Crystal structure of the human β2 adrenergic G-protein-coupled receptor

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Structural analysis of G-protein-coupled receptors (GPCRs) for hormones and neurotransmitters has been hindered by their low natural abundance, inherent structural flexibility, and instability in detergent solutions. Here we report a structure of the human β2 adrenoceptor (β2AR), which was crystallized in a lipid environment when bound to an inverse agonist and in complex with a Fab that binds to the third intracellular loop. Diffraction data were obtained by high-brilliance microcrystallography and the structure determined at 3.4 Å/3.7 Å resolution. The cytoplasmic ends of the β2AR transmembrane segments and the connecting loops are well resolved, whereas the extracellular regions of the β2AR are not seen. The β2AR structure differs from rhodopsin in having weaker interactions between the cytoplasmic ends of transmembrane (TM3 and TM6), involving the conserved E/DYR sequences. These differences may be responsible for the relatively high basal activity and structural instability of the β2AR, and contribute to the challenges in obtaining diffraction-quality crystals of non-rhodopsin GPCRs.

Challenges in crystallizing GPCRs

The difficulty in generating crystals from the wild-type β2AR and other GPCRs for diffusible hormones and neurotransmitters may be related to the observation that these molecules are conformationally complex18,21,22. In contrast to rhodopsin, many GPCRs, including the β2AR, exhibit significant basal, agonist-independent G protein activation. This basal activity has been associated with structural instability23-24, suggesting that the intramolecular interactions that maintain the receptor in the inactive state are also important for the structural integrity of the protein. Orthosteric ligands for GPCRs exhibit a spectrum of efficacies for receptor-stimulated G protein activation, ranging from inverse agonists, which inhibit basal activity, to agonists, which maximally activate the receptor.

The β2AR contains relatively unstructured regions that are involved in functionally important protein–protein interactions. Protease susceptibility and intramolecular fluorescence resonance energy transfer experiments25 indicate that the C terminus and the third intracellular loop are the most unstructured regions. The N and C-terminal ends of the third intracellular loop are involved in G protein activation and the selectivity of GPCR–G protein interactions26. The C terminus interacts with G-protein-coupled receptor kinases, arrestins and other signalling molecules27. In the case of water-soluble proteins, removal of such unstructured regions can facilitate crystallization, but for the β2AR this strategy would remove hydrophilic surfaces that are frequently observed to form lattice contacts in membrane-protein crystals.

Crystallization and structure solution

In an effort to provide conformational stability while increasing the polar surface available for crystal contacts, we generated a monoclonal antibody (Mab5) that binds to the third intracellular loop of native, but not denatured receptor protein28. Mab5 was generated

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by immunizing mice with purified β₂AR reconstituted into phospholipid vesicles at a high protein-to-lipid ratio. Binding of Mab5 to β₂AR does not alter agonist or antagonist binding affinities, and does not prevent agonist-induced conformational changes; therefore, it does not significantly alter the native structure of the receptor. Purified, deglycosylated β₂AR bound to carazolol (an inverse agonist) forms a complex with the Fab generated from Mab5 (Fab5) in detergent, and the β₂AR–Fab5 complex can be isolated by size-exclusion chromatography.

Cryosts of the carazolol-bound β₂AR–Fab5 complex were grown in DMPC bicelles using ammonium sulphate as a precipitant. The size and uniformity of the crystals were improved by removing 48 amino acids from the unstructured C terminus (β₂AR365, Fig. 1). Crystals of the β₂AR365–Fab5 complex grew as long, thin plates up to 300-μm long, approximately 30-μm wide, and less than 10-μm thick. Owing to the size and radiation sensitivity of the crystals, data collection required the use of microbeam technology in which X-ray beams are either focused (ID-13 and ID23-2 beamlines, European Synchrotron Radiation Facility, Grenoble) or moderately focused and then further collimated (23ID-B GM/CA-CAT beamline, Advanced Photon Source) to diameters between 5 and 10 μm. The initial images from the best crystals showed diffraction to 3.0 Å; however, resolution was rapidly lost in sequential images from the same crystal volume. Nevertheless, we obtained a complete data set from a single crystal, and determined the structure by molecular replacement using immunoglobulin-domain search models for the Fab. The diffraction is anisotropic, with diffraction extending to 3.4 Å in the plane of the membrane and 3.7 Å perpendicular to the plane of the membrane.

