Supplementary figure legends

Figure S1, Primary amino acid sequence of β_2AR-365N and Isoproterenol competition binding curves for methionine mutants of the β_2AR (related to Figure 2).

A) The figure illustrates the primary sequence and the features of the β_2AR-365N construct. The N-terminal FLAG epitope is shown in teal, the TEV-cleavage site is shown in pink and follows the FLAG epitope. The last 48 amino acids were truncated at Gly365. N-linked glycosylation sites are shown as small circles, the palmitoylation site on Cys341 is shown as a zig-zag line and the two disulfide bonds are shown as straight dotted lines. There are 9 methionines in the sequence; these are shown in green and purple. Methionines mutated in the β_2AR-Δ5 construct are shown in green and the remaining four methionines are shown in purple. B) Competition binding of full agonist isoproterenol with radioactively labeled antagonist [^{3}H]-dihydroalprenolol (n=3). C) Saturation binding of the radioactively labeled antagonist [^{3}H]-dihydroalprenolol (n=3). Calculated binding affinities can be found in Table S1. D) [^{35}S]GTPγS binding assays were performed to determine coupling efficiency of β_2AR and β_2AR-Δ5 to a membrane-tethered $G_s\alpha$ in response to high-affinity agonist BI-167107 and inverse agonist (ICI). Error bars represents standard error of mean for three experiments performed in triplicates. E) Emission spectra of β_2AR-Δ5 receptor labeled at Cys265 (cytoplasmic end of TM6) with monobromobimane in the absence and presence of high-affinity agonist BI-167107. Both spectra are

normalized to the spectrum of the unliganded state of β_2AR-Δ5. As previously observed for wild type β_2AR(Yao et al., 2009b), upon binding of agonist the bimane fluorescence is quenched and the maximal emission shifts from 453 nm to 459 nm. The most dramatic change in fluorescence quenching is observed at 435 nm. F) An apparent association rate for BI-167107 was determined by monitoring fluorescence intensity of bimane-labeled β_2AR-Δ5 at 435 nm after adding a super-saturating concentration (200 μM) of BI-167107. The apparent half-time of 4.4 min represents the upper limit of the true association half-time, because the change in fluorescence represents a conformational change that follows agonist binding. The dissociation rate of BI-167107 was determined by monitoring the fluorescence of BI-167107-bound, bimane-labeled β_2AR-Δ5 at 435 nm after the addition of 200 μM of the neutral antagonist alprenolol. For the dissociation experiments 100 nM bimane-labeled β_2AR-Δ5 is equilibrated with 1 μM BI-167107 prior to the addition of alprenolol. The change of bimane fluorescence associated with dissociation of BI-167107 occurs with a half-time of 403 min.

Figure S2, Methionine methyl region of carbon-HSQC spectrum β_2AR-365N and non-labeled β_2AR-365N spectrum shows only one resonance in the methyl methionine region (related to Figure 3).

A) Methionine methyl region of carbon-HSQC of β_2AR-365N expressed in ^{13}C-methyl methionine labeled media with different ligands: unliganded, alprenolol (antagonist) bound, carazolol (inverse agonist) bound and BI-167107 (full

agonist) bound. In red the unliganded spectrum is shown as a reference for the other spectra.

B) Methyl methionine region of carbon-HSQC spectrum of labeled β_2AR-Δ5 expressed in ^{13}C-methyl methionine labeled media (black) and unlabeled β_2AR expressed in regular Sf9 insect cell media (pink). We observe only one of the resonances appear in the non-labeled sample and we expect that this resonance comes from a highly flexible methyl group in the receptor and not from a methionine methyl group.

Figure S3, Assignment of methionine methyl resonances (related to Figure 3).

To better understand the structural basis for the spectral changes we assigned the remaining methionines in β_2AR-Δ5M by mutagenesis and for each mutant we obtained spectra of the unliganded receptor and of the receptor bound to the inverse agonist carazolol, the agonist BI-167107, and both BI-167107 and the G_s-mimetic nanobody 80 (Nb80). All spectra are shown overlapped with the $^{13}CH_3\varepsilon$-Met-β_2AR-Δ5M in red.

A) As expected mutating Met36$^{1.35}$ led to a loss of the most intense peak at 1.76 ppm [^{1}H] and 16.9 ppm [^{13}C], this peak didn't change chemical shift upon addition of different ligands or Nb80. Met36$^{1.35}$ is the most solvent exposed of the 4 remaining residues in β_2AR-Δ5M and we therefore expected this to be the most intense peak in the spectrum, which is also what we observe.

