Activation pathway of a G protein-coupled receptor uncovers conformational intermediates as targets for allosteric drug design

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G protein-coupled receptors (GPCRs) are the most common proteins targeted by approved drugs. A complete mechanistic elucidation of large-scale conformational transitions underlying the activation mechanisms of GPCRs is of critical importance for therapeutic drug development. Here, we apply a combined computational and experimental framework integrating extensive molecular dynamics simulations, Markov state models, site-directed mutagenesis, and conformational biosensors to investigate the conformational landscape of the angiotensin II (AngII) type 1 receptor (AT1 receptor)—a prototypical class A GPCR—activation. Our findings suggest a synergistic transition mechanism for AT1 receptor activation. A key intermediate state is identified in the activation pathway, which possesses a cryptic binding site within the intracellular region of the receptor. Mutation of this cryptic site prevents activation of the downstream G protein signaling and β-arrestin-mediated pathways by the endogenous AngII octapeptide agonist, suggesting an allosteric regulatory mechanism. Together, these findings provide a deeper understanding of AT1 receptor activation at an atomic level and suggest avenues for the design of allosteric AT1 receptor modulators with a broad range of applications in GPCR biology, biophysics, and medicinal chemistry.
As the largest superfAMILY of cell surface proteins in the human genome, G protein-coupled receptors (GPCRs) represent the therapeutic targets of nearly one-third of all approved drugs. These receptors share a conserved structural architecture of seven transmembrane (7TM) helices linked by three extra- (ECLs) and three intracellular loops (ICLs). The GPCR-mediated signal transduction is always triggered by an extracellular signal to the orthosteric site located in the extracellular region of the 7TMs bundle center, which then transduces the stimuli to the intracellular region, thereby leading to the engagement of the receptor with G proteins or β-arrerstins. In addition, some GPCRs can also transmit signals in the absence of an external stimulus or an agonist, through ‘basal’ (also known as ‘constitutive’) activity. The orthosteric site is conserved across the members of a single GPCR subfamily and, thus, poses a significant challenge in the development of selective drugs that can bind to a unique receptor subtype. As an alternative strategy, targeting a binding site outside the conserved orthosteric site, also termed as an ‘allosteric site’, may provide avenues for the design of modulators with desirable selectivity profiles, which is a long-standing bottleneck in GPCR drug discovery.

Recent technological breakthroughs in structural biology, such as cryo-electron microscopy (cryo-EM) or X-ray free-electron lasers, have led to the identification of increasing GPCR structures, either in the inactive or active conformations, thereby providing mechanistic insights into the agonist-dependent receptor activation mechanisms that are useful for investigating structure-based drug design. However, these high-resolution structures represent static snapshots obtained under specific experimental conditions; hence, they may miss important information pertinent to the conformational ensemble of GPCRs, as the receptors may have undergone a large-scale conformational transition during their (de)activation process. Therefore, mechanistic and structural elucidation of the (de)activation pathway of the GPCRs is of paramount importance as distinct conformational states, such as intermediate, metastable, or transient states, present during the inactive-to-active transition (or vice versa) of the receptors, are desired for a rational design of selective modulators.

The angiotensin II (AngII) type 1 receptor (AT1 receptor), a prototypical class A GPCR, offers an important model for mechanistic exploration as it is a prominent therapeutic target for hypertension and related cardiovascular diseases. Moreover, its high-resolution structures in both inactive, antagonist-bound, and active, agonist-bound, and nanobody-stabilized conformations have recently become available. By comparing the structures of its inactive, antagonist-bound state (ZD7155; PDB ID: 4YAY) and the active state, meaning the AT1 receptor complexed with a partial agonist S1I8 peptide, and a G protein mimetic nanobody to maintain the active conformation (PDB ID: 6DO1), the most remarkable differences in the two structures are found in the TM5–7 and helix 8 (H8) (Fig. 1C). Notably, on the intracellular side, the active state structure exhibits an outward displacement of TM5 and TM6, inward movement of TM7, and substantial repositioning of H8 parallel to the membrane, relative to the inactive state structure. On the extracellular side, the major conformational changes include an inward shift of TM5 and TM7 in the active state structure compared with the changes in the inactive state structure. Although the static, active, and inactive states of AT1 receptor exhibit marked structural divergences, it has been challenging to completely capture the large-scale conformational transitions along the activation pathway of the AT1 receptor experimentally. Therefore, it remains unclear how a dynamic pathway connects the inactive-to-active conformational transitions of the AT1 receptor, thereby hindering a deeper understanding of the comprehensive landscape of the activation mechanisms of this receptor as well as for other GPCRs. Furthermore, despite the availability of the inactive and active structures of the AT1 receptor, there are no allosteric modulators of this receptor reported to date, suggesting a challenge for targeting potential allosteric binding sites in the two available snapshots. However, a cryptic allosteric site may exist in the transition pathway. Thus, it is advisable to capture key conformational substates along the activation pathway for the purpose of allosteric drug design.

