THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 5, Issue of January 31, pp. 3162–3169, 2003
Printed in U.S.A.

Signaling States of Rhodopsin

FORMATION OF THE STORAGE FORM, METARHODOPSIN III, FROM ACTIVE METARHODOPSIN II*

Received for publication, September 20, 2002, and in revised form, October 30, 2002
Published, JBC Papers in Press, November 9, 2002, DOI 10.1074/jbc.M209675200

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Vertebrate rhodopsin consists of the apoprotein opsin and the chromophore 11-*cis*-retinal covalently linked via a protonated Schiff base. Upon photoisomerization of the chromophore to all-*trans*-retinal, the retinylidene linkage hydrolyzes, and all-*trans*-retinal dissociates from opsin. The pigment is eventually restored by recombining with enzymatically produced 11-*cis*-retinal. All-*trans*-retinal release occurs in parallel with decay of the active form, metarhodopsin (Meta) II, in which the original Schiff base is intact but deprotonated. The intermediates formed during Meta II decay include Meta III, with the original Schiff base reprotonated, and Meta III-like pseudo-photoproducts. Using an intrinsic fluorescence assay, Fourier transform infrared spectroscopy, and UV-visible spectroscopy, we investigated Meta II decay in native rod disk membranes. Up to 40% of Meta III is formed without changes in the intrinsic Trp fluorescence and thus without all-*trans*-retinal release. NADPH, a cofactor for the reduction of all-*trans*-retinal to all-*trans*-retinol, does not accelerate Meta II decay nor does it change the amount of Meta III formed. However, Meta III can be photoconverted back to the Meta II signaling state. The data are described by two quasi-irreversible pathways, leading in parallel into Meta III or into release of all-*trans*-retinal. Therefore, Meta III could be a form of rhodopsin that is storaged away, thus regulating photoreceptor regeneration.

Phototransduction in vertebrate rods starts with the isomerization of the 11-*cis*-retinal bound to opsin and the formation of the active photoproduct, metarhodopsin (Meta)[1] II. In Meta II, the Schiff base linkage between the all-*trans*-retinal and Lys^{296} is still intact but deprotonated. Catalytic activation of the G-protein, G_t or transducin, leads to a biochemical cascade of reactions, termed phototransduction. These reactions culminate in the hyperpolarization of the photoreceptor cells and ultimately in changes in the rate of neurotransmitter release at the synaptic terminus. The signaling state of Meta II is quenched rapidly by the action of rhodopsin kinase and arrestin. Equally important for vision is the metabolic cycle, which enables the visual system to take away the photolyzed chromophore, all-*trans*-retinal, and replace it with 11-*cis*-retinal, thus regenerating the pigment. The decay of Meta II thus provides an interlink among transduction, the quenching by phosphorylation and capping with arrestin, and regeneration (reviewed in Ref. 1).

During the decay of Meta II, the Schiff base linkage between the all-*trans*-retinal and the opsin apoprotein (Lys^{296}) is hydrolyzed. A side product is the bright orange ($\lambda_{max} \sim 470$ nm) Meta III, which slowly replaces the pale yellow color of the Meta II product ($\lambda_{max} = 380$ nm). Although it is not clear whether Meta III represents one homogeneous species, one may define it as the late product in which the chromophore is still bound to its original binding site. In the isolated retina and in intact rod outer segment preparations, Meta III eventually decays into all-*trans*-retinal and opsin (2–5). Compared with Meta II, opsin and Meta III adopt a more rhodopsin-like conformation (6, 7) and are either inactive or marginally active toward the G-protein. In the native system of mice, there is evidence that all-*trans*-retinal can accumulate (8), although the nature of the product between the photoisomerized chromophore and opsin has not been elucidated. *In vivo*, the decay of the Meta species is the first step in the retinoid cycle, and all-*trans*-retinal is released from opsin directly to either the cytoplasmic surface or to the intradiskal side, from where it is flipped to the cytoplasm (9). Next, all-*trans*-retinal is reduced to all-*trans*-retinol by a dehydrogenase (reviewed in Ref. 1). Moreover, all proteins that interact with photoactivated rhodopsin (*e.g.* G_t, rhodopsin kinase, and arrestin) are expected to inhibit the decay reaction (10–13).

In addition to Meta II, three distinct signaling states are described in the literature. First, retinal-free opsin has a measurable activity *in vitro* on the order of 10^{-6} that of Meta II (14); at very low pH, opsin changes its conformation toward an active state (15). Second, a signaling state is achieved by formation of a reversible Schiff base between all-*trans*-retinal and peripheral lysine group(s) of opsin, leading (preferentially at pH > 8.0) to reversible pseudo-photoproducts (11, 16). These products interact with arrestin and rhodopsin kinase, but an interaction of these products with G_t was not measured. Finally, a non-covalent opsin/all-*trans*-retinal complex can form between opsin and all-*trans*-retinal (16–20). It has a substantial activity toward the G-protein, three to four orders of mag-

* This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 366) and from the Fonds der Chemischen Industrie (to K. P. H.), National Institutes of Health Grant EY09339, a grant from Research to Prevent Blindness, Inc. (RPB) (to the Dept. of Ophthalmology at the University of Washington), and grants from Foundation Fighting Blindness, Inc. and the E. K. Bishop Foundation (to K. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Meta, metarhodopsin; FTIR, Fourier transform infrared; G_t, G-protein of the rod, transducin; $G_t\alpha$-HAA, $G_t\alpha_{(340-350)}$-high affinity analog; NRO, *N*-retinylidene opsin; bis-tris-propane, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Pipes, 1,4-piperazinediethanesulfonic acid.

nitude higher than the empty apoprotein. In this complex, the binding site for the all-*trans*-retinal appears to be different from that of Meta II (16, 20).

Fundamental questions about the nature of the decay process of Meta II still remain unanswered. For example, what are the intermediates in this process from Meta II to free opsin and all-*trans*-retinal? How active are they toward G-protein? Which products determine the sensitivity of the cell during dark adaptation and under conditions of constant illumination? What role does Meta III play in this process? How and when does arrestin dissociate from the active rhodopsin?

In this study we have investigated whether a post-Meta II photoproduct with the properties of Meta III does indeed exist. To understand how the photolyzed chromophore is handled during Meta II decay and regeneration, it is indispensable to know whether it remains bound to its original binding site in a large fraction of the receptor population. Recent accounts of the vast existing literature on the topic come to the conclusion that during the decay of Meta II into the 470-nm absorbing species, the retinal is released from its original binding pocket (21). Using an intrinsic fluorescence assay to measure chromophore release, Fourier transform infrared spectroscopy to study changes in the rhodopsin structure, and UV-visible spectroscopy to monitor formation of spectrally distinct species, we are now able to address the problem on a new experimental basis.

