Agonists and Partial Agonists of Rhodopsin: Retinals with Ring Modifications†

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ABSTRACT: Activation of the visual pigment rhodopsin is initiated by isomerization of its retinal chromophore to the all-*trans* geometry, which drives the conformation of the protein to the active state. We have examined by FTIR spectroscopy the impact of a series of modifications at the ring of retinal on the activation process and on molecular interactions within the binding pocket. Deletion of ring methyl groups at C1 and C5 or replacement of the ring in diethyl or ethyl-methyl acyclic analogues resulted in partial agonists, for which the conformational equilibrium between the Meta I and Meta II photoproduct is shifted from the active Meta II side to the inactive Meta I side. While the Meta II states of these artificial pigments had a conformation similar to those of native Meta II, the Meta I states were different. Modifications on the ring of retinal had a particular impact on the interaction of Glu 122 within the ring-binding pocket and are shown to interfere with the Glu 134-mediated proton uptake during formation of Meta II. We further found, upon partial deletion of ring constituents, a decrease of the entropy change of the transition from Meta I to Meta II by up to 50%, while the concomitant reduction of the enthalpy term was less pronounced. These findings underline the particular importance of the ring and the ring methyl groups and are discussed in a model of receptor activation.

Rhodopsin is the visual pigment responsible for dim light vision in the rod photoreceptor cells of vertebrates. It is a membrane protein with seven membrane-spanning helices and belongs to the large family of G protein-coupled receptors (GPCR)[1] (*1*−*3*). Rhodopsin gains its light sensitivity by its 11-*cis* retinal chromophore covalently linked by a protonated Schiff base to the protein. This chromophore is accommodated within the α-helix bundle in the transmembrane domain of the receptor. Absorption of a photon initiates activation of the light receptor by chromophore isomerization to an all-*trans* configuration. The resulting highly strained receptor relaxes then on the time scale of milliseconds via several still inactive intermediates to the active receptor conformation, Meta II, which couples to the G protein.

This active Meta II conformation is at room temperature in equilibrium with its still inactive precursor Meta I. The transition from Meta I to Meta II is favored by increasing temperature and, as it involves uptake of a proton from its cytoplasmic side, by lowering external pH. G protein activation assays suggested that also the apoprotein of the receptor, opsin, shows some activity at low pH in the absence of a ligand (*4*). A recent Fourier transform infrared (FTIR) study eventually showed that the apoprotein opsin forms a pH-dependent conformational equilibrium between active and inactive conformations (*5*). This conformational equilibrium was found to be similar to that of the Meta I/Meta II states, yet shifted to much more acidic pH. While the pK_A of the Meta I/Meta II equilibrium is above 8 at 30 °C and thus almost completely on the active Meta II side under neutral conditions, the pK_A of the opsin equilibrium was found to be slightly above 4 at the same temperature, being therefore on the side of the inactive opsin conformation at neutral pH. The dark state of rhodopsin, on the other hand, with its 11-*cis* retinal chromophore, remains completely inactive over the entire pH range at which the receptor remains stable.

These findings were not unexpected, considering rhodopsin is a member of the G protein-coupled receptor family. For GPCRs, an allosteric ternary complex model had been established by Lefkowitz and co-workers (*6*) to describe the influence of both ligands and G protein on the conformation of the receptor apoprotein. In this framework, the ligand-free receptor is with a certain probability, p, in an active conformation and with the probability $1 - p$ in an inactive conformation, thereby establishing a conformational equilibrium similar to that observed for opsin. Considering only receptor−ligand interactions, binding of a ligand changes the active conformation probability p. Ligand binding will probably also modify to some extent the conformation of the active state itself due to specific ligand−protein interactions. If binding of a certain ligand increases p, it is termed an *agonist* in a pharmacological sense. If it shifts the

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[1] Abbreviations: GPCR, G protein-coupled receptor; FTIR, Fourier transform infrared spectroscopy; HOOP, hydrogen-out-of-plane.

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equilibrium instead to the inactive side, it is termed an *inverse agonist*. Finally, ligands that bind to the binding site on the receptor without affecting the conformational equilibrium are termed *antagonists* or *neutral agonists*.

In the case of rhodopsin, all-*trans* retinal constitutes obviously an agonist, while 11-*cis* retinal is a strong inverse agonist, which locks the receptor in its inactive conformation. Because of the pH dependence of the involved conformational equilibria, it is helpful to characterize them by their intrinsic pK_A at a given temperature. In the past, opsin had been reconstituted with a large number of chemically modified retinals, and the resulting pigment analogues and their photoproducts had been studied. Many of these pigments were characterized as *inactive* after light absorption, as their photoproducts did not activate rhodopsin's cognate G protein transducin under certain experimental conditions. Considering the pharmacological classification scheme above and opsin's intrinsic conformational equilibrium, such a characterization seems insufficient. Instead, the pH dependence (and, if necessary, also the temperature dependence) of the Meta I and Meta II photoproducts of such pigments should be studied over a wide range, such that the apparent pK_A values of the involved photoproduct conformational equilibria can be determined. These pK_A values can then be compared to those of native Meta I/Meta II and of the opsin conformational equilibrium obtained under the same conditions. Such a procedure would allow to classify chromophore−protein interactions as *favorable* for attaining an active receptor state (in the case of an agonist) or *unfavorable* (in the case of an inverse agonist).

What can we learn from the study of such analogues? Over the past five years, we have seen several crystal structures of the dark state of rhodopsin from different crystal types (*2*, *7*, *8*), which gave important insight into the function of the receptor. They also served as starting points to explore the photoreaction and the subsequent protein reactions by, for example, molecular dynamics simulations, QM-MM calculations, or other types of modeling using distance constraints obtained by structurally sensitive experimental techniques. The results of such studies need, however, to be validated by experimental evidence. From a crystallographic point of view, there is presently only one structure of a photointermediate of rhodopsin available, that of the still inactive Meta I state at relatively low resolution (*9*). NMR methods, on the other hand, become more and more important in contributing to a detailed picture of the events leading to activation of rhodopsin. In particular, a recent study by Smith and co-workers seems to highlight a promising way (*10*). By using a combination of 2D dipolar-assisted rotational resonance NMR and ^{13}C labeling, they were able to derive distance constraints between the chromophore and specific protein residues in the dark state and in Meta II. Importantly, ^{13}C labels are sterically inert and reflect therefore the actual situation in the native pigment. Smith and co-workers were able to track the path of the retinal chromophore within its binding pocket relative to selected protein residues during activation of the receptor. It consists of a relatively large (4−5 Å) translation along its long axis toward helix 5. Their results suggest that retinal serves as a scaffold that couples the motions of different parts of the protein to achieve the active state. On the background of those findings and to enhance our understanding of the role of retinal, we study the influence of modifications on the retinal on the signal transduction from the isomerized chromophore to the protein. In this study, we focus on modifications of the ring moiety of retinal (Figure 1), which plays an important role for the docking of retinal in its binding pocket and for the conformational changes leading to activation of the receptor after photolysis. In a subsequent paper, we will focus on modifications along the polyene of retinal (Vogel, R., Siebert, F., Hirshfeld, A., and Sheves, M., manuscript in preparation). We derive pK_A values of the Meta I/Meta II equilibrium using different retinal analogues and particularly put emphasis on characterizing chromophore−protein interactions in the still inactive Meta I states of these artificial pigments. Finally, we derive the thermodynamic parameters of the equilibrium between Meta I and Meta II in some of these pigments.

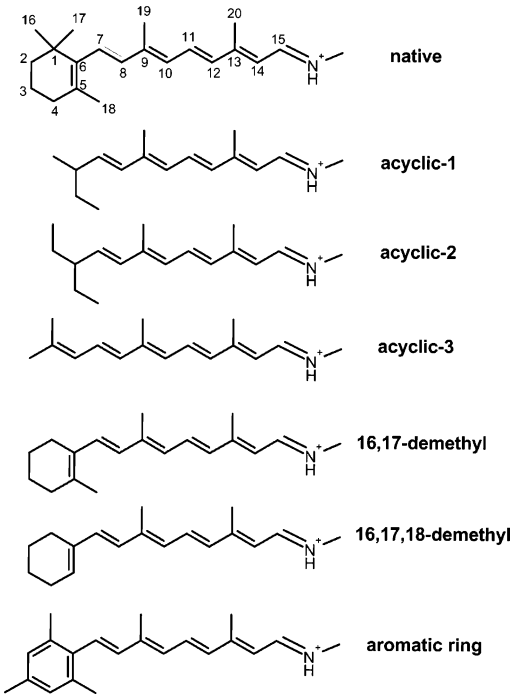

FIGURE 1: Modified retinals used in this study. The retinals are shown as all-*trans* isomers of the protonated Schiff bases. Regeneration of the pigment was achieved with the respective 9-*cis* isomer (except for the aromatic ring analogue, which was 11-*cis*).

MATERIALS AND METHODS

Pigment Preparation. Rhodopsin in washed disk membranes was prepared from cattle retinae according to standard procedures (*11*) and stored at −20 °C. Preparation of rhodopsin with modified chromophores was accomplished by regenerating opsin with synthetic retinals (0.5−1.0-fold molar excess) as described previously (*12*); approximate regeneration times and efficiencies are stated in Results. Isorhodopsin, which was used for control experiments and comparison spectra was prepared by opsin regenerated with 9-*cis* retinal (Sigma-Aldrich, Germany). Absorption peaks of retinals were obtained in ethanol. Absorption peaks of the dark states of artificial pigments were obtained from the depletion peaks upon illumination in a 1% dodecyl maltoside detergent solution in the presence of 10 mM hydroxylamine. Despite that the positive oxime peak absorbs in the UV relatively far from the dark state absorption of the pigment,

the positions determined by this method may be slightly higher than the true positions, in particular for pigments with absorption peaks more in the blue spectral range.

Mutant pigments E122Q and E134Q were prepared in the lab of Thomas P. Sakmar with the native 11-*cis* retinal as chromophore according to established methods and reconstituted into PC vesicles (*13*). Chromophore exchange was achieved by bleaching the pigment in the presence of a synthetic chromophore, which led to regeneration of the mutant pigment analogue.

Preparation of Modified Retinals. The retinal analogues were prepared according to previously described methods: acyclic-1, -2, and -3 retinals (all as 9-*cis* isomers) (*14*, *15*), 9-*cis* 16,17-demethyl, 9-*cis* 16,17,18-demethyl retinals (*16*, *17*), and 11-*cis* aromatic retinal (*18*).

FTIR Spectroscopy. FTIR spectroscopy was performed with a Bruker IFS 28 spectrometer with an MCT (mercury cadmium telluride) detector. IR 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 about 0.5 nmol pigment, that were prepared as described in detail elsewhere (*5*), with the exception that we used 40 μL of buffer at pH extremes to ensure precise pH adjustment (*19*). This sample type allows to precisely control the water content, pH value, and salt concentration in the samples and shows, in particular, Meta I/Meta II titration curves identical to membrane suspensions (*19*). As buffers, we used 200 mM citric acid, MES (2-*N*-morpholinoethanesulfonic acid), and BTP (Bis-Tris-propane), in overlapping ranges. 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.

