Binding of More Than One Retinoid to Visual Opsins

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ABSTRACT Visual opsins bind 11-cis retinal at an orthosteric site to form rhodopsins but increasing evidence suggests that at least some are capable of binding an additional retinoid(s) at a separate, allosteric site(s). Microspectrophotometric measurements on isolated, dark-adapted, salamander photoreceptors indicated that the truncated retinal analog, β-ionone, partitioned into the membranes of green-sensitive rods; however, in blue-sensitive rod outer segments, there was an enhanced uptake of four or more β-ionones per rhodopsin. X-ray crystallography revealed binding of one β-ionone to bovine green-sensitive rod rhodopsin. Cocrytallization only succeeded with extremely high concentrations of β-ionone and binding did not alter the structure of rhodopsin from the inactive state. Salamander green-sensitive rod rhodopsin is also expected to bind β-ionone at sufficiently high concentrations because the binding site is present on its surface. Therefore, both blue- and green-sensitive rod rhodopsins have at least one allosteric binding site for retinoid, but β-ionone binds to the latter type of rhodopsin with low affinity and low efficacy.

INTRODUCTION

The act of seeing begins with photon capture by visual pigments within the rods and cones of the retina. A visual pigment consists of a seven-transmembrane-helix receptor opsin protein, to which the ligand, 11-cis retinal (A1) or 3-dehydro 11-cis retinal (A2), is bound covalently. Photoexcitation isomerizes the retinal chromophore to the all trans conformation, transforming the visual pigment into a catalytically active state that sets off a G protein cascade. Later, all trans retinal dissociates from the opsin and is reduced to retinol by a dehydrogenase. Retinol moves from rods and cones to neighboring epithelial cells, is converted to 11-cis retinal, and then returns to the photoreceptors to unite with apo-opsin and regenerate rhodopsin, completing the visual cycle. In a second visual cycle pathway, Müller cells produce 11-cis retinol, which cones but not rods oxidize to 11-cis retinal to regenerate their visual pigment (reviewed in Lamb and Pugh (1) and Travis et al. (2)).

Besides their role in photoreception, retinoids have other effects. High levels are toxic. Retinal can be oxidized to retinoic acid, a transcriptional regulator. Retinoids inhibit the light-regulated channel of photoreceptors (3) and stimulate isomerization of the retinal chromophore (4,5). These latter two targets may modulate the overall sensitivity of rods and cones. The ability of a truncated retinal analog, β-ionone, to competitively inhibit the binding of 11-cis retinal to opsin led to the proposal that the chromophore-binding pocket of opsin includes a recognition site for the ionone ring (6,7). Yet all trans retinal stimulates the catalytic activity of opsin (8) but does not compete with 11-cis retinal for the chromophore-binding pocket (7). In addition, β-ionone increases the catalytic activity of some visual pigments, in which the chromophore-binding pocket is already occupied (9). Accumulating evidence suggests that rod opsin bears more than one binding site for retinoids (reviewed in Heck et al. (10)). Here we investigate the issue using β-ionone. Binding of β-ionone to rhodopsin in green (GSRs) and blue-sensitive rods (BSRs) of salamander would increase their UV absorbance, so uptake was measured by single cell microspectrophotometry. Localization of β-ionone binding to bovine rhodopsin was determined by x-ray crystallography. Some results have appeared in abstract form (11).

METHODS

X-ray crystallography

β-Ionone (Sigma Chemical, St. Louis, MO) was purified by double distillation, diluted in ethanol, and included in the mother liquor in excess over rhodopsin while keeping other conditions similar to those reported previously (12). Briefly, the solution of β-ionone in ethanol was diluted 240-fold with the rhodopsin solution prepared for crystallization as before (12). Two representative data sets (3.5 mM and 7 mM β-ionone) were collected at BL41XU of SPring-8, Hyogo, Japan. Such high concentrations of β-ionone appeared necessary to maintain the bound state because we could not confirm the binding in crystals grown with [β-ionone] = 0.5 or 1.1 mM. The data sets were processed with HKL2000 (13), and binding of β-ionone was confirmed by calculating the difference maps between the two data sets and the native (β-ionone minus) data sets using the phases from the previously obtained rhodopsin structure (PDB ID: 1U19). Because the appearance of the electron densities was better for data set 1, a β-ionone molecule was placed manually in each of the two rhodopsin models in the asymmetric unit obtained with that set and refined further with CNS (14). The refined model was validated (Ramachandran zone distribution: 81.5/13.2/3.6/1.7) using PROCHECK (15). The outliers are similar to those in the rhodopsin-only model and most of them are in the third cytoplasmic loop or in the carboxyl terminal tail.

