Conformational Selection and Equilibrium Governs the Ability of Retinals to Bind Opsin

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Background: A current hypothesis proposes that retinal binding to opsin requires transient activation of receptor.

Results: Our data show that an active conformation only promotes agonist binding; inverse agonist binding is impaired with increased relative fraction of active receptor.

Conclusion: Retinal isomers must match opsin conformation to form a stable complex.

Significance: Rhodopsin displays conformational selection for retinal binding similar to other ligand-binding GPCRs.

Despite extensive study, how retinal enters and exits the visual G protein–coupled receptor rhodopsin remains unclear. One clue may lie in two openings between transmembrane helix 1 (TM1) and TM7 and between TM5 and TM6 in the active receptor structure. Recently, retinal has been proposed to enter the inactive apoprotein opsin (ops) through these holes when the receptor transiently adopts the active opsin conformation (ops*). Here, we directly test this “transient activation” hypothesis using a fluorescence-based approach to measure rates of retinal binding to samples containing differing relative fractions of ops and ops*. In contrast to what the transient activation hypothesis model would predict, we found that binding for the agonist, 11-cis-retinal (11CR), slowed when the sample contained more ops* (produced using M257Y, a constitutively activating mutation). Interestingly, the increased presence of ops* allowed for binding of the agonist, all-trans-retinal (ATR), whereas WT opsin showed no binding. Shifting the conformational equilibrium toward even more ops* using a G protein peptide mimic (either free in solution or fused to the receptor) accelerated the rate of ATR binding and slowed 11CR binding. An arrestin peptide mimic showed little effect on 11CR binding; however, it stabilized opsin:ATR complexes. The TM5/TM6 hole is apparently not involved in this conformational selection. Increasing its size by mutagenesis did not enable ATR binding but instead slowed 11CR binding, suggesting that it may play a role in trapping 11CR.

The receptor responsible for dim light vision, rhodopsin, is unique among G protein–coupled receptors (GPCRs) as its ligand, 11-cis-retinal (11CR), is covalently attached to the protein through a Schiff base with the lysine at 296. Protonation of this linkage shifts the absorbance of the ligand from the ultraviolet (380 nm) to the visible spectrum (500 nm). The 11CR acts as an inverse agonist that quenches basal signaling by trapping the receptor in an inactive conformation. Light absorption causes isomerization of the 11CR to all-trans-retinal (ATR) (see Fig. 1 for ligand structures), which acts as an agonist and induces a series of conformational changes that ultimately push the receptor to the active, G protein-coupling conformation called metarhodopsin II (MII) (2). The MII conformation exposes a cleft on the cytoplasmic face for interactions with ancillary proteins (G protein and arrestins) and is accompanied by deprotonation of the Schiff base and thus a shift in peak retinal absorbance back to 380 nm (3, 4). Binding of arrestin blocks G protein signaling, and subsequent Schiff base hydrolysis results in the decay of the active species and release of ATR. The now empty receptor, inactive opsin (ops), has almost no basal G protein-coupling ability (5–8) and appears to have a conformation like that of the inactive 11CR-bound structure (9, 10). Rebinding of a fresh 11CR quenches the limited signaling and resets the cycle (11).

Despite rhodopsin being one of the most extensively studied members of the GPCR superfamily, it remains unclear how the very hydrophobic retinal enters or exits the protein. Numerous crystal structures along with spectroscopic and biochemical data show that rhodopsin exists primarily in two stable conformations, either a closed, inactive state (11CR-bound or ops) or an open, active form (MII or ops*) (note that other intermediates are transiently formed as the receptor converts between these two states) (6, 9, 10, 12–15). Although the closed rhodop-
sin structure shows no means for the retinal to enter or exit the binding pocket, more recent structures of the open, active receptor conformation have identified a possible ligand channel through the protein formed by rotameric shifts of bulky residues between transmembrane helix 1 (TM1) and TM7 (called Hole A) and between TM5 and TM6 (called Hole B) (11, 12, 14, 16–20) (see Fig. 1A). This structural reorientation exposes the binding pocket to the lipid bilayer and has been speculated to play a role in ligand uptake and/or release (18, 19, 21, 22). The only structure of retinal-free rhodopsin, opsin, currently available most likely does not reflect the “true” inactive opsin state in solution as it has numerous elements of the open active conformation, including the holes, and a root mean square deviation from MII rhodopsin of only ~0.4 Å (14, 15, 23). Biochemical and spectroscopic studies indicate that opsin in solution is more structurally similar to the closed, inactive 11CR-bound rhodopsin (6, 9, 10).

This conundrum (that 11CR is bound in the binding pocket of the closed, inactive receptor, but there is no clear way for it to get there) has led to the proposal that the empty state opsin must enter a transient open and active conformation (ops*) for the 11CR to gain access to the binding pocket (21) (see Fig. 1C). Evidence supporting this “transient activation” hypothesis include whole-cell electrophysiology experiments that show a brief increase in downstream activity during dark adaptation of bleached rod cells (24).

