Mechanisms of Opsin Activation*

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Rhodopsin is constrained in an inactive conformation by interactions with 11-cis-retinal including formation of a protonated Schiff base with Lys296. Upon photoisomerization, major structural rearrangements that involve protonation of the active site Glu113 and cytoplasmic acidic residues, including Glu134, lead to the formation of the active form of the receptor, metarhodopsin II b, which decays to opsin. However, an activated receptor may be generated without illumination by addition of all-trans-retinal or its analogues to opsin, as measured in this study by the increased phosphorylation of opsin by rhodopsin kinase. The potency of stimulation depended on the chemical and isomeric nature of the analogues and the length of the polyene chain with all-trans-C17 aldehyde and all-trans-retinal being the most active and trans-C12 aldehyde being the least active. Certain cis-isomers, 11-cis-13-demethyl-retinal and 9-cis-C17 aldehyde, were also active. Most of the retinal analogues tested did not regenerate a spectrally identifiable pigment, and many were incapable of Schiff base formation (ketone, stable oximes, and Schiff base derivatives of retinal). Thus, receptor activation resulted from formation of non-covalent complexes with opsin. pH titrations suggested that an equilibrium exists between partially active (protonated) and inactive (deprotonated) forms of opsin. These findings are consistent with a model in which protonation of one or more cytoplasmic carboxyl groups of opsin is essential for activity. Upon addition of retinoids, the partially active conformation of opsin is converted to a more active intermediate similar to metarhodopsin II b. The model provides an understanding of the structural requirements for opsin activation and an interpretation of the observed activities of natural and experimental opsin mutants.

Highly specific protein-protein recognition allows specific signal transduction pathways to be selected from an immense network of inter- and intracellular communications. Structural and chemical complementarities and hydrophobic and electrostatic properties of interacting domains provide precise docking of two or more proteins. Recognition domains may be permanently present in the interacting proteins, assembled because of posttranslational modifications, induced in one or both proteins by a ligand, or formed temporarily as a result of a biochemical reaction. An examination of the principles of protein-protein recognition is pivotal for understanding the relationship between structure and function of proteins, their participation in physiologically relevant processes, and their regulation.

Rhodopsin (Rho), the transducing molecule of vision and a G protein-coupled receptor, undergoes conformational changes upon illumination that ultimately lead to interaction with and activation of the retinal specific G protein (Gt) (reviewed in Ref. 1). The transiently photoactivated Rho is subsequently phosphorylated by rhodopsin kinase (RK) and binds a regulatory protein, arrestin, before it decays to opsin and free retinal (2). In native Rho, 11-cis-retinal is covalently linked via a protonated Schiff base (PSB) to the side chain amine of Lys296 (3) in the transmembrane portion of the molecule (4). In femtoseconds after absorption of a photon, newly formed and still covalently attached all-trans-retinal induces conformational changes of the surrounding protein, moieties which can be followed spectrally. Short-lived Rho intermediates, bathorhodopsin (~543 nm) and lumirhodopsin (~497 nm), sequentially rearrange to Meta I (~478 nm) and the relatively stable Meta II (~380 nm)(5), responsible for Gt activation (6). Decay of Meta II leads to opsin and all-trans-retinal. The transition from Meta I to Meta II is accompanied by deprotonation of the PSB (7), an event believed to be obligatory for Gt activation and Rho phosphorylation (8). A similar conclusion was derived from mutagenesis studies, which also showed that the covalent link between Lys296 and all-trans-retinal, which distinguishes Rho from other G protein-coupled receptors, is not necessary for Rho activation or inactivation. The opsin mutant K296C could be reconstituted with various protonated retinal derivatives, including PSBs formed between n-alkylamines and 11-cis-retinal (9, 10). More recent studies documented that a single conversion of Lys296 or Glu113, the counterion of PSB (11, 12), to a neutral or oppositely charged residue, led to constitutive activation of opsin (13, 14).

Addition of all-trans-retinal (Fig. 1) to opsin (or phosphorylated opsin) generated an active receptor as originally observed using three assays, Gt activation (15), phosphorylation by BK (16, 17), or arrestin binding (16). Furthermore, Cohen et al. (14)
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found that G₂ activation by opsin/all-trans-retinal is strongly pH-dependent with the most efficient catalysis at pH 5–6, while the activity of opsin at lower pH was attributed to spontaneous activation of G₁. Jäger et al. (18) found that opsin activated G₁ with a lower efficiency (1/250 less) than Rho*. This activity was enhanced by a factor of ~10 by the presence of all-trans-retinal. All-trans-retinal forms both non-covalent and covalent complexes with peripheral amine residues of opsin and glycerophospholipids (18).

