Decay of an active GPCR: Conformational dynamics govern agonist rebinding and persistence of an active, yet empty, receptor state

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Here, we describe two insights into the role of receptor conformational dynamics during agonist release (all-*trans* retinal, ATR) from the visual G protein-coupled receptor (GPCR) rhodopsin. First, we show that, after light activation, ATR can continually release and rebind to any receptor remaining in an active-like conformation. As with other GPCRs, we observe that this equilibrium can be shifted by either promoting the active-like population or increasing the agonist concentration. Second, we find that during decay of the signaling state an active-like, yet empty, receptor conformation can transiently persist after retinal release, before the receptor ultimately collapses into an inactive conformation. The latter conclusion is based on time-resolved, site-directed fluorescence labeling experiments that show a small, but reproducible, lag between the retinal leaving the protein and return of transmembrane helix 6 (TM6) to the inactive conformation, as determined from tryptophan-induced quenching studies. Accelerating Schiff base hydrolysis and subsequent ATR dissociation, either by addition of hydroxylamine or introduction of mutations, further increased the time lag between ATR release and TM6 movement. These observations show that rhodopsin can bind its agonist in equilibrium like a traditional GPCR, provide evidence that an active GPCR conformation can persist even after agonist release, and raise the possibility of targeting this key photoreceptor protein by traditional pharmaceutical-based treatments.

GPCR | rhodopsin | conformational dynamics | fluorescence | retinal

The superfamily of G protein-coupled receptors (GPCRs) is one of the largest targets of pharmaceutical drugs in the human genome. Classically, GPCR signaling occurs when a diffusible ligand (such as a drug) binds to the receptor and stabilizes conformations that can couple with and activate intracellular proteins. Our understanding of this process has built on the classical "ternary complex" model of receptor–ligand–G protein interaction (1), a model that, with revisions, has continued to guide our knowledge of how this critical event occurs.

However, this paradigm has faced problems when applied to rhodopsin, the dim-light visual receptor. Rhodopsin is kept in an "off" state by a covalently bound inverse agonist, 11-*cis* retinal (11CR). Light converts the 11CR to an agonist, all-*trans* retinal (ATR), which enables the receptor to activate its G protein, transducin (G_t) (2, 3). The active receptor, metarhodopsin II (MII), continues signaling until the Schiff base linking ATR to the receptor is hydrolyzed, resulting in the release of ATR and the decay of MII into an inactive apoprotein, opsin (4, 5). Binding of a new 11CR to opsin reforms the dark state (DS), enabling another round of photon detection (6).

Due to this unusual light-activated, covalently bound ligand, rhodopsin has usually been considered "different" from the larger superfamily of diffusible ligand-binding GPCRs. However, we recently discovered that rhodopsin behaves more like a traditional ligand-binding GPCR than previously thought (7). Our experiments found that inactive receptor (Ops) preferentially binds inverse agonist (11CR), whereas active-state receptor (Ops*) binds agonist (ATR) (8). We proposed these results indicate that retinal–opsin interactions are governed by the same type of a conformational selection model proposed for other GPCRs (Fig. 1) (9).

This model challenges some long-held assumptions about retinal binding, predicts some unexpected behavior, and contains two testable hypotheses. First, it suggests that, after release, ATR can rebind any receptors remaining in an Ops* conformation (Fig. 1, i). This is in contrast to current assumptions that ATR release is irreversible after Schiff base hydrolysis and directly reflects the decay of the active MII species (10, 11). The second idea builds on the first—if ATR rebinding requires an active-like Ops* conformation, then an active Ops* state may persist after ATR release (Fig. 1, ii).

Here we directly tested both hypotheses. First, we measured ATR release for samples containing different amounts of active opsins and found that the extent of ATR "release" inversely correlates to the amount of Ops* present—the more Ops*, the less free ATR. Using fluorescence assays and radioligand binding studies, we established this occurs because during MII decay ATR release and rebinding are in equilibrium.

Second, we tested whether any active Ops* conformers can persist after ATR release by simultaneously measuring ATR in the receptor and the conformational state of the receptor in real time. Our results show that ATR release and the reversion of Ops* to the inactive conformation occur sequentially, but not always simultaneously. The observation that an active-like Ops* state can exist

Significance

G protein-coupled receptors (GPCRs) represent a major pharmaceutical drug target. However, one exception has been the visual photoreceptor rhodopsin, long considered "different" due to its covalently bound, light-sensitive retinal ligands. Here we demonstrate that, in contrast to prior assumptions, release of the agonist all-*trans* retinal (ATR) is not an irreversible process. Instead, during decay of the active species, ATR can rebind any rhodopsin remaining in an active-like conformation, and this active-like state can transiently persist even after agonist dissociation. These insights demonstrate rhodopsin behaves like other diffusible ligand-binding GPCRs and raise the possibility of treating rhodopsin by pharmaceutical agents.

