Interaction with Transducin Depletes Metarhodopsin III

A REGULATED RETINAL STORAGE IN VISUAL SIGNAL TRANSDUCTION?*

Received for publication, June 18, 2004, and in revised form, August 20, 2004
Published, JBC Papers in Press, August 20, 2004, DOI 10.1074/jbc.M406856200

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In the phototransduction pathway of rhodopsin, the metarhodopsin (Meta) III retinal storage form arises from the active G-protein binding Meta II by a slow spontaneous reaction through the Meta I precursor or by light absorption and photoisomerization, respectively. Meta III is a side product of the Meta II decay path and holds its retinal in the original binding site, with the Schiff base bond to the apoprotein reprotonated as in the dark ground state. It thus keeps the retinal away from the regeneration pathway in which the photolyzed all-trans-retinal is released. This study was motivated by our recent observation that Meta III remains stable for hours in membranes devoid of regulatory proteins, whereas it decays much more rapidly in situ. We have now explored the possibility of regulated formation and decay of Meta III, using intrinsic opsin tryptophan fluorescence and UV-visible and Fourier transform infrared spectroscopy. We find that a rapid return of Meta III into the regeneration pathway is triggered by the G-protein transducin (Gt). Depletion of the retinal storage is initiated by a novel direct bimolecular interaction of Gt with Meta III, which was previously considered inactive. Gt thereby induces the transition of Meta III into Meta II, so that the retinylidene bond to the apoprotein can be hydrolyzed, and the retinal can participate again in the normal retinoid cycle. Beyond the potential significance for retinoid metabolism, this may provide the first example of a G-protein-catalyzed conversion of a receptor.

The visual pigment in retinal rods, rhodopsin, is an archetype of a G-protein-coupled receptor and belongs to the large class I of the G-protein-coupled receptor superfAMILY. Its chromophoric ligand, 11-cis-retinal, is responsible for the absorption of light and for the function as a photoreceptor. To make the receptor permanently available for light excitation, the retinal is bound to the opsin apoprotein by a covalent Schiff base linkage. However, in contrast to other retinal proteins, which serve biological functions from proton transport to archaeal phototaxis, the Schiff base is not unconditionally stable during the whole lifetime of the protein (see Ref. 1). Within minutes after light excitation and during the lifetime of the active, G-protein binding conformation, the linkage to the photoisomerized chromophore is broken by hydrolysis, and the photolyzed all-trans-retinal is released from the active site (see Refs. 2 and 3). The site is then filled with fresh 11-cis-retinal delivered by a specialized retinal metabolism that involves a complex enzymology in the distant cells of the pigment epithelium (see Ref. 4). This so-called retinoid cycle is necessary because the retinal exchange mechanism in rhodopsin is exclusive and unidirectional, so that not even the absorption of blue light allows the restoration of the original 11-cis-retinal opsin complex. This is likely to be the price to be paid for the exceedingly stable ground state provided by the tight packing of this form of retinal in the apoprotein binding pocket (5). This work and the accompanying article by Ritter et al. (43) deal with an intriguing and potentially important detail of the retinal cycle, namely, that not all of the rhodopsin activated by light is immediately subjected to regeneration and takes part in the retinal cycle. A substantial part of the retinal remains bound in a form termed metarhodopsin (Meta) III, which follows on the active Meta II intermediate.

The retinal cycle starts with light absorption in the inactive ground state of rhodopsin, in which 11-cis-retinal is bound by a protonated Schiff base linkage to Lys296 in the seventh of the transmembrane helices. After light-induced 11-cis-retinal/all-trans-retinal isomerization, the active Meta II state is formed within milliseconds. Specific interactions stabilizing the ground state are now broken in the protein and allow it to adopt a conformation in which the G-protein and also arrestin are recognized (see Refs. 2 and 3). Although the Schiff base is deprotonated in Meta II, thus breaking the central salt bridge between the Schiff base nitrogen and its counterion at Glu113, the all-trans-retinylidene linkage is still intact (see Ref. 6). A rapid quench of the catalytic activity of rhodopsin is brought about by interaction with receptor kinase, which catalyzes phosphorylation of rhodopsin at C-terminal sites and initiates tight interaction with arrestin (see Ref. 7). All these interactions occur while rhodopsin is still in its active conformation (see Ref. 3).

