Crystal structure of oligomeric β₁-adrenergic G protein–coupled receptors in ligand-free basal state

Jianyun Huang, Shuai Chen, J Jillian Zhang & Xin-Yun Huang

GPCRs are transmembrane proteins that act as key gatekeepers between external signals and cellular responses. These receptors are activated by a diverse array of ligands, including photons, odorants, chemokines, hormones, growth factors and neurotransmitters. GPCRs have critical roles in regulating many physiological functions.

Crystal structures of several GPCRs have been determined, to our knowledge, except in the unusual case of rhodopsin. It is activated by photon absorption that isomerizes rhodopsin. Rhodopsin is a special case among GPCRs because, in its ligand-free state, it is covalently bound with its inverse agonist—bound rhodopsin adopts an inactive state, the ligand-free opsin has an active-state conformation. This provides the structural basis of GPCR dimerization and oligomerization.

RESULTS

Structure determination

We determined the X-ray crystal structure of turkey β₁-AR without ligands and in the presence of synthetic lipids. Several complete data sets from individual crystals were recorded, and they gave similar structures. Only one representative data set from a single crystal is described here. Diffraction of the crystal was highly anisotropic, and the structure was solved and refined at 3.5-Å resolution. The overall quality of the electron-density map was high. The structure of the ligand-free state of β₁-AR was determined by molecular replacement using the partial agonist (salbutamol)-bound β₁-AR as a search model. Crystal packing indicated a membrane-like environment with extensive lipid impact on one packing direction.

β₁-AR packed as oligomers in parallel with two distinct dimer interfaces. Each crystallographic asymmetric unit had two β₁-AR molecules. These two β₁-AR molecules had similar configurations; the r.m.s. deviation was ~0.02 Å for all equivalent Cα atoms. Although most of the experiments including screening for crystallization conditions described in this paper were done with the construct β₁-AR(H0), the presented ligand-free structure was from the construct β₁-AR(m23) because it gave a slightly higher resolution. The β₁-AR(H0) contains deletions of amino acids 3–32, 249–283 and 366–483 and point mutations C116L and 358A.

In this structure, β₁-AR forms oligomers in a lipid membrane–like environment and adopts an inactive-state conformation.
Receptor oligomerization: the TM1-TM2-H8 dimer interface

Within the same lipid bilayer, oligomers of β1-AR were packed in a parallel arrangement with two alternating dimer interfaces (Figs. 1b,c and 2). This oligomeric architecture was remarkably similar to the proposed models for oligomeric GPCRs based on a large body of functional data. In previous crystallographic studies with β1-AR bound with antagonists or agonists, β1-AR was observed as antiparallel dimers. The lack of oligomeric arrangement in those studies might be due to the exclusion of phospholipids in the crystallization conditions. Here, in one dimer interface (dimer interface 1), the interaction was mainly through TM1 as well as some residues from the C-terminal helical domain H8, TM2 and extracellular loop 1 (Thr106, Leu108 and Trp109; Figs. 1b,c and 2). The total buried contact surface (from both receptors) was ~900 Å² (Fig. 2a,b). Residues from both TM4 (including Leu171) and TM5 (including Arg205, Ala206, Ala210, Ile218 and Arg229) contributed to the hydrophobic interaction (Fig. 2c,d). ICL2 also had a critical role in this dimer interaction (including residues Tyr140, Leu141, Thr144, Ser145, Phe147, Arg148, Ser151 and Leu152; Fig. 3d). Two residues (Trp181 and Arg183) from extracellular loop 2 also participated in this interaction (Fig. 3e). Previous studies, using the bioluminescence resonance energy transfer method, had shown that human β1-AR formed dimers and that TM4 was involved in their dimerization.

