The All-*trans*-15-*syn*-Retinal Chromophore of Metarhodopsin III Is a Partial Agonist and Not an Inverse Agonist†

Mohana Mahalingam and Reiner Vogel*

Arbeitsgruppe Biophysik, Institut für Molekulare Medizin und Zellforschung, Albert-Ludwigs-Universität Freiburg, Hermann-Herder-Strasse 9, D-79104 Freiburg, Germany

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ABSTRACT: Meta III is formed during the decay of rhodopsin's active receptor state at neutral to alkaline pH by thermal isomerization of the retinal Schiff base C15═N bond, converting the ligand from all-*trans* 15-*anti* to all-*trans* 15-*syn*. The thereby induced change of ligand geometry switches the receptor to an inactive conformation, such that the decay pathway to Meta III contributes to the deactivation of the signaling state at higher pH values. We have examined the conformation of Meta III over a wider pH range and found that Meta III exists in a pH-dependent conformational equilibrium between this inactive conformation at neutral to alkaline pH and an active conformation similar to that of Meta II, which, however, is assumed at very acidic pH only. The apparent pK_a of this transition is around 5.1 and thus several units lower than that of the Meta I/Meta II photoproduct equilibrium with its all-*trans* 15-*anti* ligand, but still about 1 unit higher than that of the opsin conformational equilibrium in the absence of ligand. The all-*trans*-15-*syn*-retinal chromophore is therefore not an inverse agonist like 11-*cis*- or 9-*cis*-retinal, which lock the receptor in an inactive conformation, but a classical partial agonist, which is capable of activating the receptor, yet with an efficiency considerably lower than the full agonist all-*trans* 15-*anti*. As the Meta III chromophore differs structurally from this full agonist only in the isomeric state of the C15═N bond, this ligand represents an excellent model system to study principal mechanisms of partial agonism which are helpful to understand the partial agonist behavior of other ligands.

Activation and deactivation of the visual pigment rhodopsin are key steps of visual perception. Rhodopsin is activated by the light isomerization of its 11-*cis*-retinal chromophore, which is covalently bound in a chromophore binding pocket in the transmembrane core of the membrane protein via a protonated Schiff base to Lys 296 on transmembrane helix 7. The isomerization to all-*trans* switches this chromophoric ligand from an inverse agonist, which stabilizes the completely inactive conformation of the dark state, to a full agonist, driving the subsequent protein changes in the conformational transition to the active state, Meta II (*1*−*6*). Meta II is formed on the time scale of milliseconds and stands in a pH- and temperature-dependent conformational equilibrium with its precursor Meta I, which has an inactive receptor conformation and which is favored at alkaline pH and lower temperature.

The active Meta II receptor conformation decays by two fundamentally different pathways. One of these pathways involves hydrolysis of the retinal Schiff base in Meta II, leading to release of all-*trans*-retinal from its binding pocket (reviewed in ref *3*). The resulting apoprotein opsin is known to adopt a pH-dependent conformational equilibrium between active and inactive conformations (*7*, *8*), of which the active conformation is formed, however, only at very acidic pH (pK_a 4.1), such that opsin is largely inactive at neutral pH. The other pathway involves a thermal isomerization of the C15═N double bond of the retinal Schiff base from an extended 15-*anti* (*trans*) geometry to 15-*syn* (*cis*) (both rhodopsin and its immediate photoproducts contain the retinal chromophore in the 15-*anti* geometry), leading to formation of Meta III[1] and deactivation of the receptor (*9*). This thermal isomerization is unique for a visual pigment. In vitro, the Meta III pathway is favored by alkaline pH (*3*), reflecting that the decay of the pH-dependent Meta I/Meta II photoproduct equilibrium to Meta III proceeds via Meta I (*10*). In Meta III the chromophore is still in its binding pocket, as was shown by CD (*11*), polarized UV−visible (*12*), and fluorescence spectroscopy (*13*), and by the ability of Meta III to undergo photochemical conversion to Meta I/Meta II and to the rhodopsin and isorhodopsin dark states (*13*−*17*). At longer time scales, Meta III finally decays by hydrolysis of the Schiff base into opsin and all-*trans*-retinal (*18*).

It was shown recently that the decay of Meta III to opsin and all-*trans*-retinal is pH-dependent, with lifetimes ranging from hours at alkaline pH to minutes at acidic pH, and that Meta III is capable of interacting with G protein or G protein-derived functional peptides (*19*). In this study, we show by FTIR spectroscopy that this pH dependence of the Meta III decay rates reflects a conformational transition of Meta III to an active state conformation at low pH, which is similar to that of Meta II. Meta III forms therefore a pH-dependent

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* Corresponding author: phone, +49 761 203 5391; fax, +49 761 203 5390; e-mail, reiner.vogel@biophysik.uni-freiburg.de.

[1] Abbreviations: Meta III, metarhodopsin III; GPCR, G protein-coupled receptor; FTIR, Fourier transform infrared spectroscopy; H, transmembrane helix.

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equilibrium between active and inactive conformations corresponding to the Meta I/Meta II photoproduct equilibrium, which is, however, shifted considerably to the side of the inactive conformation. This shift to the inactive side is reflected in a downshift of the apparent pK_a of the equilibrium from above 8 for the Meta I/Meta II equilibrium to around 5.1, implying that the active conformation of Meta III is attained only at quite acidic pH. Still, the apparent pK_a of the Meta III conformational equilibrium between active and inactive conformations is above that of the corresponding equilibrium of opsin in the absence of ligand, which was found to be 4.1 (*7*). The all-*trans*-15-*syn*-retinal ligand of Meta III is therefore not an inverse agonist, which stabilizes the inactive conformation of the receptor as compared with ligand-free opsin, but a classical partial agonist, which is capable of activating the receptor, yet with a considerably decreased efficiency as compared with the full agonist all-*trans*-15-*anti*-retinal. The isomeric state of the Schiff base C15═N double bond acts thus as a functional molecular switch between full and partial agonism. The general principle behind this functional switch could be a requirement for a certain minimal effective length of the ligand between its anchoring points at the ring and the ϵ-carbon of Lys 296 next to the Schiff base in order to achieve maximal activation of the receptor. This effective length of the ligand would be increased in the extended 15-*anti* and decreased in the 15-*syn* state of the Schiff base. This general principle is discussed in regard to other partial agonists that have deletions at the β-ionone ring at the opposite end of the ligand (*20*, *21*).