Full deactivation requires release of the photolyzed all-trans-retinal from and uptake of fresh 11-cis-retinal into the retinal binding pocket, a sequential process for which a channeling of the chromophore through entrance and exit sites of rhodopsin was recently proposed (Ref. 8; for the physiological perspective, see Ref. 9). However, parallel to hydrolysis and transition into the exit site, a fraction of the isomerized chromophore (up to 30% under cellular conditions) can also take a side tour through the Meta III state, in which the Schiff base linkage remains intact and is reprotonated. Recent work has provided evidence that Meta III arises from Meta I by isomerization

* This work was supported by Grant SFB 366 from the Deutsche Forschungsgemeinschaft (to M. H. and K. P. H.) and a grant from the Fonds der Chemischen Industrie (to K. P. H.).

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1 The abbreviations used are: Meta, metarhodopsin; P-Meta, photogenerated metarhodopsin; FTIR, Fourier transform infrared; Gt, G-protein transducin; Pipes, 1,4-piperazinediethanesulfonic acid.
around the Schiff base bond, so that the retinal polyene chain is in the all-trans configuration and the C15=N double bond is in the syn configuration (10, 11).

The presumed significance of the Meta III state lies in the fact that the chromophore is excluded for a certain time interval from the release reaction, thus avoiding accumulation of the toxic all-trans-retinal and blocking regeneration in a substantial fraction of the isomerized pigment (see Ref. 9). Intriguingly, however, Meta III is only transiently formed in situ and returns within minutes into the normal regeneration pathway, whereas it is much more stable in isolated disc membranes, and pH 7.3) for Meta III to decay, longer than any of the physiological time constants of the retinal cycle.

In this study, we will describe how the storage side pathway of the retinoid cycle is regulated. Surprisingly, we find that the G-protein transducin itself regulates the lifetime of Meta III.

EXPERIMENTAL PROCEDURES

Materials—Radioactive [α-32P]GTP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. Gα,α-(340–350), IKENLKDGLCF (12); Gα,α-(340–350)-high affinity analog peptide (Gα,α-HAA), VLEDLKSCGLF (13); and Gtα-(60–71) farnesyl, DKNPFKELKGGC-farnesyl (14) were synthesized by Dr. Peter Henklein (Humboldt University, Berlin, Germany). Isotonic buffer contains 130 mM NaCl, 1 mM MgCl2, and pH 7.3. (14) were synthesized by Dr. Peter Henklein (Humboldt University, Berlin, Germany). Isotonic buffer contains 130 mM NaCl, 1 mM MgCl2, and pH 7.3. Bovine serum albumin (BSA, Fraction V) and bovine rhodopsin (opsin complexed with all-trans-retinal) were obtained from 5°C (obtained from 5°C bleached rhodopsin; see Illumination Protocol Formation of Meta III). Difference spectra of the Meta III decay were obtained by subtracting the first spectrum recorded immediately after adjusting the pH or temperature of the samples (see Fig. 1B) or after addition of Gt or synthetic peptides to the suspensions (see Fig. 3A).

The time-dependent change of absorbance at 475 nm yields the time course of Meta III decay (see Fig. 3A, inset). The peptide-induced depletion of Meta III was also used to quantify the amount of Meta III when the decay reaction was too slow to follow directly. Briefly, aliquots were withdrawn at a given time after incubation of the membrane suspensions at the respective temperature. Immediately after addition of peptide (5 μM Gtα-HAA), a spectrum was taken and used as a baseline. The subsequent decay of Meta III was then followed spectroscopically and the amount of Meta III was calculated from the maximum change of absorbance at 475 nm using $\varepsilon = 40,000 \text{ M}^{-1} \text{ cm}^{-1}$ (24).