B In the spectra of β_2AR-Δ5M+M82V we observe the three downfield shifted peaks disappear in the unliganded and carazolol bound spectra. In the presence of the agonist BI-167107 alone or with Nb80, only one resonance is observed for Met82$^{2.53}$ at 2.05 ppm [^{1}H] and 16.8 ppm [^{13}C]. Interesting we observe a change in chemical shift for Met82$^{2.53}$ when we add inverse agonist (carazolol) compared to the unliganded sample, but we do not observe a different chemical shift of Met82$^{2.53}$ when we compare the spectra of receptor bound to agonist BI-167107 alone or with Nb80 present.

C) In the spectra of unliganded and inverse agonist bound β_2AR-Δ5M+M215I we observed the peak at 1.87 ppm [^{1}H] and 17.75 ppm [^{13}C] disappear, but also that the peak at 1.64 ppm [^{1}H] and 19.0 ppm [^{13}C] change chemical shift slightly in the proton dimension. We assign the peak at 1.87 ppm [^{1}H] and 17.75 ppm [^{13}C] to Met215$^{5.46}$. Met215$^{5.46}$ and Met279$^{6.41}$ are found in the same region of the receptor in TM5 and TM6 respectively. The methionine residues are in close proximity in the inactive structure(Cherezov et al., 2007; Rosenbaum et al., 2007) and we might therefore expect that mutating one of these methionines would affect the chemical shift of the other methionine methyl. We therefore expect that Met279$^{6.41}$ is represented by the very weak resonance at 1.64 ppm [^{1}H] and 19.0 ppm [^{13}C] in the unliganded and carazolol bound receptor samples.

In the BI-167107-bound sample of β_2AR-Δ5M the Met215$^{5.46}$ resonance weakens and shifts upfield to 1.42 ppm [^{1}H] and 17.4 ppm [^{13}C], and in the presence of both BI-167107 and Nb80 the peak the resonance shifts to 1.55 ppm [^{1}H] and 17.4 ppm [^{13}C] and intensifies. We don’t observe a clear peak representing

Met279$^{6.41}$ in the agonist bound spectrum of β_2AR-Δ5M, but in the Nb80 stabilized receptor sample we observe a resonance at 2.1 ppm [^{1}H] and 16.5 ppm [^{13}C] that we assign to Met279$^{6.41}$. This resonance is not present in the β_2AR-Δ5M+M215I spectrum, but we observe a strong resonance at 2.05 ppm [^{1}H] and 18 ppm [^{13}C]. We speculate that two resonances both represent Met279$^{6.41}$, and reflect a change in the environment around Met279$^{6.41}$ as a result of the M215I mutation.

Mutation of Met279$^{6.41}$ to Ile resulted in an increase in agonist affinity and a large decrease in expression. As a result we were not able to obtain NMR spectra on this mutant. Nevertheless, it was possible to assign resonances from Met279$^{6.41}$ based on the results of the remaining mutations.

Figure S4, Conformation of Met82$^{2.53}$ and surrounding residues (related to Figure 5).

A) Binding pocket with ligands BI-167107 and carazolol shown in the active (green, 3P0G) and inactive (orange, 2RH1) structure of β_2AR, respectively. The distances shown are to the closest aromatic ring heavy atom in the ligand to the methyl carbon of Met82$^{2.53}$. We would not expect the aromatic ring of the ligands to have a direct effect on the chemical shift of Met82$^{2.53}$**.**

B) To study the flexibility of the Met82$^{2.53}$ side chain we ran molecular dynamics simulations of the β_2AR with carazolol bound (see Experimental procedures for details). Every 18 nanoseconds during the first microsecond of the simulation we measured the three side chain torsion angles of Met82$^{2.53}$: χ_1(green), χ_2 (blue)

and χ_3 (red). We would expect that the multiple side chain conformations that we observe during the simulation would result in one peak in the NMR spectrum, because the exchange between these different conformations takes place on sub-microsecond time scale. These different side chain conformations can therefore not explain the multiple peaks we observe for this methionine methyl in the carazolol-bound spectrum.

C) In an attempt to explain the change in chemical shift seen for Met82$^{2.53}$ when we add agonist, we plotted the distance distribution to nearby aromatic residues. We plotted the distance from Met82$^{2.53}$ methyl to the nearest aromatic ring heavy atom for Tyr316$^{7.43}$, Phe282$^{6.44}$ and Trp286$^{6.48}$.

