

Novel fluorescent GPCR biosensor detects retinal equilibrium binding to opsin and active G protein and arrestin signaling conformations

Received for publication, May 29, 2020, and in revised form, September 25, 2020. Published, Papers in Press, October 6, 2020. DOI 10.1074/jbc.RA120.014631

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Edited by Henrik G. Dohlman

Rhodopsin is a canonical class A photosensitive G protein–coupled receptor (GPCR), yet relatively few pharmaceutical agents targeting this visual receptor have been identified, in part due to the unique characteristics of its light-sensitive, covalently bound retinal ligands. Rhodopsin becomes activated when light isomerizes 11-*cis*-retinal into an agonist, all-*trans*-retinal (ATR), which enables the receptor to activate its G protein. We have previously demonstrated that, despite being covalently bound, ATR can display properties of equilibrium binding, yet how this is accomplished is unknown. Here, we describe a new approach for both identifying compounds that can activate and attenuate rhodopsin and testing the hypothesis that opsin binds retinal in equilibrium. Our method uses opsin-based fluorescent sensors, which directly report the formation of active receptor conformations by detecting the binding of G protein or arrestin fragments that have been fused onto the receptor's C terminus. We show that these biosensors can be used to monitor equilibrium binding of the agonist, ATR, as well as the noncovalent binding of β -ionone, an antagonist for G protein activation. Finally, we use these novel biosensors to observe ATR release from an activated, unlabeled receptor and its subsequent transfer to the sensor in real time. Taken together, these data support the retinal equilibrium binding hypothesis. The approach we describe should prove directly translatable to other GPCRs, providing a new tool for ligand discovery and mutant characterization.

G protein–coupled receptors (GPCRs) are a superfamily of integral membrane proteins responsible for translating a diverse array of external stimuli, from light to ligands, into cellular responses. GPCR signaling was initially thought to occur only through G proteins, but it is now known that these receptors can also act through other proteins, such as arrestins (1, 2). This added signaling flexibility has encouraged extensive ligand-discovery efforts with the aim of identifying compounds that selectively elicit specific, or biased, effects (3, 4).

Rhodopsin, the photosensitive GPCR, has been conspicuously absent from most pharmaceutical efforts, in part due to the unique nature of its retinal ligands. Rhodopsin is trapped in an “off” state by its covalently bound inverse agonist 11-*cis*-retinal (11CR). Activation occurs when light isomerizes 11CR into

the agonist, all-*trans*-retinal (ATR), and it has been presumed that this active state only lasts as long as that original ATR remains bound to the receptor, attached by a Schiff base linkage. This combination of unusual ligands and mode of activation (5, 6) has so far limited pharmaceutical studies of this key receptor.

However, increasing evidence indicates that retinals interact with the rhodopsin apoprotein, opsin, much more like classical ligand binding GPCRs than previously thought (7–11). Recently, we demonstrated that rhodopsin discriminates between inverse agonist and agonist binding by a conformational selection mechanism, in which the inactive conformation (Ops) preferentially binds 11CR, whereas the active conformation (Ops*) binds ATR (7). Subsequently, we also found evidence that ATR can bind in equilibrium with the active state protein (11), and this equilibrium can be shifted to favor more bound ligand by either increasing the proportion of active receptor population or the ATR concentration (7, 11).

How then, can a ligand known to form a covalent attachment to its receptor display properties of equilibrium binding? One possibility is that the retinal Schiff base linkage transiently hydrolyzes (12) and releases the original ATR, followed by another, different ATR taking its place (11).

To further explore this hypothesis, we have created opsin-based “biosensors” to detect the formation of G protein– and arrestin–binding conformations. These chimeric opsin proteins contain key fragments of the G protein transducin or visual arrestin directly fused to the opsin C terminus, building on previous fusion studies (7, 13, 14). Because these regions only bind active receptor conformations, they can detect when the receptor scaffold has adopted a G protein– or arrestin–binding state. Binding of the fusion region is detected by monitoring the tryptophan-induced quenching of fluorescence (TriQ) (15, 16) that occurs when a Trp residue in the fusion region comes close to a fluorophore near the binding site.

Two basic types of sensors were created. To detect G protein–binding receptor conformations, the last 11 residues of the transducin G α subunit were fused to the C terminus of the receptor (GtF), a region well-known to bind to and stabilize active opsin (7, 11, 17–21). For arrestin-binding conformations, an arrestin fusion (ArrF) chimera was created that contains the “finger loop” of arrestin (residues 67–79) fused C-terminally to the receptor. Because both of these regions bind opsin at the same cytoplasmic cleft (7, 10, 22–25), we hypothesized they could be similarly used to detect active receptor conformations.

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As we show below, these fluorescently labeled fusion proteins can be used to directly test for ATR equilibrium binding, monitor shifts in conformational equilibrium, and identify diffusible ligand binding that specifically impacts Gt or arrestin binding. We also show that the usable range over which they can detect ligands can be expanded by introducing constitutively activating or inactivating receptor mutations (CAMs or CIMs, respectively) that improve or impair the ability of the receptor to adopt an active conformation. As expected, we find that ATR (agonist) binding enhances binding of both GtF and ArrF (26). Interestingly, we also find that β -ionone, a diffusible, noncovalently binding small molecule, shows expected antagonistic behavior toward GtF binding, yet shows essentially no effect on the binding or displacement of the arrestin fragment from ArrF, suggesting that β -ionone may act as a biased ligand. Finally, we note that these tools make it possible to monitor the transfer of the agonist, ATR, from an activated, WT rhodopsin to the sensor in real time. Taken together, our results present a new tool for directly observing ancillary protein interactions caused by different ligands, potentially expanding the repertoire of available agents for the visual protein and providing an additional tool for investigating other GPCRs.

Results

Sensor engineering and rationale

Our goal was to design a fluorescence-based platform for testing the idea of ATR equilibrium binding and, more broadly, for sensing GPCR conformational states, using the visual photoreceptor rhodopsin as a proof of concept. The specific mutations and constructs used to create the opsin-based fluorescent sensors are detailed in Fig. 1A. Each contains either a peptide analog of the C terminus of the $G\alpha$ subunit of transducin attached to the end of opsin sequence (GtF) (7, 11) or of the “finger loop” of bovine visual arrestin (amino acids 67–79) (ArrF) (22, 23)—both regions previously used to facilitate crystallization of active opsin (19, 26). Previously, we found that a peptide corresponding to the arrestin finger loop enhanced ATR binding to opsin (7). Other features common to all of these constructs include introduction of a stabilizing disulfide ($N2C^{N-term}/N282C^{EL3}$) to allow for detergent purification in the apoprotein form (27) and replacement of the four most reactive cysteines with serines ($C140S^{3.55}/C316S^{8.53}/C322S^{8.59}/C323S^{C-term}$) to enable introduction of individual reactive cysteines for site-specific fluorescent labeling (27, 28).