To uncover the activation pathway of GPCRs, biosensors, nuclear magnetic resonance (NMR), and computational methods have been widely applied. Among these approaches, molecular dynamics (MD) simulations have become a well-established technique for probing the conformational landscapes at an atomic level and directly uncovering biomolecular mechanisms. Integrating MD simulations with Markov state models (MSMs) has proven successful for understanding the molecular switches in β2 adrenergic receptor (β2AR), elucidating ligand-driven conformational changes in C5 chemokine receptor (CCR) 5, and revealing a cryptic allosteric pocket in dopamine D1 receptor. In the best structurally and biochemically characterized GPCRs, the rhodopsin receptor, the activation pathway, and the corresponding intermediate states have been elucidated by NMR, Fourier transform infrared spectroscopy, and MD simulations.

Here, we use a computational framework including a transition pathway generation algorithm, extensive all-atom MD simulations (300 μs) of the AT1 receptor in the membrane-embedded environment, and MSM analysis for investigating the conformational landscapes of AT1 receptor activation. We found an intermediate state during activation and identified a cryptic allosteric pocket on it. Multiple mutagenesis experiments confirm both the intermediate state and the potential regulation ability for the pocket. Our study not only offers a deep atomic-level insight into AT1 receptor activation, but also provides an opportunity for the design of allosteric AT1 receptor modulators.

**Results**

Extensive unbiased MD simulations reveal the activation pathway of AT1 receptor. To understand the inactive-to-active conformational transition pathway of the AT1 receptor, we first generated a minimum energy path (MEP) by connecting the starting, antagonist-bound inactive (PDB ID: 4YAY), and the end, both nanobody- and agonist-bound fully active (PDB ID: 6DO1) states, by inserting a series of replicas between the two AT1 receptor structures using the string method with the nudged elastic band (NEB) (see “Methods”). Both the nanobody and agonist were removed from 6DO1 and the antagonist was excluded from 4YAY. Here, we defined the GPCR structures with only agonist-bound forms as active conformations and with both agonist- and G protein- β-arrerstin-, or nanobody-bound forms as fully active conformations. After a simulated annealing process, 15 initial structures distributed on the MEP were selected and subsequently embedded with a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane and explicit water. Each structure had 2 μs × 10 independent runs with random initial velocities, leading to a cumulative simulation timescale of 300 μs. Such an extensive timescale has been proven efficient for exploring the GPCR activation process. As shown in Fig. 1, the most remarkable intracellular transmembrane domain conformational variations of the AT1 receptor during activation are arguably the outward movements of TM5 and TM6, and the inward displacement of TM7. Thus, we define two activation parameters (Fig. 1C) to project the simulated trajectories onto a
The overall structure of the inactive AT₁ receptor in complex with an antagonist ZD7155 (green) and b562RIL (yellow). B The overall structure of the fully active AT₁ receptor in complex with a partial agonist S1I8 (magenta) and a G protein mimetic nanobody (cyan). C Major conformational changes between the inactive (blue) and fully active (orange) AT₁ receptor. Red arrows show obvious transmembrane (TM) movements during activation. The zoom-in views represent the distance between the Ca atoms of L₅.₅₅ and L₇.₄₆ and the angle among the Ca atoms of §₆.₃₄, §₆.₄₇, and V².₄₁, in order to monitor the conformational rearrangements of TMs 5–7. The superscripts refer to the Ballesteros–Weinstein numbering system. All structure figures were drawn by PyMOL. AT₁R angiotensin II type 1 receptor, S1I8 S1I8 mutant angiotensin II, Nb Nanobody, TM transmembrane helices, H₈ helix 8.