MATERIALS AND METHODS

Pigment Preparation. Rhodopsin in washed disk membranes was prepared from cattle retinae according to standard procedures (*22*). Preparation of isorhodopsin with 14,15-^{13}C-labeled chromophores was accomplished by regenerating opsin with the isotopically labeled 9-*cis* retinal, which was prepared in the laboratory of Mordechai Sheves. The E134Q mutant pigment was prepared in the laboratory of Thomas P. Sakmar and reconstituted into phosphatidylcholine (PC) vesicles.

FTIR Spectroscopy. FTIR spectroscopy was performed with a Bruker IFS 28 spectrometer with an MCT (mercury cadmium telluride) detector. Spectra were recorded in blocks of 512 scans with an acquisition time of 1 min and a spectral resolution of 4 cm^{-1}. Experiments were performed with sandwich samples containing 0.5 nmol of pigment in native membranes to allow for control of water content, pH value, and salt concentration (*7*). Meta I/Meta II titration curves obtained from these samples are identical to those measured with membrane suspensions. Forty microliters of citric acid, MES [2-(*N*-morpholino)ethanesulfonic acid], or BTP (Bis-Tris-propane) was used at 200 mM to provide for precise pH adjustment particularly at pH extremes. For H/D exchange, sample films were equilibrated twice with D_2O and dried under nitrogen before the respective buffer prepared in D_2O was added.

Photochemical generation of Meta III was achieved by illumination of rhodopsin for 20 s with orange light >550 nm (through fiber optics fitted to a 150 W tungsten lamp equipped with a Schott OG 550 filter) to produce Meta II, followed by a 5 s near-UV illumination using an LED array consisting of six 10 mW UV LEDs (Yoldal Co. Ltd., Taiwan) with an emission peak at 395 nm, to produce mostly Meta III.

UV−Visible Spectroscopy. For UV−visible spectroscopy, sandwich samples identical to the IR samples were used in a Perkin-Elmer Lambda 17 spectrophotometer equipped with a temperature-controlled sample holder. Illumination conditions were similar as in the FTIR experiments. The spectral acquisition time was 1 min. Membrane suspensions were used when indicated. Meta III decay rates were alternatively determined by recording single wavelength absorption traces at 465 nm with a time resolution of 1 s and were in good agreement with the data obtained by scanning spectroscopy.

RESULTS

The Decay Rate of Meta III Is pH-Dependent. The Meta III pathway contributes significantly to the thermal decay of the Meta I/Meta II equilibrium only at neutral to alkaline pH (*9*, *13*). In order to study Meta III over a broader pH range, we have used a photochemical protocol for generation of Meta III (Figure 1A): First, we illuminate rhodopsin in disk membranes (λ_{max} 500 nm, black spectrum) for 20 s by orange light (>550 nm) to produce the Meta I/Meta II photoproduct equilibrium. At 30 °C, the apparent pK of this equilibrium is above 8, such that up to pH 7.0 essentially only Meta II (λ_{max} 380 nm, red spectrum) is produced. Meta II is then photolyzed for 5 s by near-UV light (395 nm), which produces besides small amounts of rhodopsin and isorhodopsin mainly Meta III (λ_{max} ~470 nm, blue spectrum) (*14*, *23*). We followed the thermal decay of Meta III to opsin and all-*trans*-retinal at 30 °C in the pH range from 4.0 to 7.0. This decay leads in the first line to an absorption decrease at 470 nm (Figure 1B). As the disk membranes are aligned in our samples with their membrane normal largely parallel to the measuring beam, the concurrent absorption increase at 380 nm due to released all-*trans*-retinal is marginal, as released retinal is oriented preferentially parallel to the membrane normal and thus parallel to the beam (*12*). The decay kinetics show a marked pH dependence with time constants in the range of more than 1 h at pH 7.0 down to less than 2 min at pH 4.5 (red crosses in Figure 1C), which roughly equals the decay rate of Meta II under otherwise similar conditions (*7*). At pH 4.0, the decay rate increased again, presumably due to some long-term structural instability of the photoproduct (not shown). The decay rates can be fitted to a Henderson−Hasselbalch equation with an apparent pK_a roughly around 5.1. Due to the uncertainty regarding the end point of the titration curve at low pH, pK_a values in the range between 4.9 and 5.3 were obtained, depending on the precise assumptions regarding this end point. The blue points in Figure 1C were derived from a conformational analysis of FTIR difference spectra, which will be discussed further below, and show a similar pH dependence.

In the following, we will show that this pH dependence of Meta III decay reflects a pH-dependent equilibrium of Meta III between two conformations: an active conformation at low pH, which decays by rapid hydrolysis of the retinal Schiff base similar to Meta II, and a stable inactive conformation at high pH, to some extent similar to that of Meta I. To avoid complication, we will retain the term Meta