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 530 nm (for native Iso and rhodopsin and for the demethylated pigments), 495 nm (for the aromatic ring analog), 455 nm (for acyclic-1 and acyclic-2), and 550 nm (for acyclic-3).

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.

Determination of pK_A Values of Conformational Equilibria. pK_A values were determined from pH series of FTIR spectra. Basis spectra were acquired under conditions where the conformational equilibrium was fully on either the active or the inactive side. Normalized spectra from the pH series were then fitted to the linear combination $a \cdot active + (1 - a) \cdot inactive$, where *active* and *inactive* represent these two basis spectra, to determine the position of the equilibrium. For the fitting procedure, we used the spectral range between 1600 and 1800 cm^{-1}, which contains amide I bands as well as vibrations of carboxylic acids that are all sensitive to the conformation of the protein. The coefficients *a* were then fitted to a Henderson−Hasselbalch equation for one proton, $a = 10^{(pKA-pH)}/(1 + 10^{(pKA-pH)})$, to determine the p$K_A$. This method is generally practicable, as long as pure basis spectra can be obtained. In the case of acyclic-1 and −2, where the assessment of the purity of the Meta II reference spectrum was more difficult due to extremely low pK_A values, we performed additional experiments in which a Meta II state was stabilized at higher pH by the presence of a 20-fold excess of a synthetic high affinity peptide analogue to the transducin α-subunit C-terminus (VLEDLKSCGLF) (*20*). In the case of the opsin equilibrium, a pure active state spectrum could not be obtained and we used instead the intensity of the amide II marker band of the active state at 1644 cm^{-1}. These values were normalized to that of a peptide-stabilized active state of opsin (*5*) after subtraction of peptide specific contributions, and again fitted to a Henderson−Hasselbalch equation. The error of the derived pK_A values determined by this method is estimated to be less than 0.2 units and arises mainly from uncertainties regarding the purity of the Meta II reference states and, to a small extent, the pH adjustment within the sample. Temperatures are estimated to be accurate within 0.5 °C. The buffers were pH-adjusted at 20 °C. For the pK_A analysis at other temperatures, the temperature dependence of buffer pH was taken into account by remeasuring the pH at the desired temperature and taking this value as the true sample pH. Otherwise, the stated pH values correspond to those measured at 20 °C.

Calculation of ΔH and ΔS from the Measured pK_A Values at Different Temperatures. See section A3 in the Supporting Information.

Molecular Models. Molecular graphics are based on the coordinates of the dark state by Li and Schertler (*2*) and were prepared with the software Deep View 3.7 (*21*) (available at http://www.expasy.org/spdbv) and POV Ray 3.5 (available at http://www.povray.org).

RESULTS

Conformational Equilibria of Native Meta I/Meta II and of the Apoprotein Opsin. With native rhodopsin, the pK_A of the conformational equilibrium between the active Meta II state (λ_{max} 380 nm) and its still inactive precursor Meta I (λ_{max} 480 nm) are traditionally determined using UV−visible spectroscopy (*22*, *23*). The pigment analogues examined in this study form in part Meta II states with protonated Schiff base, as we will show below, such that the Meta II states in these analogues are not easily distinguished from their inactive Meta I counterparts by UV−visible spectroscopy solely. We therefore employed instead FTIR spectroscopy to examine the pH dependence of the Meta I/Meta II conformational equilibrium (see Material and Methods and below). With FTIR spectroscopy, we monitor directly the conformation of the protein and do not need to make additional assumptions regarding the correlation between the UV−visible absorption of the chromophore and the actual conformation of the receptor. This is particularly valuable in regard of previously revealed isospectral photointermediates (*24*, *25*). To determine pK_A values of the Meta I/Meta II equilibria, we obtained for each pigment basis spectra under conditions where the respective equilibrium was fully on the Meta I and the Meta II side, respectively. Spectra obtained in an intermediate range, where both species are present, were decomposed into these basis spectra by a linear combination approach, and the position of the equilibrium could then be calculated and fitted to a Henderson−Hasselbalch equation as described in Material and Methods. Application of this method to native rhodopsin in disk membranes yielded a pK_A of the Meta I/Meta II conforma-

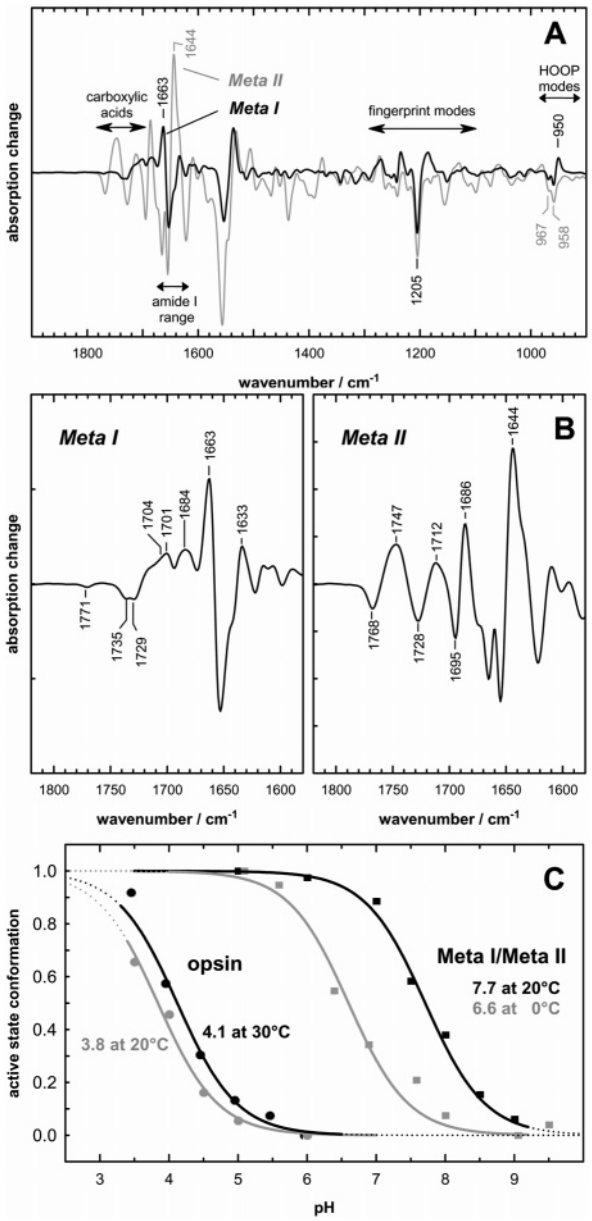

FIGURE 2: Meta I/Meta II and opsin conformational equilibrium of native rhodopsin and isorhodopsin. (A) FTIR difference spectra of pure Meta I and Meta II states were obtained at 0 °C, pH 9.0, and at 10 °C, pH 5.0, respectively. Absorption ranges of protonated carboxylic acids and amide I modes of the protein and of the fingerprint and HOOP modes of the chromophore are marked. The spectra were obtained from isorhodopsin (9-*cis*) for easier comparison with following spectra. (B) This close-up shows the spectral features of Meta I and Meta II in the spectral region of carboxylic acid and amide I bands that are sensitive to the conformation of the protein. (C) The pH dependence of the opsin and the Meta I/Meta II conformational equilibria of rhodopsin at different temperatures. The pH curves of both the opsin and the Meta I/Meta II equilibria were derived from FTIR data and reflect the conformational transition of the receptor.

tional equilibrium of 6.6 at 0°C and of 7.7 at 20 °C (Figure 2C).

For technical reasons, the chromophore analogues that were used in this study were synthesized as 9-*cis* isomers (yielding isorhodopsin pigments) instead of 11-*cis* isomers (yielding rhodopsin pigments), except for the aromatic ring analogue, which was 11-*cis*. This is not a major obstacle, as the room-temperature photoproduct equilibrium between Meta I and Meta II can be supposed to be independent of the initial state being 11-*cis* rhodopsin or 9-*cis* isorhodopsin. This is supported by same pK_A values of the respective Meta I/Meta II equilibria (see Supporting Information Figure A1). To allow an easier comparison between IR spectra obtained from pigments regenerated with 9-*cis* retinal analogues with those obtained from pigment with native retinal, we used isorhodopsin to produce FTIR control spectra of the native Meta I and Meta II states.

The FTIR difference spectra *Meta I minus dark state* and *Meta II minus dark state* (Figure 2A) reflect changes of both the chromophore and the protein occurring during the light-induced transition to the Meta I and Meta II photoproduct states, respectively. In this representation, bands of the photoproduct states are positive, while those of the initial state are negative. In Figure 2A, we have highlighted the hydrogen-out-of-plane (HOOP) range of the chromophore at the low wavenumber end with a pronounced Meta I marker band at 950 cm^{-1} and the fingerprint range of the chromophore with coupled C−C stretching vibrations of the polyene, which are specific for the chromophore isomer. Around 1650 and 1550 cm^{-1}, we observe difference bands in the amide I and amide II range, respectively, which are collective modes of the protein backbone. In the amide I range, marker bands of both the Meta I and the Meta II states can be found at 1663 and 1644 cm^{-1}, respectively. Around 1550 cm^{-1}, there is in addition the strong ethylenic (C═C stretch) mode of the chromophore. Above 1700 cm^{-1}, we observe difference bands of membrane embedded carboxylic acids, which reflect changes in hydrogen bonding and are therefore as well sensitive markers of conformational changes.

The spectral range between 1600 and 1800 cm^{-1} is of particular importance to analyze the conformations of Meta I and Meta II. We present therefore a close-up of this range in Figure 2B and will give a short summary of the difference bands observed in this range. In Meta I, the band pattern above 1700 cm^{-1} comprises a small negative band of Asp 83 on helix 2 at 1771 cm^{-1} and a difference band of Glu 122 on helix 3, consisting of a heterogeneous absorption at 1735 and 1729 cm^{-1} in the dark. In Meta I, the Glu 122 band is downshifted to around 1704 cm^{-1} in the high-frequency side of the positive peak at 1701 cm^{-1}, which itself is perhaps a high-frequency amide I mode ((*13*) and our unpublished observations) (band positions may be slightly different compared to those in the original publication, which were obtained from 11-*cis* rhodopsin). Sensitivity of these bands to replacement at the position of His 211 on helix 5 indicated a functional interaction between both side chains, which was confirmed in the X-ray structures published recently (Figure 3). The Meta I bands at 1663 cm^{-1} and at 1633 cm^{-1} are presumable amide I modes. Superimposed to the pattern of carboxylic acid modes in Meta I is a so far unreported difference band due to changes of a lipid ester C═O. Experiments performed with rhodopsin reconstituted into *sn*1,*sn*2-13C-labeled palmitoyl-oleyl PC vesicles revealed this difference band with a dark absorption at 1742 cm^{-1} and a Meta I absorption at 1721 cm^{-1}, which is insensitive to H_2O/D_2O exchange (Vogel R., unpublished observation).

