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Modulating Rhodopsin Receptor Activation by Altering the pK_a of the Retinal Schiff Base

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Abstract: The visual pigment rhodopsin is a seven-transmembrane (7-TM) G protein-coupled receptor (GPCR). Activation of rhodopsin involves two pH-dependent steps: proton uptake at a conserved cytoplasmic motif between TM helices 3 and 6, and disruption of a salt bridge between a protonated Schiff base (PSB) and its carboxylate counterion in the transmembrane core of the receptor. Formation of an artificial pigment with a retinal chromophore fluorinated at C14 decreases the intrinsic pK_a of the PSB and thereby destabilizes this salt bridge. Using Fourier transform infrared difference and UV−visible spectroscopy, we characterized the pH-dependent equilibrium between the active photoproduct Meta II and its inactive precursor, Meta I, in the 14-fluoro (14-F) analogue pigment. The 14-F chromophore decreases the enthalpy change of the Meta I-to-Meta II transition and shifts the Meta I/Meta II equilibrium toward Meta II. Combining C14 fluorination with deletion of the retinal β-ionone ring to form a 14-F acyclic artificial pigment uncouples disruption of the Schiff base salt bridge from transition to Meta II and in particular from the cytoplasmic proton uptake reaction, as confirmed by combining the 14-F acyclic chromophore with the E134Q mutant. The 14-F acyclic analogue formed a stable Meta I state with a deprotonated Schiff base and an at least partially protonated protein counterion. The combination of retinal modification and site-directed mutagenesis reveals that disruption of the protonated Schiff base salt bridge is the most important step thermodynamically in the transition from Meta I to Meta II. This finding is particularly important since deprotonation of the retinal PSB is known to precede the transition to the active state in rhodopsin activation and is consistent with models of agonist-dependent activation of other GPCRs.

Rhodopsin is the G protein-coupled receptor (GPCR) responsible for dim light vision. It is a prototypical seven-transmembrane (7-TM) helical membrane protein that specifically binds an 11-cis retinal chromophore. Unlike other GPCRs activated by diffusible ligands, rhodopsin binds its chromophore via a protonated Schiff base (PSB) with a lysine on TM helix 7. In the dark, the 11-cis chromophore acts as an inverse agonist and locks the receptor in an inactive conformation. Photoisomerization to all-trans switches the ligand to a full agonist. This initial change within the retinal binding pocket is propagated into the protein to produce a series of intermediates defined by their spectral and conformational properties. Meta II, the active receptor species, forms in milliseconds and exists at room temperature in an equilibrium with its inactive precursor, Meta I.[1] The equilibrium between Meta I and Meta II is pH-dependent, with low pH favoring Meta II.[2] Proton uptake during the transition to Meta II appears to be mediated by a partially solvent-exposed interhelical network linking the cytoplasmic ends of TM helices 3 and 6,[3] which includes Glu 134 as part of the conserved E(D)RY motif[4] (Figure 1A).

In the dark state of rhodopsin, the PSB is stabilized by the negative charge of Glu 113 on TM3 (Figure 1B),[5,6] together with the hydrogen-bonded network extending along the extracellular loop 2 (EC2, Figure 1B) to Glu 181. After photoexcitation, the dominant contribution to the counterion appears to shift from Glu 113 to Glu 181 in Meta I.[7] However, as Glu 113 remains charged in Meta I, it is possibly still part of a counterion complex.[8] In the transition to the active Meta II state, Glu 113

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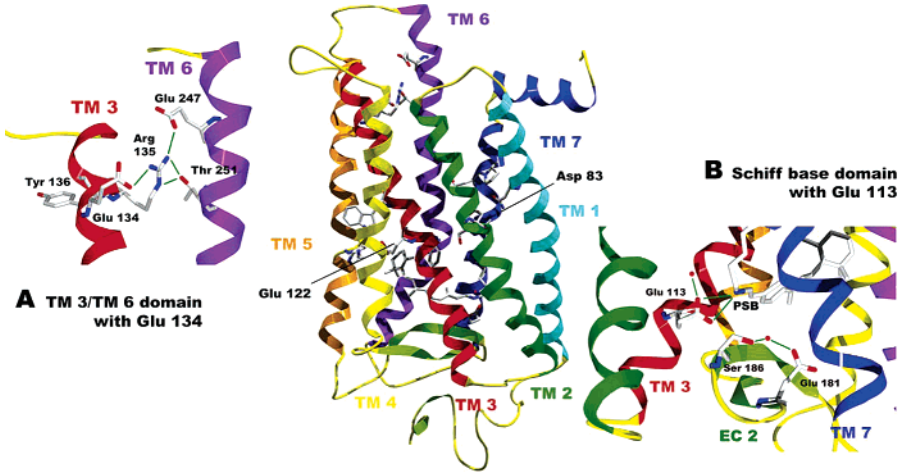

Figure 1. Molecular model of rhodopsin based on the structure of the dark state (PDB 1GZM[39]). The insets show close-ups of the cytoplasmic TM3/TM6 microdomain with the ERY motif including Glu 134 (A) and the domain around the protonated Schiff base (PSB) with the salt bridge to Glu 113 and the H-bonded network extending along extracellular loop 2 (B). The positions of the two carboxylic acids Glu 122 on TM3 and Asp 83 on TM2 are indicated.

finally becomes protonated,[9] while the retinal Schiff base deprotonates. The overall process results in a net transfer of the PSB proton to Glu 113, and the original salt bridge is abolished.

The activation of rhodopsin is known to involve a change of the helix bundle orientation due mainly to a relative repositioning of TM helices 3 and 6.[10,11] As the structure of the helix bundle does not change substantially up to Meta I,[12] this reorientation of helices takes place mostly during the transition from Meta I to active Meta II and depends on two proton-dependent steps: proton uptake by the cytoplasmic TM3/TM6 microdomain and protonation of Glu 113. Using rhodopsin artificial pigments derived from synthetic chromophores, we recently showed that the two protonation steps during receptor activation are allosterically coupled,[13] as had been suggested earlier.[14] Allosteric coupling between the cytoplasmic TM3/TM6 network around Glu 134 and the protonated Schiff base network with Glu 113 is presumably weak in the dark state and becomes considerably enhanced in the transition from Meta I to Meta II. The coupling depends in particular on the ring and 9-methyl group of the all-trans retinal, which play important roles in the concerted rearrangement of the helix bundle in the transition to Meta II.[13] Replacement of Glu 134 by Gln leads to formation of Meta II and protonation of Glu 113 independent of pH (manuscript in preparation). However, the E134Q mutant, reconstituted with acyclic or 9-demethyl retinal, displays essentially the same pH dependence of its Meta I/Meta II equilibrium as the wild type of these pigment analogues.[13,15]

Mutation of Glu 134 to glutamine mimics the proton uptake reaction without significantly perturbing the conformation of the dark state, at least in a native-like lipid environment (unpublished observation). Therefore, the E134Q mutant can be used to study both the coupling between the Glu 134 network and the Glu 113 network and the impact of the proton uptake by the TM3/TM6 network on protonation of Glu 113 and receptor activation. The influence of protonation of Glu 113 on the transition from Meta I to Meta II is much less easily mimicked by a mutation, as neutralization of Glu113 in the E113Q mutant abolishes the counterion function of Glu 113 and severely alters protein function. To circumvent this obstacle, we have used here a different approach. Instead of neutralizing the negative charge of residue 113, we lowered the intrinsic pK_a of the retinal PSB by fluorination of the polyene close to the Schiff base nitrogen.[16] The electron-withdrawing fluorine group destabilizes the positively charged PSB linkage and lowers its intrinsic pK_a. We therefore prepared 14-fluoro (14-F) isorhodopsin, 14-F acyclic isorhodopsin, and the 14-F acyclic mutant E134Q and studied the impact of a decreased intrinsic pK_a of the PSB on receptor activation, with specific attention to the properties of the Meta I/Meta II conformational equilibrium.

