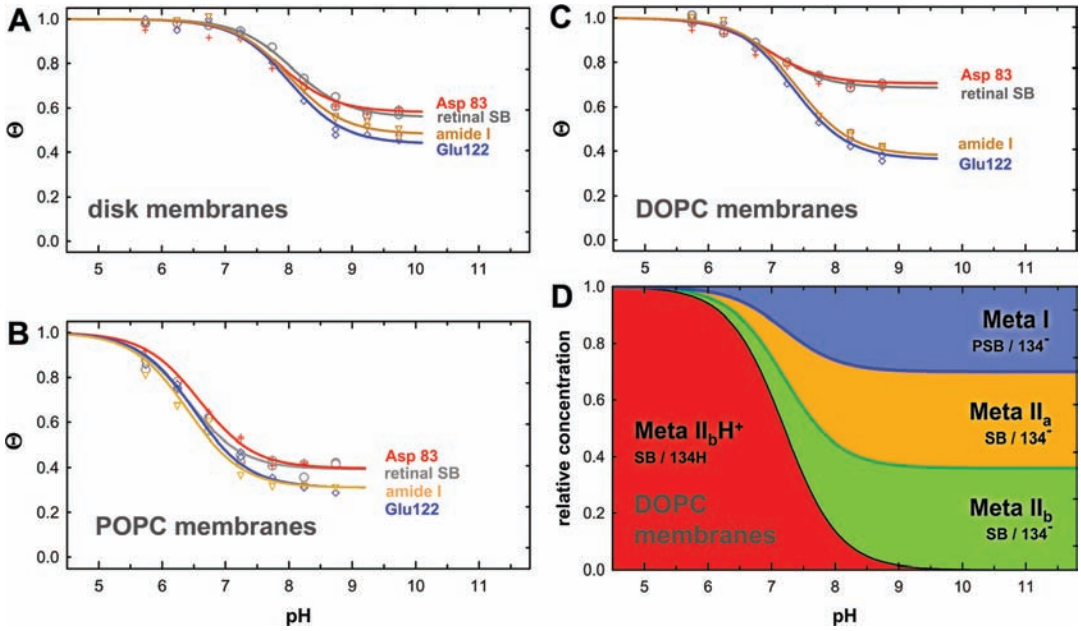

Figure 3. The Meta II_a conformational substate is selectively stabilized in DOPC membranes. The pH-dependence of FTIR marker bands of Figure 2 and of the protonation status of the retinal Schiff base ($\Theta = 0$ for fully protonated and $\Theta = 1$ for fully deprotonated, determined by UV−visible spectrometry) are compared for rhodopsin in disk membranes (A), in POPC membranes (B), and in DOPC membranes (C) (see text for details on the Θ values). While the curves obtained for disk and POPC membranes reveal only small differences in the alkaline end point value for the different markers, this does not hold for the curves obtained for DOPC membranes. Here the alkaline end point values of the Glu122 and amide marker bands are considerably lower than that of Schiff base deprotonation. This reflects substantial population of the Meta II_a conformational substate in DOPC membranes (D), which has a deprotonated Schiff base but lacks the H6 movement characteristic of the Meta II_b state. The changes sensed by Asp83, on the other hand, follow the same curve as retinal Schiff base protonation, indicating a triggering of the Asp83 activation switch already in Meta II_a.

of the lipid bilayers that affects the energetics of Glu134 protonation in the transition to Meta II_bH^+. Such an influence of the bilayer thickness had been described previously.[28,36]

The Meta II_a conformational substate is selectively stabilized in DOPC membranes. The discrepancy between the alkaline end points for Schiff base deprotonation and the Asp83 marker band on one side and the Glu122 and amide I marker bands on the other implies that besides Meta I and Meta II_b there is (at least) another spectrally distinct species contributing to the photoproduct mixture at the alkaline end point. This species has a deprotonated Schiff base and is hence by definition a Meta II state.[8,12] While its Asp83 absorption pattern is Meta II-like as well, the absorption changes of Glu122 and of the amide I marker band are Meta I-like. According to the extended reaction scheme in Figure 1B, we identify this state with Meta II_a (Figure 3C). Until now, structural studies of the Meta II_a state have been largely precluded by its transient nature and the intrinsically low steady-state populations. Notably, using its selective stabilization in DOPC membranes, the Meta II_a state now becomes experimentally accessible to study.

A very similar selective stabilization of the Meta II_a substate is also found for a recombinant of rhodopsin with DOPE/DOPC at a 1:50:50 molar ratio (results not shown). By contrast, such a selective stabilization was not evident in the case of a membrane lipid recombinant with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), having only a single unsaturated chain at the *sn*-2 position with a saturated palmitoyl *sn*-1 chain.[21] The stabilization appears therefore to be a result specific to the combination of symmetric *sn*-1/*sn*-2 unsaturated acyl groups. A further analysis of the C═O stretching bands of protonated Glu113 and Glu134 would be desirable but is hampered by compensating bands in Meta I,[37] rendering the differential absorption at 1712 cm^{-1} very small (Figure 2B). It should be further noted that the difference band pattern of Glu122 is superimposed with a lipid carbonyl difference band. The shifts of this lipid band are similar to those of Glu122 in the transitions from the dark state to Meta I and the Meta II_b states (ref 38 and unpublished data). Though we cannot fully exclude a potential compensation of the Glu122 difference band by the lipid band, such a compensation appears unlikely as it would require a stronger lipid perturbation in the Meta II_a state than in the structurally much more evolved Meta II_b states.