Dissection of the rather complex network of decay, regeneration, and storage likely requires kinetic analyses of the conversions (22–24). An appropriate biochemical system is a preparation of isolated disk membranes, which provides the correct lipid host, an important determinant of the formation and decay of Meta II (for review see Ref. 25). This measuring system is simplified by the lack of the metabolic machinery of regeneration, preventing complications from these processes on the kinetics of the decay reaction. We will present direct evidence on the formation of a stable Meta III photoproduct and propose a role for this form of rhodopsin in physiological conditions.

EXPERIMENTAL PROCEDURES

Materials—$G_t\alpha_{(340-350)}$-high affinity analog-peptide ($G_t\alpha$-HAA), VLEDLKSCGLF (26), was synthesized by Dr. Peter Henklein (Humboldt University). 11-*cis*-retinal was a generous gift from Rosalie Crouch and NEI, National Institutes of Health; the concentration was determined at 380 nm using $\epsilon = 24.400\ \text{M}^{-1}\text{cm}^{-1}$. Isotonic buffer contains 130 mM NaCl, 1 mM $MgCl_2$, and 20 mM bis-tris-propane adjusted to pH 7.5, if not otherwise noted. Phosphate buffer contains 50 mM potassium phosphate, 80 mM NaCl, and 1 mM $MgCl_2$, pH 6.5.

Rod Disk Membrane Preparation—Rod outer segments were prepared from frozen bovine retinas obtained from a local slaughterhouse by means of a discontinuous sucrose gradient method (27). Isolated disk membranes were prepared by repeated extraction of rod outer segments with low ionic strength buffer (5 mM Pipes, pH 7.0, 1 mM EDTA) as described previously (28). Rhodopsin concentration was determined from its absorption at 500 nm using $\epsilon = 40.600\ \text{M}^{-1}\text{cm}^{-1}$ (29). Opsin membranes were prepared by retinal extraction with bovine serum albumin (fatty acid-free) and hydroxylamine using illuminated disk membranes as described previously (20, 30). Opsin concentration was determined from its absorption at 280 nm using $\epsilon = 81.200\ \text{M}^{-1}\ \text{cm}^{-1}$ (19). All membrane suspensions were stored at −80 °C in isotonic buffer containing 0.3 M sucrose.

Membrane suspensions were sonicated briefly prior to the fluorescence and UV-visible measurements to reduce turbidity. All measurements were performed in isotonic buffer adjusted to the chosen pH values.

Illumination Protocol—To follow the decay of Meta II the reaction was started by complete bleaching of the membrane suspensions with a 150-watt fiber optic light source filtered through a heat filter (Schott KG2) and a 495-nm long pass filter. Samples were illuminated (15 s) at pH 6 and 20 °C, *i.e.* at conditions favoring Meta II (31). In agreement with a previous study (23) we found formation of isorhodopsin (9-*cis*-retinal form of rhodopsin) even during 15 s of illumination under conditions with significant content of Meta I in the equilibrium (alkaline pH and/or low temperatures) (data not shown). The given pH values of the samples were adjusted rapidly in the dark immediately after the photoactivation.

Fluorescence Measurements—The fluorescence assay of illuminated rhodopsin is based on the relief from the quenching of opsin Trp fluorescence exerted by the retinal ligand bound in its pocket (32). The assay was applied to study the Meta II decay in isolated disk membranes. Measurements were performed on a SPEX Fluorolog 1680 instrument. Low light intensities were used to prevent photolysis of rhodopsin. Slit settings for the fluorometer were 0.2 nm for excitation (at 295 nm) and 4 nm for emission. Emission spectra were recorded between 310 and 400 nm. For the kinetic studies, data were recorded at 330 nm. The measurements were done in stirred membrane suspensions (750 μl, 1 μM rhodopsin). Control experiments were done with closed shutter during decay to exclude an effect of the excitation light on the decay reaction (data not shown). Reaction rates were determined by a single exponential fit of the data.

UV-visible Spectroscopy—Absorbance spectra (see Fig. 7) were acquired with a Cary 50 spectrophotometer (Varian). Suspensions of isolated disk membranes (100 μl, 5 μM rhodopsin) were used in temperature-controlled microcuvettes (1-cm path length). Opsin membranes that closely matched the light scattering properties of isolated disk membranes were used as a reference.

Regeneration of opsin during decay of Meta II (see Fig. 5) was followed by recording difference spectra at pH 7.5 and 33 °C within 15 min on a Hewlett-Packard HP 8452 diode array spectrophotometer using 1-ml cuvettes equipped with a stirrer. 11-*cis*-retinal (4.3 μM) was added immediately after illumination, and pH adjustment (see above) of the membrane suspension (4.3 μM rhodopsin) and absorbance was set to zero. Retinylidene Schiff base content in disk membrane samples was estimated from the formation of the 440-nm product after acidification to pH 1.9 with 1 N HCl and solubilization of the membranes in detergent (1% dodecyl-β-maltoside).

FTIR Spectroscopy—FTIR samples were prepared using a centrifugation method as described (33). In ~40 μl of washed membranes (0.3 mM rhodopsin), the pH was adjusted by a few microliters of diluted NaOH or HCl. Subsequently the suspension was centrifuged for 20 min at 80,000 × *g*, yielding a pellet containing 2.3 mM rhodopsin (calculated from absorption at 500 nm). The buffer solution was removed, and the pellet was transferred to a 30-mm diameter transmission cell with two BaF_2 windows and a 3-μm Teflon spacer.

After equilibration for 1 h at 23 °C, a set of 256 transmission spectra was recorded. After illumination with a 150-watt fiber optic light source filtered through a heat filter (Schott KG2) and a 495-nm-long pass filter a second set of spectra was recorded. From these two sets of spectra the Meta II minus rhodopsin difference spectrum was generated (we use the convention that the spectra of the conversion A → B are calculated as B − A and termed B − A difference spectrum). The sample was then allowed to decay for 2 h at pH 5.9 and 7.5. The decay was followed by recording sets of spectra every 2 min after the first illumination. After 2 h, final spectra of the decay product were taken, and the sample was illuminated with green light (495 nm) for 30 s. Subtraction of the decay product spectra ("after" minus "before" illumination) yielded the Meta II minus Meta III FTIR difference spectrum.