However, direct mutagenesis of the proposed ligand path (shown in Fig. 1A) did not yield clear results as to a role for the open state holes during binding (21). Moreover, in contrast to the hypothesis that 11CR binds the ops* state, attempts to shift

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**FIGURE 1.** A, comparison of access to the retinal binding pocket in the inactive, closed rhodopsin state (left, gray) and active, open MII state (right, blue). To enable visualization of the pocket, the bound retinal in each has been removed and the remaining internal surface was “cast” (colored red). The models clearly show two avenues for access present in the active conformation that are absent from the inactive conformation. These have been termed Hole A (between TM1 and TM7) and Hole B (between TM5 and TM6) (19). The Cα carbon of the Schiff base lysine is shown in gold. B, structures of the chemically identical but spatially different retinal ligands, the inverse agonist 11CR and the agonist ATR. Currently, only the structure of ops* is known, and it is very similar to MII (14, 15). Because inactive opsin is thought to be structurally similar to inactive rhodopsin and thus would have no access pathway to the binding pocket, a transient activation or transient conversion of ops → ops* has been proposed to enable 11CR entry into the binding pocket (21). C, schematic of the transient activation model in which the apoprotein exists as either ops or ops*. Transition of the inactive ops to ops* is proposed to enable retinal to enter the binding pocket. This non-covalent intermediate is followed by Schiff base formation (shown here as a red star). According to the transient activation hypothesis, both 11CR and ATR enter the ops* state and form the non-covalent intermediate. The subsequent formation of the Schiff base with 11CR locks the receptor in the inactive conformation, whereas ATR forms a linkage with the active state. Light (hv) facilitates the conversion of Ops-11CR to Ops*-ATR. Coordinates from Protein Data Bank codes 1GZM and 3PXO were used for models (15, 16). Casting of the binding pocket was done with a 1.4-Å radius probe on the CASTp web server (85). Molecular graphics were created using the UCSF Chimera package (23).
the opsin to more MII-like conformations either through lowering the pH or addition of a G protein C-terminal peptide analog, Gt C-term peptide, also failed to accelerate 11CR binding (21, 25). Therefore, this transient activation model for 11CR binding, although attractive, has remained unsupported by conclusive experimental data.

Here we directly addressed the question of what governs the ability of retinal to bind opsin using a new approach. Traditionally, retinal uptake by rhodopsin has been measured by monitoring formation of the Schiff base between the retinal and the protein after it enters the binding pocket. This is accomplished by measuring the increase in the characteristic absorbance at 500 nm. However, this approach requires monitoring the formation of the protonated Schiff base (detection of the 380 nm to 500 nm shift) and so retinal binding that does not result in a protonated Schiff base goes unnoticed (as both free retinal in detergent and MII absorb maximally near 380 nm and thus cannot be easily distinguished by comparing absorbance spectra).

To circumvent this problem, we established a fluorescence assay to measure the rate of binding of retinal that is independent of protonated Schiff base formation. Essentially, this assay involves running a retinal release assay backward (26). Our approach tracks the quenching of intrinsic tryptophan fluorescence as the ligand binds in the pocket. A similar general approach has been used in several other works (21, 22, 27–31). Here, we established and calibrated our approach to ensure accurate, reproducible measurements so that retinal binding rates measured by fluorescence could be directly compared between different conditions. Additionally, we started with naïve opsin in our study rather than the common approach of using decayed MII formed after photobleaching rhodopsin as this allowed us to measure rates without contamination of other free retinal.

We then used this assay to directly test the transient activation hypothesis for retinal binding. Our strategy was to increase the amount of ops* present in a sample and then use our assay to determine whether the rates of retinal binding are faster as the above hypothesis would predict. Using the constitutively activating mutation M257Y (M257Y-CAM) (32), we shifted the conformational equilibrium from nearly fully inactive WT opsin to a mixture of ops and ops* species and compared the rates of ligand binding between these samples. Unexpectedly, rather than accelerating the rate of 11CR uptake, we found that the M257Y-CAM actually slowed 11CR binding compared with WT opsin. Moreover, stabilizing the ops* population of M257Y-CAM by conformational trapping with a peptide mimic of G protein C-terminus, Gt C-term peptide, further slowed 11CR binding (33, 34). However, we did find partial support for the transient activation hypothesis in experiments using ATR. Although ATR cannot bind to inactive WT ops, we found that ATR binding was dramatically increased as the ops* state was stabilized. The novel approach of fusing the Gt C-term peptide to the opsin C-terminus further amplified these effects.

Together, these observations indicate that the transient activation hypothesis must be modified to include a role for conformational selection. In support of this postulate, we demonstrate that stabilizing a minute, fleeting ops* population present in the WT opsin population with the Gt C-term peptide enables WT opsin to stably bind ATR. Similarly, we found that a peptide corresponding to a flexible “finger” loop of arrestin (Arr peptide; residues 67–79) (35) also enhanced ATR binding but had little effect on the binding of 11CR. Finally, we tested whether Hole B (TM5/TM6) observed in the active ops* and MII structures (14, 15) might play a role in the shift of retinal binding rates. To do this, we expanded the opening with alanine substitutions and measured the effect on 11CR and ATR binding rates. These results showed that removing the TM5/TM6 “doors” did not enable ATR uptake and unexpectedly slowed 11CR binding.