The logic underlying these sensors is shown in Fig. 1B. The sensors have a fluorescent probe attached near the cytoplasmic cleft of the receptor and a Trp residue introduced into the corresponding peptide fusion region (Fig. 1, C and D). Binding of the fusion region (either Gt or Arr) to the receptor brings the Trp residue in close proximity to the probe, resulting in decreased probe fluorescence emission (Fig. 2B) due to TrIQ (15, 16). Thus, these changes in fluorescence provide a direct method for tracking the conformational state of the opsin. As a control, identical experiments are carried out on a sensor lacking the Trp residues to identify non-TrIQ spectral artifacts and changes in emission that do not reflect fragment binding.

Binding of the fusion construct detected by TrIQ

WT opsin has a low propensity to be in the active conformation (29); thus, one might assume that the interaction between the fusion sequence and the receptor should be limited. However, as shown in Fig. 2B, the WT-GtF displays significant fluorescence quenching. In fact, comparing *A versus B* in Fig. 2 shows that the amount of WT quenching is comparable with the constitutively active mutant CAM-GtF that contains the activating $M257Y^{6.40}$ mutation (30–32). These data indicate that the Gt fusion tail is fully bound to the WT receptor, even in the absence of the CAM. This result is confirmed by analyzing components of fluorescence, which combines fluorescence steady-state and lifetime measurements to determine the amount of fluorescence quenching that occurs via static or dynamic mechanisms (Fig. 2G) and Table 1 (15, 16, 33). This analysis indicates that most of the WT quenching is the result of close, static near contact between the fluorophore and quencher.

In contrast, the WT-ArrF shows little to no quenching, indicating that the ArrF region is not bound in the apo-state (Fig. 2, D versus E). The difference may be due to the higher affinity of the optimized Gt sequence used for GtF, compared with the lower-affinity native sequence we used in the ArrF for the “finger loop.” Interestingly, results of the CAM experiments showed that ~80% quenching for GtF and 50% for ArrF were maximally achievable (Fig. 2, A and D). The differences in total quenching may be due to the orientation differences between the probe-quencher pairs used in the Gt and ArrF sensors, leading to different maximal quenching despite binding or other unknown factors (Fig. 2 (G and H)).

We also tested the effect of integrating a constitutively inactivating mutation (CIM), by introducing mutation $Y223A^{5.58}$ (34, 35) into the opsin sequence to reduce the receptor's affinity for the interacting peptide regions. Compared with the WT- and CAM-GtF discussed above, the CIM-GtF shows little difference in fluorescence between the Trp quencher-containing ($Trp_{(+)}$) sample and a Trp-less ($Trp_{(-)}$) control. This result suggests that the Gt fragment in the CIM sensors does not bind to the receptor in the apo-state and, thus, could be used to detect ligands that stabilize Gt binding (Fig. 2C). In contrast, relative fluorescence emission from the CIM-ArrF and WT-ArrF were very similar (Fig. 2, compare E and F), suggesting that neither sample showed binding of the Arr finger-loop fragment in the apo-state. Together, these various sensors provide a way to observe antagonist-induced dissociation (WT-GtF and CAM-ArrF) and agonist-promoted binding (CIM-GtF and WT-ArrF).

Agonist (ATR) equilibrium binding is detected by increased fluorescence quenching in CIM-GtF

We next tested whether the CIM-GtF could detect binding of the agonist ATR, as indicated by an increase in TrIQ (decrease in bimane probe fluorescence). Because the CIM-GtF construct lacks Gt fragment prebinding (as indicated by the lack of basal TrIQ, Fig. 2C), it has the largest potential change to fluorescence upon agonist binding.

To control for possible spectral interference between ATR absorbance and the bimane emission at high agonist concentra-