Fluorescence Measurements—The fluorescence assay of illuminated rhodopsin in isolated disc membranes is based on the quenching of opsin tryptophan fluorescence excited by the retinal ligand and was applied to study the release of the chromophore from its binding pocket (25). Fluorescence signals were detected with a SPEX Fluorolog F-30 spectrometer equipped with an integrating sphere. These measurements were performed with membrane suspensions (5 μM, 5 μM rhodopsin) in isotonic buffer were stirred continuously and incubated at 30°C during the experiment. Reactions were started by illumination with a 150-watt fiber optic light source filtered through a heat filter (Schott KG2) and a 495 nm long pass filter (Schott GG495) in the absence or presence of Gtα-HAA peptide (100 μM). The time course of light-induced fluorescence change (F) was analyzed using a simple monoexponential function (Equation 1)

$$F = [F]_0 (1 - e^{-kt})$$  \hspace{2cm} (Equation 1)

in which $[F]_0$ represents the maximum amplitude of the fluorescence change, and $k$ identifies the rate constant of the conversion of Meta II to opsin and all-trans-retinal (see the reaction scheme in Fig. 6). Addition of Gtα-HAA to Meta III induces formation of Meta II, which in turn decays to opsin and all-trans-retinal. Thus, the time course of Gtα-HAA-induced fluorescence change was analyzed using the equation describing product formation for a series of two first-order reactions (Equation 2) (see Ref. 26)

$$F = [F]_0 (1 - \frac{1}{k_1} (e^{-k_1 t} - e^{-k_2 t}))$$  \hspace{2cm} (Equation 2)

in which $k_1$ represents the rate constant of the Meta III→Meta II conversion (see Fig. 6).

GDP Dissociation Assay—To monitor dissociation of GDP from the Gα subunit, the release of [α-32P]GDP was quantified using the filter binding assay. Gtα, complexed with [α-32P]GDP was prepared by incubating Gtα (10 μM) with Gβγ (15 μM), [α-32P]GTP (2–11 nM), and illuminated disc membranes (1 μM rhodopsin) for 1 h at 22°C. After sedimentation of the membranes (min, 12000 × g), the supernatant containing the [α-32P]GDP-loaded G-protein was removed and stored on ice. The remaining membranes were washed once with Millipore cellulose nitrate filters (0.45 μm), and washed once with 1 ml of ice-cold buffer. The amount of G-protein complexed with [α-32P]GDP associated with the filter was measured by scintillation counting.
rates obtained by single exponential fit to the data are plotted as a function of the pH value (right panel with the previously reported 134 kJ/mol) (27) and significantly higher than the decay rates, namely, 3 nm was normalized to the initial amount of Meta III (usually 35–40% of the bleached pigment). Single exponential fits to the data yield values of the decay of Meta III at 475 nm were directly followed at various pH values. Selected measurements obtained at pH 5.5 (a), 6.6 (b), 7.3 (c), and 7.9 (d) are presented in the left panel. The absorption was normalized to the maximum absorbance change (i.e. the initial amount of Meta III). Decay rates obtained by single exponential fit to the data are plotted as a function of the pH value (right panel). All measurements were done at 30 °C.

counting in a Beckman LS6500. Control measurements were performed with rhodopsin in the dark and with fully decayed light-activated rhodopsin (opsin/all-trans-retinal, see Illumination Protocol: Formation of Meta III).

RESULTS

Stability of Meta III Depends on pH and Temperature—Meta III is formed by thermal decay of Meta II (or Meta I) within minutes (10, 22). Using isolated disc membranes, Meta III formation is essentially irreversible, i.e. Meta III is stable for hours at pH 8.0 (see below). This makes it easy to separate the formation and decay of Meta III from one another and to determine the rates of decay as a function of temperature or pH. Thus, a protocol was applied to which first Meta III was formed at pH 8.0 and 20 °C (i.e. where its formation is at a maximum) and then its decay was followed at various conditions. Decay of Meta III was followed spectrophotometrically by the decrease of absorbance at 475 nm (examples can be seen in Fig. 3A). Under conditions in which Meta III decay rates were too slow (t/2 > 200 min) and affected by sedimentation, the amount of Meta III present at a given time was determined by the fast and quantitative peptide-induced Meta III depletion (Figs. 1A and 3). The dependence of Meta III decay on temperature and pH is shown in Fig. 1. The temperature dependence is quite pronounced, with an increase of the rate between 0 °C and 30 °C by more than a factor of 100. Monophasic fits to the data and representation of the resulting decay rates in form of an Arrhenius plot (Fig. 1A, right panel) allow us to estimate the activation energy (E_a) as 130 kJ/mol for the overall decay of Meta III to opsin and all-trans-retinal. The value is consistent with the previously reported E_a obtained with rat retinas (E_a = 134 kJ/mol) (27) and significantly higher than the E_a of the decay of Meta II to opsin and all-trans-retinal measured under similar conditions (E_a = 67 kJ/mol; Ref. 22). The high E_a obtained gives a first hint that the decay of Meta III involves a substantial change of protein conformation. As Fig. 1B shows, Meta III depends on pH as well, with a half time of ~2 min at pH 5.5 and 60–90 min at neutral pH (30 °C). Note that the rate versus pH plot (Fig. 1B, right panel) does not allow us to determine a pK for the reaction because data for pH < 5.5 are uncertain, due to protein denaturation over the long measuring period. In any case, the pK is <6.