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Microspectrophotometry

Retinas from the tiger salamander, *Ambystoma tigrinum*, were removed under infrared illumination after dark adaptation of an animal overnight, and shredded on a quartz coverslip previously coated with poly-l-lysine. Double-sided tape lined two opposing edges and a second coverslip was placed on top. This sandwich-type chamber was mounted on the stage of the Williams-Webbers microspectrophotometer (16). Baseline measurements were taken with the polarized probe beam passing through a cell free area. The beam was then positioned on a cell with its electric vector oriented perpendicular to the long axis of the outer segment (⊥). Some outer segments were rotated 90° for a second measurement (||). Measurements were made at room temperature: 21–23°C.

Individual rods (see the Supporting Material) contained mixed pigment populations, wherein the opsin bound either an A1 or an A2 chromophore (17). The A2 visual pigment has a longer wavelength absorbance maximum and reduced extinction, compared to its A1 counterpart. The absorbance of each component was determined by fitting spectra with the sum of A1 and A2 templates (18), assuming A1 and A2 pigment maxima at 501 nm and 519 nm in GSRs and at 428 nm and 435 nm in BSRs, respectively. The fractional composition was determined using extinction coefficients: ε_{A1} = 42,000 M^{-1} cm^{-1} and ε_{A2} = 30,000 M^{-1} cm^{-1} (19, 20). For GSRs in this study, A2 content ranged from 44% to 100% in aquatic salamanders and from 23% to 82% in terrestrial salamanders. Mean values are collected in Table 1. In control experiments on nine GSRs, OD_{1, measurement} bleached 0.14 ± 0.09%/scan^{-1} (mean ± SE) of the A2 component and 0.22 ± 0.15%/scan^{-1} of the A1 component. Combined bleaches of up to 10% were considered to be acceptable.

Ringer’s contained: 108 mM NaCl, 2.5 mM KCl, 1.0 mM MgCl₂, 10 mM HEPES, 1.5 mM CaCl₂, 0.02 mM EDTA, 10 mM glucose, and in some experiments, 0.7 μM bovine serum albumin (BSA, Fraction V, γ-globulin-free, or fatty acid free, Sigma); pH 7.6. The A1 and A2 visual pigments are mediates. In control experiments on two crystals, the difference spectrum is given by

\[
\beta_{Rh} = \frac{(1.5)(OD_β/ε_β)}{OD_{A1/ε_{A1}} + OD_{A2/ε_{A2}}},
\]

where OD_{β} is the absorbance attributed to β-ionone from the difference spectrum, OD_{A1} and OD_{A2} are the absorbances of the A1 and A2 components of the visual pigment from a template fit, and the ε-values are the extinction coefficients for each component in solution. The factor of 1.5 accounts for rhodopsin dichroism in situ. The experiments yielded difference spectra whose spectral position varied from ~270 nm to 305 nm. Peaks ≤ 280 nm were encountered at low bathing [β-ionone] where the amplitudes were sometimes negative (UV absorbance was lower in cells treated with β-ionone). Those cases reflected slight variability in the ratio of the protein/visual pigment absorbance rather than a difference in β-ionone content. In Fig. 1, the absorbance of treated and untreated rods matched nearly perfectly over the range from 400 to 650 nm. More often, there were small mismatches, so the untreated rod spectrum was corrected by a multiplicative factor, before taking the difference spectrum.

In four experiments with [β-ionone] ranging from 30 to 90 μM, the mean ratio of uptake into the outer segments of intact GSRs versus that into detached GSR outer segments that lacked mitochondria was 1.02, indicating NH₂OH (Sigma), to prevent spectral interference by long-lived photointermediates.