These developments have enhanced our understanding of Rho activation at the molecular level. However, many questions remain. What is the role of deprotonation of the PSB in opsin activation? How is the signal from the SB transmitted to the surface of Rho? How are activation of Rho by light and of opsin by all-trans-retinal related? Is the complex between opsin and exogenously added all-trans-retinal non-covalent or covalent? What is the role of the chromophore in Rho? If the Lys²⁹⁶ is protonated and forms a salt bridge with Glu¹³ in opsin, what is the mechanism for regeneration with 11-cis-retinal, which requires a deprotonated Lys? Why is Rho regenerated with 11-cis-13-demethyl-retinal active without photoisomerization? Can the properties of constitutively activated opsin be explained by mechanisms of activation of Rho? How relevant are the mechanisms of opsin activation for other G protein-coupled receptors? We will try to answer some of these questions in the present study.

MATERIALS AND METHODS

Synthesis of Retinoids—All synthetic and analytical procedures with retinal and analogues were performed under dim red light and an argon atmosphere. The analogues were stored under argon at 70°C. All analogues were assayed by UV-visible absorption and NMR spectra in deuterated chloroform. HPLC was performed using Econosphere Silica SU 250 x 6 mm (Altichem), with ethyl acetatehexane as solvent.

β-Ionone was purchased from Sigma and distilled before use. All-trans-retinal was purchased from Sigma. 11-cis-retinal was a generous gift from the National Eye Institute. The purities of all three reagents were not separated.

Preparation of Bovine Rho—Rho was prepared from rod outer segments (ROS) by sequential homogenization with water, salt, and buffers (0.3 mg/ml) using a Teflon-glass tissue grinder, and collected by centrifugation. The time and the average centrifugal fields are shown in parentheses. First, ROS were homogenized twice with water (30 min; 19,000 x g); third, with 500 mM NaCl (35 min; 19,000 x g); fourth, with water (35 min; 19,000 x g); and fifth, with 10 mM BTP, pH 7.5, containing 50 mM NaCl. The oximes of all-trans-retinal were extracted with petroleum ether (10 ml/10 ml of the opsin suspension). A brief centrifugation (10 min, 18,000 x g) separated the organic and water layers, and the ether phase was discarded. The extraction was repeated four times. To facilitate opsin sediments from membranes as determined by lack of absorption at 320–380 nm, and HPLC analysis of retinoids using ethanolic/hexane extraction (24). The concentration of opsin was determined by 1) the regeneration with 11-cis-retinal and measurements of Rho concentration as described above, and 2) a colorimetric method (25) in HCOOH, using Rho as a standard. Based on these methods, 92–95% of opsin could be regenerated to Rho.

Permethylated Opsin (PM-opsin and PM-Lys-opsin)—PM-opsin was prepared from opsins (26, 27). PM-Lys-opsin was prepared by methylation of Rho, followed by bleaching and extraction of all-trans-retinal as described for opsin, thus producing opsin with all exposed Lys permethylated and Lys¹³ unmodified (18, 28). Specifically, to opsins or Rho (1 mg/ml), 300 μM NaBH₃CN (2M) was added in small aliquots (10 times) to final concentration of 30 μM NaBH₃CN and converted to A-opsin by thorough bleaching (typically 15 min) with a 180-watt lamp at a distance of 20 cm in the presence of 45 mM NH₄OH in 10 mM BTP, pH 7.5, containing 50 mM NaCl. The oximes of all-trans-retinal were extracted with petroleum ether (10 ml/10 ml of the opsin suspension). A brief centrifugation (10 min, 18,000 x g) separated the organic and water layers, and the ether phase was discarded. The extraction was repeated four times. To facilitate opsin sediments from membranes as determined by lack of absorption at 320–380 nm, and HPLC analysis of retinoids using ethanolic/hexane extraction (24). The concentration of opsin was determined by 1) the regeneration with 11-cis-retinal and measurements of Rho concentration as described above, and 2) a colorimetric method (25) in HCOOH, using Rho as a standard. Based on these methods, 92–95% of opsin could be regenerated to Rho.

