Visualization of arrestin recruitment by a G–protein–coupled receptor

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G-protein-coupled receptors (GPCRs) are critically regulated by β-arrestins, which not only desensitize G-protein signalling but also initiate a G-protein-independent wave of signalling1,2. A recent surge of structural data on a number of GPCRs, including the β2 adrenergic receptor (β2AR)–G-protein complex, has provided novel insights into the structural basis of receptor activation3–10. However, complementary information has been lacking on the recruitment of β-arrestins to activated GPCRs, primarily owing to challenges in obtaining stable receptor–β-arrestin complexes for structural studies. Here we devised a strategy for forming and purifying a functional human β2AR–β-arrestin-1 complex that allowed us to visualize its architecture by single-particle negative-stain electron microscopy and to characterize the interactions between β2AR and β-arrestin 1 using hydrogen–deuterium exchange mass spectrometry (HDX-MS) and chemical crosslinking. Electron microscopy two-dimensional averages and three-dimensional reconstructions reveal bimodal binding of β-arrestin 1 to the β2AR, involving two separate sets of interactions, one with the phosphorylated carboxy terminus of the receptor and the other with its seven-transmembrane core. Areas of reduced HDX together with identification of crosslinked residues suggest engagement of the finger loop of β-arrestin 1 with the seven-transmembrane core of the receptor. In contrast, focal areas of raised HDX levels indicate regions of increased dynamics in both the N and C domains of β-arrestin 1 when coupled to the β2AR. A molecular model of the β2AR–β-arrestin signalling complex was made by docking activated β-arrestin 1 and β2AR crystal structures into the electron microscopy map densities with constraints provided by HDX-MS and crosslinking, allowing us to obtain valuable insights into the overall architecture of a receptor–arrestin complex. The dynamic and structural information presented here provides a framework for better understanding the basis of GPCR regulation by arrestins.

To facilitate the isolation of a stable β2AR–β-arrestin complex, we used a modified β2AR construct with its C terminus replaced by that of the arginine vasopressin type 2 receptor (AVPR2). This chimaeric receptor (β2ARVPR2) maintains pharmacological properties identical to that of the β2AR, but it binds β-arrestins with higher affinity compared to wild-type β2AR11. We co-expressed β2ARVPR2, β-arrestin 1 (1–393) and GRK2CAAX (GRK2 with a membrane-tethering prenylation signal) in insect cells followed by agonist stimulation and affinity purification through the Flag-tagged receptor (Fig. 1a) However, since the isolation of a stable complex was still not feasible (Fig. 1b, lanes 1 and 2), we explored the possibility of stabilizing the complex by adding Fab30, an antibody fragment we previously reported that selectively recognizes and stabilizes the active conformation of β-arrestin 1 (ref. 13). Indeed, incubation of Fab30 with the complex in the membrane resulted in a robust purification of the β2ARVPR2–β-arrestin-1 complex (Fig. 1b, lanes 5 and 6), whereas a non-specific Fab (referred to as Fab1) did not support complex stabilization (Fig. 1b, lanes 3 and 4). Complex isolation was only possible in response to an agonist (BI-167107) and not an inverse agonist (ICI-118551) (Fig. 1b, lanes 5 and 6). Furthermore, the efficiency of complex purification using this approach directly mirrors the pharmacological efficacy of the ligand used to stimulate the cells (Fig. 1c). While stimulation of cells with inverse agonists does not yield detectable co-purification of β-arrestin 1, agonists robustly stabilize the complex and partial agonists yield co-purification of β-arrestin 1 at moderate levels. Moreover, the efficiency of complex formation also corresponds to the ligand occupancy of the receptor as reflected by the increasing amount of β-arrestin 1 co-purification with increasing agonist concentrations (Extended Data Fig. 1a, b). The direct correlation of ligand efficacy and occupancy with purification efficiency reflects the fact that this approach yields a complex that depends on both activated receptor conformation and receptor phosphorylation. The purified β2ARVPR2–β-arrestin–Fab30 complex also exhibited a robust interaction with the purified clathrin terminal domain compared to β-arrestin 1 alone, suggesting that β-arrestin 1 in this complex is in a physiologically relevant and functional conformation (Extended Data Fig. 2)14–16. Importantly, this strategy allowed preparative scale purification of a highly stable β2ARVPR2–β-arrestin–1–Fab30 complex as assessed by analytical size exclusion chromatography (Fig. 1a, bottom right, green trace, and Extended Data Fig. 1c). In addition to the Fab30-stabilized β2ARVPR2–β-arrestin-1 complex, we were also able to obtain equally stable β2ARVPR2–β-arrestin-1 complexes using the single-chain variable fragment of Fab30 (ScFv30) (Fig. 1a, bottom right, blue trace).

The interaction of β-arrestins with activated GPCRs is proposed to involve two sequential steps17. First, the phosphorylated C terminus of activated GPCRs is thought to engage the N domain of β-arrestins, a high-affinity charge–charge interaction primarily mediated between the phosphates on the receptor tail and basic residues on β-arrestins18,19. This first engagement is hypothesized to facilitate activating conformational changes in β-arrestin, leading in turn to additional interactions with the transmembrane core of the receptor17. To obtain dynamic structural information on the receptor–β-arrestin complex, we carried out HDX-MS analysis on the purified assembly18,19. In addition to the β2ARVPR2–β-arrestin–1–Fab30 complex, we used the AVPR2C-termin}

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phosphopeptide (V2Rpp)–β-arrestin-1–Fab30 complex as a reference to extract specific information about the core interaction between the receptor and β-arrestin 1.

