Opsin/*all-trans*-Retinal Complex Activates Transducin by Different Mechanisms Than Photolyzed Rhodopsin†

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Received October 6, 1995; Revised Manuscript Received December 5, 1995⊗

ABSTRACT: In rhodopsin, the 11-*cis*-retinal chromophore forms a complex with Lys[296] of opsin via a protonated Schiff base. Absorption of light initiates the activation of rhodopsin by *cis*/*trans* photoisomerization of retinal. Thermal relaxation through different intermediates leads into the metarhodopsin states which bind and activate transducin (G_t) and rhodopsin kinase (RK). *all-trans*-Retinal also recombines with opsin independent of light, forming activating species of the receptor. In this study, we examined the mechanism by which *all-trans*-retinal activates opsin. To exclude other amines except active site Lys[296] from formation of Schiff bases, we reductively methylated rhodopsin (PM-rhodopsin), which we then bleached to generate PM-opsin. Using spectroscopic methods and a G_t activation assay, we found that *all-trans*-retinal interacted with PM-opsin, producing a noncovalent complex that activated G_t. The residual nucleotide exchange in G_t catalyzed by opsin was $\sim^1/_{250}$ lower relative to that of photoactivated rhodopsin (pH 8.0, 23 °C). Addition of equimolar *all-trans*-retinal led to an occupancy of one-tenth of the putative retinal binding site(s) of opsin and enhanced the G_t activation rate 2-fold. When the concentration of *all-trans*-retinal was increased to saturation, the G_t activation rate of the opsin/*all-trans*-retinal complex was $\sim^1/_{33}$ lower compared to that of photoactivated rhodopsin. We conclude that *all-trans*-retinal can form a noncovalent complex with opsin that activates G_t by different mechanisms than photolyzed rhodopsin.

Absorption of light by the visual pigment of the rod cell, rhodopsin, leads to isomerization of its 11-*cis*-retinal chromophore to an *all-trans* conformation. This photochemical conversion occurs with a high quantum yield of 0.67, and two-thirds of the photon energy is taken up by the chromophore/protein complex (Boucher & Leblanc, 1985; Cooper, 1979; Schick et al., 1987). The energy is stored mostly in the form of distortion of the chromophore attached to the active site Lys[296] in the early Batho intermediate (Smith et al., 1991; Birge et al., 1988). Relaxation follows a series of thermal transitions through intermediates identified by their unique absorption spectra. After milliseconds, the active intermediate Meta II[1] is formed which is distinct from all its predecessors by translocation of the Schiff base (SB) proton to residue Glu[113] (Jäger et al., 1994), the counterion of the protonated SB (Sakmar et al., 1989; Zhukovsky & Oprian, 1989). The activated receptor catalyzes GTP/GDP exchange on G_t (Hofmann, 1993) when additional protons are taken up from the aqueous phase (Arnis & Hofmann, 1993). The highly conserved residue Glu[134], on the cytoplasmic border of the third helix of rhodopsin's seven-helix structure, is involved in this proton uptake and formation of the signaling state (Arnis et al., 1994). In contrast, RK activation does not require this protonation to occur, and the recognition motifs for the kinase (including the third cytoplasmic loop of rhodopsin; Palczewski et al., 1991; Shi et al., 1995) might already be exposed before the Meta states are formed. The kinase binds but does not stabilize Meta II at the expense of Meta I and therefore does not appear to prefer Meta II over its predecessor Meta I (Pulvermüller et al., 1993). This finding is consistent with earlier reports that Meta I is also a substrate for RK phosphorylation (Paulsen & Bentrop, 1983).

All known events in visual signaling are a consequence of a primary amplification, when one Meta II molecule activates thousands of G_t molecules (Pugh & Lamb, 1993). Photoactivated rhodopsin is quenched long before the spontaneous decay of Meta II to a reprotonated SB intermediate, Meta III, or directly into opsin and free *all-trans*-retinal (Baumann & Reinheimer, 1973). The current model predicts that RK phosphorylates C-terminal Ser residues of photolyzed rhodopsin (Ohguro et al., 1995) which enables tight binding of arrestin, or its splice variant p[44], and by that effectively quenches G_t activation (Palczewski, 1994). To regenerate opsin, retinol dehydrogenase (*t*-RDH) must reduce

† This research was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 60) and the Human Frontier Science Program, NIH Grant EY08161 (K.P.), and an award from Research to Prevent Blindness, Inc. to the Department of Ophthalmology at the University of Washington. K.P. is the recipient of a Jules and Doris Stein Research to Prevent Blindness Professorship.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1996.

[1] Abbreviations: BTP, 1,3-bis[[tris(hydroxymethyl)methyl]amino]-propane; G_t, transducin; MI, Meta I, metarhodopsin I; MII, Meta II, metarhodopsin II; M_{380}, pseudo $Meta_{380}$ (λ_{max} = 380 nm); PDE, cGMP phosphodiesterase; PM-opsin, permethylated opsin; PM-rhodopsin, permethylated rhodopsin; RK, rhodopsin kinase; ROS, rod outer segments; SB, Schiff base; *t*-RDH, *all-trans*-retinol dehydrogenase.

0006-2960/96/0435-2901$12.00/0

all-trans-retinal to *all-trans*-retinol, allowing arrestin to dissociate (Hofmann et al., 1992).