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Acidification of Rho and Opsin (A-Rho and A-opsin)—The acidification reaction that converted exposed Glu to Glu was done in the dark according to a general procedure introduced by Rao and Acharya (30). ROS from 100 retinas were homogenized with 40 ml of water and then with 35 ml of 20 mM MES, pH 5.8, and collected by centrifugation (20 min; 18,000 x g). The amidation reaction was carried out on Rho (32 μM) at room temperature in the presence of 0.5 mM NH₄Cl using hydroxy-2,5-dioxopyrrolidine-3-sulfonic acid sodium salt (Fluka; 2 mM) and 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC; Sigma; 40 mM) as catalysts in 20 mM MES, pH 5.8. After overnight incubation, reagents and by-products were removed by centrifugation (10 min; 28,000 x g). A-Rho was washed with 10 mM BTP, pH 7.5, containing 50 mM NaCl and converted to A-opsin by thorough bleaching (typically 15 min) with a 180-watt lamp in the presence of 0.5 mM NH₄OH in 10 mM BTP, pH 7.5, containing 50 mM NaCl at 0°C as described above. A-opsin could be fully regenerated with 11-cis-retinal. The stoichiometry of amidation was determined by the reaction of a control sample of Rho and A-Rho with 100 mM [¹⁴C]ethanolamine (1000 dpm/mmol) in con-
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Fig. 1. Structures of retinal analogues. A, 11-cis-retinal; B, all-trans-retinal; C, 7-cis-retinal; D, 11-cis-13-demethyl-retinal; E, all-trans-13-demethyl-retinal; F, 9-cis-13-demethyl-retinal; G, all-trans-C17 aldehyde; H, 9-cis-C17 aldehyde; I, all-trans-C22 aldehyde; J, all-trans-C15 aldehyde; K, trans-C12 aldehyde; L, ω-ionone.

Fig. 2. Effects of retinoids on phosphorylation of opsins, PM-Lys-opsin, and PM-opsin. Opsin, PM-Lys-opsin, or PM-opsin (80 μl; 30 μM) were preincubated for 15 min with the indicated aldehyde (120 μM) in 30 mM BTP, pH 6.5, containing 3 mM MgCl₂. The phosphorylation reaction was initiated by addition of 40 μl of RK and 5 μl of [γ-32P]ATP (5 μM; ~100,000 cpm/μml) and carried out for 10 min at 35°C. The reaction was stopped with 10% trichloroacetic acid, the membranes were extensively washed and solubilized with 88% formic acid, and radioactivity was determined using a scintillation counter (33).

Lys-opsin activities 7-fold, but had no effect on PM-opsin activity. The combination of retinals with opsins was faster than the resolution of our assay suggesting that we were always working under equilibrium conditions. Methylation of Lys⁵⁹⁶ may lead to an increase of the pKₐ of the amine group resulting in formation of a stronger salt bridge with Glu¹⁵¹ or may constrain opsin in an inactive conformation similar to that found in Rho. Alternatively, methylation of Lys⁵⁹⁶ could block the binding of all trans-retinal or perturb the opsin structure. We favor the first and second interpretations since shorter aldehydes, such as all-trans-C17, all-trans-C15, and trans-C12, which activated opsin and PM-Lys-opsin, did not activate PM-opsin (Fig. 2). Activation of PM-opsin at low pH excluded the possibility of its denaturation (discussed below). Reductive methylation is a mild modification, which converts primary amines to dimethyl tertiary amines. Such modification does not affect the interaction of modified Lys residues involved in ion pairs with carboxyl groups, and typically has little effect on the biological activity of proteins (26), but dimethyl-Lys residues do not interact with all-trans-retinal or its analogues. PM-Lys-opsin was activated, however, to a lower extent (70%) than opsin. Since PM-Rhoα also had a reduced activity (~30%) compared with Rhoα, we attributed this reduction in activity to changes in the opsin structure or lipid environment. It is likely that only multiple phosphorylation of PM-Lys-opsin was affected since this reduced activity was observed only at high kinase concentrations (data not shown).

The length of retinoids appeared to have a significant effect. All-trans-C17 aldehyde (Fig. 1) was the most effective in stimulation of opsin phosphorylation, while longer (all-trans-retinal) and shorter analogues (all-trans-C15 aldehyde) were less potent. All-trans-C22 aldehyde was ineffective in our assay, suggesting that the length of this retinoid excluded it from the...
binding to opsin, while the shortest aldehyde, trans-C12, was only modestly effective. This specificity suggested a unique interaction of opsin with retinoids, rather than a nonspecific lipid-like effect or interaction with peripheral amines.