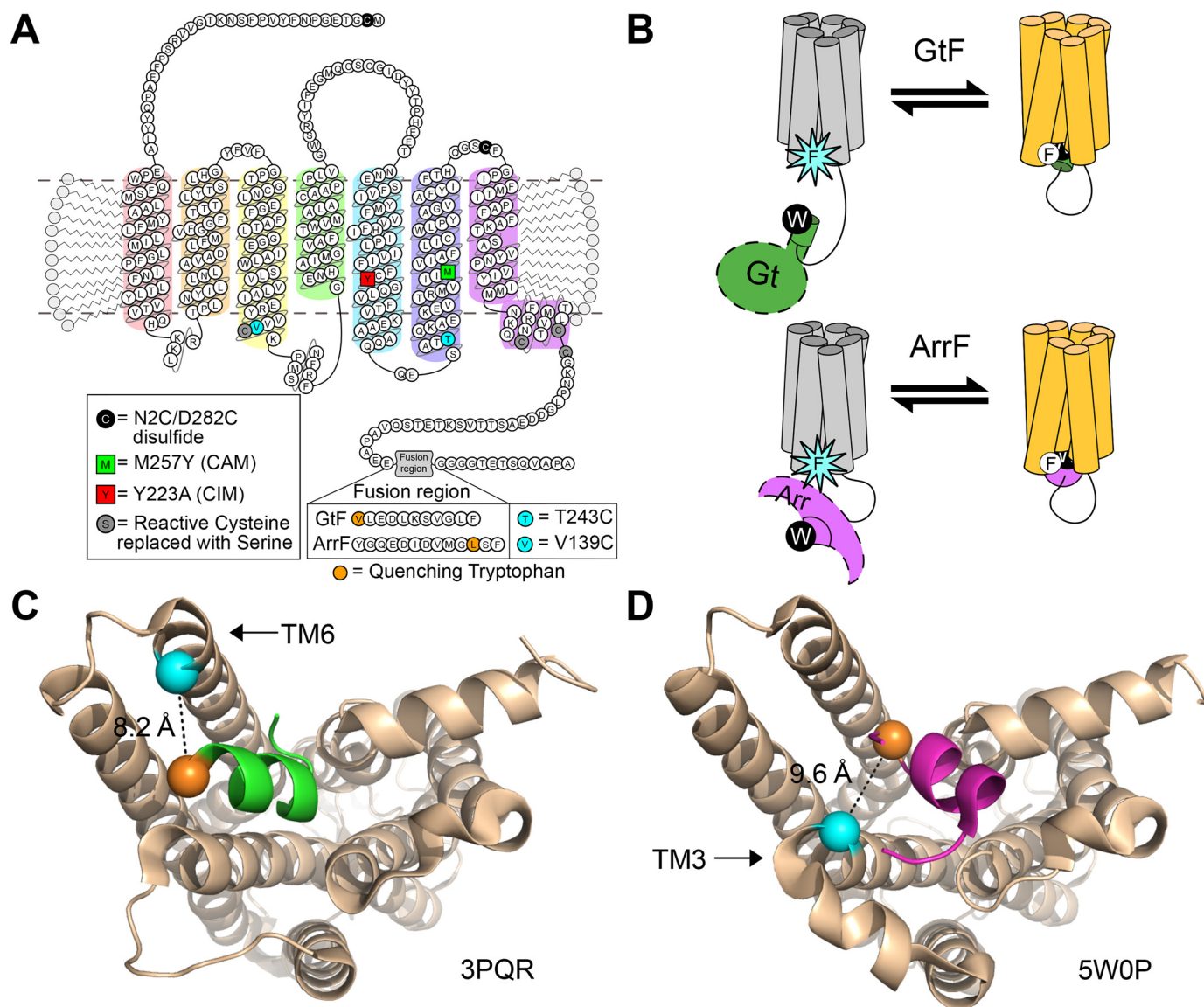


Figure 1. Cartoon scheme for opsin-based sensor that detects ligand binding by the TrIQ of fluorescence that occurs upon binding of a fused peptide corresponding to the C terminus of transducin (GtF) or the finger loop of arrestin (ArrF). A, two-dimensional plot of rhodopsin (RRID:SCR_007419), indicating the mutations made to the sensor proteins. The changes include a stabilizing disulfide (black), removal of reactive cysteines (gray), and introduction of novel cysteines (cyan) to enable site-specific attachment of fluorescent probes on the receptor (T243C^{6,26} for GtF and V139C^{3,54} for ArrF). Additional mutations used to alter basal receptor activity are also indicated: CAM M257Y^{6,40} (green box) and CIM Y223A^{5,58} (red box). The quenching tryptophan introduced in the fusion region of GtF and ArrF is shown in orange. B, cartoon of the sensor in action, showing how a change in receptor conformation allows for the peptide fusion tail to bind active opsin. Binding of the peptide fusion tail causes TrIQ (observed as fluorescence decrease as the tryptophan is brought into proximity with the probe on the receptor). C and D, view of cytoplasmic cleft of active rhodopsin (wheat) bound by fusion regions to highlight positions of tryptophans and fluorophores used for TrIQ sensor. Colors are as in A. C, structure of high-affinity G-transducin peptide (green) bound to rhodopsin (PDB entry 3PQR). The Ca-Ca distance for the TrIQ pair is 8.2 Å. D, structure of rhodopsin-arrestin complex (PDB entry 5W0P). Only the residues used in the fusion sensor corresponding to the finger loop of arrestin (magenta) are shown for clarity. The Ca-Ca distance for the TrIQ pair is 9.6 Å.

tions, we first determined the effect of increasing concentrations of the ATR on the Trp₍₋₎ CIM-GtF (Fig. 3A). As anticipated, higher ATR concentrations did cause a marked drop in bimane fluorescence, likely due to FRET from the bimane to the ATR, attenuation of bimane light excitation due to an inner filter effect, or a combination of both.

However, the ATR-induced fluorescence changes were much larger for the Trp₍₊₎ CIM-GtF sensor, indicating that ATR stabilized the GtF fragment binding as expected (Fig. 3B). Plotting the fraction quenched (calculated from a ratio of total fluorescence intensity from the Trp₍₊₎ and Trp₍₋₎ CIM-GtF

fusion proteins) as a function of the ATR concentration displays a classic sigmoidal response curve, as would be expected for a ligand binding a receptor in equilibrium (Fig. 3C). Analysis of these results indicates that the CIM-GtF sensor displays ~0.6 μ M affinity for ATR, consistent with our previous study that found that 0.5–2 μ M amounts of ATR are capable of perturbing retinal release kinetics, presumably by stabilizing Ops* through equilibrium binding of ATR (11). Importantly, the quenching ratios at the highest tested ATR concentrations are the same as observed for apo-CAM-GtF and WT-GtF, suggesting that the spectral effects of the agonist are not impeding the readout.

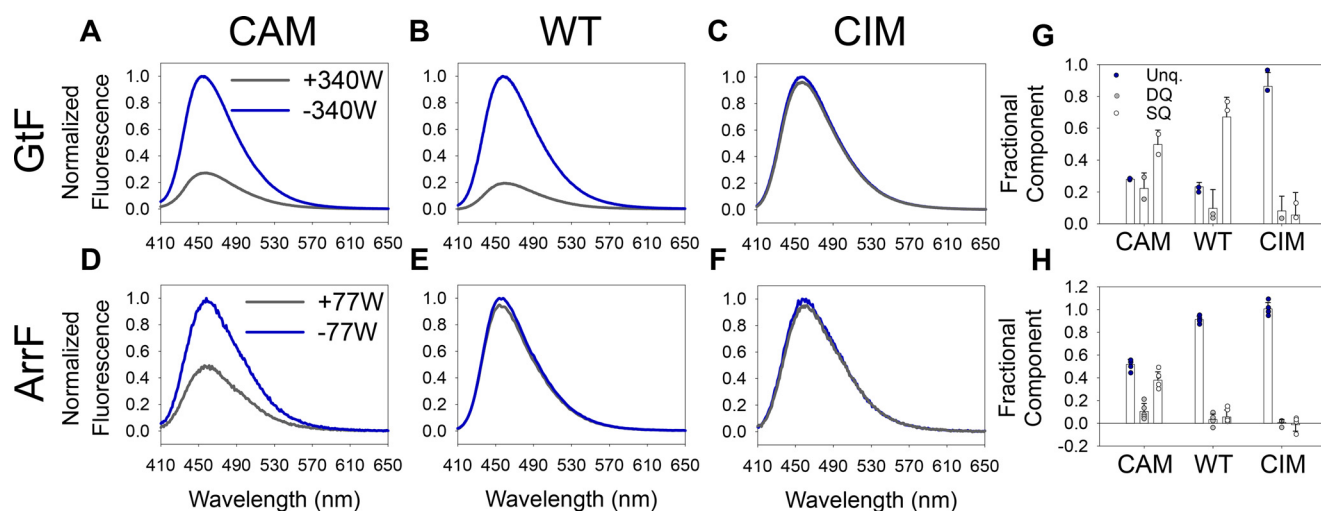


Figure 2. Fluorescence emission spectra of various bimane-labeled opsin biosensors, demonstrating how the extent of basal TrIQ in the sensor can be modulated by introducing a constitutively activating (CAM, M257Y) or a constitutively inactivating receptor mutation (CIM, Y223A). *B* and *E*, fluorescence spectra of the WT-GtF sensor protein with (gray) and without (blue) the quenching tryptophan. The extensive quenching indicates prebinding of the interacting region in the fusion tail to the receptor. *A* and *D*, to determine maximal quenching possible, a CAM was introduced in WT-GtF and WT-ArrF, and the spectra with (gray) and without (blue) the quenching tryptophan were compared. *C* and *F*, conversely, introducing a CIM to the constructs removes basal quenching and confirms that the quenching observed for the CAM and WT are caused by the fusion regions binding specifically to the receptor, rather than because of non-specific, spontaneous collisions. *G* and *H*, components of quenching analysis for Rho-GtF sensor (*G*) and Rho-ArrF sensor (*H*) in the apo-state with respective CAMs or CIMs. Bars and error bars represent mean and S.D. Individual measurements are overlaid as points. Unq., unquenched; DQ, dynamic quenching; SQ, static quenching. Note that in this analysis, CIM-ArrF did not display any static quenching.

Table 1

Components of fluorescence for CAM, WT, and CIM GtF and ArrF apoproteins (see Fig. 2, *G* and *H*)

	γ_0^a	γ_{DQ}^b	γ_S^c
CAM-GtF	0.280 ± 0.006 ^d	0.213 ± 0.097	0.507 ± 0.091
WT-GtF	0.232 ± 0.028	0.074 ± 0.119	0.694 ± 0.124
CIM-GtF	0.864 ± 0.086	0.074 ± 0.092	0.062 ± 0.141
CAM-ArrF	0.517 ± 0.045	0.100 ± 0.069	0.383 ± 0.073
WT-ArrF	0.912 ± 0.030	0.030 ± 0.048	0.058 ± 0.060
CIM-ArrF	1.006 ± 0.056	0.007 ± 0.025	-0.013 ± 0.056

^aFractional unquenched fluorescence.