Materials and Methods

Preparation of Modified Retinals. 14-F 9-cis retinal was synthesized as previously described.[17] 14-F acyclic retinal (2-fluoro-3,7-dimethyl-10-ethyl-2,4,6,8-dodecatetraenal) was prepared by condensation of the 2-cis isomer of 6-ethyl-3-methyl-2,4-octadienal with acetone (in the presence of sodium hydroxide), followed by reaction with the sodium salt of diethyl phosphono-2-fluoroethylacetate and subsequent reduction with diisobutylaluminum hydride and oxidation with MnO_2.

Pigment Preparation. Rhodopsin artificial pigments were prepared in their native disk membrane environment as isorhodopsins by regeneration of opsin with the synthetic 9-cis ligands.[18] For control experiments, isorhodopsin was prepared similarly using unmodified 9-cis retinal. Mutant pigments were purified, reconstituted into phosphatidyl choline (PC) vesicles, and regenerated with synthetic chromophores as described previously.[15]

Fluorination of C14 of retinal (Chart 1) decreases the pK_a of the retinal Schiff base by 2.3 units compared to that of the unfluorinated

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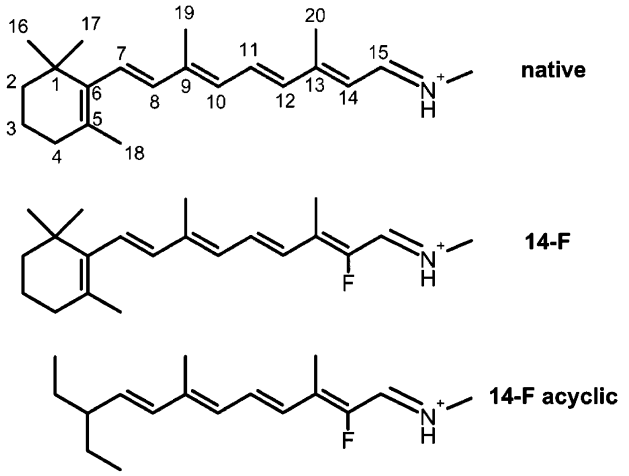
Chart 1. Modified Chromophores Used in This Study[a]



[a] The chromophores are shown as all-trans isomers of the protonated Schiff bases. Regeneration of the pigment was achieved with the respective 9-cis retinals.

Schiff base in methanol.[16] 9-Cis 14-F retinal absorbed at 377 nm in ethanol. It reacted readily with opsin, regenerating within 2 h at room temperature the 14-F isorhodopsin analogue with a visible absorption peak at 511 nm at full yield, in agreement with previous studies.[17]

FTIR Spectroscopy. Fourier transform infrared (FTIR) spectroscopy was performed with a Bruker IFS 28 spectrometer with a mercury−cadmium−telluride (MCT) detector. Spectra were recorded in blocks of 512 scans with an acquisition time of 1 min and a spectral resolution of 4 cm^{-1}. Experiments were performed with sandwich samples containing 0.5 nmol or less of pigment in native membranes to allow for control of water content, pH value, and salt concentration.[19] Meta I/Meta II titration curves obtained from these samples are identical to those measured with membrane suspensions.[20] Aliquots of 40 μL of citric acid, 2-*N*-morpholinoethanesulfonic acid (MES), or Bis-Tris-propane (BTP) were used at 200 mM to provide for precise pH adjustment, particularly at pH extremes.[20] For H/D exchange, sample films were equilibrated twice with D_2O and dried under nitrogen before addition of the respective buffer prepared in D_2O. Buffer pH was adjusted at 20 °C, and effective pH was measured again at the specific temperature of an experiment to account for the temperature dependence of buffer pK_a. Stated pH values are always effective pH values unless noted otherwise.

Samples were photolyzed for 20 s through fiber optics fitted to a 150 W tungsten lamp equipped with a long-pass filter. The cutoff wavelengths were 530 nm for 14-F and native isorhodopsin and 495 nm for 14-F acyclic isorhodopsin. pK_a values for Meta I/Meta II equilibria at different temperatures were determined as described previously,[15] employing the conformationally most sensitive spectral region between 1800 and 1600 cm^{-1}, which comprises mostly bands of structurally important protonated Glu and Asp residues in the transmembrane core of the receptor and the amide I bands of the protein backbone.

The Meta I/Meta II equilibrium was verified to be sensitive to the presence of a 20-fold excess of a peptide analogue to the C-terminus of the transducin α-subunit, peptide 23 (VLEDLKSCGLF),[21] which shifted the equilibrium in all cases toward Meta II.

UV−Visible Spectroscopy. For UV−visible spectroscopy, sandwich samples identical to the IR samples were used in a Perkin-Elmer Lambda 17 spectrophotometer equipped with a temperature-controlled sample holder. Illumination was the same as in the FTIR experiments. Low-temperature spectroscopy was performed using a custom-made liquid-nitrogen-cooled cryostat. Samples for UV−visible spectroscopy at −20 °C were supplemented with 50% glycerol (v/v) to reduce scattering.

Results

In the following, all photoreactions of the artificial pigments started from the 9-cis isorhodopsin dark state and are compared to those of isorhodopsin obtained by regeneration of opsin with unmodified 9-cis retinal.

We will distinguish between Meta I and Meta II on the basis of their functionality rather than merely on their visible absorption properties, as those may be misleading (see, e.g., refs 22 and 23 for active receptor states with aberrant absorption characteristics). We refer to Meta II as the photoproduct state with an active state conformation similar to that of native Meta II, being in conformational equilibrium, with characteristic anomalous pH dependence,[2] with a Meta I state that is shifted toward Meta II by binding of G protein or of G protein-derived functional peptides (as in this study). While the Meta II states of the artificial pigments studied here generally have conformations very similar to that of native Meta II, the conformations of the respective Meta I states are often distinctly different from that of native Meta I, reflecting the altered ligand−protein interactions. The anomalous pH dependence and the sensitivity to G protein-derived peptides of their associated conformational equilibria with their respective Meta II states, as well as a conformational distinction from their Lumi precursor states, are therefore used to identify these states as Meta I states.

14-F Isorhodopsin. 14-F isorhodopsin (λ_{max} = 511 nm) formed, at acidic pH, a Meta II photoproduct absorbing at 392 nm. The FTIR difference spectrum of the transition from the isorhodopsin dark state to Meta II (Figure 2A) shows all Meta II marker bands. In particular, in the range above 1700 cm^{-1}, the absorption changes of the C=O stretch vibrations of protonated carboxylic acids are practically identical to those in *Meta II minus dark state* spectra of unmodified isorhodopsin, while other bands have small alterations of their intensities (e.g., the Meta II bands at 1644 and 1532 cm^{-1} and the dark-state bands at 1665 and 1557 cm^{-1}). The dark-state fingerprint vibrations of the 14-F chromophore, the coupled C−C stretch and the CH in-plane bending modes in the range between 1100 and 1400 cm^{-1}, are considerably different from those of unmodified isorhodopsin, reflecting the influence of the 14-F group on position and coupling of the chromophore vibrational frequencies. Also, the position of the C11=C12 hydrogen out-of-plane (HOOP) mode of the chromophore is shifted from 958 in unmodified isorhodopsin to 952 cm^{-1} in 14-F isorhodopsin.