D) The two panels show time traces from temperature accelerated molecular dynamics simulations in which a lower (left) and higher (right) fictitious temperature was applied to the distance between the backbone centers-of-mass of Leu75$^{2.46}$ and Pro323$^{7.50}$ (see Experimental procedures). The dashed lines denote the corresponding values of the distances in the inactive crystal structure (2RH1).

Figure S5, Slice of BI-167107 and BI-167107+NB80 bound β_2AR-Δ5 spectrum (related to Figure 6).

1D slice of β_2AR-Δ5 spectrum with BI-167107 and BI-167107+NB80 bound. The red line shows the carbon chemical shift where the 1D proton slice is taken.

Table S1 (related to Figure 2)

	K_D (nM) ±SE	K_i (μM) ±SEM
β_2AR-365N	2.12±0.33	0.38±0.04
β_2AR-Δ5	2.40±0.60	1.47±0.05
β_2AR-Δ5+M36L	2.94±0.60	0.96±0.09
β_2AR-Δ5+M82V	1.38±0.50	1.12±0.03
β_2AR-Δ5+M215I	1.95±0.47	2.69±0.05
β_2AR-Δ5+L272M	4.29±0.91	0.37±0.06

Competition binding curves and saturation binding curves can be found in Figure S1. Ki values were calculated based on the equation:
$K_i=IC_{50}/(1+ [ligand]/K_D)$

Table S2. NMR experiments (related to Figures 3 and 4)

		Temp (°C)	900MHz	800MHz	WET	Number of increments	Number of scans
β_2AR-365N	Alprenolol	25		X	N	32	320
	BI-167107	25		X	N	32	512
	Carazolol	25		X	N	32	512
	Unliganded	25		X	N	32	288
β_2AR-Δ5	Unliganded	25	X		N	64	320
	Carazolol	25	X		N	64	320
	BI-167107	25	X		N	64	320
	BI-167107+Nb80	25		X	N	64	384
β_2AR-365N Non-labeled	Unliganded	25	X		N	64	320
β_2AR-Δ5-M82V	Unliganded	25		X	N	64	320
	Carazolol	25		X	Y	64	208
	BI-167107	25		X	Y	64	256
	BI-167107+Nb80	25		X	Y	64	96
β_2AR-Δ5-M36L	Unliganded	25		X	Y	64	320
	Carazolol	25		X	Y	64	320
	BI-167107	25		X	Y	64	320
	BI-167107+Nb80	25		X	Y	64	320
β_2AR-Δ5-M215I	Unliganded	25		X	N	64	320
	Carazolol	25		X	N	64	352
	BI-167107	25		X	N	64	320
	BI-167107+Nb80	25		X	Y	64	256
β_2AR-Δ5-L272M	Unliganded	25		X	Y	64	320
	Carazolol	25		X	Y	64	352
	BI-167107	25		X	Y	64	320
	BI-167107+Nb80	25		X	Y	64	256

Table S3. Simulations performed (related to Figures 5 and 6)

# note	Duration, μs	Initial conformation	Ligand	TAMD (k_B), kcal/mol	Mutant
1	15.0	Inactive β_2AR	Carazolol	-	-
2	15.0	Inactive β_2AR	Carazolol	-	-
3	10.0	Inactive β_2AR	Unliganded	-	-
4	10.0	Inactive β_2AR	Unliganded	-	-
5	2.0	Inactive β_2AR	Carazolol	7.2	-
6	2.0	Inactive β_2AR	Carazolol	9.5	-
7	5.0	Active β_2AR	BI-167107	-	-
8	5.0	Active β_2AR	BI-167107	-	-
9	10.0	Active β_2AR+Nb80	BI-167107	-	-
10	5.0	Active β_2AR+Nb80	BI-167107	-	-
11 *a*	2.7	Alt. Inactive β_2AR	Carazolol	-	-
12	10.0	Inactive β_2AR	Carazolol	-	-
13	10.0	Inactive β_2AR	Unliganded	-	-
14 *b*	2.0	Active β_2AR	BI-167107	-	M215I
15 *b*	15.4	Inactive β_2AR	Carazolol	-	-

Only previously unpublished simulations are listed here. All simulations used β_2AR(N187E) residues 29 to 342 except for unresolved residues in ICL3. The "inactive" (i.e., crystallographic inactive) β_2AR conformation was taken from the crystal structure of carazolol-bound β_2AR (PDB entry 2RH1) (Cherezov et al., 2007; Rosenbaum et al., 2007), while the active β_2AR conformation was taken from the crystal structure of β_2AR bound to BI-167107 and Nb80 (PDB entry 3P0G) (Rasmussen et al., 2011a). Certain trajectories omitted carazolol or Nb80. In the TAMD simulations, a Brownian particle with the indicated reference temperature was tethered to Leu75–Pro323 backbone distance. All histidines were protonated on their epsilon carbons only (neutral) and Glu122 was protonated (neutral) in these simulations. Asp79 was protonated in simulations 1-10, and Asp130 was protonated in the simulations starting with active β_2AR. All other acidic residues were deprotonated (charged). Proteins were capped with peptide bonds to N-methylamide and acetyl groups. Cys341 was palmitoylated in every trajectory.

