Agonists and Partial Agonists of Rhodopsin: Retinal Polyene Methylation Affects Receptor Activation†

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Received October 27, 2005; Revised Manuscript Received December 12, 2005

ABSTRACT: Using Fourier transform infrared (FTIR) difference spectroscopy, we have studied the impact of sites and extent of methylation of the retinal polyene with respect to position and thermodynamic parameters of the conformational equilibrium between the Meta I and Meta II photoproducts of rhodopsin. Deletion of methyl groups to form 9-demethyl and 13-demethyl analogues, as well as addition of a methyl group at C10 or C12, shifted the Meta I/Meta II equilibrium toward Meta I, such that the retinal analogues behaved like partial agonists. This equilibrium shift resulted from an apparent reduction of the entropy gain of the transition of up to 65%, which was only partially offset by a concomitant reduction of the enthalpy increase. The analogues produced Meta II photoproducts with relatively small alterations, while their Meta I states were significantly altered, which accounted for the aberrant transitions to Meta II. Addition of a methyl group at C14 influenced the thermodynamic parameters but had little impact on the position of the Meta I/Meta II equilibrium. Neutralization of the residue 134 in the E134Q opsin mutant increased the Meta II content of the 13-demethyl analogue, but not of the 9-demethyl analogue, indicating a severe impairment of the allosteric coupling between the conserved cytoplasmic ERY motif involved in proton uptake and the Schiff base/Glu 113 microdomain in the 9-demethyl analogue. The 9-methyl group appears therefore essential for the correct positioning of retinal to link protonation of the cytoplasmic motif with protonation of Glu 113 during receptor activation.

The visual pigment rhodopsin is the most extensively studied G protein-coupled receptor (GPCR). Unlike GPCRs with diffusible ligands, rhodopsin employs the chromophore ligand 11-*cis*-retinal, which is covalently bound via a protonated Schiff base in its active site. Activation of the receptor is initiated by the isomerization of the retinal chromophore from the inverse agonist 11-cis isomer to the agonist all-trans isomer. The protein responds to this initial, light-dependent step by proceeding through several spectrally distinct intermediates and on the order of milliseconds comes to a conformational equilibrium between the active state, Meta II, and its inactive precursor, Meta I. Under physiological conditions, the Meta I/Meta II equilibrium is largely on the side of Meta II, and the receptor pool is therefore fully active. At lower temperature and/or alkaline pH, the equilibrium shifts toward the inactive receptor conformation Meta I. To determine the ligand characteristics essential for receptor activation, modified synthetic retinals can be regenerated with opsin apoprotein to produce functional artificial pigments that can be probed by various methods (*1*, *2*).

This study is part of a series in which we systematically modified certain moieties of retinal and examined by Fourier transform infrared (FTIR) difference spectroscopy the impact of these modifications on receptor activation and the Meta I/Meta II equilibrium. FTIR-difference spectroscopy is an ideal method for this purpose since it is sensitive to both the conformation of the protein as well as to chromophore changes. In a previous paper (*3*), we focused on the ring portion of retinal and found that demethylation or partial deletion of the cyclohexenyl ring rendered all-trans retinal only a weak partial agonist. In particular, interaction between the methyl group at C5 of the ring (see Scheme 1) and the protein proved to be of particular importance for attaining an active receptor state. We further measured the apparent pK_A values of Meta I/Meta II titration curves at different temperatures and established a method to derive the thermodynamic parameters that govern this equilibrium: the

† This work was supported by grants from the DFG (Si 278/16-3.4 to F.S. and R.V.), the Israel Academy of Sciences and Humanities Fund, and the Human Frontier Science Program (to M.S.). T.P.S. is a senior scholar of The Ellison Medical Foundation. M.S. holds the Katzir-Makineni professorial chair in chemistry.

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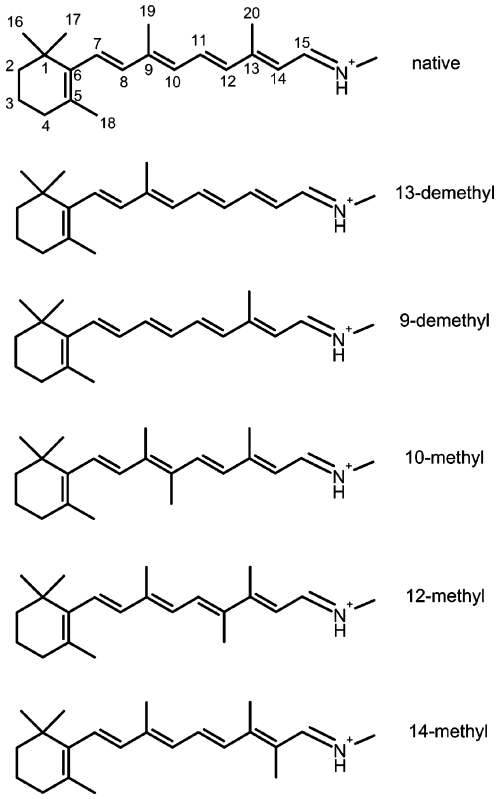
[1] Abbreviations: GPCR, G protein-coupled receptor; FTIR, Fourier transform infrared spectroscopy; HOOP, hydrogen out-of-plane; Rho, rhodopsin; Iso, isorhodopsin; PC, phosphatidyl choline; TM, transmembrane helix; EC, extracellular loop; 9-dm, 9-demethyl; 13-dm, 13-demethyl.

10.1021/bi052196r CCC: $33.50
Published on Web 01/17/2006

Scheme 1[a]



[a] Modified retinals used in this study. The retinals are shown as the all-trans isomers of the protonated Schiff bases. Regeneration of the pigment was achieved with the respective 9-cis isomers (for 13-demethyl, 9-demethyl, 10-methyl, and 14-methyl) and 11-cis isomers (13-demethyl and 12-methyl).

enthalpy change ΔH and the entropy change ΔS. In native rhodopsin, the transition from Meta I to Meta II is driven by a large positive entropy change (*4*, *5*). In the Gibbs' free energy change of the transition, $\Delta G = \Delta H - T\Delta S$, this large positive contribution of the entropy term $T\Delta S$ is largely compensated by a positive enthalpy term ΔH. Ring demethylation or deletion of the closed ring structure substantially decreased both ΔH and ΔS, with the decrease of $T\Delta S$ being generally more pronounced than that of ΔH (*3*), thereby shifting the Meta I/Meta II equilibrium toward the inactive Meta I conformation.

In this study, we focus on the role of methyl groups along the polyene of retinal in the activation process of rhodopsin. Specifically, we studied the roles of the native 13- and the 9-methyl groups by using 13- and 9-demethyl retinal analogues, and of additional methyl groups placed at C10, C12, and C14 by using 10-, 12-, and 14-methyl analogues (Scheme 1). All five artificial pigments formed Meta II photoproducts with FTIR difference spectra that were quite similar to spectra of native Meta II. Their Meta I states, however, differed from native Meta I to a much larger extent. At 20 °C, the conformational equilibrium between Meta I and Meta II was shifted progressively toward inactive Meta I, following the order 14-methyl, 13-demethyl, 12-methyl, 10-methyl, and 9-demethyl from most to least active. The structural basis of these observations was further elucidated by studying the opsin mutant E134Q regenerated with retinal analogues. While replacement of Glu 134 by glutamine shifted the Meta I/Meta II equilibrium more to the Meta II side of the 13-demethyl analogue, it did not enhance significantly Meta II formation of the 9-demethyl analogue. The 9-methyl group therefore appears to be essential for correct positioning of retinal to allow efficient coupling of protonation-induced conformational changes in the cytoplasmic ERY microdomain around Glu 134, to protonation of Glu 113, which forms the counterion to the protonated Schiff base in the dark state (Figure 1).

MATERIALS AND METHODS

Pigment Preparation. Preparation of rhodopsin analogues in their native membrane environment and preparation of mutant pigments reconstituted into phosphatidyl choline membranes and regenerated with synthetic retinals was achieved as described previously (*3*).

Preparation of Modified Retinals. The retinal analogues 13-demethyl-9-*cis*-retinal (*6*), and 14-methyl-9-*cis*-retinal (*7*) were prepared according to previously described methods. 12-Methyl 11-*cis*-retinal was prepared from 2,3-dimethyl-5-(2′,6′,6′-trimethyl-1′-cyclohexen-1′-yl)-2,4,6-heptatrienenitrile via methylation with methyl iodide (*8*). The product was transformed to 12-methyl-11-*cis*-retinal by conventional methods. 10-Methyl 9-cis retinal was prepared by methylation of all-*E*-3-methyl-5-(2′,6′,6′-trimethyl-1′-cyclohexen-1′-yl)-2,4-pentadienenitrile (*8*). The cis isomer was separated by chromatography and was transformed to 10-methyl-9-*cis*-retinal by conventional methods. The all-trans isomers are shown in Scheme 1, while the cis-isomers used for regeneration are shown in Supporting Information, Figure A5.