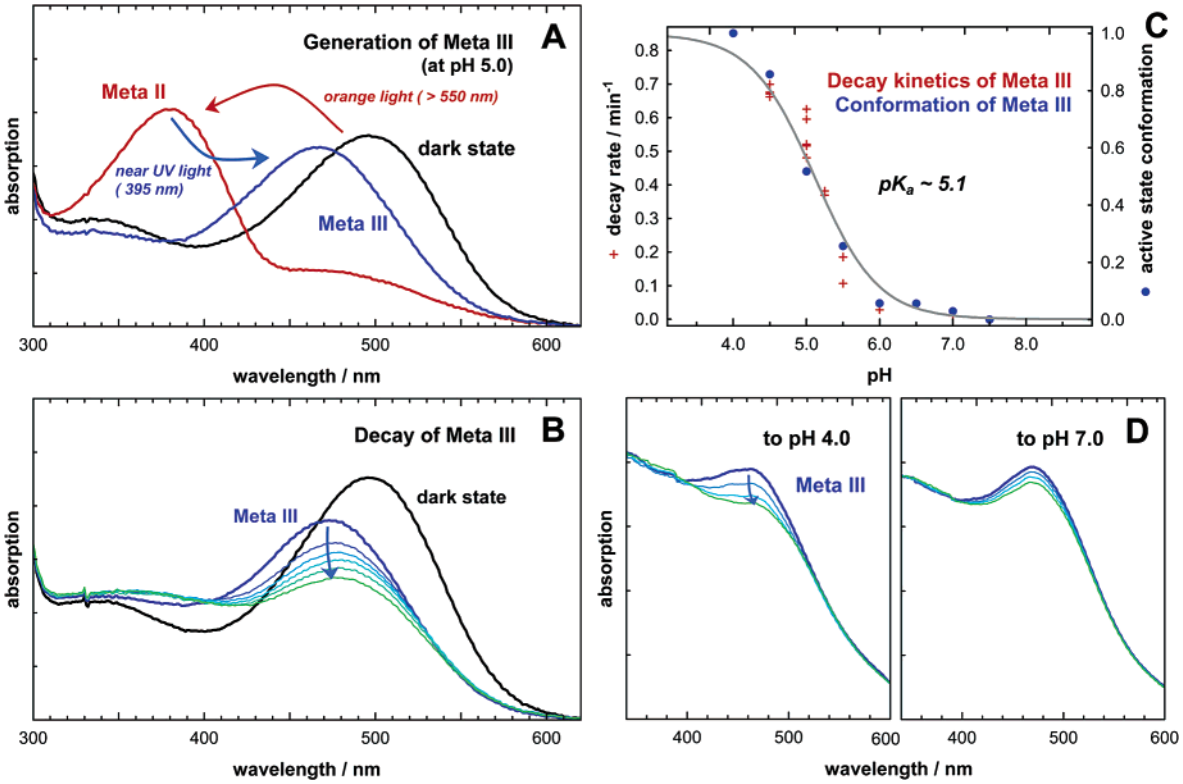

FIGURE 1: The decay kinetics of Meta III are pH-dependent. (A) For studying Meta III over a broad pH range, Meta III was produced by photochemical conversion of the dark state to Meta II by orange light (>550 nm), followed by photochemical conversion of Meta II to Meta III by near-UV light (395 nm). UV−visible spectra were recorded at pH 5.0 and 10 °C, where the respective photoproduct states were stable during the time required for spectrum acquisition. (B) The decay of Meta III was studied at 30 °C. UV−visible spectra of Meta III decay were recorded at $t = 0$, 1, 2, 4, 8, and 16 min after photochemical production of Meta III by the same protocol as in (A). (C) The decay kinetics of Meta III (red crosses, left axis) show a marked pH dependence, which follows a Henderson−Hasselbalch titration curve. The transition from a stable Meta III species at alkaline pH to a rapidly decaying Meta III species at low pH has an apparent pK_a roughly around 5.1 at 30 °C. A conformational analysis of FTIR difference spectra Meta III minus dark state (blue dots, right axis), as described in the text, shows a similar pH dependence. (D) Meta III was produced in a membrane suspension by the same photochemical protocol as in (A) at 30 °C in 20 μL of buffer at pH 5.0 (20 mM citrate, 180 mM NaCl), and the pH was then shifted by adding 180 μL of 200 mM citrate buffer (pH 4.0) or of 200 mM MES buffer (pH 7.0). The subsequent decay of Meta III was monitored at $t = 1$, 2, 4, and 8 min. By switching the pH, the initially identical Meta III product is converted to the Meta III species with slow decay at more alkaline pH and to the Meta III species with rapid decay at more acidic pH, indicating that both species form a conformational equilibrium.

III for both all-*trans* 15-*syn* species and distinguish between both species by specifying for the active low-pH form and the inactive high-pH form.

Low- and High-pH Conformations of Meta III Form a pH-Dependent Equilibrium. We first examined whether these two species can be interconverted by changing the pH. We illuminated 1 nmol of rhodopsin by the same protocol as above to produce Meta III in 20 μL of 20 mM citrate buffer at pH 5.0, added then 180 μL of either 200 mM citrate buffer at pH 4.0 or MES buffer at pH 7.0, and followed the decay kinetics of Meta III by UV−visible spectroscopy. As evident from Figure 1D, the Meta III state produced under identical conditions was converted to the rapidly decaying and to the slowly decaying Meta III form by switching the pH of the sample to more acidic or more alkaline values, respectively.

Low- and High-pH Forms of Meta III Have Active and Inactive Receptor Conformations. In order to study the conformational changes involved in the two light reactions from the dark state to Meta II and from Meta II to Meta III, we followed these reactions by FTIR difference spectroscopy (Figure 2). In order to avoid the implications of rapid photoproduct decay at higher temperatures, these experiments were performed at 10 °C. Control experiments performed with UV−visible spectroscopy on identical samples as used for FTIR spectroscopy confirm both at pH 6.0 and at pH 4.0 the conversion of the dark state to mostly Meta II by orange light and the subsequent conversion to Meta III by near-UV light (Figure 2A). The small peaks around 480 nm in the Meta II spectra are due to small contributions of Meta I at pH 6.0 and to pH-induced formation of some Meta II with protonated Schiff base at pH 4.0. The Meta III spectra appear very similar at both pH values and indicate a protonated Schiff base for both the low- and high-pH form of Meta III.