Recent work has drawn attention to a new class of signaling states of the receptor which are generated without light, by mere addition of the chromophore *all-trans*-retinal to opsin (in the dark). These states are of interest in mechanistic terms because they lack the high-energy photointermediates and opsin might be activated analogously to an agonist-activated receptor. Furthermore, these complexes of opsin could be involved in a phenomenon called bleaching adaptation. Fukada and Yoshizawa (1981) were the first to describe a weak activation of PDE in native opsin membranes by *all-trans*-retinal. Hofmann et al. (1992) have found a "pseudo" photoproduct M_{380}, produced by addition of exogenous *all-trans*-retinal to phosphorylated opsin. Phosphorylated opsin formed protonated SBs (absorbing in the same range as the photoproducts Meta I or Meta III) which were destabilized by arrestin but not by G_t. However, the opsin/*all-trans*-retinal complex must interact with G_t independently of light, as has been shown for the opsin mutant E113Q (Sakmar et al., 1989), for wild type and different mutants of opsin in COS cell membranes (Cohen et al., 1992, 1993), and also recently for opsin in its native disk membrane environment (Palczewski et al., 1994; Surya et al., 1995). The *all-trans*-retinal/opsin complex is also phosphorylated by RK (Hofmann et al., 1992; Palczewski et al., 1994). These observations are difficult to reconcile with only one form of opsin/*all-trans*-retinal complex.

In this study, we examined the photoproduct M_{380} in the disk membranes (referred to as the opsin/*all-trans*-retinal complex) using spectrophotometric and fluorescence G_t activation assays. To prevent random SB formation between *all-trans*-retinal and peripheral amines, we reductively methylated rhodopsin and membrane lipids in the dark (Longstaff & Rando, 1985) and after bleaching produced PM-opsin. We found that the opsin/*all-trans*-retinal complex activated G_t independently of light and that retinal was bound to opsin without SB formation with Lys^{296}.

MATERIALS AND METHODS

all-trans-Retinal was purchased from Sigma, and β-ionone was from Fluka. 11-*cis*-Retinal was a generous gift from Hoffman-LaRoche. The concentrations of the retinoids in ethanol were determined spectrophotometrically using the extinction coefficients: 11-*cis*-retinal (380 nm), 24 400 M^{-1} cm^{-1}; *all-trans*-retinal (380 nm), 43 400 M^{-1} cm^{-1}; and β-ionone (300 nm), 8700 M^{-1} cm^{-1}.

Protein Preparation. ROS disk membranes were purified from fresh, dark-adapted bovine retinas. Rhodopsin in the native disk membrane (referred to as native rhodopsin) was prepared by removal of the soluble and membrane-associated proteins using repetitive washes with a low-ionic strength buffer as described by Heck and Hofmann (1993). Opsin was prepared from native rhodopsin by thorough bleaching with white light for 45 min on ice in 10 mM BTP buffer (pH 7.5) containing 130 mM NaCl. To remove *all-trans*-retinal, opsin was incubated with Hydrazide AvidGel Ax (BioProbe International), an aldehyde-selective gel, that specifically coupled retinal via a hydrazone bond to the gel matrix. The reaction was carried out at 23 °C in 50 mM sodium acetate buffer (pH 5.0) containing 130 mM NaCl. Alternatively, ROS membranes were bleached with white light for 45 min on ice in 10 mM BTP buffer (pH 7.5) containing 130 mM NaCl and 10 mM NH_2OH. The addition of NH_2OH led to conversion of retinal to its oximes. Membranes were extensively washed with 10 mM BTP buffer (pH 7.5) containing 130 mM NaCl. The residual activity of Hydrazine AvidGel-treated and NH_2OH-treated opsin (mostly employed in this study) had comparable residual activity toward G_t.

G_t was extracted from isotonically washed ROS using GTP (Heck & Hofmann, 1993). Any remaining traces of opsin were removed by a Concanavalin-A column. G_t concentration was determined using the Bradford method (1976) with bovine serum albumin as the standard. The rhodopsin concentration was calculated from the change in absorption at 500 nm before and after bleaching, assuming an absorption coefficient $\epsilon = 40\,000$ M^{-1} cm^{-1}; the opsin concentration was measured at 280 nm assuming $\epsilon = 64\,000$ M^{-1} cm^{-1} (Applebury et al., 1974).

Methylation Procedures. Native rhodopsin was methylated employing procedures similar to those described by Longstaff and Rando (1985). Specifically, two rounds of methylation in the dark were performed using 20 mM formaldehyde (added from a stock solution of 0.2 M formaldehyde prepared by hydrolyzation of paraformaldeyde) and 0.2 M $NaCNBH_3$ (10 times more than used in the original procedure). PM-rhodopsin was desalted using a G-25 Sephadex column. PM-opsin was prepared from bleached PM-rhodopsin as described above for opsin.

Spectrophotometry. A spectrophotometer (Shimadzu UV 3000) and a quartz cuvette with a 2 mm pathway were used to record the spectra of photoproducts. For the fast regeneration kinetics of opsin, a Hewlett-Packard HP 8452 diode array spectrophotometer was used. After addition of 5 μM 11-*cis*-retinal to 2.5 μM opsin or PM-opsin [in 50 mM BTP buffer (pH 6.0 and 8.0) containing 130 mM NaCl], the spectra were recorded at chosen time points ($T = 23$ °C). In all measurements, the reference wavelength was 600 nm.

Intrinsic G_t Fluorescence. The fluorescence assay is based on the rise of the intrinsic tryptophan fluorescence (W207, Faurobert et al., 1993), resulting from conversion of the Gα subunit into its active state (Higashijima et al.,1987). G_t (400 nM) and rhodopsin or opsin (40 nM) were mixed at 23 °C in 50 mM BTP buffer (pH 6.0 or 8.0) containing 130 mM NaCl and 5 mM $MgCl_2$. The membranes were sonicated before the measurements (Branson Sonifier S 125, 80 W, 10 s) to obtain uniform size particles. Immediately before the measurement, rhodopsin was illuminated with yellow light for 10 s to produce Meta II. For the opsin measurements, the apoprotein was allowed to incubate with retinoids for 10 min at 23 °C in the same buffer (pH 6.0 or 8.0). G_t activation was initiated by the addition of 20 μM GTPγS. The fluorescence changes were observed using a spectrofluorometer Spex Fluorolog-2, employing excitation and emission wavelengths of 300 and 340 nm, respectively. The slit of the double monochromator facing the xenon lamp was kept open at 0.4 mm (out of the maximal 8 mm), to reduce the effect of 300 nm light on the sample. At this condition, photoisomerization of the retinal was not observed.