G_a C-terminal Peptide Accelerates the Decay of Meta III—FTIR spectroscopy is sensitive not only to the environment of the chromophore, but also to alterations of the protein structure that accompany the formation of an intermediate. Spectrum a in Fig. 2A represents the Meta II minus rhodopsin difference spectrum obtained when the rhodopsin dark ground state is photoactivated. It shows the typical fingerprint pattern around 1238 cm⁻¹ characteristic for 11-cis/all-trans isomerization. Illumination of Meta II with blue light results in formation of P-Meta III (for details, see the accompanying article by Ritter et al., Ref. 43). The P-Meta III content in the sample was probed by its absorption at 475 nm of aliquots taken after incubation of the sample at the respective temperature (see Fig. 3 and “Experimental Procedures”). The overall change in absorbance at 475 nm was normalized to the initial amount of Meta III (usually 35–40% of the bleached pigment). Single exponential fits to the data yield values of the decay rates, namely, 3 × 10⁻⁵ s⁻¹ (0 °C), 1.12 × 10⁻³ s⁻¹ (20 °C), and 9.6 × 10⁻³ s⁻¹ (30 °C). Representation of these rates in Arrhenius coordinates (right panel, squares) yields a value of 130 kJ mol⁻¹ for the activation energy of the Meta III decay. The triangle identifies the Meta III decay rate obtained at 30 °C and pH 8.0 by the fluorescence assay (see “Experimental Procedures” and Fig. 2B). B, spectral changes during the decay of Meta III at 475 nm were directly followed at various pH values. Selected measurements obtained at pH 5.5 (a), 6.6 (b), 7.3 (c), and 7.9 (d) are presented in the left panel. The absorption was normalized to the maximum absorbance change (i.e. the initial amount of Meta III). Decay rates obtained by single exponential fit to the data are plotted as a function of the pH value (right panel). All measurements were done at 30 °C.
The family of time-dependent absorption change measured without peptide (Fig. 2B, trace a), the onset of the peptide-induced fluorescence increase is significantly delayed, demonstrating that at least two consecutive reaction steps with similar rate constants are involved in the release of the chromophore. The sigmoidal shape of fluorescence change is indeed well fitted by Eq. 2, which is based on a process with two irreversible consecutive reaction steps (see “Experimental Procedures” and Fig. 6). Thus, a mechanism can be excluded in which the peptide induces the direct decay of Meta III to opsins and all-trans-retinal.

When G\(_{\alpha}\)-HAA was added after partial decay of Meta III, the amplitude of fluorescence increase was reduced. Thus, analogous to the protocol applied in UV-visible spectroscopy, the dependence of the peptide-induced fluorescence change on the incubation time could be used to analyze the stability of Meta III; the data thereby obtained (data not shown) yield a value of 77 min for the half-time of Meta III decay in the absence of G\(_{\alpha}\)-HAA (30 °C, pH 8.0), which is consistent with the results obtained by UV-visible spectroscopy under identical conditions (Fig. 1A, right panel).

We note that the fast peptide-induced depletion of Meta III cannot be explained by a simple back-shift from Meta III, as a consequence of the known stabilization of Meta II by interaction with the peptide (12, 28). This would be analogous to the formation of “extra Meta II,” which is seen when Meta II is enhanced at the expense of its predecessor, Meta I (29). According to such a model, the intrinsic rate of the Meta III → Meta II conversion would be fast (\(t_{1/2} \approx 100\) s). The observed stability of Meta III in the absence of the peptide (\(t_{1/2} > 100\) min) could then only be explained by a very fast intrinsic rate of Meta III formation (\(t_{1/2} < 100\) s). Such rapid formation of Meta III is not observed experimentally, providing the first evidence for a direct interaction between the peptide and the Meta III species.

