A crystal clear view of the β 2 - adrenergic receptor

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A crystal clear view of the β2-adrenergic receptor

Robert J Lefkowitz, Jin-Peng Sun & Arun K Shukla

The crystal structure of a second G protein–coupled receptor sheds light on these key pharmaceutical targets.

G protein–coupled receptors (GPCRs), also known as seven-transmembrane receptors, are the largest family of plasma membrane receptors (~1,000 members) and the most important class of targets for current therapeutics[1]. Nonetheless, despite years of effort in many laboratories, the crystal structure of only a single GPCR has been solved: the visual sensory protein rhodopsin, in its ground state and photoactivated state[2,3]. Now, with the appearance of three papers[4–6] in *Science* and *Nature* presenting structures of the human β2-adrenergic receptor (β2AR), the way at last appears cleared for more rapid progress.

GPCRs mediate their effects through stimulation of members of the ubiquitously expressed family of heterotrimeric G proteins or β arrestins[1,7], thereby regulating the intracellular levels of various second messengers. It has been just over 20 years since cloning of the gene and cDNA for the β2AR unexpectedly revealed its homology with rhodopsin and the presence of seven stretches of presumably transmembrane-spanning hydrophobic residues in the deduced sequence[8]. The generality of this conserved primary structure has been repeatedly confirmed over the ensuing years as our understanding of the vast size and diversity of the receptor superfamily has rapidly expanded.

Although β2AR and rhodopsin have been central in unraveling the properties of the receptor superfamily, many of the properties of rhodopsin are in fact unique. Rhodopsin contains a covalently bound 'ligand', cis-retinal, which stabilizes the opsin protein in an inactive state, reducing intrinsic conformational flexibility[2]. Rhodopsin is activated by absorption of a photon of light, leading to cis-trans isomerization of the retinal, which induces a series of conformational changes in the opsin that activate signaling. Moreover, structural studies of rhodopsin have been facilitated by its great abundance; as much as 90% of the protein in purified retinal rod outer segments is rhodopsin, making the preparation of large amounts of nonrecombinant protein relatively straightforward. Even so, and despite the first reports of rhodopsin crystals in 1982 (ref. 9), it was not until 2000 that the first structure of inactive rhodopsin was reported[2], not until 2004 that the first high-resolution structure was described[10], and not until 2006 that the structure of the photoactivated deprotonated state was determined[3].

In contrast, β2AR and essentially all of the other GPCRs are present at very low concentrations in membranes. Even in the era of recombinant protein production, until recently obtaining more than a few milligrams has been heroic work. Moreover, other factors conspire to make these receptors extraordinarily difficult to crystallize. Like all membrane proteins, they must be solubilized with detergents before purification, which disrupts the organization of stabilizing membrane lipids, and the structural flexibility required for their signaling function produces conformational heterogeneity that hinders formation of well-ordered crystals.

To overcome these daunting obstacles, Brian Kobika's group used various protein-engineering techniques[5,6] and antibodies[4] while collaborating with structural biologists Gebhard Schertler, William Weiss and Ray Stevens, who used state-of-the-art techniques for obtaining crystals and solving structures of membrane proteins[4–6]. β2AR was modified by truncating the unstructured carboxy-terminal tail, reducing conformational flexibility and microheterogeneity due to phosphorylation in this region. A site for N-linked glycosylation was also mutated.

To stabilize the third intracellular loop, that connects transmembrane helices 5 and 6 (**Fig. 1**)—one of the most flexible regions, and a major site of interaction with G proteins—the authors took two approaches. In one, a Fab antibody fragment that recognizes a conformational epitope with determinants at both ends of the loop was developed[11]. In the second, the loop was entirely replaced with T4 lysozyme. This soluble globular protein is known to be easily crystallizable and can form various crystal contacts[5]. Replacement of the loop also reduces conformational flexibility. The structure was further stabilized by the binding of a very high-affinity antagonist, carazolol, in the ligand-binding site.

Although only the second approach yielded a high-resolution structure (2.3 Å), the lower-resolution structure solved in the first approach (3.4–3.7 Å) is also valuable because the protein is closer to the native receptor, allowing exclusion of artifacts that may have been introduced by the insertion of T4 lysozyme. In addition to these design features, the authors took advantage of the latest crystallographic technologies, including incorporation of the receptor into two different types of membrane environments (so-called 'bicelles', or lipidic cubic phase), microfocused synchrotron beams to analyze tiny crystals, automated technologies for screening and harvesting diffraction-quality crystals, and incorporation of cholesterol into the lipid phases.