**Structure of the β₂AR–Fab5 complex**

Figure 2a shows the packing of the β₂AR365–Fab5 complex in the crystals. The crystals seem to be formed from stacks of two-dimensional crystals, as previously reported for bacteriorhodopsin crystallized in bicelles. There are few contacts between adjacent receptor molecules within a bicelle layer, indicating that the receptor is monomeric in the crystal. This is somewhat surprising considering that, in all reported crystals of rhodopsin, rhodopsin exists as antiparallel or parallel dimers. Moreover, evidence from a variety of biochemical and biophysical studies suggest that the β₂AR and many other GPCRs exist as dimers or higher-order oligomers in the plasma membrane of cultured cells, and there may be a role for dimers in the export of properly folded receptor protein from the endoplasmic reticulum.

It is important to note, however, that β₂AR dimerization is not required for G protein activation. Purified β₂AR exists as monomers, and monomeric β₂AR reconstituted into recombinant high-density lipoprotein particles couples efficiently to Gα—its preferred heterotrimeric G protein.

The best-resolved regions of the crystal are the Fab5 fragments and cytoplasmic ends of the transmembrane segments of the receptor (Supplementary Fig. 1). In contrast to the cytoplasmic side of the receptor, the electron density is uninterpretable in the extracellular domain (Supplementary Fig. 2), even though this region of two receptor molecules packs together in a head-to-head manner around the crystallographic two-fold axis. The poor packing in this interface probably explains the significant anisotropy and poor overall resolution of the crystals. In an effort to improve the packing of the extracellular domains, we further modified β₂AR365 by inserting a TEV cleavage site after amino acid 24 (β₂AR24/365, Fig. 1). However, crystals of this construct are isomorphous to those made with β₂AR365, and the structure (Supplementary Table 1) is virtually identical to that obtained from β₂AR365–Fab5.

As expected, the overall structure of the β₂AR (Fig. 2b) is similar to rhodopsin, with seven transmembrane helices and an eighth helix that runs parallel to the cytoplasmic face of the membrane. Several of
the transmembrane helices are broken by non-helical kinks, most prominently TM7. Residues not included in the β2AR model, owing to absent or uninterpretable electron density, are indicated in Fig. 1. In the transmembrane helices, the majority of the missing side chains face the lipid environment. The loss of electron density occurs just above the ligand-binding site, near the predicted lipid-water interface, suggesting that ligand binding and/or the lipid environment contributes to the order of the transmembrane segments. Specific interactions between the variable domains of Fab5 and the β2AR occur over a sequence of nine amino acids at the N-terminal end of intercellular loop 3 (I233–V242) and two amino acids at the C-terminal end (L266 and K270) (shown in green in Fig. 2b). Therefore, Fab5 recognizes a three-dimensional epitope on the β2AR, which is in agreement with the observation that Fab5 binds to native, but not denatured β2AR protein28. Additional lattice contacts occur between the constant domain of a symmetry-related Fab5 molecule and the second intracellular loop of β2AR (shown in magenta in Fig. 2b).

**Structural insights into basal activity**

The ligand-binding site can be identified by an extended flat feature in the electron-density maps close to the extracellular side of the transmembrane helices (Fig. 2b, c). This is the only large feature in residual electron-density maps and is adjacent to Asp 113, Val 114, Phe 289, Phe 290 and Asn 312—residues identified from mutagenesis studies as being involved in ligand binding in the β2AR34,35. This region corresponds to the retinal-binding site of rhodopsin. The weak electron density in this region precludes definitive modelling of carazolol. It is unlikely that the crystallization conditions resulted in dissociation of carazolol from β2AR. Carazolol bound to the β2AR has a distinct fluorescence emission spectrum36, and β2AR crystals and associated protein precipitate harvested from equilibrated hanging-drops showed no significant loss of carazolol binding, as detected by fluorescence spectroscopy (data not shown).

Figure 3 shows a comparison of transmembrane segments of the β2AR superimposed with the homologous structure of rhodopsin. The root mean squared deviation for the alpha carbon backbone of the transmembrane segments is 1.56 Å. Although the overall arrangement of the transmembrane segments is similar, the β2AR has a more open structure. The difference in the arrangement of the cytoplasmic ends of the transmembrane segments of β2AR and rhodopsin may provide structural insights into basal receptor activity. Rhodopsin has no detectable basal activity, a feature essential for vision. In contrast, even when bound to the inverse agonist carazolol, the comparatively high basal activity of the β2AR is suppressed by only 50% (Supplementary Fig. 4). Therefore, the carazolol-bound β2AR is not functionally equivalent to dark rhodopsin. Figure 3b compares the β2AR and two rhodopsin structures at the level of the conserved (E/D)R(V/Y)W sequence (found in 72% of rhodopsin family members)37. In the high-resolution structure of inactive (dark) rhodopsin, E134 and R135 in TM3 and E247 in TM6 form a network of hydrogen bonds and charge interactions referred to as the ‘ionic lock’38. These interactions maintain rhodopsin in an inactive conformation. The ionic lock residues seem to have a similar role in the β2AR because mutations of these amino acids in the β2AR or other adrenergic receptors lead to constitutive activity17,38. Moreover, evidence from biophysical studies suggests that movement of the cytoplasmic end of TM3 relative to TM6 on activation is similar for the β2AR and rhodopsin17,39. However, as shown in Fig. 3b, the transmembrane segments of the β2AR have a more open structure in this region, and R131 in carazolol-bound β2AR is not close enough to E268 to form a hydrogen bond. The structure of carazolol-bound β2AR around the ionic lock is more similar to the structure of light-activated rhodopsin40 (Fig. 3b), in which R135 and E247 are separated by 4.1 Å. This light-activated rhodopsin structure may not represent the fully active conformation because the spectral properties of these crystals are similar, but not identical, to those of metarhodopsin II41. Nevertheless, given the role of TM3, TM6 and the adjacent cytoplasmic loops in G protein coupling, the more open structure of the β2AR may account for the residual basal activity of the β2AR bound to the inverse agonist carazolol.