The 9-cis-isomer of the potent all-trans-C17 aldehyde also exhibited activity when recombined with opsin or PM-Lys-opsin, but the stimulation was decreased relative to the trans isomer (Fig. 2).

A Special Case of 13-Demethyl-Rho—Ebrey et al. (34) observed that 13-demethyl-Rho, opsin regenerated with 11-cis-13-demethyl-retinal, activated Gt as measured by phosphodiesterase activity in the dark. This finding was surprising, since 13-demethyl-retinal lacks only the methyl group in position 13. When 11-cis-13-demethyl-retinal was preincubated for 15 min with opsin in the dark, significant phosphorylation was observed. The activity was increased when the all-trans isomer was used, but decreased with 9-cis-13-demethyl-retinal (Fig. 2). These results were consistent with the observations of Ebrey et al. (34).

The maximum stimulation was observed at ~10-fold excess of 11-cis-13-demethyl-retinal over opsin or PM-Lys-opsin. Higher concentrations of the retinal led to decreased phosphorylation (Fig. 3A). The significant difference between PM-Lys-opsin and opsin, and the biphasic nature of the stimulation, suggested that at least two distinct processes were taking place, for example activation and regeneration. Lack of peripheral amine residues that could interact with free retinal would increase its effective concentration, enhance regeneration, and thus lower the stimulation, consistent with the findings. In contrast, the effect of all-trans-retinal was maximal at 40-fold excess with an activity of 0.7 nmol of phosphate transferred/min (Fig. 3B). Higher concentrations of all-trans-retinal had no further effect and were avoided to eliminate nonspecific effects of ethanolic solutions of aldehydes on membranes (data not shown). Stimulation of opsin by all-trans-retinal showed a different type of biphasic effect. At low concentrations of retinal (Fig. 3B, inset), there was significantly higher stimulation of opsin than PM-Lys-opsin, suggesting that the reaction of all-trans-retinal with peripheral amines may further stimulate activity (18), or that permethylation lowers the affinity for retinal. The half-maximal effect was seen at 7–8 retinals/opsin with a maximal activity of ~0.07 nmol of phosphate/min. This was ~5 times higher activity than that observed with 11-cis-13-demethyl-retinal. Simulation by all-trans-C17 aldehyde was comparable with that observed for all-trans-retinal, but lower stimulation was observed with PM-Lys-opsin. In contrast, no stimulation was found when Rho was used in the presence of either all-trans-retinal or all-trans-C17 aldehyde.

These differences between opsin incubated with 11-cis-13-demethyl-retinal and 11-cis-retinal were not a consequence of differences in the properties of the corresponding Rho. When opsin was fully regenerated with a 10-fold excess of 11-cis-
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Fig. 4. Characterization of opsin and PM-Lys-opsin phosphorylation by 13-demethyl-retinals. A, opsin phosphorylation in the presence of different retinals preincubated for various times. Opsin (80 μl; 30 μM) was preincubated with a 4-fold molar excess of the indicated retinal in 30 mM BTP, pH 6.5, containing 3 mM MgCl₂. At the indicated time, the phosphorylation reaction was initiated by addition of 40 μl of RK and 5 μl of [γ-32P]ATP (5 mM; ~100,000 cpm/nmol) and carried out for 10 min at 35 °C in the dark. The dashed line indicates opsin activity without retinoids. B, PM-Lys-opsin phosphorylation in the presence of 11-cis-13-demethyl-retinal and all-trans-13-demethyl-retinal preincubated for the indicated times. C, time course of phosphorylation of Rho* and 13-demethyl-Rho* of PM-Lys-opsin (100,000 cpm/nmol) in 20 mM BTP, pH 7.5, containing 3 mM MgCl₂, 100 mM NaCl, and 200 μM [γ-32P]ATP were phosphorylated by RK under a 180-watt lamp at a distance of 20 cm. The reaction was stopped with 10% trichloroacetic acid and analyzed as described under “Materials and Methods.” In a control experiment, the samples were incubated with RK under identical conditions in the dark (○). The dark, no phosphorylation of Rhos was observed. Rho and 13-demethyl-Rho were prepared by regeneration of the corresponding opsins with a 10-fold excess of 11-cis-retinal or 11-cis-13-demethyl-retinal for 1 h at room temperature, then overnight in 5 °C. Inset, a double-reciprocal plot of the activity at different concentrations of Rho* ( ), and 13-demethyl-Rho* ( ).