Fluorination of C14 shifts the Meta I/Meta II equilibrium toward Meta II (Figure 2C). This is particularly pronounced at 0 °C, where the apparent pK_a of Meta I/Meta II equilibrium is increased by about 1 unit. At 20 °C, an increase of the pK_a from 7.7 in native pigment to approximately 8.2 in the 14-F pigment is still noticeable. The pK_a value for 14-F pigment at 20 °C is only an approximate value, as it was not possible to extend the titration curve sufficiently into the alkaline range to obtain a reasonably pure Meta I state. To obtain a pure Meta I

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reference spectrum, we had to resort to lower temperatures (−10 °C at a pH of approximately 9.3).

As reported previously,[13] the apparent pK_a values of Meta I/Meta II titration curves (pK_1 and pK_2) measured at two different temperatures (T_1 and T_2) can be used to calculate both the enthalpy change (ΔH) and the entropy change (ΔS) of the transition from Meta I to Meta II using the expressions

$$\Delta H = R(2.303)(\mathrm{p}K_1 - \mathrm{p}K_2)\frac{T_1T_2}{T_1 - T_2}$$

and

$$\Delta S = R(2.303)\left(\frac{\mathrm{p}K_1T_1 - \mathrm{p}K_2T_2}{T_1 - T_2}\right)$$

where R is the gas constant. The calculated thermodynamic parameters are listed in Table 1. A striking difference between isorhodopsin and 14-F isorhodopsin was the considerably decreased temperature sensitivity of the Meta I/Meta II photoproduct equilibrium in the 14-F pigment, implying a reduced enthalpy increase accompanying the transition (46 kJ/mol in 14-F Meta I/Meta II, compared with 84 kJ/mol observed in native Meta I/Meta II). The decrease of ΔH of 14-F Meta I/Meta II is, to a considerable extent, offset by a concomitant decrease of ΔS of the transition.

The *Meta I minus dark state* difference spectrum of 14-F isorhodopsin at −10 °C and pH ∼9.3 reveals several pronounced changes compared with that of isorhodopsin (Figures 2B and 3A). We observe a strong alteration of the difference band of the ethylenic C═C stretching mode of the chromophore (at 1553(−)/1537(+) cm^{-1} in *Meta I minus dark state* spectra of unmodified isorhodopsin), which is correlated with an altered visible absorption in Meta I, as will be shown below. Further, there are considerable changes above 1700 cm^{-1} involving Asp 83 on TM2, which takes part in a hydrogen-bonded network between TM2, TM6, and TM7, and Glu 122 on TM3, which is part of a network between TM3 and TM5 (Figure 1). With isorhodopsin, we observe a dark-state band of Asp 83 at 1771 cm^{-1} and a difference band of Glu 122 with Meta I absorption around 1704 cm^{-1} in the high-frequency side of the band pattern, peaking at 1701 cm^{-1} and with split bands at 1735 and 1728 cm^{-1} in the dark. With 14-F isorhodopsin, the dark absorption band of Asp 83 is slightly downshifted to 1768 cm^{-1}, while Glu 122 shows a peculiar absorption pattern in 14-F pigment: the dark absorption at 1728 cm^{-1} corresponds to the low-frequency part of the split absorption band in the dark state of the native pigment. In the Meta I state of 14-F, there is more heterogeneous hydrogen bonding of Glu 122 than in native Meta I, leading to two positive absorption bands at 1745 and 1710 cm^{-1}. This heterogeneous absorption indicates at least two distinct populations of Glu 122 rotamers in 14-F Meta I. The identification of these Meta I bands with the C═O stretch of Glu 122 is based on their H_2O/D_2O sensitivity (Figure 3B), allowing the assignment to a carboxylic acid, and the disappearance of the band pattern in the *Meta I minus dark state* difference spectrum of the E122Q mutant of 14-F isorhodopsin (Figure 3C). Further, the Meta I state of 14-F pigment is distinctly different from its Lumi precursor, in particular in regard to the conformationally sensitive C═O stretching vibration of Glu 122 close to the ring of retinal, which shows only very small absorption changes in the 14-F Lumi, similar to the native Lumi (not shown).

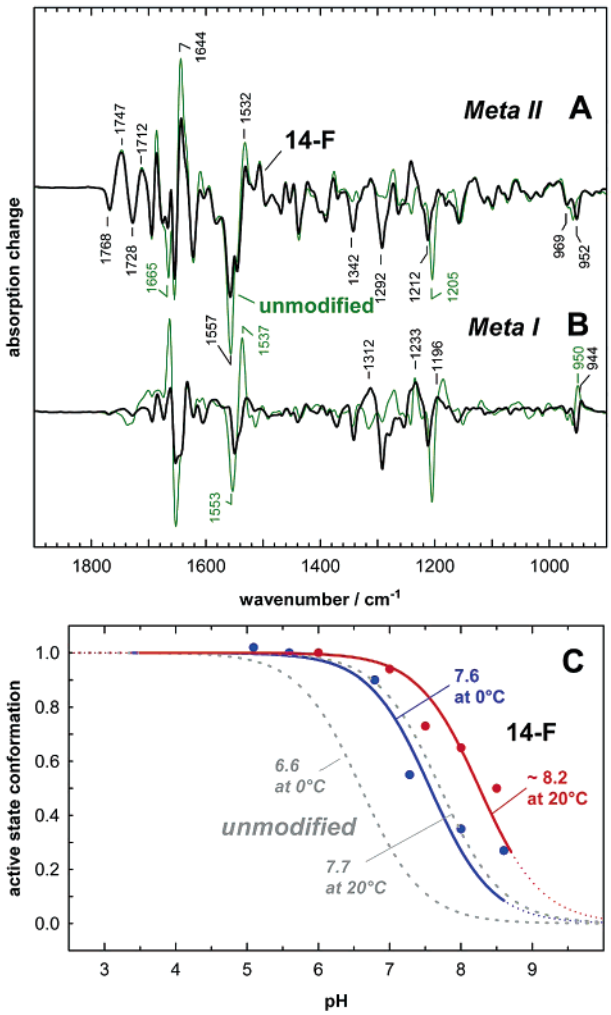

Figure 2. Meta II and Meta I of 14-F isorhodopsin. FTIR difference spectra *photoproduct minus dark state* of 14-F isorhodopsin (black) were obtained under conditions favoring either Meta II (at 0 °C, pH 5.1, A) or Meta I (at −10 °C, pH ∼9.3, B). These difference spectra are compared with corresponding spectra of the transition from unmodified isorhodopsin to the native Meta I/Meta II photoproduct states (green) obtained at 10 °C, pH 5.0 for Meta II and at −10 °C at pH ∼9.3 for Meta I. In the *photoproduct minus dark state* representation, photoproduct bands are positive, while contributions of the dark state are negative. (C) The apparent pK_a of the Meta I/Meta II equilibrium of 14-F isorhodopsin (colored curves) is approximately 0.5 unit higher at 20 °C (red) and 1.0 unit higher at 0 °C (blue) than that of native Meta I/Meta II (gray dotted curves). The all-trans 14-F ligand is therefore a more potent agonist than the native all-trans ligand. In contrast to those for Meta I/Meta II of unmodified isorhodopsin, the pK_a values of 14-F Meta I/Meta II are less dependent on temperature in the range between 0 and 20 °C, indicating a reduced enthalpy change in the transition.