The light-induced FTIR difference spectra are shown as photoproduct minus initial state spectra, such that photoproduct bands are positive, while those of the respective initial state are negative. In Figure 2B, the difference spectra of the first illumination with orange light (red spectra) show both at pH 6.0 and at pH 4.0 the distinct band pattern of the transition from the dark state to Meta II, reflecting the conformational changes going along with receptor activation. These band patterns comprise that of the C=O stretches of protonated carboxylic acids above 1700 cm^{-1}. They include the positive band at 1712 cm^{-1} due to protonation of Glu 113 on transmembrane helix (H) 3, the counterion to the protonated Schiff base in the dark state, as well as a distinct band pattern with positive and negative bands at 1727, 1747,

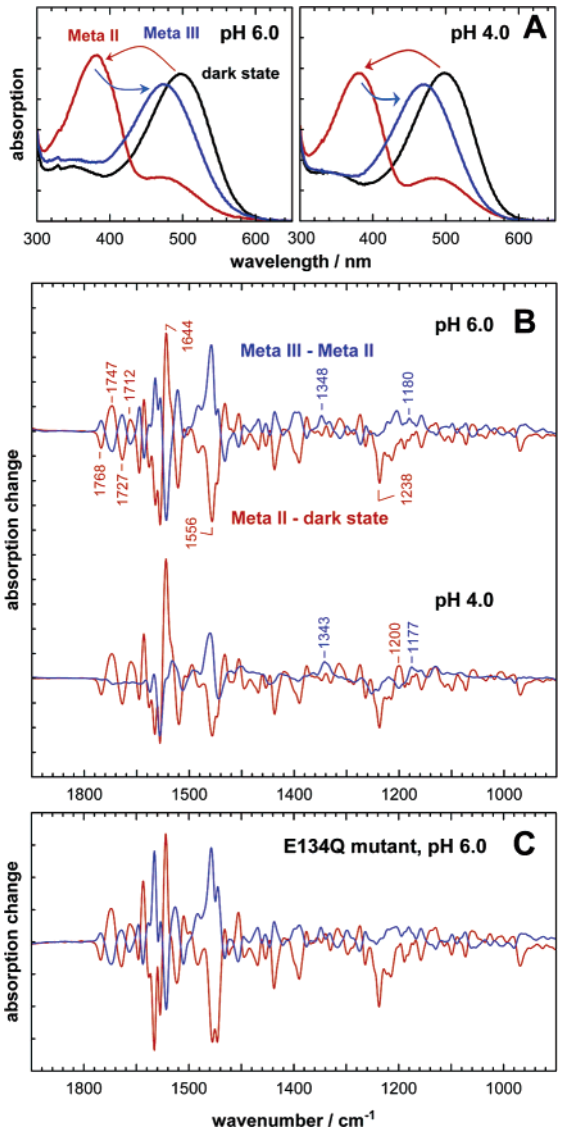

FIGURE 2: The low-pH form of Meta III adopts an active state conformation. (A) High- and low-pH forms of Meta III were produced at 10 °C at pH 6.0 (left panel) and pH 4.0 (right panel), respectively, by the same photochemical protocol as in Figure 1A, involving successive orange (>550 nm) and near-UV (395 nm) illumination steps. The UV−visible spectra of the product (blue spectra), consisting mainly of Meta III, are at both pH values very similar and indicate in particular a protonated Schiff base in both cases. (B) FTIR difference spectra photoproduct minus initial state were recorded for the transitions from the dark state to Meta II (red spectra) and from Meta II to Meta III (blue spectra), induced by the orange and near-UV illuminations, respectively. At both pH 6.0 and pH 4.0, the red spectra of the transition from the dark state to Meta II reveal the conformational changes due to the activation of the receptor, leading to the distinct carboxylic acid band pattern in the range above 1700 cm^{-1} and the pronounced amide I marker band of Meta II at 1644 cm^{-1}. The transition from Meta II to Meta III (blue spectra) involves a deactivation of the receptor at pH 6.0, as evident from the reversal of the conformationally sensitive Meta II bands. At pH 4.0, on the other hand, the active state conformation of Meta II is maintained in Meta III, as evident from the absence of this band reversal. The high-pH form of Meta III corresponds therefore to an inactive receptor conformation, while the low-pH form of Meta III has an active state conformation similar to that of Meta II. (C) The experiments of panel B were repeated with the E134Q mutant of rhodopsin reconstituted into lipid membranes. Similar to the native pigment, a deactivation of the receptor is observed in the transition from Meta II to Meta III (blue spectrum) at pH 6.0. The E134Q mutation does therefore not significantly increase the apparent pK_a of the Meta III conformational equilibrium.

and 1768 cm^{-1}, which reflect hydrogen-bonding changes of Glu 122 on transmembrane helix H3 and Asp 83 on H2 (*24*) due to conformational changes in interhelical hydrogen-bonded networks between H3 and H5 and between H1, H2, and H7, respectively (*4*, *25*) (compare Figure 4B), and of a lipid molecule (*26*). There is further a prominent positive amide I marker band of Meta II at 1644 cm^{-1}, reflecting changes of the protein backbone during receptor activation.

At pH 6.0, conversion of Meta II to Meta III by near-UV light (blue spectrum) reverts most of these conformational changes, as evident from the mirror symmetry of the spectra in the range between 1600 and 1800 cm^{-1}. This is in line with previous experiments (*16*), indicating that the transition from Meta II to Meta III involves a deactivation of the receptor at this pH. The band patterns in the range between 900 and 1400 cm^{-1}, which include in particular vibrational modes of the chromophore, are not mirror symmetric, indicating that not the dark state with its 11-*cis* 15-*anti* chromophore is restored with its characteristic fingerprint band at 1238 cm^{-1} but mainly Meta III with the all-*trans* 15-*syn* isomer of the chromophore with characteristic positive bands at 1348 and 1180 cm^{-1}.