RESULTS

Meta II Decay Monitored by Fluorescence Change—To measure the release of all-*trans*-retinal from photoactivated rhodopsin, we have adapted the intrinsic Trp fluorescence assay (34, 35), which was later established by Farrens and Khorana (32) as a means of monitoring all-*trans*-retinal release from solubilized rhodopsin. The data in Fig. 1 show that a similar change in Trp fluorescence of opsin can be measured in native isolated rod disk membranes. In the lipid bilayer, within milliseconds of light activation, rhodopsin reaches an equilibrium of Meta I and Meta II tautomers. All subsequent conversions start from the pool of Meta intermediates, which we will term Meta I/II. The fluorescence increased concurrently with a decrease in Schiff base concentration, as measured at 440 nm after acid denaturation. This indicates that it is indeed the release of the chromophore that gives rise to the fluorescence change (32). Under these conditions the absorbance decrease leveled out at ~25% of the starting absorbance, which is because of the presence of other all-*trans*-retinal Schiff bases formed with peripheral residues other than Lys^{296} (*N*-retinylidene opsin, NRO).

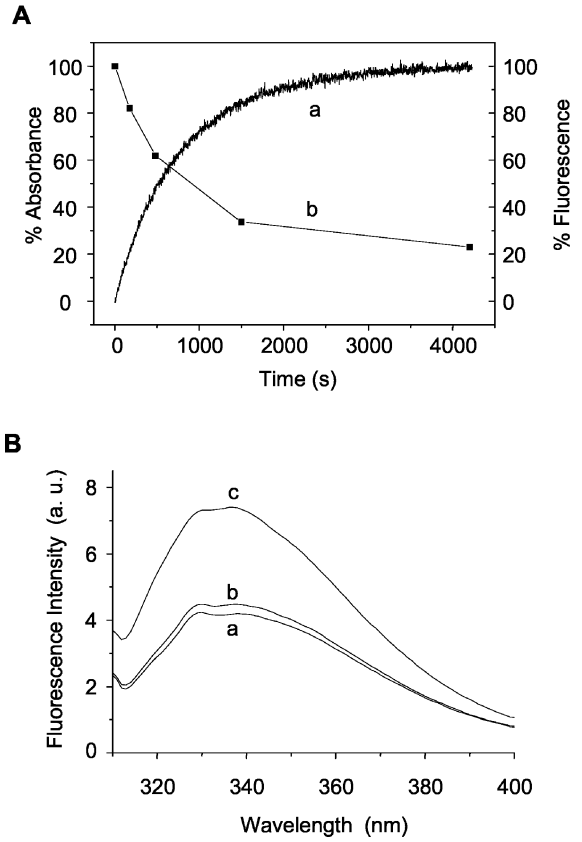

FIG. 1. **Change of intrinsic fluorescence monitoring the decay of photoactivated rhodopsin (R*) in bovine rod disk membranes.** *A*, kinetics of fluorescence increase (*a*) after saturating photoactivation of rhodopsin (1 μM) in disk membranes (λ_{ex} = 295 nm, λ_{em} = 330 nm, pH 6.5, 20 °C). The amount of protonated Schiff base left at the indicated times (*b*) was estimated from the absorption at 440 nm after acid denaturation (pH 1.9) and solubilization of the samples. *B*, fluorescence emission spectra (λ_{ex} = 295 nm) of rhodopsin membranes (pH 6.5, 20 °C) in the dark (*a*), immediately after photoactivation (*b*) and after 60 min (*c*).

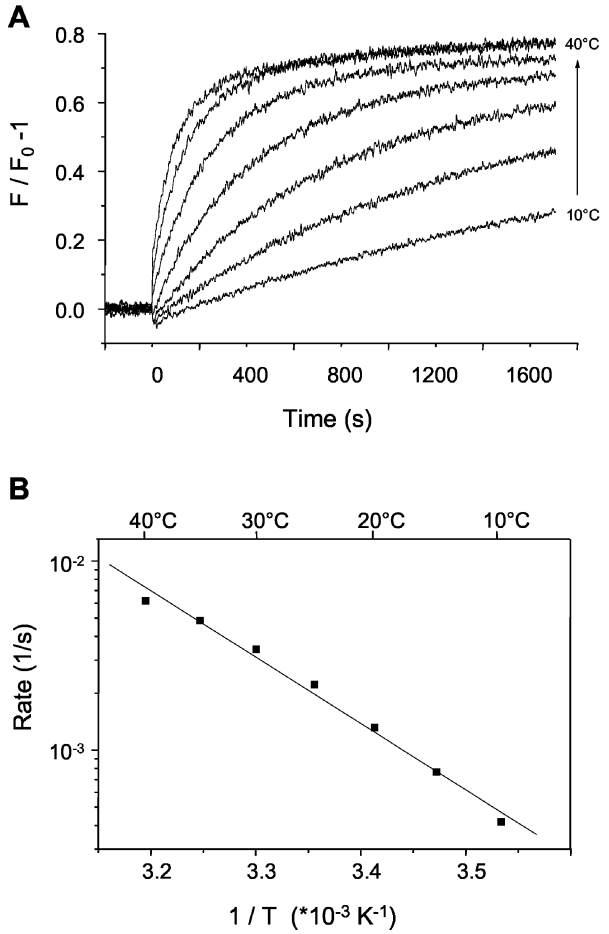

FIG. 2. **Temperature dependence of fluorescence change.** *A*, fluorescence increase after photoactivation of rhodopsin (1 μM) at different temperatures (pH 6.5). *B*, Arrhenius plot of the rates of fluorescence increase. *Solid line* is a linear least squares fit to the data.

The fluorescence spectra taken in the dark (rhodopsin), after illumination for 15 s (Meta I/II), and after complete decay of Meta I/II are shown in Fig 1*B*. The strong quenching of Trp fluorescence exerted by the all-*trans*-retinal bound in its pocket persists with only minor change immediately after illumination. This is interesting in light of the proposed flip of the β-ionone ring out of its location in the ground state (see "Discussion"). After complete decay of Meta I/II, the fluorescence of the resulting opsin is twice as large, as compared with the initial Meta II (the dominant species at pH 6.5).