G\(_{\alpha}\) C-terminal Peptide Interacts Directly with Meta III—Further experimental evidence for a direct interaction between peptide and Meta III was obtained by concentration-dependent measurements of the back-reaction Meta III → Meta II. Such data are shown in Fig. 3. The UV-visible difference spectra monitor Meta III decay as the decrease of absorbance at 475 nm with a concomitant increase of absorbance in the range of 360–380 nm (Fig. 3A). The family of time-dependent original records in Fig. 3B reveals that the Meta III decay reaction depends on G-protein peptides and demonstrates that the rate of the reaction (the initial slope of the traces) depends on the peptide concentration.

G\(_{\alpha}\) Catalyzes Metarhodopsin III Depletion

Fig. 2. Characterization of Meta III by FTIR and fluorescence spectroscopy. A, FTIR difference spectra measured at 23 °C and pH 7.2. a, Meta II minus rhodopsin difference spectrum obtained on illumination of rhodopsin. Typical fingerprint pattern includes the negative band at 1238 cm\(^{-1}\) and its satellites, which are characteristic for 11-cis-retinal/all-trans-retinal isomerization. b, Meta III minus Meta II difference spectrum. This conversion was induced by illumination of Meta II with 400 nm light. For better comparison, the spectrum is inverted. The retinal fingerprint pattern is completely different from a; bands assigned to retinal cistrans isomerization are absent; instead, a new band at 1348 cm\(^{-1}\) is present, c, Meta II minus P-Meta III difference spectrum obtained in the absence of G\(_{\alpha}\)-HAA peptide. The spectrum was recorded 210 min after light-induced formation of P-Meta III. The pronounced negative band at 1348 cm\(^{-1}\) indicates the presence of Meta III in the sample. The intensity of this band is significantly reduced in the difference spectrum recorded 120 min after light-induced formation of P-Meta III in presence of G\(_{\alpha}\)-HAA peptide (d). The bands at 1238 and 1206 cm\(^{-1}\) are assigned to 11-cis and 9-cis ground state. B, decay of Meta II (generated by saturating photoactivation of 5 μm rhodopsin, first flash) was monitored by the change of intrinsic fluorescence accompanying the release of all-trans-retinal from the active site at 30 °C and pH 7.5 (22). G\(_{\alpha}\)-HAA (100 μM) was added either before photoactivation of rhodopsin (trace a) or at two times after completion of the Meta II decay (traces b and c, arrows). After 60 min, all samples were illuminated again (second flash). Inset, enlarged view of traces b and c showing the time course of the fluorescence change induced by the addition of G\(_{\alpha}\)-HAA (trace b) or by the second illumination after addition of G\(_{\alpha}\)-HAA (trace c). The smooth lines plot Eq. 2 using \(k_1 = 0.0063\) s\(^{-1}\) and \(k_2 = 0.0024\) s\(^{-1}\) (trace b) or Eq. 1 using \(k_2 = 0.0024\) s\(^{-1}\) (trace c; see Fig. 6 for identification of these rate constants).

indicated by the absence of the 1348 cm\(^{-1}\) absorption change. Light-induced changes in this spectrum can be explained by cis/trans isomerization of residual 11-cis-retinal and 9-cis-retinal identified by the bands at 1238 and 1206 cm\(^{-1}\). These cis-isomers may be generated by the illumination of free all-trans-retinal initially present in the dark-adapted sample. The result indicates that the peptide can influence the amount of P-Meta III once formed.