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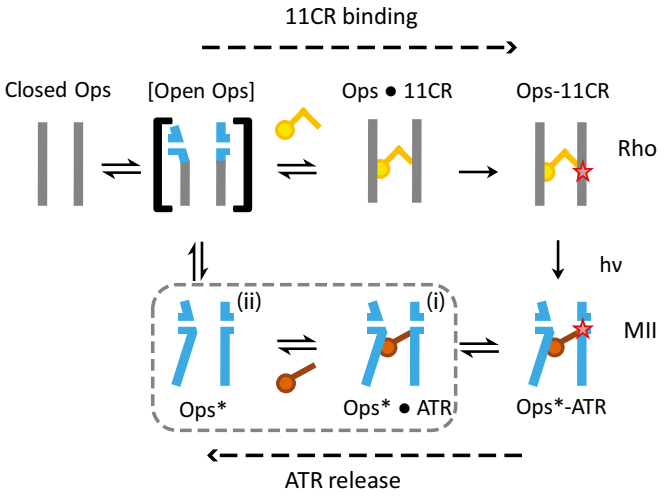

Fig. 1. Conformational selection model for retinal binding to opsin (8). The focus of the current work is on the process of ATR dissociation from opsin (enclosed in the dashed rounded rectangle) testing two hypotheses: first, that ATR will bind in equilibrium depending on the conformation of the opsin (i), and second, that the reversion of Ops* to Ops is distinct from the ligands presence, resulting in the possibility of an active Ops* state can transiently persist in the absence of ligand (ii).

even after agonist release may have wider implications for other GPCRs, and our finding that rhodopsin can bind its agonist ATR in equilibrium opens the possibility of using classical pharmacological methods to both study and ultimately target this key photoreceptor.

Results

Stabilization of Ops* Prevents Full Retinal Release. A conformational selection model (Fig. 1) predicts that, after release, ATR will rebind to any receptor remaining in an active (Ops*) conformation. We tested this idea using opsin samples with varying amounts of active Ops*, monitoring the protein fluorescence increase that occurs as retinal dissociates (10).

For light-activated WT rhodopsin (11CR-bound receptor), these assays show that ATR release is complete—tryptophan fluorescence increases and ultimately reaches a plateau that does not increase when hydroxylamine (HA) is added to convert ATR to ATR-oxime (which does not rebind opsin), as well as to cleave any remaining retinal–Schiff base attachments (Fig. 2*B*) (10, 12, 13).

In contrast, rhodopsin samples that contain active Ops* due to a constitutively activating mutation M257Y (CAM) (14, 15) do not show full ATR release. Instead, ~60% of the CAMs have ATR still bound under steady-state conditions (Fig. 2*C*) and thus show a large fluorescence increase upon HA addition.

Even more dramatic results are observed for rhodopsin samples that have a high-affinity analog of the $G_{t\alpha}$ C terminus fused to their end (GtF) (8), which stabilizes the active Ops* conformation. After light activation, these samples show almost no apparent ATR release. All rate values and percent ATR retention for Fig. 2 are reported in Table 1.

Equilibrium Binding Explains the Lack of Apparent ATR Release for Ops*-Containing Samples in the Retinal Release Assays. Two possibilities could explain the results above. Either release is blocked in the samples with more Ops* or ATR is continually releasing and rebinding in equilibrium to the stabilized active receptors. To determine which is correct, we exploited a key difference between these two scenarios: Retinals involved in equilibrium binding would become exposed to bulk solvent, whereas trapped retinals would not.

Thus, we repeated the ATR release assays in the presence of *o*-tert-butyl HA (tbHA). An alkylated derivative of HA, tbHA is too big to enter the receptor binding pocket and therefore can only react with retinals that have dissociated (16). As expected, tbHA did not affect WT rhodopsin (compare Fig. 2 *E* and *B*). However, tbHA caused complete ATR release for the CAM sample (Fig. 2*F*) and even induced ATR release from GtF, although at a slower pace, presumably because some ATR rebinds to Ops* faster than it can collide and react with tbHA (Fig. 2*G*).