In summary, we found no evidence to support the hypothesis that transient activation of opsin is required for 11CR binding. In fact, we observed that increasing ops* actually impaired 11CR binding. However, we did find that increasing the amount of ops* enhanced ATR binding, providing partial support of the transient activation hypothesis. We also discuss how together these data clearly show that, in our purified system, classical conformational selection between retinal isomers plays a key role for stable retinal binding and how this concept can be used to propose a conformational selection-based model for retinal binding.

**EXPERIMENTAL PROCEDURES**

**Buffers**—11-cis-retinal was generously provided by Dr. R. Crouch (Medical University of South Carolina and the National Eye Institute, National Institutes of Health), Gt C-term peptide (VLEDLKSVGLF) (33), Arr peptide (YGQEDIDVMGLSF), and 1D4 peptide (TETSQVAPA) were purchased from GenScript. All other chemicals were purchased from either Sigma-Aldrich or Fischer. The following buffers were used in this study: PBSSC (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.0 mM Na2HPO4, pH 7.2), Buffer B (PBSSC, 1% DDM), Buffer C (PBSSC, 1 mM NaCl, 1 mM MgCl2, 1% DDM), Buffer D (PBSSC, 0.05% DDM), Buffer E (5 mM MES, 0.05% DDM, pH 6.0), and Elution Buffer (E4, 40 mM NaCl).

**Mutant Generation**—Mutagenesis was performed by overlap extension PCR to generate single site mutants in a synthetic rhodopsin gene (36) and subcloned into a modified version of the original PMT4 expression vector (37). All constructs were confirmed by sequencing. Additionally, each contained the engineered, stabilizing disulide (N2C and D282C) (38) and minimal reactive cysteines (C140S, C316S, C322S, and C323S) (2, 39). This construct, without any additional mutations, has been well characterized and is termed WT throughout (2, 37, 39). The θ subscript is used to differentiate between the minimal cysteine construct and wild type (WT) with only the stabilizing disulide. The minimal cysteine construct was used for direct comparison with previous conformational data determined using site-directed labeling (10). These results were then confirmed with WT containing all native cysteines. The construct referred to as opsin-Gt C-term peptide fusion had the Gt C-term peptide sequence (described above) fused to the C-terminal tail of the receptor. Four glycines were included after the Gt C-term peptide sequence, and an additional 1D4 tag for purification finished the sequence. The entire added sequence

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is as follows: EEVLEDKSVGLFGGGGTETSQVAPA. The gene containing this sequence was purchased from GenScript.

Protein Expression and Purification—Expression and purification of opsin were adapted from methods described previously (10, 37). Briefly, COS-1 cells were transiently transfected using PEI and 30 μg of DNA/15 cm plate. After 50–65 h, the plates were washed with 10 ml of PBSSC and scraped free of the surface. The cells were then pelleted and resuspended in 0.5 ml of PBSSC/plate with 0.5 mM PMSF. Harvested cells were snap frozen in liquid nitrogen and stored at −80 °C until use. The thawed cell mass was solubilized in 1% DDM for 1 h. The solubilized slurry was spun at 100,000 g for 45 min. The supernatant from this spin was incubated with 1D4 antibody beads in Buffer C for 3 h. Beads were then transferred to columns with Buffer D and washed with at least 100 column volumes in Buffer D followed by washing with Buffer E. Elutions were in Elution Buffer with 200 μM 1D4-specific peptide (TETSQVAPA) (10).

Absorbance Spectroscopy—Proper regeneration of the receptors, purity, and rates of Schiff base formation were measured by absorbance spectroscopy using a Shimadzu 1601 spectrophotometer. Temperature was controlled by a VWR water bath. For experiments well below room temperature, the sample chamber was filled with dry air to prevent condensation on the cuvette viewing window. Testing of newly purified opsin was done with a 5 × molar excess of 11CR to opsin (2.5 μM 11CR added to 0.5 μM opsin; concentration determined by ε360 = 56,500 M−1 cm−1) at 20 °C to determine maximal regeneration for the sample by observing increases to the absorbance at 500 nm. All samples were capable of greater than 75% regeneration as indicated by the final absorbance at 500 nm. The rate-determining experiments using absorbance spectroscopy were conducted using 1 μM opsin and 1 μM 11CR in 0.1% DM at 10 °C. Spectra were measured from 700 to 350 nm in 0.5 nm intervals on the “fast” setting, giving 35 s/spectrum. Time between spectra varied between 60 and 300 s depending on the expected length of the experiment. The increase in absorbance at 500 nm was fit by a monoexponential rise to maximum using SigmaPlot and the following equation.

\[ F(t) = A \times (1 - e^{-kt}) + C \]  

(Eq. 1)

Acid protonation was achieved by addition of 4 μl of 0.4 M H2SO4 to 77 μl of the above binding reactions. Pre- and post-acid addition spectra were dilution-adjusted by matching 280 nm absorbance.