So what have we learned from the new structures? There are no major surprises. Most of the features are as might have been expected from several decades of biochemical, biophysical and mutagenesis studies. But there are some unexpected findings, and validation of many previous findings by atomic-resolution structural information is of course gratifying.

As expected, the receptor consists of seven transmembrane helices connected by extra- and intracellular loops. The receptor fold is very similar to that of rhodopsin in its inactive state. The root-mean-square (r.m.s.) deviation between the two structures, which belong to the same subfamily of GPCRs (Class A), is 2.3 Å, and for the transmembrane helices only it is 1.6 Å. The sequence identities are 21% overall and 23% in the transmembrane domains. For comparison, the r.m.s. deviation for the kinase domains of the closely related protein kinases PKA and PKC is also ~2.3 Å, but their sequence identities are higher at 31%. The r.m.s. deviation for the kinase domains of two very closely related PKC isoforms, theta and iota, is 1.6 Å, but the sequence identity is much higher at 50%. This speaks to the very high level of conservation in the three-dimensional architecture of GPCRs. This common architecture hints that GPCRs may signal by a stimulus-induced structural rearrangement of the helical bundle that is itself highly conserved.

Several other features in the new structures support this interpretation. Similar to the rhodopsin mechanism, the conserved tryptophan side chain W286 in transmembrane helix 6 of the β2AR serves as a 'toggle' switch that stabilizes the inactive conformation. A network of water-mediated hydrogen bonds is also observed in both structures, extending from the ligand-binding pocket down through the space between

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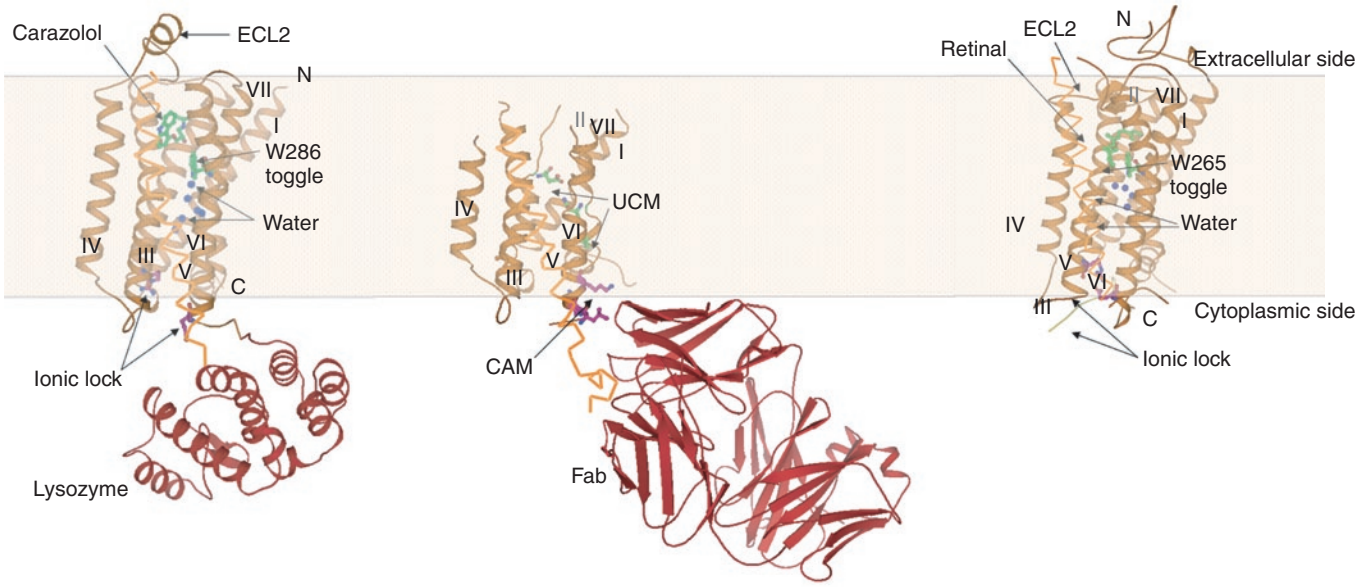