These results suggest the apparent incomplete release is due to ATR leaving and rebinding the receptor, rather than being "trapped" inside the binding pocket. Consistent with this interpretation, we found the amount of ATR bound to the CAM increased with increasing amounts of exogenous ATR (Fig. 2*I*), as would be expected for equilibrium binding. Intriguingly, at higher ATR concentrations even WT opsin showed increased binding (Fig. 2*H*), suggesting this phenomenon is not exclusive to mutants with increased Ops* populations. As expected, the ATR release profile for GtF shows no change with extra ATR, because the receptor is already fully bound with ligand (Fig. 2*I*).

Radioactive Ligand-Binding Studies Further Confirm an ATR-Binding Equilibrium Can Occur After Receptor Photoactivation. We next tested whether ATR could bind in equilibrium by monitoring exchange of ATR produced inside the protein by light with exogenously added radioactive ATR (Fig. 3*A*). In these experiments, we added an equimolar amount of [^{3}H]ATR to each sample

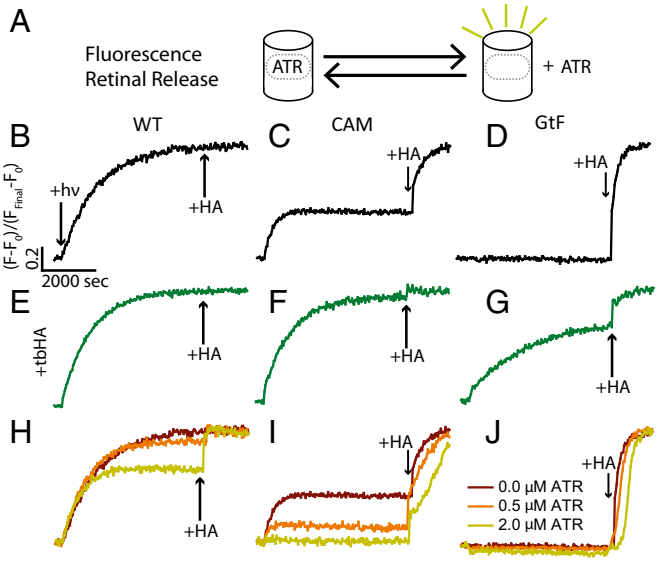

Fig. 2. Evidence that ATR released during decay of photoactivated MII rhodopsin can rebind in an Ops*-dependent manner. (*A*) Cartoon depiction of how retinal release following light activation can be monitored by an increase in intrinsic protein fluorescence. (*B*) Release trace from WT opsin shows a monoexpontential rise to a plateau. HA treatment yields no additional release, indicating full ATR dissociation. (*C*) Release from the CAM instead results in some ATR remaining bound to the receptor in an equilibrium that is only relieved when HA is added. (*D*) Photoactivation of GtF, a rhodopsin sample with the $G_{t\alpha}$ C terminus fused on its end, shows no apparent ATR release until HA is added. (*E*) Addition of tbHA, an HA derivative that cannot enter the binding pocket (16), had no effect on retinal release for the WT sample (compare with *B*). (*F*) In contrast, tbHA induces full retinal release from the CAM, resulting in data nearly identical to the WT opsin. (*G*) Similarly, the presence of tbHA induced a (slower) retinal release from GtF. Subsequent assays measured in the presence of increasing amounts of added ATR show a shift in the equilibrium to more bound ATR. (*H*) Results for increasing [ATR] for WT rhodopsin release. (*I*) Extra ATR further shifts the CAM toward the bound state. (*J*) Experiments with GtF and increasing amounts of ATR show no change, because the receptor is already fully bound at the lowest ATR concentration. Release experiments were conducted at 20 °C in 0.05% *N*-dodecyl-β-D-maltopyranoside (DDM) at pH 6.0.

Table 1. Time to half-maximal ATR release and percent remaining complexes before HA addition

	+0.0 μM ATR[†]		+0.5 μM ATR		+2.0 μM ATR		+tbHA[‡]	
Sample	$t_{½}$, min	% Bound	$t_{½}$, min	% Bound	$t_{½}$, min	% Bound	$t_{½}$, min	% Bound
WT[§]	12.5 ± 1.0[¶]	4.63 ± 0.78	10.9 ± 0.52	12.1 ± 2.2	7.17 ± 0.10	30.4 ± 5.8	10.0 ± 2.3	−0.03 ± 2.1
CAM	3.26 ± 0.37	62.5 ± 3.1	n.d.[#]	85.6 ± 1.4	n.d.	96.0 ± 3.1	11.2 ± 0.5	2.76 ± 1.0
GtF	n.d.	103 ± 4.3	n.d.	104 ± 1.4	n.d.	107 ± 0.38	24.9 ± 0.97	23.0 ± 4.2

[†]Exongenous ATR was added before photoactivation.
[‡]Ten millimolar tbHA was added before photoactivation.
[§]All experiments at 20 °C, 0.5 μM 11CR incubated overnight with 0.75 μM opsin.
[¶]Errors reported are SDs.
[#]Not determined (n.d.); rates of release from experiments showing too little initial release for a reliable fit are not reported.

then measured how much of it had bound to the receptors after light activation. As shown in Fig. 3*B*, the amount of receptor-bound [^{3}H]ATR mirrored the amount of active Ops* present in the samples (Fig. 3*B*).