We observed a reduction in the HDX rate in the three major loops—the finger loop (55%), the middle loop (16%) and the lariat loop (23%)—of β-arrestin 1 when we compared the HDX-MS profile of the β2V2R–β-arrestin-1–Fab30 complex with that of the V2Rpp–β-arrestin-1–Fab30 complex (Fig. 2 and Extended Data Fig. 3a). Thus, these regions, and especially the finger loop, are likely to be buried (or have reduced solvent exposure) in the β2V2R–β-arrestin-1–Fab30 complex, probably through an intricate engagement with the transmembrane receptor core. This finding is consistent with previous electron paramagnetic resonance (EPR) studies on rhodopsin–arrestin interactions, which revealed a crucial involvement of the finger loop of arrestin with the core of rhodopsin17,20–22. Interestingly, several regions in both the N and the C domains of β-arrestin 1, in contrast, reveal enhanced HDX rates, indicating that they become more dynamic upon interaction of β-arrestin with the agonist-bound phosphorylated receptor. This observation suggests that the core interaction between β2V2R and β2V2R probably has long-range effects on β-arrestin 1 structure. Previous studies mapping interactions between GPCRs and arrestins suggested that receptors may also interact with the broad concave surfaces of the N and C domains of arrestins21,23–25. However, peptides representing these surfaces are not fully represented in our HDX-MS studies, thus limiting our ability to detect these interactions. We also note that our previously published high-affinity agonist radioligand binding data on the T4 lysozyme (T4L)–β2V2R–β-arrestin-1–Fab30 complex in membranes, which provides a readout of the fully engaged β-arrestin conformation, suggested that approximately 32% of the receptor is in a high-affinity agonist binding state23. This indicates that our HDX-MS data represent an average of two mixed complex populations, one with fully engaged β-arrestin 1 with the receptor and the other displaying partially engaged β-arrestin 1.

Our previous crystal structure of V2Rpp bound to activated β-arrestin 1 revealed a marked repositioning of the finger loop compared to when it is bound to the inactive β-arrestin 1, presumably because it is primed to engage with the transmembrane core of the activated receptor23. To test this we carried out MS-based mapping of the T4L–β2V2R–β-arrestin 1 interface using the homobifunctional, primary amine reactive chemical crosslinker disuccinimidyl adipate (DSA). We found that Lys 77 on β-arrestin 1 (towards the distal end of the finger loop) crosslinks with Lys 235 in the third intracellular loop of the β2AR (Extended Data Fig. 3b–e). These findings are in line with previously published biochemical and biophysical data suggesting an intricate interaction of the receptor core and the finger loop in arrestins. As an additional control for the close proximity of these residues, we created a series of mutants with single cysteine substitutions around Lys 235 in the N-terminal end of the third intracellular loop of the β2V2R (amino acids 231–236) and in the finger loop around Lys 77 of β-arrestin 1 (amino acids 75–79) and evaluated the formation of disulphide-trapped complexes in pairs of receptor and β-arrestin 1 mutants. Consistent with our chemical crosslinking data, cysteines engineered at position 235 of the receptor and at position 78 in β-arrestin 1 yielded the most robust disulphide-trapped complex, suggesting a close proximity of these two residues in the complex (Extended Data Fig. 4). Taken together these findings demonstrate a direct interaction of the finger loop with the receptor core.

We next employed single-particle electron microscopy (EM) to examine the architecture and conformational dynamics of β2V2R–β-arrestin 1 complexes. Owing to the asymmetric nature and small size of these complexes (~150 kilodalton (kDa) and ~125 kDa for the Fab and ScFv complexes, respectively) characterization attempts with cryo-EM were
regions with significant HDX rate changes are enlarged in the ‘hanging’ arrestin conformation based on the V2Rpp–β-arrestin-1. a. Differential HDX rates of β-arrestin 1 in the β2V2–β-arrestin-1–Fab30 versus V2Rpp–β-arrestin-1–Fab30 complexes were mapped onto the β-arrestin-1 crystal structure (Protein Data Bank (PDB) accession 4JQ1). Blue and red colour coding indicate the β-arrestin-1 regions that exchange slower and faster, respectively, in the β2V2–β-arrestin-1–Fab30 complex when compared to the V2Rpp–β-arrestin-1–Fab30 complex. Boxed regions with significant HDX rate changes are enlarged in a–c. The HDX rates of the finger loop (residues 63–75) (a), middle loop (residues 129–140) and lariat loop (residues 274–300) (b) became slower, whereas those of other regions, for example, β-strand I, II and X in the N domain (c) became faster in the β2V2–β-arrestin-1–Fab30 complex when compared to the V2Rpp–β-arrestin-1–Fab30 complex.

not successful and we thus applied negative-stain EM, which provides adequate contrast for alignment of small particle projections. This approach also enabled a direct comparison with our earlier negative-stain EM analysis of the β2AR–GFPs protein complex. As in that work, here we used a T4L fusion at the N terminus of the receptor (referred to as T4L–β2V2) to provide a marker for the receptor orientation. The negative-stain EM visualization showed a monodisperse particle population (Fig. 3a and Extended Data Fig. 5) and we applied reference-free alignment and classification to obtain two-dimensional averages of the complex.