The UV−visible spectra of the 14-F photoproducts reveal a deprotonated Schiff base for Meta II (Figure 4) with an absorption peak at 392 nm in the difference spectrum. At pH 9.5, we observe a Meta I photoproduct with a protonated Schiff base and a red-shifted absorption peak around 516 nm (compared with its dark-state absorption at 511 nm) that appears to have a reduced absorption coefficient. The small light-dependent absorption increase at 390 nm might reflect residual Meta II formed at this pH, while the light-independent absorption in the spectra around 365 nm is due to retinal-oxime and unreacted retinal present in the pigment preparation. Formation of a Meta

Table 1. Enthalpy and Entropy Changes in the Meta I/Meta II Equilibria of Selected Pigments[a]

	pK_a					
	at 20 °C	at 0 °C	ΔpK_a	ΔH (kJ/mol)	ΔS (J/(mol K))	$T\Delta S$ at 20 °C (kJ/mol)
native[b]	7.7	6.6	1.1	84	434	127
14-F	8.2	7.6	0.6	46 (−38)	314	92 (−35)
14-F acyclic	5.0	5.3	−0.3	−25 (−107)	17	5 (−122)
acyclic[c]	5.0	4.1	0.9	69 (−15)	331	97 (−30)
opsin[b,d]	3.8			48 (−36)	236	69 (−58)

[a] ΔpK_a is the difference between the pK_a values measured at 0 and 20 °C. The estimated error margins for ΔH and ΔS are 15 kJ/mol and 54 J/(mol K), respectively. In the case of the 14-F pigment, these error margins are larger due to the less precisely determined pK_a value at 20 °C. The values in parentheses for ΔH and $T\Delta S$ indicate the difference from the respective value of the native pigment. [b] From ref 15. [c] Diethyl acyclic-2 from ref 15. [d] Opsin conformational equilibrium;[19] see text for details.

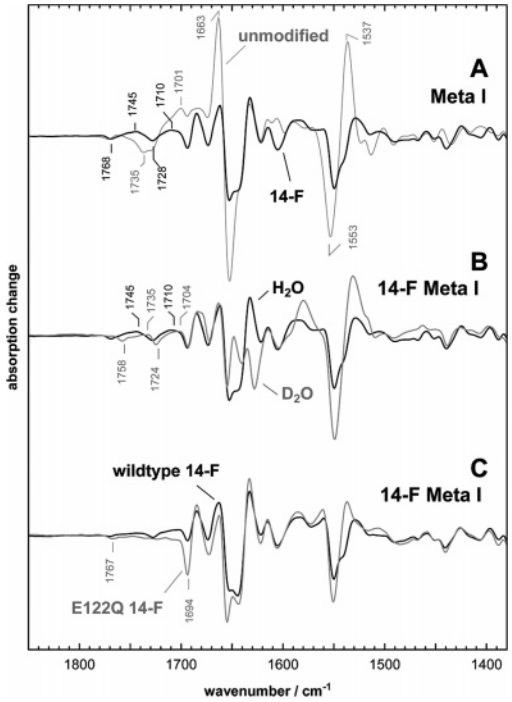

Figure 3. Meta I of 14-F isorhodopsin. (A) Close-ups of the *Meta I minus dark state* difference spectra of 14-F isorhodopsin (black) and unmodified isorhodopsin (gray) of Figure 2B in the spectral range of amide I and carboxylic acid vibrations. (B) *Meta I minus dark state* difference spectra of 14-F isorhodopsin obtained in H_2O (black, as in A) and D_2O (gray) at −10 °C at pH ∼9.3, allowing the identification of the C=O stretches of carboxylic acids in 14-F Meta I at 1745 and 1710 cm^{-1} by their deuteration-induced downshifts to 1735 and 1704 cm^{-1}, respectively. (C) *Meta I minus dark state* difference spectra of wild-type 14-F isorhodopsin (black) and the E122Q mutant of 14-F isorhodopsin (gray), obtained at −20 °C and pH 7, revealing Meta I bands at 1744 and 1709 cm^{-1} in the wild type that disappear in the E122Q mutant.

I state with a completely protonated Schiff base is also observed at −20 °C, where Meta I is stable over the entire accessible pH range.

Fluorination of C14 withdraws electrons from the protonated Schiff base, reducing its intrinsic pK_a and consequently increasing the pK_a of its protein counterion. This leads to a weakening of the protonated Schiff base salt bridge in a thermodynamic sense, such that neutralization of the salt bridge becomes enthalpically more favorable. The fluorination-induced change of the protonated Schiff base does, however, also lead to small

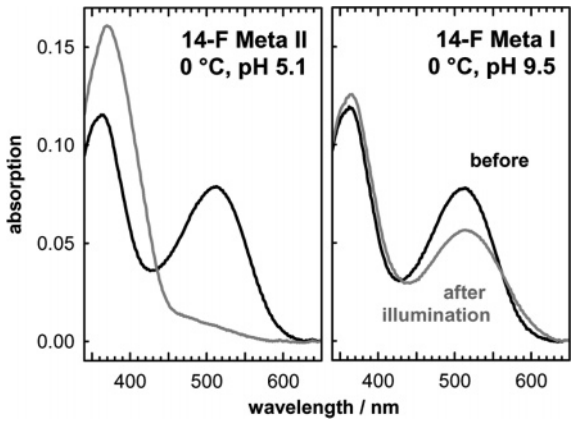

Figure 4. UV−visible spectra of 14-F Meta II and Meta I. Dark-state isorhodopsin (black) and photoproduct spectra (gray) were obtained at 0 °C. The Meta II photoproduct formed at pH 5.1 has a completely deprotonated Schiff base with a photoproduct peak at 392 nm. At pH 9.5, the photoproduct is largely Meta I with a protonated Schiff base and an absorption peak that is red-shifted compared with that of the isorhodopsin dark state.

structural alterations of the hydrogen-bonded network involving the protonated Schiff base and its complex counterion formed by Glu 181 and Glu 113 (and presumably other additional residues and bound water molecules) in Meta I. Due to allosteric coupling of the Schiff base region to the cytoplasmic TM3/TM6 proton uptake region,[13] disruption of the Schiff base salt bridge in Meta I is strictly coupled to the conformational transition to Meta II, such that a shift of the equilibrium toward Meta II is observed rather than a deprotonation of the Schiff base in Meta I. We therefore prepared another fluorinated analogue, 14-F acyclic, in which we additionally deleted the retinal β-ionone ring (Chart 1). As we had shown previously, deletion of the ring portion largely abolishes this coupling between the Schiff base region and the cytoplasmic TM3/TM6 region around Glu 134.[15] It appeared therefore plausible that, by combining the 14-F and the acyclic modification, a disruption of the Schiff base salt bridge in Meta I could be uncoupled from the conformational transition to Meta II.

14-F Acyclic. The 9-cis 14-F acyclic retinal absorbed at 362 nm in ethanol and reacted with opsin at a much slower rate than the 14-F or previously studied acyclic analogues.[15] The complete regeneration of 14-F acyclic isorhodopsin (visible absorption peak at 481 nm) required incubation for at least 6 h at room temperature. Removal of the β-ionone ring in acyclic pigments had been shown recently to shift considerably the Meta I/Meta II equilibrium toward inactive Meta I (Table 1).[15,24] Likewise, in the 14-F acyclic pigment, the apparent pK_a of the equilibrium is shifted by roughly 3 units compared with that of the 14-F pigment (Figures 2C and 5C). The temperature dependence of the pK_a of 14-F acyclic is very unusual, as the pK_a decreases slightly as the temperature is increased, implying that the transition from Meta I to Meta II is enthalpically downhill in the analogous pigment, unlike in native Meta I/Meta II (Table 1). The associated entropy change, on the other hand, which is the driving force of the transition in the native pigment, is practically zero in the 14-F acyclic analogue.