In spectra obtained from rhodopsin crystals, absorbance was sometimes so high that there were distortions due to nonlinearity in the instrument. OD values exceeding 1.1 were therefore deleted and spectra with very broad bandwidth were rejected. In control experiments on two crystals, the bleaching rate per scan was <0.2%. Absorbance maxima of the main rhodopsin band were determined from fits of spectra in which <5% of the rhodopsin had been bleached, with a fourth-order polynomial for OD > 0.8 of the maximum (Igor Pro v. 5.03; Wavemetrics, Lake Oswego, OR). For all other analyses, a bleach of <10% was deemed acceptable.

**RESULTS**

**Uptake of β-ionone into rods determined by microspectrophotometry**

Perfusion of GSRs with β-ionone increased their ultraviolet (UV) absorbance (Fig. 1). Baseline measurements were made with β-ionone present in the bathing solution so the spectral change indicated an accumulation of the substance within the cells. The time course of the absorbance change was not resolved because uptake equilibrated in <5 min—within the time taken to deliver the β-ionone. There was uptake into the ellipsoid (not shown), an area densely packed with mitochondria (21), as well as into the outer segment, where all of the rhodopsin is localized. For five GSRs bathed in 347 μM β-ionone, the ratio of uptake into the outer segment to that in the ellipsoid area of the inner segment was 1.2.

The molar ratio of β-ionone to visual pigment in the outer segment was estimated as

\[
\beta_{Rh} = \frac{(1.5)(OD_β/ε_β)}{OD_{A1/ε_{A1}} + OD_{A2/ε_{A2}}},
\]
that the accumulation did not require a cellular energy source. Measurements of each type were therefore pooled in all experiments. Inclusion of 0.7 μM BSA in the Ringer’s to facilitate delivery of β-ionone had little or no effect on the results. Uptake of β-ionone increased in proportion to its bath concentration, attaining β/Rh ratios that surpassed 7 (Fig. 1 B). These observations accord with the avid partitioning of a hydrophobic molecule into membranes. Assuming [rhodopsin] ≈ 3.3 mM in the outer segment (22), the slope of the concentration dependence of uptake implied a partition ratio of (0.049 μM⁻¹)/(3300 μM) = 162. At low bathing [β-ionone], minor variability in measurements of the protein absorbance introduced scatter in the determination of uptake; however, restricting the analysis to [β-ionone] ≥ 30 μM did not alter the slope or intercept of the uptake relation appreciably. Because a nonzero y intercept would obtain if β-ionone bound to rhodopsin, these results preclude specific binding of even a single β-ionone to rhodopsin in GSRs with very low Kₘ.

Visual pigments orient in the disk-membranes such that light absorption is optimal with the electric vector of the measuring beam aligned perpendicular to the long axis of the outer segment (ODₚ). The absorbance measured in this configuration, divided by that with the rod oriented orthogonally (ODₚ), yielded a dichroic ratio of 3.1 (Table 1), comparable to a previous report (22). In contrast, the mean dichroic ratio for β-ionone was 1.0 with little evidence for any dependence on bathing concentration. The absence of dichroism provided further support for membrane partitioning, given that β-ionone distributes randomly throughout different depths of the lipid bilayer (23).

Washing GSRs with Ringer’s containing BSA after treatment with 33 μM β-ionone removed >90% of the β-ionone (Fig. 2), matching the reversibility reported in physiological experiments (4,5,9). As with uptake, washout occurred rapidly, within the period of time elapsing for chamber perfusion. However, after treatment with 50–150 μM β-ionone, 25–55% of the β-ionone was retained, even after more than one wash, for at least 2 h.