Expanding on this result, we tested how fast the exchange could occur for the GtF rhodopsin sample. On the surface, the GtF rhodopsin samples seem to have a stably bound ATR after light activation—they show no apparent ATR release in the fluorescence retinal release assay (Fig. 2*D*), and they have a stable Schiff base linkage as determined by acid protonation (Fig. 3*D*). However, within minutes after light activation, these samples show full exchange with exogenously added [^{3}H]ATR (Fig. 3*C*). Interestingly, the receptors show complete expected exchange (calculated from the expected maximal binding, Fig. S1) that occurs even faster than retinal release from the WT protein ($t_{½}$ GtF exchange of ∼5min vs. $t_{½}$ WT ATR release of ∼13 min).

Method for Simultaneously Measuring Retinal Release and the Conversion from Active Ops* to Inactive Ops Conformation. Along with equilibrium binding, the conformational selection model predicts another unusual possibility: that an active-like Ops* conformation can persist following the release of bound agonist (Fig. 1, ii). We tested this hypothesis by modifying the retinal release assay to simultaneously monitor the receptor conformational state and ATR release (described above). The Ops* state was monitored by tracking the large movement of transmembrane helix 6 (TM6) that occurs during receptor activation, using the Trp-induced quenching (TrIQ) technique (17). TrIQ monitors the quenching of the small fluorescent probe bimane, which only occurs when the Trp and probe are in near contact (18).

Specifically, we attached a bimane to a cysteine on the cytoplasmic end of TM6 (V250C) and introduced a Trp across from it on TM3 (V139W). Previous studies of rhodopsin (19) and the β2 adrenergic receptor (B2AR) (20) have shown activation moves this pair into close contact, resulting in a large decrease in probe fluorescence (see Fig. 4*A*, Ops*). During decay of MII, TM6 moves back to its starting, inactive position, resulting in a fluorescence increase as the bimane quenching is relieved (Fig. 4*A*, Ops).

Importantly, the absorbance spectra are essentially identical for the V139W/V250B and the V250B control sample. Both show complete conversion to active MII rhodopsin (indicated by the shift from 500-nm to 380-nm absorbance) and no optical artifacts that could affect direct comparison of their fluorescence properties (Fig. 4 *B* and *C*).

However, their fluorescence emission spectra are strikingly different. For the control, V250B, the bimane fluorescence increases immediately after photoactivation to a maximal value and stays constant (Fig. 4 *D* and *F*). This increase is caused by the 500- to 380-nm shift in retinal absorbance, which removes FRET from the bimane to 11CR in DS rhodopsin (21). In contrast, the V139W/V250B fluorescence does not increase immediately after photoactivation. Rather, it slowly grows with a rate closely matching the profile of retinal release (Fig. 4 *E* and *G*), as the probe on TM6 moves close to (and is quenched by) the Trp on TM3 during MII formation (19, 20), then over time returns to its initial inactive position, relieving the TrIQ (19–21).

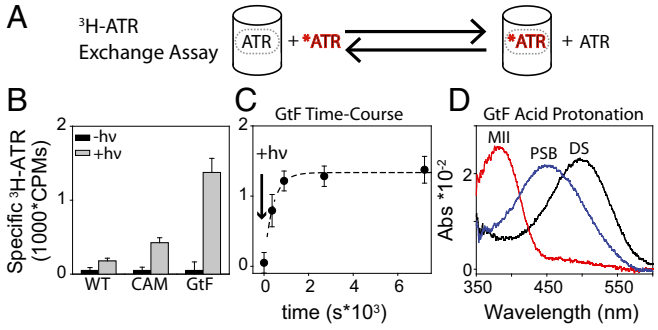

Fig. 3. ATR produced inside rhodopsin by light bleaching can exchange with an equimolar amount of exogenously added, radioactive [^{3}H]ATR. (*A*) Cartoon depiction of the ATR exchange experiments. (*B*) The amount of radioligand exchange for WT, CAM, and GtF rhodopsins at 2 h after photoactivation correlates with the amount of active Ops* and retinal trapping seen in Fig. 2. (*C*) Time-course measurement for the GtF construct shows complete and rapid ATR exchange ($t_{½}$ ∼4.7 min). (*D*) Surprisingly, although GtF clearly shows exchange with exogenous [^{3}H]ATR, acid protonation experiments of identical samples show that the light-activated ATR–Schiff base linkage in GtF remains "stable" for the entire (2-h) length of the exchange experiment [note the ∼440-nm absorbing species indicating a protonated retinal Schiff base (PSB)]. Experiments were performed at room temperature in 0.05% DDM at pH 6.0.