The conformation of the Meta II state of 14-F acyclic, which is formed only at very acidic pH, is very similar to that of native

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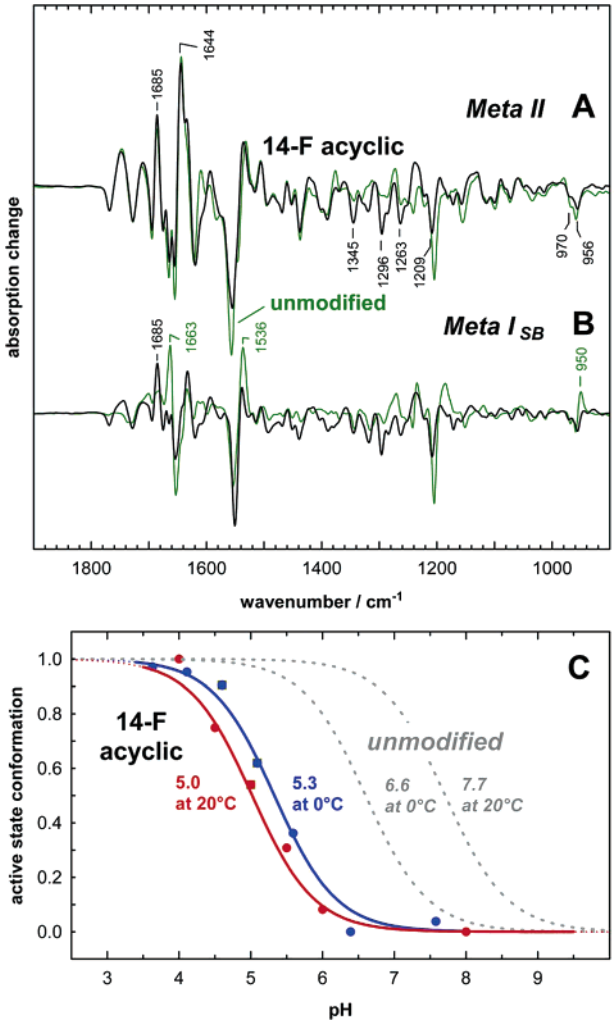

Figure 5. Meta II and Meta I of 14-F acyclic isorhodopsin. FTIR difference spectra *photoproduct minus dark state* of 14-F acyclic isorhodopsin (black) were obtained under conditions favoring either Meta II (at 0 °C, pH 4.6, A) or Meta I_{SB} (at 0 °C, pH 7.6, B) and are compared with corresponding spectra of the native states (gray) obtained from unmodified isorhodopsin (green) at 0 °C at pH 5.1 and pH 9.5, respectively. C. The apparent pK_a values of the Meta I/Meta II equilibrium of 14-F acyclic isorhodopsin (colored curves) are, both at 0 °C (blue) and at 20 °C (red), considerably lower than those of unmodified isorhodopsin (gray dotted curves). The all-trans 14-F acyclic ligand shows, therefore, a partial agonist behavior. Compared with the Meta I/Meta II photoproduct equilibrium of unmodified isorhodopsin, the pK_a of 14-F acyclic Meta I/Meta II has a weak, inverse dependence of temperature in the range between 0 and 20 °C, indicative of an enthalpy decrease during the transition from Meta I to Meta II.

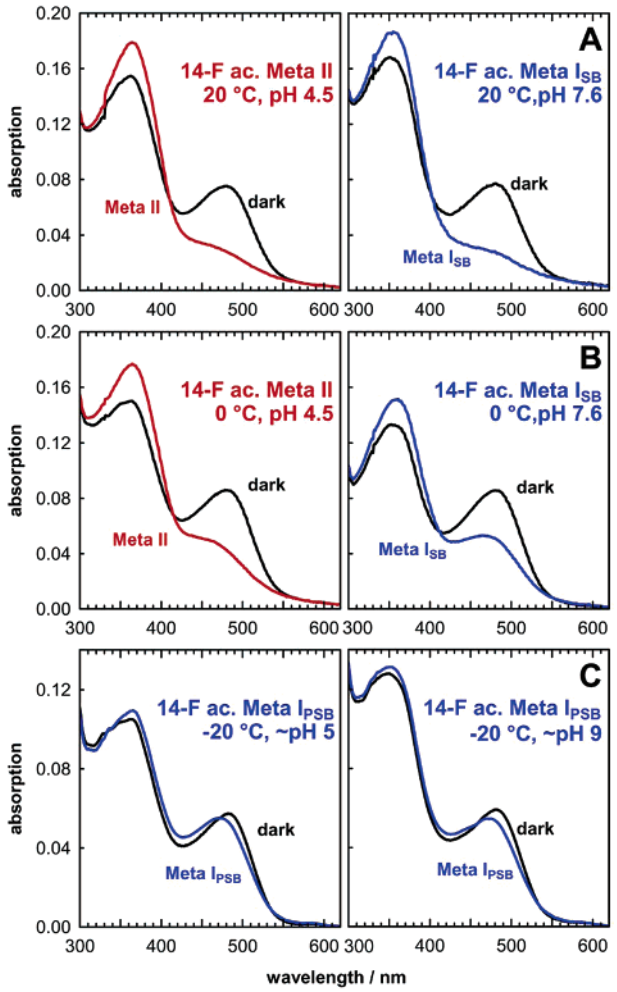

Figure 6. 14-F acyclic forms an inactive Meta I photoproduct with a deprotonated Schiff base. UV−visible spectra of the 14-F acyclic isorhodopsin dark state (black spectra) and its photoproduct states (red for Meta II and blue for Meta I) were obtained at 20 °C (A) and 0 °C (B) under conditions that favor either Meta II (at pH 4.5) or Meta I (at pH 7.6). The spectra show a Meta I photoproduct with a retinal Schiff base that is completely deprotonated at 20 °C and still largely deprotonated at 0 °C (Meta I_{SB}). At −20 °C (C), a low-temperature Meta I photoproduct state with largely protonated Schiff base (Meta I_{PSB}) is formed at both acidic and alkaline pH.

Meta II (Figure 5A). Differences between the Meta II FTIR difference spectra of isorhodopsin and 14-F acyclic isorhodopsin are restricted mainly to the fingerprint modes of the chromophore.

Meta I of the artificial pigment, on the other hand, shows a very surprising behavior. At −20 °C, where the transition to Meta II is completely blocked, a low-temperature Meta I state with a PSB (Meta I_{PSB}) absorbing at 477 nm is observed (Figure 6C). In the temperature range between −10 and 20 °C, however, where Meta I equilibrates with Meta II in the pH-dependent conformational equilibrium (Figure 5C), a Meta I state with a largely deprotonated Schiff base (Meta I_{SB}) is formed, absorbing at 370 nm, similar to the absorption of the artificial pigment Meta II (375 nm, Figure 6A,B). The thermodynamic parameters derived above apply, therefore, to the transition from Meta I_{SB} to Meta II. Despite the similarity of their UV−visible spectra, the *photoproduct minus dark state* difference spectra of the Meta I_{SB} and Meta II photoproducts are distinctly different. Most pronounced are the differences in the absorption range of protonated carboxylic acids above 1700 cm^{-1}, where the difference bands in the Meta I_{SB} spectrum generally attain only about half of their intensity in the Meta II spectrum, which is further examined below. Further, the amide I marker band of Meta II at 1644 cm^{-1} is entirely lacking in Meta I_{SB}. For a functional characterization, we tested the sensitivity of the conformational equilibrium between Meta I_{SB} and Meta II to the presence of a G protein-derived peptide analogue (see Materials and Methods). The presence of a high-affinity peptide analogue to the C-terminus of the transducin α-subunit shifted the equilibrium toward Meta II at the expense of Meta I_{SB}. This indicates that the peptide favors the Meta II state of the 14-F acyclic analogue over its Meta I_{SB} state for binding and stabilization, suggesting that the receptor conformation of Meta I_{SB} is not an active conformation.