Fluorescence Spectroscopy—Protein fluorescence was monitored by steady-state fluorescence using a modified the Photon Technology International Quantamaster instrument described below. The standard arc lamp excitation source was replaced under the cuvette viewing window. Testing of newly purified opsin allowed for the detection of any chromophore in the binding pocket and not just those retinals that have formed a protonated Schiff base. Thus enabling the study of both 11CR and ATR binding (the latter of which is “spectroscopically silent” in traditional absorbance binding assays). Moreover, the sensitivity of this approach is also at least 10× greater than that of the traditional absorbance assay.

Before embarking on experiments using this assay, we established optimal conditions to enable reproducible binding measurements and removal of nonspecific binding signals. We found that bringing the ratio of opsin to ligand to equimolar amounts (1 retinal:1 opsin) and increasing the detergent concentration to 0.1% (w/v) brought the binding rates into a measurable window that yielded reproducible results and, impor-
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FIGURE 2. Rates of retinal binding to opsin can be measured by monitoring quenching of the protein intrinsic tryptophan fluorescence. A, example of the traditional approach for monitoring 11CR binding to naïve opsin in which absorbance spectroscopy is used to observe the shift in the maximum absorbance from 380 to 500 nm as 11CR retinal forms a protonated Schiff base linkage with the protein. Inset, the increase of the 500 nm absorbance as a function of time can be used to determine the rate of binding. B, example of 11CR (brown) and ATR (green) retinal binding by monitoring fluorescence of tryptophan residues in WT opsin. The observed decrease indicates that 11CR binds to the empty opsin, but ATR does not. Addition of retinal is shown by an arrow. C, Arrhenius analysis of the absorbance (Abs) and fluorescence (Fluor) binding data for 11CR binding to opsin at different temperatures. The plots show that the two methods report essentially the same activation energies within experimental error (12.75 ± 2.1 and 13.0 ± 0.31 kcal/mol, respectively). Fluorescence binding assays except where noted were with 0.5 μM opsin and 0.5 μM retinal in 5 mM MES, 40 mM NaCl, 0.1% DDM at pH 6.0 at 10 °C. Absorbance assays were conducted with 1 μM opsin and 1 μM retinal, which was necessary to increase signal over noise. To enable comparison with absorbance data, the Arrhenius analysis of 11CR binding by fluorescence was performed using 1 μM opsin and 1 μM retinal.

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this peptide stabilizes the M257Y-CAM<sub>θ</sub> opsin in the active conformation, ops* (10, 17, 43, 44) (Fig. 4A). Indeed, as would be expected if a conformational selection model were correct, adding G<sub>i</sub> C-term peptide to the M257Y-CAM<sub>θ</sub> sample resulted in very rapid agonist ATR binding and even slower binding of 11CR compared with M257Y-CAM<sub>θ</sub> alone (<sup>k</sup><sub>M257Y-CAM<sub>θ</sub> + Gt C-term peptide(11CR) = 0.363e−3 ± 1.7e−5 s<sup>−1</sup></sup> and <sup>k</sup><sub>M257Y-CAM<sub>θ</sub> + Gt C-term peptide(430nm) = 6.67e−3 ± 4.3e−4 s<sup>−1</sup></sub>; Fig. 4B and C).

We sought to further increase the amount of ops* trapped by increasing the apparent concentration of the G<sub>i</sub> C-term peptide. This was accomplished by fusing it to the C-terminal tail of the opsin, an approach inspired by a similar protocol used by Kobilka and co-workers (45) to study agonist binding to β<sub>2</sub>-adrenergic receptor. Surprisingly, the M257Y-CAM<sub>θ</sub>-G<sub>i</sub> C-term peptide fusion did not further exacerbate the ligand affinity shift, suggesting that the free peptide was already at saturating conditions. Interestingly, the amount of binding of ATR to the M257Y-CAM<sub>θ</sub> does not appear to go to 100% completion but rather is about 25% less than that of 11CR to the same protein (Fig. 4C). This likely reflects a dynamic equilibrium between bound and unbound states resulting from the mixed conformational population.

Stabilizing the ops* conformation through incubation with G<sub>i</sub> C-term peptide either free in solution or as a fusion caused the maximal binding of ATR to reach 11CR levels (Fig. 4C). These results are reflected in the acid protonation spectra in Fig. 4D. M257Y-CAM<sub>θ</sub> alone did not show a complete shift to 440 nm, the characteristic absorbance of a protonated Schiff base, with the addition of acid. This indicates that free ATR is still present and that there is an incomplete occupation of the binding pocket. Free G<sub>i</sub> C-term peptide stabilized the bound complex, and the 440 nm shift was more complete. G<sub>i</sub> C-term peptide incubated with ATR in the absence of opsin showed no absorbance shift with acid (data not shown). Together with the change in rates, these results suggest that the conformational state of opsin dictates the affinity for different retinal isomers.