To obtain the catalytic G_t activation rates k, the records were fit by the function

$$C(t) = C_{max}(1 - e^{-kt}) \quad (1)$$

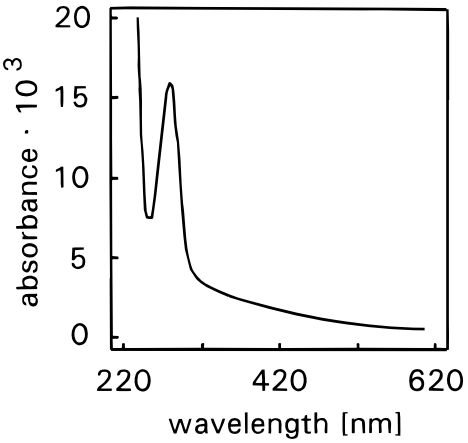

FIGURE 1: Absorption spectrum of opsin in native membranes. Opsin was prepared from rhodopsin by thorough bleaching with white light in the presence of Hydrazide Avid Gel Ax as described in Materials and Methods. The spectrum was recorded in 50 mM sodium acetate (pH 5.0) containing 130 mM NaCl at 23 °C. Note the lack of typical absorption for retinoids at 320−380 nm.

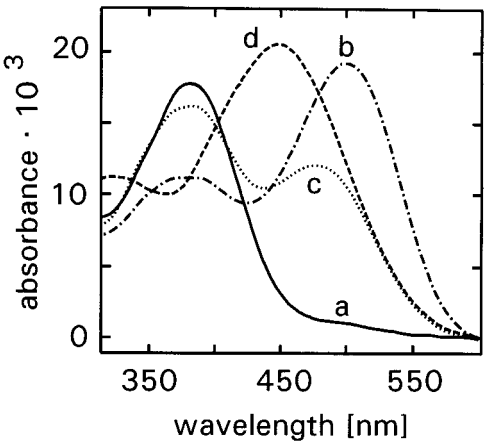

FIGURE 2: Absorption spectra of PM-opsin regenerated with 11-*cis*-retinal. The difference spectra of 2.5 μM PM-opsin in 50 mM BTP buffer (pH 8.0) containing 130 mM NaCl at 10 °C were measured immediately after the addition of 5 μM 11-*cis*-retinal (the reference sample had 2.5 μM PM-opsin) (trace a), 30 min later (trace b), after illumination (white light) (trace c), and after acidification with 1% (v/v) 12 N HCl (trace d). Note that after 30 min PM-opsin was regenerated (peak at 498 nm) with 11-*cis*-retinal (380 nm); after bleaching, photolyzed PM-rhodopsin produced two peaks at ∼480 nm (MI) and at ∼380 nm (MII) which were converted to one peak absorbing at ∼450 nm upon acidification.

where C_{max} is the maximal amplitude of the fluorescence increase and k is the catalytic G_t activation rate in s^{-1}. In the case of the reaction starting by the addition of retinoid instead of the nucleotide at $t = 0$, the measurements were fit by the consecutive reaction scheme A → B → C:

$$C(t) = C_{max}[1 - 1/(k_2 - k_1)(k_2 e^{-k1t} - k_1 e^{-k2t})] \quad (2)$$

where C_{max} is the maximal amplitude of the fluorescence increase, k_1 (A → B) is the rate constant of the formation of the active receptor in s^{-1}, and k_2 (B → C) is the catalytic G_t activation rate.

PDE Assay. PDE activity was determined by measuring proton release from cGMP hydrolysis using a pH electrode. We carried out all assays at room temperature (20 ± 1 °C) in a final volume of 200 μL and in a buffer containing 7.5 mM BTP (pH 7.5) containing 130 mM NaCl, 5 mM $MgCl_2$, 0.5 mM GTP, and 3 mM cGMP. The protein concentrations were 50 nM PDE, 0.5 μM G_t, and 0.5 μM rhodopsin. The reactions were started with bleaching of 0.1% of rhodopsin. When opsin/*all-trans*-retinal was used, the reaction was started by the addition of a mixture of cGMP and GTP. PDE activity was determined as the first time derivative of the pH records. For details of the monitor, see Heck and Hofmann (1993).

RESULTS

PM-Opsin Regenerated with 11-cis-Retinal. In these studies, we used rhodopsin in native ROS membranes to produce opsin by thorough bleaching; the resulting *all-trans*-retinal was converted into oximes with NH_2OH or mostly removed with a hydrazide gel (Figure 1). To block 10 peripheral Lys residues of rhodopsin (and other minor proteins) and amino group-containing phospholipids, rhodopsin was reductively methylated with formaldehyde (Longstaff & Rando, 1985). PM-rhodopsin was bleached to produce PM-opsin. In agreement with Longstaff and Rando (1985), PM-opsin regenerated with 11-*cis*-retinal to PM-rhodopsin at least to 90% (Figure 2). After illumination, the 380 nm (Meta II) and 480 nm (Meta I) peaks were found. Protonated SB was observed after acid trapping [addition of 1% (v/v) 12 N HCl], suggesting that the isomerized retinal was bound covalently via SB to Lys^{296}. These experiments showed that PM-opsin could be regenerated to form functional PM-rhodopsin.

Endogenous all-trans-Retinal Did Not Form a SB with PM-Opsin. When a 2-fold molar excess of *all-trans*-retinal was added to opsin, we observed formation of a shoulder in the UV−vis spectrum at ∼460 nm at pH 6.0 ($SB \rightarrow SBH^+$), but not at pH 8.0 ($SB \leftarrow SBH^+$). After acid denaturation, all SBs formed were trapped and a peak at ∼440 nm was produced (Figure 3A,B). We obtained similar results when rhodopsin substituted for opsin (data not shown).