As expected, the time course of the decay reaction depends strongly on temperature (Fig. 2*A*). The Arrhenius plot of the rate constants yields 16.0 kcal/mol as the activation energy of the decay reaction (Fig. 2*B*), similar to the 20.2 kcal/mol obtained with rhodopsin solubilized in dodecyl-β-maltoside (32). The results validate the rhodopsin fluorescence assay of all-*trans*-retinal release in membrane-bound rhodopsin (Fig. 1*A*).

pH Dependence of Meta I/II Decay in Vitro: Formation of Meta III—Both the kinetics and the final level of the change of fluorescence during Meta I/II decay depend significantly on pH (Fig. 3*A*). With increasing pH, the reaction becomes slightly faster (Fig. 3*D*), with a half-time of ~600 s at pH 6.0 and ~400 s above pH 8.0 (20 °C). The maximum amplitude of the signal is reduced by a factor of 2 over the range of pH tested (Fig. 3*C*). To distinguish between the pH dependences of the actual decay reactions and of the fluorescence monitor, the decay was analyzed in the presence of $G_t\alpha$-HAA. This peptide binds to the Meta II conformation (26, 36). The $G_t\alpha$-HAA strongly reduces the pH dependence of the fluorescence change (Fig. 3, *B* and *C*). Because of the fact that Meta II decays quantitatively to opsin in the presence of the peptide (see below), the residual pH dependence is likely reflecting the pH effect on Trp fluorescence. The difference between the fluorescence changes with and without $G_t\alpha$-HAA yields the relative fraction of a photoproduct in which the Trp fluorescence is quenched persistently. From the resulting curve, shown in the *inset* of Fig. 3*C*, it can be estimated that this photoproduct is formed with an apparent pK_a of 7.0. Under the conditions of the experiments, its maximum fraction is 33% of the initially bleached rhodopsin. Anticipating the results described below, this photoproduct contains all-*trans*-retinal bound via a protonated Schiff base to Lys^{296} and is here defined as Meta III.

Similar results were obtained at 33 °C (*insets* in Fig. 3, *A* and *B*), except the decay is faster (half-time of ~130 s), and the maximum fraction of Meta III formed is slightly smaller. However, even at 33 °C, no significant loss of Meta III is seen within 30 min, as reflected in the stable level of fluorescence.

Interestingly, the Meta II/$G_t\alpha$-HAA complex decays above pH 6.0 in a pH-independent manner, with a half-time of 620 ± 17 (S.E.) s (20 °C). A similar rate is found for the Meta I/II decay without the peptide between pH 6.0 and 6.5. Although the peptide mimics the effect of G_t on Meta II stabilization, it does not significantly influence the fluorescence change and, hence, hydrolysis of the Schiff base and loss of the chromophore from the binding pocket (see "Discussion").

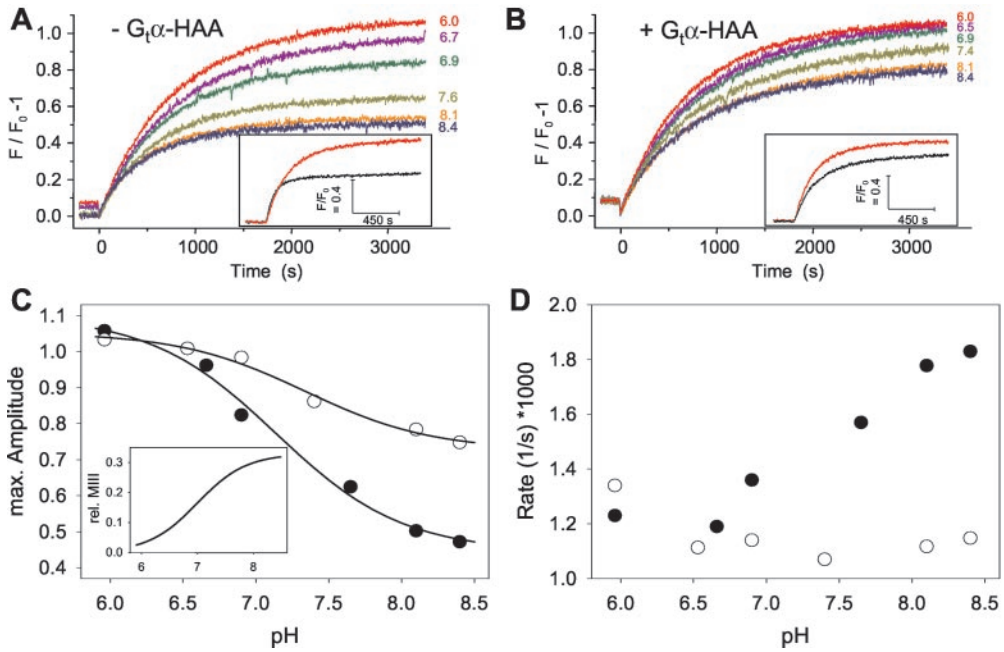

FIG. 3. **pH dependence of fluorescence change.** Families of fluorescence traces measured at 20 °C and the indicated pH in the absence (*A*) and in the presence (*B*) of 100 μM $G_t\alpha$-HAA. Samples (1 μM rhodopsin in disk membranes) were illuminated at pH 6.0 for 15 s and immediately adjusted to the respective pHs. *Insets*, fluorescence traces measured at 33 °C and pH 6.4 (*red traces*) and pH 8.1 (*black traces*). *C*, pH dependence of the maximum amplitude of the fluorescence increase (20 °C) with and without $G_t\alpha$-HAA (*open* and *closed circles*, respectively). *Solid lines* are fits to the titration data using linear hyperbolic functions. The difference between the fitted curves yield the pH dependence of Meta III formation (*inset*). *D*, pH dependence of the reaction rates of fluorescence increase (20 °C) with and without $G_t\alpha$-HAA (*open* and *closed circles*, respectively).

Enzymatic Reduction of All-trans-retinal Has No Effect on Meta II Decay—Using the fluorescence assay, we also studied the influence of NADPH on the Meta I/II decay to opsin and Meta III. Addition of NADPH, the cofactor of an endogenous all-*trans*-retinol dehydrogenase, results in reduction of all-*trans*-retinal to all-*trans*-retinol (see Fig. 5, *B* and *C*). As seen in Fig. 4, neither the time course nor the final level of the fluorescence change are influenced by NADPH. Under the conditions of the experiments (pH 7.5, 20 °C), a significant fraction of Meta III is formed that is seen directly in the larger fluorescence increase in the presence of $G_t\alpha$-HAA-peptide (see Figs. 3 and 4). The lack of any additional opsin formation in the presence of NADPH indicates a (quasi-)irreversible formation of Meta III.

Decay of Meta II in the Presence of 11-cis-retinal—To probe the accessibility of the retinal binding pocket in the decay products, regeneration with 11-*cis*-retinal during Meta I/II decay was followed by UV-visible difference spectroscopy (Fig. 5, *A-C*). The records reflect an increase in absorbance around 500 nm and a parallel decrease around 380 nm, consistent with the formation of rhodopsin at the expense of free 11-*cis*-retinal. In the control membranes (Fig. 5*A*), the absorption maximum of rhodopsin is apparently blue-shifted because of the contribution of other photoproducts with similar absorption. These include Meta III, as defined above, and protonated Schiff bases (*e.g.* NRO; see "Discussion"), which prevent a quantitative evaluation of the spectra. However, despite the complexity of the decay reactions the spectra maintain an isosbestic point. This implies that the species involved exhibit only two distinct spectra. This condition is met when all conversions occur with the same apparent rate (see "Discussion").