FTIR Spectroscopy. FTIR difference spectroscopy was performed with a Bruker IFS 28 spectrometer with a mercury cadmium telluride (MCT) detector. The spectra shown in this study are generally photoproduct minus dark state difference spectra. FTIR spectra were recorded in blocks of 512 scans with a spectral resolution of 4 cm^{-1} and an acquisition time of 1 min and corrected for temporal baseline drifts. Experiments were performed with sandwich samples with 0.5 nmol or less pigment in membranes, that were prepared as described in detail elsewhere (*9*). This sample type allows for controlling water content, pH value, and salt concentration in the samples. In particular, the measurements indicate Meta I/Meta II titration curves identical to those measured with membrane suspensions (*10*). Forty microliters of either 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 (*10*). For H/D exchange, we twice equilibrated the sample film with D_2O and dried it under nitrogen before adding 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. Specified pH values are always effective pH values unless stated differently.

Samples were photolyzed for 20 s through a fiber optics fitted to a 150 W tungsten lamp equipped with long-pass filters. The cutoff wavelengths were 475 nm for 9-demethyl Iso and 530 nm for all others. pK_A values of Meta I/Meta II equilibria at different temperatures were determined by fitting of FTIR difference spectra to Meta I and Meta II reference spectra of the analogue pigments as described previously (*3*).

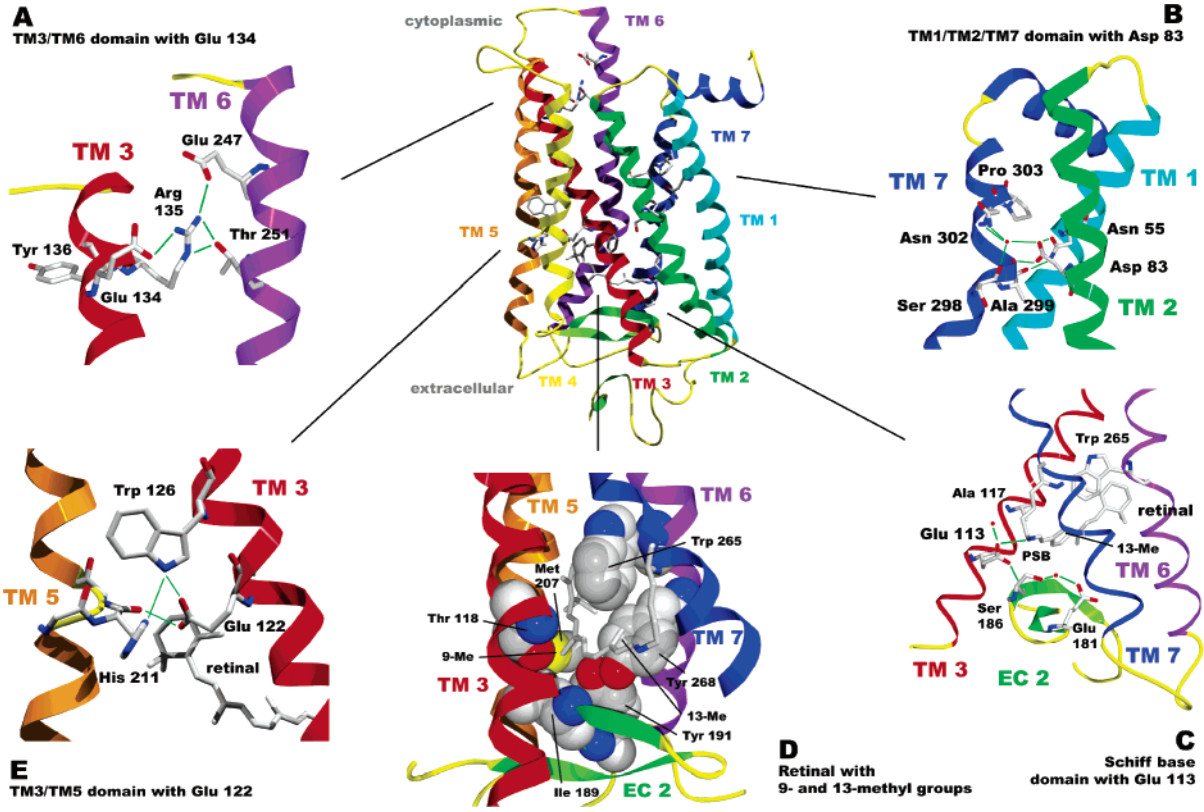


FIGURE 1: Molecular model of rhodopsin and its microdomains based on the structure of the dark state, PDB 1GZM (*11*). The insets show close-ups of functional domains of rhodopsin together with reporter groups specifically relevant for analysis of the FTIR difference spectra: the cytoplasmic TM 3/TM 6 domain with the ERY motif including Glu 134 (A), the TM 1/TM 2/TM 7 domain with Asp 83 (B), the Schiff base domain with the salt bridge between Glu 113 and the protonated Schiff base (PSB), and the network extending along extracellular loop 2 (EC 2) (C), the retinal binding pocket around the 9- and 13-methyl groups (D), and the TM 3/TM 5 domain with Glu 122 (E), close to the ring of retinal.

In cases in which it was not possible to obtain a definitely pure Meta II state, pure Meta II was stabilized in a complex with a synthetic peptide (VLEDLKSCGLF) being a high-affinity analogue to the C-terminus of the transducin α-subunit.

UV−Visible Spectroscopy. For UV−visible spectroscopy sandwich samples identical to the infrared samples were used in a Perkin-Elmer Lambda 17 spectrophotometer equipped with a temperature-controlled sample holder. Illumination was similar as in the FTIR experiments. Spectroscopy on detergent-solubilized pigment was performed with 100 μL microcuvettes with 10-mm path length.

Molecular Models. Molecular graphics are based on the coordinates of the dark state by Li and Schertler (*11*) and were prepared with the software Deep View 3.7 (*12*) (available at http://www.expasy.org/spdbv) and POV Ray 3.5 (available at http://www.povray.org).

RESULTS

Below we provide a description of the Meta I and Meta II states of the artificial pigments in the native disk membrane environment. Each of the pigments examined formed a pH-dependent Meta I/Meta II equilibrium, which responded to addition of a peptide (VLEDLKSCGLF) being a high-affinity analogue to the C-terminus of the transducin α-subunit (*13*) by a shift to the Meta II state. The Meta I and the Meta II states of native rhodopsin and isorhodopsin (Iso) and the pK_A values of their conformational equilibria in native membranes have been described in detail previously (*3*).

13-Demethyl Analogues. 13-Demethyl has been investigated both as 11-*cis*-rhodopsin and 9-*cis*-isorhodopsin. 11-*cis*-13-Demethyl retinal absorbed at 377 nm in ethanol, and 9-*cis*-13-demethyl retinal absorbed at 370 nm. The 9-*cis*-isomer reacted with opsin readily and completely within 4 h of incubation, regenerating 13-demethyl Iso absorbing at 488 nm. The 11-cis isomer reacted with opsin to only about 50% within 4 h at room temperature (at 1:1.5 opsin/retinal stoichiometry and 40 μM opsin concentration) yielding 13-demethyl rhodopsin absorbing at 500 nm. The resulting pigments have therefore similar absorption peak positions as the respective native isomers, isorhodopsin, and rhodopsin. Absorption maxima and 11-cis isomer partial regeneration are in agreement with previous studies (*14*).

In the following, we will focus on the photoproducts obtained from 13-demethyl rhodopsin. 13-Demethyl Iso produced identical Meta I and Meta II photoproducts with same associated pK_A (see Supporting Information, Figure A1).

At 20 °C, pH 4.5, 13-demethyl Rho forms a Meta II state, which corresponds to native Meta II (Figure 2A). The retinal Schiff base is deprotonated in Meta II as evident from parallel UV−visible experiments (Figure 4). The pK of the conformational equilibrium with Meta I is at 6.0 at 20 °C (Figure 2C) and is thus 1.7 units shifted toward more acidic values compared with native rhodopsin. This shift is almost halfway to the pK_A of the equilibrium between active and inactive conformation of the apoprotein opsin in the absence of ligands (*3*, *9*). The all-trans

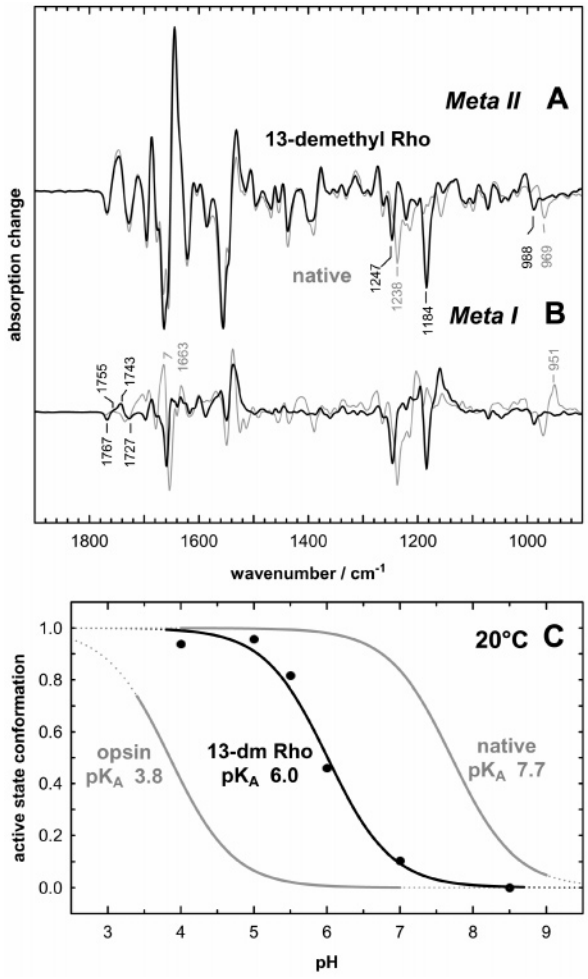


FIGURE 2: Meta II and Meta I of 13-demethyl rhodopsin. Meta II (A) and Meta I (B) FTIR difference spectra photoproduct minus dark state of 13-demethyl rhodopsin (black spectra) were obtained at 20 °C at pH 5.0 (A) and at 0 °C at pH 8.6 (B), respectively. In comparison, corresponding FTIR difference spectra of the transition to Meta II and Meta I from native rhodopsin, obtained at 10 °C at pH 5.0 (A) and at 0 °C at pH 9.5 (B), respectively, are shown in gray. In this representation, photoproduct bands are positive, while negative bands belong to the dark state. While the 13-demethyl Meta II state is very similar to native Meta II, there are pronounced alterations in the 13-demethyl Meta I state. The pK_A of the Meta I/Meta II equilibrium of 13-demethyl rhodopsin was determined at 20 °C to be 6.0 (C), which is about halfway between that of native Meta I/Meta II and that of ligand-free opsin at the same temperature (gray curves), rendering all-trans 13-demethyl a ligand with partial agonist behavior.