Disulfide trapping of β1-AR dimers

Next we used disulfide-trapping experiments to biochemically test some representative residues identified from our structural studies for their involvement in β1-AR dimerization in cells. Cysteine replacement of an appropriately disposed pair of residues at the dimer interfaces is expected to generate a disulfide bridge. In our structural model, we selected a few residues from the two interfaces and mutated these residues into cysteines on the background of β1-AR(H0). In dimer interface 1, Lys354 from one protomer interacted with Lys354 from the second protomer (Fig. 2e). In dimer interface 2, Arg148 from one protomer interacted with Arg148 from the other protomer.

Table 1 Data collection and refinement statistics

| Data collectiona | Ligand-free β1-ARb |
|------------------|--------------------|
| Space group      | C2                 |
| Cell dimensions  | a, b, c (Å)        |
|                  | 229.66, 79.59, 69.04 | 90, 101.83, 90 |
| α, β, γ (°)      | 90, 101.83, 90     |
| Resolution (Å)  | 67.57–3.35 (3.44–3.35)c |
| Rmerge           | 0.141 (>1.0)     |
| I / σ (I)       | 6.3 (1.7)       |
| Completeness (%) | 98.2 (97.0)     |
| Redundancy       | 4.3 (4.2)       |
| Refinement       |                    |
| Resolution (Å)  | 29.78–3.50       |
| No. reflections (test set) | 13,006 (642) |
| Rwork / Rfree (%) | 30.99 / 35.46   |
| No. atoms        | Protein            |
|                  | 4,442              |
| Overall B factor (Å²)d | 79.3             |
| r.m.s. deviations | Bond angles (°)   |
|                 | 0.006              |
|                 | 1.136              |

*aOne crystal was used for data collection and refinement. bThe data set was anisotropically truncated to 3.3 × 3.3 × 4.3 Å after merging and scaling. cValues in parentheses are for highest-resolution shell. dAn additional isotropic B factor of –54.13 was applied to the scaled data for map sharpening.
protomer (Fig. 3d). As a negative control, we also mutated Phe112 to cysteine. Phe112 is on the extracellular side of TM3 and was not involved in the dimer interfaces, on the basis of our crystal structure. We transfected these mutants individually into CHO cells, and stable cell lines were selected. After exposing the membrane preparations to the hyperoxidizing environment of copper phenanthroline (CuP), dimer formation was assessed by western blot analysis of detergent-solubilized protein samples with antibodies to β1-AR (Fig. 3e). Wild-type β1-AR (having no cysteine residues in the two dimer interfaces) and the F112C mutant showed only monomers with or without CuP.
Ligand-free basal state of β1-AR in an inactive conformation

One of the characteristics of the inactive state of class A GPCRs is the presence of the ionic-lock salt bridge between the highly conserved D(E)R3.50Y motif in TM3 and an E/D6.30 residue in TM6 (Ballesteros-Weinstein numbering system is in superscripts)14,16,42. This ionic-lock salt bridge between Arg1393.50 and Glu2856.30 was present in the ligand-free state of β1-AR (Fig. 4a,b). Hence, our data are consistent with the ligand-free basal-state β1-AR being in an inactive state.

In the first report of the crystal structure of β1-AR bound with the antagonist cyanopindolol, the ionic lock was absent10. In a subsequent report of the crystal structures of β1-AR with cyanopindolol, the ionic lock was present in some structures but absent in others13. In the structure of cyanopindolol-bound β1-AR with the ionic lock, the cytoplasmic end of TM6 (the G protein–interacting region) was in a bent conformation (Fig. 4c)13. In the cyanopindolol-bound β1-AR without the ionic lock, the cytoplasmic end of TM6 was in a straight conformation (Fig. 4d)13. Thus, it was proposed that the presence of the ionic lock was associated with the bent conformation of the cytoplasmic end of TM6 (ref. 43). However, in the ligand-free basal-state structure of β1-AR described here, the ionic lock existed concomitantly with the straight conformation of TM6 (Fig. 4c,d).