At pH 4.0, this reversal of conformationally sensitive bands in the range between 1600 and 1800 cm^{-1} is not observed in the transition from Meta II to Meta III (blue spectrum). Instead, hardly any changes are observed in the range above 1700 cm^{-1} of protonated carboxylic acids and at 1644 cm^{-1} at the position of the amide I marker band of Meta II. This clearly indicates that the active state conformation of Meta II persists in Meta III. In particular, the receptor microdomains between H3 and H5 around Glu 122 and between H1, H2, and H7 around Asp 83 are not substantially changed by the transition. Further, Glu 113 on H3 remains protonated as in Meta II, and the protein backbone changes responsible for the amide band at 1644 cm^{-1} in Meta II, which had been tentatively assigned to those associated with relative movement of H3 and H6 (*27*), also persist in Meta III at this low pH.

At both pH 4.0 and pH 6.0, the near-UV illumination of Meta I/Meta II produces besides Meta III also 11-*cis*-rhodopsin and 9-*cis*-isorhodopsin, which can be quantified by photolysis of these stable products after complete decay of Meta III. At pH 4.0, this contribution is small, amounting to less than 20% of the total photoproduct and consisting predominantly of rhodopsin. At pH 6.0, this contribution increases to a total of about 45% of the photoproduct of the near-UV illumination, consisting of about equal amounts of both isomers. This will be important when turning to the high-pH form of Meta III, as will be detailed below. Interestingly, rhodopsin and isorhodopsin appear to be formed not only by a single light isomerization of the C11 or C9 polyene double bond but to some extent also by light isomerization of both the Schiff base C15═N and one of either the C11 or C9 polyene double bonds, followed by a thermal back-isomerization of the C15═N to 15-*anti* on a time scale of few minutes (unpublished observations). Similar combinations of consecutive light-induced and thermal isomerizations had been reported previously in the photoreactions of Meta III (*17*).

The low- and the high-pH forms of Meta III, which had been identified above by their distinctly different decay properties, correspond therefore to different protein confor-

mations of Meta III. The stable high-pH form, which is also formed during the thermal decay of Meta I/Meta II at neutral to alkaline pH, corresponds to an inactive receptor conformation, while the low-pH form corresponds to an active receptor conformation similar to that of Meta II, with which it also shares the rapid decay pathway by Schiff base hydrolysis.

The E134Q Mutation Does Not Favor the Active Meta III Conformation. In order to study the influence of the E134Q mutation on the conformation of Meta III, the experiments described in the previous section were repeated with the E134Q mutant of rhodopsin reconstituted into PC lipid membranes. As evident from Figure 2C, the conformational changes of the transition from the dark state to Meta II (red spectrum) are reverted in the transition to Meta III by the near-UV illumination at pH 6.0 to a similar extent as in the native pigment at the same pH (Figure 2B). Similar results were obtained for the wild-type pigment in PC lipid membranes, revealing little influence of the specific lipid environment (not shown). The E134Q mutation does therefore not significantly extend the pH range in which Meta III is present in its active low-pH form to higher pH values, which bears similarity to other partial agonists (*20*, *27*, *28*).

Analysis of the Chromophore in the Active Meta III Conformation at Low pH. The chromophore marker bands of Meta III are at slightly different positions (1343 and 1177 cm^{-1}) in the low-pH form of Meta III as compared with Meta III obtained at pH 6.0 or with classical Meta III obtained by thermal decay of Meta I/Meta II at alkaline pH. For classical Meta III, the isomerization of the Schiff base to 15-*syn* in the transition to Meta III had been revealed using isotopic labeling in combination with a quantum chemical vibrational analysis (*9*), which had been reproduced as well for Meta III formed photochemically by near-UV illumination of Meta II at neutral pH (*23*). In a 15-*syn* geometry, the NH in-plane bending mode of the Schiff base and the C14−C15 stretching mode of the polyene are kinetically coupled, leading to a downshift of the C14−C15 stretch in H_2O and an upshift in D_2O (*29*). In Meta III with its 15-*syn* geometry of the Schiff base, the C14−C15 stretch experiences therefore an enormous upshift upon H/D exchange by more than 50 cm^{-1} (*9*).

In Figure 3, the positions of the chromophore bands are compared for Meta III produced photochemically at pH 4.0 (Figure 3B, bands belonging to Meta III being positive) with those of Meta III produced in the thermal decay of Meta I/Meta II (Figure 3A, bands belonging to Meta III being negative), both in H_2O (red) and in D_2O (blue). The band at 1343 cm^{-1} in the active state conformation of Meta III at pH 4.0 in H_2O is the NH bending mode of the Schiff base, which is slightly downshifted from 1349 cm^{-1} in inactive thermal Meta III obtained at pH 8.0 and which disappears in D_2O. The band at 1177 cm^{-1} is the C14−C15 stretching mode, which had been assigned using ^{13}C-labeling of C14 and C15 of the retinal (not shown) and which is as well slightly downshifted as compared with the inactive Meta III form at alkaline pH. Upon H/D exchange, the C14−C15 stretch in the low-pH form of Meta III shows the same ~50 cm^{-1} upshift as in the high-pH form. In the high-pH form, the C14−C15 stretch appears to be split in D_2O into two bands at 1247 and 1236 cm^{-1}, which had been proposed to be due to an interfering Meta II protein band of opposite sign (*9*). In the low-pH form of Meta III, which shares the active state conformation of Meta II, this interfering band is lacking, such that the C14−C15 stretch appears in D_2O as a single band at 1238 cm^{-1}. This confirms the implicit assumption that low- and high-pH forms of Meta III share the same 15-*syn* chromophore geometry.