Two-dimensional (2D) space to comprehensively capture the conformational landscape of AT₁ receptor activation. Notably, L₅.₅₅ (superscripts indicate the Ballesteros–Weinstein numbering for GPCR residues) undergoes a large and conserved rearrangement during GPCR activation and N₇.₄₆ localizes at the TM7 twist position, which reflects the inward movement of TM7 during activation. Thus, one parameter for defining activation was the distance between the Ca atoms of L₅.₅₅ and N₇.₄₆, which represented the conformational changes of TM5 and TM7. The other parameter was the angle among the Ca atoms of F₆.₃₄, S₆.₄₇, and V².₄₁, which reflected the outward movement of TM6, a crucial hallmark of class A GPCR activation that provides space to accommodate downstream signal proteins including G proteins or β-arrestins. As shown in Fig. 1C, the corresponding distance and angle values were markedly distinct between the inactive and fully active AT₁ receptor crystal structures, thereby highlighting the discriminatory power of the activation parameters.

Based on these activation parameters, we calculated the corresponding value of each snapshot during the simulations and plotted a free-energy landscape (Fig. 2A), depicting the inactive-to-active transition pathway of AT₁ receptor activation. Since the initial inactive crystal structure of the AT₁ receptor was located at a distance and angle of 21.3 Å and 36.7°, respectively, the largest free-energy basin with a distance of ~19–23 Å and an angle of ~35–42° represented the inactive region. Further, owing to the outward movement of TM6, the angle gradually increased, reflecting an open degree of TM6, whereas the movement of TM5 and TM7 caused a decrease in the interhelical distance. During the activation process, the AT₁ receptor overcome a relatively low-energy barrier and enters the intermediate state located at a free-energy basin with a distance of ~17–19 Å and an angle of ~46–51°, and it then crosses a high-energy barrier at ~17 Å and 63° to arrive at the fully active state (17.4 Å, 69.4°), representing the coordinates of the fully active AT₁ receptor crystal structure (PDB 6DO1). Overall, the convergence of the landscape was proven in both the timescale of a single trajectory and the number of rounds (Supplementary Note 1), confirming that the sampling has been sufficient to explore AT₁ receptor activation.

Because of the recent breakthroughs in receptor crystallization, more than 440 class A GPCR structures of >70 receptors have been determined by X-ray or cryo-EM crystallography (Supplementary Note 2 and Supplementary Data 1). These structures represent the GPCR conformational ensemble covering all inactive, active, and intermediate states, including different inverse agonist- or antagonist-bound inactive conformations, agonist-bound active conformations, and their effector G

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**Fig. 1** The distinction between the inactive and fully active AT₁ receptor crystal structures. A Overall structure of the inactive AT₁ receptor in complex with an antagonist ZD7155 (green) and b562RIL (yellow). B Overall structure of the fully active AT₁ receptor in complex with a partial agonist S1I8 (magenta) and a G protein mimetic nanobody (cyan). C Major conformational changes between the inactive (blue) and fully active (orange) AT₁ receptor. Red arrows show obvious transmembrane (TM) movements during activation. The zoom-in views represent the distance between the Ca atoms of L₅.₅₅ and L₇.₄₆ and the angle among the Ca atoms of §₆.₃₄, §₆.₄₇, and V².₄₁, in order to monitor the conformational rearrangements of TMs 5–7. The superscripts refer to the Ballesteros–Weinstein numbering system. All structure figures were drawn by PyMOL. AT₁R angiotensin II type 1 receptor, S1I8 S1I8 mutant angiotensin II, Nb Nanobody, TM transmembrane helices, H₈ helix 8.