The basal state with a contracted ligand-binding pocket

On the basis of comparisons of the crystal structures of several GPCRs in inactive and active states, it has been proposed that, although the overall GPCR structures did not change significantly, an outward movement of the cytoplasmic end of TM6 (and, to a lesser degree, TM5 as well) relative to the receptor helix-bundle core is a hallmark of the active state13,17,22–24. The ligand-free basal state of β1-AR did not display this characteristic outward movement of TM6 and TM5, consistent with its inactive conformation. Furthermore, agonist binding
to β₁-AR induces the contraction of the ligand-binding pocket by ~1 Å (as measured between the Cα atoms of Ser211 and Asn329)\(^{18}\). The ligand-binding pocket in the ligand-free state of β₁-AR was empty (Fig. 4e and Supplementary Fig. 3). Moreover, the ligand-binding pocket of the ligand-free state of β₁-AR was narrower than that of the antagonist-bound structure and was similar to that of the agonist-bound structure of β₁-AR (Fig. 4f–h). Thus, the contraction of the ligand-binding pocket may not be an essential feature of the binding of full agonists to β₁-AR.

**DISCUSSION**

**The ligand-free basal state of GPCRs**

Before ligand binding, GPCRs are in a basal state. As agonists or inverse agonists could shift the ligand-free state to an activated state or an inactive state, respectively, the ligand-free state is probably conformationally flexible. This may partly explain the difficulty in crystallizing ligand-free GPCRs. However, many GPCRs, including β₁-AR, have a low basal activity in the absence of ligands, which suggests that, in the ligand-free state, a large fraction of the receptor population is in the inactive state. For the ligand-free GPCRs, although opsin is in an active state, the ligand-free β₁-AR is in an inactive state. These differences might reflect the different crystallization conditions such as the presence of membrane-like environment in the β₁-AR structure or different stabilized conformations caused by different crystal packings. The crystal structures only provide a snapshot of the lowest-energy conformations that these receptors could adopt under the specific crystallization conditions.

It might also be argued that the ligand-free β₁-AR observed here in the inactive state was stabilized by the thermostabilizing mutations. However, that is unlikely because these thermostabilizing mutations, although making β₁-AR proteins more stable at higher temperatures, do not stabilize β₁-AR(m23) in an inactive conformation. As recently reported, this mutated β₁-AR(m23) is still a functional receptor capable of binding agonists and antagonists and activating intracellular agonist responses (Supplementary Fig. 4; ref. 44). Furthermore, most crystal structures of this thermostabilized β₁-AR(m23) with agonists or antagonists displayed intermediate conformations without the ionic lock and without the outward movement of the cytoplasmic end of TM6 (refs. 10,18,43). The structure presented here was determined from β₁-AR proteins purified with alprenolol-affinity purification (eluted with cyanoindol and the crystallization condition was at pH 4, which reduced antagonist binding to β₁-AR (Supplementary Fig. 4), we could not completely exclude the possibility of a very low occupancy of cyanoindol in the presented structure. However, use of β₁-AR proteins purified with two rounds of nickel-affinity purifications (without the alprenolol-affinity purification step) resulted in similar structures, although at lower resolutions.

It is known that some GPCRs display varying levels of constitutive activity (that is, the basal activity in the absence of any ligands), which are critical for their physiological functions. Structural determinations of the ligand-free states of these GPCRs should provide molecular insights into the activation processes of GPCRs, the basal activities and the development of agents for therapeutic applications, as the ligand-free state is the starting state and offers a point of comparison.