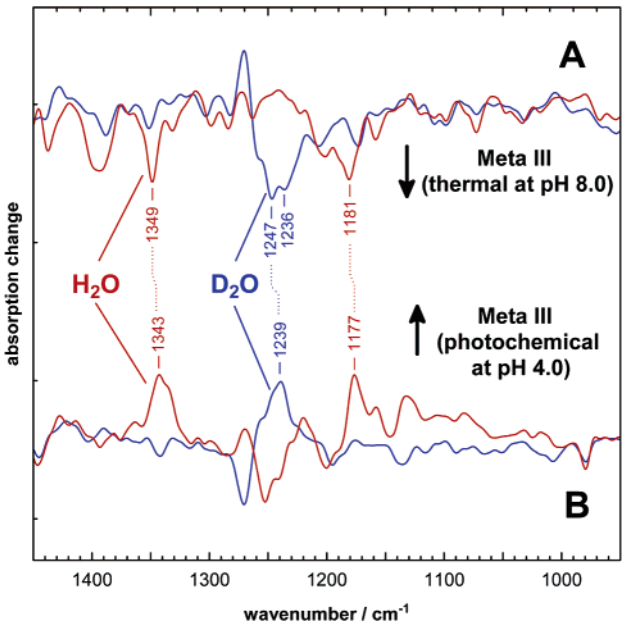

FIGURE 3: The active state conformation of Meta III shares the all-*trans* 15-*syn* chromophore geometry of classical Meta III. (A) Classical (thermal) Meta III, which had been produced by thermal decay of Meta I/Meta II at 30 °C and pH 8.0, was photoconverted back to Meta I/Meta II, producing a Meta I/Meta II minus Meta III difference spectrum (bands of thermal Meta III being negative). This difference spectrum reveals in H_2O (red spectrum) the characteristic chromophore bands of the 15-*syn* chromophore of Meta III, the NH bending mode of the Schiff base at 1349 cm^{-1}, and the C14−C15 stretching mode of the polyene at 1181 cm^{-1}, of which the NH bending mode disappears in D_2O (blue spectrum), while the C14−C15 stretch shows a pronounced upshift to a doublet at 1247/1236 cm^{-1} (*9*). (B) Meta III minus Meta II difference spectra of the photochemical production of Meta III by near-UV illumination of Meta II at pH 4.0 (Meta III bands being positive) reveal a very similar band pattern with NH bending and C14−C15 stretching modes slightly downshifted to 1343 and 1177 cm^{-1}, respectively, in H_2O, while the C14−C15 doublet in D_2O is replaced by a single band at 1238 cm^{-1}. This confirms that the low-pH active Meta III form has indeed the same all-*trans* 15-*syn* chromophore isomer as classical inactive Meta III.

Analysis of Active and Inactive Conformations of Meta III. As shown above in Figure 2B, the active Meta III conformation corresponds to that of Meta II and features in particular a protonated Glu 113, a similar hydrogen bonding of Asp 83 and Glu 122 in the H1/H2/H3 and the H3/H5 microdomains, respectively, and a similar structure of the protein backbone.

For a characterization of the inactive high-pH form of Meta III, we will not use the photochemical protocol for generation of Meta III, as this protocol produces in particular at alkaline pH also substantial amounts of rhodopsin and isorhodopsin, which might interfere with a structural analysis. We therefore generated Meta I/Meta II by orange illumination of rhodopsin in disk membranes at 30 °C and pH 8.0 (conditions under which this photoproduct equilibrium is largely on the Meta II side) and let this photoproduct equilibrium decay completely over 20 min by the thermal decay pathways to Meta III (in its inactive high-pH conformation) and opsin. Meta

III was then selectively photolyzed for 20 s by a >475 nm long pass filter, which converted it back to Meta I/Meta II, as described before (*9*, *23*). All of these reactions were followed by FTIR and UV−visible spectroscopy, yielding two light-dependent difference spectra: the Meta I/Meta II minus dark state spectrum of the first illumination and the Meta I/Meta II minus Meta III difference spectrum of the second illumination. Importantly, the position of the Meta I/Meta II photoproduct equilibrium does not depend on the initial state and is therefore identical in both difference spectra. We could now subtract the latter difference spectrum of the second illumination from that of the first illumination, such that contributions of Meta I/Meta II cancel out, ending up with a Meta III minus dark state difference spectrum. The spectrum of the second illumination is, however, substantially smaller than the spectrum of the first illumination, as only part of the initial Meta I/Meta II photoproduct of the first illumination decays to Meta III. We therefore derived a normalization parameter for the subtraction by analyzing the amplitude of the 380 nm photoproduct peak of Meta II in the two light reactions by UV−visible spectroscopy. Under our experimental conditions, this normalization parameter was found to be 35−40%, corresponding to the contribution of the Meta III pathway in the thermal decay of Meta I/Meta II.

Using this scaling factor, a Meta III minus dark state difference spectrum is obtained that can be compared to a Meta I minus dark state difference spectrum for comparison of the protein conformation in these two inactive product species (Figure 4A). The Meta III minus dark state spectrum is shown with two limiting scaling factors in light blue and dark blue, corresponding to a 35% and 40% yield of Meta III in the thermal decay, respectively. Glu 122 experiences an increase of its hydrogen bonding in Meta I as compared to the dark state, leading to a difference band with absorption at 1735 and 1728 cm^{-1} in the dark state and a shoulder at the high-frequency side of the peak at 1704 cm^{-1} in Meta I. In the inactive Meta III state, the absorption of Glu 122, which is positioned close to the ring of retinal (Figure 4B), is upshifted toward 1741 cm^{-1}, indicating a weakening of its hydrogen bonding in inactive Meta III and a perturbation of the H3/H5 network different from that in Meta I. Also, the absorption change of Asp 83 is in Meta III hardly changed as compared to the dark state, while a small but distinct negative absorption peak is produced in the transition to Meta I at 1769 cm^{-1}. Further, the amide I band of Meta I at 1664 cm^{-1} is absent in inactive Meta III.

