Rhodopsin's active state is frozen like a DEER in the headlights

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That G protein-coupled receptor (GPCR) signaling complexes are allosteric machines par excellence is not in dispute. Agonist receptor ligands outside the cell induce catalytic guanine–nucleotide exchange on a heterotrimeric G protein inside the cell, where the ligand binding site on the receptor and the nucleotide binding site on the G protein are on the order of 8–10 nm or more apart. Although the precise molecular pharmacology and chemical basis of ligand binding and specificity are becoming clearer—especially from recent reports of high-resolution crystal structures of engineered β_2-adrenergic receptors (ARs) (1, 2) and earlier structures of rhodopsin (3, 4)—what we need to know is how the signaling complex works in space and time. What dynamic receptor conformational changes are induced by an agonist ligand? Or simply, how does the "active" state structure of a receptor differ from its "inactive" state structure?

In this issue of PNAS, Altenbach *et al.* (5) have gone to extraordinary lengths to map the surface movement of rhodopsin upon photoactivation, using a newly emerging electron pair spin resonance (EPR) technology called "double electron–electron resonance" (DEER) spectroscopy, which interrogates pairs of nitroxide spin labels. The label pairs were introduced by site-directed mutagenesis and chemical modification, using the rhodopsin crystal structure as a guide. The authors present a quantitative triangulation of relative interhelical distance changes between transmembrane (TM) helices as rhodopsin converts to its active form. What is most significant here, beyond the sheer technical achievement, is that the work provides a solid foundation for the so-called "helix movement model" of receptor activation.

Since the initial reports of substantial movement of certain TM helices of rhodopsin during photoactivation from EPR experiments of site-directed, spin-labeled (SDSL) mutants (6) and from engineered metal-ion binding sites that can block photoactivation of rhodopsin (7), several studies have seemed to corroborate the helix movement model, at least qualitatively. Notably, extensive SDSL EPR studies of rhodopsin more carefully mapped the conformational changes in rhodopsin (8). In addition, fluorescence studies of other GPCRs by using site-specific fluorophore labeling or, alternatively, fluorescent protein fusion constructs, suggested that the agonist-dependent conformational change in the cytoplasmic domain might be a general feature of GPCR activation (9, 10).

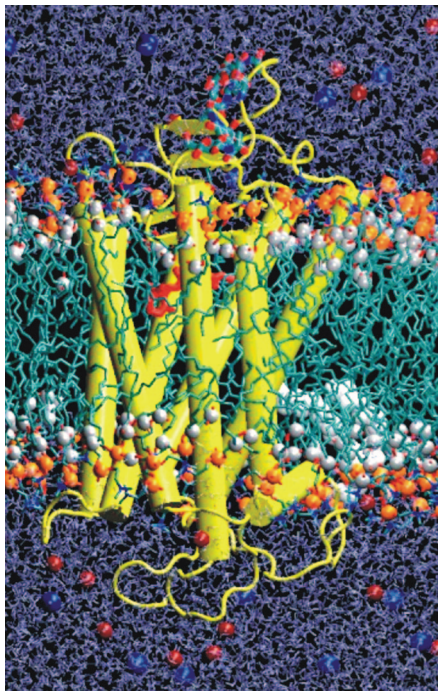

Fig. 1. Dynamic receptors. Rhodopsin is depicted in a phospholipid bilayer membrane. The structure was simulated in a model bilayer environment (15-ns and 45,000 atoms) starting from the crystal structure (1HZX) (19). The retinylidene ligand is shown in red; the lipid acyl carbonyl carbons representing the hydrophobic boundary are shown as white balls; and the phosphates representing the headgroups are in orange. The extracellular surface is toward the top and the cytoplasmic surface toward the bottom. DEER shows that the cytoplasmic end of TM6 moves away from the center of the receptor upon activation, which likely requires rigid-body movement of the helix.

A hallmark of family A (rhodopsin-like) GPCRs is the sequence motif (D/E)R(Y/W) at the cytoplasmic end of TM3. In rhodopsin and a few other GPCRs, such as the β_2-AR, formation of the active state depends on proton uptake activity (probably protonation of the acidic residue in the highly conserved motif). In rhodopsin, this motif has been implied in the uptake of a proton during the transition from inactive photoproduct to the active species meta II (11). Although the exact mechanism rhodopsin activation is still a matter of debate, rhodopsin activation appears to alter the interaction between the cytoplasmic ends of TM3 and TM6, resulting in outward movement of TM6, as demonstrated here by Altenbach *et al.* (5). Fluorescence studies of the pH-dependent, agonist-induced conformational change suggest that a similar proton uptake is involved in β_2-AR (12). Internal water molecules that connect functional microdomains in the TM region of the receptors appear to be involved in the activation mechanism, as indicated by the effect of osmotic pressure on meta II formation (13). Moreover, the lipid bilayer—and possibly higher-order molecular complexes in the membrane—appear to modulate the receptor activity (14).