The majority of averages of the β2V2–β-arrestin-1–Fab30 complex revealed distinct projection profiles of an ovoid density, attributed to the receptor in partially flattened detergent micelle, with an attached T-like density attributed to the Fab30–β-arrestin-1 complex (Fig. 3b and Extended Data Fig. 6a). Comparisons with averages of the β2V2–β-arrestin-1–ScFv30 complex identify the Fab30 density engaging the middle of β-arrestin 1, in agreement with our recent crystal structure of β-arrestin-1–Fab30 co-crystallized with the V2Rpp (Fig. 3b and Extended Data Fig. 6b). In this conformation β-arrestin 1 appears to hang off the receptor via a single point interaction presumably involving only the flexible V2Rpp fused on β2AR. The flexible nature of this interaction is further supported by the variable receptor orientation in these averages, as judged by the T4L domain positioning. It is possible that this ‘hanging’ arrestin conformation based on the V2Rpp–β-arrestin-1 interaction represents a transient intermediate step in the recruitment process that has been stabilized by Fab30. Strikingly, we also observe a substantial number of class averages, representing ~ 37% of particles, in which β-arrestin 1 forms a much more extensive interface with the receptor, employing roughly the opposite face of the Fab30 binding region (Fig. 3b, bottom). The observed fraction of particles displaying the extensive interface is in agreement with our previous radioligand binding results on the T4L–β2V2–β-arrestin-1–Fab30 complex in membranes, which suggested that approximately 32% of the receptor is in a high-affinity agonist binding state. This observation also raised the possibility that β-arrestin 1 fully engages the receptor through a second set of weak interactions.

To stabilize this weak interaction, we developed an approach whereby the β2V2–β-arrestin-1–Fab30/ScFv30 complex is crosslinked by exposure to a glutaraldehyde–containing buffer zone while migrating through a size exclusion column (Extended Data Fig. 7a). This method facilitated near complete crosslinking of preformed complexes at relatively high concentrations and simultaneously enabled the isolation of highly monodisperse sample (Extended Data Figs 7b, c, 8, 9).

EM classification and averaging of the crosslinked β2V2–β-arrestin-1–Fab30/ScFv30 complexes revealed distinct views of a uniform particle architecture, suggesting that crosslinking stabilized a single complex conformer (Fig. 3c). More importantly, the averages show that arrestin interacts extensively with the receptor in a configuration that appears very similar to the one observed in the smaller fraction (~37%) of the
native complex. The conformational stabilizing action of the crosslinking is also evidenced by the consistent position of the T4L projection profile, in contrast to the variable positioning observed in averages of the native complex. To better characterize the β₂V₂R–β-arrestin-1 assembly, we employed the random conical-tilt approach⁹ to calculate low-resolution three-dimensional maps (~29 Å) from selected classes of the crosslinked complex (Extended Data Fig. 10). The three-dimensional reconstructions show distinct densities for the main complex components, in full agreement with our domain assignment in the two-dimensional projections averages (Fig. 4a and Extended Data Fig. 10). The receptor-containing region appears ovoid due to the large micelle ‘belt’ characteristic of the lauryl maltose neopentyl glycol (LMNG) detergent, as we also observed in the case of the β₂AR–Gαs complex⁹. A protrusion on one end of the receptor–micelle globular density represents the T4L domain that marks the receptor extracellular region. On the opposite side, the β-arrestin 1 density lies longitudinally on the receptor, engaging roughly the opposite side of the Fab30-interacting region. In this configuration, both β-arrestin domains appear to engage the receptor but one of the domains lies mostly outside the interacting zone.

The HDX-MS, chemical crosslinking and disulphide trapping data allowed us to constrain the modelling of the T4L–β₂AR and β-arrestin-1–Fab30 crystal structures within the density of the EM three-dimensional maps and generate a low-resolution model for the overall conformation of the β₂AR–β-arrestin-1 complex (Fig. 4b). This model can accommodate limited rotations and translations of the individual crystal structures, which are also expected to undergo conformational changes upon complex formation. Lys 77 of β-arrestin 1 in our model is placed in close proximity to β₂AR Lys 235, which is located at the end of a helical extension of transmembrane (TM)5 in the β₂AR–Gαs complex¹⁰. This prompted us to use this structure to model the β₂AR–β-arrestin-1 complex. In our model, β-arrestin 1 forms an extensive interface with the receptor through its N-terminal domain, which includes interactions with the phosphorylated receptor tail and the insertion of the finger loop directly in the receptor core, involving the space between TM3, 5 and 6. We note that the finger loop insertion is probably associated with outward shifts in the positioning of TM helices 3, 5 and 6 and also helix 8. The middle and lariat loops of β-arrestin 1 do not participate in major interactions but reside close to the interface, as suggested by the modest reduction in their HDX rates observed by HDX-MS (Fig. 2c). The relative positioning of these loops is also in agreement with previous EPR studies on visual arrestin in complex with activated and phosphorylated rhodopsin²⁰,²¹.