In the Meta II spectrum, we observe a distinctive band pattern in the range above 1700 cm^{-1}, consisting of the −1768/+1749 cm^{-1} difference band of Asp 83 and the −1728/+1745 cm^{-1} difference band of Glu 122 (*26*, *27*),

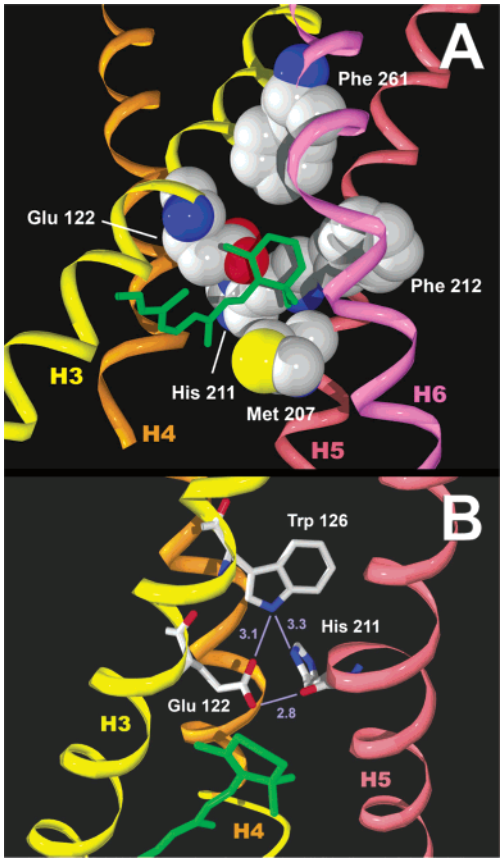

FIGURE 3: Molecular model of the environment of the ring in the dark state. (A) The retinal chromophore is shown as a green stick model together with part of the residues (in full sphere representation) that line the retinal binding pocket. Glu 122 on helix 3, hydrogen-bonded to His 211 on helix 5, contacts one side of the ring; Trp 265 and Tyr 268 on helix 6 line the other side, but have been omitted in this graph. Met 207 and Phe 212 on helix 5 and Phe 261 further contribute to confine the ring. In this view, helices 1, 2, and 7 have been omitted for clarity. The cytoplasmic side is at the top. (B) Hydrogen-bonding network between Glu 122 and Trp 126 on helix 3 and His 211 on helix 5. Glu 122 is in hydrogen-bonding distance to the ring NH of Trp 126 and to the backbone carbonyl of His 211.

which form together the peak at 1747 cm^{-1} in Meta II. Superimposed is again the absorption change of a lipid ester C=O at −1727/+1744 cm^{-1} (*28*, *29*). The positive band at 1712 cm^{-1} reflects protonation of Glu 113 upon formation of Meta II (*30*), and the strong Meta II band at 1644 cm^{-1} reflects an amide I mode. The positive bands at 1684 cm^{-1} in Meta I and 1686 cm^{-1} in Meta II have not been assigned yet and could be amide I modes or C=O modes of Asn or Gln side chains. This band pattern of Meta II in the 1600−1800 cm^{-1} range with its distinct amide I and carboxylic acids changes is characteristic of the active state conformation.

To obtain information about the conformational equilibrium of the apoprotein opsin, we used a previously published method (*5*). We illuminated rhodopsin at either 20 or 30 °C with a 530 nm long-pass filter and followed the decay of the photoproducts via hydrolysis of the retinal Schiff base and dissociation of all-*trans* retinal to the final product opsin. To avoid interference of all-*trans* retinal with the apoprotein either by re-binding into the original binding pocket or to other binding sites (*31*), which might influence the conformation of the protein, these experiments were performed in the presence of 50 mM hydroxylamine. This considerably accelerates the hydrolysis reaction and scavenges all-*trans* retinal as inert retinaloxime. Quantitative conversion to oxime was verified in parallel UV−visible experiments, which was particularly important at pH < 4.5, where the reaction is slowed due to a decreased reactivity of hydroxylamine. The resulting pK_A of the opsin equilibrium was calculated to be 3.8 at 20 °C and 4.1 at 30 °C (Figure 2C) in agreement with previously published data obtained under somewhat different sample conditions (*5*).

Acyclic-1 (Methyl,ethyl Acyclic). Acyclic-1 9-*cis* retinal absorbed at 361 nm in ethanol. It regenerated within 2 h at room temperature quantitatively with opsin to form a pigment absorbing at 456 nm, in agreement with published data (*14*, *15*).

Despite of the replacement of the ring by a methyl and an ethyl group, the acyclic-1 pigment is capable of forming both Meta I and Meta II states (Figure 4A), albeit the latter only at higher temperature and more acidic pH. The acyclic-1 Meta II FTIR difference spectra obtained at pH 4.0, 30 °C, and similarly also at 20 °C, are comparable to those of native pigment. The positive Meta II band at 1686 cm^{-1} and the −1664/+1645 cm^{-1} Meta II difference band in the amide I range, of which the latter may also contain contributions of the C=N stretching mode of the chromophore, are slightly larger than in the case of native Iso. The lack of an uncompensated ethylenic mode of the dark state (which is found for native Iso at 1556 cm^{-1}) indicates that the Schiff base remains protonated in Meta II of acyclic-1 at pH 4.0 (compare ref *32*), in agreement with UV−vis spectra. In a series of experiments, acyclic-1 Meta II was stabilized also at higher pH by the presence of a transducin-derived peptide analogue (VLEDLKSCGLF) (*20*). This allows to monitor the Schiff base protonation state in the Meta II/peptide complex also at higher pH. In the complex, the Schiff base was again found to be fully protonated at pH 4.0, but largely deprotonated at pH 7.0, indicating a titratable Schiff base in the active state, at least in its peptide complexed form (spectra not shown). Importantly, the positive band at 1712 cm^{-1} reflecting protonation of the Schiff base counterion in the dark, Glu 113, is present irrespective of the protonation state of the Schiff base.

In Figure 4B, we show the pH dependence of Meta II formation of the acyclic-1 pigment at 20 °C, as determined by FTIR spectroscopy. The pK_A of the Meta I/Meta II equilibrium was found to be 4.8. It is therefore by almost 3 units lower than that of pigment with native retinal and only 1 unit above that of ligand-free opsin. The all-*trans* acyclic-1 ligand constitutes therefore a weak partial agonist in regard of receptor activation.

This property allows to study the Meta I state of acyclic-1 over a very broad range of temperatures, which is in native pigment handicapped by the transition to Meta II.

In the temperature range between −7 and 20 °C, the Meta I state of acyclic-1 differs in several respects from native Meta I (Figure 4A). We notice the lack of the Meta I C11=C12 HOOP band, which is found in native Meta I at 950 cm^{-1} (*33*). HOOP vibrations acquire large IR intensity only if the polyene is twisted around single bonds next to the double bond (*34*). The lack of the C11=C12 HOOP in Meta I of acyclic-1 indicates that removal of the ring removes torsion along the polyene and renders the chromophore planar

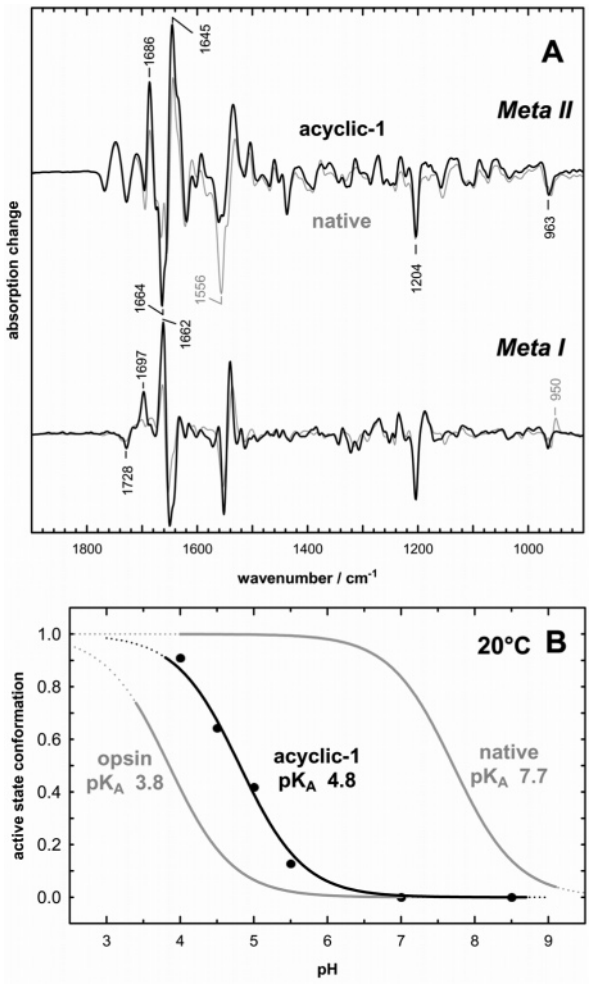

FIGURE 4: Meta I and Meta II with the acyclic-1 ligand. (A) Meta I and Meta II FTIR difference spectra of acyclic-1 (black) were obtained at 0 °C, pH 7.0, and at 30 °C, pH 4.0, respectively, and are compared to spectra of the native states (gray) obtained from Iso under conditions as in Figure 2A. The lack of a pronounced negative ethylenic mode at 1556 cm^{-1} in Meta II reveals a largely protonated Schiff base in Meta II, while the lack of a positive HOOP mode at 950 cm^{-1} in Meta I indicates less torsion in the polyene compared to Meta I with native retinal. In the range above 1700 cm^{-1}, Meta I of acyclic-1 differs considerable from native Meta I, which is enlarged in Figure 5. (B) The pK_A of the Meta I/Meta II equilibrium of acyclic-1 is shifted to more acidic values by almost 3 units compared to that of the native Meta I/Meta II equilibrium and only 1 unit higher than that of the opsin equilibrium in the absence of ligand. Similar spectra and a similar shift of the Meta I/Meta II equilibrium were obtained for acyclic-2 (see text).

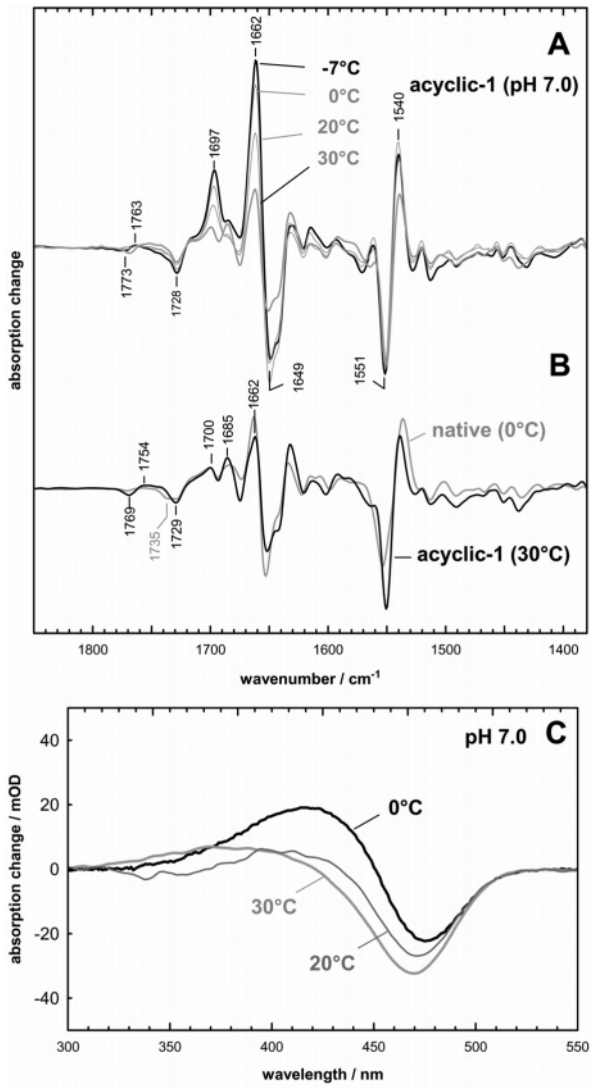

FIGURE 5: Temperature dependence of the Meta I conformation of acyclic-1. (A) Meta I FTIR difference spectra of acyclic 1 obtained at pH 7.0 in the range from −7 to 30 °C show a marked temperature dependence, which is particularly pronounced for the band of Glu 122 at 1697 cm^{-1} and the amide I band at 1662 cm^{-1}. (B) A close-up of the Meta I spectra of Figure 4A in the high-frequency region allows a more detailed comparison between Meta I obtained from acyclic-1 at 30 °C and pH 8.5 and Meta I obtained from native Iso at 10 °C and pH 9.0. The spectra reveal a relatively close similarity of native Meta I to the high-temperature Meta I state of acyclic-1, in contrast to its low-temperature Meta I state. (C) UV−visible difference spectra of acyclic-1 reveal at 0 °C a blue-shifted Meta I state compared to the initial state. The absorption coefficient of Meta I decreases as the temperature is raised, such that the negative depletion peak becomes more prominent. In addition, at 30 °C, the photoproduct has also some UV absorption. All spectra were recorded at pH 7.0 and were normalized to a dark state absolute absorption of approximately 90 mOD.