^bFractional dynamically quenched fluorescence.

^cFractional statically quenching fluorescence.

^dValues are mean ± S.D.

We further validated the results above by repeating these studies using a different fluorescent probe, BODIPY, attached to the same site on the receptor (15, 16). As anticipated, repeating these ATR-binding experiments with the Trp(-) control (Fig. 3*D*) showed little change in fluorescence, even at high concentrations of ATR, presumably because the red-shifted spectral properties of BODIPY eliminate spectral overlap between the fluorophore and the ATR and thus remove the background FRET observed in bimane-labeled sensors. Additionally, the more red-shifted excitation wavelength (380-nm bimane *versus* 480-nm BODIPY) reduces the inner filter effect observed at high concentrations of ATR. Repeating the ATR titration with the Trp(+) CIM-GtF sensor (Fig. 3*E*) showed an ATR-dependent TrIQ response similar to bimane. Analysis of the data again revealed a similar sigmoidal dose-response curve (Fig. 3*F*) with ~0.5 μ M affinity.

Noncovalent binding of a G protein antagonist (β -ionone) causes a decrease in fluorescence quenching in the WT-GtF sensor

We next tested the ability of a putative antagonist for visual rhodopsin, β -ionone, to bind and stabilize the inactive opsin

state, thus dislodging the Gt fusion tail and decreasing the TrIQ quenching. β -Ionone is an unusual retinoid ligand. Although it has a molecular scaffold similar to that of retinal, it cannot form a covalent Schiff base with opsins and has been reported to have opposing effects on different types of opsins—sometimes acting as an agonist and other times as an antagonist for G protein activation in studies on salamander photoreceptors (36–38). Interestingly, for bovine visual rhodopsin, β -ionone can induce phosphorylation by GRK1, but not Gt activation (39, 40); thus, for our purposes, here we are classifying it as a Gt antagonist. Unfortunately, we could not test the most physiologically relevant inverse agonist ligand for rhodopsin, 11CR, as its high absorbance and strong spectral overlap with both the bimane and BODIPY probes result in massive amounts of FRET, overwhelming the emission from the sensors.

Note that β -ionone primarily binds in the retinal binding pocket at a single site, suggesting that β -ionone acts as a competitive inhibitor for 11CR binding (41, 42). To monitor β -ionone antagonism of Gt binding, we used the WT-GtF sensor to detect the loss of Gt binding, observed as the increase in bimane fluorescence that occurs when TrIQ is alleviated as the GtF tail dissociates from the receptor. Compared with ATR, β -ionone has far less spectral overlap and FRET with bimane, and thus higher concentrations could be used with little effect on the fluorescent emission as determined by limited change to the Trp-less control (Fig. 4*A*). These experiments showed that the WT-GtF sensor exhibits a dose-dependent increase in the fluorescence of the probe with increasing β -ionone concentration, indicating that binding of this compound disrupts the receptor-fragment fusion complex (Fig. 4*B*). Plotting the ratio quenched compared with the β -ionone concentration reveals an increase in fluorescence (Fig. 4*C*). Unfortunately, we could

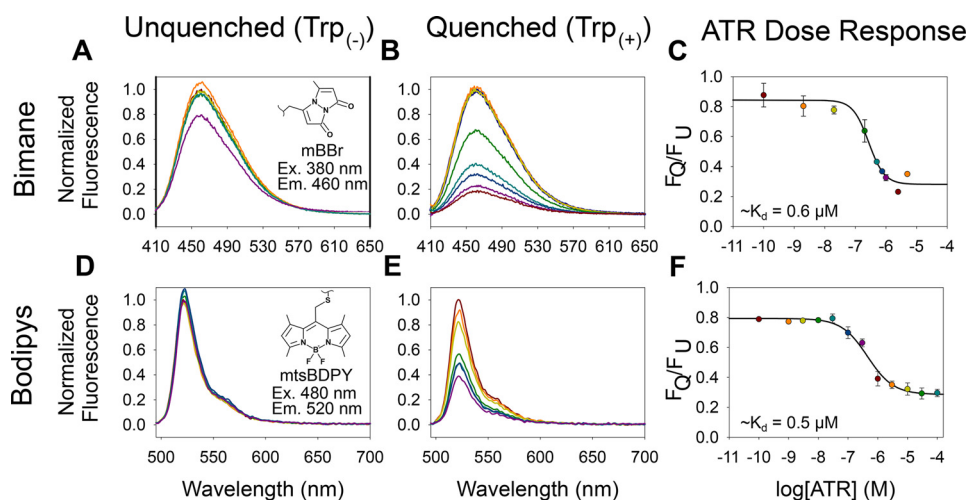


Figure 3. The addition of agonist, ATR, induces the fused Gt C-terminal peptide region to bind to the sensor in a dose-dependent manner. A, increasing ATR concentrations show little effect on fluorescence emission on the bimane-labeled control that lacks a quenching tryptophan, CIM-GtF (Trp₍₋₎). The decrease in fluorescence results from a combination of FRET from the probe to the retinals and the inner-filter effect due to absorbance of retinal. B, in contrast, the Trp-containing CIM-GtF sensor (Trp₍₊₎) shows a marked decrease in fluorescent emission upon the addition of increasing ATR. C, ATR dose-response plot for the addition of ATR to the CIM-GtF sensor yields an apparent K_d of 0.6 μM. F_Q/F_U was calculated as a ratio of total fluorescence intensity (area under the trace) from the quenched versus unquenched sample at each concentration measured. D and E, same experiment as in A and B, except using a BODIPY-labeled control CIM-GtF (Trp₍₋₎). BODIPY is a red-shifted fluorophore that alleviates FRET to the retinal, thus enabling better resolution at higher ATR concentrations (>3 μM). Note that the fluorescence decrease from the BODIPY CIM-GtF sensor (Trp₍₊₎) upon ATR addition is similar to that observed for the bimane-labeled sensor. F, ATR dose response of the BODIPY-labeled CIM-GtF sensor (K_d ~0.5 μM) is in close agreement with that of the bimane-labeled CIM-GtF sensor.