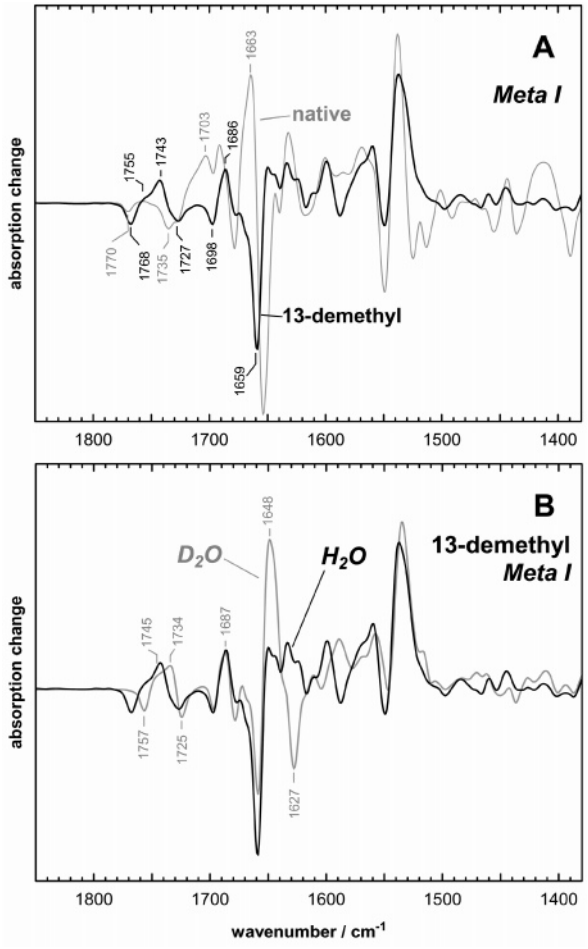


FIGURE 3: Meta I of 13-demethyl rhodopsin. (A) A close-up of the Meta I minus dark state FTIR difference spectrum of 13-demethyl rhodopsin of Figure 2B in the region of carboxylic acid and amide vibrations is compared to the corresponding native Meta I difference spectrum (gray), revealing the conformational alterations in 13-demethyl Meta I compared with native Meta I. (B) The Meta I difference spectrum of 13-demethyl shown in A (in H_2O, black) is compared with a corresponding spectrum obtained in D_2O (gray).

13-demethyl chromophore exhibits therefore only a partial agonist behavior.

The Meta I state itself is very different from native Meta I (Figure 2B). In the range of the hydrogen-out-of-plane (HOOP) vibrations of the chromophore, the positive C11═C12 HOOP mode of native Meta I at 951 cm^{-1} is lacking (*15*). HOOP modes of retinal gain high IR intensity from twists of the polyene around single bonds adjacent to the respective double bond of the HOOP mode (*16*). This implies that, in contrast to native Meta I, the polyene is planar in the segment around C11═C12 in Meta I of the 13-demethyl pigment.

In the region above 1700 cm^{-1}, we observe generally the C═O stretches of lipid esters (*17*) and of protonated carboxylic acids (*18*, *19*). The carboxylic acid C═O stretch frequency depends on the hydrogen bonding strength of both its C═O and OH groups. It absorbs above 1770 cm^{-1} in the absence of hydrogen bonding or down to 1697 cm^{-1} in the presence of two strong hydrogen bonds (*3*, *18*, *20*). In rhodopsin, membrane-embedded carboxylic acids are very sensitive markers of the overall Meta I to Meta II conformational change. In FTIR difference spectra, we observe contributions of Asp 83 on transmembrane (TM) helix 2 and Glu 122 on TM helix 3. Asp 83 participates in an interhelical hydrogen-bonded network between helices 1, 2, and 7 (Figure 1B), while Glu 122 hydrogen bonds to Trp 126 on TM helix 3 and the backbone carbonyl of His 211 on TM helix 5 (Figure 1E) (*11*, *21*−*23*). Both networks seem to play a profound role in receptor activation and undergo a rearrangement during the transition to Meta II. This rearrangement leads to a strengthening of the hydrogen bonding of Asp 83 and a weakening of that of Glu 122 (*19*, *24*). In the transition from the dark state to the still inactive Meta I state of native rhodopsin, the C═O stretch of Asp 83 is only slightly downshifted from its position at 1770 cm^{-1}. Glu 122, which has a split absorption peak at 1735 and 1727 cm^{-1} in the dark, absorbs at around 1704 cm^{-1} and contributes to the high-frequency lobe of the positive 1703 cm^{-1} peak in Meta I (Figure 3A). Both downshifts reflect a rearrangement

of these residues leading to slightly strengthened hydrogen bonding of Asp 83 and considerably stronger hydrogen bonding of Glu 122 in Meta I in the native pigment.

In Figure 3A, an enlarged view of the same Meta I difference spectra as in Figure 2B allows a comparison of the difference bands of 13-demethyl rhodopsin with those of native rhodopsin in the region of the amide I and II vibrations of the protein backbone around 1650 and 1550 cm^{-1}, as well as in the region of protonated carboxylic acids above 1700 cm^{-1}. Compared with native Meta I, the absorption pattern of Asp 83 and Glu 122 is considerably changed in 13-demethyl Meta I. The downshift of Asp 83 is much more pronounced, giving rise to a stronger negative band at 1768 cm^{-1} and a Meta I absorption at 1755 cm^{-1}, indicating a somewhat stronger hydrogen-bonding than in native Meta I. The C═O stretch of Glu 122, on the other hand, does not undergo the downshift observed in native Meta I, but is instead upshifted to 1743 cm^{-1}, indicating a weakening of the hydrogen bonding of Glu 122 in 13-demethyl Meta I. The assignment of these C═O vibrations to carboxylic acids has been verified using their sensitivity to H_2O/D_2O exchange (Figure 3B) and the assignment of the Glu 122 vibration has been further verified using the E122Q mutant regenerated with 13-demethyl retinal (not shown). These alterations show that changes of amino acid side chains and/or helix arrangement are considerably different in 13-demethyl Meta I relative to native Meta I. Possibly some of the conformational changes that are otherwise observed in the transition to Meta II only (where the absorption of Asp 83 shifts further down to 1750 cm^{-1} and that of Glu 122 shifts further up to 1749 cm^{-1}) are anticipated in certain microdomains already in the Meta I state of 13-demethyl to a limited extent.

Besides these changes detected by the absorptions of carboxylic acids, the amide band at 1663 cm^{-1}, which is an amide I marker band of native Meta I, is lacking in 13-demethyl Meta I. Instead, a strong negative band at 1659 cm^{-1} is present in 13-demethyl Meta I, which is only slightly H/D sensitive, indicating that it might be an amide I mode. Upon H/D exchange, a pronounced difference band at −1627/+1648 cm^{-1} is noticed in D_2O (Figure 3B), which is not evident in H_2O. Presumably, this difference band reflects the Schiff base C═N stretch mode, which undergoes a larger change of frequency during the transition from the dark state to Meta I in D_2O than in H_2O due to different coupling to the ND and NH bending modes.

In Figure 4, UV−visible spectra obtained at 20 °C at pH 5 and pH 8.0 show 13-demethyl Meta I and Meta II absorbing at similar positions as the respective native states. The spectrum obtained at pH 6.0 (Figure 4B) is composed of about equal parts of the spectra obtained at pH 5.0 and pH 8.0, as to be expected if the photoproduct equilibrium observed in the UV−visible range corresponds to the conformational equilibrium monitored in the IR range.

Glu 134 at the cytoplasmic end of TM helix 3 (Figure 1A) was reported to control proton uptake during formation of Meta II (*25*). To examine whether this proton uptake path is disturbed in the 13-demethyl analogue, we regenerated the mutant E134Q reconstituted in phosphatidyl choline (PC) membranes with 13-demethyl retinal. Because of the higher regeneration yield, we used the 9-cis isomer for this purpose.