Release of the chromophore from the active site can be monitored by the change of intrinsic tryptophan fluorescence of opsin (Fig. 2B) (22). Importantly, the strong quenching of Trp fluorescence exerted by all-trans-retinal bound in its original pocket is similar in all metarhodopsin intermediates (Meta I, Meta II, and Meta III). Using the fluorescence monitor, it was previously shown that interaction of G\(_{\alpha}\)-HAA with Meta II effectively blocks its conversion to Meta III, which is directly seen in the larger fluorescence increase in the presence of the peptide (Fig. 2B, trace a) as compared with the fluorescence change measured without peptide (Fig. 2B, trace b). A second illumination with green light after 60 min of decay converts the fraction of Meta III formed to Meta II, whose subsequent decay to opsins/all-trans-retinal gives rise to a further increase in fluorescence (Fig. 2B, trace c). The initial fast fluorescence decrease reflects the fast light-induced conversion of Meta III to Meta II (see Fig. 2A and the accompanying article by Ritter et al., Ref. 43), which is similar to the initial fast fluorescence change that accompanies the light-induced conversion of rhodopsin to Meta I and Meta II. These changes are small compared with the fluorescence change that accompanies the release of the chromophore, confirming that the chromophore maintains its quenching of Trp fluorescence through Meta II and Meta III. Trace b of Fig 2B shows that addition of G\(_{\alpha}\)-HAA after formation of Meta III induces an increase of Trp fluorescence to a level similar to that obtained when Meta II quantitatively decays into opsin and all-trans-retinal. As seen in the inset of Fig. 2B, the onset of the peptide-induced fluorescence increase is significantly delayed, demonstrating that at least two consecutive reaction steps with similar rate constants are involved in the release of the chromophore. The sigmoidal shape of fluorescence change is indeed well fitted by Eq. 2, which is based on a process with two irreversible consecutive reaction steps (see “Experimental Procedures” and Fig. 6). Thus, a mechanism can be excluded in which the peptide induces the direct decay of Meta III to opsin and all-trans-retinal.

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This is most likely due to a lower affinity of these peptides, roughly in parallel to their lower affinity toward Meta II.

Interaction of Meta III with G-protein—The peptide data yield a first criterion that Meta III is not an inactive species, as was thus far generally assumed (see Refs. 3 and 30). The data presented in Fig. 4 show that addition of purified Gt induces a similar depletion of Meta III as that seen with the Gt peptides. Increasing amounts of Gt gradually accelerate Meta III depletion. As described above, this finding excludes a previously suggested mechanism (31) in which Gt acts via its stabilizing interaction. Accordingly, the slow phase reflects the slow recycling of Meta III complexed by peptide. Consistently, the data points shown in Fig. 4 reflect roughly in parallel to their lower affinity toward Meta II.

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interact with Meta III. However, in the presence of excess GDP, the G-protein-induced Meta III depletion is inhibited (Fig. 4C). Increasing amounts of GDP in the sample result in a suppression of predominantly the fast component of the depletion signals. Thus, we may conclude that Meta III-G-protein complexes are dissociated by GDP. The effect is seen at a few micromolar GDP, i.e. much less than was found for the GDP-dependent destabilization of the Meta II-G-protein complex.

The finding that addition of Gt causes the depletion of Meta III poses the question of how Meta III would build-up and decay at concentrations of Gt as they prevail in the rod cell. The experiments show (Fig. 4D) that in the absence of G-protein, Meta III is formed within 10 min and decays in a slower reaction (t1/2 ~100 min at pH 7.5 and 30 °C; ○ in Fig. 4, B and D). When the G-protein is present on the membrane at a ratio of 1:10 to rhodopsin, Meta III is transiently formed (Fig. 4D). As expected from the data in Figs. 1 and 4, Meta III decay is substantially accelerated, whereas the initial rate of Meta III formation is not affected by Gt. The depletion reaction cuts in before the maximum amount of Meta III is formed. This is because most G-protein is initially trapped by Meta II, thereby allowing the rest of the photoactivated rhodopsin to decay. Due to the high density of the rhodopsin receptor in the disc membrane, the complex with the G-protein is never quantitative, even with excess G-protein. Thus, in contrast to the Gt peptides, even a high concentration of G-protein cannot fully block Meta III formation. The negative absorbance differences at points late in the reaction are likely to arise from the initially formed Meta I, which causes a negative baseline.