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retinal or 11-cis-13-demethyl-retinal and the corresponding properties of Rho and 13-demethyl-Rho were tested, the extent, the rate of phosphorylation, lack of phosphorylation in the dark, and the affinity of RK for Rho* (Kₐ 15 μM) were indistinguishable (Fig. 4). The sites of phosphorylation were also found to be identical (data not shown). These data suggest that once opsin was regenerated with 11-cis-13-demethyl-retinal, its properties were similar, if not identical, to native Rho. This was consistent with findings that the bleaching of 13-demethyl-Rho from Meta II to opsin is similar to native pigment (35, 36).

The effect of 11-cis-13-demethyl-retinal was time-dependent and decayed by 50% in ~30 min. This correlated with the rate of regeneration of opsin with 11-cis-13-demethyl-retinal, which was 1/9 the rate of regeneration of opsin with 11-cis-retinal (Ref. 37; data not shown). The basal activity of 13-demethyl-Rho was higher than that for opsin or Rho, consistent with the observation that 13-demethyl-Rho readily hydrolyzed to free retinal and opsin (38). Predictably, PM-Lys-opsin was inactivated faster with 11-cis-13-demethyl-retinal (with 50% of the effect at ~10 min), likely because of an increase in the effective retinal concentration as discussed above. Retinals that rapidly regenerated Rho, 11-cis-retinal and 9-cis-13-demethyl-retinal, only stimulated opsin phosphorylation at the first point of the time course. The activity stimulated by all-trans-retinals decayed slowly over several hours (Fig. 4).

Non-covalent Association of Retinoids with Opsin—These studies have shown that all-trans-retinal or its analogues form complexes with opsin; however, the nature of this association is ambiguous. Shorter retinoids, which do not produce a pigment with the spectral characteristics of opsin, may still interact covalently with Lys by generating a pronounced bathochromic absorption shift, for example by formation of SB or carbinalamine intermediates. There was also a possibility that the non-covalent-intermediate occurred only at very low concentrations. However, three sets of experiments showed that opsin forms non-covalent complexes with retinoids: 1) Aldehydes shorter than all-trans-retinal could not extend to Lys by the binding of the ionone-ring is fixed (39). This is also consistent with the finding by Towner et al. (40) that shorter all-trans-retinal analogues did not form a covalent link with opsin via SB, as reduction with NaBH₄ did not covalently attach the retinals to opsin, and with observations made by Jager et al. (18), that only peripheral amine groups were reactive with aldehydes but not the active site Lys. 2) Stable oximes of all-trans-C17, all-trans-C15, and trans-C12 stimulated opsin in the phosphorylation assay, while longer oximes of all-trans-retinal were ineffective. Use of O-alkyl-substituents of oximes with different lengths provided an additional way to probe the active site. For example, the length of all-trans-C17 appeared to be optimal, as an increase in the length of the aldehyde by generation of hydroxyl, O-methyl, or O-ethyl oximes progressively lowered the efficiency of these oximes in the activation of opsin. Similarly, the O-methyl oxime of all-trans-C15 and the O-ethyl oxime of trans-C12 were optimal, suggested again that the optimal length of the retinoid was comparable to the length of all-trans-C17 aldehyde (Fig. 5A). To prove that oximes were chemically stable during the incubation, all-trans-C17 oxime was incubated with opsin, extracted, and analyzed by HPLC. The results indicate that the all-trans-C17 oxime was not hydrolyzed to all-trans-C17 aldehyde during the incubation with ROS (Fig. 5B). 3) Non-aldehyde analogues, which could not react with opsin, such as the ketone, β-ionone (Fig. 5A), or all-trans-retinol (17), also stimulated opsin. This stimulation was unaffected by the presence of hydroxylamine, suggesting that β-ionone activation did not result from aldehyde impurities, but from unique interaction with opsin. Finally, opsin was stimulated by a SB of 9-cis-C17 aldehyde and methylamine (Fig. 5). This activity was increased by illumination of the sample, bringing the activity to a level similar to that found for the all-trans-C17 aldehyde SB with methylamine. Interestingly, illumination of the 9-cis-C17 aldehyde was less effective in this stimulation, possibly because of a lower efficiency of photosomerization of the aldehyde as compared with the SB derivative. Thus, the effectiveness of stimulation was dependent on the chemical and structural nature of the aldehyde or its analogues.