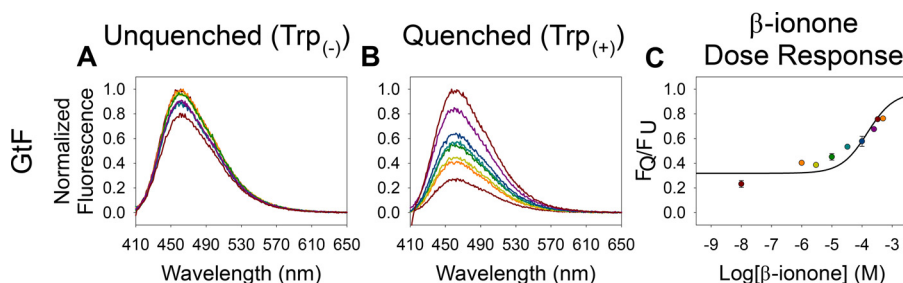


Figure 4. Noncovalent antagonist (β-ionone) addition to the WT-GtF sensor results in an increase in fluorescent emission, indicating decreased Gt fusion region binding. A, β-ionone has less spectral overlap with the bimane probe, enabling much higher concentrations (>100 μM) to be used before influencing the emission of the unquenched control (Trp₍₋₎). B, relief of bimane quenching occurs with increasing amounts of β-ionone (antagonist), indicating decreased Gt fusion region binding. C, dose-response curve of β-ionone. Unfortunately, β-ionone began precipitating out at concentrations above ~1 mM; thus, a full-saturation could not be obtained.

not achieve full saturation conditions with β-ionone before it precipitated out of our detergent-buffer solution.

ArrF sensor can detect binding of ATR yet shows no response to β-ionone

We next tested the effect of ATR and β-ionone on the ArrF sensor. The effect of β-ionone on arrestin has not previously been reported, and the interaction of this region of arrestin with receptor is less understood than for the C-terminal tail of Gt; thus, we first screened all three sensors (CAM, WT, and CIM) against a relatively high concentration of either ATR (2 μM; Fig. 5A) or β-ionone (300 μM; Fig. 5C) to identify those sensors that showed the largest changes. Incubation with ATR elicited a response from all of the sensor variants. The WT-ArrF showed a substantially greater change in fluorescence compared with the CIM and CAM sensors, and thus it was chosen for further testing (Fig. 5B). The response from this sensor is clearly sigmoidal and displays an affinity of ~1.2 μM, further supporting the hypothesis that ATR can bind opsin in equilib-

rium. In contrast, the Gt antagonist β-ionone showed no significant change in binding of any of the ArrF sensors, suggesting that this ligand does not facilitate or disrupt arrestin binding (Fig. 5C) at least under these conditions; therefore, a dose response was not conducted.

Measuring release of ATR from photoactivated rhodopsin and transfer to the CIM-GtF sensor in real time

Because our results further support the idea that ATR can bind to opsin in equilibrium, we next tested whether we could detect the opposite—the release of ATR from photoactivated rhodopsins and subsequent transfer to the biosensor in real-time. A schematic representation of this approach is shown in Fig. 6A. In these experiments, bimane-labeled CIM-GtF sensor was mixed with a 4-fold molar excess of DDM-solubilized native-source rhodopsin, and then the samples were photo-bleached and potential ATR transfer was simultaneously monitored. The experiments were carried out as follows. First, to detect the release of ATR from bleached rhodopsin, we used

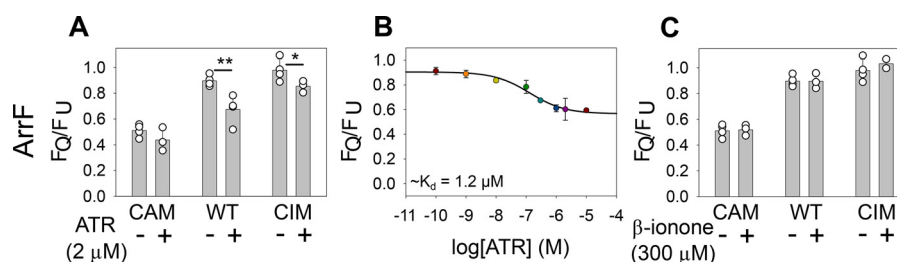


Figure 5. Arrestin fusion sensor (ArrF) detects binding of the agonist ATR but not antagonist β -ionone. A, bar chart comparing changes in the ratio of quenched (F_0) and unquenched (F_U) fluorescence for the three ArrF sensors (CAM, WT, and CIM) with and without the addition of 2 μ M ATR. WT-ArrF showed the greatest changes upon agonist addition and thus was used for an ATR dose-response curve. B, ATR dose response on the bimane fluorescence from the WT-ArrF sensor containing the quenching tryptophan compared with WT-ArrF without the tryptophan ($K_d = 1.2 \mu$ M). C, change in the ratio of the ArrF sensors (CAM, WT, and CIM) with and without 300 μ M β -ionone. Bars and error bars, mean and S.D.; individual points are overlaid in circles. The lack of significant change in fluorescence indicates that β -ionone does not facilitate binding of the arrestin fusion fragment. A and C, statistical significance between ligand- and vehicle-treated sensors was assessed by an independent one-way t test (*, $p < 0.05$; **, $p < 0.01$).

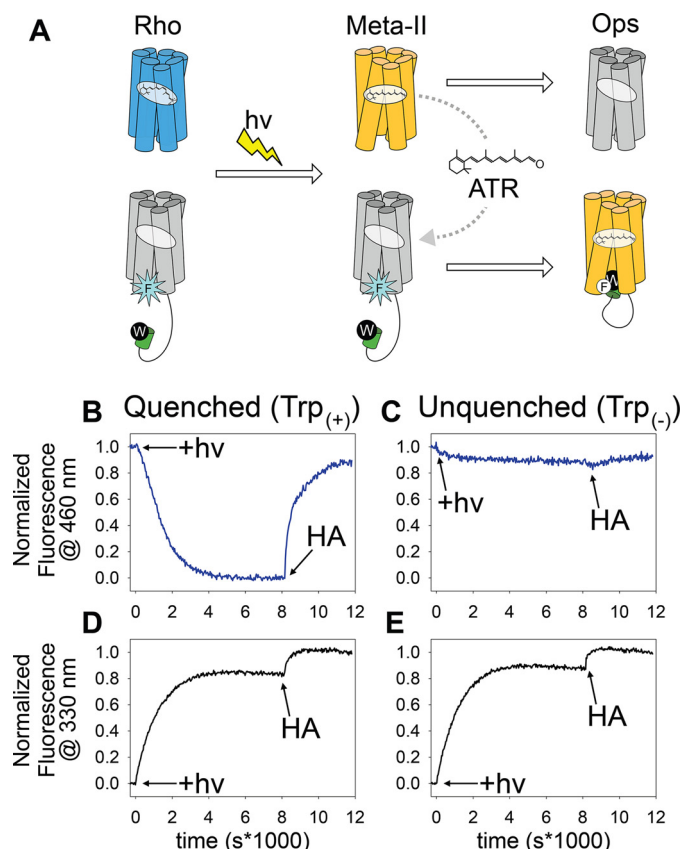


Figure 6. Release of ATR from unlabeled rhodopsins and subsequent transfer to the CIM-GtF sensor monitored in real time. A, cartoon of experimental setup. Briefly, ATR from photoactivated rhodopsin dissociates from its original receptor, resulting in an increase in exogenous ATR concentration. The subsequent binding of this ATR by the sensor is reflected by a decrease in the bimane signal from the sensor. B and C, neither the sensor with (B) nor the sensor without (C) the quenching tryptophan affects the intrinsic protein fluorescence, indicating that the sensors do not impact ATR release from the rhodopsins following light activation ($t = 0$). D, the sensor containing the quenching tryptophan ($\text{Trp}_{(+)}$) shows a steady decrease in fluorescence as the exogenous ATR concentration increases. The addition of hydroxylamine (HA) reverts the bimane fluorescence back to near the original fluorescence. E, the unquenched control ($\text{Trp}_{(-)}$) shows little to no effect on the bimane fluorescence over the course of the experiment.

our established assay (11, 43) that measures the increase in intrinsic protein fluorescence as ATR is released (note the increase in Trp fluorescence in Fig. 6 (B and C) after photoactivation). Subsequent treatment with hydroxylamine (HA) com-

pletes the release, by both severing any remaining Schiff bases attaching ATR and reacting with ATR to form ATR-oxime (the latter cannot rebind as it has much lower affinity for the receptors (43)). The further emission increase after HA treatment corresponds to vacating of residual ATR from the CIM-GtF sensors.