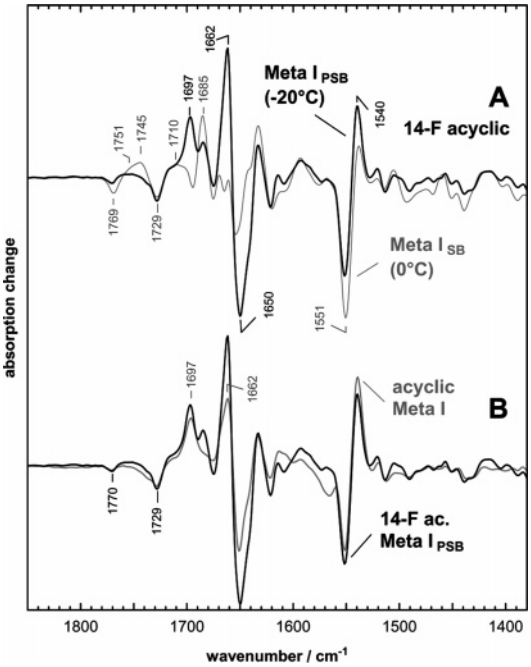

Figure 7. Meta I of 14-F acyclic isorhodopsin. (A) Comparison between *photoproduct minus dark state* difference spectra of the Meta I_{SB} photoproduct (gray) formed at 0 °C, pH 7.6, and of the low-temperature Meta I_{PSB} photoproduct (black) formed at −20 °C, pH 9, of 14-F acyclic isorhodopsin. (B) The *photoproduct minus dark state* difference spectra of the low-temperature Meta I_{PSB} photoproduct of 14-F acyclic isorhodopsin (black, as in A) bears similarity to that of the Meta I photoproduct of non-fluorinated acyclic isorhodopsin (gray, at 0 °C, pH 7.0).

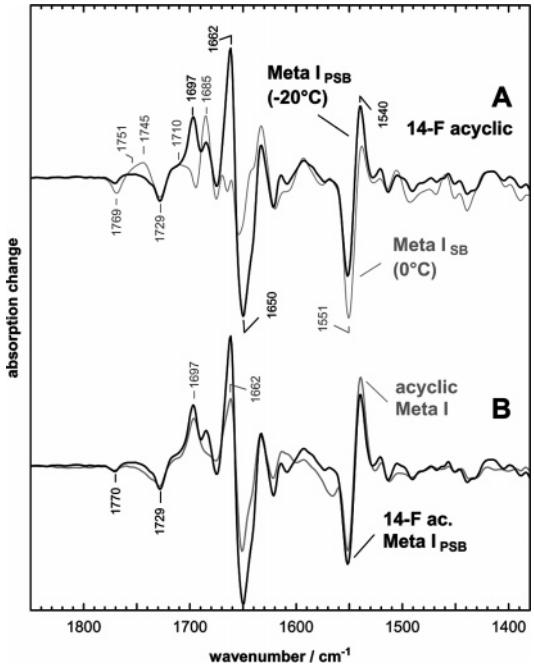

Figure 8. Meta I_{SB} of 14-F acyclic isorhodopsin. (A) A close-up of the *photoproduct minus dark state* difference spectra of the Meta I photoproduct of unmodified isorhodopsin (gray) and of the Meta I_{SB} photoproduct of 14-F acyclic isorhodopsin (black) in the range of amide I and carboxylic acid bands is reproduced from Figure 5B. (B) A comparison between *Meta I_{SB} minus dark state* FTIR difference spectra of wild-type 14-F acyclic isorhodopsin (black) and of the E122Q mutant of 14-F acyclic isorhodopsin (gray) at 0 °C and pH 7.6 allows the assignment of the 1745(+)/1729(−) cm^{-1} difference band to the C═O stretch of Glu 122. (C) *Meta I_{SB} minus dark state* difference spectra of 14-F acyclic isorhodopsin obtained in either H_2O (black) or D_2O (gray) at 0 °C, pH 7.6 reveal the H/D sensitivity not only of the C═O stretch vibrations of Asp 83 and Glu 122, but also of an additional photoproduct band shifting from 1710 cm^{-1} in H_2O to 1704 cm^{-1} in D_2O, which is possibly due to protonation of Glu 113 in Meta I_{SB} of 14-F acyclic.

Meta I_{SB} and the low-temperature Meta I_{PSB} product display considerably different receptor conformations as well. The FTIR *photoproduct minus dark state* difference spectrum of the low-temperature Meta I_{PSB} photoproduct is very similar to that of Meta I of the acyclic artificial pigment described earlier[15] (Figure 7B). In particular, a photoproduct band at 1697 cm^{-1} is reproduced, which is a unique feature in the Meta I spectra of non-fluorinated acyclic pigment. Using site-directed mutagenesis, this band could be assigned in the acyclic pigments to the C═O stretch of Glu 122, which experiences unusually strong hydrogen-bonding in Meta I. Further, the C11═C12 HOOP mode of Meta I at 950 cm^{-1} is lacking in Meta I_{SB} of the analogue (not shown), implying that removal of the ring renders the chromophore geometry planar in the C11═C12 region of the polyene, again similar to Meta I of non-fluorinated acyclic pigments,[15] but unlike native Meta I. It should be noted that this low-temperature Meta I_{PSB} photoproduct of the 14-F acyclic analogue does not represent an actual Lumi state, as Lumi of this analogue differs considerably in several respects and, in particular, does not show the strong hydrogen-bonding of Glu 122 (data not shown).

At 20 °C, the photoproduct obtained at pH 7.6 consists entirely of Meta I_{SB}. At 0 °C, the Schiff base in Meta I is still largely deprotonated, and only a small fraction has a PSB (Figure 6B). The contribution of this small fraction of Meta I with PSB remained roughly constant over the pH range from 6.5 to 9, in which the Meta I conformation is stable. This behavior implies that the Schiff base moiety in 14-F acyclic Meta I is not accessible to titration from the solvent. Deprotonation of the retinal Schiff base in Meta I_{SB} leads to considerably altered amide I and protonated carboxylic acid vibrations in the FTIR difference spectra, indicating a protein conformation of Meta I_{SB} that is distinctly different from both that of Meta I_{PSB} of the analogue (Figure 7A) and that of native Meta I (Figure 8A). The amide I marker band of native Meta I at 1663 cm^{-1} is largely absent in Meta I_{SB} of 14-F acyclic, and the band pattern above 1680 cm^{-1} bears strong similarity to the band pattern of Meta II without, however, attaining its full intensity. In the photoproducts of unmodified isorhodopsin, a photoproduct band at position and intensity similar to those of the positive band at 1685 cm^{-1} of 14-F acyclic Meta I_{SB} is observed in Meta II only (Figure 5A).