Since the landmark report of the inactive-state structure of rhodopsin (4), several crystal structures of rhodopsin photoproducts have been reported. Among them, a photoproduct of rhodopsin with a deprotonated retinylidene Schiff base linkage—a *sine qua non* of the active state—showed surprisingly few, if any, structural changes at the cytoplasmic surface (15). How might this so-called active-state rhodopsin structure be reconciled with the present study and with earlier hypotheses concerning receptor activation dynamics? The structure reported by Salom *et al.* (15) was prepared from bleached rhodopsin in crystals, and an absorbance spectrum indicated a deprotonated retinylidene Schiff base. Although it is tempting to associate a metastable photointermediate containing a deprotonated Schiff base with the active state, FTIR experiments have revealed cases in which packing constraints block the gross conformational change character-

Author contributions: T.H. and T.P.S. wrote the paper.

The authors declare no conflict of interest.

See companion article on page 7439.

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istic of meta II formation while having no effect on Schiff base deprotonation (16). In this light, the absence of large-scale conformational changes in the cytoplasmic domain in the Salom *et al.* structure does not contradict the findings of Altenbach *et al.* (5).

Would computational methods such as molecular dynamics (MD) simulations be of use in generating an active-state structure of rhodopsin or other receptors, starting from a model of the inactive-state crystal structure? Extensive simulations of a rhodopsin model with an isomerized chromophore have been reported in the microsecond time scale (17); however, it appears to be kinetically impossible to reach even the inactive meta I receptor state in 1 μs. Meta I does not form on a microsecond time scale; at least orders of magnitude, longer times are required (18). Therefore, even the most elaborate MD simulations are unlikely to provide useful information about true active-state receptor structures any time soon. However, the simulations are exceedingly useful for determining the proper orientation and structure of the receptor within the bilayer environment (Fig. 1) (19).

Most of the meaningful structural dynamics information on rhodopsin obtained to date stems from cryogenic studies with either trapping or quenching of conformational states. Studies under ambient conditions are rare and are typically limited to concentration/temperature/pressure-jump UV-vis or FTIR difference (dark state minus light state) spectroscopy. But conclusions from cryogenic studies cannot always be reconciled with detailed kinetic studies at more physiologically relevant ambient temperature. Methods involving cryoquenching of high-temperature intermediates might exhibit similar problems. The underlying assumption of cryoquenching from high temperature is that rapid cooling from conditions favoring, for example, meta II should result in a distribution of states similar to the quasiequilibrium distribution of the high-temperature condition but without exchange. Complicating these kinetically determined relaxation pathways is the fact that it is probably impossible to obtain pure states because slow cooling will change the distribution according to van't Hoff. Therefore, one might ask with respect to cooling from cryogenic methods, "How fast is fast enough?" Faster cooling is obviously better, provided the correct distribution of conformational states can be guaranteed. Therefore, Altenbach *et al.* (5) carefully developed a protocol using the synergistic action of three meta II-promoting factors (the detergent dodecylmaltoside, low-pH buffer, and highly osmotically active concentrations of glycerol) together with rapid cooling of the samples in capillaries plunged into liquid nitrogen. This protocol was designed to cause the receptor to proceed directly into a glass phase of the glycerol-containing sample medium and seems to be ideal for maintaining the meta II conformation generated originally at high temperature. In contrast, solid-state NMR studies of rhodopsin samples in phospholipid membranes oriented on stacks of glass plates, or in detergent solution in magic-angle spinning rotors, might suffer from insufficiently fast cooling of the relatively large samples. This might lead to shifting of the temperature-dependent meta I/meta II equilibrium toward a significant fraction of meta I, even in cases where the high-temperature form is almost completely meta II.

Conclusions from cryogenic studies cannot always be reconciled with detailed kinetic studies.

Other advanced methods for probing receptor structural dynamics are on the horizon. We might expect, even in the coming months, to see reports of additional GPCR structures, and possibly even receptor structures in complex with G proteins or allosteric modulators. Fluorescent and other probes, including advanced spin-label probes, might be introduced into expressed receptors by unnatural amino acid mutagenesis employing the amber codon suppression strategy (20) and interrogated by using single-molecule detection technology. The longstanding strategy of introducing amino acid replacements by site-directed mutagenesis to cause a loss-of-function—to infer function—is being rapidly replaced by mutagenesis strategies designed to stabilize various receptor states and introduce nonperturbing but informative chemical probes. Almost exactly 50 years after E. W. Sutherland and T. W. Rall proposed a biochemical basis for hormone signaling, we may be on the verge of actually understanding how it all works.

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