The binding pocket for 11-cis retinal in opsin accommodates β-ionone (6,7), so to demonstrate that our method could detect pocket occupancy, the β-ionone content was followed in nine GSRs as they were treated with 107 μM β-ionone, washed with BSA, bleached, washed with BSA again, and finally treated with β-ionone a second time (data not shown). The initial exposure to β-ionone produced a β/Rh of 4.6. Washout was incomplete; β/Rh dropped to 1.0. After bleaching, the second treatment with β-ionone increased β/Rh to 5.5, presumably by allowing one β-ionone

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**FIGURE 1** β-Ionone uptake into rod outer segments. (A) Subtraction of the average absorption spectrum (ODₚ; see Methods) of 10 untreated GSRs (thick gray line) from that of 16 GSRs treated with 66 μM β-ionone (thin black line) yielded a difference spectrum (thick black line) with a peak at 292 nm (Gaussian fit, red line). After a template fit of the untreated GSR spectrum (dashed red line) to determine the rhodopsin content, the β/Rh ratio was estimated to be 3.2 (see text). (B) Linear regression of β-ionone uptake as a function of [β-ionone] in the bath for GSRs (green diamonds) yielded a slope = 0.049 μM⁻¹ and a y intercept = 0.26 (continuous green line). For [β-ionone] ≥ 30 μM (dashed green line), the slope = 0.048 μM⁻¹ and y intercept = 0.38. For BSRs (blue squares), linear regression for [β-ionone] ≥ 30 μM (dashed blue line) returned a slope of 0.055 μM⁻¹ and an intercept of 3.84. Fitting all BSR results with a Michaelis-Menten function summated with the linear relation obtained for GSRs (dotted gray line) suggested that eight molecules of β-ionone bound at saturation, Kₚ near 90 μM. Substituting a Hill function for the Michaelis-Menten produced a slightly better fit (continuous gray line) with a Hill coefficient of 4.8 and Kₚ of 30 μM. β-Ionone uptake was determined from ODₚ and ODₚ measurements, whereas pigment content was taken from ODₚ measurements. For some experiments with low bathing concentrations of β-ionone, the Gaussian fit to the difference spectrum was poor, so uptake was evaluated as the mean absorbance of the difference spectrum between 285 and 300 nm minus that between 350 and 365 nm.

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**FIGURE 2** Washout of β-ionone. Seventeen GSRs were treated with 33 μM β-ionone (thin black line). Subtraction of the scaled spectrum of 21 untreated GSRs (continuous gray line) gave a difference spectrum revealing the extent of uptake (thick black line). Perfusion with Ringer’s containing BSA removed nearly all of the β-ionone from the 17 GSRs (broken gray line).
rhodopsin plus β-ionone (Rβ) was similar, 491.7 ± 0.5 nm (six measurements on four crystals). In addition, the main absorption band for both types of crystals was broad, conforming more closely to an A2 rather than an A1 template (18). These attributes may have arisen from photoexcitation of some of the rhodopsin by red light used for evaluating crystal growth and for manipulating the crystal during sample preparation. As expected from previous descriptions of P41 crystals of rhodopsin (12,25), absorption by the rhodopsin band was optimal with the electric vector of the measuring beam oriented orthogonally to the long axis of the crystal (Fig. 4, A–C). For very large crystals, the maximal absorbance of the main pigment band was estimated by fitting both sides of the peak with a template, disregarding the high absorbance values which were subject to nonlinearity in measurement. The highest dichroic ratios were then 2.9 for an Ro crystal and 3.7 for an Rβ crystal. The mean dichroic ratios were: 2.0 ± 0.5 for five Ro crystals and 2.9 ± 0.3 for six Rβ crystals, with 1–3 determinations made per crystal. The difference in dichroism, although statistically significant, probably reflected distortion of crystals during sample preparation rather than the presence of β-ionone. There was also a secondary maximum near 330 nm in OD1 spectra (Fig. 4 B). This feature, which was not observed in absorption spectra of intact rods (22), may be a better resolution of the cis peak due to greater homogeneity in bond angles within 11-cis retinal and alignment of partial chromophores (26) perpendicular to the long axis of the crystal. The relatively large size of the secondary maximum shifted the peak of the main band in OD1 spectra to slightly shorter wavelength: 489.2 ± 0.7 nm for Ro (14 measurements on five crystals) and 487.7 ± 0.7 nm for Rβ crystals (13 measurements on seven crystals).