Opsin Activation Depends on Protonation of a Cytoplasmic Residue

Phosphorylation of the opsin/all-trans-C17 aldehyde complex (or PM-opsin/all-trans-C17 aldehyde) was strongly pH-dependent (Fig. 6A). To insure buffering of our phosphorylation mix-
ture and to stabilize RK, phosphate/BTP buffer was chosen, even though phosphate strongly inhibited phosphorylation at low pH. The effect of denaturation of RK or opsin was 10%.

These data suggest that inhibition of Rho* phosphorylation at low and high pH was affected by a protonation state of RK/Rho and by the inhibitory effects of salts, but not by inactivation of the receptor or the kinase.

Since these inhibitory effects remain constant, a plot of the
The pH of the kinase assay mixture was adjusted by an addition of different amounts of 100 mM H₃PO₄ (final concentration of H₃PO₄ was 1.2 mM at pH 9, and 30 mM at pH 4) to 10 mM BTP base. The assay was carried out as described under "Materials and Methods" and the legend to Fig. 2. Rho, PM-opsin or opsin concentrations were 20 μM with 80 μM all-trans-C17 aldehyde as indicated. The maximal activity for Rho⁺ at pH 7.0 was taken as 100%. Specific enzymatic activity of RK was comparable to that described in Fig. 2. A, the activity of Rho⁺ (●) was measured under continuous illumination. The activity of opsin with all-trans-C17 aldehyde (○) and PM-Lys-opsin with all-trans-C17 aldehyde (□) in the dark. The dashed line represents the phosphorylation of Rho⁺ measured at pH 7.0 after the sample was prepared at the indicated pH, incubated for 10 min at 37°C in the dark (in the standard assay conditions, but without ATP), and adjusted to neutral pH. The activity was measured with [γ-32P]ATP after illumination (see "Materials and Methods") for 10 min in 37°C. Inset, the ratio between activity for opsin stimulated by all-trans-C17 aldehyde to the activity of Rho⁺. B, the kinase activity toward opsin (without chromophore; ○) and Rho with all-trans-C17 aldehyde (○), C, the kinase activity toward PM-Lys-opsin (○) and PM-opsin (□) without chromophore.

ratios of the activities of Rho⁺ and opsin/all-trans-C17 aldehyde revealed the magnitude of the stimulation (Fig. 6A, inset). These data suggest that phosphorylation of opsin/all-trans-C17 aldehyde was dependent on protonation of a group(s) in opsin with a pKₐ of ~4.8 (Fig. 6A, inset), such as Glu or Asp. For Rho, this group was protonated as a consequence of changes occurring during conversion of Rho to Meta II (41). Similarly opsin or PM-Lys-opsin were activated 30–50-fold at low pH as compared with the activity at neutral pH levels (Fig. 6, B and C). This phenomenon was not observed for Rho, because 11-cis-retinal linked via Lys296 constrained the receptor in an inactive conformation even in the presence of exogenous retinals. The activity of PM-opsin was significantly reduced, further suggesting that methylation prevents opsin from assuming a more relaxed and active conformation. These data are in agreement with findings reported by Jäger et al. (18), but in conflict with Surya et al. (42) who found that opsin maximally activated Gₛ at pH 6.5–7.0.

Conversion of cytoplasmic Glu and Asp in Rho to Gln and Asn (A-Rho⁺) did not affect phosphorylation (Fig. 7A). In contrast, A-opsin was active at all pH levels tested, in contrast to opsin which was phosphorylated only at low pH (Fig. 7B). The activity of A-opsin was stimulated by the addition of all-trans-C17 aldehyde at a weakly pH-dependent manner; however, opsin activation by all-trans-C17 aldehyde at different pH levels. D, ratio of the activities for opsin and A-opsin, and opsin and A-opsin stimulated with all-trans-C17 aldehyde at different pH levels.

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Based on numerous published reports and the results of this study, we propose a mechanism of opsin activation (Scheme 1).