G Protein C-terminal Peptide Is Sufficient to Stabilize ATR-bound WT<sub>θ</sub> Opsin—We also found that the G<sub>i</sub> C-term peptide alone enabled WT<sub>θ</sub> opsin to bind ATR (Fig. 4F). It has been extensively noted that ATR can interact with opsin, inducing guanine nucleotide exchange in G proteins and phosphorylation by rhodopsin kinase (8, 41, 46–51). Only recently has it been shown that interactions with the G protein actually induce proper ATR binding into the binding pocket (31). Here we show that the C-terminus alone is sufficient to form a stable complex with ATR in the retinal binding pocket of WT<sub>θ</sub> opsin as evidenced by fluorescence data (Fig. 4F) and acid protonation absorbance spectra (Fig. 4G). In agreement with previous reports, we found that WT<sub>θ</sub> opsin does not show any observable ATR uptake (Fig. 4C) (41), and we previously have not seen accumulation of stable interactions of WT<sub>θ</sub> opsin with G<sub>i</sub> C-term peptide (10). However, combining all three components (opsin, ATR, and G<sub>i</sub> C-term peptide) enabled robust ATR binding to inactive WT<sub>θ</sub> opsin. Similar to what was observed with the M257Y-CAM<sub>θ</sub> alone, the WT<sub>θ</sub> opsin-ATR-G<sub>i</sub> C-term peptide complex was relatively unstable compared with 11CR, resulting in an incomplete drop in fluorescence (~80% that of

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**FIGURE 3.** A CAM of opsin, M257Y, binds 11CR slower than WT but also gains the ability to bind ATR. A, pictorial representation of the effect the M257Y mutation (denoted by the purple star) has on the conformational equilibrium between ops and ops*. The M257Y-CAM<sub>θ</sub> shifts the equilibrium of the opsin samples from nearly completely inactive (WT) to a mixture of active (ops*) and inactive (ops) receptors. B and C, WT<sub>θ</sub> opsin readily binds 11CR stably but is unable to bind ATR. D and E, in contrast, the M257Y-CAM<sub>θ</sub> can stably bind both 11CR and ATR. Interestingly, 11CR binding is substantially slower for this mutant than it is to the fully inactive WT<sub>θ</sub>. Experimental conditions were the same as previously described in Fig. 2 and under “Experimental Procedures.”
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11CR). The free Gt C-term peptide had no effect on the rate of 11CR binding to WTopsin (Fig. 4E).

In comparison with M257Y-CAM, which was already saturated by the free peptide, the WTopsin Gt C-term fusion showed greatly enhanced ATR binding, and the opsin-peptide interaction was strong enough to slow 11CR binding (Fig. 4, E and F). This construct also completely stabilized the ATR-bound complex, resulting in a fluorescence drop comparable with that induced by 11CR. Acid protonation spectra confirmed Schiff base formation between the ATR and the receptor in the presence of Gt C-term peptide (Fig. 4G). Additionally, because WT opsin without Gt C-term peptide showed no presence of a Schiff base upon addition of acid, these data further support our conclusion that the ATR does not form a nonspecific Schiff base with a peripheral lysine under our experimental conditions.

Arrestin “Finger Loop” Peptide Also Enhances ATR Binding—We also tested whether a peptide corresponding to the finger loop of bovine arrestin (Arr peptide; residues 67–79) also affected 11CR or ATR binding to opsin. Similar to the G protein, arrestin has been shown to inhibit ATR release from the photoactivated receptor (4, 52, 53). As depicted in Fig. 4A, the Arr peptide binds in the same cleft as the Gt C-term peptide, shown here as peptide fragments bound in opsin crystal structures (4, 17, 53–56). Interestingly, we found that the Arr peptide increased the rate and amount of ATR binding to M257Y-CAM (Fig. 4L) and showed some stabilization of ATR binding to WTopsin (Fig. 4I). The presence of a Schiff base with both samples was confirmed by acid protonation spectra (Fig. 4, J and M). The Arr peptide showed minimal effects on the binding rate of 11CR to either form of opsin (Fig. 4, H and K) compared with the robust changes observed with Gt C-term peptide, which might be due to the lower affinity and non-optimized state of the Arr peptide (53).

Conformational Selection of Retinal Isomers by Opsin Is Not an Artifact of the Minimal Cysteine Construct—To eliminate the possibility that the minimal cysteine background (WTopsin) used for all the experiments in Fig. 4 might be influencing the results, we repeated the experiments with WTopsin containing all native cysteines in addition to the stabilizing disulfide (Fig. 5). The WTopsins showed identical results across the different conformations (Fig. 5, A and B). Additionally, we did not observe FRET between WT and ATR, indicating that the native cysteines constituting the palmitoylation sites (Cys-322 and Cys-323) do not, at least under our detergent conditions, provide a high affinity secondary ATR binding site (Fig. 5B). The only clear deviation between the minimal cysteine constructs and WT came from 11CR binding to the M257Y-CAM alone (Fig. 5A). With all native cysteines, the rate of 11CR binding was slowed to levels similar to those in the Gt C-term peptide experiments. This observation might indicate a smaller proportion of ops in the M257Y-CAM sample compared with M257Y-CAM.<ref>

For all other conditions tested, the WT variants performed identically to the WTopsin and the interpretation of the results from the minimal cysteine construct is valid for the WT protein. See supplemental Table 1 for complete rate results.