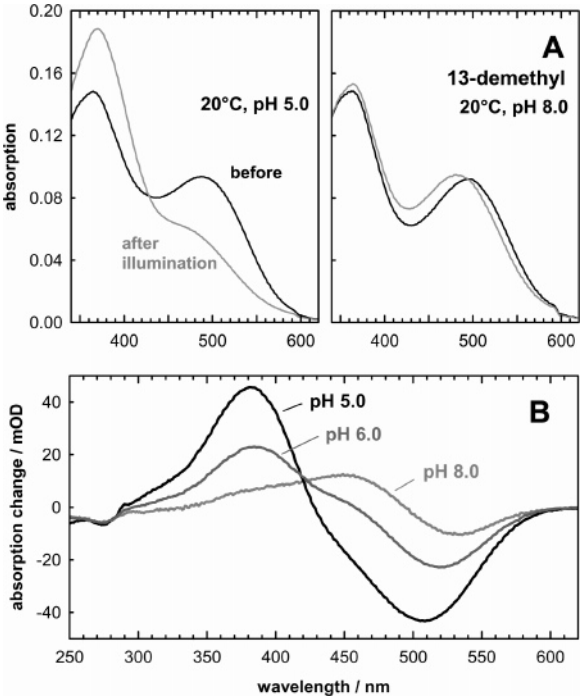


FIGURE 4: UV−visible spectra of 13-demethyl rhodopsin. (A) UV−visible spectra before (black) and after illumination (gray) were recorded at 20 °C, pH 5 and 8, producing predominantly Meta II and Meta I, respectively. (B) UV−visible difference spectra photoproduct minus dark state of the transition to Meta II at pH 5 and to Meta I at pH 8 are shown together with a difference spectrum obtained at pH 6.0, close to the pK_A of the Meta I/Meta II equilibrium.

At 0 °C, E134Q 13-demethyl Iso formed Meta II-like photoproducts both at pH 5.1 and 8.6 (Figure 5), albeit the amplitudes of Meta II marker bands were reduced at pH 8.6, indicating already contributions of Meta I at this pH. Wildtype 13-demethyl Iso in PC membranes formed Meta II only at pH 5.1, while the photoproduct at pH 8.6 was pure Meta I. The effect of PC lipids compared with disk membrane lipids on Meta I/Meta II is relatively small (S. Lüdeke, M. Schmitt, and R. Vogel, unpublished results). Replacement of Glu 134 by Gln increases therefore the pK_A of Meta I/Meta II of 13-demethyl by approximately 3 units.

We further analyzed by UV−visible spectroscopy the photoreaction of 13-demethyl Iso solubilized in 1% (w/v) dodecyl maltoside (DM) at 10 °C. In the pH range from 5.0 to 9.0, 13-demethyl Iso formed exclusively a 380 nm photoproduct state indicative of Meta II (Figure A2 in Supporting Information). Therefore, in the detergent environment, no Meta I photoproduct could be observed, similarly as for detergent solubilized native rhodopsin, but different from, for example, detergent-solubilized 9-demethyl rhodopsin or acyclic pigment (*26*−*28*).

9-Demethyl Analogue. 9-*cis*-9-Demethyl retinal absorbed at 366 nm in ethanol. It reacted readily with opsin within 4 h at room temperature and good yield to the 9-demethyl Iso pigment absorbing at 457 nm.

The Meta I and Meta II states of 9-demethyl have been studied earlier by FTIR spectroscopy (*26*, *29*) and both low- and room-temperature photoproducts were examined in detail. The thermodynamics and the pH-dependence of the Meta I to Meta II transition in 9-demethyl pigments, however, has been described only in a preliminary way (*26*, *28*).

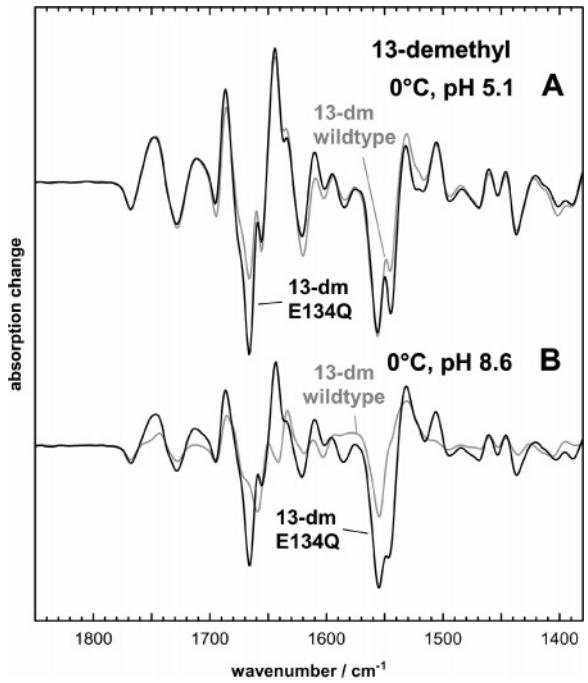


FIGURE 5: Photoproducts of the 13-demethyl E134Q mutant. FTIR difference spectra photoproduct minus dark state of E134Q 13-demethyl isorhodopsin (black spectra) reconstituted in PC membranes show a Meta II-like photoproduct at pH 5.1 (A) and a mostly Meta II-like photoproduct at pH 8.6 (B), both at 0 °C. In comparison, the photoproduct of wildtype 13-demethyl isorhodopsin in PC membranes (gray spectra) was Meta II-like at pH 5.1 only (A), while it was pure Meta I at pH 8.6 (B). The pH-dependence of Meta I/Meta II observed with wildtype 13-demethyl is therefore considerably shifted in the E134Q mutant of the 13-demethyl pigment.

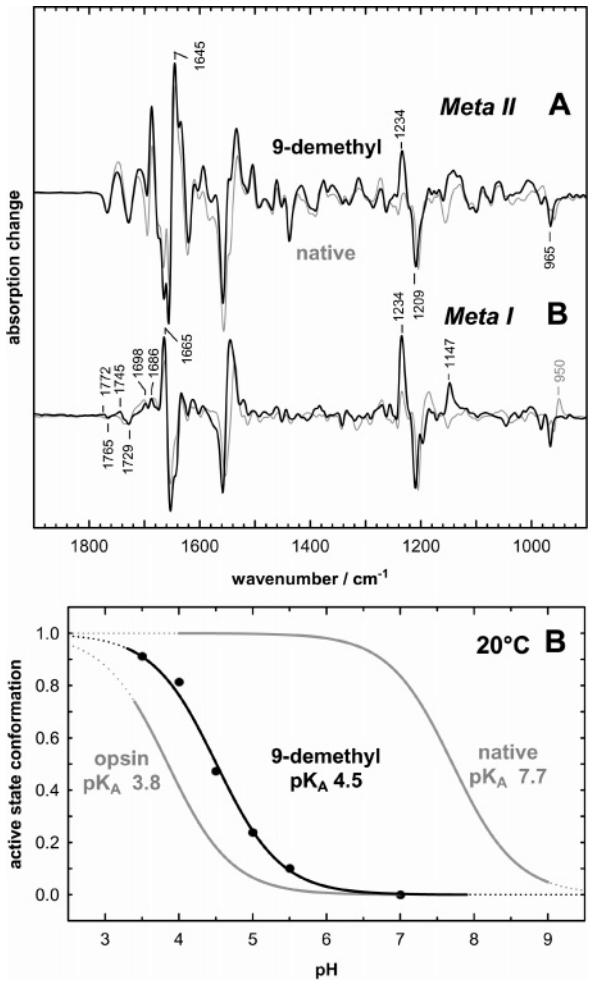


FIGURE 6: Meta II and Meta I of 9-demethyl Iso. Meta II (A) and Meta I (B) FTIR difference spectra photoproduct minus dark state were obtained from 9-demethyl Iso at 30 °C at pH 4.0 (A) and at 0 °C at pH 7.0 (B), respectively. The corresponding native spectra (gray) were obtained from native Iso under conditions as in Figure 2. The pK_A of the Meta I/Meta II equilibrium of 9-demethyl is at 4.5 at 20 °C (C) and thus only 0.7 units above that of the opsin equilibrium in the absence of ligand. All-trans 9-demethyl is therefore a very weak partial agonist only.

9-Demethyl forms a Meta II photoproduct only at very acidic pH (Figure 6 A,C). The pK_A value of 4.5 at 20 °C is the lowest of the pigments that we have studied so far and is only 0.7 units above that of the opsin conformational equilibrium in the absence of ligand.

The Meta I photoproduct shows some noteworthy differences from native Meta I (Figure 6B). Remarkably, the Meta I HOOP band at 950 cm^{-1} is missing, indicating a planar chromophore geometry around C11=C12, similarly as described above for 13-demethyl rhodopsin and previously for acyclic-1 and -2 pigments (*3*). Further differences are observed for the absorption changes of Asp 83 and Glu 122. Glu 122 is upshifted in Meta I, leading to a −1729/+1745 cm^{-1} difference band. Asp 83 is slightly upshifted in Meta I, giving rise to a very small −1765/+1772 cm^{-1} difference band. The assignment of both differences to the C=O stretch of carboxylic acids has been verified using H/D exchange.

We further regenerated the E134Q mutant reconstituted in PC membranes with 9-demethyl-9-*cis*-retinal and measured FTIR difference spectra at 0 °C at pH 4.1 and 6.1 (Figure 7). The difference spectra show that E134Q 9-demethyl Iso formed a pH-dependent Meta I/Meta II equilibrium, which is hardly shifted to Meta II compared with wildtype 9-demethyl in PC membranes. Comparison of the mutant spectra with spectra from wildtype 9-demethyl indicate that an increase of the pK_A of Meta I/Meta II introduced by the E134Q mutation is less than one unit.