Besides the active site Lys^{296} (Bownds, 1967; Ovchinnikov et al., 1982; Hargrave et al., 1983), opsin contains nine peripheral Lys residues on the cytoplasmic and one Lys on the intradiskal surface. These Lys residues, ∼30 phosphatidylethanolamines, and 10 phosphatidylserines per rhodopsin molecule (Miljanich et al., 1981) are candidates for SB formation with exogenously added retinal (DePont et al., 1968; Plack & Pritchard, 1969). In contrast to opsin, most of PM-opsin did not form a SB between Lys^{296} and added *all-trans*-retinal (Figure 3C,D). Spectra recorded at higher concentrations of *all-trans*-retinal gave the same results (data not shown), suggesting that Lys^{296} was inactive or that *all-trans*-retinal was sterically hindered from forming a SB bond. To study these two possibilities, regeneration of PM-opsin with 11-*cis*-retinal was carried out in the presence of *all-trans*-retinal or β-ionone.

all-trans-Retinal Did Not Compete with 11-cis-Retinal for Lys^{296}. The kinetics of regeneration were measured for native opsin and PM-opsin as an increase in the 500 nm absorption (Figure 4). For the regeneration of PM-opsin (open squares), a single exponential fit (curve a) yielded a regeneration rate constant $k = 0.015 \pm 0.001$ s^{-1}, while for native opsin (open circles), the regeneration rate was decreased after the first 2−3 min; therefore, no single exponential fit these data. This rate corresponded well with 11-*cis*-retinal forming random SBs with native opsin which proceeded with a rate of $k = 0.0086 \pm 0.0004$ s^{-1} (data not shown). When only the first

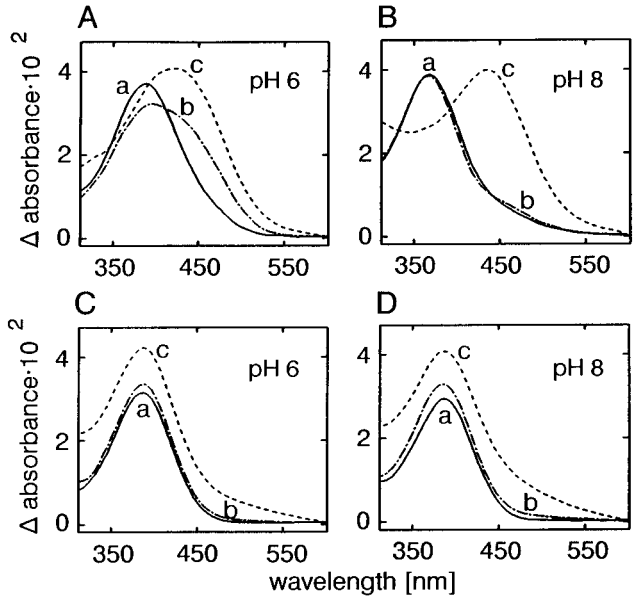

FIGURE 3: Absorption spectra of opsin (A and B) and PM-opsin (C and D) in the presence of *all-trans*-retinal. (A) The difference spectra of 2.5 μM opsin in 50 mM BTP (pH 6.0) containing 130 mM NaCl at 10 °C were measured immediately after the addition of 5 μM *all-trans*-retinal (the reference sample had 2.5 μM opsin) (trace a), 15 min later (trace b), and after acidification with 1% (v/v) 12 N HCl (trace c). (B) The difference spectra of 2.5 μM opsin were measured as in A, but in 50 mM BTP buffer (pH 8.0) containing 130 mM NaCl. (C) The difference spectra of 2.5 μM PM-opsin were measured as in A; the reference sample had PM-opsin. (D) The difference spectra of 2.5 μM PM-opsin were measured as in C, but in 50 mM BTP buffer (pH 8.0) containing 130 mM NaCl.

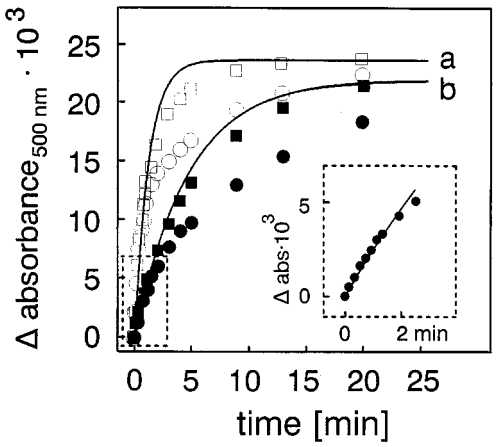

FIGURE 4: Regeneration of native opsin and PM-opsin in the presence of β-ionone. The regenerations of opsin and PM-opsin were measured as a rise of the 500 nm absorption in 50 mM BTP buffer (pH 8.0) containing 130 mM NaCl at 20 °C; the reference wavelength was 600 nm. Regeneration of opsin (2.5 μM) was carried out in the presence of 5 μM 11-*cis*-retinal, without (open circles) or in the presence of (closed circles) 100 μM β-ionone; the regeneration had a fast and a slow component, and no single exponential could fit the experimental data. The inset shows a fit of the first 2−3 min by a single exponential for the regeneration in the presence of β-ionone. Regeneration of PM-opsin (2.5 μM) was carried out in the presence of 5 μM 11-*cis*-retinal, without (open squares) or in the presence of (closed squares) 100 μM β-ionone; a single exponential fit the data, without (curve a) or with (curve b) β-ionone.

2−3 min of the opsin regeneration was considered, a single exponential fit the data ($k = 0.0155 \pm 0.001$ s^{-1}), yielding comparable regeneration rates for opsin and PM-opsin.