ATR Dissociation and the Conformational Change from Ops* to Ops Do Not Always Occur Simultaneously. Although TM6 movement and ATR release seem to coincide for the WT (V139W/V250B) rhodopsin (Fig. 4*G*), a closer examination reveals a short, but reproducible, delay between the events (Fig. 5*A*). To see whether this lag was real, and could be increased, we repeated these experiments under conditions that accelerate ATR dissociation. Two different approaches were used. First, we carried out the experiments in the presence of HA (Fig. 5*C*) to cleave the retinal–Schiff base linkages immediately after photoactivation (10). As expected, HA treatment dramatically increases the rate of ATR release. However, although TM6 movement was also accelerated in the presence of HA (Fig. 5*C*), it was noticeably delayed in comparison with ATR release.

We also tried decoupling ATR release and TM6 movement by introducing a mutation that promotes ATR dissociation. The mutation, A295S, lies immediately next to the Schiff base lysine at position 296 and greatly increases retinal release rates (22). Indeed, TM6 movement for A295S, although also accelerated, substantially lags behind ATR release (Fig. 5*E*). Together, these results support the model in Fig. 1 suggesting that retinal release and conversion of Ops* to Ops are sequential, yet distinct, events, with an active-like Ops* conformation transiently persisting after retinal release. All of these experiments were repeated at different temperatures, and the nearly

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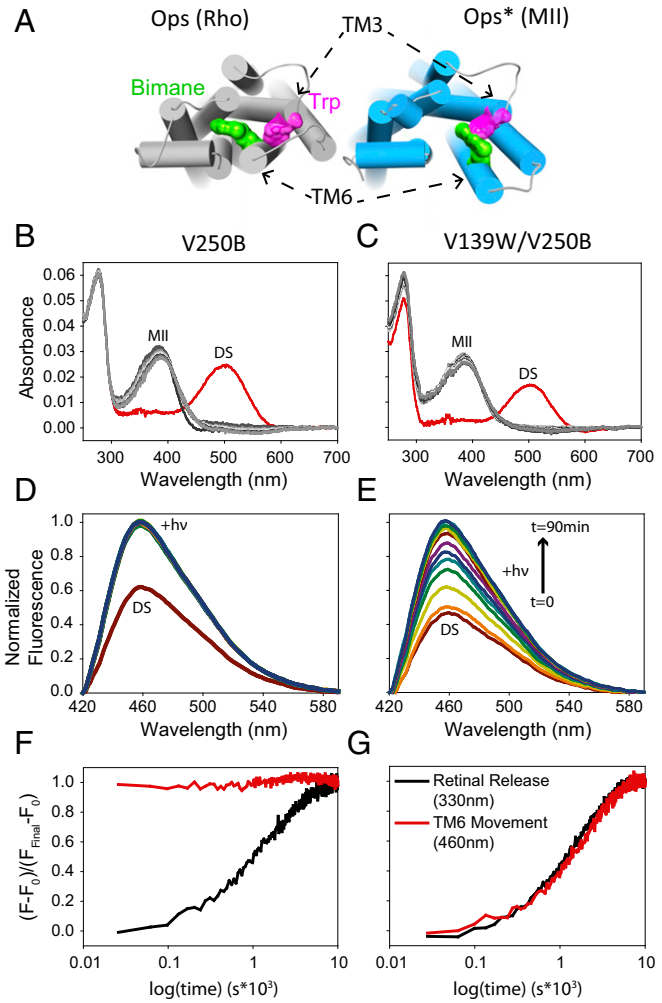