12-Methyl Analogue. 11-*cis*-12-Methyl retinal absorbed at 375 nm in ethanol. Incubation at room temperature with opsin for 4 h regenerated 12-methyl rhodopsin at full yield, absorbing at 487 nm.

12-Methyl rhodopsin formed a Meta II photoproduct only at very acidic pH. At 20 °C and pH 4.0, the 12-methyl Meta II photoproduct state is similar to native Meta II, albeit with altered intensities of some of the Meta II peaks (Figure 8A). Some of these alterations, in particular, the reduced intensity of the positive bands at 1750 and 1644 cm^{-1} may be due to some Meta I being still present under these conditions. In 12-methyl Meta II, the Schiff base is titratable and protonated at pH 4.0, as verified by UV−visible spectroscopy, while it is already partially deprotonated at pH 5.0.

The pK_A of the transition to Meta I is 5.1 at 20 °C (Figure 8C), which is 2.6 units lower than that of native Meta I/Meta II and only 1.3 units above that of the opsin conformational equilibrium. The all-trans 12-methyl chromophore is therefore only a weak partial agonist.

The protein conformational changes following light absorption by 12-methyl pigment and its transition to Meta I affect Glu 122 and Asp 83 in 12-methyl in a similar way as

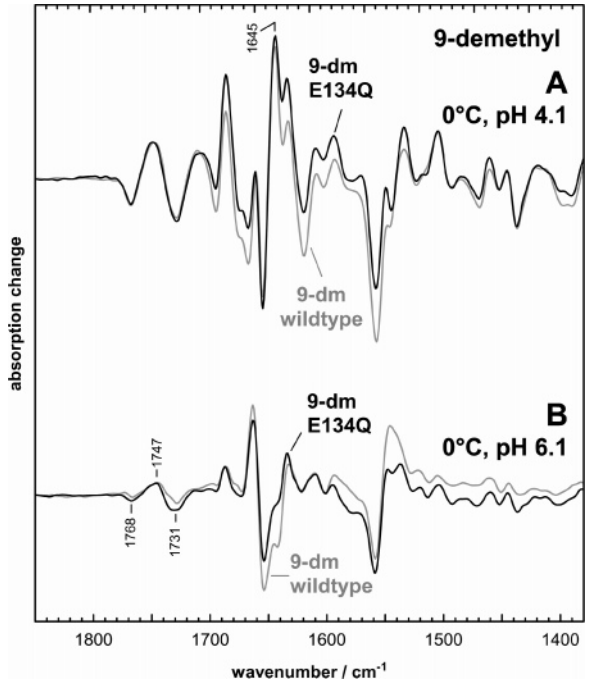


FIGURE 7: Meta I/Meta II of wildtype and E134Q 9-demethyl Iso. FTIR difference spectra photoproduct minus dark state were obtained at 0 °C at pH 4.1 (A) and at pH 6.1 (B) from wildtype 9-demethyl Iso (gray) and the E134Q mutant of 9-demethyl Iso (black), both in PC membranes. The spectra indicate that the E134Q mutation does not substantially shift the position of the Meta I/Meta II equilibrium of the 9-demethyl analogue.

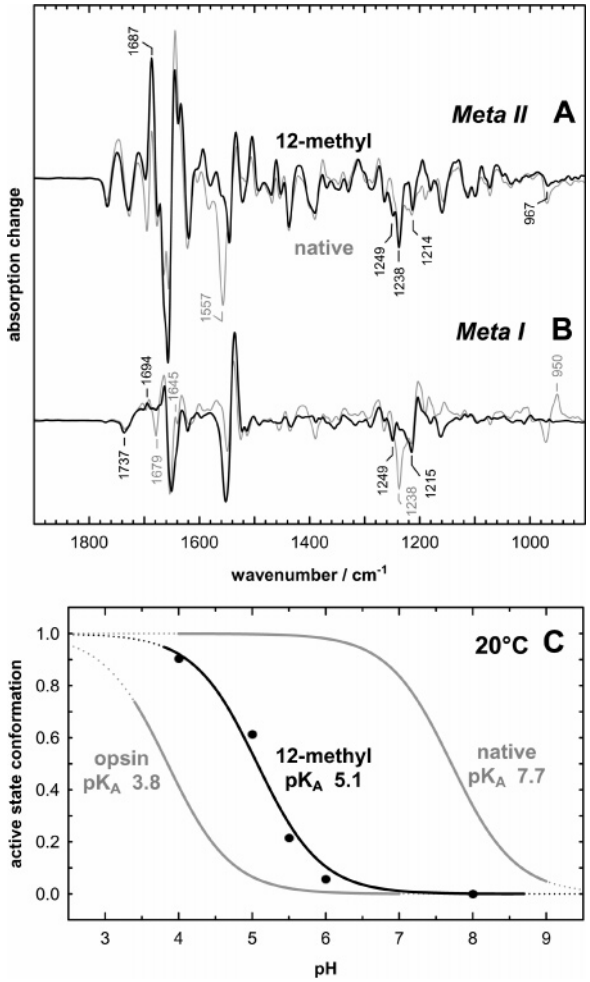


FIGURE 8: Meta II and Meta I of 12-methyl rhodopsin. Meta II (A) and Meta I (B) FTIR difference spectra photoproduct minus dark state of 12-methyl rhodopsin (black) were obtained at 20 °C at pH 4.0 (A) and at 10 °C at pH 7.3 (B), respectively. The corresponding native spectra (gray) were measured with native rhodopsin under conditions as in Figure 2. The pK_A of the Meta I/Meta II equilibrium of 12-methyl is considerably downshifted to more acidic values (C). All-trans 12-methyl retinal shows therefore only a weak partial agonist behavior.

in native pigment, yet the pattern of the heterogeneous absorption of Glu 122 in the dark state is somewhat different from that in native rhodopsin (Figure 8B). Further differences are observed at lower frequencies, such as a positive, unassigned peak at 1694 cm^{-1}, the lack of a pronounced negative band at 1679 cm^{-1}, which might indicate a changed interaction of a Gln or Asn side chain carbonyl in Meta I, and an altered band pattern around 1550 cm^{-1}. Negative difference bands in the fingerprint range (Figure 8A,B) look at first sight very much changed compared with Meta II difference spectra: In the 12-methyl Meta II difference spectrum, fingerprint modes of the dark state are noticed at 1249, 1238, and 1214 cm^{-1}. In the Meta I difference spectrum, a strong Meta I-specific photoproduct band compensates the dark state mode at 1238 cm^{-1}. In the HOOP range, there are no pronounced difference bands in the 12-methyl Meta I difference spectrum, suggesting that infrared HOOP modes are either lacking in both the dark and the Meta I states, or coincidentally overlap in the difference spectrum. The latter is probably the case, as a small dark state HOOP mode can be detected in the Meta II difference spectrum at 967 cm^{-1}. This implies that a HOOP mode (which could be the isolated C11 HOOP or the C7=C8 HOOP) is present in Meta I, but that it is quite small.

10-Methyl Analogue. 9-*cis*-10-Methyl retinal absorbed at 376 nm in ethanol. Incubation at room temperature with opsin for 4 h regenerated 10-methyl isorhodopsin at full yield, absorbing at 500 nm, somewhat red-shifted from native Iso.

The Meta I/Meta II equilibrium of the 10-methyl analogue was found to be shifted toward the inactive Meta I state with a p*K* of 5.0 at 20 °C (Figure 9C), similar to the 12-methyl pigment. The Meta II difference spectrum is slightly altered with an increased intensity of the Meta II band at 1687 cm^{-1} (Figure 9A). The Meta II Schiff base was found to be protonated at pH 4.0, as also verified by UV−visible spectroscopy. In the presence of G protein-derived peptide, a Meta II state could be stabilized over a wider pH range, revealing a titratable Schiff base in Meta II, which was deprotonated at neutral pH. The Meta I state of the 10-methyl pigment (Figure 9B) has a strong alteration with an additional intense band at 1686 cm^{-1}, which might be an amide I mode reflecting additional backbone distortions. The reduced intensity of the dark state C11=C12 HOOP mode at 947 cm^{-1} in the Meta I difference spectrum compared with the Meta II difference spectrum indicates a compensating Meta I HOOP mode at around the same position as in the 9-cis dark state, in agreement with previous studies of 10-methyl rhodopsin (*30*). The absorption bands attributable to Asp 83 and Glu 122 above 1700 cm^{-1} appear to be similar to those in native pigment both in the dark and in the photoproduct states.