The presence of β-ionone in Rβ crystals was indicated by a higher absorbance near 290 nm (Fig. 4 D). With spectra taken away from the dichroic maximum of the main rhodopsin band, there was a larger secondary maximum for Rβ than for Ro crystals, a feature presumed to be related to the higher dichroic ratio of Rβ. The contribution of the secondary maximum was removed before estimating the molecular ratio of β-ionone to rhodopsin (e.g., Fig. 4 D) at orthogonal orientations of the crystals as

\[
\text{Horizontal} \quad \beta/\text{Rh} = (\epsilon_{A1})/(\epsilon_{A1} \cos \theta) = 0.4,
\]

\[
\text{Vertical} \quad \beta/\text{Rh} = (\epsilon_{A1})/(\epsilon_{A1} \sin \theta) = 1.5,
\]

where \(\epsilon_{A1}\) is the mean absorbance ratio for Ro and Rβ crystals and \(\theta\) is 63.5° (the angle of the vector drawn from the aldehyde group of β-ionone to C5 in the ring relative to the long axis of the crystal). The mean of the two estimates was 0.9, denoting that a single β-ionone was bound to each rhodopsin molecule.

Specific binding was confirmed by solving the x-ray crystallographic structure for Rβ (Fig. 5). The space group,
lattice constants, and arrangement of the helices were nearly the same as for Ro crystals, making it possible to use the previously solved crystal structure of rhodopsin at 2.2 Å (12) to better define the features attributable to β-ionone.

Parameters for the crystals are summarized in Table 2.

Electron density corresponding to β-ionone was discovered near the third extracellular loop connecting helices VI and VII in both data sets 1 and 2. Each rhodopsin had a β-ionone bound to the site; occupancy refinement with data set 1 yielded values of 0.96 and 0.91 for the pair of rhodopsins comprising the asymmetric unit. A hairpin structure of the Gly-Pro-Ile-Phe residues 284–287 formed the binding site. The interaction between the cyclohexenyl ring and the opsins moiety was predominantly hydrophobic. Other residues in the vicinity that may contribute to binding include V271, D282, F283, and I290. No specific interaction was found for the aldehyde group of β-ionone with rhodopsin.

Locating β-ionone into this site displaced only a single water molecule in the 2.2 Å structure model (12). The arrangement of the helical segments of rhodopsin, helices III and VI in particular, were unchanged by the presence of β-ionone.

**DISCUSSION**

Visual opsins are G-protein-coupled receptors for retinoids whose low basal catalytic activity is quenched by the covalent binding of 11-cis retinal as it regenerates rhodopsin. Light isomerizes 11-cis retinal to the all trans conformation, converting an inverse agonist to a full agonist. Other retinoids lacking the full polyene side chain and/or the terminal aldehyde moiety do not bind covalently and their identity as agonist or inverse agonist is dependent upon opsin type (4,5,27,28). For example, β-ionone extinguishes the activity of red-sensitive cone (RSC) opsin but stimulates the activities of blue- (BSC) and UV-sensitive cone (UVSC) opsins.

**FIGURE 4** (A) Micrograph of a rhodopsin crystal digitally captured using infrared light. (Small white square to the right of center) Measuring beam. (B) Dichroism of an Ro crystal. Absorbance was highest for crystals oriented horizontally (as shown in A), i.e., with their long axes perpendicular to the electric vector of the measuring beam (OD<sub>H</sub>). Spectra (thick gray traces) were fit with A2 (dashed black lines) templates. The absorbance maximum at the dichroic minimum (OD<sub>D</sub>) was typically a few nm shorter than at the dichroic maximum. (C) Variation in absorption of the main band as a function of the tilt of the long axis of the crystal from horizontal. (Open circles) Absorbance of the main rhodopsin band near 500 nm. (Solid circles) Absorbance of the protein band near 280 nm, divided by the absorbance of the main rhodopsin band and averaged. The difference (thick continuous black line) was taken from the average spectra for Rβ (thin black line with error bars) crystals minus that for Ro (thin gray line with error bars). A spectral component absorbing near 330 nm (dashed black line) was removed to reveal the absorbance of β-ionone in isolation (thick continuous gray line). Values near 280 nm were disregarded due to the limited number of usable observations (hence large SEs in the Ro and Rβ spectra).