a Simulation 11 was initialized by taking a frame after 10 μs in simulation 1, which was in the alternative inactive state (see classification above), and removing the acidic proton from Asp79.

b Simulations 14 and 15 are older simulations that were run without the electrostatic adjustments to Asp, Glu, and Arg. Simulation 14 was run with Rcut = 12.79 Å, σ = 2.77 Å, and σ_s = 1.85 Å. Simulation 15 used a POPE bilayer; was

run with Rcut = 9.00 Å, σ = 2.01 Å, and σ_s = 1.41 Å; and was equilibrated for 5 ns.

Materials and Methods

Expression and Purification

The construct referred to as β_2AR-365N contains the human wild-type coding sequence of the β_2AR starting at Gly2 with a FLAG epitope tag at the amino terminus. A sequence containing a TEV cleavage site was introduced between the FLAG epitope and the start of the receptor. The third glycosylation site was removed by mutating N187E and the receptor sequence was terminated after Gly365 (Fig. S1A) (Rasmussen et al., 2007). The sequence was cloned into the pFastBac1 Sf9 insect cell expression vector (Invitrogen).

Mutations were made using the Quickchange Multi protocol from (Stratagene). We designed close to 40 base pair primers that contained the desired base pair substitution, after running PCR reactions, digestion of template DNA was performed using DpnI, as described in the Quickchange Multi protocol.

Recombinant baculovirus was made using the Bac-to-Bac system (Invitrogen). For some constructs the recombinant baculovirus was made by first subcloning the construct into the pVL1392 transfer vector and then producing virus using the BestBac system (Expression Systems).

Between 15-20 L of Sf9 cells were infected by baculovirus encoding one of the β_2AR constructs described above and grown in methionine deficient media (Expression system) with ^{13}C methyl labeled methionine (Cambridge Isotopes)

added into the media at 250 mg/L concentration. Cells were grown in suspension for 48 h at 27 ^{0}C. The cells were spun down and cell pellets were stored at -80 ^{0}C until used. Cell pellets were resuspended in a lysis buffer (10 mM Tris, 1 mM EDTA) without salt to lyse the cells by hypotonic lysis. The membranes were then spun down and solubilized using buffer (20mM HEPES pH 7.5, 100 mM NaCl) containing 1% DDM and protease inhibitors (Leupeptin at 100 μM and benzamidine at 1 mM). The solubilized receptor was loaded onto a M1 column (Sigma) and washed thoroughly with buffer containing 0.1% DDM and 2 mM $CaCl_2$, the receptor was then eluted of the column using an elution buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 0.2 mg/mL FLAG-peptide, 5 mM EDTA, 0.1% DDM, 0.01% cholesterol hemisuccinate).

The receptor was purified by ligand affinity chromatography using an alprenolol column as previously described (Kobilka, 1995). Before loading onto the column the concentration of NaCl in the sample was increased to 350 mM. After loading the sample onto the column the column was washed with washing buffer (20 mM HEPES pH 7.5, 0.1% DDM, 0.01% cholesterol hemisuccinate, 350 mM NaCl) until no protein was seen in the flow-through from the column. The protein was eluted with elution buffer (20 mM HEPES pH 7.5, 0.1% DDM, 0.001% cholesterol hemisuccinate, 350 mM NaCl, 300 uM alprenolol, 2 mM $CaCl_2$) and directly loaded on a M1 column (Sigma). The column was washed and the protein was eluted off the column with the buffers described above. Finally the protein was

dialysed twice against a buffer containing 20 mM HEPES pH 7.5, 100 mM NaCl, 0.1 % DDM and prepared in 98% D_2O.

The receptor was then concentrated with a 100 KDa cutoff Vivaspin concentrator (Vivascience) to a final volume of 270 µL and a final concentration of around 100 µM (see figure captions for exact concentrations). The final concentration of the NMR samples was determined by Bradford protein assay. The final NMR samples were loaded into Shigemi microtubes (Shigemi Inc.). Ligands were dissolved in perdeuterated dimethyl d_6-sulfoxide (Cambridge Isotopes) to a final concentration of 100 mM.