In regards to β₂AR, TM5 and the third intracellular loop in this model locate above the concave β-sheet region of the N-terminal domain of

Figure 4 | Structural model of the β₂V₂R–β-arrestin-1–Fab30 complex. a, Views of the T4L–β₂V₂R–β-arrestin-1–Fab30 complex three-dimensional reconstruction with modelled T4L–β₂AR (green–orange; PDB accession 3SN6), β-arrestin-1 (blue; PDB accession 4JQI), and Fab30 (purple; PDB accession 4JQI) crystal structures. The density surrounding β₂V₂R represents the LMNG detergent micelle and is marked by ‘m’. βarr, β-arrestin. Scale bar, 5 nm. b, Views of the β₂V₂R–β-arrestin-1 interface within the dashed line square of a. Areas of β-arrestin 1 with reduced HDX are shown in cyan. Crosslinked Lys 235 of β₂V₂R and Lys 77 of β-arrestin 1 are highlighted. c, Illustration of the two-step GPCR–β-arrestin-1 interaction using surface representations of the structures of β₂AR (orange), the phosphorylated C-terminal tail of V₂R (yellow) and β-arrestin 1 (blue). The C-terminal portion of the V₂R peptide (Glu 335–Asp 367) in the right model is positioned as found in the β-arrestin-1–Fab30–V₂Rpp structure (PDB accession 4JQI), whereas the N-terminal portion (Ala 342–Pro 352) was remodelled to connect to the β₂AR C terminus.
β-arrestin 1. The placement of these receptor elements implies that the N terminus of V2Rpp cannot be in the position observed in the crystal structure of V3R–β-arrestin–1–Fab30 (ref. 13), suggesting that the V2R C terminus in the β2V3R chimeraic receptor is mobile and repositions itself markedly upon β-arrestin–1 interaction with the receptor core. In contrast to the N-terminal domain, the C-terminal domain of β-arrestin 1 lies mostly outside the interaction zone, apart from the loop of residues 242–246 that is at interacting distance from the short α-helical segment connecting TM3 and TM4 of β2V3R. This observation is intriguing considering that mutation of the residues distal to the DRY motif (at the end of TM3) have been reported to directly affect β-arrestin recruitment for a number of GPCRs including the β2AR23.

Our results suggest that arrestin probably employs a biphasic mechanism to engage the receptor (Fig. 4c). The first phase involves an interaction between the phosphorylated C-terminal tail of the receptor and the N-terminal domain of arrestin. Given the flexibility and the length of the C-terminal receptor tail, it is expected to act like a fishing line, sampling a wide interaction space at a high rate. The second point of interaction appears weak and involves primarily the insertion of the finger loop within the receptor core, resulting in a longitudinal arrangement of arrestin on the receptor (Fig. 4a, c). This arrangement would most certainly preclude GPCR engagement of G-protein heterotrimers, thereby blocking classical GPCR signalling and inducing desensitization. While it is not yet clear whether the single point interaction resulting in a hanging arrestin configuration has other physiological functions, it seems possible that these might involve recruitment and complex formation with components of the receptor endocytosis and signalling machinery such as clathrin and Gβγ.

**METHODS SUMMARY**

β2V3R, β-arrestin 1 and GRK2CAAX were co-expressed in SP9 cells. Sixty-six hours post-infection, cells were stimulated with the high-affinity agonist BI-167107 for 30 min at 37°C. Cells were harvested and lysed by douncing, followed by incubation with purified Fab30. One hour post-incubation, cells were solubilized and purified on a Flag M1 affinity column followed by size exclusion chromatography. The purified complex was subjected to HDX-MS analysis by incubating it with D2O for various time points followed by pepsin digestion and liquid chromatography (LC)/MS-based identification of peptides. Purified T4L–β2V3R–β-arrestin–1–Fab30/ScFv30 complex was embedded in negative stain and visualized by EM. EM two-dimensional reconstructions of the complexes were obtained by ISAC28 and three-dimensional reconstructions were obtained through the random conical-tilt method28.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Author Contributions** A.K.S. and G.H.W. performed HDX-MS and crosslinking mapping experiments with assistance from P.T.-S. R.I.R. and L.-Y.H. provided the linker sequence, vector and advice on the HDX-MS experiments assisted by S.L., J.Q., A.W.K. and A.B., performed the crosslink biochemistry and pharmacology characterization of the complex. G.H.W. performed the HDX-MS experiments assisted by S.L., J.Q., A.W.K. and A.B., provided the crosslinking mapping experiments assisted by J.Q. and A.W.K., and designed the disulphide trapping experiments carried out by M.C. V.L.W. Jr supervised the initial phase of the HDX-MS experiments. C.-R.L., L.-G., J.-M.S. and X.C. synthesized the high-affinity agonist BI-167107. R.H. and S.S.S. contributed the linker sequence, vector and advice on ScFv conversion and expression. X.J.Y. and B.U.K. contributed in assessing various methods of complex formation. P.A.P. provided advice on implementation of ISAC28 and three-dimensional reconstructions were obtained through the random conical-tilt method. 28.