Using the E122Q mutant in combination with the 14-F acyclic chromophore, the *Meta I_{SB} minus dark state* difference band of Glu 122 is identified at 1728(−)/1743(+) cm^{-1} (Figure 7B), reflecting an upshift of this band in Meta I_{SB}, similar to that in Meta II. The difference band of Asp 83 at 1769(−)/1751(+) cm^{-1} is much more intense in the *Meta I_{SB} minus dark state* spectrum of 14-F acyclic than in the control pigment (Figure 8A) or the Meta I_{PSB} spectrum (Figure 7A). The positive photoproduct band at 1710 cm^{-1} appears to be sensitive to H_2O/D_2O exchange (Figure 8C), suggesting that this band is due, at least in part, to another protonated carboxylic acid in Meta I. On the basis of FTIR data on site-directed counterion mutants,[9] a band at the same position in Meta II spectra is usually assigned to Glu 113, which becomes protonated in the transition to Meta II. The intensity of that Meta II band appears, however, to be

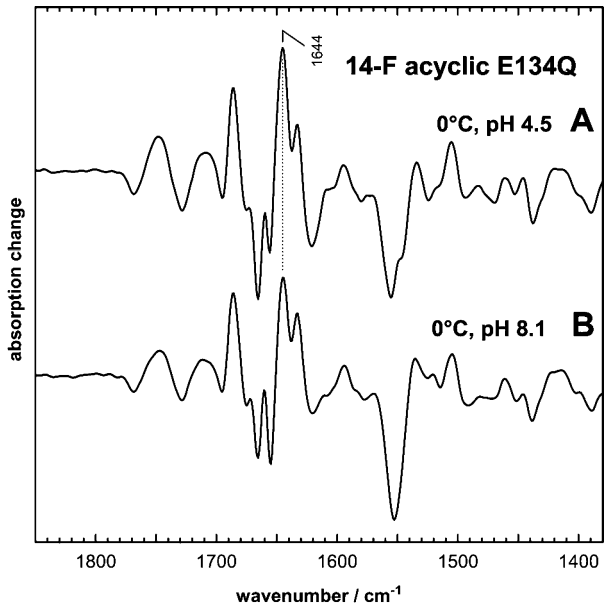

Figure 9. The E134Q mutant of 14-F acyclic isorhodopsin. *Photoproduct minus dark state* difference spectra of the E134Q mutant of 14-F acyclic reveal a Meta II-like photoproduct at both pH 4.5 (A) and pH 8.1 (B) at 0 °C, as indicated by the prominent Meta II marker band at 1644 cm^{-1}. The E134Q mutation therefore strongly favors the Meta II state of 14-F acyclic pigment also at alkaline pH.

higher than the intensity of this Meta I band in 14-F acyclic. This band could therefore reflect (partial) protonation of Glu 113 or possibly also Glu 181 already in Meta I_{SB} of the 14-F acyclic analogue. This (partial) neutralization of the counterion would complement the deprotonation of the Schiff base. A rigorous assignment and quantitative evaluation of the extent of such counterion protonation reaction is, however, not possible on the basis of these data.

Finally, using the E134Q mutant of the 14-F acyclic analogue, we have evaluated the effect of neutralization of Glu 134, located in the cytoplasmic TM3/TM6 microdomain, on the position of the Meta I/Meta II equilibrium of this pigment. Presumably due to the very slow reactivity of the retinal analogue, only small amounts of the mutant pigment analogue could be obtained. FTIR difference spectra revealed a Meta II-like photoproduct at both pH 4.5 and pH 8.1 in the mutant analogue, with formation of the characteristic amide I marker band of Meta II at 1644 cm^{-1} (Figure 9), in contrast to the wild-type analogue, which formed Meta II only at low pH. This indicates that the E134Q mutation strongly favors the formation of the Meta II state of the 14-F acyclic pigment, even at alkaline pH.

Discussion

The transition from the still inactive Meta I photoproduct state to the active Meta II state of rhodopsin appears to depend on two distinct protonation steps. One involves proton uptake by a cytoplasmic network between TM3 and TM6 around the conserved E(D)RY motif including Glu 134, and the other corresponds to a net proton transfer from the protonated Schiff base to Glu 113, which accounts for the major part of its dark-state counterion function. In the transition from the early photointermediates to Meta I, the contribution of Glu 181 to the counterion is increased significantly,[7] such that both Glu 181 and Glu 113 contribute to the complex counterion in Meta I.[8] In this study, we tried to reduce the stability of the salt bridge between the protonated Schiff base and its protein counterion in the Meta I state by substituting the polyene C14 with a fluorine group, which decreases the intrinsic pK_a of the retinal Schiff base. It is plausible that, thereby, the hydrogen-bonded network associated with the PSB and the carboxylate will be affected such that the apparent pK_a of the counterion will be increased.[25,26] In the dark state, the apparent pK_a of the Schiff base has been found to be above 16.[16] A lowering of the intrinsic pK_a by 2 or 3 units will therefore not be sufficient to neutralize the salt bridge with Glu 113 in the dark state. In Meta I, on the other side, the pK_a of the Schiff base is substantially lower and drops even below 2.5 in Meta II,[23] such that fluorination of the Schiff base should strongly affect the transition between these late intermediates.

As we have previously shown, there seems to exist no strict steric constraint at the C14 position affecting the Meta I/Meta II equilibrium, as addition of a considerably larger methyl group at C14 led to only moderate changes in the properties of the equilibrium.[13] Furthermore, the van der Waals radius of a fluorine group (1.35 Å) is only slightly larger than that of a hydrogen (1.20 Å), while the radius of a methyl group is considerably larger (2.0 Å).[27] We therefore expect that the alterations introduced by the fluorine group are mostly electronic rather than steric in nature.

In the 14-F artificial pigment, the decrease of the intrinsic pK_a of the retinal Schiff base due to fluorination did not neutralize the Schiff base salt bridge in Meta I. Weakening of the Schiff base salt bridge led instead to a shift of the equilibrium between Meta I and Meta II toward the active Meta II conformation. This is not surprising, as the two proton-dependent steps of the transition to Meta II, proton uptake by the cytoplasmic TM3/TM6 domain around Glu 134 and the net proton transfer from the Schiff base to Glu 113, are strictly coupled during this transition in native rhodopsin.[13] By facilitating specifically one of these two steps, the other step is favored, leading to the full conformational transition to Meta II. According to recent 2D dipolar-assisted rotational resonance NMR experiments, the transition to Meta II involves a 5 Å movement of the chromophore along its polyene axis toward TM5,[28] leading to a perturbation of the hydrogen-bonding pattern of Glu 122 on TM3 to residues on TM5 by the β-ionone ring of retinal,[29] which was also suggested by previous FTIR studies.[30] This retinal movement is further coupled via Trp 265 to a rotation of TM6 to its active-state conformation.[31,32] The concerted rearrangement of chromophore, TM5, TM6, and presumably also TM7, to which the chromophore is covalently bound via Lys 296, appears to be the link between the disruption of the Schiff base salt bridge and the proton uptake by the cytoplasmic TM3/TM6 network. A lowering of the enthalpy required for disruption of the Schiff base salt bridge due to fluorination of the chromophore would therefore shift the Meta

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I/Meta II conformational equilibrium toward Meta II, which is equivalent to increasing the apparent pK_a of the pH-dependent transition.

To gain further insight into these protonation-dependent steps, we examined the 14-F acyclic analogue, a fluorinated artificial pigment in which the retinal β-ionone ring had been largely deleted. Deletion of the ring had been shown in previous experiments to completely abolish the allosteric coupling between the Schiff base region and the TM3/TM6 microdomain, presumably by changing the interaction between the retinal and the TM3/TM5 microdomain around Glu 122 (TM3) and His 211 (TM5).[15] We therefore expected that net proton transfer from the protonated Schiff base to Glu 113 and proton uptake by the TM3/TM6 motif would occur as two independent events in the 14-F acyclic artificial pigment. This was, indeed, the case. Net proton transfer from the PSB to Glu 113 was no longer coupled to cytoplasmic proton uptake and the full conformational transition to Meta II. Instead, disruption of the PSB salt bridge led to formation of a Meta I state with deprotonated Schiff base, Meta I_{SB}. Simultaneously, its complex counterion, consisting of Glu 113 and Glu 181, appeared to be partially neutralized in this Meta I_{SB} state. The transition to full Meta II was achieved in a separate pH-dependent step. This second step involved proton uptake by the TM3/TM6 motif, as shown using the E134Q mutant of the 14-F acyclic pigment, which extended formation of Meta II into the extreme alkaline range.