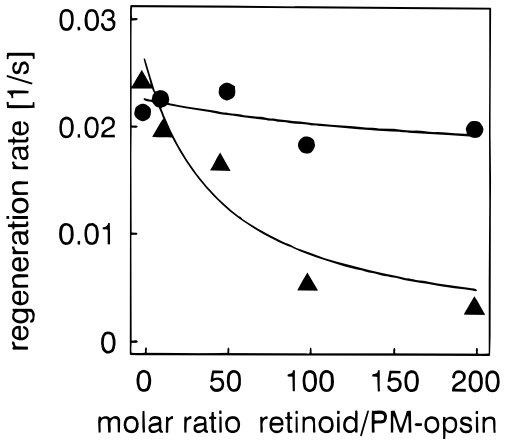

FIGURE 5: First-order rate constants of the PM-opsin regeneration in the presence of *all-trans*-retinal and β-ionone. Regeneration of PM-opsin (2.5 μM) was carried out in the presence of 5 μM 11-*cis*-retinal and increasing amounts of β-ionone (closed triangles) or *all-trans*-retinal (closed circles) (0−200, expressed as a ratio to PM-opsin), as described in Figure 4.

These data suggest that for opsin random SB[2] formation reduced the effective concentration of 11-*cis*-retinal to react with Lys^{296}. Preincubation of membranes with a 100-fold molar excess of β-ionone resulted in a reduction of the regeneration rate constant k by a factor of 3.75 for PM-opsin (filled squares in Figure 4, curve b; $k = 0.004 \pm 0.0003$ s^{-1}) or 5.1 for opsin (filled circles; $k = 0.0031 \pm 0.0003$ s^{-1}). These data suggest that β-ionone competed with 11-*cis*-retinal for the retinal binding site as observed earlier (Matsumoto & Yoshizawa, 1975).

When we increased concentrations of β-ionone, expressed as a ratio of [β-ionone]/[PM-opsin], from 0 to 200, the suppression followed a hyperbolic curve (closed triangles in Figure 5). In contrast, *all-trans*-retinal did not affect the regeneration rate (filled circles, Figure 5). These data suggest that *all-trans*-retinal was excluded from the chromophore binding pocket, most likely as a result of steric hindrance.

Binding of all-trans-Retinal to Opsin Generated Active Products toward G_t. We used a fluorescence assay (Higashijima et al., 1987; Faurobert et al., 1993) to measure the efficiency of G_t activation by photolyzed PM-rhodopsin and the PM-opsin/*all-trans*-retinal complex. When PM-opsin was regenerated with increasing 11-*cis*-retinal at the molar ratio [retinal]/[PM-opsin] from 0 to 1, G_t activation curves (inset in Figure 6) could be fit using eq 1. The catalytic activation rates k were plotted as a function of the molar ratio (triangles in Figure 6), and again this plot was fit by a single exponential. Photolyzed PM-rhodopsin activated G_t exclusively in a light-dependent manner, while G_t alone was inactive (open triangle, trace a). The G_t activation saturated at ∼20−30% regenerated and photoactivated rhodopsin (Figure 6). We assume that, at higher than 20% photoisomerized retinal, the membrane association of G_t was the rate-limiting step. This idea was also supported by light-scattering measurements which showed that the activation of G_t was faster than its association with the membrane (Hofmann, 1993). Due to a small amount of bleaching of

[2] We do not know the nature of these SBs. Likely, they are formed between amino group-containing lipids rather than Lys residues due to their high pK_a. In this study, we refer to them collectively as random SBs.

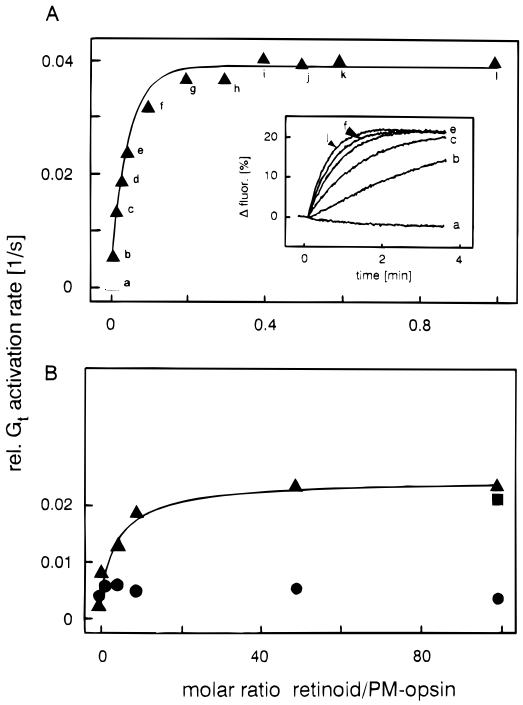

FIGURE 6: Activation of G_t by photoactivated PM-rhodopsin (A) and the PM-opsin/*all-trans*-retinal complexes (B) triggered by the addition of GTPγS. (A) A fluorescence assay was employed to measure the rate of receptor-catalyzed G_t/GTPγS formation. The increase in the fluorescence is proportional to the activation of G_t (Materials and Methods). The inset shows selected traces from the original fluorescence measurements produced by 400 nM G_t and 40 nM PM-opsin regenerated with increasing concentrations of 11-*cis*-retinal (molar ratio r = [retinal]/[PM-opsin] = 0−1). The reaction was initiated by illumination and 20 μM GTPγS, and all measurements were performed in 50 mM BTP buffer (pH 8.0) containing 130 mM NaCl at 23 °C. Trace a represents the measurement of a control sample without PM-opsin. All data could be fit by a single exponential for a first-order kinetics, yielding the activation rates k. The activation rates k for PM-opsin regenerated with 11-*cis*-retinal (closed triangles) were plotted as a function of the molar ratio r = [retinal]/[PM-opsin]; $k(r)$ was fit by a single exponential. The open triangle represents the k for the control sample without PM-opsin (trace a). (B) The activation rates k for PM-opsin in the presence of different retinoids. The measurements were done in a manner similar to that in A, but instead of 11-*cis*-retinal, PM-opsin was incubated with increasing concentrations of *all-trans*-retinal (closed triangles) (the data were fit by a hyperbolic function of r = [retinoid]/[PM-opsin] = 0−100), β-ionone (closed circles), or a mixture of *all-trans*-retinal and β-ionone (both at a 100-fold molar excess over PM-opsin) (closed square).

rhodopsin by the excitation light of 300 nm, we could not perform these measurements at low illumination levels.