in the C10 to C13 segment already in Meta I. In native pigment, this planarity is reached only upon transition to Meta II. Figure 5A,B allow a more detailed comparison in the range between 1400 and 1800 cm^{-1}, comprising the amide I and II ranges at around 1650 and 1550 cm^{-1}, respectively, and the C═O stretch vibrations of protonated carboxylic acids above 1700 cm^{-1} at different temperatures. At low temperature, we notice a sharp Meta I amide band at 1662 cm^{-1} and the appearance of an intense band at 1697 cm^{-1}. The identity of this latter photoproduct band with the C═O stretch of Glu 122 was verified using the E122Q mutant regenerated by acyclic-1 retinal (Figure 6A). Replacement of Glu 122 by Gln abolishes the difference band at −1728/+1697 cm^{-1} and introduces a new difference band in the mutant located at −1695/+1672 cm^{-1} due to the C═O stretch of the glutamine. In the mutant pigment, we see a residual difference band at −1744/+1726 cm^{-1}, which presumably corresponds to the lipid band of native Meta I (see above).

The absorption of Glu 122 in acyclic-1 Meta I at 1697 cm^{-1} is unusually low for a carboxylic acid C═O stretch. It is known that the position of the C═O stretch of carboxylic acids depends on the hydrogen-bonding properties of both their C═O and OH groups ((*35*) and references therein). A recent study examined these properties by use of density

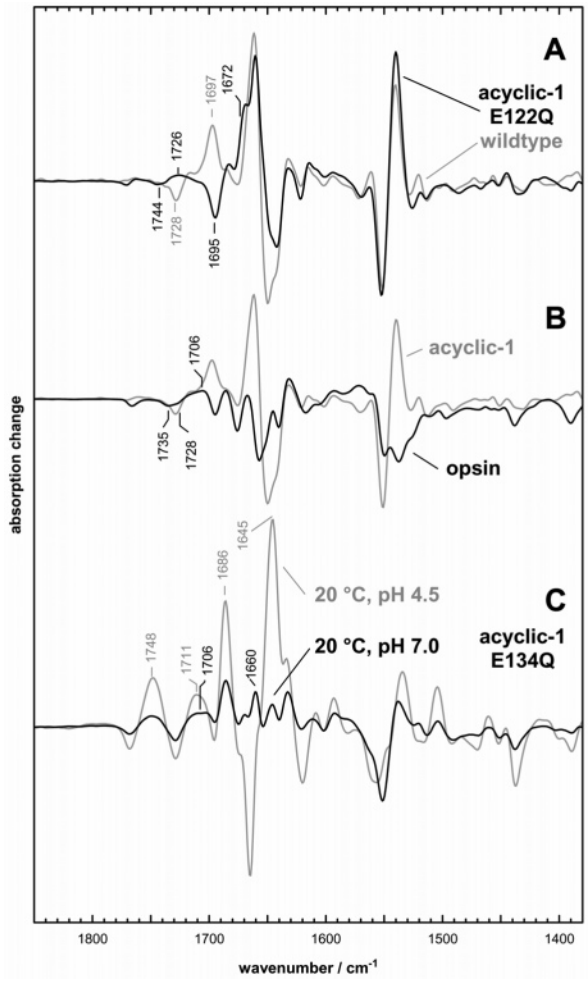

FIGURE 6: Role of Glu 122 and Glu 134 in the Meta I to Meta II transition of acyclic-1. (A) Meta I difference spectra from acyclic-1 wild-type and acyclic-1 E122Q were recorded at 0 °C and pH 7.0 and 6.5, respectively. The difference band at $-1728/+1697$ cm^{-1} of the C═O stretch of Glu 122 in wild-type is replaced by the difference band at $-1695/+1672$ cm^{-1} of the C═O stretch of Gln 122. (B) Comparison of the Meta I difference spectrum of acyclic-1, obtained at 20 °C and pH 7.0, with an inactive opsin state minus dark state difference spectrum of rhodopsin obtained at 20 °C and pH 6.5 in the presence of 50 mM hydroxylamine. In the inactive opsin state, the C═O stretch of Glu 122 is at 1706 cm^{-1}, similar as in native Meta I, but different from Meta I of acyclic-1. (C) Replacement of Glu 134 by glutamine considerably changes the Meta I conformation of acyclic-1 but does not abolish the pH dependence of Meta I/Meta II. At 20 °C and pH 4.5, a Meta II photoproduct is obtained (gray spectrum), while at pH 7.0, the photoproduct is in a Meta I conformation (black spectrum). In contrast to this, E134Q with native retinal forms under the same conditions a Meta II photoproduct irrespective of pH.

function theory and normal-mode analysis (*36*). They found that strong hydrogen bonds to the C═O or the hydrogen of OH shift the position of the C═O stretch from 1776 cm^{-1} in a vacuum by each about $30-35$ cm^{-1} to lower wavenumbers, while hydrogen bonding to the OH oxygen may increase the C═O frequency by up to 11 cm$\cdot^{-1}$. The very low position of the C═O stretch of Glu 122 at 1697 cm^{-1} indicates strong hydrogen bonding with both the C═O and the OH group as hydrogen-bonding acceptor and donor, respectively.

Glu 122 is positioned next to the ring in the binding pocket (Figure 3A). Is this special interaction of Glu 122 a mere consequence of removal of bulk volume from the ring? To probe this possibility, we may compare a Meta I spectrum of acyclic-1 with a corresponding difference spectrum of the inactive opsin state (*5*), where the binding pocket is empty (Figure 6B). In the inactive opsin state, the C═O stretch of Glu 122 is found at 1706 cm^{-1}, which was further verified using the E122Q mutant (data not shown). This is close to its position in native Meta I but not in Meta I of acyclic-1. This indicates that the ring modification creates a special environment for Glu 122, which is different both from that in native Meta I and that in the ligand-free inactive opsin state.

As the temperature is increased, the Meta I conformation of acyclic-1 changes. Both the Glu 122 band at 1697 cm^{-1} and the amide I band at 1662 cm^{-1} considerably lose intensity (Figure 5A). At 30 °C, the band pattern around 1700 cm^{-1} changes and becomes similar to that in native Meta I (obtained at 0 °C) (Figure 5B), but has an altered band shape in the dark state compared to native pigment. Besides these pronounced features, the small difference band of Asp 83 changes as well from $-1773/+1763$ cm^{-1} at low temperature to $-1769/+1754$ cm^{-1} at 30 °C.

These findings of two (or a continuum of) different Meta I conformations are supported by UV−visible spectroscopy, which indicates that the Meta I absorption is blue-shifted compared to the Iso dark state to about 442 nm. While at 0 °C, the absorption coefficient of Meta I is comparable to that of the dark state (Figure 5C), it decreases at higher temperatures. At 30 °C, the photoproduct absorption extends to the UV-range, which is in part due to photoproduct decay.

From these findings we can conclude that the Meta I conformation of acyclic-1 depends on temperature. We therefore have to distinguish between a low-temperature Meta I state, that can be obtained with small gradual changes up to 20 °C, and a high-temperature Meta I state, that is formed at 30 °C. The low-temperature Meta I state of acyclic-1 state is clearly different from native Meta I obtained in the same temperature range and involves specifically a changed interaction of Glu 122. Importantly, the low-temperature Meta I state is considerably different from the Lumi state of acyclic-1 stabilized at around −100 °C, as shown previously (*37*). The high-temperature Meta I state of acyclic-1 at 30 °C cannot be directly compared to native Meta I, as Meta I cannot be stabilized in native pigment for steady-state experiments at 30 °C. Possibly, there is a similar transition to a high-temperature Meta I state also in native pigment. Upon replacement of the relatively fluid environment offered by the disk membrane by more rigid lipid bilayers or even a two-dimensional crystal lattice, an inactive precursor of Meta II can be stabilized also in native rhodopsin at higher temperature. This inactive state is similar but not identical to Meta I stabilized at, for example, 0 °C (*38*, *39*). Interestingly, the high-temperature Meta I state of acyclic-1 is more similar to native Meta I (obtained at 0 °C) than the low-temperature Meta I state of acyclic-1.

In regard of the amide difference band at 1662 cm^{-1}, we note that its increased intensity at lower temperature does not necessarily imply larger conformational changes at low temperature compared to high temperature, which would be counterintuitive. In particular for α-helical membrane proteins, amide difference bands reflect localized distortions of secondary structure only and are quite insensitive to the thereby induced changes of tertiary structure (*19*, *40*, *41*).

Possibly, the conformational change leading to the strong difference band at 1662 cm^{-1} at lower temperature is due to a relatively large but sharply localized distortion of secondary structure, having a strong influence on the coupling of the neighboring amide C═O oscillators. At higher temperature, this distortion, that still leads to a similar change of tertiary structure, is possibly spread over several peptide bonds and thus smaller per single amide unit. This could allow continuous mixing of the single oscillators and thus change little in their overall coupling, resulting in an amide *difference* band with much smaller intensity.

It should be further noted that in a previous FTIR study of the acyclic-2 pigment, which behaves very similarly as acyclic-1 (see below), Meta I spectra had been measured at 7 °C in either a membrane or a fluid detergent environment (*37*). The Meta I state of the detergent-solubilized acyclic-2 pigment shows some similarities to our high-temperature Meta I of acyclic-1. The high-temperature Meta I state of the acyclic-1 pigment could therefore reflect a higher conformational flexibility of the protein at the elevated temperature. In the native pigment, existence of such a high-temperature state would be hidden by the transition to Meta II in steady-state experiments (see above).