Fig. 4. Time-resolved fluorescence assay based on TrIQ for simultaneously monitoring receptor conformational changes and retinal release after rhodopsin photoactivation. (*A*) Model showing activation moves the bimane fluorophore (green) on TM6 into near contact with the quenching Trp (purple) on TM3. Reversion back to the inactive conformation relieves the TrIQ, causing a rise in the bimane emission. (*B* and *C*) Absorbance spectra indicate both V250B (Trp-less control, *B*) and V139W/V250B (*C*) are WT-like in their photoactivation properties. Spectra taken before and then every 5 min after photoactivation. (*D* and *E*) Bimane emission spectra following photoactivation of V250B and V139W/V250B. Spectra were taken first in the dark then every 5 min after bleach. The immediate increase for V250B is due to relief of bimane FRET to the 11CR. Note that for V139W/V250B the initial increase is absent, and instead the emission only slowly increases over time. (*F* and *G*) Time course for tryptophan ATR release (Trp fluorescence monitored at 330 nm, black trace) with simultaneous measurement of bimane fluorescence (at 460 nm, red trace) for V250B and V139W/V250B. A log time scale is used to enable comparison over a wide time range. For V139W/V250B, note the ATR release rate and TM6 movement are very similar, with a very small time lag. Models produced using Chimera and Protein Data Bank ID codes 1GZM and 3PXO (44–46). Experiments were conducted in the same conditions as Fig. 2.

identical results from Arrhenius analyses suggest that the underlying mechanism involved is not altered by either the introduced chemical (HA) or mutation (A295S) (Fig. 5 *B*, *D*, and *F*). Other mutations known to impair G protein interaction [Y223A,F (23, 24)] were also investigated and showed a similar delay (Fig. S2). Table S1 reports the retinal release rates and Arrhenius values for the tested conditions.

Discussion

In the present work, we tested two questions concerning retinal–rhodopsin interactions: Can ATR released from activated rhodopsin rebind to the receptor, and can an empty, yet active, receptor conformation (Ops*) transiently persist after ATR is released? Our results and their implications are discussed below.

After Rhodopsin Photoactivation, an Equilibrium of ATR Release and Rebinding Can Occur If Ops* Is Present. Binding of either Gt or arrestin can cause incomplete ATR release from photoactivated rhodopsin (12, 25). The presumption has been that the retinal release is blocked, causing the ATR to be "trapped" inside the receptor binding pocket (12, 13). However, our results with the CAM, M257Y, made us rethink what actually causes retinal "trapping." Similar to release in the presence of Gt or arrestin, the CAM does not show complete retinal release, but instead reaches an initial plateau that persists until HA is added. Based on this result, we asked whether the phenomenon of retinal "trapping" could in some cases actually be caused by ATR releasing and rebinding in equilibrium.

We first tested this idea using the HA derivative tbHA. Like HA, tbHA reacts with ATR to form retinal-oxime that cannot rebind to the receptor. However, because tbHA is too large to enter the ligand-binding pocket (16), and thus can only modify ATR that has exited the protein, retinal release would only be affected if release and rebinding is occurring. In agreement with previous observations, tbHA has little effect on the release of ATR from WT rhodopsin (Fig. 2*E*) (16). However, for the CAM, tbHA completely abolished the initial incomplete release (Fig. 2*F*), and even greatly increased the rate of ATR release from the GtF sample (Fig. 2*G*).

Clearly, these results suggest retinal "trapping" is actually due to an equilibrium of ATR release and rebinding for both the CAM and the GtF. What could cause this equilibrium? A