**Dimer interfaces and G-protein interaction**

In our crystal structure of β₁-AR oligomers, there are two dimer interfaces: one involves TM1-TM2-H8, and the other engages TM4-TM5-ICL2 (Fig. 1). Among the published crystal structures of GPCRs, there are four other GPCRs showing parallel dimers. In rhodopsin and κ-opioid receptor, the dimer interface involves residues from TM1-TM2-H8 (refs. 12,13,45,46; Supplementary Fig. 5a,b). This dimer interface is similar to dimer interface 1 in our β₁-AR structure. In the CXCR4 structure, there is a dimer interface involving residues from TM5 and TM6 (ref. 15; Supplementary Fig. 5c). However, dimer interface 2 of β₁-AR involves TM4 and TM5. Compared to β₁-AR, one monomer of CXCR4 rotates ~40° toward another monomer (Supplementary Fig. 5c). In a recent crystal structure of oligomeric µ-opioid receptor, two dimer interfaces were observed: one involves TM1-TM2-H8, and the other involves TM5-TM6 (ref. 47). Hence, the TM1-TM2-H8 interface is rather conserved in various GPCRs. In contrast, the TM5 interface sometimes functions with TM4 and other times with TM6. Notably, the crystal structures of GPCRs so far only displayed these two types of dimer interfaces, which are in agreement with a large body of experimental data, indicating that these two dimer interfaces are likely to be physiological relevant.

In addition to the TMs, intracellular regions contribute significantly to the dimer interfaces. There are four residues from H8 involved in the TM1-TM2-H8 dimer interface. Eight residues from ICL2 contribute to the TM4-TM5-ICL2 dimer interface. ICL2 is critical for interacting with G proteins on the basis of the structural model of the complex of β₂-AR and Gs (ref. 24). A Gs trimer could be docked onto a β₁-AR dimer formed through the TM1-TM2-H8 dimer interface (Fig. 5a,b). However, it was not possible to dock a Gβγ trimer onto the β₁-AR dimer formed by the TM4-TM5-ICL2 dimer interface.
interface without steric collisions (Fig. 5c,d). Participation of ICL2 in this dimer interface may prevent G-protein coupling to the dimer formed through the TM4-TM5-ICL2 interface, or G-protein binding may disrupt this dimer interface. Therefore, we propose that, if the signaling unit is a pentamer (two GPCRs and one trimeric G protein), the GPCR dimer interface in this signaling unit is TM1-TM2-H8 (Fig. 5a,b). In this model, only one β1-AR contacts the G-protein trimer, and the other β1-AR is ‘spared’ or could function through trans-protomer allosteric regulation (discussed below).

**Fig. 6** Comparison of the β1-AR oligomer with the μ-opioid receptor oligomer. (a) Oligomer structures, with β1-AR in green and μ-opioid receptor (PDB 4DKL) in magenta. Top, side view of the oligomers. Bottom, top view (from the extracellular surface) of the oligomers. The alignment was performed between molecule A of β1-AR and one molecule in μ-opioid receptor, using all seven TM segments. (b) Docking of Gs onto the β1-AR tetramer. The complex of β2-AR and Gs (PDB 3SN6) was aligned with molecule B of the β1-AR dimer with the TM1-TM2-H8 interface.

**GPCR oligomerization and receptor activation**

In our crystal structure, β1-AR forms oligomers in a membrane-like environment. Although the physiological functions are not clear, studies using various approaches have indicated that GPCRs could form oligomers in cells. While our manuscript was under review, a recent crystal structure of μ-opioid receptor also showed oligomers. The β1-AR oligomers show some similarities and some differences from the oligomers of μ-opioid receptors (Fig. 6a). Both oligomers have two dimer interfaces and share the same TM1-TM2-H8 dimer interface (Fig. 6a). However, in the second dimer interface, TM5 works together with TM4 in β1-AR, whereas TM5 acts together with TM6 in μ-opioid receptors (Fig. 6a). Furthermore, β1-AR oligomers form a linear array in one direction, but μ-opioid receptors are in a sine-wave arrangement (Fig. 6a).