To pinpoint the group responsible for the pH dependence of the Meta I/Meta II equilibrium in acyclic-1, we regenerated the E134Q mutant with acyclic-1 retinal and tested the sensitivity of Meta II formation of this pigment at 20 °C. As evident from Figure 6C, replacement of Glu 134 by a glutamine is not sufficient to abolish the pH dependence of Meta I/Meta II of acyclic-1. While at pH 4.5, a photoproduct with Meta II conformation is formed; this is not the case at more alkaline pH. The Meta I photoproduct formed at pH 7.0 does not show the Meta II marker band at 1645 cm^{-1}, and in addition, the intensity of the bands above 1700 cm^{-1} is considerably reduced. The Meta II absorption of protonated Glu 113 at 1711 cm^{-1} is missing and replaced by a shallow broad absorption band at 1706 cm^{-1}. Clearly, Meta I of acyclic-1 in E134Q is considerably different from Meta I of acyclic-1 of wild-type, which implies that Glu 134 plays an important role in maintaining the particular protein conformation of acyclic-1 Meta I in wild-type. The persistent pH dependence of Meta I/Meta II in acyclic-1 in E134Q shows, however, that Glu 134 does not control proton uptake in the regulation of the Meta I/Meta II equilibrium in the acyclic pigment, which is in contrast to native pigment. In native pigment, the Glu 134 Gln mutation abolishes the classical Meta I/Meta II pH dependence ((*42*) and Lüdeke, S., Sakmar, T. P., Siebert, F., and Vogel, R., unpublished observation), indicating that the acyclic chromophore perturbs the causality of proton uptake of the Meta I/Meta II conformational transition of native rhodopsin.

Acyclic-2 (Di-ethyl Acyclic). Acyclic-2 9-*cis* retinal absorbed at 360 nm in ethanol. As acyclic-1, it regenerated within 2 h at room temperature quantitatively with opsin and formed a pigment absorbing at 461 nm, in agreement with published data (*14*).

Acyclic-2 behaves very similar as acyclic-1 and forms in particular Meta I and Meta II states with FTIR difference spectra virtually identical to those of acyclic-1. Again, we observe the existence of different Meta I conformations at low and high temperatures. The pK_A of the Meta I/Meta II equilibrium of acyclic-2 at 20 °C was found to be 5.0 and thus very similar as for acyclic-1. Spectra of acyclic-2 are shown in Figure A2 in the Supporting Information.

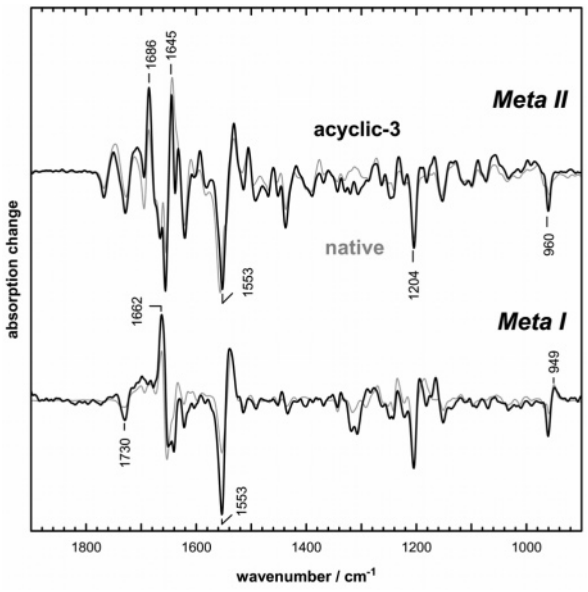

FIGURE 7: Meta I and Meta II with the acyclic-3 ligand. Meta I and Meta II FTIR difference spectra were obtained at 10 °C, pH 8.0, and 20 °C, pH 4.0, respectively. In contrast to the other acyclic analogues, the Meta I state of acyclic-3 shows a HOOP mode at 949 cm^{-1}. The spectra obtained for native Iso (gray) are the same as in Figure 2A.

Acyclic-3 (Di-methyl Acyclic with 5 C═C Bonds). Acyclic-3 9-*cis* retinal absorbed at 380 nm in ethanol. In contrast to the other two acyclic 9-*cis* retinals, pigment formation was very slow and incomplete. In a 2:1 retinal/opsin stoichiometry with 50 μM opsin, there was only 5% regeneration within 2 h at room temperature. Additional 20 h at room temperature increased the yield to 15%, but no further increase was observed upon additional incubation for 20 h. The position of the absorption peak was roughly at 510 nm. Slow and incomplete regeneration and peak position are in agreement with published data (*15*, *43*). One of those studies further showed that regeneration efficiency was even lower for the respective 11-*cis* analogue (*43*).

Acyclic-3 forms a Meta II photoproduct at 20 °C and pH 4.0 (Figure 7). The Meta II FTIR difference spectrum of acyclic-3 corresponds largely to that of native Meta II, with alterations in the amide I range around 1640 cm^{-1} and at 1686 cm^{-1} and above. Possibly, these alterations are due to residual Meta I being still present under the chosen conditions. The strong uncompensated negative ethylenic mode at 1553 cm^{-1} suggests that the Schiff base is deprotonated in acyclic-3 Meta II. The Meta I difference spectrum of acyclic-3 obtained at 10 °C and pH 8.0 shows a stronger negative band at 1730 cm^{-1} of presumably Glu 122. In contrast to acyclic-1 and -2, there is a positive Meta I HOOP mode at 949 cm^{-1}, which is similar to that in native Meta I. The somewhat stronger negative band at 1553 cm^{-1} suggests that the Schiff base may possibly be deprotonated already in Meta I of acyclic-3 at alkaline pH.

Because of the low regeneration yield, we did not measure a complete titration curve of the Meta I/Meta II equilibrium with this pigment, yet additional experiments place the pK_A in the range around 5, similar as for the two other acyclic pigments.

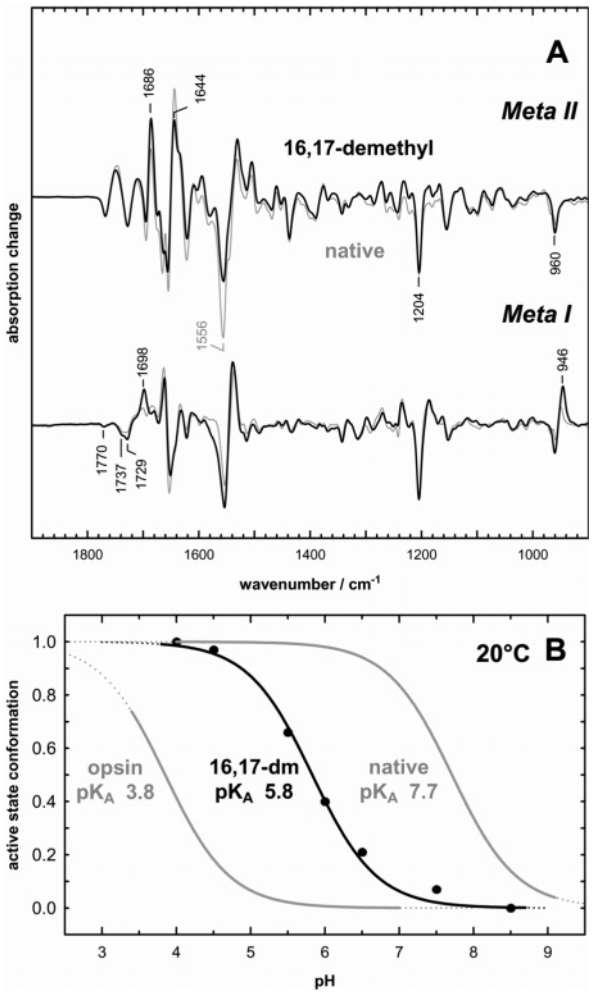

FIGURE 8: Meta I and Meta II with the 16,17-demethyl ligand. (A) Meta I and Meta II FTIR difference spectra were obtained at 0 °C, pH 8.0, and 20 °C, pH 4.0, respectively. The Meta I state of 16,17-demethyl shows the unusual strong Glu 122 band as acyclic-1; it has, however, a strong HOOP mode at 946 cm^{-1}. The spectra obtained for native Iso (gray) are the same as in Figure 2A. (B) The pK_A of the Meta I/Meta II equilibrium of 16,17-demethyl is at 5.8 at 20 °C and thus halfway between that of native Meta I/Meta II and the ligand-free conformational equilibrium.

16,17-Demethyl. 9-*cis* 16,17-Demethyl retinal absorbed at 389 nm in ethanol. Pigment formation with opsin was slow and only incomplete, yielding about 10% regeneration with 50 μM opsin after 6 h at room temperature. Further incubation did not increase the pigment yield substantially. Interestingly, preparation of IR sandwich samples seemed to enhance the pigment yield to maybe 50%, presumably due to concentration of the reactants during sample film preparation (millimolar range). This might indicate slow binding and unbinding of the chromophore from the binding pocket leading to a dynamic equilibrium. The resulting pigment absorbed at 505 nm.

16,17-Demethyl forms, at 20 °C and pH 4.0, a Meta II photoproduct with similar FTIR spectrum as native pigment (Figure 8A). Slight alterations are observed for the amplitudes of amide I bands at 1686 and 1644 cm^{-1}. The lack of a strong uncompensated negative ethylenic mode (at 1556 cm^{-1} for native Iso) indicates that the Schiff base in Meta II of 16,17-demethyl is at least partially protonated, and the positive band at 1712 cm^{-1} indicates that Glu 113 becomes protonated, as to be expected in a Meta II state. The Meta I spectrum of 16,17-demethyl is as well quite similar to that of native pigment except for the carbonyl range above 1700 cm^{-1}. Focusing on this range, the absorption changes of Asp 83 are well-reproduced and also the absorption of Glu 122 in the dark state is similar as in native pigment with its split absorption peaks at 1737 and 1729 cm^{-1}. The Meta I absorption of Glu 122, however, is different, with a strong peak at 1698 cm^{-1}, which is similar to that observed in acyclic-1 and -2. In marked contrast to the acyclic-1 and -2 pigments, where no Meta I HOOP band was observed, there is a strong Meta I HOOP band for 16,17-demethyl at 946 cm^{-1}. Removal of the two methyl groups at C1 seems therefore to have a similar impact on the interaction between the chromophore and the Glu 122/His 211 network in Meta I, yet the presence of the ring in 16,17-demethyl maintains the twisted polyene geometry of wild-type around C11═C12. At 30 °C, the Meta I features in the region above 1700 cm^{-1} change similarly as with acyclic-1 and -2 (spectra not shown). The HOOP band, however, is still present in this high-temperature Meta I state of 16,17-demethyl, albeit at a somewhat reduced intensity compared to lower temperature.

The pK_A of the Meta I/Meta II equilibrium is found to be 5.8 at 20 °C and thus midway between that of native Meta I/Meta II and the opsin conformational equilibrium (Figure 8B). All-*trans* 16,17-demethyl retinal is therefore again a partial agonist only, yet stronger than either acyclic-1 or -2.

16,17,18-Demethyl. 9-*cis* 16,17,18-Demethyl retinal absorbed at 381 nm in ethanol. Pigment formation was slow and only incomplete, similar as with 16,17-demethyl, yielding only 5% regeneration with 50 μM opsin within 4 h at room temperature and about 12% after 20 h. Further incubation did not increase the regeneration yield. Again, as with 16,-17-demethyl, the pigment yield increased considerably during preparation of the sample films, which may be similarly interpreted as in the case of 16,17-demethyl. The resulting pigment absorbed at around 500 nm.