Figure 1 Structures of the β_2-adrenergic receptor and rhodopsin. The left structure is an engineered β_2AR–T_4 lysozyme fusion protein, the middle structure is the β_2AR in complex with a Fab, and at the right is the structure of rhodopsin. Transmembrane helices are numbered sequentially from I–VII. For clarity, helix V is shown as a ribbon. The N terminus and C terminus of each structure are labeled as N and C, respectively. ECL2, extracellular loop 2. Carazolol and retinal stabilize β2AR and rhodopsin, respectively, in conformations that do not activate G proteins through interactions with a tryptophan in transmembrane helix 6, which serves as a 'toggle switch'. From this region, a series of water molecules (blue) form a network of hydrogen bonds extending to the cytoplasmic region. Many mutants that uncouple the receptors from G proteins involve residues that are found in this region. Most mutations leading to constitutive activity are found in proximity to the ionic lock described in the text. Colors indicate oxygen (red), nitrogen (blue) and carbon (green at extracellular surface and magenta at cytoplasmic surface).

the helical bundle formed by transmembrane helices 2, 3, 6 and 7 to the cytoplasmic surface (**Fig. 1**). This network involves residues that are highly conserved in Class A GPCRs.

One might expect that features of the ligand-binding pocket would differ more significantly between β2AR and rhodopsin. In fact, the position of the pocket and its very hydrophobic nature are similar. However, an important difference was found in the accessibility of the binding pocket to the extracellular milieu. In rhodopsin, the covalently bound retinal is covered by a buried β sheet in extracellular loop 2. This effectively shields the binding site from the extracellular compartment (**Fig. 1**). In contrast, this extracellular loop in β2AR contains a previously unappreciated short helix that permits extracellular ligands access to the binding site.

An interesting question about the new structures is the nature of the receptor conformation that was crystallized. It has long been thought that inactive GPCR conformations are constrained by intramolecular interactions that are abrogated by binding of stimulatory ligands, which induce or stabilize activating conformational changes[12]. In fact, the first clues about these interactions emerged from a study of constitutively active mutant adrenergic receptors containing point mutations that also appeared to abrogate constraining intramolecular interactions, mimicking agonist stimulation[13]. Such mutagenesis studies with several GPCRs[12] and the structure of rhodopsin in the ground state indicated that a key element of the constraint is the so-called 'ionic lock' formed by electrostatic interactions and hydrogen bonds between residues at the cytoplasmic ends of transmembrane helices 3 and 6 (ref. 14). The lock is closed in the published inactive rhodopsin structures, with a distance of 2.9 Å between the carboxylate group of E247 and guanidine group of R135. This is consistent with the completely inactive state of cis-retinal–occupied rhodopsin. In the light-activated state of rhodopsin, the ionic lock is broken, with a distance of 4.1 Å between R135 and E247 (ref. 3).

In the β2AR structure, the ionic lock is disrupted; the distance between R131 and E268 is 6.2 Å (**Fig. 1**)[4]. This result is initially quite surprising, as the bound antagonist carazolol is thought to be an 'inverse agonist'. This means that, similar to cis-retinal bound to opsin, it lowers the constitutive activity of β2AR. Unlike retinal, however, carazolol lowers the basal activity of β2AR by only ~50%. Thus, the authors reasoned that the broken ionic lock in β2AR may reflect this residual constitutive activity. They also suggest that insertion of the T4 lysozme structure between transmembrane helices 5 and 6 may have caused subtle structural alterations that contributed to the partially activated state of the receptor. This state is reflected by the enhanced affinity of the receptor-T4 chimera for agonists, but not antagonists—a feature of constitutively active mutant receptors[13]. However, another possibility is that carazolol has positive agonist activity toward signal transducers other than G proteins (for example, β-arrestins)[7] and that the observed conformation reflects this activity. Such a situation has recently been reported for the structurally very closely related β-blocker carvedilol, which weakly activates β arrestin–mediated signaling through β2AR[15]. Additional structures of the receptor occupied by ligands with well-defined signaling properties will be necessary to resolve these issues.

There is excellent agreement between several features of the recently reported receptor structures and previous insights gained from mutagenesis and biophysical studies. For example, mutations that lead to constitutive activity, such as those in the highly conserved D/ERY motif at the cytoplasmic end of transmembrane helix 3 or those in transmembrane helix 6, are seen to either involve or surround the ionic lock (**Fig. 1**). Mutations that lead to the loss of agonist and antagonist binding are seen to interact with carazolol in the structure, and mutations that uncouple the receptors from G proteins, either directly or indirectly, participate in the water-mediated hydrogen-bond network in the helical bundle.