When the PM-opsin/*all-trans*-retinal complex was used instead of photolyzed PM-rhodopsin, *all-trans*-retinal at a concentration of 100 times higher was necessary to obtain the activation rates of photolyzed PM-rhodopsin (Figure 6B). Furthermore, the catalytic activation rates never exceeded k = 0.025 s^{-1}, and a hyperbolic function could fit the data. We assume that this finding reflects the binding isotherm for *all-trans*-retinal reaching full occupancy at the maximal k, and from this curve, the occupancy of the binding site(s) was calculated. At the ratio of [*all-trans*-retinal]/[PM-opsin] of 2, as used in the spectroscopic assays above, the occupancy of the binding site was 20% of the putative active site(s). If the retinal had formed a SB, this would be well within the sensitivity of our assay after acid denaturation (Figure 3C,D). How much exogenous *all-trans*-retinal must

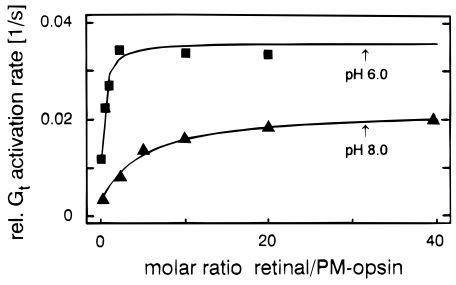

FIGURE 7: Activation of G_t by the PM-opsin/*all-trans*-retinal complexes at pH 6 and 8. PM-opsin was preincubated for 15 min with increasing amounts of *all-trans*-retinal, and a fluorescence assay was employed to measure the rate of receptor-catalyzed G_t/GTPγS formation as described in Materials and Methods and Figure 6. The activation rates k were obtained at pH 6.0 (closed squares) and pH 8.0 (closed triangles), using 50 mM BTP containing 130 mM NaCl. The data were fit by a hyperbolic function of r = [retinoid]/[PM-opsin]. The residual G_t activation rate for PM-opsin was between k = 0.010−0.012 ± 0.002 s^{-1} at pH 6.0 and k = 0.0024−0.0032 ± 0.0005 s^{-1} at pH 8.0.

be added to PM-opsin to express the same G_t activation rates as photolyzed PM-rhodopsin? By comparison in the linear range of panels B and A of Figure 6, a 5-fold excess of added *all-trans*-retinal over PM-opsin was needed to reach the same k produced by a 0.02-fold excess of 11-*cis*-regenerated and photoactivated rhodopsin (a factor of 250). At pH 6.0 (Figure 7), $^{0.6}/_{0.02}$ = 30-fold more exogenously added *all-trans*-retinal was required to reach the same activation level as photolyzed rhodopsin. The rate of G_t activation versus the *all-trans*-retinal concentrations was considered to be linear at low concentrations (2-fold excess over PM-opsin). In this range, the catalytic activation rate was higher by a factor of ∼8 at pH 6.0 than at pH 8.0 (Figure 7). Importantly, the activation of G_t by photolyzed PM-rhodopsin was only 2-fold higher at pH 6.0 compared to that at pH 8.0 (data not shown), as predicted from the pH-dependent MII/MI + MII ratio (Parkes & Liebman, 1984). These data suggest that photolyzed rhodopsin and opsin/*all-trans*-retinal are two different G_t active states. When opsin substituted PM-opsin, similar G_t activation curves were obtained (data not shown). This result suggests that *all-trans*-retinal involved in the noncovalent complex and SB's formation with peripheral amines is interchangeable.

We excluded the possibility that photoisomerization of *all-trans*-retinal to 11-*cis*-retinal during the fluorescence assay could account for significant activation for the following reason. When PDE activity was measured in complete darkness, we found that opsin/*all-trans*-retinal activated G_t and in turn PDE (data not shown), in agreement with an earlier report (Fukada & Yosizawa, 1981). These data are also consistent with earlier reports of the opsin activation by retinoids that become substrate for RK (Palczewski et al., 1994), bound arrestin (Hofmann et al., 1992), and activated G_t (Fukada & Yoshizawa, 1981; Cohen et al., 1992; Palczewski et al., 1994; Surya et al., 1995).

Residual Activity of Opsin. We compared the catalytic activity of opsin and the opsin/*all-trans*-retinal complex with that of photolyzed rhodopsin. The average level of residual opsin activity was k = 0.0028 s^{-1} (at pH 8.0). To calculate the activity of the opsin/*all-trans*-retinal complex, we found from Figure 6B an increase of G_t activation to k = 0.007 s^{-1} at ∼33% occupation, which gave us the relative activity of 0.007 s^{-1}/0.33 = 0.021 s^{-1} for full occupancy, indeed as

found experimentally (Figure 6). For light-activated rhodopsin (Figure 6A), an analogous consideration gives 0.0082 $s^{-1}/0.0125 = 0.67\ s^{-1}$. This means that photoactivated rhodopsin was ~250 times and the opsin/*all-trans*-retinal complex was ~7.5 times as active as opsin without any chromophore (pH 8.0; $T = 23$ °C). Therefore, relating photoactivated rhodopsin (covalently bound retinal) to the opsin/*all-trans*-retinal complex (noncovalently bound retinal), we found the G_t activation rate differs by a factor of $^{250}/_{7.5} = 33$. In physiological terms, it may be interesting to ask about the activity when *all-trans*-retinal and opsin are present in a 1:1 ratio. Because the occupancy then was 10% (Figure 6B), the sample then was only 2 times more active than unliganded opsin. Recently, Surya et al. (1995) compared G_t activation rates of opsin and Meta II using a filter binding assay. Their second-order rate constants for both species differed by a factor of ~30 (pH 7.4). This value differs from our data (pH 8.0) by a factor of 8. Furthermore, addition of *all-trans*-retinal resulted in comparable activation levels of Meta II and opsin/*all-trans*-retinal (Surya et al., 1995). We could not confirm this finding, which may be due to saturation effects of their assay.