**GDP Is Transiently Released during Depletion of Meta III**—To provide an extension and final proof of the interactions derived above, the release of GDP from the G-protein was measured. We had already seen that depletion of Meta III was inhibited by GDP, indicating release of GDP by either Meta III or Meta II interaction. The release of GDP during G-protein-induced depletion of Meta III was directly measured applying the filter binding assay and G-protein preloaded with [α-32P]GDP (Fig 5A; see “Experimental Procedures” for details). Binding of GDP to Gtα is very tight in the absence of light-activated rhodopsin. Consistently, no nucleotide release is detected on the time scale of the experiments in the absence of active receptor (Fig. 5A, ○). Interaction between Meta II and G-protein leads to almost quantitative dissociation of GDP from the Gtα subunit (Fig. 5A, ◊). However, the resulting GDP-free Meta II-Gt complex is not stable under the experimental conditions, which is seen in the slow reassociation of the released GDP to Gt. When Gt is added to Meta III-containing membranes, a substantial fraction of the GDP is slowly released (Fig. 5A, ▼). After correction of the data for the small but significant GDP release induced by the decay product opsin/all-trans-retinal (Fig. 5A, ○) also present in the sample, the relative fraction of released GDP per Meta III at a given time is obtained. The resulting curve shows that GDP is transiently released, with a maximum of 40% after 10 min. Comparison with the UV-visible data measured on aliquots under identical conditions reveals an identical onset of both reactions Meta III conversion to Meta II and GDP release, respectively (Fig. 5B).

**DISCUSSION**

The long-lived Meta III intermediate forms as a side product of the normal retinoid cycle, thus providing a mechanism to store away a fraction of the photoisomerized chromophore. Because in Meta III the chromophore remains bound to its original binding site, both the release of the photolysed all-trans-retinal and the entry of fresh 11-cis-retinal are blocked. The present investigation was motivated by our recent analysis of the molecular nature and lifetime of Meta III, in which we found an essentially irreversible formation of Meta III, with a lifetime longer than any of the time constants of the physiological retinoid cycle (22). On the other hand, the appearance of Meta III is a transient phenomenon under physiological conditions, with a rise and decay within minutes (see Refs. 9, 24, and 32). Our previous results were obtained with samples of isolated disc membranes that contain their native lipid and integral membrane inventory but are devoid of any regulatory proteins. The apparent conflict is solved by the results of this study. We show that the G-protein transducin, otherwise involved in transmitting the signal from rhodopsin to its phosphodiesterase effector, is also competent to empty the Meta III storage.

The G-protein Transducin Regulates Meta III Lifetime—The data presented here demonstrate that in the presence of Gt, the blockade of Meta III decay is resolved, and the rhodopsin receptor is put back into the active Meta II state, thus participating again in the normal retinoid cycle. Importantly, any effect of the G-protein by its well-known interaction with Meta II cannot explain the experimental data because Gt would have to “wait” for this species to be formed. In other words, the intrinsic Meta III →Meta II back-reaction would be rate-limiting, thereby dictating the pace at which the Meta III pool can...
Gt Catalyzes Metarhodopsin III Depletion

The data in Fig. 5 indicate that it is not an inactive species, as was in situ Gt-induced Meta III conversion back to Meta II is likely the Meta III decay rate depends on the concentration of Gt and all-forms in an essentially irreversible reaction (22) from Meta I (10). Meta II reaction to all-retinal and opsin. This irreversible reaction transm with deprotonated Schiff base (RhD) reactions and reactants shown in gray, including the rhodopsin species. The overall effect is enhanced by the pH dependence of both the Meta I—Meta III reaction and the subsequent Meta III conversion to Meta II by Gt. Under acidic conditions, formation of Meta III is very slow (22) and its Gt-induced depletion is fast, whereas at alkaline pH, formation of Meta III is faster than its decay (22), even in the presence of Gt, (see Fig. 4D). The overall effect is enhanced by the pH dependence of the Meta I-Meta II equilibrium (35).

The measured decay of Meta III in the absence of Gt (Fig. 1) shows that the Gt-independent thermal conversion of Meta III to the final products opsin and all-trans-retinal is slow but nevertheless significant. Because the interconversion of Meta I and Meta II as well as the decay of Meta II is much faster, none of these intermediates accumulates during the decay of Meta III, and it is hence not yet possible to analyze the reaction pathway. The data do not exclude hydrolysis of the Schiff base bond in Meta III itself (see Refs. 11 and 24), which might even be the predominant path in the absence of Gt. In any case, Gt-induced Meta III conversion back to Meta II is likely the predominant path in situ.