In this model, Rho is constrained in an inactive conformation because binding of 11-cis-retinal to Lys296 via the PSB (–NH⁺ = ) induces changes in Rho's helical transmembrane domain and/or cytoplasmic surface (B:3) that prevent interaction with native RK or Gₛ. Upon photoisomerization of 11-cis-retinal to all-trans-retinal, the receptor undergoes major structural rearrangements that include displacement of the positively charged SB from its interaction with negatively charged Glu113 (43). Low stability of the uncompensated, positively charged SB from its interaction with negatively charged Glu113 at the main and/or cytoplasmic surface (B:3) induces changes in Rho to Meta II (41). Similarly to Meta II, Rho and A-Rho (16 μM) in 20 mM BTP/25 mM Bis-Tris, at pH 5, 6, 7, or 8 containing 3 mM MgCl₂, 100 mM NaCl, and 200 μM [γ-32P]ATP were phosphorylated by RK with illumination from a 180-watt lamp from a distance of 20 cm. In a control experiment, the samples were incubated with RK under identical conditions in the dark. Note, Rho and A-Rho had low activity. B, opsin or A-opsin (16 μM) in 20 mM BTP/25 mM Bis-Tris, at pH 5, 6, 7, or 8 containing 3 mM MgCl₂, 100 mM NaCl, and 200 μM [γ-32P]ATP, were phosphorylated by RK in the presence or absence of a 4-fold excess of all-trans-C17 aldehyde. C, ratio of the activities for opsin and A-opsin stimulated with all-trans-C17 aldehyde at different pH levels. D, ratio of the activities for opsin and A-opsin, and opsin and A-opsin stimulated with all-trans-C17 aldehyde at different pH levels.
Mechanisms of Opsin Activation

Receptor is converted with high efficiency (ΔH° = -20 kJ/mol) to Meta II b by protonation of cytoplasmic Glu residue(s) (opsin[N = all-trans-retinal][BH]⁺[Nac]) (41, 80), producing a form of the receptor active toward RK or Gt. The hydrolysis of a SB at neutral pH requires its protonation and accessibility to water. Low levels of PSB may be in equilibrium with other species either as active or inactive forms (opsin[-NH⁺ = all-trans-retinal][BH]⁻[Nac]; opsin[-NH⁺ = all-trans-retinal]-[B: Y Nac]). Reprotonation of the SB and its hydrolysis leads to active (with protonated cytoplasmic Glu residues) [opsin[-NH₂; all-trans-retinal][BH]⁺[Nac]; opsin[-NH₂; all-trans-retinal]⁻[B: Y Nac]) non-covalent complexes of all-trans-retinal with opsins. These complexes exist in equilibrium with free opsins (partially active opsin[-NH₂; BH]⁺[Nac] or inactive opsin[-NH₂; BH]⁻[Nac]). In vivo, reduction of free all-trans-retinal by all-trans-retinal dehydrogenase and NADPH (17) completes quenching of the activated receptor. This model highlights two elements in the opsins structure that are critical for its activation: 1) interaction of the chromophore with the transmembrane domain and of active site residues Lys296 (as the PSB) and Glu113.

Our model is based on data from this study employing exclusively the phosphorylation assay and from other published studies employing transducin activation as an indication of receptor activity. While we cannot rule out uncoupling of these two parameters by some opsins modifications, the results of previous studies of opsin activation by several retinoids were comparable when examined by phosphorylation or transducin activation assays (17). Thus, our model is likely to reflect opsin activation generally.

Rhodopsin—Interaction between opsins and 11-cis-retinal via a PSB or SB is critical for inactivation of the receptor (Scheme I). Even at low pH where opsin was weakly active, Rho or A-Rho were completely inactive (Fig. 6). In addition, Rho generated from the constitutively active opsin mutant E113Q does not exhibit any catalytic activity (11, 44) either in PSB or SB forms and is transformed to the SB form by illumination (45). Another example is mutant K296G, which is inactivated by 11-cis- but not by all-trans-isomers of positively charged retinaline analogues (10, 13). Although Glu113 strikingly affects the absorption maximum and pKₐ for the PSB (11, 43), it is not the salt bridge between PSB and Glu113 that keeps Rho in an inactive form but the bound chromophore in its 11-cis configuration. Positioning of 11-cis-retinal in the active site may be critical and could result from either formation of a linkage with Lys296 (Rho, E113Q mutant), or positioning PSB analogues in close proximity of Glu113 (as in K296G) (9). This is in agreement with the report that the double mutant E113Q K296G is not inactivated by the protonated analogues of retinal (10). If this positioning is not precise, retinals will exert their activating effects even in a 11- or 9-cis configuration (Fig. 2).