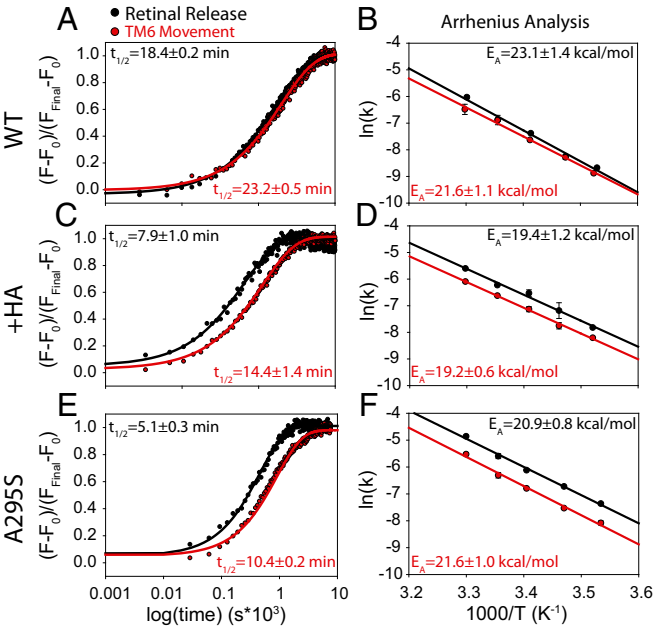

Fig. 5. The time lag between ATR release and TM6 movement back to the inactive state is increased when retinal release is accelerated. As with Fig. 4 *F* and *G*, the plots show simultaneous measurement of tryptophan (black traces) and bimane fluorescence (red traces) as a function of time (note log time scale). (*A*) Data for "WT" (V139W/V250B) receptor. (*B*) Arrhenius analyses show similar activation energies for both events. (*C*) Addition of HA accelerates retinal release faster than the TM6 movement. (*D*) HA does not have a drastic effect on the activation energies of either process. (*E*) Same measurements carried out on rhodopsin mutant, A295S, reveal a similar separation of ATR release and TM6 movement. (*F*) Arrhenius analysis of this construct again shows little change in the activation energy of the two events. Errors reported as SDs. Experiments were conducted in the same conditions as Fig. 2.

likely explanation is that the ATR is in flux between the active and inactive opsin populations present in both mutant samples. Thus, in samples where the completely inactive Ops state is favored (WT), ATR seems to fully release (Fig. 2*B*). In contrast, when an active conformation is stabilized, such as the CAM, an intermediate plateau is observed (Fig. 2*C*). For GtF (the sample with the most Ops*) ATR release seems blocked (Fig. 2*D*).

Our radioligand binding experiments further confirm retinal release–rebinding equilibrium is occurring. The ATR produced inside the receptor (by light activation) clearly exchanges with externally added, radioactively tagged [^{3}H]ATR (Fig. 3), with the amount of exchange correlating with the amount of stabilized Ops* present (Fig. 3*B*).

The most striking example is seen for the GtF sample. ATR seems to be fully "trapped" in GtF by the fluorescence retinal release assay (Fig. 2*D*). However, the ATR produced in GtF by light activation rapidly exchanges with externally added [^{3}H]ATR with full exchange achieved within minutes (Fig. 3*C*). Thus, ATR only seems to be "fully bound" because, under steady-state conditions, most of the ATR is bound to receptors in Ops* conformation—the "stable" Schiff base linkage attaching ATR to GtF is actually rapidly breaking and reforming as the retinal releases and rebinds the receptor (discussed below).

In retrospect, our results are perhaps not so surprising. Several groups have shown a dose response for ATR-induced G protein activation and arrestin binding (26, 27), and the ability of ATR to bind to active-stabilized opsins has also been shown (8, 14, 28–30). Our results with WT protein (Fig. 2*H*) suggest that this ability is not unique to mutants with stabilized Ops* but is a property inherent to opsin, dependent only on relative agonist concentration. How other rhodopsin photoproducts, metarhodopsin I and III, might affect the binding equilibrium is not clear, because our detergent-purified system favors MII formation (Fig. 3*D*). However, although this process may be more complicated in native rod cells, the fundamental conclusions should not change.

Is "Retinal Release" Due to the Protein's Reverting Back to an Inactive Ops Conformation Faster Than the Released ATR Can Rebind? Our results show that the extent of apparent ATR release depends on the amount of Ops* present. What insights does this give about the mechanism underlying ATR dissociation? One intriguing possibility is that "release" simply reflects the protein's inability to rebind the ATR agonist—when the Ops* conformation is stabilized, more ATR binding is observed in the fluorescence release assay (10, 12, 22).

This interpretation also raises the possibility that ATR could be in flux from the moment of photoactivation, as suggested by the rapid exchange with exogenously added [^{3}H]ATR seen in GtF (Fig. 3*C*). If ATR can exchange much more frequently than previously thought, then it may be possible to outcompete the rebinding of ATR with drugs designed to either temper the activity of rhodopsin after light exposure, or alternatively, encourage ATR dissociation from hyperactive rhodopsin mutants and thus enable regeneration with 11CR and proper photocycling.

These results also suggest interesting implications about the instability of the covalent Schiff base linkage. Previously, we postulated that the Schiff base connecting the 11CR to the dark-state protein may spontaneously hydrolyze but then rapidly rebind to the receptor due to a "kinetic trap," based on our studies of the retinitis pigmentosa mutant D190N (31). Our current results highlight the instability of the ATR–opsin covalent bond and bolster this hypothesis. They also imply that exchange of the dark-state 11CR chromophore should be possible if the "trap" is broken, an event that has been observed for cone opsins (32).

Opsin Can Transiently Retain an Active-Like Conformation Following Agonist Dissociation. Implicit in both the discussion above and the conformational selection model (Fig. 1) is the concept that ATR release and the reversion of Ops* to Ops are sequential, but distinct, events. Our results simultaneously monitoring the Ops* conformation and ATR release support this idea, showing the decay of Ops* can lag behind agonist dissociation (Fig. 5*A*). Accelerating the release further exaggerates the disparity (Fig. 5 *C* and *E*).