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**Data availability** Readers are welcome to retain an version of the paper. Correspondence and requests for materials should be addressed to R.J.L (leko001@receptor-biol.duke.edu), B.K.K. (kobika@stanford.edu) or G.S. (skinchj@umich.edu).

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ScFv30 was expressed using the same protocol as Fab30. It was either purified on Ni-NTA resin or used directly as periplasmic extract to stabilize the T4L–β2R–β-arrestin-1 complex.

**Gluaraldehyde crosslinking of the preformed complex.** In order to mildly cross-link the complex, an ‘on-column’ crosslinking method was used. First, a bolus of glutaraldehyde was injected to a pre-equilibrated Superdex 200 (10/300 global in 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.01% LMNG, 100 mM BI-167107) column and run at 0.25 ml min⁻¹ for 20 min (that is, a total of 5 ml buffer). Subsequently, the column flow was paused, and the injection loop was flushed using buffer followed by injection of purified complex (200 μl volume, at 5–10 μM concentration). Subsequently, the column was run at 0.25 ml min⁻¹ and 0.3 ml fractions were collected. Crosslinking efficiency was visualized by running the individual fractions on a 4–20% SDS gel, followed by staining with ‘Simply Blue’ stain. Various concentrations of glutaraldehyde were tested in order to identify an optimal concentration (0.25% in this case) that yields maximal crosslinking efficiency and minimal protein aggregation.

**HDX-MS Fragmentation tuning for β-arrestin 1.** We first performed fragmentation tuning experiments in order to optimize experimental conditions to achieve the best peptide fragmentation pattern for β-arrestin 1. In brief, 3 μl of β-arrestin 1 protein solution was diluted with 9 μl of a buffer containing 150 mM NaCl, 8.3 mM Tris-NC buffer in H₂O and then divided into equal sized aliquots. Each aliquot was mixed individually with 18 μl of a series of quench solutions containing 0.8% formic acid, 16.6% glycerol, and various concentrations of GuHCl (ranging from 0.05 to 4 M) and TCEP (ranging from 0.015 to 0.5 M). The protein–buffer–quench solution mixtures were incubated at 0°C for 1 min, frozen on dry ice and then stored at −80°C until subjected to on-line pepsim digestion and liquid chromatography (LC)/MS analysis. Protein fragmentation maps under different conditions were generated and compared. The condition that produced the best peptide fragmentation pattern (the best coverage along the amino acid sequence, the most number of fragments, and highest number of high quality peptides) was used for all the HDX-MS experiments described in this study.

**HDX experiments.** The V₃₉₆₋β-arrestin1–Fab30 and T4L–β₂R–β-arrestin1–Fab30 complexes were prepared as described earlier. Free β-arrestin 1 protein was used as a control. For each protein complex, three sets of samples were prepared: (1) non-deuterated (ND), (2) fully deuterated (FD), and (3) time-dependent on-exchange samples. A protein:buffer:quench solution ratio of 1:3:6 (volume) was used for all sample preparations. The FD sample sets were prepared by mixing protein samples with D₂O buffer (0.8% Formic acid in 100% D₂O) and incubating at room temperature for 12 h before quenching. The ND sample sets were prepared using a similar procedure with H₂O buffer (150 mM NaCl, 8.3 mM Tris-HCl, pH 7.2) in H₂O without the incubation step. The on-exchange sample sets were prepared by adding 3 volumes of D₂O buffer (150 mM NaCl, 8.3 mM Tris-HCl, pH 7.2) in D₂O at 0°C and incubating for varying time points (10 s, 10² s, 10³ s, 10⁴ s and 10⁵ s). Six samples of ice-cold quench solution were then added to each sample, followed by snap-freezing on dry ice and storage at −80°C.

**On-line pepsim digestion, LC/MS analysis and data processing.** Samples prepared as described earlier were thawed right before the on-line pepsim digestion at 0°C using a cyrogenic autosampler and immediately passed over an immobilized porcine collagen column (16 μl bed volume). Peptide fragments were collected contemporaneously on a C₁₈ trap desalting column, separated by a Magic C₁₈ AQ column using a linear gradient of acetonitrile from 6.4 to 38.4% over 30 min, and followed by LC/MS analysis using an Orbitrap Elite mass spectrometer (Thermo Scientific). Both MS1 and MS2 spectra were collected using the data-dependent acquisition mode. Peptide identification was performed using LC/MS data sets collected from the above database search experiment with ProteinProphet (Thermo Fisher Scientific). The SEQUEST database search results were submitted to D克斯M Explorer (version 2.0, Sierra Analytics), filtered using several threshold parameters to create an initial peptide pool. The quality of the MS1 data for each filtered peptide was then checked by assigning an initial quality score by DックスM Explorer software, followed by a quality control process which included manual inspection of peptide sequence for potential artifacts and modifying/improving the quality score. Only peptides with high quality in the MS1 spectra were kept in the final peptide pool. The retention times and m/z ranges of each peptide from the final peptide pool were manually verified and adjusted across all LC/MS data sets from on-exchange samples and FD samples to ensure that D克斯M Explorer had selected the correct peptide for all experiments. Results from FD samples were used to monitor the back-exchange rates during on-line pepsim digestion and LC/MS analysis. The centroids of isotopic envelopes of non-deuterated, partially deuterated and fully deuterated peptides were measured using DックスM Explorer, and then to deuteration level with corrections for back-exchange. A deuteration accumulation plot was created for each peptide as a further quality check and data refinement analysis. The peptide sequence determination was achieved by the above database search method.
process. Rainbow maps were generated and DXMS data comparison was performed using different macros in Excel.