Meta II of 16,17,18-demethyl was similar to that of 16,-17-demethyl with the exception of a slightly larger amide I band at 1644 cm^{-1} (Figure 9A). Again, the Schiff base in 16,17,18-demethyl Meta II is at least partially protonated. The Meta I state of 16,17,18-demethyl is as well similar to that of 16,17-demethyl. There are some small changes in the carbonyl range regarding the dark state. The dark absorption of Asp 83 is slightly downshifted to 1769 cm^{-1}, and that of Glu 122 is less heterogeneous. The HOOP bands of both the dark state and Meta I are upshifted by about 3 cm^{-1} compared to those of 16,17-demethyl. 16,17,18-Demethyl forms the additional high-temperature Meta I state similarly as 16,17-demethyl (spectra not shown) with a persisting HOOP band.

The pK_A of the Meta I/Meta II equilibrium is 5.2 at 20 °C and thus another half unit lower than for 16,17-demethyl (Figure 9B). All-*trans* 16,17,18-demethyl is therefore also a partial agonist, albeit somewhat weaker than all-*trans* 16,-17-demethyl.

Aromatic Ring. The retinal analogue with aromatic mesityl ring was synthesized as 11-*cis* isomer and absorbed at 376 nm in ethanol. It reacted with opsin only very slowly and incompletely, yielding 6% pigment after 4 h at room temperature, which increased to about 20% after 20 h. Further incubation did not further increase pigment yield, which is

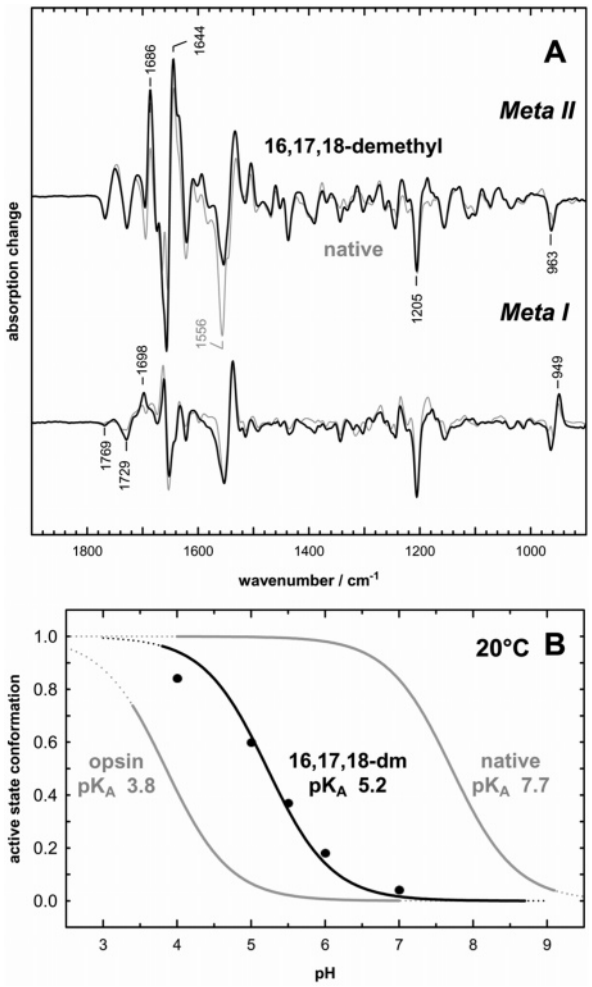

FIGURE 9: Meta I and Meta II with the 16,17,18-demethyl ligand. (A) Meta I and Meta II FTIR difference spectra were obtained at 10 °C, pH 7.0, and at 20 °C, pH 4.0, respectively. The Meta I spectrum corresponds to that of 16,17-demethyl with minor modifications. The spectra obtained for native Iso (gray) are the same as in Figure 2A. (B) The pK_A of the Meta I/Meta II equilibrium of 16,17,18-demethyl is at 5.2 at 20 °C and thus 0.6 units lower than that of 16,17-demethyl.

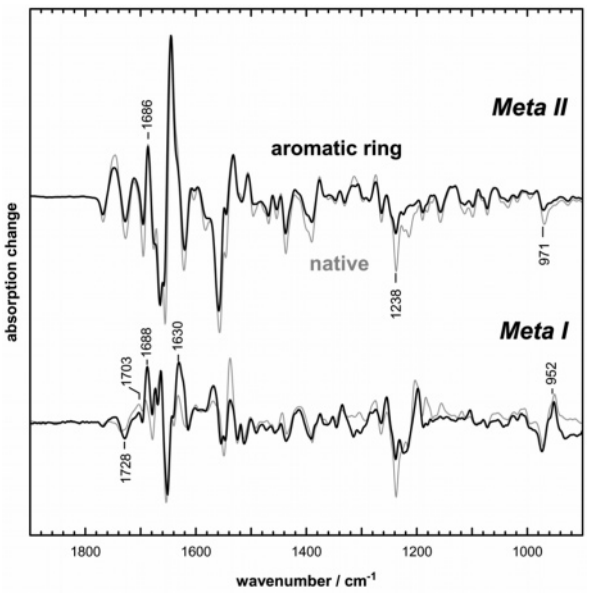

FIGURE 10: Meta I and Meta II with the aromatic ring analogue. Meta I and Meta II FTIR difference spectra were obtained at −7 °C, pH 9.0, and 10 °C, pH 5.0, respectively. The corresponding native spectra (gray) were measured with native rhodopsin at 0 °C, pH 9.0, and 10 °C, pH 5.0. Note that spectra were measured with 11-*cis* pigments in this particular case and that unequivocal normalization of the analogue spectra to those of native pigment is not possible in this case.

in agreement with previous studies (*44*). The resulting pigment had its absorption peak at 475−478 nm.

The Meta II spectrum of the aromatic ring analogue obtained at 10 °C and pH 5.0 looks regular (Figure 10), with no obvious bandshifts, yet the intensities of single bands differ from native Meta II. There is no straightforward way to normalize the spectra, as the negative fingerprint band at 1238 cm^{-1}, which is usually suitable for normalization of 11-*cis* dark states, seems to have different absorption coefficients in the two pigments, possibly due to overlap with a photoproduct band. It was not possible to obtain a pure Meta I spectrum of the aromatic ring analogue at 10 °C, as even at pH 9.0 there was still considerable contribution of Meta II to the photoproduct equilibrium. The Meta I/Meta II equilibrium of the aromatic pigment is therefore shifted toward Meta II compared to native rhodopsin, rendering the all-*trans* aromatic ring ligand a stronger agonist than native all-*trans* retinal. The pH dependence, however, did not seem to follow a regular single-proton titration curve, such that a pK_A value could not be determined.

We measured a Meta I spectrum of the aromatic ring analogue at −7 °C, pH 9.0, where a seemingly pure Meta I state could be stabilized. The Meta I spectrum of the aromatic ring analogue differs from native Meta I in several respects. There are pronounced changes compared to native pigment in the amide I range around 1630 cm^{-1}, and there is a quite intense band at 1688 cm^{-1} (possibly also an amide I mode identical to that observed otherwise in Meta II at 1686 cm^{-1} only). These changes may indicate that the aromatic ring structure leads to deformations of the helix backbones in Meta I which are quite different from those in native Meta I. In addition, the Glu 122 difference band has a much stronger negative (dark) absorption at 1728 cm^{-1}. Further differences can be observed in the amide II range, while the HOOP mode at 952 cm^{-1} is similar as in native Meta I.

ΔH and ΔS of Meta I/Meta II of Ring-Modified Pigments. The pK_A of the Meta I/Meta II equilibrium of native pigment responds sensitively to temperature and rises from 6.6 at 0 °C to 7.7 at 20 °C, yielding a shift by 1.1 units over this 20 °C range (Figure 2C). This temperature sensitivity reflects the energetics of the Meta I to Meta II reaction, which is driven by its large entropy gain ΔS, compensating the enthalpy increase ΔH of the transition to the Meta II state (*22*). A shift of the Meta I/Meta II equilibrium toward Meta I corresponds to an increase of the Gibbs free-energy difference $\Delta G = \Delta H - T\Delta S$ of the transition from Meta I to Meta II, where T is the absolute temperature. To distinguish between associated changes of either ΔH or ΔS, we measured the temperature dependence of the Meta I/Meta II equilibrium for some of the studied ring-modified pigments, namely acyclic-1 and -2, and the two demethylated pigments (Table 1). All four tested pigments have a reduced temperature sensitivity compared to native rhodopsin. From the measured pK_A values at 20 and 0 °C, we can calculate the enthalpy and entropy changes of the transition using the following expressions, which were derived in section A3 in

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	ΔpK_A	ΔH (kJ/mol)	ΔS (J/(mol·K))
native	7.7	6.6	1.1	84	301
acyclic-1	4.8	4.0	0.8	61	167
acyclic-2	5.0	4.1	0.9	69	197
16,17-demethyl	5.8	4.8	1.0	77	238
16,17,18-demethyl	5.2	4.5	0.7	54	148
opsin[b]	-	-	-	48	102

[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. ΔS values were calculated for pH 7.0. [b] Opsin conformational equilibrium, see text for details.

the Supporting Information:

$$\Delta H = -R2.303\frac{\mathrm{p}K_2 - \mathrm{p}K_1}{\frac{1}{T_2} - \frac{1}{T_1}}$$

and

$$\Delta S = R2.303\left(\frac{\mathrm{p}K_2T_2 - \mathrm{p}K_1T_1}{T_2 - T_1} - \mathrm{pH}\right)$$

The calculated values are shown in the last two columns of Table 1. It is interesting to note that ΔH is completely defined by the difference of the pK_A values measured at the two temperatures and does not depend on their absolute positions. The latter are determined by the entropy change of the transition.

Importantly, the relatively small net ΔG that drives the reaction is composed of relatively large counteracting contributions of ΔH and ΔS, which mostly compensate each other. For instance, at 20 °C and pH 5.0, typical conditions to stabilize Meta II, the free-energy gain is −15 kJ/mol. This stems from an enthalpy change of 84 kJ/mol and a $T\Delta S$ term of 99 kJ/mol (note that the ΔS terms in Table 1 were calculated for pH 7). The position of the Meta I/Meta II equilibrium depends therefore on quite subtle differences between the enthalpy and the entropy term. Looking at the pigments with ring deletions, we note that for most of them both ΔH and ΔS are reduced. Notably, the relative decrease of the entropy gain is in all cases much larger than the relative decrease of the enthalpy change. This leads to a more positive value of ΔG and thus to the observed shift of the Meta I/Meta II equilibrium toward Meta I for these four pigments.

Turning to the single pigments, we can order them according to the degree to which ΔH and ΔS deviate from their respective values for native pigment. In the resulting list, 16,17-demethyl with only the methyl groups at C1 deleted is closest to native pigment, with a similar ΔH and a ΔS of about 80% of that of native pigment. It is followed by acyclic-2 with the ring replaced by two ethyl groups and acyclic-1 with an ethyl and a methyl group. In the latter, ΔH is reduced to 73% and ΔS to 55%. 16,17,18-Demethyl is at the very end with ΔH and ΔS values that are reduced to 64% and 49% of those of native pigment, respectively. The decrease of ΔH and ΔS follows the degree of ring deletion, with a notable exception: presence of the 18-methyl group is more important for receptor activation than presence of a closed ring structure, as suggested from a comparison between 16,17,18-demethyl and the acyclic pigments.