We monitored ATR transfer to the sensor molecules by simultaneously probing the bimane fluorescence in the opsin sensor (to detect Gt fragment binding and concomitant TrpIQ) while also monitoring ATR release from photobleached rhodopsin. As can be seen, the decreasing bimane fluorescence (ATR transferring to and binding to the sensors) mirrors the increase in Trp fluorescence (release of ATR from the photoactivated nonsensor rhodopsins), indicating that the ligand is transferring to the sensors (Fig. 6D). Interestingly, the decrease in bimane fluorescence was slightly delayed compared with the Trp increase, possibly reflecting the transit time required for ATR to change detergent micelles or when sufficient free ATR is present for binding. The addition of HA returns the bimane fluorescence to the initial level, confirming that the quenching was due to ATR binding. Identical experiments with the $\text{Trp}_{(-)}$ CIM-GtF control showed little change in bimane signal for the duration of the experiment (Fig. 6E), yet the protein signal matched that of the $\text{Trp}_{(+)}$ samples, indicating that the quenched samples are not altering ATR transfer (Fig. 6C).

Discussion

Monitoring specific receptor conformations with the sensor can detect ligand binding and provides clear evidence of ATR equilibrium binding to opsin and the role receptor conformational states can play in this process

GPCRs are known to induce both G protein- and arrestin-dependent signaling. Some ligands can preferentially trigger these separate pathways, a process referred to as biased agonism. We explored this phenomenon at a molecular level using two distinct opsin fusion constructs that enable observation of ligand interactions that stabilize G protein (GtF) or arrestin (ArrF) binding. The first ligand we tested, ATR, is a well-characterized agonist for both G protein and arrestin interactions (39). As expected, incubation with increasing ATR concentrations stabilizes a receptor conformation capable of binding either fusion sequence (Figs. 3 and 5). Additionally, our results

provide further evidence supporting the emerging hypothesis from our laboratory (7, 11) and others (44) that opsin can bind retinals in equilibrium. Our measurements using two individual opsin-sensor proteins and two different fluorescent TrIQ probes indicate that opsin displays a $\sim 0.5 \mu\text{M}$ affinity for ATR.

Interestingly, our studies with β -ionone provided different results. Whereas β -ionone is reported to induce phosphorylation of bovine rhodopsin by rhodopsin kinase (GRK1), it can apparently also act as an antagonist for G protein activation (39, 40). Further complicating this observation are conflicting reports that β -ionone can show both G protein agonism and antagonism on salamander photoreceptors (36, 37). To our knowledge, the effect of β -ionone on arrestin binding has not previously been documented.

We thus tested the effect of β -ionone on our biosensors and observed that it strongly inhibits binding of the $G\alpha$ C terminus and thus presumably G protein coupling, consistent with the previous results (39, 40). Interestingly, β -ionone did *not* affect the interaction with the fusion tail of ArrF, indicating that it is not acting as an efficient arrestin agonist. Conversely, β -ionone also does not appear to act as an inhibitor of arrestin finger-loop binding. Of course, these results do not exclude the possibility that another region of the arrestin protein is interacting with the GPCR as has been previously speculated by other structure-based experiments (2, 24). The interface and conformational change for this latter type of arrestin interaction could be different from the one occupied by the arrestin finger loop (the region tested in our sensor) and has been proposed to involve different types of movement in TM7/H8 (45, 46).

ATR released from photoactivated rhodopsin can transfer to a different opsin

Our opsin-based sensor could detect increasing ATR concentration following photobleaching of rhodopsins in real time (Fig. 6). These data clearly demonstrate that released ATR can transfer and bind to another opsin, thus supporting the ATR equilibrium binding hypothesis. This observation is relevant in the context of the outer segment of the rod cell, where the concentration of rhodopsin molecules reaches as high as 4.5 mM (47). Therefore, activation of a subset of proteins could produce a chain reaction of ATR dissociating and rebinding, resulting in a prolonged photocascade. Previously, we noted that one mechanism that prevents continued signaling by ATR-bound opsin is the reversion of the active opsin back to the native inactive apoprotein before ATR rebinding can occur (7). Subsequently, we also showed that sufficiently high ATR concentration can overcome the conformational mismatch and bind to the WT opsin (11). Whereas such interactions might extend signaling, they would also provide a temporary sink for liberated ATR, affording time for retinal dehydrogenase 8 to begin clearance of ATR through initiating the retinal regeneration process, as has been proposed previously (48–50). Relatively low ATR concentrations can induce cellular death (51), so careful control over free ATR is needed due to the immensely high potential concentration of ATR in a photoactivated rod cell (52). The sensors presented here could be used to track the release and metabolism of ATR in photoreceptor cell lysates by reporting the

amount of available free ATR. With some modification, the sensors could even be implemented in living retinal cells.

Applications to drug discovery for other GPCRs

Although immediately applicable to rhodopsin, the general design of our sensors should be applicable for GPCR drug discovery that is complementary to currently used cell-based assays that detect interactions between the receptor and ancillary proteins, generally through a proximity-based fluorescent reporter (*e.g.* genetically encoded FRET or BRET probes) (53). Indeed, another class A GPCR, β_2 -adrenergic receptor, has been shown to be amenable to both peptide fusions (14) and TrIQ (54) techniques although, to our knowledge, never coupled in this way. Our *in vitro* sensor takes a more direct approach by focusing exclusively on the isolated receptor, thus avoiding obstructions due to off-target binding or passage through the cellular membrane, and potentially yielding hits that would otherwise be missed in a cellular context. For example, an intracellularly binding molecule that cannot cross the membrane might be missed in a traditional cell-based assay but would be caught by our reductive assay and could potentially be modified to overcome the membrane barrier.

Screening mutations and ligands for specific protein interactions

Whereas the interaction between the GPCR and G protein has been well-established (55), only recently has the GPCR-arrestin complex been described in high resolution (24, 56). The receptor-arrestin complex is complicated by the presence of alternate tail binding, which is conformationally heterogeneous and therefore not resolvable to high-resolution (2), although this interaction is resolvable within larger complexes, such as the recently described GPCR-G protein- β -arrestin megacomplex (57) and could theoretically be detected via a sensor similar to the one described here to detect C-terminal interactions with arrestin, although the phosphorylation requirement could pose a problem in implementation. In the context of the GPCR-arrestin interaction, the core “finger-loop” interaction is necessary for stability. However, to ensure homogeneity, successful complexes for structure determination often require artificial modifications, including cross-linking (58), the addition of a high-affinity C terminus to the receptor (59), or fusion of the arrestin to the receptor (24, 56, 60), to select for the core interaction. The ArrF sensor described here could be helpful in identifying ligands that not only promote arrestin interactions but also discriminate against those that fail to stabilize the finger loop-core binding. Thus, these additional criteria for ligand choice could eliminate the need for further modifications to the arrestin complex to obtain high-resolution structures.