**FIGURE 5** Structure of bovine rhodopsin with β-ionone bound. (A) 11-cis retinal + K296 (red), β-ionone (green), and disulfide bond between C110 and C187 (orange line). (B) β-Ionone (space-filled) docked onto rhodopsin (yellow, residues 284–287; red, a part of transmembrane helix; green, irregular helical region; white, extracellular loop). (C) A simulated-annealed omit map was calculated using the structure factor amplitudes from an Rβ crystal (7.0 mM β-ionone, data set 1) and phases from the model excluding β-ionone and nearby region within 2.5 Å. The map was contoured at the 4σ level.
TABLE 2  Crystallographic data collection and refinement statistics of the rhodopsin-β-ionone complex

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More Than One Retinoid Can Bind to Opsin

Two considerations were ruled out before inferring that there were binding sites for β-ionones on the BSR rhodopsin.

First, the lipid composition of gecko photoreceptor outer segment membranes deviates from that of frog and bovine rods perhaps because cone pigments require a different lipid environment than rod pigments (36). BSRs express a cone pigment (37), so if their outer segment lipids differ from those in GSRs, β-ionone may partition more completely in BSR membranes. But then uptake would increase in greater proportion to bathing concentration for BSRs than for GSRs. Our observations indicated otherwise; at bath concentrations ≥30 μM, the intercept of the linear relation for BSRs was displaced from the origin and the slope did not differ significantly from that for GSRs (Fig. 1B).

Second, β-ionone bleaches BSR rhodopsin, albeit at a very slow rate (9). BSRs were usually measured within minutes after application of β-ionone to minimize the extent of bleach. Some apo-opsin could have been present even before treatment with β-ionone because pigment regeneration in BSRs is often incomplete, even after extensive dark adaptation of salamanders (9,38). Dividing the absorbance due to β-ionone in the membrane plus that residing in the chromophoric binding pocket of opsin by the absorbance of a less-than-full complement of rhodopsin would then give a spuriously high uptake ratio. However, the amount of free opsin would have to have been equal to or greater than the amount of pigment in order to explain the high β/Rh observed in BSRs. Another argument may be advanced as follows. GSR opsin has five tryptophans and 21 tyrosines, whereas BSR opsin has seven and 13, respectively (39,40). Assuming extinction coefficients of 5690 M⁻¹ cm⁻¹ for tryptophan and 1280 M⁻¹ cm⁻¹ for tyrosine at 280 nm (41), BSR and GSR opsins absorb similarly at 280 nm. Because rod and cone rhodopsins have very similar extinction coefficients at their absorbance maxima (42–45), the ratio of absorbance near 280 nm to that at λmax was used to gauge rhodopsin content. The ratios for GSRs and BSRs were similar (Table 1), supporting the notion that the bleached visual pigment content was low in BSRs. Given that physiological experiments demonstrated activation of rhodopsin and the phototransduction cascade by β-ionone in intact, dark-adapted BSRs and BSCs as well as in UVSCs (9), we conclude that the enhanced uptake of β-ionone into BSRs arose at least in part from binding to the visual pigment.

Cascade activation in BSRs was saturated at bath concentrations of ~10 μM β-ionone (9). Uptake measurements suggest that only one or two β-ionones bind each rhodopsin under those conditions (Fig. 1B). The mechanism of action is not known. Because β-ionone also caused pigment bleaching, it could lower the activation energy of the visual pigment and accelerate the rate of thermal isomerization. Alternatively, β-ionone could stabilize the active conformation of the visual pigment with pigment bleaching.
as a secondary consequence. RSC pigment will transfer its 11-cis retinal chromophore to GSR opsin (46) or to a retinal binding protein, CRALBP (38) without isomerization. The rate of chromophore release by RSC pigment is accelerated by the presence of \( \beta \)-ionone (9) or 9-cis retinal (38, 46). Thus, a third possibility is that binding of \( \beta \)-ionone(s) near the chromophore binding pocket of opsin (Fig. 5) simply increases the local concentration of \( \beta \)-ionone, enabling it to compete with and more effectively displace the native chromophore.