A docking exercise revealed that with the oligomeric arrangement, trimeric G proteins could not be fitted in without steric hindrance (Supplementary Fig. 6). Even though this docking was speculative, it suggests that either G-protein binding may disrupt the oligomers or change the oligomeric arrangement, or the oligomeric architecture would prevent the pronounced sideways rotation and upward translation of the helical domain of the Gαs subunit relative to the Ras-like domain of the Gρs subunit, as observed in the crystal structure of the complex of β2-AR and Gs24. Indeed, the extent of membrane-driven oligomerization of a GPCR (such as D2 receptors and 5HT2c receptors) in the inverse agonist–bound state may be larger than in the agonist-bound state40,41. Moreover, inverse agonists stabilize β2-AR oligomers, whereas Gs reduced the extent of oligomerization of β2-AR50. Hence oligomerization of GPCRs is sensitive to ligand binding. That is, agonist binding may disrupt the oligomerization of GPCRs into dimers and/or tetramers.

It is possible to dock two trimeric G proteins into a β1-AR tetramer (Fig. 6b). To know whether this model is physiologically relevant requires further experimental testing. In this model, the two promoters through the TM4-TM5-ICL2 dimer interface were spared (not interacting with G proteins; Fig. 6b). An asymmetric function for GPCR dimers has been proposed54,51. Previously, a dimer interface involving TM1 has been shown to be insensitive to ligand binding and the receptor-activation state, as shown for dopamine D2 receptors and serotonin 5HT2c receptors31,41. The similarity of dimer interface 1 (involving TM1) in the inactive β1-AR and the active rhodopsin is consistent with the notion that this dimer interface involving TM1 does not undergo significant conformational changes from inactive to active states of GPCRs (Supplementary Fig. 5a). In contrast, the dimer interface involving TM4 makes structural rearrangements during the GPCR activation process, at least in the cases of dopamine D2 receptors and serotonin 5HT2c receptors40,41. These imply a possible role for the TM4-TM5 dimer interface in GPCR transactivation, even though the two promoters do not directly interact with G proteins in the proposed model (Fig. 6b). On the basis of the structures of active GPCRs, the intracellular end of TM5 moves away from the TM bundle core24. Therefore it is possible that, upon the agonist’s binding, the configuration change at the TM4-TM5 dimer interface is part of the receptor-activation process.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Atomic coordinates and structure factor files for the oligomeric turkey β1-AR have been deposited in the PDB, with accession number 4GPO.

**Note:** Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS J.H. performed the protein purification, set up crystallization trials, grew crystals for data collection and participated in data collection. S.C. processed the diffraction data, and solved and refined the structures. J.J.Z. participated in project strategy and manuscript preparation. X.-Y.H. was responsible for the overall project strategy and management and participated in data collection and wrote the manuscript.