Expanding Hole B (TM5/TM6) Does Not Enable ATR Uptake and Actually Slows 11CR Binding—As discussed in the Introduction, crystal structures show that an opening forms Hole B (TM5/TM6) in MII and the active ops* conformation (Fig. 6A) (14, 15, 17, 43, 57). This opening has been proposed to form part of a channel for retinal uptake or release (11, 18, 19). Therefore, we tested whether this hole might play a role in the shift in retinal preference that we observed with ops* stabilization (Fig. 4, B and C). Hole A was not pursued because of its close proximity to the Schiff base.

To inhibit closure of Hole B, we mutated the flanking phenylalanines to alanines (F208A and F273A) and also constructed a double alanine mutant (F208A/F273A). As shown in Fig. 6, C, E, and G, these hole constructs failed to show any detectable ATR binding despite their having a more active-like opening to the pocket, suggesting that expanding Hole B alone is not sufficient to induce the shift in retinal preference. Moreover, increasing the size of this hole did not increase the rate of 11CR binding (as the transient activation hypothesis would predict) but rather slowed the rate of binding with the double mutant being slower than the single sites combined (Fig. 6, B, D, and F).

**DISCUSSION**

In this work, we probed the role of structural dynamics in retinal binding to opsin. Our work was initially designed to test a recently proposed transient activation hypothesis that postulates that retinal binding requires formation of ops* for the ligand to gain entry into the binding pocket (21). For 11CR, our results are the exact opposite of what this model would predict: as the equilibrium of receptor conformations was shifted from primarily ops (inactive WT opsin) to a mixture of ops and ops* (M257Y-CAM), 11CR binding was increasingly slower. How-
ever, we also obtained results for ATR that are consistent with the transient activation model: the more we increased the ops* pool, the faster ATR bound.

As a result of these findings, we propose a new model for retinal binding to naïve opsin with 11CR and ATR binding to different conformations of opsin, consistent with our conformational selection data (Fig. 7). Below, we discuss the potential implications of the results of our experiments and new model.

**Conformational Selection of Retinal Isomers by Opsin**—Is ops* formation necessary or required for retinal binding? As shown in Figs. 3, 4, and 5, increasing the amount of ops* impaired 11CR binding but enabled binding of the agonist ATR. Thus, ops* is clearly important for ATR binding as...
inactive ops showed no ability to bind ATR. Therefore, we propose that the binding of retinals to opsin is primarily dictated by the conformational state of the receptor. This result is consistent with the conformational selection model discussed below (Fig. 7).

Why does ATR ever leave the activated photoreceptor if its affinity is higher for the ops* state than 11CR? We think the answer is the following. Although the protein conformation of ops* prefers the ATR agonist, the binding is less stable than for 11CR due to the open nature of the ops* form. In the ops* state, the retinal-Schiff base linkage becomes exposed to water due to conformational changes in the protein that open up a solvent channel leading from the retinal binding pocket to the cytoplasmic face. Waters traversing this channel hydrolyze the retinal-opsin Schiff base linkage, resulting in the release of ATR (58). If the ATR leaves the protein after the cleavage event and the active protein reverts back to the inactive ops form, the ATR will be unable to rebind until the opsin flickers back to the ops* state, which is a very low probability event for WT opsin (9). Thus, the ops*-ATR form is in constant flux and, in the absence of stabilization by G protein or arrestin, the ligand is in equilibrium between the bound and unbound states (31, 52, 59). This
Our proposed conformational selection model for ligand binding is in agreement with other well studied GPCRs, such as the β2-adrenergic receptor (64–67). Additionally, we recapitulate the extended ternary complex model originally established to explain ligand binding to CAM β2-adrenergic receptor for rhodopsin, greatly implying that the dim light receptor behaves like a ligand-binding GPCR rather than being a special case (68). Interestingly, activation of rhodopsin has been proposed to be an extreme example of an induced fit mechanism as the inverse agonist isomerizes to the agonist while inside the binding pocket and appears to actively push the inactive conformation to active MII (69). Taken with the data presented here, it appears that rhodopsin compartmentally exhibits both classical models of ligand-receptor interactions. The receptor shows clear conformational selection when binding exogenous ligand but induced fit when prebound 11CR is isomerized by light.

Can the photostationary state also be interpreted within the confines of a conformational selection model? For example, one can imagine that, after photoisomerization of the 11CR, the protein is no longer restricted to the inactive conformation and can sample other structural states, including the ops* conformation. Transition to the active state would be quickly stabilized by the high apparent affinity of the agonist covalently bound in the binding pocket. In this model, rather than an induction of the active state, the agonist selects the active conformation as the rhodopsin explores the conformational landscape. An understanding like this might help explain the molecular mechanism behind agonist-induced activation of other GPCRs, such as the AT1 angiotensin receptor (70).

An aspect of the detergent system is an increased flexibility of the opsins that allows for more consistent occupation of the two conformational extremes and importantly the open conformation. Previous work has shown that a lipid bilayer system increases the energy barrier for the transition between ops and ops* and an impaired conversion to MII upon photobleaching (10, 71). Therefore, the conformational selection observed here might be less dramatic in membranes due to a smaller population of ops* present at any point in time.