Discussion

Membrane lipids shift conformational equilibria in rhodopsin activation. Membrane lipids are known to significantly perturb the light-induced conformational changes of rhodopsin.[21,27−30] This affords an important avenue into investigating the activation mechanism, complementary to the use of site-directed mutants in dissecting biochemical or biological pathways. Here we tested the applicability of the extended reaction scheme Meta I ⇌ Meta II_a ⇌ Meta II_b ⇌ Meta II_bH^+ to rhodopsin in a membrane lipid environment, e.g. as opposed to dodecyl maltoside detergent micelles where the majority of structural work and studies of mutants has been undertaken. Unexpectedly, by varying the lipid composition of the membrane environment, we found that the Meta II_a conformational substate is selectively stabilized in recombinant membranes with synthetic lipids having unsaturated acyl chains at both the glycerol *sn*-1 and *sn*-2 positions, such as DOPC or DOPE (Figure 3C).

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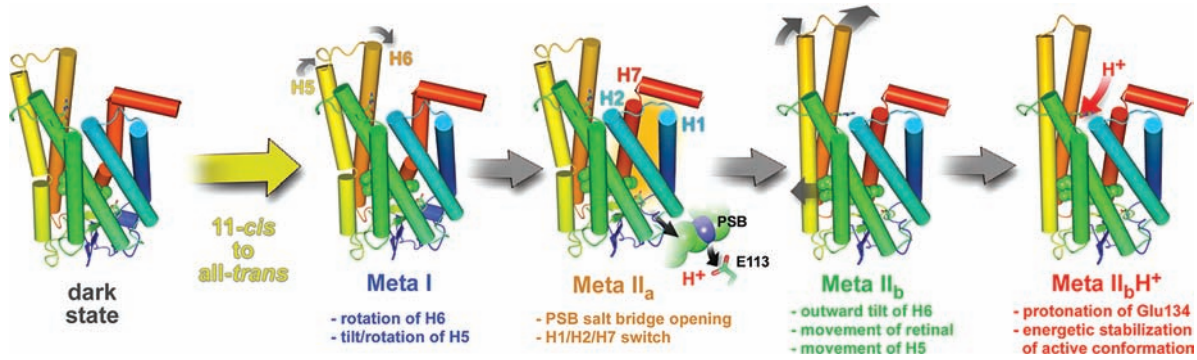

Figure 4. Structural and functional transitions of rhodopsin intermediates. Representations of the Meta substates summarize results of complementary biophysical studies[8,11,12,21−24] and highlight structural and functional changes in the activation pathway of rhodopsin. This study adds essential structural information for the Meta II_a intermediate: opening of the PSB salt bridge is accompanied by activating conformational changes in the water-mediated H1/H2/H7 network around Asp83, while movement of retinal toward H5, which triggers the larger movements of H5 and H6, occurs only in the transition to Meta II_b.

By contrast, in asymmetric membrane lipids with *sn*-1 saturated and *sn*-2 unsaturated acyl groups, such as POPC or POPE, or in native disk membrane lipids, such an effect is not as obvious and the steady state concentration of Meta II_a in the Meta I/Meta II equilibria is low.[21] Though a precise nature of this effect remains to be elucidated, we attribute this selective stabilization to the symmetric *sn*-1/*sn*-2 diunsaturated acyl group structure of DOPC versus the asymmetric *sn*-1/*sn*-2 saturated/unsaturated acyl groups of the disk membrane phospholipids. Possible mechanisms by which this difference in chain composition might influence the conformational transitions include an altering of the packing stability of the transmembrane helix bundle or a modulation of the bilayer curvature stress in a previously discussed flexible surface model.[28,30]

Structural changes occur in a conserved water-mediated interhelical network in the Meta II_a conformational substate. Our experiments indicate that the deprotonation of the retinal Schiff base in the transition from Meta I to Meta II_a leads in the first place to structural changes in the interhelical network around Asp83. Besides Asp83 on H2 this network involves other conserved residues, such as Asn55 on H1 and the NPxxY motif on H7, and it is linked by several water molecules, extending into the ligand-binding pocket to Trp265 on H6 (Figure 1A). This interhelical water-mediated network is assumed to be functionally conserved in other members of family A (rhodopsin-like) GPCRs.[39] In Meta II_a, the absorption changes of Asp83 are already Meta II-like, indicating that the activating conformational changes in this H1/H2/H7 network take place already in the transition from Meta I to Meta II_a. Other microdomains of the receptor, on the other hand, remain in a Meta I-like inactive conformation. This concerns in particular the hydrogen-bonded network between Glu122 and Trp126 on H3 and His211 on H5, which interacts with the ionone ring of retinal. In the transition to the active receptor conformation, the ring of retinal moves toward H5,[14] rearranges this hydrogen-bonded network, and weakens in particular the hydrogen bonding to Glu122.[20,37,40] The absorption changes of Glu122 indicate that this rearrangement due to retinal movement takes place only in the transition to Meta II_b, such that in Meta II_a the hydrogen-bonded network and the position of all-*trans* retinal in its binding pocket are still Meta I-like. Similar results were obtained for distinct amide I bands, reflecting structural changes of the protein backbone. The most intense amide I marker band for Meta II at 1644 cm^{-1} rises in the transition from Meta II_a to Meta II_b only, in parallel to the outward tilt of H6 observed by EPR spectroscopy.[12]