β-Ionone Did Not Affect Generation of the Catalytically Active Opsin/all-trans-Retinal Complex. To study the influence of β-ionone on the G_t activation by opsin/*all-trans*-retinal, we have chosen pH 8.0. At this pH, β-ionone did not affect the G_t activation by opsin (Figure 6B, closed circles), but slowed regeneration (Figure 4, closed squares). If the sites occupied by β-ionone and *all-trans*-retinal are identical, β-ionone should also be able to slow G_t activation by opsin/*all-trans*-retinal. However, addition of β-ionone to opsin/*all-trans*-retinal (or vice versa, *all-trans*-retinal to the opsin/β-ionone complex) did not affect significantly the rate of G_t activation ($k = 0.020 \pm 0.003\ s^{-1}$, closed square in Figure 6B), and β-ionone alone did not exert any effect ($k = 0.0035 \pm 0.0004\ s^{-1}$) (closed circles). These findings are consistent with the suppression of regeneration by β-ionone but not by *all-trans*-retinal and lack of covalent linkage between *all-trans*-retinal and Lys^{296}. It should be noted that β-ionone promotes G_t activation at pH 6.0 in agreement with an earlier observation (Palczewski et al., 1994).

Kinetics of Formation of the PM-Opsin/all-trans-Retinal Complex. To determine the time constant for the formation of the opsin/*all-trans*-retinal complex, G_t activation was started by addition of *all-trans*-retinal instead of the nucleotide. In this case, the active receptor first must be formed before the activation of G_t can occur. The measurements were fit by a consecutive reaction scheme A → B → C (Materials and Methods). For the formation of the active receptor, k_1 (A → B), we found values of ~0.05 and ~0.02 s^{-1} for 11-*cis*- and *all-trans*-retinal, respectively. The value for 11-*cis*-retinal agreed well with the spectrophotometrically determined rate of regeneration (Figure 8). These results suggest that regeneration of opsin was faster than the formation of the opsin/*all-trans*-retinal complex. For the G_t activation rates, k_2 (B → C) was 0.036 ± 0.004 and $0.012 \pm 0.002\ s^{-1}$ for 11-*cis* retinal and *all-trans*-retinal, respectively.

Is the Low-Level Activity a Nonspecific Membrane Effect of the Retinoid? It is conceivable that *all-trans*-retinal does not enter the active site, but acts nonspecifically with membranes and thereby raises the residual activity of opsin.

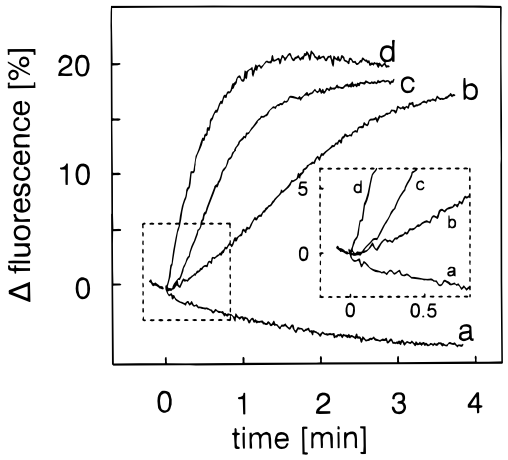

FIGURE 8: Activation of G_t by the PM-opsin/*all-trans*-retinal complexes triggered by the addition of retinoids. A fluorescence assay was employed to measure the rate of receptor-catalyzed G_t/GTPγS formation. The increase in the fluorescence is proportional to the activation of 400 nM G_t produced by 40 nM PM-opsin in the presence of 20 μM GTPγS (the nucleotide was added immediately before the reaction was started). The reaction was initiated by the addition of 80 nM *all-trans*-retinal (at time zero; trace b), or 80 nM 11-*cis*-retinal, concurrent with the illumination of the sample (20 s) (trace c). In control experiments, PM-opsin was omitted (trace a), or the reaction was initiated by addition of 20 μM GTPγS at time zero to a sample containing 40 nM photolyzed PM-rhodopsin (trace d). All measurements were performed in 50 mM BTP buffer (pH 8) containing 130 mM NaCl at 23 °C. The inset shows the initial time course (sigmoidal) of the G_t activation.

However, the increase of the opsin activity at pH 6.0 was already pronounced at a ratio of [retinal]/[opsin] = 0.5, so that 0.5 retinal should exert such an effect on ca. 80 lipids per opsin, which would be difficult to believe. Furthermore, PDE activity was measured in complete darkness with samples containing opsin and *all-trans*-retinal or rhodopsin and *all-trans*-retinal. Opsin/*all-trans*-retinal activated G_t and in turn PDE, whereas rhodopsin/*all-trans*-retinal did not (M. Heck and K. P. Hofmann, unpublished results). Importantly, not only did interaction of *all-trans*-retinal with rhodopsin in the dark not produce G_t activation, but we also did not detect arrestin binding or phosphorylation by RK. These experiments demonstrate that *all-trans*-retinal reacts specifically with opsin by forming an active state and that 11-*cis*-retinal locks the receptor in a completely inactive conformation. It should be noted that G_t is not activated by *all-trans*-retinal without membranes (Figure 8, trace a).

DISCUSSION

Photoactivated rhodopsin thermally relaxes to a series of intermediates which can be distinguished by their absorption properties. The fast initial reaction steps (Photo → Batho → BSI → Lumi) likely involve the chromophore portion, while the succeeding intermediates (Lumi → Meta I → Meta II) include changes in the protein conformation (Lewis & Klinger, 1992). All these intermediates have in common that the agonist *all-trans*-retinal is attached covalently to Lys^{296} via a SB bond. Hydrolysis of this SB is generally assigned to Meta II decay, either via Meta III or directly from Meta II (Baumann & Reinheimer, 1973). The resulting opsin and free *all-trans*-retinal form a "low-level active state" which is characterized in the present study.