Activity of Meta III—The finding that Meta III interacts with transducin indicates that it is not an inactive species, as was generally assumed in previous work (see Refs. 10, 22, and 24). The data in Fig. 5B show that the time course of GDP dissociation coincides with that of the spectrosopically measured depletion of Meta III and that GDP is quantitatively released from the fraction of Gt that is involved in the transition. On the other hand, GDP effectively inhibits Meta III depletion (Fig. 4), indicating a very high apparent affinity of GDP. This apparent contradiction is solved by the following reaction mechanism: Meta III + GtGDPsMeta III-Gt + GDP = Meta II-Gt + GDP.

The reaction is initiated by a direct interaction of Gt with Meta III. In contrast to Gt-Meta II interaction, Meta III-Gt complex formation is slow and thermodynamically not favored. Subsequent formation of the well-known Meta II-Gt complex is fast and essentially irreversible, which is consistent with the findings that Gt blocks the back reaction (i.e. Meta III formation) and that no Meta III is formed from Meta II-Gt in the presence of excess GDP. The observed inhibition of Meta III depletion by GDP is due to the dissociation of the transiently formed Meta III-Gt complex by GDP before it relax into the Meta II-Gt complex, i.e. by a kinetic competition mechanism. According to this reaction scheme, the GDP-free complex Meta III-Gt would never accumulate. Thus, Meta III is an active species with respect to its ability to induce a slow release of GDP from Gtα. On the other hand, Meta III itself could act as a true catalyst only when GTP uptake by the Meta III-Gt complex is faster than its conversion to Meta II. In any case, the finding that Meta III interacts with Gt, and is thereby converted to an active species adds a mechanistically interesting and potentially important new aspect for the role of metarhodopsins in dark adaptation and bleaching desensitization.

Possible Significance of Regulated Meta III in Visual Physiology—Besides its putative role as an active species, the dependence of Meta III depletion on Gt and GDP provides an as yet unknown possibility to regulate the amount of retinal storage. Notably, the rise and decay of Meta III found under physiological conditions (see Ref. 32) are correctly reproduced for a concentration of the G-protein that approximates the amount present in situ (Fig. 4D). However, it is not clear whether the visual cycle in situ makes use of the variability of Meta III lifetime, thus adjusting the retinoid cycle to conditions of long-term illumination. The case measured by Baumann and Bender (32) would be just one of the possible cases, and Meta III formation and disappearance would mark the time-dependent availability of the retinal binding site for retinal release and hence regeneration of this fraction of the receptor. High nucleotide (GTP and GDP) concentration in the rod could prevent depletion of Meta III by G-protein (see Fig. 4), so that changes of physiologically relevant parameters would regulate the amount of Meta III formed. The data on amphibian rod outer segments presented by Kolesnikov et al. (24) would rather argue that Meta III remains in its time course independent of alterations of the energetic state of the cell (i.e. concentration of GTP, GDP, and GMP). On the other hand, Donner and Hemilä (36) have reported that the amount of Meta III in isolated perfused frog retinas depends on bleaching, so that no Meta III forms in rods for bleaches lower than 10% of the rhodopsin. It is obvious that the dependence on G-protein would easily explain such a variable formation of Meta III. Furthermore, transducin may not be the only protein that interacts with Meta III. Other proteins known to bind to light-activated rhodopsin, including rhodopsin kinase, arrestin, and its splice variant, p44, may also induce depletion of Meta III. It will be the subject of future studies to analyze the influence of such proteins on Meta III formation and decay.

Meta III is also generated by illumination of Meta II with blue light (T-Meta II; see the accompanying article by Ritter et al., Ref. 43) and is photoconverted to Meta I by green light (Fig. 6). Thus, its amount depends on the intensity and spectral
distribution of the light that is absorbed in the retinal photoreceptors. Furthermore, under conditions of continuous illumination, the amount of Meta III depends on the yield of the photochemical conversions and the relative rates of the thermal reactions involved. This could result in a steady state in which a fraction of the receptor is continuously in the storage form. Such a mechanism would also link the dynamically stored retinal. Such a mechanism would also link the

Acknowledgments—We thank Peter Henklein for providing the peptide. We also thank Jana Engelmann and Ingrid Semjonow for excellent technical assistance and Alexander Pulvermüller for helpful discussions.

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