NMR

The HSQC pulse sequence used is a modified version of the basic HC-HSQC experiment as described in Bokoch et al(Bokoch et al., 2010). Data were acquired on the 800 MHz Varian INOVA spectrometer at Stanford Magnetic Resonance Laboratory (SMRL) and on the 900 MHz Bruker Avance 2 at the Central California 900 MHz NMR Facility.

Spectral widths used for the methionine experiments recorded on the 800 MHz spectrometer were 6033.1 Hz in the proton dimension (ω_1) and 4525.9 Hz in the carbon dimension (ω_2) centered at 20 ppm in the ^{13}C-dimension. Spectral widths used for the experiments recorded on the 900 MHz spectrometer were 10901.2

Hz in the proton dimension (ω_1) and 6788.9 Hz in the carbon dimension (ω_2) centered at 20 ppm in the ^{13}C-dimension.

For all the samples, except the β_2AR-365N-construct samples, 128 complex t_1 points were collected, for the β_2AR-365N sample only 64 complex t_1 points were collected (See Table S2). To allow temperature equilibration 64 steady state scans preceded data acquisition and a relaxation delay of 1.5 s were inserted to allow spin to relax back to equilibrium. The spectra were processed and visualized using NMRPipe/NMRDraw (Delaglio et al., 1995) software and NMRViewJ software, respectively. To eliminate horizontal noise from the very intense detergent methyl peak at 0.85 ppm [^{1}H] and 16.8 ppm [^{13}C] we used WET suppression of this methyl peak in some of the experiments (See Table S2). A common threshold, based upon a natural abundance peak at 1.4 ppm [^{1}H] and 19.25 ppm [^{13}C], was chosen for all spectra.

Competition- and saturation-binding

[^{3}H]DHA-competition binding was performed as previously described (Rasmussen et al., 2011b; Swaminath et al., 2002). The data were fitted to a single-site binding model using GraphPad prism. Saturation binding experiments were performed as described in Rosenbaum et al (Rosenbaum et al., 2007).

[^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding assays were performed as previously described (Zou et al., 2012).

Fluorescence spectroscopy experiments

Fluorescence spectroscopy experiments were performed on a FluoroLog-3 spectrofluorometer (Jobin Yvon Inc.). Bimane labeled β2AR-Δ5 was prepared as previously described (Yao et al., 2009a) and was used at a concentration of 50 nM. For association experiments, β2AR-Δ5 was incubated at 25 °C with a saturating concentration of BI-167107 (100 μM) and the change in fluorescence intensity at 435 nm was measured in 15 second intervals for 30 min. The resulting data was fit to a mono-exponential association reaction as no change in the apparent rate was observed for different concentrations of BI-167107, suggesting that the rate of change in fluorescence intensity is slower than the rate of BI-167107 association. For dissociation experiments, 1 μM BI-167107 was added to β2AR-Δ5, and the resulting mixture was incubated at 25 °C for 1 hr to reach equilibrium. The neutral antagonist alprenolol was added to 200 μM to begin the dissociation time course and fluorescence emission spectra were acquired over 2660 min. The resulting data was fit to a mono-exponential dissociation reaction. Data analysis was performed using Prism 5.0 (GraphPad software).

Molecular Dynamics Simulation Methods

In this work, we analyzed over 100 simulations of β_2AR totaling 588 μs. In addition to performing over 100 μs of previously unreported simulations (see Table S3), we performed new analyses on 89 simulations totaling 236 μs were

from a previously published study investigating drug binding to β_2AR (Dror et al., 2011b) and 42 simulations totaling 233 μs were from a study investigating the activation mechanism of the β_2AR (Dror et al., 2011a).

Initial coordinates and system setup. We prepared the all-atom molecular dynamics simulations of β_2AR listed in Table S3 following a protocol described previously (Dror et al., 2011a). Briefly, trajectories were initiated from a model based on coordinates from the crystal structure of either β_2AR bound to carazolol (PDB entry 2RH1) or β_2AR bound to BI-167107 and Nb80 (PDB entry 3P0G). Using Maestro (Schrödinger LLC, New York NY) and Dowser (Zhang and Hermans, 1996), the T4L was removed, hydrogens and structural waters were added, termini were capped, and Cys341 was palmitoylated. Carazolol was removed to prepare apoprotein, and Nb80 was removed to prepare β_2AR bound to BI-167107 only. Simulations retained Glu187 (N187E), a mutation used during crystallization to eliminate glycosylation. For some simulations, an additional point mutation was introduced (see Table S3). Prepared protein was placed in an equilibrated palmitoyl-oleoyl-phosphatidylcholine (POPC) bilayer and solvated with 0.15 M NaCl. Some sodium ions were removed to neutralize the system, and the lipid and water molecules clashing with the protein were removed. Carazolol-bound and apoprotein systems comprised approximately 82,000 atoms in an 95 × 95 × 90 Å periodic box, including roughly 15,000 water molecules, 240 lipid molecules, 41 chloride ions, and 36 sodium ions. BI-bound systems with Nb80 removed comprised approximately 76,000 atoms in a 90 × 90 × 95 Å box,