In addition to this Meta I_{SB} state, another Meta I state with PSB, Meta I_{PSB}, is formed at lower temperature (−20 °C), where the equilibrium with Meta II is thermally blocked. This Meta I_{PSB} state of 14-F acyclic was found to be very similar to the Meta I state of the non-fluorinated analogue. The existence of this low-temperature Meta I_{PSB} state implies that the pK_a difference between the PSB and the counterion, which is responsible for a stable salt bridge, is progressively decreased as the temperature is increased. This is similarly observed in native rhodopsin, where the pK_a difference is, however, higher such that a Meta I state with deprotonated Schiff base is observed only above 20 °C as a transient species on the pathway to Meta II.[33]

The disruption of the salt bridge between the PSB and its protein counterion in Meta I_{SB} of the 14-F acyclic pigment does, therefore, considerably alter the conformation of the protein as compared with those of native Meta I and Meta I_{PSB} of the analogue. This is indicated by structurally sensitive spectral features in the FTIR difference spectrum of Meta I_{SB} that appear Meta II-like, without, however, attaining the same intensity as in the spectrum of the corresponding Meta II state. Several of the microdomains of the receptor are therefore in conformations that are intermediate between those of native Meta I and Meta II. These microdomains include the TM3/TM5 network with Glu 122, the TM1/TM2/TM7 network with Asp 83, and also the chromophore with its more relaxed conformation. These conformational changes are not sufficient to render the Schiff base moiety titratable from the solvent in Meta I_{SB}, which is the case only in the fully activated Meta II state.[23] A most striking exception to this general behavior concerns the intense Meta II marker band at 1644 cm^{-1}, which is an amide I mode reflecting presumably localized changes of the protein backbone. This band is entirely lacking in the difference spectrum of Meta I_{SB} of the 14-F acyclic analogue. It appears, therefore, to reflect structural changes that are associated with the proton uptake reaction of the cytoplasmic TM3/TM6 domain, in agreement with previous studies.[8] In a tentative assignment, this pronounced Meta II band could reflect structural changes in a putative hinge region around conserved Pro 267 on TM6, which are associated with the cytoplasmic rigid body motion of helix 6 away from the receptor core during the transition to the active state.[10,11]

In contrast to the 14-F acyclic analogue studied here, the unfluorinated acyclic analogue had revealed only negligible effects of the E134Q mutation on the position of the Meta I/Meta II equilibrium in the membrane environment.[15] In that pigment, neutralization of Glu 134 was not sufficient to break the Schiff base salt bridge in Meta I and to induce thereby the conformational transition to Meta II. This behavior indicated the importance of the retinal β-ionone ring for the allosteric coupling between the Schiff base region and the cytoplasmic TM3/TM6 network during receptor activation. In Meta I_{SB} of the 14-F acyclic pigment, on the other hand, the Schiff base salt bridge is already broken, such that neutralization of residue 134 in the E134Q mutant is sufficient to shift the Meta I/Meta II equilibrium completely toward Meta II, even in the absence of this allosteric coupling between the cytoplasmic and the Schiff base microdomains.

It is instructive to inspect the impact of fluorination on the thermodynamic parameters of the Meta I/Meta II equilibria of the14-F and 14-F acyclic analogues. In the 14-F analogue, the enthalpy increase in the transition from Meta I to Meta II was reduced to about 55% of that of native rhodopsin. A large part of the positive reaction enthalpy of the transition from Meta I to Meta II appears, therefore, to be required for breaking the salt bridge between the PSB and its protein complex counterion, Glu 181 and Glu 113. This becomes even more obvious with the 14-F acyclic analogue, where the salt bridge between the Schiff base and the protein counterion is neutralized already in Meta I_{SB}. In that case, the enthalpy change of the transition from Meta I_{SB} to Meta II becomes negative.

We can further try to dissect the transition from Meta I to Meta II of native rhodopsin into two partial reactions: the neutralization of the Schiff base salt bridge (and the thereby induced rearrangement of the receptor in a hypothetical Meta I_{SB} state) and the cytoplasmic proton uptake reaction, leading to completion of the conformational transition to Meta II in a pH-dependent manner. Using the thermodynamic data of the Meta I/Meta II transitions of 14-F acyclic with those of non-fluorinated acyclic pigment (Table 1), we can derive rough estimates for the enthalpy and entropy changes involved in each of the partial reactions in the acyclic pigment. By subtracting the values obtained for the transition from Meta I_{SB} to Meta II of the 14-F acyclic analogue from those of Meta I/Meta II of the non-fluorinated acyclic analogue, we calculate about 94 kJ/mol being required for the first partial reaction, disruption of the Schiff base salt bridge and the thereby induced rearrangement of the receptor to Meta I_{SB}. This partial reaction is accompanied by a similarly large positive entropic contribution, $T\Delta S$, of about 92 kJ/mol (at 20 °C). The second partial reaction, cytoplasmic proton uptake and completion of the transition to Meta II, corresponds to the transition from Meta I_{SB} to Meta II

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of 14-F acyclic, with an experimentally determined enthalpy release of about 25 kJ/mol and a negligible entropy change. These energetic values for the partial reactions apply actually only for the acyclic analogue. However, the thermodynamic parameters of the non-fluorinated acyclic analogue deviate from those of native rhodopsin only by roughly 20−25%, which is little compared with the extremely strong alterations observed for the 14-F acyclic pigment. These data are therefore also suitable to give a qualitative view of the thermodynamics of the corresponding partial reactions for native rhodopsin. They underline that the energetically most relevant step in the transition from Meta I to Meta II consists of the neutralization of the Schiff base salt bridge.

In opsin, Lys 296 on TM7 replaces the PSB in the inactivating salt bridge to Glu 113 on TM3.[34] A putative interhelical salt bridge between homologous residues is suggested to be broken during activation of the α_{1b}-adrenergic receptor[35] and to free the glutamate on TM3 for productive interaction with the positively charged amino group of the catechol ligand.[36]

In summary, the 14-F and the 14-F acyclic artificial pigments show strikingly different behaviors. In the 14-F pigment, with its otherwise native retinal chromophore, destabilization of the Schiff base salt bridge in Meta I is coupled to a shift of the Meta I/Meta II conformational equilibrium toward the active state Meta II. Additional deletion of the ring disrupts this coupling, such that the Schiff base salt bridge may be broken without inducing the full conformational transition to Meta II, giving rise to the structurally altered Meta I_{SB} state with a deprotonated Schiff base. In native pigment, deprotonation of the retinal Schiff base is known to precede receptor activation, as shown by time-resolved UV−visible spectroscopy at physiological temperature, leading to the transient formation of a species termed Meta I_{380}.[37] This species cannot be trapped thermally, which hampers investigation of its structural properties. In detergent solution, a possibly related Meta II precursor, Meta II_a, has been described, in which the Schiff base salt bridge is broken prior to the proton uptake reaction, leading to the active receptor state.[38] By combination of fluorination with ring deletion, a Meta II precursor state with deprotonated Schiff base can be stabilized, such that structural information can be obtained using steady-state FTIR spectroscopy. Our studies show that deprotonation of the retinal Schiff base in Meta I leads to conformational changes in certain receptor microdomains, including the networks between TM1, TM2, and TM7 and between TM3 and TM5, which are approximately halfway in the transition to Meta II. Another conformational change, on the other hand, which comprises a strong distortion of the protein backbone, is shown to be linked to the proton uptake reaction by the cytoplasmic network between TM3 and TM6 around Glu 134, and is observed only in the full transition to Meta II. This study provides, therefore, useful information for unraveling the sequence of the structural changes taking place in the activation of rhodopsin and other GPCRs with conserved cytoplasmic hydrogen-bonded networks.

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