The G-protein-coupled receptor (GPCR) family includes more than 1,000 members that mediate the actions of a diverse group of hormones, neurotransmitters, and other signal molecules. These receptors are characterized by seven transmembrane helices that span the cell membrane, and they are activated by the binding of a ligand to an extracellular domain. The activation of a GPCR typically leads to the stimulatory or inhibitory coupling of a G-protein, which in turn affects the function of a variety of downstream targets, including enzymes, ion channels, and transcription factors.

In our model, the 11CR molecule enters into this open, inactive conformation (Ops-11CR) and then the covalent Schiff base is formed (depicted here by a red star). Ops-11CR is linked to Ops*-ATR by activation of the complex by light (hv). Note that the ATR branch of retinal binding is the same as proposed in the transient activation hypothesis.

**FIGURE 7. A modified model for retinal binding to opsin.** Our data indicate that both ATR and 11CR do not bind to the same active receptor conformation and are consistent with this new model. The model proposes that an open but inactive opsin form exists that enables 11CR binding (shown in brackets), one that is an intermediary between the closed, inactive and open, active receptor states. In our model, the 11CR molecule enters into this open, inactive conformation (Ops-11CR) and then the covalent Schiff base is formed (depicted here by a red star). Ops-11CR is linked to Ops*-ATR by activation of the complex by light (hv). Note that the ATR branch of retinal binding is the same as proposed in the transient activation hypothesis.
positions 208 and 273 to alanines, thereby forcing a hole to be present regardless of receptor conformation. Experiments on this perforated opsin clearly showed that the presence of a hole between TM5 and TM6 is not sufficient for ATR binding, indicating that ATR likely does not use Hole B for entry but rather might enter through Hole A. Interestingly, 11CR binding rates were drastically slowed by these mutations (Fig. 6). This latter observation might indicate that Hole B must be closed to help stabilize or “trap” 11CR in the binding pocket.

Could the slow 11CR binding to the Hole B mutants explain why the ops* conformation slows inverse agonist binding? We speculate that, in the two-state model for receptor activity, this result might indicate that during binding of 11CR (either entering through Hole A or Hole B) Hole B must “snap shut” to trap 11CR in the pocket. Forcing Hole B open (as we did here) would prevent the trap from operating correctly, enabling 11CR to “escape” before the Schiff base can form. Alternatively, a third receptor state could be invoked to explain these results: one that has a closed Hole B but an open Hole A. In this model, after entering through Hole A, 11CR may simply exit through the enlarged Hole B (caused by the alanine mutation) before proper binding and Schiff base formation can occur. In either case, the TM5/TM6 Hole B appears to be essential for efficient and rapid stable 11CR binding. This interpretation is in agreement with the proposal that the TM5/TM6 hole might be the avenue for retinal exit from the receptor following activation (11, 19, 20).

All of our mutants tested here formed a wild type-like chromophore upon addition of 11CR as indicated by a 500 nm peak (data not shown). Thus, we favor the interpretation that Phe-208 and Phe-273 stabilize the retinal for proper binding and do not perturb protein folding. However, we cannot formally rule out the possibility that the effects we saw are in part caused by non-localized effects as has been proposed previously (21). Furthermore, although mutations F208A, F273Q, and F273L have been shown previously to be non-disruptive to the spectroscopic properties of WT, the F273A and F208A/F273A mutants might have unexpected consequences (21, 74).

Occupancy of the Cytoplasmic Binding Cleft of Opsin by Either G Protein C-Terminus or Arrestin Finger Loop Peptide Mimics Is Sufficient to Enhance ATR Binding—The peptides used in this study, Gt C-term and Arr peptide, both stabilized ATR binding to opsin. These results confirm previous speculations about the role of these protein regions based on studies using full-length G protein and arrestin (31, 75). Importantly, our observations indicate that the well known phenomenon of MII “trapping” by G protein and arrestin needs to be reevaluated (3, 33, 34, 52, 56, 59, 76). Our results indicate that G protein and arrestin trapping cannot simply be ascribed to these proteins preventing ATR release from the activated MII receptor. Rather they suggest that some of the trapping is almost certainly due to the fact that these proteins stabilize ops* and thus enable released ATR to rebind due to an increased affinity of the agonist for the receptor by stabilization of the active form.

Interestingly, the increase of ops* and affinity for ATR appears to be a common consequence of binding something into the cytoplasmic cleft in opsin because both the G protein and arrestin peptide fragments display this effect (3, 4, 17, 52, 56, 59, 77). Furthermore, these small fragments of the proteins are sufficient to promote an increased affinity for the agonist, suggesting that the whole protein is not necessary for the effect to occur but may increase the magnitude. Our results also show that the slowing effect of G, C-term peptide on 11CR binding was not reciprocated by Arr peptide (Figs. 4, H and K, and 5, G and J) and therefore might indicate a unique receptor conformation (78) that has a higher affinity for ATR potentially to keep the agonist bound and to protect the rod cell from free ATR accumulation (60, 76) but does not impair binding of 11CR. This is consistent with a model of physiological arrestin interaction where the arrestin is released from phosphorylated opsin only when the opsin is regenerated by 11CR (79).