**Fig. 2** The free-energy landscape of AT₁ receptor. A Conformational landscape of the AT₁ receptor generated using the Ca atom distance between L₅.₅₅ and L₇.₄₆, and the angle among the Ca atoms of §₆.₃₄, §₆.₄₇, and V².₄₁ as the order parameters along the activation pathway. Arrows point out different states on the landscape. Color scales of the landscape are shown on the right. MATLAB is applied to draw the landscape. B Projection of all reported fully active (red), inactive (blue), and active (green) structures of class A GPCRs onto the AT₁ receptor conformational landscape. The unit of free-energy values is kcal/mol.
The outliers inactive structures have mostly similar TM5 crystal structures. This can perhaps be ascribed to the fact that the and suggesting that it is thus suitable for further investigations. Our simulations to reproduce the overall class A GPCR activation agreement with the experimental data, highlighting the ability of result suggests that the computational model is in reasonable experimental structures within the reaction path (Fig. 2B). This landscape of GPCR ensemble with the major distribution of AT1 receptor activation pathway samples a wide conformational registrations (Supplementary Note 4). Since our simulations are based on protein-, β-arrestin-, or nanobody-stabilized fully active conformations. Considering the conformational heterogeneity of class A GPCR structures, we projected these experimentally solved structures onto the 2D conformational landscape of the AT1 receptor sampled by the simulations. The corresponding residues at positions 5.55, 7.46, 6.34, 6.47, and 2.41 of each structure were selected to calculate the distance and angle using their Ca atoms. Then, the fully active, inactive, and active states were mapped to provide insights into the dynamic conformational landscape of the receptors. The projection suggests that the AT1 receptor activation pathway samples a wide conformational landscape of GPCR ensemble with the major distribution of experimental structures within the reaction path (Fig. 2B). This result suggests that the computational model is in reasonable agreement with the experimental data, highlighting the ability of our simulations to reproduce the overall class A GPCR activation and suggesting that it is thus suitable for further investigations.

Interestingly, the inactive energy basin largely matched its structural features. This can perhaps be ascribed to the fact that the inactive structures have mostly similar TM5–TM7 conformation. The outliers—platelet-activating factor receptor (PDB 5ZKP) and P2Y12 receptor (PDB 4NTJ)—however, have an unusual anchor (TM2) movement and distinct TM6 twist, respectively (Supplementary Note 3). Of note, receptor-Gs complex has a larger angle than receptor-Gi complex, which is consistent with the different volumes of the two downstream proteins. The position of the G protein complex has a similar distribution with nanobody-stabilized structures, suggesting that the nanobodies used in crystallization have a minor influence on its conformational landscape (Supplementary Note 4). Since our simulations are based on the apo structure, the active cloud is not highly correlated with fully active non-rhodopsin structures, indicative of the instability of active structures and the necessity for transducers to stabilize the fully active conformations. Collectively, the free-energy landscape illustrates the activation process of the AT1 receptor and uncovers a hidden intermediate state along the pathway linking the inactive and active receptors.