Two Complexes between Opsin and all-trans-Retinal: Covalent and Noncovalent

Covalent. *all-trans*-Retinal forms reversible SBs with opsin, rhodopsin, and lipids containing reactive amino groups. Binding of *all-trans*-retinal to phosphorylated opsin produces "pseudo-photoproducts" (Hofmann et al., 1992), capable of interacting with arrestin. This interaction shifts the equilibrium between $SBH^+ \rightleftarrows SB$ toward the deprotonated form (Hofmann et al., 1992). Furthermore, as shown in this study, PM-opsin does not form SBs to a significant degree, suggesting that *all-trans*-retinal interacts with peripheral amine residues, rather than Lys^{296}.

Noncovalent. We were unable to detect SB formation between PM-opsin and *all-trans*-retinal using spectroscopic methods, suggesting that the complex is noncovalent. The complex exhibits stronger pH dependence for the G_t activation than photolyzed rhodopsin (Cohen et al., 1992; Figure 7). For example, for the spectrophotometrically defined intermediates Meta I and Meta II of the light-induced pathway, 2 times more Meta II is formed at pH 6.0 than at pH 8.0 (T = 20 °C; Parkes & Liebman, 1984). Because MII is the basis for the G_t active conformation (Emeis et al., 1982; Hofmann et al., 1983; Kibelbek et al., 1991), the catalytic G_t activation rates should differ by a factor of 2, but for opsin/*all-trans*-retinal, they differ by 8-fold. These data suggest a direct coupling of bound *all-trans*-retinal with the highly pH-dependent cytoplasmic interaction domain (see below), bypassing the conformation change linked to SB deprotonation. The complex is also phosphorylated by RK (Palczewski et al., 1994). In addition, β-ionone binds to the chromophore binding pocket of the apoprotein (Matsumoto & Yoshizawa, 1975; Jin et al., 1993) and competes with 11-*cis*-retinal but not with *all-trans*-retinal, as measured using the regeneration and G_t activation assays. Consistently, *all-trans*-retinal did not influence regeneration.

A Form of Seven-Helix Receptor with Intrinsically Low Activity

In principle, the low activity of a receptor can be formed by two mechanisms that are not necessarily mutually exclusive. First, an active (Meta II-like) conformation is formed, and the overall low activity resulted from an equilibrium fundamentally shifted toward the inactive form (Samama et al., 1993). Second, an active receptor of intrinsically low activity is formed (Surya et al., 1995).

Our experiments with opsin and *all-trans*-retinal have shown that the latter possibility does really exist for opsin. These data agree with recent studies, which have shown that the chromophore domain (domain A) and the cytoplasmic G_t interacting domain (domain B) in rhodopsin can be variably coupled (Arnis & Hofmann, 1993, 1995). On the basis of the analysis of mutant pigments, Fahmy et al. (1995) have moreover defined "on" and "off" states (with respect to G_t activation) of individual molecular groups. It remains to be examined how the activity described here fits into the framework given by these analyses of the light-induced pathway.

Physiological Implications

It has long been known that the presence of bleached rhodopsin in the living eye depresses the sensitivity of the visual apparatus to subsequent illumination to a higher degree than expected from mere quantum catch reduction. This extra sensitivity loss, called bleaching adaptation, is directly related to bleached pigment (Pepperberg et al., 1978; Baylor & Lamb, 1982). Light-scattering assays of G_t activation *in situ* have suggested that bleached pigment does not diminish the biochemical gain (G_t turnover) of fresh photolyzed rhodopsin formed in the adapted condition (Kahlert et al., 1990). In current models, it is assumed that the shutoff reactions after bleaching leave rhodopsin in a state capable of activating the transduction cascade, so that the effect of real light is reduced. In a study using the patch clamp technique, Jin et al. (1993) have concluded that free opsin generates such a state. They have shown that the extra sensitivity loss can be reversed by mere superfusion of the cells with β-ionone, suggesting that noncovalent occupation of opsin's retinal binding site is sufficient to relieve the signal (leading to the extra sensitivity loss) from opsin. This argues strongly against any of the forms where the extra sensitivity loss is explained by a back reaction of the bleached pigment to the Meta II state. Indeed, *all-trans*-retinal did not form a covalent bond with Lys^{296} of opsin. In further agreement with our finding that both retinoids do not influence each other, superfusion of the cells with *all-trans*-retinal instead of β-ionone did not affect the extra sensitivity loss in either direction (Corson et al., 1990).

Finally, we may discuss our results in the context of receptor noise. Baylor et al. (1980) already presented evidence that noise of dark-adapted rods, which is quantal in nature, results from spontaneous thermal activation of native rhodopsin molecules. A quantal event rate of 1 per 50 s was found. Leibrock et al. (1994) showed that, following a small bleach (0.2%), this noise increased and proposed [as already suggested by Lamb (1980, 1981)] that R* (active form of rhodopsin) was formed by reversible back reactions from later intermediates (referred to as R′ and/or R″). Comparing their kinetics of quantal events (1 per 60 s at 22 °C) with our kinetics of formation of the opsin/*all-trans*-retinal complex (1 per 100 s at 23 °C, when extrapolated to the ratio [*all-trans*-retinal]/[opsin] = 1), we found that the rates are in the same range. But because the catalytic G_t activation rate of the noncovalent complex once formed is at least a factor of 45 slower than that of R* (pH 8.0, T = 23 °C), the complex can hardly account for the noise (which requires high G_t activation rates; Leibrock et al., 1994). We must admit, however, that protonation speeds the rate dramatically (Figure 8); thus, the explanation of the noise increment after bleaching remains an open question.

In conclusion, we found that opsin interacts with *all-trans*-retinal, generating a product with increased G_t-activating properties. *all-trans*-Retinal binds to opsin noncovalently in a binding pocket different from that occupied by 11-*cis*-retinal in rhodopsin. The physiological significance of the opsin/*all-trans*-retinal complex requires further studies.

ACKNOWLEDGMENT

We thank Inge Bäumle for technical assistance and Oliver Ernst, Tina Mah, Jack Saari, and Jim Lewis for critical reading of the manuscript.

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BI9524068