In native Rho, the active site around Lys296/Glu113 is sequenced (10, 44) as shown by its resistance to protonation from the aqueous bulk medium (47) or penetration by hydroxylamine or water, a factor that increases Rho stability. Lack of Glu113 increases accessibility of the SB in the dark to hydroxylamine or anions (48). This tight steric interaction between different groups and chromophore in the active site is also suggested from work with an analogue of retinal lacking only the 9-methyl group. 9-Demethyl-Rho does reach a Meta II-like conformation but shows decreased activation properties upon photolysis (49–51). Methylation of the active site SB (8) may prevent Meta II formation by steric hindrance rather than by affecting its protonation. Indeed, the methylated active site has lost its ability for deprotonation when illuminated and Meta I rearranges directly to a Meta III-like intermediate (36). The ability of PM-opsin for this activation is limited as the conformation that requires displacement of Lys296 from the chromophore binding sites is likely inhibited by methylation.

Activation—The transition of Rho to Meta II involves a major conformational rearrangement as observed by infrared spectroscopy (52), and the ionone ring of the chromophore may be critical for the formation of the Meta II state (53). During Meta II formation, Glu113 is protonated (54), perhaps by accepting a proton from the PSB (55), and conformational changes occur in the chromophoric domain of opsins (56). Is the deprotonation of the PSB obligatory for opsin activation (8) or is the deprotonation a consequence of photoisomerization of the retinal? If the protonation status of the SB does not impose a critical restriction on the activation of Rho*, is the protonation of Glu113 a prerequisite for the receptor activation (57)?

During activation there is a movement of Lys296 (43), which is consistent with an observed increase in the volume of Meta II (58). Consequently, the PSB may move into an environment that does not support protonation of this linkage, for example, a hydrophobic, low dielectric medium. It is known that an uncompensated, positively charged group is extremely unstable in the transmembrane domain of proteins, and residues bearing these groups are frequently found at the boundary between transmembrane and exposed segments (59). Interestingly, a mutant with the counterion moved by one helical turn in the transmembrane segment IV (60, 61) contains a PSB and an active Meta II-like conformation (62). Thus, isomerization of the chromophore determines the activation of opsins. In native receptor, this activation is followed by changes in protonation in the active site that blue-shifts the absorption, preventing capture of a subsequent photon. Furthermore, all-trans-retinal, which does not react with opsins' Lys296 under various experimental conditions (18), and other analogues activated opsins apparently without changes in the ionization of the Glu113 and Lys296 (Fig. 2). Our data show that A-opsin has significant activity over the whole range of pH levels, suggesting that further protonation of the active site may facilitate an increase in the activation of opsins, but is not absolutely required. Thus, in our model (Scheme I), Meta II a, with a deprotonated SB, is shown in an inactive conformation (41). However, because of...
photosomerization and decoupling of Glu\textsuperscript{113} from Lys\textsuperscript{196}, a cytoplasmic region of opsin also undergoes changes resulting in protonation of Glu\textsuperscript{134} (Meta II b), that occurs with high efficiency (41, 63, 64). It is unlikely that Glu\textsuperscript{134} is moved into a transmembrane domain, as UV-visible (11) and Raman (65) spectra of Glu\textsuperscript{134} mutants are not different from that of native Rho before or after photolysis.

Cytoplasmic Domain—How does the protonation of Glu\textsuperscript{134} in native Rho\textsuperscript{*} take place at pH 7–8? We suggest that Glu-Glu ionic paring could be responsible for increasing the pK\textsubscript{a} of Glu\textsuperscript{134}. Such pairing has been found in several crystal structures (66), and is also known for many organic compounds. The transformation of Rho to Meta II b determines the timing of the appearance and the lifetime of Meta II, and is energetically unfavorable. However, there is sufficient energy of the photon, 35\% of which is stored in bathorhodopsin, to account for the energetics of these transitions (67). Thus, mutation in Glu\textsuperscript{134} caused more rapid formation of Meta II from photolyzed Rho (monitored as an equilibrium between Meta I and II) (68, 69).

Furthermore, mutant E134Q opsin was constitutively active, (74, 76). According to our model, interaction of the PSB and residues in the intact photoreceptor is not known, but partial constitutive activity of these mutants is due to an Rho*-like inactive form. No effect on the sensitivity has been observed on the addition of all-trans-retinal to either intact rods or cones (74, 79), or of all-trans-C17 aldehyde to cones (78). A possible explanation of these latter results is that the trans forms are not taken up efficiently in these physiological preparations.

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