In Fig. 5, *B* and *C* the influence of NADPH and $G_t\alpha$-HAA-peptide on Meta I/II decay and opsin regeneration, respectively, is shown. Reduction of all-*trans*-retinal by endogenous all-*trans*-retinol dehydrogenase is seen directly as an increase in absorbance at 320 nm (all-*trans*-retinol formation). The loss of an isosbestic point in these spectra indicates that the additional reaction introduced, namely the reduction of all-*trans*-retinal after its release from photoactivated rhodopsin, becomes kinetically visible. This is readily explained by a transient accumulation of free all-*trans*-retinal and/or its complexes with opsin (all-*trans*-retinal/opsin and NRO). Eventually, the pool of released all-*trans*-retinal is reduced, which is seen as a further decrease in absorbance at 380 nm (all-*trans*-retinal/opsin). In addition, the absorption shifts toward 500 nm in the late spectra, indicating complete removal of transiently formed NRO.

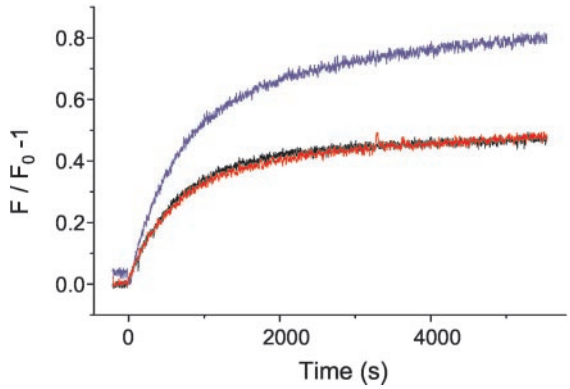

FIG. 4. **Effect of enzymatic reduction of all-*trans*-retinal on the changes in fluorescence.** Fluorescence increase of illuminated disk membranes (pH 7.5, 20 °C) in the absence of NADPH (*black*), in the presence of 70 μM NADPH (*red*), and in the presence of both 70 μM NADPH and 100 μM $G_t\alpha$-HAA (*blue*), respectively.

Consistent with the results obtained above (see Figs. 3 and 4), more all-*trans*-retinal is released and eventually reduced in the presence of $G_t\alpha$-HAA-peptide, which makes opsin available that would have otherwise escaped into the Meta III storage (further decrease in 380 nm absorption). Any additional absorption change in the 470–500-nm range is masked by a simultaneous loss of Meta III, but a final shift toward 500 nm is evident (Fig. 5*C*).

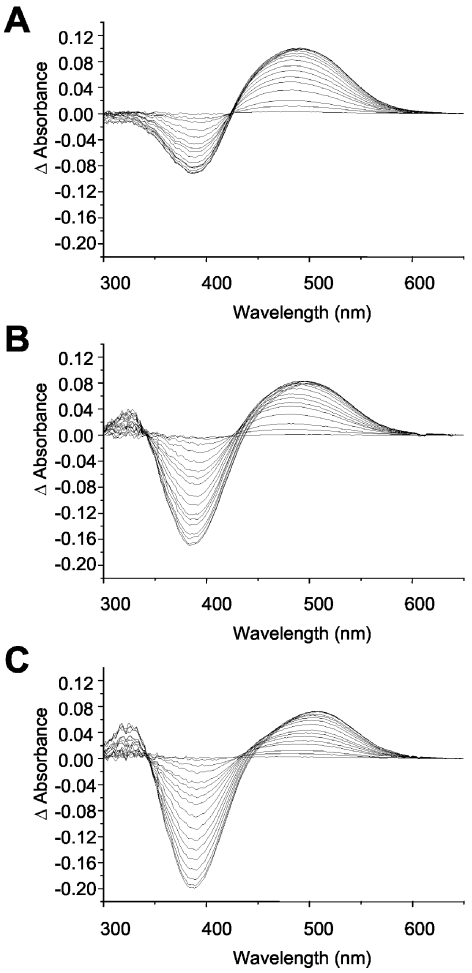

FIG. 5. **UV-visible difference spectra recorded during decay of Meta II with concurrent regeneration of opsin to rhodopsin with 11-*cis*-retinal.** Time course of spectral changes during rhodopsin decay and regeneration at pH 7.5 and 33 °C without NADPH (*A*), with 70 μM NADPH (*B*), and with both $G_t\alpha$-HAA-peptide (100 μM) and NADPH (70 μM) (*C*). Disk membranes (4.3 μM rhodopsin) were fully bleached at pH 6.0 and adjusted immediately to pH 7.5. After addition of equimolar 11-*cis*-retinal, the absorption was set to zero. The difference spectra were taken at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 15 min, respectively.

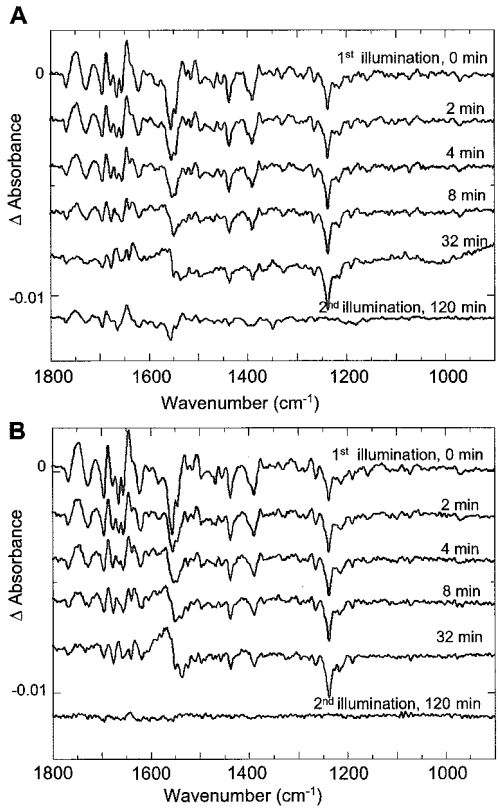

FIG. 6. **Characterization of Meta III by FTIR spectroscopy.** FTIR difference spectra were recorded immediately after Meta II formation (Meta II minus rhodopsin) and after the times indicated (decay products minus rhodopsin) at 23 °C, pH 7.5 (*A*) and pH 5.9 (*B*). A second illumination with green light after 120 min yields the difference spectra between a form of Meta II (see text and Ref. 37) and Meta III (Meta II-like minus Meta III).