Markov state model discovers a synergistic transition mechanism of AT1 receptor activation. To elucidate the mechanism of AT1 receptor activation, we built a kinetic network MSM using the activation parameters. From a statistical viewpoint, MSM provides summarized insights of the conformational ensemble of biomacromolecules at equilibrium. Therefore, the key intermediate states are identified more precisely and the thermodynamic properties, such as transition timescale, are also quantified. Upon validation of the Markovian properties by Fig. 3 The three macrostates divided by MSM, and constitutive activity. A The distribution of active (green), inactive (yellow), and intermediate (purple) states on the free-energy landscape. The attribution and probability of each macrostate are shown on the right. B The transition time among the active (green), inactive (yellow), and intermediate (purple) states, represented by the mean first passage time. Constitutive activities of AT1 receptor in Gq (C), Gi (D), and G12 (E) pathways (red). Vasopressin 2 receptor (V2R) was used as a negative control (black). The gradient cell surface expression levels of AT1 receptor and V2R were achieved by adjusting the transfecting amounts of plasmids encoding the respective receptor in HEK293 cells (Supplementary Fig. 3). Data were from three independent experiments. The bars indicate the mean ± SEM values. The absolute luminescence intensity values are provided in source data. If no special instructions were shown, histograms were drawn by GraphPad. △BRET: the change of bioluminescence resonance energy transfer value.
Fig. 4 The synergistic activation mechanism of AT1 receptor. A–C The comparison of representative inactive (A, blue), intermediate (B, cyan), and active (C, orange) structures. Zoom-in views of key structures are depicted on the right of (A), (B), and (C). Top left: the breaking of hydrophobic lock during activation. Top right: the activation renders the approach of Yγ6.45 and Yγ5.58. Bottom left: polar interaction on the position of common ionic lock is broken on the process of activation. Bottom right: ratchet past Fγ6.44 and Fγ6.45 with the outward movement of TM6. Distances between atoms are shown in dashed yellow lines. Dynamic variations of these activation properties during a representative simulation trajectory are shown in (D) for the distance between the guanidine carbon atom of Rγ3.50 and the Cβ atom of Nβ6.30 (red), (E) for the area of the triangle composed of the Cβ atom of Vγ6.41, and the Cβ atom of Wγ6.40 (blue), and (F) for the angle among the centroids of the benzene ring of rγ5.51, rγ6.44, and rγ6.45 (yellow).
We then clustered the representative conformations of the macrostates according to the root mean square deviation (RMSD) between structures (see “Methods”). The micro-switches are also shown in the inactive, intermediate, and active AT1 receptor states (Fig. 4A–C). Based on a representative simulation trajectory across the three states, we observed the dynamic positioning of our switches (Fig. 4D–F). Globally, TM6 moved outward, while TM5 and TM7 approached the inactive-to-active conformational transition. Meanwhile, the landscape coordinates of the inactive, intermediate, and active representative conformations were located at (23.3 Å, 40.3°), (19.0 Å, 50.6°), and (17.1 Å, 71.3°), respectively; matching the low-energy conformer and further strengthening the accuracy of our cluster methodology.

Other structural elements also appear functionally significant. Upon activation, ECL2 and extracellular TM6 moved toward the center of the endogenous AngII pocket and nearly closed the pocket in the active state, indicating that ECL2 and TM6 stabilize the pocket and maintain the binding of AngII during activation (Supplementary Note 7). The closure of the orthosteric pocket has also been observed in the active β2AR-nanobody complex structure. Moreover, intracellular TM5 formed more α-helices in the active state, and the length of ICL2 decreased accordingly to help the binding of the downstream proteins, which is in line with the common GPCR activation process. In addition, H8 moved upward from the inactive to the active state to provide space for the downstream effectors. This upward movement produces a suitable accommodation for ligand binding among TM1, TM7, and H8, which may offer an opportunity for the formation of a cryptic pocket for drug design (Supplementary Note 7). We further explored the variation of secondary structural elements among macrostate trajectories using the Definition of Secondary Structure of Proteins algorithm (Supplementary Note 8) and community analysis for the signal transfer (Supplementary Note 9). During receptor activation, the ECL2 became stable, the intracellular TM5 was elongated, and connections to the transducer pocket increased. The upward movement of H8 was also observed.

In agreement with the observations revealed by comparing the crystal structures, the micro-switches generally and synergistically changed from the inactive state to the intermediate and active states. For example, V6.41 moved toward the membrane step-by-step and finally broke the hydrophobic lock in the active structure. This movement provided space for Y7.53, which finally approached Y5.58 (inactive: 18.9 Å, intermediate: 13.1 Å, active: 5.1 Å). In addition, the distance between R3.50 and N9.30 increased upon the outward movement of TM6 (inactive: 5.4 Å, intermediate: 15.3 Å, active: 22.8 Å), which in turn, rendered F6.44 and F6.45 to ratchet over E5.51. Meanwhile, the relative positions of the residues in the intermediate state were in between the active and inactive states, indicating that the activation process generally occurs. In a single spontaneous activation trajectory (Fig. 4D–F), the variations of the three micro-switches—breaking of the ionic lock (Fig. 4D), the opening of the hydrophobic lock (Fig. 4E), and rearrangement of phenylalanine ratchet (Fig. 4F)—synchronize, and thus reflect the synergistic activation mechanism of the AT1 receptor. The rationale for the measurement of residue pairs describing the hydrophobic lock is provided in Supplementary Note 10. Thus, the zoom-in views of the residue