The availability of the new β2AR structures is a great step forward for the field of GPCR biology that represents more of a beginning than a conclusion. Similar approaches will likely be applicable to many other members of the various GPCR subfamilies. Structures of

the receptors in their multiple active conformations, stabilized with ligands, should ultimately elucidate the molecular mechanisms of receptor activation. These problems will be challenging and may well necessitate solving structures of receptors in complex with their signaling partners, such as G proteins and arrestins. Moreover, determination of the structures of constitutively active receptor mutants and biased receptor mutants that signal via one or the other signaling pathway may provide interesting correlations between receptor structure and activation mechanisms.

Crystal structures of therapeutically important proteins provide an excellent template for drug discovery and design, as they can be used for *in silico* screening of complex chemical libraries. Previous efforts on *in silico* screening for GPCR ligands used receptor-homology models based on the inactive rhodopsin structure. The newly determined crystal structures of β2AR provide a better tool for ligand-docking attempts, especially as the ligand-binding pocket and ligand-receptor interactions for β2AR show some substantial differences with rhodopsin. However, as mentioned earlier, the exact conformation that the receptor is in is not clear (inactive or β-arrestin signaling). Therefore, although these structures can be used for screening carazolol-like compounds, they may be of limited use for screening and identifying classical or biased agonists. Clearly, structures of β2AR bound to other ligands such as agonists, antagonists and partial agonists will be required to fully understand the ligand-binding and activation mechanisms and to carry out more useful and widely applicable screening of functionally specific ligands.

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Regulating rejection with cell therapy

Mohamed H Sayegh & Howard L Weiner

Regulatory T cells may prevent acute and chronic rejection of organ transplants.

Transplantation is one of the strongest challenges to the immune system. Although organ transplantation across histocompatibility barriers can be successful, lifelong administration of immunosuppressive drugs is often required to mitigate immune rejection. In a recent paper in *Nature Medicine*, Joffre *et al.*[1] show that regulatory T cells (Tregs) can be used to prevent acute and chronic rejection of skin and cardiac allografts.

Several publications have demonstrated a role for Tregs in mediating transplantation tolerance in experimental animal models[2]. Joffre *et al.* show for the first time that such cells can promote bone marrow chimerism and prevent subsequent acute and chronic allograft rejection of tissue and solid transplants in a donor-specific manner in mice. These findings are timely, as pilot trials to induce tolerance in humans are taking place under the auspices of the Immune Tolerance Network (http://www.immunetolerance.org/)[3].

Joffre *et al.* begin by showing that administration of *in vitro* allostimulated and expanded host $CD4^+CD25^+Foxp3$ Tregs induce donor-specific tolerance to allogeneic bone marrow and subsequent skin or cardiac allografts in recipients subjected to a nonmyeloablative irradiation regimen. They then tested the ability of Tregs alone without bone marrow infusion to induce tolerance to skin allografts. However, such a protocol failed to induce tolerance. Finally, infusion of directly allostimulated Tregs prevented acute rejection but not chronic rejection; prevention of chronic rejection required administration of directly plus indirectly allostimulated Tregs.

To explain these results, the authors propose two independent mechanisms: a direct effect of Tregs on alloreactive T cells, and an effect of Tregs on enhancing bone marrow chimerism. Interestingly, infusion of Tregs alone without bone marrow did not cause tolerance to skin allografts. However, because this was not tested for cardiac allografts, it is not possible to predict whether infusion of Tregs alone, without bone marrow transplant and with or without irradiation, will prevent rejection of solid organ allografts.

More importantly, the authors provide further proof that directly alloreactive T cells (which recognize intact allo-major histocompatibility complex (MHC) molecules on donor antigen-presenting cells) play a dominant role in mediating acute rejection, whereas indirectly alloreactive T cells (which recognize donor-derived allopeptides presented by recipient antigen-presenting cells) mediate chronic rejection[4]. Indeed, several studies in experimental animal models and in human transplant recipients have provided evidence to support this theory, but definitive proof has been lacking.

The finding that directly allostimulated Tregs cannot protect against indirect alloreactivity and chronic rejection is fascinating in and of itself, and indicates that in the context of alloantigen the function of Tregs is restricted by the mode of allorecognition. This has important therapeutic implications for the future design of Treg-based therapies as it suggests that optimal efficacy to prevent acute and chronic rejection will require the generation and infusion of Tregs with both direct and indirect allospecificities (**Fig. 1**).

It would have been interesting to test whether indirectly allostimulated Tregs alone can directly crossregulate alloreactive T cells, because this would obviate the need for donor cells to stimulate Tregs as these donor cells could be replaced by donor MHC peptides presented by recipient antigen-pre-

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