Identification of Meta III by FTIR Spectroscopy—Meta I/II decay was also followed using FTIR difference spectroscopy. The first record in Fig. 6, *A* and *B* is the Meta II minus rhodopsin difference spectrum (see "Experimental Procedures"). At pH 7.5 and 23 °C, the Meta I/Meta II equilibrium is to 70% and at pH 5.9 and 23 °C, to more than 90% on the Meta II side. Thus, the spectra are dominated by typical Meta II features, including bands at 1768 cm^{-1} and 1745 cm^{-1} (indicating a change in hydrogen bonding of Asp^{83} and Glu^{122}) and 1713 cm^{-1} (indicating the protonation of the Schiff base counterion, Glu^{113}).

At both pH 7.5 (Fig. 6*A*) and pH 5.9 (Fig. 6*B*), the decay is virtually complete after 32 min, as indicated by the absence of the Meta II features. This difference spectrum represents a mixture of the opsin/all-*trans*-retinal minus rhodopsin and Meta III minus rhodopsin difference spectra (see "Experimental Procedures"). There is a weakly expressed feature that arises specifically at pH > 6.0, namely the positive band at 1348 cm^{-1}, which was recently assigned to Meta III formation (37). When the sample is illuminated after 2 h of decay, a Meta II minus Meta III difference spectrum can be recorded. This spectrum shows the Meta III band at 1348 cm^{-1} with negative polarity and the features around 1700 to 1750 cm^{-1} with positive polarity, indicating the formation of Meta II (or Meta II-like product in terms of protein conformation) (Fig. 6*A*), as described previously (37). This identifies Meta III as the only decay product that can be converted by light to the active species Meta II.

In contrast to the observation at pH 7.5, no photolysis products are seen at pH 5.9, in conditions when virtually no Meta III is present (Fig. 6*B*). This observation further supports the existence of Meta III as a decay product. This species is the major (or even the only) decay product that undergoes photoreversal to Meta II.

$G_t\alpha$-HAA-Peptide Prevents Meta III Formation—UV-visible spectroscopic data on the formation and decay of Meta I/II are shown in Fig. 7. Illumination of rhodopsin at pH 6.0 and 20 °C results in an almost quantitative formation of Meta II (Fig. 7*A*, *traces a* and *b*). After adjusting the same sample to pH 8.0 (to avoid photoconversion of Meta I to isorhodopsin; see "Experimental Procedures"), Meta I is formed rapidly at the expense of Meta II (Fig. 7*A*, *trace c*). The subsequent decay of Meta I/II results in only minor spectral changes (Fig. 7*A*, *traces c-f*) because of the spectral overlap between reactants and products. Species in the 360–380-nm range include Meta II, free all-*trans*-retinal, and opsin/all-*trans*-retinal complex with unprotonated Schiff base. In the 460–480-nm range are Meta I, Meta III, and all other protonated Schiff bases (reviewed in Ref. 25).

The overall time course of the decay products can be followed from the spectral changes (Fig. 7*A*), yielding the same common rate for all conversions, as was seen in the fluorescence and FTIR experiments described above. Meta III can be identified

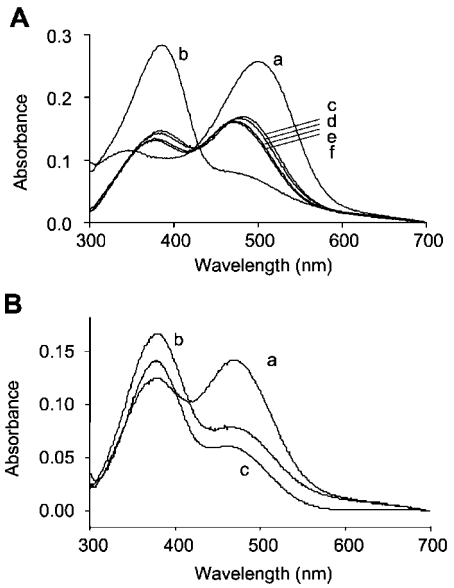

FIG. 7. **Characterization of Meta II decay products by UV-visible spectroscopy.** The measurements were carried out as described under "Experimental Procedures." *A*, spectrophotometric characterization of the formation and decay of Meta I/II. Absorption spectrum of a disk membrane suspension in the dark (*a*) and after 15 s of illumination at pH 6.0 (*b*). After adjusting the sample to pH 8.0 by addition of NaOH, further spectra were recorded 1.5 (*c*), 5 (*d*), 15 (*e*), and 35 (*f*) min after illumination of the suspension. Note the shift of the baseline upon addition of NaOH. *B*, after complete decay of Meta II (40 min) 100 μM $G_t\alpha$-HAA was added to the sample shown in *A*, and spectra were taken before (*a*) and after (*b*) a second illumination of the suspension with green light. A second sample was allowed to decay at pH 8.0 in the presence of 100 μM $G_t\alpha$-HAA-peptide, and a spectrum was taken after 45 min (*c*).

spectrally using its photoconvertibility to Meta II (see Fig. 6*A*). The experiment starts with the completely decayed sample (Fig. 7*A*, *trace f*). Addition of $G_t\alpha$-HAA-peptide yields *trace a* in Fig. 7*B*; it coincides with *trace f* in Fig. 7*A* apart from a small dilution effect. Subsequent illumination results in a spectrum (Fig. 7*B*, *trace b*) that is very similar to the spectrum obtained from an aliquot that had decayed in the presence of $G_t\alpha$-HAA (Fig. 7*B*, *trace c*). The experiment confirms that the peptide blocks formation of Meta III.

DISCUSSION

The visual process in the vertebrate retina depends not only on the signal transduction machinery in rods and cones and on neuronal processing but also on an extended network of metabolic reactions. They produce the necessary energy, intermediates, and 11-*cis*-retinal to regenerate bleached visual pigments to their light-sensitive ground state. This regeneration process was underscored recently, when it was shown that a direct, light-induced reversal from all-*trans*- to 11-*cis*-retinal (so-called photoregeneration) does not occur to any measurable degree (37). Light absorption of the active Meta II state of the rhodopsin in the rods does not, as in cyclic proteins such as sensory rhodopsin in bacteria, lead back to photoregenerated rhodopsin but to new forms with properties different from native rhodopsin (37). Replacement of all-*trans*-retinal with fresh 11-*cis*-retinal requires that the retinylidene Schiff base be hydrolyzed and the all-*trans*-retinal be released from the opsin chromophore pocket. All-*trans*-retinal release occurs by decay of the active, G-protein binding Meta II intermediate, in which the Schiff base is intact but deprotonated.