It is unlikely that the observed structural differences between the β2AR and rhodopsin are due to distortion of the β2AR owing to interactions between Fab5 and the third intracellular loop, because binding of Fab5 had no effect on agonist or antagonist binding affinity, and does not effect agonist-induced movement of TM3 relative to TM6 (ref. 28). However, we cannot exclude the possibility that crystal packing interactions between Fab5 and the second extracellular loop (Fig. 2b) contribute to these structural differences.

Another set of intramolecular interactions known to be important for minimizing the basal activity of the β2AR involves L272 in TM6. Mutation of L272 to alanine was the first reported constitutively active mutant of the β2AR41. As seen in Fig. 4, L272 forms extensive van der Waals interactions with I135 in TM3; V222 and Y219 in TM5; and Y141 in intracellular loop 2 (Fig. 4, and Supplementary Fig. 5). Because L272 is adjacent to E268, disruption of the packing interactions by mutation to alanine may have an effect similar to
disruption of the ionic lock in rhodopsin. It is likely that this mutation would produce a more loosely packed, dynamic structure in this region, shifting the equilibrium towards a more active state.

It is interesting that packing interactions around L272 are observed while the ionic lock interactions are absent. Because mutation of either E268 or L272 leads to elevated basal activity, it is likely that both are involved in maintaining the basal state of the receptor. From the current structure, we can conclude that formation of the ionic lock and the tight packing of L272 are not interdependent, and might even be structurally incompatible. It is possible that the ionic lock and L272 interactions stabilize two of several distinct substates in the unliganded β2AR, and that these two substates have lower activity towards Gs than the others. Carazolol binding may further stabilize the substate that favours packing around L272, and therefore reduce basal activity relative to the ensemble of substates in the unliganded receptor. The residual activity in the carazolol-bound receptor may be due to the failure to stabilize ionic lock interactions.

The limitations of this crystal structure of the β2AR can be attributed to the poor crystal packing and the inherent structural flexibility of this GPCR relative to rhodopsin. Different crystallographic approaches will be needed to stabilize and visualize the extracellular domain and provide a more detailed picture of extracellular loops as well as the ligand-binding site. Nevertheless, this structure of the β2AR in a lipid environment provides structural insights into the basis of basal activity, a feature of many GPCRs that may have both physiologic and therapeutic relevance.

METHODS SUMMARY

β2AR was expressed in S9 insect cells using recombinant baculovirus. S9 cell membranes were solubilized in dodecylmaltoside and purified by sequential antibody and ligand affinity chromatography. Fab5 was generated by papain digestion of Mab5 and purified by ion-exchange chromatography. The β2AR–Fab5 complex was formed by mixing purified β2AR with a stoichiometric...
excess of Fab5, and then isolated by size-exclusion chromatography. The purified βAR–Fab5 complex was mixed with biciples composed of the lipid DMPC and the detergent CHAPSO. The final βAR–Fab concentration ranged between 8 and 12 mg ml−1. Crystals were grown by hanging-drop vapour diffusion in a mixture of ammonium sulphate, sodium acetate and EDTA over a pH range of 6.5 to 7.5. Crystals grew within 7 to 10 days. They were cryoprotected in 20% glycerol before freezing in liquid nitrogen. Owing to the size and radiation sensitivity of the crystals, diffraction images were obtained by microcrystallography. The structure of the βAR365–Fab5 complex was solved by molecular replacement, using separate constant and variable Fab domain structures as search models. Coordinates and structure factors are deposited in the Protein Data Bank (accession codes 2GR4 for the βAR365–Fab5 and 2GR5 for the βAR243–Fab5).