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β1-AR constructs and purification of β1-AR proteins. A cDNA plasmid for the 
turkey β1-AR was obtained from E. Ross53. For crystallization screening of 
β1-AR constructs for structural studies, we used the fluorescence-detection 
size-exclusion chromatography (FSEC) method for integral membrane pro-
teins developed previously53. In this screening method, the target proteins are 
covaently fused to GFP. The resultant fusion proteins are monitored first for 
expression level and pattern in whole cells by epifluorescence microscopy. After 
solubilization of whole cells or crude membranes, the resulting unpurified pro-
tein is analyzed by FSEC. A monodispersed and folded protein would generally 
and used for crystallization. In other preparations, 
molecular weight of a monomer of 
β1-AR was eluted by using 3× bed volume buffer C (20 mM Tris-HCl, pH 8.0, 
0.35 M NaCl, 10 mM imidazole, protease inhibitors and 0.025% DDM). We purified the recombinant β1-AR proteins from 
High5 insect cells. The stability of these 
β1-AR proteins in different detergents was tested at 18 °C. Most of the studies were with a β1-AR construct (β1-AR(H0)) with deletions of amino acids 3–32, 249–286 and 363–483 and point mutations 
Cl16L and C358A (Supplementary Fig. 1). We purified the recombinant β1-AR proteins from 
β1-AR mutants (with a C-terminal His6 tag) were subcloned into baculoviral expression vector pVL1393. Recombinant baculoviruses were picked and ampli-
fied. High5 insect cells were grown in suspension in High5 Express Medium 
(Invitrogen) at 27 °C with shaking at 110 r.p.m. Cells were infected at a multiplicity 
of infection of 5–10. Following shaking for one hour, an equal volume of fresh 
medium was added. Cells were harvested by centrifugation 48 h after infection. 
Infected cells from cultures were harvested by centrifugation, and the resulting 
pellet was resuspended in 20 mM Tris-HCl, pH 8.1, 8 mM EDTA. Cells were flash 
frozen in liquid nitrogen and stored at ~80 °C. Cells were broken by sonication. 
After centrifugation at 2,000 g for 10 min, the supernatant was collected and centrifuged for 1 h at 45,000 r.p.m. at 4 °C in a Beckman Ti45 rotor. Membrane 
pellets were resuspended in the same volume of buffer, and the centrifugation 
was repeated. The final pellet was resuspended in a buffer with a reduced EDTA 
concentration (0.2 mM) at 10–20 mg protein/ml and frozen in liquid nitrogen 
and stored at ~80 °C. Membranes containing 2 g of total proteins were thawed and 
diluted to 10 mg/ml protein in ice-cold 20 mM Tris-HCl, pH 8, with 0.35 M NaCl, 
2% DDM and protease inhibitors and then stirred at 4 °C for 1 h. After centrifuga-
tion for 1 h at 45,000 r.p.m. in a Ti45 rotor (4 °C), solubilized membrane proteins 
were mixed with Ni-NTA beads preequilibrated with buffer A (20 mM Tris-HCl, 
pH 8, 0.35 M NaCl, 10 mM imidazole, protease inhibitors and 0.025% DDM). 
The mixture was rolled at 4 °C for 6 h. The protein-loaded resin was washed with 
buffer B (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.025% DDM), and the bound 
protein was eluted by using 3× bed volume buffer C (20 mM Tris-HCl, pH 8.0, 
50 mM NaCl, 250 mM imidazole, 0.025% DDM). In some preparations, β1-AR 
proteins were purified again with a second round of Ni-NTA affinity purification 
and used for crystallization. In other preparations, β1-AR proteins were puri-
fied by alprenolol-affinity purification. For alprenolol-affinity purification, after 
dilution of the protein sample with four volumes of buffer D (Buffer C without 
imidazole), the sample was incubated with alprenolol–Affi-Gel beads overnight 
at 4 °C. Alprenolol-NH2 was synthesized at Cornell’s chemistry core facility, 
following a published protocol55. Alprenolol was cross-linked to Affi-Gel-15. 
After the alprenolol beads were washed with buffer D, β1-AR was eluted with 
buffer D containing 50 μM cyanopindolol and then dialyzed, concentrated and 
changed to a buffer of 10 mM Tris, pH 7.7, 50 mM NaCl, 0.02% DDM and 0.1 mM 
EDTA with Centriconos (100 kDa cutoff; Millipore)56,57. SDS-PAGE showed that 
β1-AR protein was >90% pure. The yield was 2–mg of purified β1-AR proteins 
from 1 l of insect cells.