The conversions of Meta II have been the subject of intense investigations, mainly in intact retina and in rod outer segment preparations. An exceedingly complex picture emerged from these studies, introducing elements of retinal metabolism (*e.g.* all-*trans*-retinol dehydrogenase, the retina-specific ABC transporter) and regulation (including G_t, rhodopsin kinase, and arrestin). Even in membrane preparations, which contain only the pigment and no additional protein, several products of thermal decay appear in variable amounts. These include non-covalent opsin/all-*trans*-retinal complexes (16, 20) and Meta III-like pseudo-photoproducts (*N*-retinylidene-opsin, *i.e.* opsin/all-*trans*-retinal complexes with Schiff bases; see Refs. 5 and 11). All these forms have the potential to serve as a "storage" for the photolyzed chromophore.

The goal of this study was to identify some of the intermediates that arise during Meta II decay. Using a preparation of isolated photoreceptor disk membranes and biophysical techniques, we present conclusive evidence for the parallel decay of the active Meta II into two species: (i) Meta III, an inactive storage form with all-*trans*-retinal bound in or near the original binding site, and (ii) an all-*trans*-retinal opsin complex, in which the all-*trans*-retinal has been translocated to second binding site(s). Both products are formed in parallel through essentially irreversible pathways, *i.e.* they remain present at constant concentration when the decay reaction has gone to completion. We will first discuss the species involved and then the way they are concatenated.

Metarhodopsin III—Definitions of Meta III based on visible absorbance around 460–470 nm are ambiguous because of the various products that form on a quite different chemical basis but with similar UV-visible properties. The UV-visible spectrophotometric results are consistent with the presence of a protonated Schiff base. This, by definition, is the classical Meta III product (see Refs. 21 and 25). However, previous work has suggested the existence of protonated Schiff bases that are formed with Lys residues other than the active Lys^{296}, and the photolyzed chromophore is also known to form adducts with phosphatidyl-ethanolamine (38). Our results, based on the combined application of fluorescence and FTIR assays, allow us to identify the decay products and to separate products that are isochromic in the UV-visible range. Therefore, we now define Meta III as the fraction of decay products that (i) does not go through the fluorescence change, (ii) is resistant to NADPH-dependent reduction, and (iii) is photoconverted to a Meta II-like intermediate (37). Thus, the most obvious assumption is that Meta III contains the all-*trans*-retinal in the original site (37).

All-trans-retinal/Opsin Complexes—According to the classical scheme, the main path of Meta II decay leads via Schiff base hydrolysis to opsin and the photoisomerized chromophore, all-*trans*-retinal. There is general agreement that this conversion is irreversible (39, 40). In apparent contradiction to this notion, recombination of purified opsin with exogenous all-*trans*-retinal leads to substantial activity toward the G-protein (16–20), rhodopsin kinase (11, 41), and arrestin (11). However, the activity toward G_t was also seen with a permethylated active site (16), and regeneration with 11-*cis*-retinal was not inhibited in the presence of all-*trans*-retinal added in excess (20). This has led to the conclusion that a non-covalent complex is formed in which the all-*trans*-retinal has virtually no access to the original binding site occupied in Meta II (16, 20). Whether the residual activity, which is measured after completion of Meta II decay (data not shown) (42), is because of the formation of similar non-covalent complexes remains to be elucidated.

Reaction Scheme—To derive a reaction model for Meta II decay, we start from two experimental findings: (i) Schiff base hydrolysis and/or all-*trans*-retinal release are irreversible, and (ii) Meta III is not affected by NADPH-dependent reduction of all-*trans*-retinal to all-*trans*-retinol (Fig. 4). We therefore con-

clude that the decay of Meta I/II comprises two parallel conversions that are irreversible within the time window (1 h) of the experiments (shown in Reaction 1). Kinetic theory says that parallel reactions proceed with one common observed reaction rate. In the simplest case (shown in Reaction 2), the observed reaction rate (k_{obs}) is the sum of the individual reaction rates ($k_{obs} = k_1 + k_2$) (43).

Thus Meta III formation and all-*trans*-retinal release must necessarily occur with the same observed reaction rate. The scheme also implies that the relative amounts at which the two species are formed depend on kinetic competition, *i.e.* the intrinsically faster pathway produces the higher yield. This is, under our conditions, always the formation of all-*trans*-retinal/opsin. The higher the pH, the more the decay runs into Meta III formation, with a maximum of ~35% at pH > 8.0 (*inset* of Fig. 3). The reaction scheme (Reaction 1) does not exclude more complicated schemes that arose from detailed analyses of UV-visible difference spectra (24). In particular, we cannot exclude the existence of isochromic species formed during the decay of Meta II. However, the results of this study expand existing approaches by showing that a substantial amount of the species formed is Meta III, in which the chromophore is still bound to its original binding pocket. Meta III is a stable species, formed in a quasi-irreversible, although pH-dependent, manner. Even at 33 °C, the stable level of fluorescence (Fig. 3) indicates that the Meta III, as defined above, is stable within the physiologically relevant time range of 30 min.

Meta I / II → Meta III
Meta I / II → opsin / all-*trans*-retinal

REACTION 1

$$A \xrightarrow{k_1} P_1 \qquad A \xrightarrow{k_2} P_2$$

$$[P_1] = \frac{k_1}{k_1 + k_2}[A]_0\left(1 - e^{-(k_1+k_2)t}\right) \qquad [P_2] = \frac{k_2}{k_1 + k_2}[A]_0\left(1 - e^{-(k_1+k_2)t}\right)$$

REACTION 2

The stability of Meta III is in apparent conflict with the partial loss within minutes of Meta III-like absorbance in membrane preparations (23, 24). We can now suggest that these unstable species were not Meta III but rather NRO-like species, as discussed above. Under cellular conditions, Meta III eventually decays to opsin and all-*trans*-retinal (2–5). Here, other factors (*e.g.* G_t) come into play that may prevent (see this work and Ref. 10) or even reverse (4, 42) Meta III formation. In addition, direct hydrolysis of the Schiff base in Meta III itself was suggested (3, 5, 44) to explain the loss of 470-nm absorbance.