In summary, the fluorescent conformational sensors provide an excellent platform for detecting ligand binding and conformational changes to rhodopsin with potential application to other class A GPCRs. It is increasingly clear that many GPCRs are capable of signaling through both G protein- and arrestin-dependent signaling, and thus the previous image of GPCRs existing in either a discrete “on” or “off” state is quickly becoming more complicated and nuanced (61, 62). Currently, the conformational heterogeneity leading to this phenomenon and the

resulting implications on receptor behavior remains widely uncharacterized. We propose that the general sensor design we describe here can be implemented to measure conformational dynamics of specific receptor-ligand interactions in GPCRs in a manner similar to established techniques, such as DEER (63). Given the moderately precise resolution (~ 10 Å) of TrIQ, local perturbations in conformation (detected as changes in TrIQ) can be measured to glean information on binding energy and conformational stability. Thus, use of these types of fluorescent biosensors provides a rapid way to define changes in receptor function in the context of point mutations, specific receptor-ligand interactions, and receptor-signaling partner interactions for both the visual receptor rhodopsin and other members of the GPCR protein family.

Experimental procedures

Buffers and reagents

11-*cis*-Retinal was generously provided by Dr. R. Crouch (Medical University of South Carolina and NEI, National Institutes of Health). 1D4 peptide (TETSQVAPA) and fusion genes were purchased from GenScript. ConA-Sepharose and CNBr-activated Sepharose beads were purchased from GE Healthcare. Fluorophores used in TrIQ experiments were purchased from Toronto Research Chemicals. 1D4 antibody was produced and purified by the Oregon Health and Science University antibody core. All other chemicals were purchased from either Sigma-Aldrich or Fisher. Buffers used in this report are as follows: PBSSC (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 1 mM Na_2HPO_4 , pH 7.2), Buffer B (PBSSC, 1% DDM), Buffer C (PBSSC, 1 M NaCl, 2 mM MgCl_2 , 1% DDM), Buffer D (PBSSC, 0.05% DDM), Buffer E (5 mM MES, 0.05% DDM, pH 6.0), Elution Buffer (Buffer E, 40 mM NaCl, 200 μM 1D4 peptide), Buffer F_{LowD} (low-detergent labeling buffer) (5 mM MES, 50 mM HEPES, 1 mM EDTA, 0.025% DDM, pH 6.7), Buffer F_{HiD} (high-detergent labeling buffer) (5 mM MES, 50 mM HEPES, 1 mM EDTA, 0.2% DDM, pH 6.7), and ConA buffer (20 mM HEPES, 140 mM NaCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 1 mM MnCl_2 , 1.46% octyl glucoside, pH 7.0).

Mutant generation

Where applicable, mutations are denoted as the native residue first, followed by residue number, the mutation to be introduced, and finally, Ballesteros-Weinstein nomenclature in superscript (64). For example, swapping the native valine at position 139 to cysteine is denoted as V139C^{3,54}. Site-directed mutagenesis of a synthetic bovine rhodopsin gene was conducted by overlap extension PCR and subcloned into a modified version of the PMT4 expression vector and confirmed by sequencing (65). Fusion constructs were made in the background of a thermally stabilized (27), minimal-cysteine opsin as described previously (7). In both cases, a 2 $\times$ glutamate linker precedes the fusion sequence appended to the rhodopsin C terminus to mimic the native C-terminal carboxyl group (Fig. 1A). Immediately after the fusion sequence is a 4 $\times$ glycine linker and an additional 1D4 sequence to facilitate immune-purification with anti-1D4 antibody. (Note that this additional epitope sequence is necessary because the 1D4 epitope must be C-ter-

minal for optimal antibody binding.) Specifically, the Gt C-terminal fusion sequence was EEVLEDLKSGLFGGGGTETSQVAPA, whereas the ArrF “finger-loop” fusion sequence was as follows: EEYGQEDIDVMGLSFGGGGTETSQVAPA. Location of the TrIQ fluorophore/quenching tryptophan pairs was rationally guided by examination of the PDB files 3PQR (19) and 5W0P (56) for GtF and ArrF, respectively.

Purification, labeling, and quality control

All proteins were transiently expressed in COS-1 cells and then purified in the apoprotein form, as described previously (7, 66). Briefly, the cells were scraped free from 15-cm plates 50–65 h post-transfection. After solubilization with Buffer B, the samples were spun at $100,000 \times g$ to remove insoluble particulate. The supernatant from this spin was then incubated >2 h with CNBr-Sepharose beads precoupled to 1D4 antibody (1D4 resin) in Buffer C. The beads were transferred to columns and washed with 100 column volumes of Buffer D followed by 100 column volumes of Buffer E. Elutions were performed in batch with Elution Buffer.

Fluorescent labeling of the samples was done while the receptors were bound to the 1D4 resin as follows. After beads were washed with Buffer D, beads were then washed with 50 column volumes of Buffer F_{LowD} . The columns were then capped, and the beads were incubated with ~ 20 -fold molar excess of either monobromobimane or BODIPY-methanethiosulfonate in Buffer F_{LowD} for 2 h while nutating at 4 °C. The bead pack was then washed with 50 column volumes of Buffer F_{LowD} , 100 column volumes of Buffer F_{HiD} , 50 column volumes of Buffer F_{LowD} , and finally 100 column volumes of Buffer E. To facilitate the removal of excess, unreacted free label, the column flow during all post-labeling washes was slowed by affixing a 22-gauge needle to the column tip. Finally, elutions of pure, labeled samples were collected by the addition of Elution Buffer containing 200 μM 1D4-specific peptide.

The extent of fluorescence labeling was determined by absorbance spectroscopy. Concentration of label was approximated by absorbance at either 380 or 518 nm using extinction coefficients of $5000 \text{ cm}^{-1} \text{ M}^{-1}$ or $70,165 \text{ cm}^{-1} \text{ M}^{-1}$ for monobromobimane or BODIPY-methanethiosulfonate, respectively. Additionally, all labeled samples were tested for the presence of free label by TCA precipitating the protein (67) and measuring any remaining fluorescence in the supernatant following centrifugation. Less than 10% free label was present in all samples. Proper folding of the opsins was verified by incubating the opsin in a molar excess of 11CR and measuring the absorbance at 500 nm. All samples showed $>75\%$ regeneration, indicating an abundance of properly folded and functional opsin molecules.