At 37 °C, the time lag for "WT" rhodopsin (V139W/V250B) is ~1 min (extrapolated from the Arrhenius value). Although this value is significantly longer than the subsecond lifetimes recently estimated for some B2AR conformations (33), and is likely exaggerated by our nonphysiological conditions, it is reminiscent of the slow movements induced in the B2AR upon binding of some agonists (34), ascribed to a sequence of conformational changes these ligands induce upon binding and activating the receptor. Perhaps the time lag we observe reflects a similar process, in reverse. Regardless, our data do clearly show that aspects of the active conformation can persist even in the absence of bound agonist.

This time lag between agonist release and collapse of Ops* is intriguing. Retinal-free rhodopsin (Ops) is extremely inactive, showing very little constitutive activity (5), and hence is normally assumed to only exist as an inactive conformation (4). How, then, could an empty, active-like receptor conformation transiently persist following activation? Presumably, this reflects some sort of "protein memory," where the photoreceptor briefly stays active-like in the absence of the agonist.

Such persistence of protein memory might also play a role in signal amplification by other GPCRs. Conformational selection-based ligand-binding mechanisms as well as multiple receptor signaling states are well documented for other GPCRs, including the B2AR and CB1 receptor (7, 20, 35–38). Hence, the delayed reversion to the inactive receptor conformation we observe for rhodopsin might play a heretofore unappreciated role here and in other GPCR signaling systems.

Implications for Other Visual GPCRs. Our results also have interesting implications for the cone opsins responsible for color vision. Recently, Knox and coworkers (39) showed that the process of ATR release from cone opsins is faster than rhodopsin but exhibits the same activation energy, and thus concluded both photoreceptors share a similar mechanism of activation for Schiff base hydrolysis, with the differences in release rates indicating different noncovalent interactions between the retinal and the binding pocket. Our data are consistent with this idea.

Moreover, our results might also help explain other differences between rhodopsin and cone opsins. Cone opsins operate in high-light conditions and thus have to rapidly respond to new photons without losing sensitivity. Therefore, their faster recovery to the inactive state (25, 40, 41), due to a rapid collapse to an inactive (Ops) conformation, would discourage ATR rebinding (and thus persistence of signaling) and be essential for maintaining the ability to discriminate differences in light intensity during daylight conditions (25). In contrast, rhodopsin, the dim-light photoreceptor, needs to convert a single photon into a maximal neuronal signal. Thus, opsin lingering in an active conformation after agonist release, and thus possibly activating more G proteins, would not be detrimental and may act to facilitate kinase or arrestin binding.

Conclusions

Our data provide further evidence that rhodopsin behaves like a ligand-binding GPCR. Despite the uniqueness of its covalently bound ligand, rhodopsin seems to interact with its retinal ligands in a way consistent with a conformational selection model (8), opening the possibility of using pharmacological approaches to modulate the activity of this key photoreceptor. Currently, drugs targeting rhodopsin only seek to improve receptor biogenesis. However, our data suggest that it may be possible to develop drugs that target and competitively displace ATR causing inappropriate signaling. Replacing the ATR with a less efficacious ligand could be used to quell inappropriate signaling and promote the deactivation

of the photoreceptors, thereby improving general visual sensitivity in some cases.

Experimental Procedures

Buffers, Cloning, Mutagenesis, Transfection, and Purification of Rhodopsin Mutants. Site-directed mutagenesis was carried out by overlap extension PCR into a rhodopsin background containing minimal reactive cysteines (17). Opsin experiments contained a stabilizing disulfide (N2C/D282C) to facilitate apoprotein purification (42). Proteins were expressed by transient transfection in COS-1 cells and either purified as opsin or regenerated with 11CR and labeled with the fluorophore monobromobimane as previously described (8, 15). See *SI Experimental Procedures* for full details.

Fluorescent Measurements. Fluorescence spectroscopy was carried out as previously described using a modified Photon Technology International steady-state fluorometer with the excitation source replaced with OceanOptics LEDs, LLS-295 and LLS-405 (8, 13). A bifurcated fiber optic cable allowed for dual excitation of both intrinsic tryptophans and a bimane probe. See *SI Experimental Procedures* for full details.

Radioligand Binding. Labeled ATR exchange was done by photoactivating regenerated opsins in the presence of exogenous [^{3}H]ATR. Unbound radiolabeled retinals were removed using mini size-exclusion chromatography columns and the incorporated [^{3}H]ATR was counted by liquid scintillation (43). See *SI Experimental Procedures* for full details.

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