**Chemical crosslinking and mass spectrometry**

**Chemical crosslinking reaction.** In order to identify a potential interaction interface between the β2V2R and β-arrestin 1, we performed chemical crosslinking experiments on the preformed T4L–β2V2R–β-arrestin 1–Fab30 complex. To facilitate the identification of crosslinked peptides, we used an equimolar mixture of light (13C6) and heavy (13C6) DSA (a homobifunctional amine-reactive crosslinker, spacer arm = 8.6 Å) to crosslink the complex. The crosslinked peptides were characterized with ‘doublet’ peak signatures in mass spectra. The T4L–β2V2R–β-arrestin 1–Fab30 complex was prepared in buffer containing 20 mM HEPES, 150 mM NaCl, 0.01% LMNG, 100 mM TCEP. Crosslinker solution was freshly prepared in DMSO at a concentration of 10 mM. For the crosslinking reaction, 100-fold excess of DSA–C6:DSA–C6 equimolar mixture was added to the T4L–β2V2R–β-arrestin 1–Fab30 complex solution and incubated for 30 min at 25 °C. Unreacted crosslinker was quenched by incubation with 100 mM ammonium bicarbonate for 20 min. The crosslinked T4L–β2V2R–β-arrestin 1–Fab30 complex was separated by SDS–PAGE, and the corresponding protein band was in-gel digested overnight at 37 °C with trypsin at a final concentration of 10 ng μl−1 and subjected to LC/MS analysis.

**LC/MS analysis and crosslinked peptide identification.** LC/MS analyses were performed on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) with a Finnigan Nanospray II electrospray ionization source. Tryptic peptides were injected onto a 75 μm×150 mm BEH C18 column (particle size 1.7 μm, Waters) and separated using a Waters nano ACQUITY Ultra Performance LC (UPLC) System. The LTQ Orbitrap XL was operated in the data dependent mode using the TOP10 strategy. In brief, each scan cycle was initiated with a full MS scan of high mass accuracy 375–1,800 and automatic gain control (AGC) target of 1000, which was followed by MS/MS scans (AGC target 5,000; threshold 3,000) in the linear ion trap on the ten most abundant ions. The LC/MS/MS data were acquired in DDA experiments with 100–fold excess of DSA–C6:DSA–C6 equimolar mixture. LC/MS/MS data were first converted to mgf files using Mascot Distiller or Proteowizard 3.0 and saved under a pLink master directory, where a composite database containing protein sequences of T4L–β2AR, T4L–β-arrestin 1 and Fab30 in FASTA format is also stored. In the pLink configuration file, both the names and paths of the mgf and database file were indicated correspondingly. The filter for precursor mass accuracy was set to 1 ppm, and automatic gain control (AGC) target of 5,000, which was followed by MS/MS scans (AGC target 5,000; threshold 3,000) in the linear ion trap on the ten most abundant ions. Selected ions were dynamically excluded for 30 s. Singly charged ions were excluded from MS/MS analysis. The LC/MS/MS data were processed and analysed by pLink software. In brief, acquired raw data from LTQ Orbitrap XL mass spectrometer were first converted to mgf files using Mascot Distiller or Proteowizard 3.0 and saved under a pLink master directory, where a composite database containing protein sequences of T4L–β2AR, T4L–β-arrestin 1 and Fab30 in FASTA format is also stored. In the pLink configuration file, both the names and paths of the mgf and database file were indicated correspondingly. The total numbers of fixed modifications was set to 1 for cysteine carbamidomethylation. The name and number of crosslinkers used in the experiment were indicated as well in the configuration file. The filter for precursor mass accuracy was set to ±10 ppm. After pLink analysis, identified crosslinked peptides were manually inspected in the raw file for the appearance of a pair of doublet MS peaks.

**Disulphide trapping of β2V2R and β-arrestin 1 complex.** Cysteine mutants of the β2V2R and β-arrestin 1 co-expressed in HEK-293 cells. Forty-eight hours post-transfection, cells were stimulated with the β2AR agonist isoprotrenol (10 μM) and treated with H2O2 (1 mM) at different time points to induce the formation of disulphide bond and trap the complex. Cells were washed and lysed in lysis buffer (50 mM HEPES, 250 mM NaCl, pH 7.4, 2 mM EDTA, 10% glycerol, 0.5% NP40, 1 mM NaF, 57 mM NaF EDTA-free complete protease inhibitor). Cell lysates were used for anti-Flag antibody immunoprecipitation (N-terminal Flag-tagged β2V2R). Beads were washed and eluted proteins were separated by SDS–PAGE followed by detection using western blotting. Expression levels of the receptor and β-arrestin 1 mutants were measured by radioligand binding and western blotting, respectively. Densitometry analysis of the β-arrestin 1 bands was done using ImageJ software.