The pH dependence of the Meta III conformation, which was characterized in Figure 1C already by monitoring the decay kinetics, was also studied by FTIR spectroscopy by producing Meta III by the photochemical protocol with orange and near-UV illumination at a series of pH values. This approach is somewhat hampered by the side reactions in the near-UV illumination step, which produce besides Meta III also rhodopsin and isorhodopsin in a pH-dependent manner, as reported above. Nevertheless, the obtained spectra can be evaluated to obtain a rough estimate of the apparent pK_a of the conformational equilibrium of Meta III, using the conformationally most sensitive spectral range between 1800 and 1600 cm^{-1} of the FTIR spectra as described previously (*20*). This analysis indicates a pK_a of the transition roughly around 4.7 at 10 °C and 5.1 at 30 °C. The data obtained at 30 °C are shown in Figure 1C in blue and are in agreement with the pH dependence of the decay kinetics presented in the same graph in red.

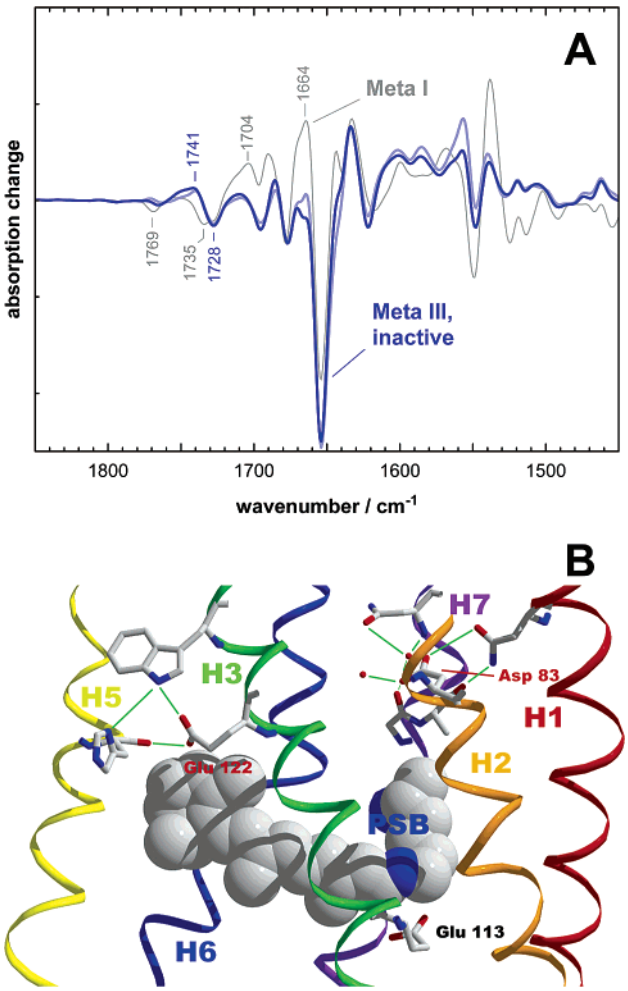

FIGURE 4: The all-*trans* 15-*syn* chromophore of inactive Meta III interacts with the microdomains around Glu 122 and Asp 83 differently as compared with the all-*trans* 15-*anti* chromophore in Meta I. (A) An FTIR difference spectrum Meta I minus dark state (gray spectrum), obtained at 10 °C and pH 9.0, is compared to difference spectra of inactive high-pH Meta III minus dark state (blue spectra). The latter spectra were obtained at 30 °C and pH 8.0 from Meta III produced in the thermal decay of Meta I/Meta II and show two limiting cases, corresponding to 40% (dark blue) and 35% (light blue) yield of Meta III in the thermal decay process (see text for details). The high-pH form of Meta III shows the general features of an inactive receptor conformation with some distinct differences to Meta I in the range above 1700 cm^{-1} due to changed interactions of Asp 83 in the H1/H2/H7 microdomain and Glu 122 in the H3/H5 microdomain. (B) A structural model of the retinal binding pocket with the protonated retinal Schiff base (PSB) and Glu 113 on H3, the H3/H5 microdomain with Glu 122, and the H1/H2/H7 microdomain with Asp 83 is shown, which is based on the coordinates 2HPY (*32*) of the inactive Lumi conformation with its all-*trans* 15-*anti* chromophore (the cytoplasmic side points upward).

DISCUSSION

Meta III is formed during the thermal decay of the Meta I/Meta II photoproduct equilibrium at neutral to alkaline pH in parallel to decay of Meta II by hydrolysis of the retinal Schiff base. Meta III can likewise be produced photochemically by near-UV illumination of Meta II. In both cases the transition to Meta III is triggered by an isomerization (either

thermal or light-induced) of the Schiff base C15═N double bond leading to an all-*trans* 15-*syn* isomer of the chromophore. In this study, we have shown that Meta III exists in a pH-dependent conformational equilibrium between active and inactive conformations similar to the Meta I/Meta II photoproduct equilibrium, however, with an apparent pK_a that is considerably downshifted into the range around 5.1. The inactive Meta III conformation formed at neutral to alkaline pH is stable and corresponds despite some distinct differences to a Meta I-like conformation. The active conformation of Meta III formed at pH values below the pK_a, on the other hand, corresponds to that of Meta II, with conformational rearrangements of receptor microdomains, protonation of Glu 113, and decay properties similar to that of Meta II. The Schiff base in this active form of Meta III remains, however, protonated, at least in the low-pH range in which this receptor conformation is stabilized.

Also, the apoprotein opsin forms in the absence of a ligand a conformational equilibrium between active and inactive conformations. This equilibrium is as well pH-dependent (*7*, *30*), indicating that the pH dependence of rhodopsin's photoproduct conformational equilibria is an intrinsic property of the receptor protein. The presence of a ligand in the retinal binding pocket merely modulates the position of this intrinsic equilibrium by specific ligand−protein interactions, which alter the energetics of the associated conformational states and thereby the apparent proton affinity (see, e.g., ref *28*). A shift of the position of the equilibrium is therefore synonymous to changing the apparent pK_a of the equilibrium. In the case of the Meta III conformational equilibrium, the apparent pK_a is at 30 °C around 5.1 and thus higher than that of opsin in the absence of ligand, which was found to be 4.1 (*7*), but lower than that of Meta I/Meta II in the presence of the full agonist all-*trans*-15-*anti*-retinal, where the apparent pK_a is above 8. As compared with the full agonist all-*trans*-15-*anti*-retinal, the all-*trans* 15-*syn* ligand of Meta III is therefore a classical partial agonist. In the thermal decay of the Meta I/Meta II equilibrium to Meta III at neutral pH, the thermal isomerization of the Schiff base C15═N double bond is therefore sufficient to switch the receptor from an active to an inactive conformation. Such an isomerization is, however, not sufficient to completely lock the receptor in an inactive conformation, as inverse agonists like 11-*cis* or 9-*cis* ligands would do.