After finding that \( \beta \)-ionone failed to bind (this study) or activate (9) salamander GSR rhodopsin, it was surprising to discover that \( \beta \)-ionone incorporated into the crystal structure of bovine GSR rhodopsin. A species difference between bovine and salamander rhodopsins seems unlikely, given that the residues 284–287 in bovine rhodopsin that interact with the ring portion of \( \beta \)-ionone are absolutely conserved in salamander GSR rhodopsin. The binding sequence, GPxF where \( x \) is either I or V, is also present in the GSR opsin of other species (Fig. S1 in the Supporting Material). Among the other residues that may contribute to binding, i.e., V271, D282, F283, and I290, there is an I290V substitution in salamander GSR opsin. We therefore presume that \( \beta \)-ionone binds to all GSR rhodopsins at a site that can only be occupied at exceedingly high salt and/or ligand concentrations. The structure of bovine rhodopsin was unchanged by the binding of \( \beta \)-ionone. In particular, positioning of the sixth helix resembled that in catalytically inactive rhodopsin (12) and differed from that in the partially active opsin (47, 48). Thus, in GSR rhodopsin, binding either did not lead to activation or activation was suppressed by crystal lattice contacts.

If the corresponding residues participate in binding \( \beta \)-ionone to BSR rhodopsin, several substitutions could detract from the hydrophobicity of the site, i.e., G284D, P285L, I286R, and F287M, whereas V271F and D282L might enhance it (GSR opsin numbering). P285L also disrupts the hairpin turn. Interestingly, locating \( \beta \)-ionone on the helix 6–7 linker poises it near one of the lower probability sites of retinal egress from GSR suggested by molecular dynamics (49), which may be relevant to its mode of BSR pigment activation. The binding of additional \( \beta \)-ionones to BSR rhodopsin could involve four other proposed retinoid binding sites on GSR rhodopsin. Two sites within opsin, along with the chromophore-binding pocket, form a retinoid channel through the protein (50). The channel-forming sites are not accessible in GSR rhodopsin; however, the situation could differ in BSR rhodopsin. For example, the Schiff base linkage of 11-cis retinal to lysine in BSR pigment is vulnerable to chemical attack by hydroxylamine, whereas that in GSR pigment is not (37, 51, 52). Limited access to the channel in GSR rhodopsin may help to confer its extraordinary thermal stability. Two other potential sites were located on the intracellular surface (53). One site nestles alongside the palmitates attached to C322 and C323. Palmitoylation may be important for binding because the activation of bovine GSR opsin by all trans retinal decreases upon their removal (31). Both cysteines are conserved in salamander GSR opsin (39). One is conserved in BSR opsin (40), although recombinant BSR protein expressed in COS exhibits heterogeneity in the palmitoylation status (54). There are also sites in GSR rhodopsin crystals occupied by detergent and small amphiphilic molecules (e.g., (55)) that could have a higher affinity for retinoids in BSR rhodopsin. Finally, nonspecific binding of \( \beta \)-ionones to BSR rhodopsin may have been greater than to GSR rhodopsin.

Differences in the allosteric modulation of G-protein coupled receptors sometimes exist between members of a group that share the same orthosteric ligand (35). Thus, in future studies, it will be important to find out how many retinoid binding sites are present on each type of opsin; which retinoids bind to them; under what circumstances they are occupied; and the physiological significance of each site.

SUPPORTING MATERIAL

One figure is available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(10)00969-0.

The coordinates and structure factors have been deposited with the accession code 30AX.

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REFERENCES


26. Sperling, W., and C. N. Rafferty. 1969. Relationship between...


22. Ha´rosi, F. I. 1975. Absorption spectra and linear dichroism of some...

12. Okada, T., M. Sugihara, ...

15. Laskowski, R. A., M. W. MacArthur, ...

24. Edwards, P. C., J. Li, ...

224:590–594.