**Specimen preparation and EM imaging of negative-stained samples.** T4L–β2V2R–β-arrestin 1–Fab30 complex was prepared for EM using the conventional negative-staining protocol13, and imaged at room temperature with a Tecnai T12 electron microscope operated at 120 kV using low-dose procedures. Images were recorded at a magnification of ×71,138 and a defocus value of −1.5 μm on a Gatan US4000 CCD camera. All images were binned (2 × 2 pixels) to obtain a pixel size of 4.16 Å on the specimen level. Particles were manually excised using Boxer (part of the EMAN 1.9 software suite)13 apart from tilt pairs (0° and 60°) where particles were selected using WEB4.

**Two-dimensional classification and three-dimensional EM reconstructions.** Two-dimensional reference-free alignment and classification of particle projections was performed using ISAC12. Sixteen-thousand two-hundred and eighty-six particles were subjected to classification using ISAC, producing 186 classes accounting for 9,193 particle projections and 145 classes of 8,127 projections, respectively. Fourteen-thousand one-hundred and nine particles were subjected to classification using ISAC, producing 111 classes accounting for 8,011 particle projections and 149 classes accounting for 8,127 projections, respectively. To determine the particle distribution of native T4L–β2V2R–β-arrestin 1–Fab30 conformations, each class average was designated as ‘tight’, ‘loose’ or ‘unassigned’ and the number of projections contributing to the class averages for each designation were added to calculate percentages.

For native and crosslinked Fab30 complexes the random conical tilt technique was used to calculate a first back projection map from individual classes using the 60° tilted particle projections. After angular refinement of the 60° projections, untilled particle projections were added to the data set and the images were subjected to another cycle of refinement. For final reconstructions, particle projections from similar class averages were pooled together, corrected for contrast transfer function (CTF) according to local defocus values obtained by CTFIT11 and subject to further angular refinement and reconstruction in FREALIGN10. Two-thousand eight-hundred and twenty-five particle projections from 0° and 60° images contributed to the crosslinked T4L–β2V2R–β-arrestin 1–Fab30 complex reconstruction. Two-thousand one-hundred and ninety-six particle projections from 0° and 60° images contributed to the native T4L–β2V2R–β-arrestin 1–Fab30 three-dimensional reconstruction. The resolution for each map was determined by the conventional Fourier shell correlation (FSC) approach at FSC = 0.5 (Extended Data Fig. 10).

**Molecular modelling.** For modelling the T4L–β2V2R–β-arrestin 1–Fab30 complex we used the crystal structure of T4L–β2AR from the T4L–β2AR–GxG complex (PDBe accession 3SN6) and the crystal structure of V2Rpp–β-arrestin 1–Fab30 (PDBe accession 4QI). Owing to the low resolution of the EM map and the presence of the considerable detergent micelle density surrounding the receptor region, all docking operations within the EM maps were performed manually with visual inspection of best fit while taking into account additional constraints from crosslinking and HDX-MS data. The Fab30 and T4L structures were independently shifted from the original position in the corresponding crystal structures to obtain an improved fit into the EM density map, reflecting both the flexible nature of their association with β-arrestin 1 and β2AR, respectively, and a partial deformation of the complex on the carbon support of the EM grid.
Extended Data Figure 1 | Formation of the β2V2R-β-arrestin-1–Fab30 complex follows agonist occupancy of the receptor and is biochemically stable. a, Sf9 cells co-expressing the β2V2R, β-arrestin 1 (βarr1) and GRK2CAAX were stimulated with varying doses of the high-affinity agonist BI-167107 followed by addition of Fab30 and purification of the complexes. Stimulation of cells with increasing concentration of BI-167107 results in increasing amount of β-arrestin 1 co-purification, indicating a direct correlation between occupancy of the receptor with agonist and complex formation. b, Quantification of agonist-dependent complex formation from seven independent experiments normalized with respect to the β-arrestin-1 signal at the highest agonist concentration. c, Purified T4L-β2V2R-β-arrestin-1–Fab30 complex was stored either at 4 °C or at room temperature for 4 days followed by size exclusion chromatography on a Superdex 200 (10/300) column (flow rate 0.5 ml min⁻¹). No substantial dissociation of the complex was detected as monitored by appearance of a peak corresponding to the receptor (13.5 ml) or β-arrestin 1 (14.5 ml).
Extended Data Figure 2 | Functionally relevant conformation of β-arrestin 1 in the T4L-β2V2R-β-arrestin-1–Fab30 complex as revealed by enhanced clathrin-TD interaction. Purified glutathione S-transferase (GST)-tagged clathrin-TD was added to the purified complex or an equivalent amount of β-arrestin 1 (βarr1) alone. Interaction of clathrin-TD with the complex or β-arrestin 1 was measured by subsequent co-immunoprecipitation and western blot analysis. Quantification of four independent experiments shown as a bar graph. The relative intensities of the β-arrestin-1 bands are normalized with respect to β-arrestin 1 alone (set as 1). A Coomassie-stained gel indicating comparable amounts of β-arrestin 1 for complex versus β-arrestin 1 alone conditions in clathrin-TD co-immunoprecipitation experiments is shown on the left. Error bars show standard error of the mean (s.e.m.). P < 0.05 for paired t-test.
Extended Data Figure 3 | HDX-MS analysis and MS-based mapping of the crosslinking site in T4L–β2V2R–β-arrestin-1–Fab30 complex. 