with roughly 15,000 water molecules, 210 lipid molecules, 39 chloride ions, and 33 sodium ions. Systems retaining Nb80 were elongated, having approximately 110,000 atoms in a 90 × 90 × 140 Å box, including roughly 26,000 water molecules, 210 lipid molecules, 70 chloride ions, and 63 sodium ions.

Force field parameters. We used the CHARMM27 parameter set with CMAP terms for protein molecules and salt ions, with the CHARMM TIP3P water model (MacKerell et al., 1998; Mackerell et al., 2004). Simulations 1–13 of Table S3 incorporated modified parameters for the Asp, Glu, and Arg side-chains (Piana et al., 2011). A modified CHARMM lipid force field (Klauda et al., 2010) was used. Force field parameters for carazolol, palmitoyl-cysteine, and BI-167107 were designed previously (Dror et al., 2009; Rosenbaum et al., 2011). Full parameter sets are available upon request.

Simulation protocols. Simulations were performed as described previously (Dror et al., 2011a). Each simulation consisted of a 50-ns equilibration run followed by a 2–15 μs production run, except for simulation 15 of Table S3. Systems were equilibrated in the NPT ensemble (310 K, 1 bar; Berendsen coupling scheme), with initial velocities sampled from the Boltzmann distribution; 5 kcal $\cdot$ mol^{-1} $\cdot$ Å^{-2} harmonic position restraints were applied to all non-hydrogen atoms of the protein and ligand, and tapered off linearly over 50 ns. Production simulations were initiated from the final snapshot of the equilibration run. Both equilibration and production runs were performed on Anton (Shaw et al., 2009;

Shaw et al., 2010), a special-purpose computer designed to accelerate standard molecular dynamics simulations by orders of magnitude. Simulation 15 of Table S3 followed an earlier equilibration protocol with restraints tapered off over 5 ns. All bond lengths to hydrogen atoms were constrained using M-SHAKE (Kräutler et al., 2001). An r-RESPA integrator (Tuckerman and Berne, 1992) was used with a time step of 2 fs, and long-range electrostatics were computed every 6 fs. Van der Waals and short-range electrostatic interactions were cut off at a specified distance (Rcut). Long-range electrostatics were computed using the *k*-space Gaussian split Ewald method (Shan et al., 2005) with a 64 × 64 × 64 grid and spreading parameters σ and σ_s. Parameters for simulations 1–13 of Table S3 were as follows: Rcut = 9.72 Å, σ = 2.17 Å, and σ_s = 1.50 Å for carazolol and unliganded simulations; Rcut = 9.45 Å, σ = 2.10 Å, and σ_s = 1.47 Å for BI-167107 simulations; and Rcut = 13.06 Å, σ = 2.82 Å, and σ_s = 1.98 Å for simulations with BI-167107 and Nb80.

Temperature-accelerated molecular dynamics. Temperature-accelerated molecular dynamics (TAMD) (Maragliano and Vanden-Eijnden, 2006) is a method for enhancing sampling along a chosen set of collective variables (CVs) (e.g., the distance between two groups of atoms). The acceleration is achieved by tethering a CV to a fictitious particle undergoing Brownian motion at a higher temperature. With a proper choice of parameters, the sampling of the chosen CV is accelerated such that the fictitious particles still obey Boltzmann statistics at the higher, fictitious temperature, $\bar{T}$, while the non-accelerated orthogonal

degrees of freedom of the real system remain properly distributed at the real temperature. In the TAMD simulations, the system was accelerated along the distance between the centers of mass of the backbone atoms of Leu75$^{2.46}$ and Pro323$^{7.50}$. For both TAMD simulations (simulations 5 and 6 in Table S3), the spring constant tethering the CV to the fictitious particle was 200 kcal mol^{-1} Å^{-2} and the friction coefficient for the fictitious particle was 200 ps kcal mol^{-1} Å^{-2}. The fictitious temperature, $k_B\overline{T}$, was 7.2 kcal/mol in one simulation and 9.5 kcal/mol in the other.