Mechanism of Schiff Base Hydrolysis—The decay reaction proceeds from a pool of Meta I/II. Because these species rapidly interconvert on a millisecond time scale, our kinetic data do not allow us to decide whether the decay reactions start from Meta I or Meta II. It is known that hydrolysis of free Schiff bases requires their protonation. If this is true for the all-*trans*-retinal Schiff base in rhodopsin, one would argue that hydrolysis cannot start from Meta II. On the other hand $G_t\alpha$-HAA-peptide stabilizes Meta II but has only a minor effect on all-*trans*-retinal release and thus Schiff base hydrolysis (see Figs. 3, 4, and 7). A solution for the problem would be an additional active, peptide-binding intermediate with reprotonated Schiff base (45–47) as the actual precursor of hydrolysis. In the scheme (Eq. 1), we consider all these species in a pool of conformations that form and equilibrate within milliseconds after light activation. They all have in common the photoisomerized chromophore, all-*trans*-retinal, that is still in its original binding site, with the Schiff base bond to Lys^{296} intact.

Structural Basis of the Fluorescence Change—Rhodopsin contains five Trp residues in positions 35, 126, 161, 175, and 265 (Fig. 8*A*). These residues are located in different parts of

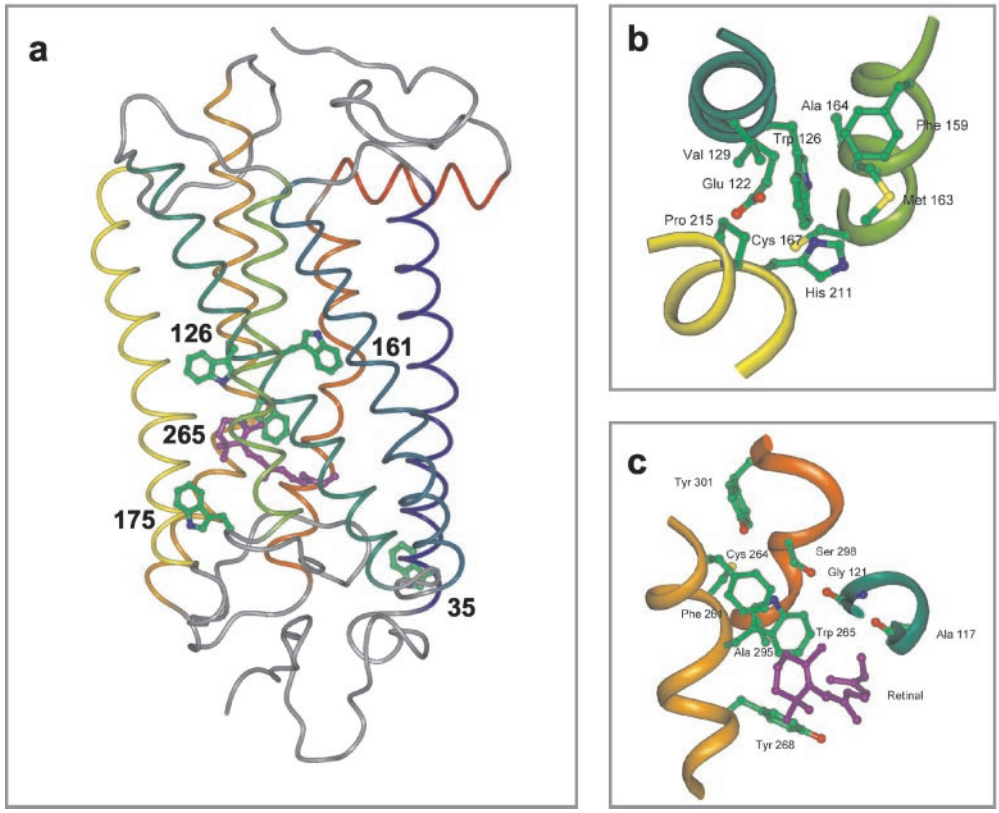

FIG. 8. **Model of rhodopsin.** The model is based on a high resolution crystal structure (1HZX) (56). *a*, rhodopsin with indicated positions of Trp residues. *b*, close-up of the vicinity of Trp^{126}. *c*, close-up of the vicinity of Trp^{265}.

the molecule and play different roles. Trp^{126} is sequestered within a hydrophilic part of the transmembrane bundle of helices (Fig. 8, *A* and *B*), in close vicinity of Glu^{122}. The substitution of this Glu residue resulted in changes in both the decay rate of Meta II and the rate of regeneration (48, 49). In addition, it was postulated that changes in helix III are associated with formation of Meta II (reviewed in Ref. 50). Trp^{126} is too distant from the chromophore to be impacted when chromophore is released. Trp^{265}, located on helix VI, which is critical for the activation process (50), is closest to the β-ionone of the chromophore (51) (Fig. 8, *A* and *C*). By virtue of this location and isomerization, Trp^{265} should undergo the most profound changes during the activation process. It was noted that isomerization of the chromophore may cause changes in the location of the β-ionone during formation of Meta II (52, 53). Based on molecular dynamics, Röhrig *et al.* (54) proposed that such changes occur even earlier, just nanoseconds after photon absorption. In the new orientation, Trp^{265} comes closer to Phe^{261}, and such interaction would have a similar quenching effect on the Trp emission as one provided by β-ionone in rhodopsin. Therefore, changes in fluorescence during formation of Meta II are small (only ~10% difference between Meta II and rhodopsin) and could be attributed to Trp^{265} and possibly Trp^{126}, in agreement with Trp absorbance studies (55). Upon chromophore release, large fluorescence changes could result from relaxation of Trp^{265}, now in the empty binding pocket of rhodopsin. Meta III would have preserved quenching properties either by the chromophore or by Phe^{261}. These assertions require further experimental proof.

Physiological Implications—Our finding that NADPH-dependent removal of all-*trans*-retinal does not accelerate the decay of Meta II extends previous results that the reduction of all-*trans*-retinal limits the rate of regeneration in mice (8). The present study now provides the evidence that the actual rate-limiting step for the reduction, and thus the pacemaker for the overall regeneration process in the vertebrate retina, is an intrinsic transformation in the active photopigment itself. It is the step that makes all-*trans*-retinal available for all-*trans*-retinal reduction and/or transport (reviewed in Ref. 1). Parallel to this reaction, the Meta III form arises to an amount dictated by kinetic competition. Meta III has the properties to be a storage form of rhodopsin that has a photoisomerized chromophore but is inactive for G_t stimulation. Factors that can regulate the level of Meta III formation include G_t and green light. Storing aside rhodopsin that is in Meta III could lower the probability of quantum catch of the rod cell, if the quantum efficiency of Meta III is reduced compared with rhodopsin. Reducing further excitation of already light-saturated photoreceptor cells would contribute significantly to light adaptation.

Acknowledgments—We thank Dr. Peter Henklein for providing the peptide. We also thank Jana Engelmann and Ingrid Semjonow for excellent technical assistance. We thank Sławomir Filipek for preparation of Fig. 8.

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