In an attempt to compare the results obtained for Meta I/Meta II of the ring modified pigments with the opsin conformational equilibrium (*5*), we also calculated the thermodynamic parameters of the latter. Because of the experimental procedure, we were able to measure pK_A values for the opsin equilibrium only at 30 and 20 °C, which were fitted to pK_A values of 4.12 and 3.84, respectively. The resulting thermodynamic parameters are ΔH 48 kJ/mol and ΔS 102 J/(mol·K). In this case, however, the error margins, in particular for the value of ΔS, are large.

DISCUSSION

The retinal chromophore of rhodopsin plays a remarkable role for the functioning mechanism of the photoreceptor: in the dark, the 11-*cis* retinal isomer (and similarly also the non-native 9-*cis* isomer, which was in parallel used in this study) locks the receptor in an almost completely inactive conformation and prevents spontaneous activation of the protein, which would occur in the absence of the ligand particularly at low pH (*4*, *5*). The light-induced isomerization of rhodopsin is very efficient (quantum yield of 0.67) and extremely fast (completed within 200 fs) (*45*) in transforming the chromophore from an inverse agonist into an agonist. In its agonist all-*trans* form, the ligand subsequently drives the protein conformational changes that ultimately lead to the active receptor species Meta II, which is situated at the beginning of the visual signal transduction cascade. How does all-*trans* retinal accomplish receptor activation? To answer this question, opsin would be regenerated with synthetic retinals that had been modified selectively to probe the influence of the modified groups on the signal transduction properties of the receptor (reviewed in refs *46*, *47*). Many of these studies focused on the early steps of signal transduction, the formation of the Batho, BSI, Lumi, and Meta I states. As these states evolve subsequently, they prepare the receptor for the final activation step that leads from still inactive Meta I to active Meta II. However, the changes of the protein remain very small up to formation of Meta I, as revealed by the previously published mesoscopic structure of Meta I (*9*). The larger helix movements, that lead to the active receptor conformation ((*48*) and references therein), occur during the transition from Meta I to Meta II only, and it is the influence of a synthetic ligand on this equilibrium that classifies it as a full agonist or as an only weakly activating partial agonist.

In this study, we focus on the role of the ring moiety of retinal on the Meta I state of the receptor and on the subsequent Meta I/Meta II conformational equilibrium. Previous studies have shown that the retinal ring is important for docking the chromophore in its binding pocket (*49*). However, synthetic retinals, in which the ring structure had been replaced by two short hydrocarbon groups, have been reported to form pigments as well (*14*, *43*, *50*, *51*), albeit sometimes at low yield only and without leading to an active Meta II state after illumination. In an attempt to determine the constituents of the ring that are important for attaining the signaling state, we stepwise deleted the ring: we prepared retinals in which two (C16 and C17) or all three (C16, C17, and C18) ring methyl groups were deleted (Figure 1). We

Table 2: Properties of Pigments with Ring-Modified Retinals

pigment	regeneration (with 9-*cis* isomer)	activation[a]	Meta I characteristics
acyclic-1	rapid and complete	weak partial agonist	planar C10 to C13 segment (no HOOP mode) low-temperature Meta I with strongly hydrogen-bonded Glu 122 presence of a high-temperature Meta I state at 30 °C
acyclic-2	rapid and complete	weak partial agonist	similar as acyclic-1
acyclic-3	slow and only partial	weak partial agonist	HOOP mode present
16,17-demethyl	slow and only partial	partial agonist	similar as acyclic-1, but with strong HOOP mode present in both Meta I states
16,17,18-demethyl	slow and only partial	partial agonist (<16,17-demethyl)	similar as 16,17-demethyl
aromatic ring	slow and only partial [b]	strong agonist (>native)	HOOP mode present, backbone distortion in Meta I

[a] Ligands with similar pK_A as for native retinal were characterized as *agonists*, those with lower pK_A as *partial agonists*. Ligands with a pK_A close to that of the intrinsic opsin conformational equilibrium were classified as *weak partial agonists*. [b] With 11-*cis* isomer.

further replaced the closed ring structure by combinations of alkyl groups to yield the acyclic-1 and acyclic-2 chromophores and also tested an acyclic chromophore with 5 C=C bonds (acyclic-3). Finally, we replaced the cyclohexenyl ring by a planar aromatic ring. For all six artificial pigments, we characterized the Meta I and Meta II states by FTIR difference spectroscopy and determined the position of the Meta I/Meta II conformational equilibrium (Table 2). For four of them, we analyzed the temperature dependence of this equilibrium and calculated the enthalpy change ΔH and the entropy change ΔS of the transition to compare them with those of native pigment (Table 1).

The Acyclic Pigments. In a previous study, a dimethyl acyclic retinal was shown to be unable to form a pigment with opsin, while the methyl,ethyl acyclic-1 and the diethyl acyclic-2 retinals formed as 9-*cis* isomers photoreactive pigments (*14*). The diethyl acyclic-2 had been further characterized by UV−visible and FTIR spectroscopy, but failed to show substantial receptor activation (*37*, *50*). In this study, both the methyl,ethyl acyclic-1 and the diethyl acyclic-2 behaved very similar in regard of the pK_A of the Meta I/Meta II equilibrium and of their FTIR difference spectra of both Meta I and Meta II. Both pigments are only weak partial agonists with a pK_A of the Meta I/Meta II equilibrium only about 1 unit higher than that of the ligand-free opsin conformational equilibrium (measured at 20 °C).

Acyclic-1 and -2 form at temperatures around 20 °C and below a low-temperature Meta I state, which changes to a high-temperature Meta I state at 30 °C (see below). The low-temperature Meta I spectra of the acyclic pigments reveal a significantly changed interaction of Glu 122 on helix 3 with its environment in comparison to native Meta I and also compared to the ligand-free inactive opsin state. In the dark state of native pigment, Glu 122 is hydrogen-bonded to the ring NH of Trp 126 and to the backbone carbonyl of His 211, as revealed in the crystallographic structures (Figure 3B). In FTIR spectra, we observe a split absorption band with peaks at 1735 and 1729 cm^{-1} in the dark state (Figure 2B), which indicate hydrogen bonds of intermediate strength. This hydrogen bonding seems to persist and is slightly intensified in Meta I, as the C=O stretch of Glu 122 becomes more downshifted to around 1704 cm^{-1}. This pattern depends on the presence of His 211, as shown by site-directed mutagenesis (*13*). In acyclic-1 and -2, we observe in the dark state only a single absorption band of Glu 122 at 1728 cm^{-1}, which becomes extremely downshifted to a very intense and narrow band at 1697 cm^{-1} in low-temperature Meta I, as verified using the E122Q mutant regenerated with acyclic-1. The only single absorption band in the dark state implies that the acyclic-1 and -2 ligands do not evoke the same special hydrogen-bonding network around Glu 122 as observed with native retinals (being either 11-*cis* or 9-*cis* in the dark state). But what about the extremely low position in Meta I?

The position of the C=O stretch of a carboxylic acid may provide information about hydrogen bonding of the carbonyl group as well as the OH group (*35*, *36*). According to Nie et al. (*36*), hydrogen bonding to either the C=O or the OH hydrogen shifts the position of the C=O stretch of a carboxylic acid by each 30−35 cm^{-1} from its position in a vacuum at around 1776 cm^{-1}. The extremely low position of the Glu 122 C=O stretch in the low-temperature Meta I states of these acyclic pigments indicates therefore that Glu 122 forms two defined and very strong hydrogen bonds with both its C=O and OH group as hydrogen-bonding acceptor and donor, respectively. In native pigment, the ring of retinal has been suggested to be retained in its binding pocket in a position similar as in the dark state up to Meta I (*52*). Local changes in the ring environment may therefore be small, such that in native Meta I, Glu 122 is still hydrogen-bonding to the ring NH of Trp 126 on helix 3 (one turn away from Glu 122) and the backbone carbonyl of His 211 on helix 5. In the acyclic pigments, the changed interaction of the synthetic chromophores with the protein seems to allow for stronger hydrogen bonding of Glu 122 to possibly the same residues in the low-temperature Meta I states of these modified pigments. As at least the hydrogen bond to His 211 was reported to be broken upon receptor activation (*53*), this stronger hydrogen bonding would be expected to decrease the enthalpy of Meta I, thereby increasing the enthalpy change of the transition to Meta II, and thus contributing to the inhibition of receptor activation in acyclic-1 and -2. However, considering the experimental data on ΔH and ΔS of the transition from Meta I to Meta II in Table 1, it is evident that ΔH is smaller than for native Meta I/Meta II, such that this putative decrease of the enthalpy of Meta I must be overcompensated by other effects. These effects act by either increasing the enthalpy of Meta I or decreasing the enthalpy of Meta II. Possible mechanisms for this will be discussed further below.

The low-temperature Meta I state of the acyclic-1 and -2 pigments is thus considerably different from native Meta I. This difference, however, vanishes as the temperature is increased. The acyclic pigments produce pure Meta I states

even at 30 °C. At this temperature, the particularly strong hydrogen bonding of Glu 122 is weakened and/or becoming less defined such that spectral features in these high-temperature Meta I states of acyclic- 1 and -2 become similar to those of native Meta I. However, even at this higher temperature, the Meta I/Meta II equilibrium remains shifted to acidic pH to a similar extent as at lower temperature.

Another interesting feature of acyclic-1 and -2 is the absence of the C11═C12 HOOP mode in Meta I, which causes a positive band at 950 cm^{-1} in native Meta I. This indicates that the single bonds adjacent to C11 and C12 are not twisted in Meta I of the acyclic pigments, such that the entire C10 to C13 segment is planar in Meta I. The cyclohexenyl ring constitutes therefore an important contact region between native retinal and the protein, which maintains a twist on the chromophore that seems to be required to direct the protein into the active state conformation. This notion is in agreement with low-temperature experiments on acyclic-2 (*37*) and with results from the Meta I state of 9-dm rhodopsin (*12*), which also lacks the C11═C12 HOOP mode and is similarly inactive as acyclic-1 and -2.

In an attempt to rescue this inhibition of activation, we studied another acyclic analogue, acyclic-3, in which another C═C bond had been added to the polyene compared to the other acyclic analogues (Figure 1). This was found to be not sufficient to restore activity, yet it recovered the C11═C12 HOOP mode in Meta I.

Deletion of the Ring Methyl Groups. Deletion of C16 and C17 or of all three methyl groups (C16, C17, and C18) leads to a considerable decrease in regeneration speed and efficiency, of which the latter might indicate a slow equilibrium between binding and unbinding. The ring methyl groups seem therefore to constitute important anchoring points of retinal in its binding pocket making binding a quasi-irreversible reaction for native retinal. Both the 16,17- and 16,17,18-analogues have not been studied before.