14-Methyl Analogue. 9-*cis*-14-Methyl retinal absorbed at 374 nm in ethanol. Regeneration was slow with ~20% yield after 4 h and ~70% regeneration after 20 h at room

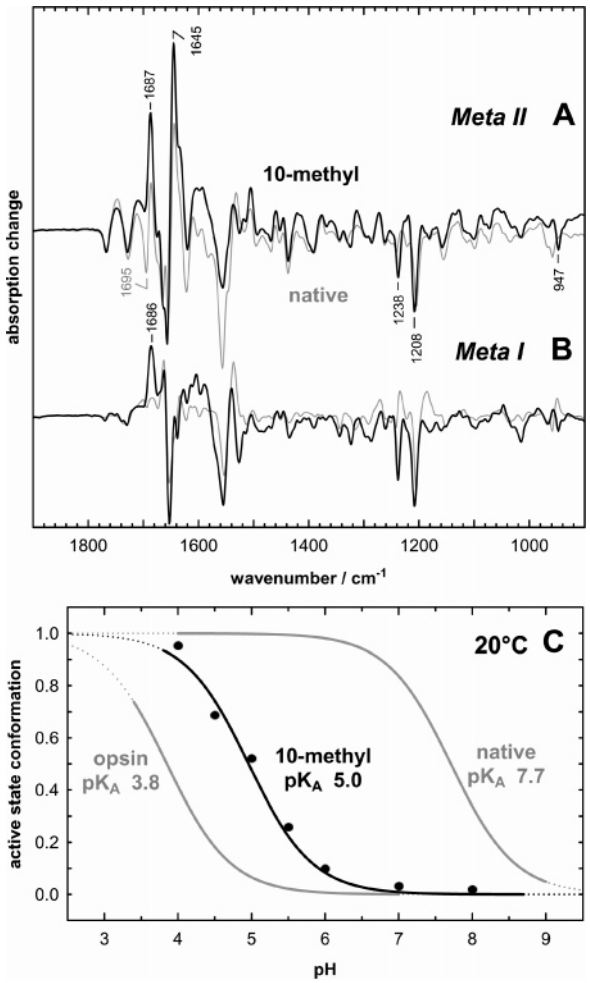


FIGURE 9: Meta II and Meta I of 10-methyl isorhodopsin. Meta II (A) and Meta I (B) FTIR difference spectra photoproduct minus dark state of 10-methyl isorhodopsin (black) were obtained at 20 °C at pH 4.0 (A) and at 0 °C at pH 7.6 (B), respectively. The corresponding native spectra (gray) were measured with native isorhodopsin under conditions as in Figure 2. The pK_A of the Meta I/Meta II equilibrium of 10-methyl is shifted to 5.0 (C), such that all-trans 10-methyl retinal is a weak partial agonist only, similar as all-trans 12-methyl retinal.

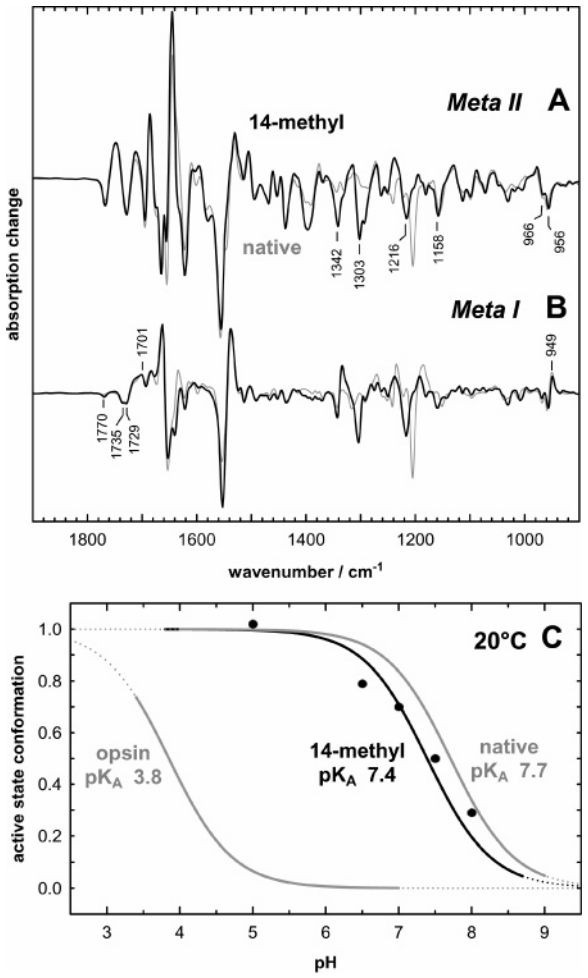


FIGURE 10: Meta II and Meta I of 14-methyl isorhodopsin. Meta II (A) and Meta I (B) FTIR difference spectra photoproduct minus dark state of 14-methyl Iso (black) were obtained at 20 °C at pH 5.0 (A) and at 0 °C at pH 8.6 (B), respectively. The corresponding native spectra (gray) were obtained under conditions as in Figure 2. The pK_A of the Meta I/Meta II equilibrium of 14-methyl is shifted by 0.3 units to more acidic values (C), rendering all-trans 14-methyl retinal an only slightly less potent agonist compared with native all-trans retinal.

temperature. The resulting pigment 14-methyl isorhodopsin absorbed at 492 nm in agreement with published data (*31*).

At 20 °C, the Meta I/Meta II equilibrium of 14-methyl Iso is only slightly shifted toward Meta I. The pK_A of the transition is 7.4 compared with 7.7 of native Meta I/Meta II (Figure 10C). 14-Methyl forms a regular Meta II state with deprotonated Schiff base. Besides differences regarding the fingerprint bands of the chromophore, 14-methyl Meta II is very similar to native Meta II (Figure 10A). The same holds for 14-methyl Meta I, which corresponds largely to native Meta I (Figure 10B). The similar band pattern above 1700 cm^{-1} implies that Asp 83 and Glu 122 sense the same changes during the transition from the dark state to Meta I in 14-methyl Iso as in native Iso. In addition, the HOOP modes of the dark state and Meta I are very similar to those of native pigment.

Thermal Decay of Photoproducts. Native Meta I/Meta II is known to decay via two pathways, either to all-trans retinal and the apoprotein opsin by hydrolysis of the retinal Schiff base or to a species termed Meta III via thermal isomerization of the Schiff base C═N bond from 15-anti to 15-syn (*32*, *33*). The decay pathway to Meta III is relevant only under conditions that allow accumulation of Meta I to appreciable concentration, indicating that formation of Meta III proceeds via Meta I only (*34*). This was interpreted to reflect steric incompatibility of the all-trans 15-anti chromophore of Meta I with the binding pocket in the inactive Meta I conformation, which is relieved in the all-trans 15-syn isomer of Meta III.

Among the pigments studied in this paper, appreciable formation of Meta III was detected only in 10-methyl and 12-methyl pigments. Decay of the Meta I/Meta II photoproduct equilibria of these two pigments to Meta III was observed at 20 °C and neutral to alkaline pH at a rate roughly similar to that detected in native pigment. The identity of Meta III in both pigments had been verified by using the Meta III Schiff base NH bending mode vibration absorbing around 1350 cm^{-1} and its H/D sensitivity (*32*).

The Meta I/Meta II photoproduct equilibrium of the other pigments decayed via hydrolysis of the Schiff base only. Thermal isomerization of the Schiff base C═N bond is not induced in the Meta I states of either the 9- and 13-demethyl pigments, where the lack of HOOP modes indicates a more

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

	pK_A at 20 °C	pK_A at 0 °C	Δ pK_A	ΔH in kJ/mol	ΔS in J/(mol K)	TΔS in kJ/mol at 20 °C	ΔG in kJ/mol at 20 °C
native[c]	7.7	6.6	1.1	84	434	127	−43
13-demethyl	6.0	5.7	0.3	23 (−61)	193	57 (−70)	−34 (+9)
9-demethyl	4.5	3.9	0.6	46 (−38)	243	71 (−56)	−25 (+18)
10-methyl	5.0	4.4	0.6	46 (−38)	252	74 (−53)	−28 (+15)
12-methyl	5.1	4.7	0.4	31 (−53)	202	59 (−68)	−28 (+15)
14-methyl	7.4	5.8	1.6	122 (+38)	559	164 (−37)	−42 (+1)
opsin[b,c]	3.8			48 (−36)	236	69 (−58)	−21 (+22)

[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. Values in brackets for ΔH, TΔS, and ΔG indicate the difference to the respective values of native pigment. [b] Opsin conformational equilibrium, see text for details. [c] From ref *3*.

relaxed fit of their chromophores into the inactive protein conformation of Meta I. In the case of 14-methyl Meta I with its similarity to native Meta I, the lack of Meta III formation might reflect a specific inhibition of the thermal isomerization of the Schiff base C15=N bond by the 14-methyl group.

ΔH and ΔS of the Meta I/Meta II Equilibria of the Polyene-Modified Pigments. In native rhodopsin, the transition from Meta I to Meta II is driven by a small gain of the Gibbs' free energy, $\Delta G = \Delta H - T\Delta S$, where T is the absolute temperature and ΔH and ΔS are the enthalpy and the entropy change of the transition. The transition from Meta I to Meta II in native rhodopsin at 20 °C is characterized by ΔG of −43 kJ/mol. This value is composed of a positive enthalpy change of 84 kJ/mol and a TΔS term of 127 kJ/mol (*3*), in agreement with previous studies (*5*). Up to now, we have considered only the position of the Meta I/Meta II equilibrium at 20 °C. This allows a classification of a ligand as a full agonist or as a partial agonist, but gives no clue whether, for examples, a shift of the equilibrium toward Meta I is due to an increase of the ΔH term or rather due to a decreased entropy gain, ΔS.

In a previous study on ring-modified pigments, we established a method to obtain the enthalpy change ΔH and the entropy change ΔS of the transition from Meta I to Meta II from pK_A values of the equilibrium, pK_1 and pK_2, measured at two different temperatures, T_1 and T_2 (*3*). We derived the following expressions for ΔH and ΔS (see section A4 in Supporting Information):

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

$$\Delta S = R(2.303)\left(\frac{\mathrm{p}K_1 T_1 - \mathrm{p}K_2 T_2}{T_1 - T_2}\right)$$

where R is the gas constant 8.314 J/K·mol.