Purification of native rhodopsin from bovine retinas was carried out as described previously (68). Briefly, the retinas were extensively Dounce-homogenized, and the rod outer segment was separated by sucrose gradient. The outer segments were then solubilized in 1% DDM before being spun at $100,000 \times g$. The proteins were then bound to ConA-Sepharose beads (purchased from GE Healthcare), washed with 50 column volumes of ConA buffer, and eluted with ConA buffer containing 300 mM methyl- α -D-mannopyranoside. Rhodopsin concentration

Fluorescent biosensor for GPCR conformations

was determined by absorbance at 500 nm using an extinction coefficient of $40,600 \text{ cm}^{-1} \text{ M}^{-1}$.

Steady-state fluorescence spectroscopy

Steady-state fluorescence measurements were conducted using a PTI Quantamaster fluorometer with a water-jacketed cuvette housing. Sample temperatures were maintained by a VWR circulating water bath and continuously monitored using an OMEGA thermistor. Measurements of bimane emission used the following parameters: $0.5 \mu\text{M}$ bimane-labeled sensor, 380-nm excitation (slit = 2-nm bandpass), 410–650-nm emission scan (slit = 10-nm bandpass), 2-nm step size, 0.5-s integration time. Measurements of BODIPY emission used the following parameters: $0.5 \mu\text{M}$ BODIPY-labeled sensor, 480-nm excitation (slit = 0.5-nm bandpass), 495–700 nm emission scan (slit = 4-nm bandpass), 2-nm step size, 0.5-s integration time. Total fluorescence for each emission scan was determined by integrating the area under the resulting spectra.

Titration points were prepared individually and incubated for 30–60 min to ensure equilibrium had been reached. Each point had corresponding Trp₍₋₎, Trp₍₊₎, and buffer with just the ligand samples prepared identically. Sensor spectra with and without Trp were baselined to buffer without sensor prepared exactly with an equivalent ligand concentration to account for dilution and inner filter effects before normalization. In the case of the ATR experiments in Fig. 3, the samples were normalized to apo-spectra for both Trp₍₋₎ and Trp₍₊₎; however, for the β -ionone experiments with the prebound sensor in Fig. 4, the apo-spectrum for Trp₍₋₎ was used in both cases as maximum emission.

Time-resolved fluorescence experiments were conducted using a different, modified PTI Quantamaster fluorometer in which the standard arc lamp excitation source was replaced with OceanOptics LLS-295 and LLS-405 LEDs, as described previously (11). These LED excitation sources have a spectral peak at 295 and 405 nm, respectively. The timing of the LED was tied into the shutter control by TTL signaling logic. Fluorescence measurement used both the 295- and 405-nm LEDs simultaneously to excite intrinsic tryptophans and the fluorescent label, respectively. In these experiments, the light from these LEDs was attenuated by a neutral density filter (ND 1.7) to minimize bleaching of rhodopsin. Fluorescent emissions were detected by monochromators set to 330 nm for intrinsic tryptophan fluorescence (slit = 10-nm bandpass), 460 nm for bimane fluorescence (slit = 10-nm bandpass), or 520 nm for BODIPY fluorescence (slit = 10-nm bandpass). Retinal uptake experiments were conducted on the same instrument monitoring only tryptophan fluorescence, as described previously (7, 11).

Fluorescence lifetime spectroscopy

Fluorescence lifetimes of the labeled opsins were measured on a FluoTime 200 TCSPC system (PicoQuant) with a 405-nm diode laser. The emission was measured at 490 nm using 2.0-nm slits and tempered with a 470-nm long-pass filter. The instrument response function was determined using a dilute solution of Ludox, yielding a full width at half-maximum of ~ 64 ps. Decays were analyzed using the PicoQuant FluoFit

Table 2

Abbreviations used for Steady-state emission spectra and fluorescence lifetimes for samples containing the TrIQ pair and a control protein

	Probe/Trp (TrIQ pair)	Probe only (control)
Steady-state emission	Fw	Fo
Fluorescence lifetime	τ_w	τ_o

software by Lorentzian distribution to one or two exponents. The quality of the fit was assessed by a χ^2 of between 0.9 and 1.2. From this analysis, the amplitude-weighted lifetimes were used to calculate the components of fluorescence, as detailed previously (15, 16, 66).

Calculation of components of fluorescence

In a TrIQ study, the relative population of fluorophores in a nonstatically quenched state (γ) and those in a statically quenched state ($1 - \gamma$) can be calculated as follows.

First, steady-state emission spectra and fluorescence lifetimes are measured for both of the samples containing the TrIQ pair (F_w and τ_w , respectively) and a control protein including the fluorophore but lacking the quenching tryptophan (F_o and τ_o , respectively) (Table 2). Next, γ is calculated using the following equation,

$$\gamma = \left(\frac{F_w}{F_o} \right) \times \left(\frac{\tau_o}{\tau_w} \right) \quad (\text{Eq. 1})$$

As γ represents the population of Probe/Trp pairs not in a static complex, the amount of static quenching can be calculated as follows.

$$\% \text{ Static quenching} = \gamma - 1 \quad (\text{Eq. 2})$$

Within γ , the fraction of fluorophores that experience their full fluorescent lifetime without being quenched can be calculated as follows.

$$\% \text{ Unquenched} = \left(\frac{\tau_w}{\tau_o} \right) \times \gamma \quad (\text{Eq. 3})$$

Similarly, the fraction of fluorophores in γ that experience a shortened lifetime by dynamic quenching can be calculated as follows.

$$\% \text{ Dynamic quenching} = \left(1 - \frac{\tau_w}{\tau_o} \right) \times \gamma \quad (\text{Eq. 4})$$

A full derivation of these equations can be found in Ref. 15.

Data availability

All data for these studies are contained within this article.

Acknowledgments—We thank Adam Oken, Dr. Emily Platt, Triona Matheson, and Yizhou Yao for careful reading of the manuscript and thoughtful feedback.

Author contributions—C. T. S., A. S., and D. L. F. conceptualization; C. T. S. and A. S. data curation; C. T. S. and A. S. formal analysis; C. T. S. and A. S. investigation; C. T. S., A. S., and D. L. F. methodology;

C. T. S. and D. L. F. writing-original draft; C. T. S., A. S., and D. L. F. writing-review and editing; A. S. validation; A. S. and D. L. F. visualization; D. L. F. resources; D. L. F. supervision; D. L. F. funding acquisition; D. L. F. project administration.

Funding and additional information—This work was supported, in whole or in part, by National Institutes of Health Grants R01EY029343, R21EY027959, and R21DA043001 (to D. L. F.); F31DA049438 (to A. D. S.), and T32 EY023211 and T32 GM071338 (to C. T. S.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: GPCR, G protein-coupled receptor; GtF, GPCR with region of G-protein fused C-terminally; ArrF, GPCR with region of arrestin -fused C-terminally; HA, hydroxylamine; 11CR, 11-cis-retinal; ATR, all-trans-retinal; TrIQ, tryptophan-induced quenching of fluorescence; CAM, constitutively activating receptor mutation; CIM, constitutively inactivating receptor mutation; DDM, dodecyl maltoside; ConA, concanavalin A; PDB, Protein Data Bank.

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J. Biol. Chem. 2020, 295:17486-17496.

doi: 10.1074/jbc.RA120.014631 originally published online October 6, 2020

Access the most updated version of this article at doi: [10.1074/jbc.RA120.014631](https://doi.org/10.1074/jbc.RA120.014631)

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