Meta II_a links the inactive Meta I intermediate with the activated Meta II_b substates formed by H6 rearrangement. Our study links crystallographic results on the early inactive intermediates Batho,[7] Lumi,[6] and Meta I[41] with recent FTIR work on azido-labeled rhodopsin mutants to track helix movements in Meta I,[22] spin-label EPR studies of Hubbell and coworkers on the Meta II intermediates,[11−13] and crystallographic studies of Ernst and Hofmann on opsin in a G protein interacting conformation[1,24] (Figure 3B). In the inactive dark state conformation, 11-*cis* retinal acts as an inverse agonist that stabilizes H6 through its interaction with Trp265.[3,19] Photoisomerization switches the retinal ligand to a full agonist, which subsequently drives the conformational changes associated with receptor activation. These conformational transitions involve the sequential movement of transmembrane helices that lead to eventual formation of a G protein-binding pocket[24,42] (Figure 4).

The first movements of transmembrane helices are observed for Meta I,[22] which has a lifetime of milliseconds. The side chain of Trp265 on H6 is released by isomerization of the chromophore[15,19] and allows a small rotational movement of H6 and an additional tilt/rotational movement of H5 away from H3, which lead to a first set of rearrangements on the cytoplasmic receptor side.[22] During these transitions, the interaction of the PSB with its complex counterion, consisting of Glu113 and Glu181, is altered. The main counterion function of Glu113 is gradually weakened, while that of Glu181 is strengthened.[43]

The subsequent transition from Meta I to Meta II_a eventually involves disruption of the PSB salt bridge to its complex counterion, which must evidently involve a change of the pK_a of the PSB. It has been previously hypothesized that the negative charge of Glu181 contributes to the extremely high pK_a of the PSB in the dark state,[43] which was found to be above 16 experimentally.[44] A possible explanation for the substantial

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decrease of the pK_a of the PSB in the transitions to Meta I and Meta II_a could therefore be an altered interaction of Glu181 with the chromophore induced by retinal isomerization, and the subsequent local changes of the binding pocket. This view is supported by FTIR investigations of the E181Q mutant,[43] which revealed an uncoupling of pH-dependent spectroscopic features at 0 °C that parallel those observed here at much higher temperature due to the presence of Meta II_a. As we show here, this disruption of the PSB salt bridge is accompanied by structural changes of the water-mediated network between helices H1, H2, and H7 involving Asp83 as a key residue and reporter group.

Movement of retinal along its long axis toward H5[14] and rearrangement of the hydrogen-bonded H3/H5 interhelical network involving Glu122 and His211 takes place only in the subsequent transition from Meta II_a to Meta II_b, as is evident from our analysis of the Glu122 marker band in the present study. The hallmark of this transition is the outward tilt of H6 out of the helix bundle, as first detected by spin-label EPR spectroscopy,[11−13] thereby creating a site for G protein binding exposed to the aqueous solvent.[24,42] The transition to Meta II_bH^+ involves uptake of a proton from the aqueous solvent[8] and protonation of Glu134 of the ERY motif of the cytoplasmic H3/H6 network.[23] This enthalpically favorable protonation step further stabilizes the active state of the photoreceptor in its native membrane environment.[21,45] At some point during formation of the two Meta II_b intermediates, the internal salt bridge between Glu134 and neighboring Arg135 is broken. This enables Arg135 to engage in new intramolecular interactions with Tyr223 on H5 and Tyr306 on H7, as well as in intermolecular interactions upon binding of the C-terminus of the α subunit of the G protein.[24] Furthermore, a salt bridge to Glu247 designated as a cytoplasmic "ionic lock" and present in the dark state crystal structures is broken at another point of the activation pathway.

Beyond the relevance for rhodopsin activation, knowledge of the structural details of these intermediates may be pertinent to GPCRS that are activated by binding of diffusible ligands.[1,25] Recent studies have shown that agonists and partial agonists are able to trigger a ligand-specific set of activation switches in the β_2-adrenergic receptor, leading to the formation of ligand-specific conformational substates.[46] Furthermore, GPCR signaling goes clearly beyond a linear signal transduction scheme. Rather it involves differential signaling toward different G protein subtypes and even other signal transduction molecules, such as kinases and arrestins.[47,48] A detailed understanding of the various activation intermediates of rhodopsin sets the stage for greater insight into the role of these conformational substates in other rhodopsin-like GPCRs.

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