We have measured titration curves of all five pigment analogues, as well as those of native pigment, at both 0 and 20 °C (Figure A3 in Supporting Information). In Table 1, the pK_A values of these titration curves are listed together with the respective values of ΔH and ΔS derived from it using the above expressions. Opsin in the last row signifies the equilibrium between the active and the inactive conformation of opsin in the absence of ligand (*9*). In that case, the thermodynamic parameters have been derived from pK_A values measured at 20 and 30 °C, which somewhat decreases the reliability in particular of the estimate for ΔS (*3*).

Examination of the thermodynamic parameters of the single pigments indicates that 9-demethyl, 13-demethyl, 10-methyl, and 12-methyl behave differently from 14-methyl. In all four pigments, the Meta I/Meta II transition is shifted toward Meta I, at least in the temperature range considered here (0 to 20 °C). For 14-methyl, the position of the Meta I/Meta II equilibrium becomes more sensitive to temperature compared with native pigment. This is due to an increase of ΔH. This also implies that at higher temperature, 14-methyl all-trans retinal is expected to be an equally strong or even stronger agonist than native all-trans retinal. The other pigments have a less pronounced temperature sensitivity of the positions of their respective Meta I/Meta II equilibria. This behavior reflects their lower enthalpy changes during the transition, which is the most diminished in 13-demethyl to 23 kJ/mol compared with 84 kJ/mol for native pigment. This decrease of ΔH, which was favorable for formation of Meta II, is offset by an even more pronounced decrease of the entropy gain TΔS of the transition to only 57 kJ/mol in 13-demethyl compared with 127 kJ/mol for native Meta I/Meta II. It is evident that these values are in the same range as those of the opsin conformational equilibrium (Table 1).

DISCUSSION

We have examined in this study the conformational equilibria between the active Meta II and the inactive Meta I states of five artificial pigments derived from retinal analogues bearing altered side chains along the polyene. These alterations of retinal included both deletion of the native methyl groups at C9 and C13 (comprising carbon 19 and 20, respectively, see Scheme 1) in the 9-demethyl and 13-demethyl pigments, as well as addition of methyl groups at either C10, C12, or C14 in the 10-methyl, 12-methyl, and 14-methyl pigments. Similarly, as in a previous study, in which we had examined pigments regenerated with retinals modified at the ring, all these pigments form Meta II states with a more or less similar conformation as native Meta II. Their Meta I states differ, however, in part considerably from native Meta I due to altered retinal-protein interactions, with the single exception of 14-methyl Meta I, which exhibits a nativelike conformation. The positions of the respective Meta I/Meta II equilibria are in all cases shifted toward the inactive Meta I state, which is reflected in lowering of the apparent pK_A values of the transitions. The 9- and 13-demethyl and the 10- and 12-methyl analogues have a much less pronounced dependence of this pK_A on temperature compared with native pigment, while that of 14-methyl is more pronounced. From the positions of the apparent pK_A values obtained at two different temperatures, we can calculate the enthalpy change ΔH and the entropy change ΔS of the transition from Meta I to Meta II for each pigment. In native

rhodopsin, the reaction is determined by positive values of both ΔH and $T\Delta S$ (Table 1), which cancel each other to a large extent. In the 9- and 13-demethyl and the 10- and 12-methyl pigments, both ΔH and $T\Delta S$ are strongly reduced compared with the values of native pigment. This reduction is in all four cases more pronounced for the $T\Delta S$ term, which is responsible for the shift of the equilibrium toward Meta I. In the 14-methyl pigment, on the other hand, we have observed a nearly equal increase of both ΔH and $T\Delta S$ terms.

13-Demethyl Analogues. The photoproducts of 13-demethyl pigment had been the subject of several previous studies. It has been argued that the pretwist of the C11=C12 double bond in 11-*cis*-retinal due to steric clash of the 13-methyl group with the hydrogen at C10 is in part responsible for the rapid and selective photoisomerization around this double bond (*35*). Low-temperature FTIR spectroscopy has revealed that the twisting of the chromophore is different in 13-demethyl Batho compared with native Batho (*15*), and time-resolved UV−visible spectroscopy suggested a higher flexibility of the chromophore in the early intermediates (*36*). A recent resonance Raman analysis has shown that steric interaction of the 13-methyl group (and also of the 9-methyl group) is crucial for the distortion of the chromophore polyene in Batho (*37*). UV−visible experiments have been performed at 2 °C, pH 8.5 and 5.1 and revealed Meta I and Meta II states with inconspicuous absorption characteristics (*38*). The position of the Meta I/Meta II equilibrium in those experiments possibly deviates somewhat from our pK_A measurements. Another study reported similar activity toward phosphodiesterase for the photoproduct of 13-demethyl as for that of native rhodopsin at 30 °C (*39*). Much of the research on 13-demethyl had focused on the transient activation of opsin by binding of 11-*cis*-13-demethyl retinal (*40*, *41*); however, the properties of Meta I/Meta II of this analogue have not been examined in detail. In particular, all-trans 13-demethyl retinal had not been recognized previously as a pure partial agonist.

Our results show that removal of the 13-methyl group abolishes the C11=C12 HOOP mode of the chromophore in Meta I, implying that the chromophore is much more planar in the C11=C12 range in 13-demethyl Meta I than in native Meta I. The 13-methyl group appears to maintain strain on the chromophore in native Meta I, which might be necessary for efficient receptor activation. The lack of an amide I marker band of native Meta I at 1663 cm^{-1} indicates that the conformation of the protein backbone in 13-demethyl Meta I is changed as well. We have also observed a considerable alteration of hydrogen bonding of Glu 122 on TM helix 3, which forms an interhelical hydrogen-bonded network with Trp 126 on helix 3 and His 211 on helix 5, and which is part of the binding site of the ring of retinal. In native rhodopsin, this hydrogen bonding of Glu 122 is strengthened in the transition to Meta I, while it is weakened in 13-demethyl. This influence of the 13-methyl group on the hydrogen bonding properties of Glu 122 is presumably mediated by the ring of retinal. The altered polyene conformation in 13-demethyl Meta I does therefore also affect the positioning of the ring in its binding pocket and thereby the ring-Glu 122 interaction. Importantly, this alteration of the ring-protein interaction in 13-methyl Meta I is considerably different from that observed recently with pigments regenerated with ring-modified retinals, as partial deletion of the ring led to a pronounced strengthening of the hydrogen bonding pattern of Glu 122 (*3*).

The reduced steric interaction of the 13-methyl group with the protein in 13-demethyl Meta I, which leads to a more relaxed polyene conformation, increases the entropy of Meta I and reduces the entropy gain of the transition to Meta II. Possibly, the changed positioning of the ring weakens favorable interactions with hydrophobic residues in the ring binding pocket already in Meta I and not only in the transition to Meta II, which would result in the observed reduction of the enthalpy change.

The 13-methyl group has been postulated to rotate in Meta II from its position in the dark state by more than 90 ° toward Ala 117 (Figure 1C) and to translate (together with the retinal) by 4 to 5 Å along the retinal long axis toward helix 5 (*42*). Such a lateral movement could also involve steric interaction of the 13-methyl group with the protein, as for instance with the bulky side chain of Trp 265 on TM helix 6 (Figure 1D), thereby assisting the rotational outward motion of helix 6 observed during the transition to the active receptor conformation (*43*, *44*). Such a movement takes place on a much smaller scale possibly already in Meta I, as suggested by the recently published structure of Meta I (*45*).

9-Demethyl Analogue. 9-Demethyl is the classical partial agonist and has been examined in detail by a variety of methods including FTIR spectroscopy (*26*, *29*), UV−visible spectroscopy (*28*), and mutagenesis (*28*, *46*, *47*). In comparison to native pigment, electrophysiological experiments revealed a reduced, yet more persistent response of this pigment to photolysis (*48*, *49*). Recent studies have explained the reduced activity of 9-demethyl toward transducin by a shifted Meta I/Meta II equilibrium (*26*, *28*) and proposed an increased entropy of the Meta I state and a therefore diminished entropy change of the transition to Meta II as a dominant factor for this shift (*26*). This view has been corroborated in this study revealing a reduction of the $T\Delta S$ term by about 57 kJ/mol. The latter is only partially compensated by a concomitant reduction of ΔH by ca. 38 kJ/mol (Table 1).

The crystal structure of the dark state of rhodopsin reveals a special binding site for the 9-methyl group (*11*), which restricts rotational freedom of this group by interaction with residues on TM helices 3 (Thr 118) and 6 (Tyr 268) and on the extracellular loop 2 (EC2) (Ile 189 and Tyr 191), while translational motion along the retinal long axis toward Met 207 on helix 5 appears to be allowed (*42*) (Figure 1D). Anchorage of the 9-methyl group in this channel prevents relaxation of the chromophore prior to formation of Meta II and might help directing translation of the retinal toward helix 5 during receptor activation (*42*). In 9-demethyl, this locally tight interaction between retinal and the binding pocket is lost, resulting in relaxation of the chromophore already in Meta I and thus inefficient signal transduction (*26*). This view is supported by the lack of HOOP modes in 9-demethyl Meta I, which indicates a planar chromophore geometry similar as in 13-demethyl Meta I. Absence of the 9-methyl group also perturbs the positioning of the ring of retinal in its binding site, leading to an altered hydrogen bonding pattern of Glu 122. The presence of a defined binding site accommodating the 9-methyl group has been confirmed recently using retinal analogues with C-9 side

chains of varying size (*47*, *50*). Interestingly, this interaction of the 9-methyl group with the binding pocket is a special property of rod opsin only and is absent in cone pigments (*51*).