**a**, The differential HDX between the T4L–β2V2R–β-arrestin-1–Fab30 complex and the V2Rpp–β-arrestin-1–Fab30 complex are mapped on the sequence of β-arrestin 1 (βarr1). 

**b**, DSA, a homobifunctional amine-reactive crosslinker, was used to crosslink the preformed T4L–β2V2R–β-arrestin-1–Fab30 complex. 

**c**, A representative SDS–PAGE showing the DSA crosslinking efficiency of the preformed complex. 

**d**, The crosslinked peptides were characterized with ‘doublet’ peak signatures in mass spectra as described in Methods and revealed a crosslink between K235 of the β2V2R and K77 at the distal end of the finger loop in β-arrestin 1. 

**e**, Structural model of the β2V2R–β-arrestin-1 complex highlighting the crosslinking site.
Extended Data Figure 4 | Disulphide trapping strategy reveals close proximity of residue 235 of the β2V2R and residue 78 at the distal end of the finger loop in β-arrestin 1. a, Structural model of the β2V2R–β-arrestin-1 complex depicting the proximity of K235 on the β2V2R and D78 on β-arrestin 1 (βarr1). b, Single cysteine insertion mutants of the β2V2R (covering residues 231–236) and β-arrestin-1(D78C) were co-transfected in HEK-293 cells and complex formation was induced by stimulating the cells with an oxidizing agent, H2O2 and agonist (isoproterenol (Iso)). Subsequently, a co-immunoprecipitation assay was performed using Flag M2 beads (Flag-β-arrestin 1). Formation of disulphide trapped complex was visualized by western blotting. c, Quantification of β-arrestin 1 in S–S trapped complex from three independent experiments with s.e.m.
Extended Data Figure 5 | Raw EM images of negative-stained native T4L–β2R–β-arrestin-1–Fab 30/ScFv30 complex. a, Raw EM image of T4L–β2R–β-arrestin-1–Fab complex. b, Raw EM image of T4L–β2R–β-arrestin-1–ScFv complex. Scale bar, 100 nm.
Extended Data Figure 6 | Two-dimensional classifications of the T4L-β2V2R-β-arrestin-1–Fab30/ScFv30 complex. a, b, Reference-free two-dimensional class averages were obtained using ISAC. a, Two-dimensional classification of the T4L–β2V2R–β-arrestin-1–Fab30 complex. b, Two-dimensional classification of the T4L–β2V2R–β-arrestin-1–ScFv30 complex. Scale bar, 10 nm.
Extended Data Figure 7 | ‘On-column’ glutaraldehyde crosslinking of the preformed complex. a, Schematic representation of the on-column crosslinking strategy. A glutaraldehyde solution is injected to a size exclusion chromatography column, followed by injection of the purified complex protein. As the complex protein passes through the glutaraldehyde bolus, the receptor and the β-arrestin (βarr) components of the complex are crosslinked through proximal primary amine groups. This procedure allows only brief exposure of the complex to glutaraldehyde and serves as an ‘in-line’ purification of homogenously crosslinked protein from any aggregation that may arise from non-specific crosslinking. b, On-column crosslinking of the T4L–β2V2R–β-arrestin-1–ScFv30 complex. Purified complex (approximately 20 μM) was injected onto a 24 ml Superdex 200 gel filtration column after a pre-injection of 200 μl of 0.25% glutaraldehyde bolus. Individual fractions were collected and analysed by SimplyBlue-stained SDS–PAGE. c, On-column crosslinking of the T4L–β2V2R–β-arrestin-1–Fab30 complex performed as described for the ScFv complex earlier.
Extended Data Figure 8 | Raw EM images of negative-stained crosslinked T4L–β3,5R–β-arrestin-1–Fab30/ScFv30 complex. a, Raw EM image of T4L–β3,5R–β-arrestin-1–Fab30 complex. b, Raw EM image of T4L–β3,5R–β-arrestin-1–ScFv30 complex. Scale bar, 100 nm.
Extended Data Figure 9 | Two-dimensional classifications of crosslinked T4L–β2V2R–β-arrestin-1–Fab30/ScFv30 complex. a, b, Reference-free two-dimensional class averages were obtained using ISAC. a, Two-dimensional classification of crosslinked T4L–β2V2R–β-arrestin-1–Fab30 complex. b, Two-dimensional classification of crosslinked T4L–β2V2R–β-arrestin-1–ScFv30 complex. Scale bar, 10 nm.
Extended Data Figure 10 | Three-dimensional EM reconstructions and resolution indications by FSC. The top panel shows the three-dimensional map from particles representing the fully engaged β2V2R–β-arrestin-1 conformation of the T4L–β2V2R–β-arrestin-1–Fab30 complex. The bottom panel shows the three-dimensional reconstruction from particles displaying the loose, hanging arrestin conformation of the same complex. Representative two-dimensional averages of particles used for the calculation of initial models by the random conical tilt method are shown on the left of each respective three-dimensional map.