**Fig. 5 The intermediate-specific micro-switches and their importance for activation.** A The intermediate-specific micro-switches. Involved residues are shown in sticks and corresponding distances are depicted by yellow dashed lines. B–D Constitutive and AngII-induced activities of WT AT1 receptor and mutants in Gq (B), Gi (C), and G12 (D) pathways. WT, K5.42A, Y5.58A, W5.62A, F6.34A, and F8.50A mutations are shown as black, cyan, red, orange, green, and yellow points. Data were from three independent experiments and representative dose-response curves were shown in AngII-induced activity. The summary of data was shown in Supplementary Note 14. The absolute luminescence intensity values are provided in source data. **ΔBRET:** the change of bioluminescence resonance energy transfer value. ***P < 0.001, HEK293 cells transfected with AT1R mutants compared with those transfected with WT AT1R. The bars indicate the mean ± SEM values. Statistical differences between WT and mutants were analyzed using two-way ANOVA with Dunnett’s post hoc test. (The p values in B–D are < 0.0001, < 0.0001, < 0.0001).
rrearrangements were consistent with the global secondary structural movements.

To demonstrate the transition pathway, we employed gaussian accelerated MD (GaMD) simulations on the holo AT1 receptor systems using the intermediate structure as a model system. The inverse agonist olmesartan and the endogenous agonist AngII were docked to the orthosteric pocket of the intermediate state, respectively. Then, the two holo systems underwent 2 μs conventional MD simulations followed by 1 μs GaMD simulations for three independent runs. In the corresponding free-energy landscapes, AngII binding pushed the open of TM6 along with the inward movement of TM5 and TM7, resulting in the active state, while olmesartan binding led to a smaller TM6 angle and a larger TMS–TM7 distance, shifting the receptor conformation to the inactive state (Supplementary Note 11). Together, these results suggest the likely existence of an intermediate structure as captured from the transition pathway.

Since the AT1 receptor activates multiple G protein subtypes in its apo and holo states, we further explored whether this intermediate state could be commonly required by different G protein downstream signal pathways. By comparing the intermediate structure with the inactive and active macrostate structures, we identified several specific micro-switches for the intermediate state, such as polar contacts among K5.42, H6.51, and T6.55; a hydrogen bond between Y5.58 and I6.37; hydrophobic network among M6.38, W5.62, and F6.34; and hydrophobic contacts among V1.53, V1.56, and F8.50 (Fig. 5A and Supplementary Note 12). Based on these intermediate-specific interactions, we designed K5.42A, Y5.58A, W5.62A, F6.34A, and F8.50A mutations to specifically disrupt the intermediate state. The protein level of the variants were similar to that of the WT receptor (Supplementary Note 13). Constitutive and AngII-induced activities for G proteins were then measured using BRET assays to evaluate the influence of these mutations (Fig. 5B–D).

Based on BRET assays, the disruption of micro-switch interactions in the intermediate state inhibited both the constitutive and AngII-induced activities of the AT1 receptor for G proteins, including Gq, Gi, and G12. The decrease of constitutive activity was more obvious than the AngII-induced activity. In the AngII-induced activity, K5.42A decreased the activity of Gq and Gi more than G12, suggesting that Gq and Gi activity may need a larger movement of extracellular TM5. Although W5.62A and F6.34A did not influence the Gq and Gi signal in Emax, the increase of the EC50 value reflected a weaker activation upon AngII binding (Supplementary Note 14). The remaining Y5.58A and F8.50A mutations similarly modulated the activity of the AT1 receptor in the three G proteins. In summary, these results indicated that the intermediate state is required for all G protein signaling pathways, highlighting its possibility to become a drug target.

**Distinct active conformations from global movement can induce biased signals.** Apart from the dimension reduction accomplished by the features extracted from the biological process (such as the activation parameters shown in Fig. 1C), we also applied time-structure-based independent component analysis (tICA) to our system to analyze the global movement of the receptor during activation. tICA employs linear combinations to particular features, such as the phi/psi angle of the backbone, to maximize the decorrelation time among these features. Thus, tICA enables the capture of slow dynamic processes during simulations. With the help of tICA, we projected the trajectories onto another 2D landscape, which was representative of the global phi/psi movement. Through the implied timescale test, dimensionality reduction using tICA has been proven to have Markovian properties. The tICA landscape was separated into eight macrostates according to the PCCA+ algorithm, and its prediction met the requirements of the Chapman–Kolmogorov test (Supplementary Note 15). To analyze the global movement during simulations, the representative conformation of each tICA macrostate was extracted and projected onto the distance-angle landscape based on their corresponding activation parameters (Fig. 6A). Finally, the TPT method was applied to calculate the transition dynamics between six major macrostates (Fig. 6B).