How does the C15═N isomerization to 15-*syn* achieve this switch from a full to a partial agonist? Recent NMR studies revealed a longitudinal movement of retinal toward H5 during receptor activation (*31*). The β-ionone ring of retinal had been shown to interact closely with the interhelical network between Glu 122 and His 211 on TM3 and TM5, respectively (Figure 4B). This key interaction couples allosterically the disruption of the salt bridge between protonated Schiff base and Glu 113 on TM3 in the transmembrane core of the receptor with proton uptake by a cytoplasmic microdomain between H3 and H6 around Glu 134 (*20*, *27*). Partial or complete removal of retinal's β-ionone ring weakens this key interaction, uncouples the Schiff base and the cytoplasmic microdomains, and renders receptor activation inefficient. This uncoupling becomes obvious in a deficiency of the E134Q mutation to counteract inefficient receptor activation by acyclic or 9-demethyl partial agonist ligands (*20*, *28*), which is observed similarly here for the all-*trans* 15-*syn* partial agonist.

In the all-*trans* 15-*anti* full agonist, the chromophore maintains its extended geometry of the polyene beyond the Schiff base C15═N double bond to the ϵ-carbon of Lys 296 (Figure 4B), before the lysine side chain turns sharply in the direction to the cytoplasmic side, as suggested by the recently published structure of lumirhodopsin (*32*). In this structure, the ϵ-carbon of Lys 296 is docked between Met 44 on H1, Phe 91 on H2, and Phe 293 on H7. The all-*trans* 15-*anti* chromophore represents therefore a quite rigid linker connecting the H1/H2/H7 domain at one end close to the Schiff base with the ring binding pocket and the H3/H5 domain.

In the all-*trans* 15-*syn* isomer of Meta III, this fully extended geometry is lost, and the effective length of the chromophore becomes shorter. This shortening of the rigid part of the chromophore could impair receptor activation by weakening tight interactions between the ring and H3/H5 microdomain at the opposite end. Such a change in interaction is seen in the altering of the hydrogen bonding of Glu 122 in this microdomain (Figure 4B). In line with such an effective shortening of the ligand, the network between H1, H2, and H7 at the Schiff base end of the ligand appears to be unperturbed by the all-*trans* 15-*syn* chromophore as compared with the dark state, while the extended all-*trans* 15-*anti* geometry of the full agonist leads to a small, but distinct signature in the absorption of Asp 83 (H2) in the Meta I minus dark state difference spectrum (Figure 4A).

Obviously, the partial agonist behavior of the all-*trans* 15-*syn* ligand in Meta III could further be expected to be influenced considerably by the reorientation of the protonated Schiff base in the 15-*syn* isomer. However in Lumi, the farthest evolved photoproduct state of which an atomic resolution structure is available up to now, Glu 113 is oriented to the side of the polyene plane (*32*) (Figure 4B), such that the influence of the isomeric state of the C15═N double bond on a PSB−Glu 113 salt bridge could in fact be rather marginal. This situation might be somewhat changed again in Meta I or in the inactive Meta III conformation, as retinal's motion seen in the transition to Meta II (*31*) might be anticipated to some extent already in these inactive conformations and as the influence of Glu 181 on extracellular loop 2 to the counterion function would become stronger (*25*, *33*).

The general principle of a minimal effective length of the ligand required for maximal activation of the receptor is likely also relevant for other partial agonists that have, for instance, deletions at the β-ionone ring at the opposite end of the ligand. As mentioned above, deletions of ring methyl groups or of the closed ring structure of retinal severely impaired receptor activation and rendered the ligands classical partial agonists (*20*, *21*). The partial agonism of these acyclic or ring-demethylated retinal analogues could be at least partially caused by an effective shortening of the length of the ligand similar to that of the 15-*syn* ligand of Meta III. Of course, the precise molecular interactions at the ring binding pocket interface are different for the ring-modified ligands as compared with the Meta III ligand. This is evident from the entirely different hydrogen-bonding pattern of Glu 122 (Figure 4B), showing an extremely strong hydrogen bonding in the inactive conformation of the acyclic analogues

(*20*), which is not seen in the inactive Meta III state. Nevertheless, we observe with both ligand systems a similar shift of the conformational equilibrium to the inactive side going along with the characteristic breakdown of the allosteric coupling between the Schiff base region and the cytoplasmic H3/H6 microdomain around Glu 134. This suggests that a shortening of the effective length of the ligand could be a common mechanism for partial agonism that is shared by both the acyclic ligands and the all-*trans* 15-*syn* ligand of Meta III.

In summary, our results show that the 15-*anti* to 15-*syn* isomerization of the all-*trans* chromophore Schiff base in the thermal decay from Meta I/Meta II to Meta III accomplishes a partial deactivation of the receptor by switching the ligand from a full agonist to a partial agonist. An effective shortening of the ligand could be the molecular mechanism behind this functional switch. This switch leads to a silencing of the receptor at neutral to alkaline pH, that does, however, not restore the complete, pH-independent inactivity of the dark state. Complete deactivation of the receptor is achieved only after release of all-*trans*-retinal from its binding pocket and binding of 11-*cis*-retinal supplied by the visual cycle, which act as an inverse agonist locking the receptor in its inactive conformation.

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