Both ligands are after photoisomerization only partial agonists, with 16,17,18-demethyl being weaker than 16,17-demethyl. Nevertheless, they are both stronger than the acyclic ligands. The Glu 122 absorption in the dark in the 16,17-demethyl is similarly split into two bands as in native pigment, while in 16,17,18-demethyl we observe only a single band at 1729 cm^{-1}, similar as in acyclic-1 and -2. The 18-methyl group seems therefore to be involved in interaction with Glu 122, which is also supported by their close proximity in the crystal structure of rhodopsin (Figure 3A). It should, however, be kept in mind that the conformation of the polyene/ring connection (6-*s*-*cis* in rhodopsin) does not need to be the same in the analogue, as an 18-demethyl (or 5-demethyl) analogue had been proposed to have possibly a 6-*s*-*trans* conformation (*44*). The Meta I vibrational bands of both pigments are quite similar and show an intense downshifted band of Glu 122 at 1698 cm^{-1}, similar as in acyclic-1 and -2. Removal of the 16- and 17-methyl groups at C1 is therefore already sufficient to allow for this special interaction of Glu 122 with its strong hydrogen bonding in Meta I. In contrast to acyclic-1 and -2, however, both demethylated analogues have an intense HOOP band in Meta I, indicating that the twist of the polyene in the C10−C13 region is maintained also after removal of the ring methyl groups and disappears only after removal of the closed ring structure.

Removal of the 18-methyl group lowers both ΔH and ΔS of the Meta I/Meta II equilibrium to values below those of even acyclic-1. The 18-methyl group seems therefore to constitute an important anchoring point being more relevant for the transition from Meta I to Meta II than, for example, the closed ring structure. These results further suggest that the function of the 18-methyl group is mimicked by the ethyl substitution of acyclic-1 and one of the two ethyl groups in acyclic-2. This is supported by the finding that dimethyl acyclic retinal does not form a pigment (*14*).

The Aromatic Ring Analogue. This pigment was studied to explore the role of the ring conformation on receptor activation. The aromatic ring analogue does not regenerate well, indicating possibly mild steric incompatibility with the binding pocket. In this analogue, the ring and its connection to the polyene is planar, in contrast to the cyclohexenyl ring of retinal. This changes the positioning of the ring in the binding pocket and induces distortions of the protein backbone, leading to considerably changed amide bands in the Meta I minus dark state difference spectra of this analogue. Compared to native pigment, the aromatic ring analogue is hyperactive; that is, it favors Meta II over Meta I, such that pure Meta I can be stabilized only at lower temperature.

A Model for Receptor Activation. Recent 2D dipolar-assisted rotational resonance NMR studies have shown that upon transition to the active state, the chromophore translates along its long axis toward helix 5 (*10*). Simultaneously, the interhelical hydrogen bond between the backbone carbonyl of His 211 on helix 5 and Glu 122 is broken, as shown by solid-state NMR (*53*). UV-spectroscopy on site-directed mutants revealed further that this transition to Meta II is coupled as well to a weakening of the hydrogen bonding of Trp 126 on helix 3 (*54*), which is one of the hydrogen-bonding partners of Glu 122 (Figure 3B). These results, indicating a breaking of the hydrogen bonding of Glu 122 and a shift of the side chain to a less polar environment in the transition to Meta II, are consistent with FTIR results, which show an upshift of the C═O stretch absorption of Glu 122 to 1745 cm^{-1} (*26*), corresponding to an only weakly hydrogen-bonded carboxylic acid. As the Meta II band pattern in this range is very similar for all pigment analogues studied here, Glu 122 seems to undergo a similar change also in these analogues, albeit the hydrogen bonding in Meta I is intensified compared to native Meta I.

The transition from still inactive Meta I to active Meta II is known to involve an increase in enthalpy (*55*) and is driven by the positive entropy balance of the reaction. Retinal can be considered a scaffold that coordinates helix movements and that directs the free-energy gain stemming from the increase of entropy into the disruption of interactions that stabilize the inactive protein conformation of Meta I. The interhelical networks between helix 3 and 5, maintained by Glu 122, Trp 126, and His 211, and between helix 3 and 6, involving the ERY motif (Glu 134, Arg 135, and Try 136) and residues on the cytoplasmic side of helix 6, including Glu 247 and Thr 251 (*7*), contribute among others substantially to these inactivating constraints. The translational motion of the retinal chromophore couples to Trp 265 (*10*), which is close to a putative, proline-induced hinge at position 267 on helix 6 (*2*, *7*), initiating thereby the experimentally found rigid body outward motion of helix 6 relative to helix

3 (*48*, *56*, *57*). This view is supported by a small motion of Trp 265 already present in the Meta I state (*9*). Simultaneously, the motion of retinal toward helix 5 disrupts the hydrogen-bonding network around Glu 122 between helix 3 and helix 5. As we could show, the methyl groups at C1 and particularly at C5 are important for this action and an efficient activation of the receptor. Deletion of the two methyl groups at C1 and even more of all ring methyl groups leads to an inefficient positioning of the ring and inhibits thereby signal transduction. This becomes even worse upon deletion of the ring structure in acyclic-1 and -2. Both alterations seem to even strengthen this interhelical network that stabilizes Meta I.

This rather mechanistic point of view would predict a decrease of the enthalpy of Meta I in the demethylated pigments and in acyclic-1 and -2 due to strengthening of interhelical hydrogen bonding. The ΔH of the transition from Meta I to Meta II was, however, found to be decreased compared to native pigment. The effect of stronger hydrogen bonding must therefore be overcompensated, for example, by a lack of favorable interactions between the ring and the ring-binding pocket, which are weakened or broken upon transition to Meta II. Alternatively, the experimentally observed decrease of ΔH of the transition from Meta I to Meta II could also imply a reduced enthalpy of the Meta II state in these analogue pigments. In this regard, it is astonishing that the Meta II spectra of the analogues with ring deletions are very similar to native Meta II as far as conformationally sensitive bands are concerned. In particular, the band patterns of protonated carboxylic acids, which sense the conformational changes of the protein, are almost identical to those observed in native pigment. Small alterations are observed for the intensities of some of the amide difference bands, while their positions are unchanged. These observations imply that the protein conformation of the Meta II states of these analogue pigments is similar as in native pigment. This is further corroborated by the ability of these Meta II states to bind transducin-derived peptides, leading to largely similar spectral changes as observed for native Meta II. Therefore, while it cannot be excluded rigorously, there is no experimental evidence for a considerably changed conformation of Meta II in the analogue pigments compared to native Meta II. Perhaps the changed thermodynamic parameters reflect changes of specific interactions between chromophore and protein that have little impact on the conformation of the receptor, such that they remain largely unobtrusive in the FTIR spectra. Such interactions could include hydrophobic interactions of the ring with the ring binding pocket, which are highly modified in the analogue pigments with ring deletions. Whatever the reason for the decrease of ΔH in the analogue pigments, an increase of the enthalpy of Meta I, a decrease of the enthalpy of Meta II, or both, our results show that the interactions underlying it specifically involve the ring constituents and in particular the ring methyl groups.

This decrease of ΔH for the transition from Meta I to Meta II in the pigments with partial ring deletion, which would favor the active Meta II conformation in the Meta I/Meta II equilibrium, is overcompensated by a concomitant, much more pronounced decrease of the entropy gain. It is this large decrease of ΔS which shifts ΔG to values more positive than in native pigment and which thereby stabilizes the inactive Meta I state leading to the partial agonism in the acyclic and demethylated pigments. This decrease of ΔS may in principle again be caused by changes of both Meta I and Meta II. For instance, in Meta I, lack of specific interactions between ring components and here again specifically the ring methyl groups may increase the conformational freedom of the protein and of the chromophore, thereby increasing the entropy of Meta I. A similar mechanism, an increased entropy of the Meta I state, had been suggested to be one of the causes of the only very weak partial agonism of 9-demethyl rhodopsin (*12*).

In our study, we have measured apparent pK_A values of the conformational transition between Meta I and Meta II, and we should therefore comment on the group or groups responsible for this pH dependence. From previous studies, it is known that Glu 134 is involved in the pH-dependent regulation of Meta I/Meta II (*42*, *58*), which had been verified also in a membrane environment (Lüdeke, S., Sakmar, T. P., Siebert, F., and Vogel, R., unpublished observations). The inactivity of ligand-free opsin, on the other hand, is primarily controlled by the salt bridge between Glu 113 and Lys 296, as indicated by studies on constitutively active opsin mutants (*59*). In the opsin conformational equilibrium, the effect of Glu 134 was reported to be secondary only (*4*). Further, protonation of Glu 113 had been shown to be a requirement for attaining the active state conformation (*60*) (in keeping with that, all analogue Meta II spectra in this study feature a protonation of Glu 113). This indicates that Glu 113 plays its role as well in the pH dependence of Meta I/Meta II. A working model could be that the proton uptake responsible for this pH dependence is controlled by Glu 134, while Glu 113 modulates the apparent pK_A by its influence on the conformational change, without being the primary proton acceptor. In the case of acyclic-1, this interdependence of proton uptake and conformational change is considerably disturbed. The pH dependence of Meta I/Meta II is not abolished in the E134Q mutant regenerated with acyclic-1 chromophore, indicating that Glu 134 is not primarily responsible for proton uptake. This distinguishes the acyclic pigment from the 9-demethyl pigment, which is a partial agonist equally weak as acyclic-1, but whose activation potential is restored in the E134Q mutant (*61*). This might imply that the ring of retinal is responsible for pushing the Glu 134-mediated apparent pK_A of Meta I/Meta II to physiological values. In the acyclic pigment, this interaction is lost (possibly due to the special situation around Glu 122, which might prevent correct positioning of helix 3), and the transition to Meta II cannot be achieved, unless a second constraint is broken as well. Breaking of this second constraint is achieved as well by proton uptake, which determines the pH dependence of Meta I/Meta II in the acyclic pigment. A possible candidate is Glu 113, whose protonation would break the salt bridge to the protonated Schiff base in Meta I. Rupture of this constraint lowers then the barrier for unlocking the cytoplasmic network between helix 3 and helix 6, such that the full transition to Meta II can be achieved also in the acyclic pigment. This would further indicate a close similarity of Meta I/Meta II of the acyclic pigment to the opsin conformational equilibrium (*5*) rather than to native Meta I/Meta II.

CONCLUSION

We have used modifications of the ring moiety of the retinal chromophore to explore their impact on the steps leading to receptor activation, namely, on the structure of Meta I and on the transition to Meta II. Deletion of the ring methyl groups at C1 was found to be sufficient to considerably change the hydrogen-bonding network between helix 3 and 5 in the Meta I state and to impair the transition to the active state. Further deletion of the ring methyl group at C5 and replacement of the ring structure by short alkyl chains lead progressively to further inhibition of receptor activation. Positioning of the ring in Meta I further induces a twist along the polyene chain, which is not abolished by demethylation of the ring, but only in the acyclic analogues. Together with the indication for a different proton uptake path in acyclic-1, these findings contribute to our current understanding of receptor activation and highlight in particular the importance of retinal/protein contacts for efficient conversion of the initial chromophore isomerization into the protein conformational changes.

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SUPPORTING INFORMATION AVAILABLE

Figures on isorhodopsin and the acyclic-2 pigment, as well as a derivation of expressions for ΔH and ΔS values. This material is available free of charge via the Internet at http://pubs.acs.org.

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