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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Preparation of $k^5$ perpendicular to $u$ values of 0.447 and 0.452, electron density scores of 5.3 and 10.6 for the constant domain, and 4.5 and 21.7 from this $k^5$ weighted 2Z light chain but within the plane of the membrane and 3.7 Å of data measured using a 43˚1581 set was transferred to the higher-resolution 3Zb light chain and 3760 of data collected in this manner, measured at beamline ID23-2 of the APS were processed with HKL2000

The unit cell parameters used for subsequent analysis (Supplementary Table 1) were obtained from initial indexing and refinement from one wedge of the ESRF data, and were subsequently found to be sufficient for processing the remaining data without unit cell constant refinement. Using a partial specific volume of 1.21 Å³/Da for protein, the unit cell would have 66% lipid, detergent and aqueous solvent for one $b^5$–Fab5 complex in the asymmetric unit.

Structure solution and refinement. The structure of the $b^5$AR Fab5 complex was solved by molecular replacement, by searching with separate constant and variable domain models against a low-resolution (4.1 Å) data set measured at ESRF beamline ID-13. The Fab was derived from a murine IgG antibody containing a κ light chain and γ1 heavy chain28. At the time of these calculations the sequence of the heavy chain was not known, and the crystal structure of a Fab containing a κ light chain but γ2 heavy chain44 (PDB code 1HTG) was used as a search model. Molecular replacement was performed with the program PHASER45, using data between 12 and 4.5 Å. The constant domain was placed first, followed by the variable domain. The constant domain model retained all side chains, whereas the variable domain was reduced to polyalanine. All atomic temperature factors were set to 50 Å². The best solution had rotation and translation function $Z$ scores of 5.3 and 10.6 for the constant domain, and 4.5 and 21.7 for the variable domain. An electron density map calculated to 6 Å from this solution revealed rods of density corresponding to the transmembrane helices of the receptor. A model of the transmembrane portion of rhodopsin made by removing the cytoplasmic and extracellular loops, retinal and water molecules, and replacing those residues non-identical with $b^5$AR with alanine could be manually placed into this density. To obtain a convenient starting model for building the receptor, the molecular replacement calculation was re-run to include the rhodopsin transmembrane helices model as a third search model after placing the two Fab domains. Although the top solution was not very strong statistically (rotation function $Z = 2.5$, translation function $Z = 7.0$), after rigid body refinement the rhodopsin model was very close to that placed manually into the 6 Å map. This molecular replacement solution was then subjected to rigid-body refinement between 20 and 5 Å in CNS46, using five rigid bodies (the Fab constant domain light and heavy chains, the variable domain light and heavy chains, and rhodopsin). This gave $R$ and $R_{free}$ values of 0.447 and 0.452, respectively.

Electron-density maps made with phases either from the Fab model alone or the rigid-body refined Fab5–AR model indicated significant differences between rhodopsin and $b^5$AR, and extensive manual rebuilding was required to refine the structure. The structure was initially refined at 4.1 Å resolution. The test set from the 4.1 Å set was transferred to the higher-resolution $b^5$AR Fab5 set measured at the ESRF (Supplemental Table 1) and additional test set reflections added in the 4.1–3.4 Å range. Multiple rounds of manual rebuilding, positional and grouped temperature factor refinement were performed using the maximum likelihood amplitude target in CNS. The electron density of the Fab is very well defined owing to its tight packing in the crystal, whereas the receptor is poorly packed and has much higher temperature factors (Supplementary Fig. 1 and Supplementary Table 1). Because the receptor density is poor, we also refined against a second data set from a single crystal of the $b^5$AR Fab5 complex (Supplementary Table 1), to ensure that any densities observed in the receptor region are not due to noise in the first data set. The $b^5$AR Fab5 data set was obtained from 225˚ of data measured using a 4-μm × 6-μm beam at beamline 23ID-B of the APS. Although there is electron density in the extracellular region, the final model retains only those residues that could be unambiguously assigned (Fig. 1).

The high-temperature factors and weak electron density for the receptor raises concerns about model bias. However, the Fab represents 50% of the scattering mass and, because of its better order, contributes even more to the total scattering and so represents a significant source of phase information independent of the receptor. Simulated annealing omit maps confirmed the interpretation presented here. Moreover, alternative sequence registers or backbone paths were considered in several portions of the receptor, but these models could be eliminated based on inspection of $σ_A$ weighted $2F_o – F_c$ and $F_o – F_c$ electron density maps.

On the basis of the average $F(F_o) of reflections near the three crystallographic axes (as defined by the program TRUNCATE42), we estimate the effective resolution to be 3.4 Å within the plane of the membrane and 3.7 Å perpendicular to the membrane for the $b^5$AR Fab5 structure, and 3.4 Å/3.8 Å for the $b^5$AR Fab5 structure.