Analysis protocols. Trajectory snapshots were saved every 180 ps during production simulations. Time series data shown in figures were smoothed by applying a 9.9-ns (55-snapshot) running average.

Snapshots of the β_2AR were characterized as being in the active, intermediate, inactive, or alternative inactive state using alpha carbons in the G protein–binding site. A 9.9-ns (55 frame) running average was determined for the distance between Arg131$^{3.50}$ and Leu272$^{6.34}$ (R131–L272) and the distance between Leu75$^{2.46}$ and Pro323$^{7.50}$ (L75–P323.) The frame in the middle of the 9.9-ns window was classified as active if R131–L272 was at least 10 Å and L75–P323 was between 7.5 Å and 10 Å. The frame was classified as intermediate if R131–L272 was at least 10 Å and L75–P323 was less than 7.5 Å or at least 10 Å. The frame was classified as crystallographic inactive if R131–L272 was less than 10 Å and L75–P323 was at least 8 Å and alternative inactive if R131–L272 was less

than 10 Å and L75–P323 was less than 8 Å. This approach differs slightly from our previously published classification (Dror et al., 2011a) but is simpler and results in essentially the same classification.

Distance probability distributions shown in figures were computed by kernel density estimates with Gaussian kernels (SciPy 0.9.0 with default settings), using raw (unsmoothed) distance time series.

VMD (Humphrey et al., 1996) was used to visualize trajectories, and both VMD and PyMol (DeLano, 2002) were used to produce molecular renderings.

References

Bokoch, M.P., Zou, Y., Rasmussen, S.G., Liu, C.W., Nygaard, R., Rosenbaum, D.M., Fung, J.J., Choi, H.J., Thian, F.S., Kobilka, T.S.*, et al.* (2010). Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. Nature *463*, 108-112.

Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G., Thian, F.S., Kobilka, T.S., Choi, H.J., Kuhn, P., Weis, W.I., Kobilka, B.K.*, et al.* (2007). High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science *318*, 1258-1265.

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., and Bax, A. (1995). NMRPipe: a multidimensional spectral processing system based on UNIX pipes. Journal of biomolecular NMR *6*, 277-293.

DeLano, W. (2002). The PyMOL molecular graphics system (DeLano Scientific, San Carlos, CA). http://www.pymol.org.

Dror, R.O., Arlow, D.H., Borhani, D.W., Jensen, M.O., Piana, S., and Shaw, D.E. (2009). Identification of two distinct inactive conformations of the beta2-adrenergic receptor reconciles structural and biochemical observations. Proc Natl Acad Sci U S A *106*, 4689-4694.

Dror, R.O., Arlow, D.H., Maragakis, P., Mildorf, T.J., Pan, A.C., Xu, H., Borhani, D.W., and Shaw, D.E. (2011a). Activation mechanism of the beta2-adrenergic receptor. Proc Natl Acad Sci U S A *108*, 18684-18689.

Dror, R.O., Pan, A.C., Arlow, D.H., Borhani, D.W., Maragakis, P., Shan, Y., Xu, H., and Shaw, D.E. (2011b). Pathway and mechanism of drug binding to G-protein-coupled receptors. Proc Natl Acad Sci U S A *108*, 13118-13123.

Humphrey, W., Dalke, A., and Schulten, K. (1996). VMD: visual molecular dynamics. Journal of molecular graphics *14*, 33-38, 27-38.

Klauda, J.B., Venable, R.M., Freites, J.A., O'Connor, J.W., Tobias, D.J., Mondragon-Ramirez, C., Vorobyov, I., MacKerell, A.D., Jr., and Pastor, R.W. (2010). Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. The journal of physical chemistry B *114*, 7830-7843.

Kobilka, B.K. (1995). Amino and carboxyl terminal modifications to facilitate the production and purification of a G protein-coupled receptor. Anal Biochem *231*, 269-271.

Kräutler, V., van gunsteren, W.F., and Hünenberger, P.H. (2001). A fast SHAKE algorithm to solve distance constraint equations for small molecules in molecular dynamics simulations. J Comput Chem *22*, 501-508.

MacKerell, A.D., Bashford, D., Bellott, Dunbrack, R.L., Evanseck, J.D., Field, M.J., Fischer, S., Gao, J., Guo, H., Ha, S.*, et al.* (1998). All-Atom Empirical Potential for Molecular Modeling and Dynamics Studies of Proteins†. The Journal of Physical Chemistry B *102*, 3586-3616.