Allosteric Coupling between Glu 134 and Glu 113 in 13-Demethyl and 9-Demethyl. Previous experiments on rhodopsin solubilized in DM detergent indicate proton uptake during the transition from Meta I to Meta II, which is abolished in the E134Q mutant, where Glu 134 of the conserved ERY motif at the cytoplasmic terminus of TM helix 3 (Figure 1A) is replaced by a neutral Gln (*25*). Preliminary experiments performed in lipid membranes indicate that replacement of Glu 134 by Gln leads to pH-independent formation of the Meta II state (S. Lüdeke, T. P. Sakmar, F. Siebert, and R. Vogel, manuscript in preparation).

While replacement of Glu 134 by Gln considerably enhanced Meta II formation in 13-demethyl pigment by increasing the pK_A of its Meta I/Meta II equilibrium by about 3 units, the E134Q mutation was inefficient in that respect for the 9-demethyl analogue. The latter observation seems in conflict with the previous report of rescue of Meta II formation of 9-demethyl pigment in the E134Q mutant (*28*). Those experiments, however, were performed with pigment solubilized in DM and not in lipid membranes as in the present study. Solubilization of native rhodopsin in DM is known to perturb the energetics of the Meta I/Meta II equilibrium considerably, leading to exclusive formation of Meta II over the entire accessible pH range. Pigments solubilized in detergents such as DM show therefore a behavior considerably different from that of pigment in a native environment, as it is offered by lipid membranes. The current results even suggest a synergistic effect of the E134Q mutation and solubilization in DM, as the upshift of the pK_A by the E134Q mutation is considerably larger in DM than in membranes. This synergistic effect hints to a specific interaction of Glu 134 with the lipid environment during receptor activation. Similar discrepancies regarding the effect of the E134Q mutation were observed previously for pigments regenerated with acyclic retinals, which also show only partial agonist behavior (*27*). In membranes, the shift of the Meta I/Meta II equilibrium of an acyclic pigment to the side of inactive Meta I was hardly affected by replacement of Glu 134 by Gln(*3*), while in detergent rescue of Meta II formation was observed (*27*).

9-Demethyl and 13-demethyl respond to a different degree to the E134Q mutation. The pK_A shifts introduced by the mutation of less than one unit in 9-demethyl and about 3 units in 13-demethyl correlate to the different extent to which their Meta I/Meta II equilibrium is shifted toward the inactive side: all-trans 9-demethyl retinal is a considerably weaker partial agonist compared with all-trans 13-demethyl retinal. 9-Demethyl all-trans retinal and similarly the previously studied acyclic all-trans retinal are therefore deficient in coupling protonation changes at the ERY network at the cytoplasmic side of TM helices 3 and 6 to another group involved in the transition from Meta I to Meta II.

Which is this second group? The persistent pH-dependence of Meta I/Meta II in the E134Q mutants of both 9-demethyl and acyclic pigments reflects proton uptake during this transition by a group apart from the cytoplasmic microdomain around Glu 134. In case of the acyclic pigment, we have speculated recently that this residue could be Glu 113 (*3*) (Figure 1C), the counterion to the protonated Schiff base in the dark state (*52*, *53*), which is known to become protonated in the transition to Meta II not only in rhodopsin (*54*), but also in a series of mutant pigments and pigment analogues (*3*, *26*, *55*), as well as in the transition from the inactive to the active conformation of ligand-free opsin (*9*, *56*). The identification of Glu 113 as the second proton accepting group mentioned above is reasonable, but, of course, not stringent on basis of the data presented here.

These findings may contribute to our understanding of rhodopsin activation. In native rhodopsin (in membranes) the transition to Meta II is coupled to proton uptake (*57*) and depends on protonation of a presumably cytoplasmic group of the ERY network (*25*), and of Glu 113 (*55*), which forms the salt bridge to the protonated Schiff base. Protonation of one of these groups increases allosterically the pK_A of the other group sufficiently to induce protonation of the other group and to allow the transition to Meta II. In the Meta I/Meta II equilibrium, the pK_A values of the cytoplasmic group and Glu 113 are therefore highly coupled by an allosteric mechanism. Such a model had been already proposed and extensively examined in previous studies (*58*). It is in agreement with studies on active-site methylated rhodopsin, where the salt bridge to Glu 113 is stabilized by a permanent positive charge on the nitrogen, concomitantly decreasing the pK_A of Glu 113. This inhibited protonation of Glu 113 in the photoproducts and locked the pigment in a Meta I-like conformation (*59*, *60*).

Our results show that in 13-demethyl the coupling between the cytoplasmic group and Glu 113 is weaker than in native rhodopsin but still quite dominant. It is considerably weakened for 9-demethyl (this study) and for acyclic pigment (*3*). This implies that retinal and in particular portions of its ring and the 9-methyl group of the polyene are crucial elements of this coupling mechanism. Deletion of one of these elements impairs the coupling between the cytoplasmic ERY network and the Schiff base region and results in partial agonism. Possibly, the coupling between these two groups becomes significant particularly in the transition from Meta I to Meta II and is less pronounced in the dark state. Recent NMR results suggest that this allosteric coupling may be induced by retinal translational motion and helix rearrangement (*42*).

Introduction of Additional Methyl Groups. We introduced additional methyl groups at either C10, C12, or C14 with very different consequences. Addition of a methyl group at C14 had only minor impact on the FTIR photoproduct spectra and on the position of the Meta I/Meta II equilibrium at 20 °C, even though both ΔH and ΔS were increased. The speed of regeneration was very slow, indicating interference of the additional methyl group at C14 with docking in the retinal binding site. On the other hand, the visible absorption maximum in the 9-cis dark state was similar to that of native Iso. In the crystal structure of the dark state of rhodopsin, a C14-methyl group would be positioned close to Glu 113 and Ala 117 on TM helix 3 and Cys 187 on the E2 loop, which does, however, not necessarily reflect the true situation with the 9-cis isomer that we used in our study. Our data indicate that the additional volume of this group at the position of C14 is not critical in the all-trans photoproduct states and does not substantially interfere with receptor activation.

In contrast, the effect of an additional methyl group at either C10 or C12 was very pronounced. The Meta I/Meta II transition is considerably perturbed in both the 10- and 12-methyl pigments with ΔH and ΔS values that are similarly reduced as in 9- and 13-demethyl and a pK_A of around 5 at 20 °C. In contrast to the demethylated pigments, the hydrogen-bonding pattern of Glu 122 is only slightly changed compared with native pigment, indicating a rather regular positioning of the ring in the binding pocket in the dark and in the photoproduct states. Therefore, additional methyl groups at either C10 or C12 likely perturb the packing of the transmembrane helices around the polyene in the photoproduct states. This might interfere with the correct anchoring of the 13- or also 9-methyl groups in the binding pocket and thus lead to a similarly defect receptor activation as with the demethylated pigments.

Conclusions. Removal of either the 9- or 13-methyl group of native all-trans retinal allows a more relaxed fit of the resulting chromophore into the inactive binding pocket of Meta I. This renders Meta I in these pigments a high-entropy state and makes the transition to the enthalpically higher Meta II state less favorable, despite the observation that ΔH of Meta I/Meta II is reduced as well. Together with the ring methyl groups at C5 and C1 (*3*), both the 9- and the 13-methyl groups constitute important anchoring points of retinal to the binding pocket during receptor activation. Despite that deletion of the 13-methyl group leads to a less deficient pigment than deletion of the 9-methyl group in regard of the position of the Meta I/Meta II equilibrium at 20 °C, the impact on both ΔH and ΔS of this equilibrium are more pronounced in the 13-demethyl analogue than in the 9-demethyl analogue, which has not been appreciated up to now. Partial agonism in these pigments is at least in part due to an impaired coupling between proton uptake at the cytoplasmic ERY motif and the pK_A of presumably Glu 113 during the transition to Meta II. While this allosteric coupling is only mildly perturbed in the 13-demethyl pigment, it is strongly impaired in 9-demethyl and in a previously studied acyclic pigment (*3*). Finally, while introduction of an additional methyl group at C14 led to only mild perturbations of Meta I/Meta II, resulting in an increase of both ΔH and ΔS of the transition, but not a shift of the equilibrium position, addition of methyl groups at either C10 or C12 had a similar impact as removal of the 9- or 13-methyl groups.

ACKNOWLEDGMENT

We thank K. Gunnarsson, T. Huber, E.C.Y. Yan, M.A. Kazmi, and B. Mayer for their help in preparation of the pigments. We further thank W. Sevenich, W. D. Schielin, P. Merkt, and K. Zander for their technical support.

SUPPORTING INFORMATION AVAILABLE

Supporting Information with additional figures on 13-demethyl Iso, the temperature dependence of the Meta I/Meta II equilibrium of the studied pigments, a derivation of the expressions for ΔH and ΔS, and a scheme with the retinal isomers used for regeneration. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI052196R