Crystalization. β1-AR(H0) proteins were initially used for screening crystal-
ization conditions. β1-AR at a final concentration of ~8 mg/ml in 20 mM Tris-
HCl, pH 7.7, 50 mM NaCl, 0.1 mM EDTA, 0.02% DDM, 0.1 mg/ml lipid (3:1:1:1 
PPOC/POPE/POPG/cholesterol) was incubated on ice for 1 h before setup of the 
tray. Crystals were obtained in several crystallization conditions. To further 
ensure that there were no ligands in the final crystals, we selected conditions with 
low pH, which decreased the binding of ligands from β1-AR38 (Supplementary 
Fig. 4). Crystalization was performed by the vapor-diffusion hanging-drop 
method at 18 °C. A volume of 1 μl β1-AR protein sample was mixed with 1 μl 
crystallization buffer (0.1–0.3 M (NH4)2SO4, 0.02 M NaAc, pH 3.6–4.2, 26–30% 
PEG 200). With the β1-AR(H0) construct, the screened crystals yielded diffraction 
to ~8 Å. We then introduced the six point mutations (R68K, M90V, Y227A, 
A282L, F327A and F383M) and generated a construct the same as the thermostabi-
lized β1-AR(m23) (Supplementary Fig. 1)18. Thus the ligand-free structure 
described here was for β1-AR(m23), and this thermostabilized β1-AR mutant 
was used in previous crystal structural studies with antagonists or agonists18,19. 
This mutant β1-AR is able to adopt different conformations, to bind antagonists, 
partial agonists and agonists, as well as to activate G proteins and increase cAMP 
levels in cells in response to agonists18,19,44. Crystals were formed within one week 
and were directly frozen in liquid nitrogen. Crystals were screened, and diffrac-
tion data were collected at the National Synchrotron Light Source (beamlines 
X6A and X25) of the Brookhaven National Laboratory or the Advanced Photon 
Source beamline NE-CAT 24E at Argonne National Laboratory.

Data collection, structure determination and refinement. Diffraction data 
presented in this paper were collected at 100 K by using synchrotron radiation 
(λ = 1.1000 Å) at beamline X25, National Synchrotron Light Source, by using 
a PILATUS 6M CCD detector. The crystal diffracted to ~3.3 Å, and diffraction 
data were indexed and integrated with XDS, followed by merging and scaling 
with XSCALE39. The crystal belongs to space group C2221, and the corresponding 
data-collection statistics are shown in Table 1. Analysis of the final data set by 
the UCLA diffraction anisotropy server indicated that the diffraction was highly 
anisotropic, strong data in two directions while weak in the third direction along 
the reciprocal space axis c* (ref. 60). As guided by an χ2 < χ2_g < χ2_g cutoff of 
3.0 along each reciprocal space axis, reflections were anisotropically truncated 
to 3.3 × 3.3 × 4.3 Å along a*, b*, c* and sharpened by application of a negative 
isotropic B factor of ~54.13 before use in refinement. 
The structure of the ligand-free state of β1-AR was solved by molecular replacement 
with PHASER of the CCP4 suite by using a monomer of the salbutamol-
bound β1-AR-m23 structure (PDB 2Y04, chain A) as the search model41. A total 
of two copies of the monomer were observed per asymmetric unit. Model refine-
ments were performed with REFMAC5 and PHENIX followed by employing 
Coot for iterative cycles of rebuilding based on F2-weighted 2Fo – Fc and 
Fc – Fomaps, as well as noncrystallographic symmetry (NCS) averaged and anaveraged 
maps26,44. During refinement, reflections within the resolution range 30–3.5 Å 
were selected, and tight two-fold NCS restraints were applied to chains A and B, 
with a notable reduction in Rmerge, with good geometry. Refinement statistics are 
also presented in Table 1, and the stereochemical quality of the refined structure 
was validated by using MolProbity55. The Ramachandran-plot distribution for 
residues in the structure was 95.6% in the favored region and 4.4% in allowed 
region. The high R factors might be partially attributed to the unmodelled discon-
tinuous density maps in the gaps between protein molecule layers, which could 
not be fitted into any ordered lipid molecules or solvents, owing to the resolution 
limit of 3.5 Å. The interfaces of two β1-AR dimers were analyzed by using the 
EBI PDBe PISA server web site46. Global alignment of various structural models of β1-AR 
was performed by using PyMOL (super_align) (http://www.pymol.org/), and all 
structural-model figures were created with PyMOL as well.

Cysteine cross-linking. Disulfide-trapping experiments were performed as previously described30,41. Additional details are in Supplementary Note.
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