Mackerell, A.D., Jr., Feig, M., and Brooks, C.L., 3rd (2004). Extending the treatment of backbone energetics in protein force fields: limitations of gas-phase quantum mechanics in reproducing protein conformational distributions in molecular dynamics simulations. Journal of computational chemistry *25*, 1400-1415.

Maragliano, L., and Vanden-Eijnden, E. (2006). A temperature accelerated method for sampling free energy and determining reaction pathways in rare events simulations. Chem Phys Lett *426*, 168-175.

Piana, S., Lindorff-Larsen, K., and Shaw, D.E. (2011). How robust are protein folding simulations with respect to force field parameterization? Biophysical journal *100*, L47-49.

Rasmussen, S.G., Choi, H.J., Fung, J.J., Pardon, E., Casarosa, P., Chae, P.S., Devree, B.T., Rosenbaum, D.M., Thian, F.S., Kobilka, T.S*., et al.* (2011a). Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. Nature *469*, 175-180.

Rasmussen, S.G., Choi, H.J., Rosenbaum, D.M., Kobilka, T.S., Thian, F.S., Edwards, P.C., Burghammer, M., Ratnala, V.R., Sanishvili, R., Fischetti, R.F*., et al.* (2007). Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. Nature *450*, 383-387.

Rasmussen, S.G., Devree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., Kobilka, T.S., Thian, F.S., Chae, P.S., Pardon, E., Calinski, D*., et al.* (2011b). Crystal structure of the beta(2) adrenergic receptor-Gs protein complex. Nature.

Rosenbaum, D.M., Cherezov, V., Hanson, M.A., Rasmussen, S.G., Thian, F.S., Kobilka, T.S., Choi, H.J., Yao, X.J., Weis, W.I., Stevens, R.C*., et al.* (2007). GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. Science *318*, 1266-1273.

Rosenbaum, D.M., Zhang, C., Lyons, J.A., Holl, R., Aragao, D., Arlow, D.H., Rasmussen, S.G., Choi, H.J., Devree, B.T., Sunahara, R.K*., et al.* (2011). Structure and function of an irreversible agonist-beta(2) adrenoceptor complex. Nature *469*, 236-240.

Shan, Y., Klepeis, J.L., Eastwood, M.P., Dror, R.O., and Shaw, D.E. (2005). Gaussian split Ewald: A fast Ewald mesh method for molecular simulation. The Journal of chemical physics *122*, 54101.

Shaw, D.E., Dror, R.O., Salmon, J.K., Grossman, J.P., Mackenzie, K.M., Bank, J.A., Young, C., Deneroff, M.M., Batson, B., Bowers, K.J*., et al.* (2009). Millisecond-scale molecular dynamics simulations on Anton. In Proceedings of the Conference on High Performance Computing Networking, Storage and Analysis (Portland, Oregon: ACM), pp. 1-11.

Shaw, D.E., Maragakis, P., Lindorff-Larsen, K., Piana, S., Dror, R.O., Eastwood, M.P., Bank, J.A., Jumper, J.M., Salmon, J.K., Shan, Y*., et al.* (2010). Atomic-level characterization of the structural dynamics of proteins. Science *330*, 341-346.

Swaminath, G., Steenhuis, J., Kobilka, B., and Lee, T.W. (2002). Allosteric modulation of beta2-adrenergic receptor by Zn(2+). Molecular pharmacology *61*, 65-72.

Tuckerman, M., and Berne, B.J. (1992). Reversible multiple time scale molecular dynamics. J Chem Phys *97*, 1990-2001.

Yao, X.J., Velez, R.G., Whorton, M.R., Rasmussen, S.G., DeVree, B.T., Deupi, X., Sunahara, R.K., and Kobilka, B. (2009a). The effect of ligand efficacy on the formation and stability of a GPCR-G protein complex. ProcNatlAcadSciUSA *106*, 9501-9506.

Yao, X.J., Velez Ruiz, G., Whorton, M.R., Rasmussen, S.G., DeVree, B.T., Deupi, X., Sunahara, R.K., and Kobilka, B. (2009b). The effect of ligand efficacy on the formation and stability of a GPCR-G protein complex. Proceedings of the National Academy of Sciences of the United States of America *106*, 9501-9506.

Zhang, L., and Hermans, J. (1996). Hydrophilicity of cavities in proteins. Proteins *24*, 433-438.

Zou, Y., Weis, W.I., and Kobilka, B.K. (2012). N-terminal t4 lysozyme fusion facilitates crystallization of a g protein coupled receptor. PloS one *7*, e46039.