Moreover, these observations also lead to the question: in vivo, what prevents ATR from simply rebinding opsin in the presence of G protein or arrestin, such as in the context of high light bleaching conditions in the retina (63)? One answer may lie in the reduction of the retinal to retinol by retinol dehydrogenase, which pulls the ATR out of the binding reaction. Thus, retinol dehydrogenase proteins may play a key step in preventing futile cyclic ATR binding as has been proposed previously (47, 49, 61, 80, 81).

A Modified Conformational Selection Model for Retinal Binding to Opsin—Our data clearly show that the conformational state of the receptor plays a critical role in determining the rate of retinal binding. The simplest way to fit these results would be to invoke a classical two-state binding model in which the inverse agonist, 11CR, binds directly to ops and the agonist, ATR, binds to the open ops* (67).

The problem with this assumption is that it describes the process but does not provide a mechanism for how retinal can access the binding pocket. As discussed earlier (Fig. 1A), there is no pathway to the binding site in the inactive rhodopsin structures. In contrast, the active rhodopsin conformation does show an access route to the binding pocket, which motivated the proposal (shown in Fig. 1C) that both ATR and 11CR bind to the same active receptor conformation (what we refer to here as the transient activation hypothesis (TAH)) (12, 14, 21).

Our results both support and contradict this idea. We found that stabilizing the active ops* state accelerated agonist binding in agreement with the TAH model. However, in direct contradiction to the TAH model, our data also show that creating more active ops* slowed binding of 11CR, the inverse agonist. The latter result strongly suggests that the TAH model cannot fully explain all forms of retinal binding.

Why not simply modify the TAH model to include a conformational selection step after retinal binding? Such a model could explain a slower rate of apparent binding and Schiff base formation for 11CR because after 11CR enters into the ops* pocket the protein would have to revert back to the ops conformation to form a proper Schiff base and that could take time. However, such an argument breaks down for the simple reason that the TAH model still assumes that 11CR and ATR can only enter the ops* conformation and thus cannot explain why we saw a rate of 11CR binding that was inversely related to the
amount of ops*. In other words, it does not make sense that the less ops* there is the faster 11CR binds as that would suggest that the fastest rate of 11CR binding would occur when there is no ops* at all, which becomes a nonsensical extrapolation within the confines of the TAH model due to the ops* requirement for binding.

However, as noted above, there is also an inherent problem with a classical, simple two-state conformational selection model: it does not explain how 11CR enters opsin. Opsin has usually been assumed to be a closed conformation based on the fact that opsin is functionally inactive and that all inactive rhodopsin structures show no access pathways into the binding pocket. However, there is no experimental evidence of which we are aware indicating that inactive opsin has no access pathways to the binding pocket.

Thus, we propose a relatively simple but little discussed alternative possibility that would circumvent this problem and explain our results, a model in which inactive opsin contains unidentified opening(s) that enable 11CR entry (these could be the same holes observed in the active opsin structure but do not have to be). Importantly, this hypothetical conformer would lack the conformational changes in the cytoplasmic face that enable G protein and arrestin binding, rendering it still functionally inactive and thus distinct from ops*.

Therefore, our new model for retinal binding (presented in Fig. 7) contains this new putative, open opsin conformation (in brackets) placed between the fully active ops* and fully closed off ops. As discussed above, we speculate that this new state is an intermediary between the two previously defined conformations. A similar explanation has been invoked to explain results deviating from the simple two-state model in other GPCRs (82–84). Although our model depicts the retinals binding to only one conformation, our results do not preclude the possibility that they can also non-preferentially bind to the other opsin state.

Conclusions—In summary, we found that shifting the conformational equilibrium in favor of the more active state (ops*) reduced 11CR binding rates but enhanced the rate of ATR binding. Thus the current transient activation hypothesis for 11CR binding to rhodopsin needs modification (21) to include contribution from conformational selection.

However, a number of questions remain unanswered by our present work. Our binding data only measure stable retinal-opsin interactions. Possible roles for transient shifting of the transmembrane helices, rapid flickering of the residues surrounding the binding pocket, or even formation of a third receptor state as discussed above and proposed for other GPCRs (82) cannot yet be ruled out as means for retinal entry.

A mechanistic explanation of how the retinal enters and exits the receptor still has not been established and may ultimately require the inactive opsin crystal structure to provide structural context to rule out or reveal another possible entry pathway into the protein. Interestingly, our data also suggest that using 11CR to shut off a rhodopsin CAM, proposed as a treatment for some retinal diseases, may prove difficult because the presence of ops* lowers 11CR affinity. Allosteric ligands may be a better approach for modulating activity in such mutants. Experiments are underway to further test our model of conformational selection, to elucidate what molecular events govern it, and to better understand retinal-opsin interactions. These topics have clear implications in drug design and protein engineering both for vision and for GPCRs.

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REFERENCES

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