As shown in Fig. 6A, macrostates 1 and 5 were in the intermediate state, macrostates 2–4 and 8 were in the inactive state, and macrostates 6 and 7 were in the active state. Notably, the macrostates 3–8 occupied more than 96% of the snapshots during our simulations (Supplementary Note 15) and stayed close to the energy basins in our landscape (Fig. 2A), thereby suggesting that the coordinate parameters (distance and angle) elucidated the activation process just as the global movement. Thus, the mechanism according to the activation parameters is credible. In addition, the representative conformations of macrostates 4 and 6 corresponded to the area with the most inactive and active crystal structures, respectively (Figs. 2B and 6A). Pathway 4–5–6 also takes the shortest transition time, thus it is the most preferential way to activate the AT1 receptor among all the analyzed pathways (Fig. 6B). Hence, macrostates 4 and 6 are crucial conformers along the activation pathway, that is, macrostate 4 is encountered before entering the activation process, and the typical activation process involves the macrostates 4–6.

Since the AT1 receptor has a constitutive activity for G proteins (Fig. 3C–E) and shows β-arrestin activity upon AngII binding or specific agonists, we explored the connection between different active states and biased signaling. Because Gq is the major G protein activated by the AT1 receptor and β-arrestin 2 is commonly used in biased signaling, we first constructed AT1 receptor–Gq and AT1 receptor–β-arrestin 2 complexes based on the active macrostates 6 and 7 to determine whether the two active conformations had a bias for these transducers (Supplementary Note 16). The models showed that macrostates 6 and 7 tend to initiate Gq and β-arrestin 2, respectively, suggesting that biased conformations naturally exist in the ensemble of the AT1 receptor.

Supported by this preliminary analysis, we further investigated biased signaling in the AT1 receptor based on tICA analysis. From the representative structures, we explored specific micro-switches of each macrostate (Fig. 7A). Y7.53 is close to hydrophobic residues at TM1 and TM2 in both macrostates 5 and 6, while it...
forms a hydrogen bond with R^{3.50} or Y^{5.58} in macrostates 1 and 7, respectively. Thus, by introducing a hydrophobic residue, the Y^{7.53}I mutation could maintain the hydrophobic interactions, but disrupted the polar interactions, leading to the stability of macrostates 5 and 6, and the instability of macrostates 1 and 7. In addition, N^{3.35} and D^{2.50} form a tight hydrogen bond in macrostate 7, whereas it is weak in macrostate 6 and diminishes in macrostates 1 and 5. N^{3.35}A mutation disrupted the hydrogen bonding interaction, preferred the conformation with a long distance between the residue at 3.35 and the polar residue at 2.50 (macrostates 1, 5, and 6). In turn, a moderate mutation D^{2.50}N changes the charge of its sidechain, which may weaken the interaction between residue at 3.35 and residue at 2.50. Because macrostate 7 has a strong hydrogen bond, its conformation may maintain in response to the D^{2.50}N mutation. However, macrostate 1, 5, and 6 may be disturbed owing to this mutation. Our mutation experiments for biased signaling were benchmarked against these micro-switches and the corresponding results (Fig. 7B–E). An operational model was also applied to determine the biased signaling with normalized E_{max} values (Fig. 7F).

In Fig. 7F, blue and light pink represent a weak signal and red reflects a strong signal, compared with the WT AT_{1} receptor. In particular, N^{3.35}A mutation (benefits macrostates 1, 5, and 6,
Fig. 8 The predicted pockets on the intermediate state and comparison of them with other GPCR allosteric sites. A All the pockets predicted by Fpocket in the intermediate state. Red circles show the overlap pockets with the prediction of inactive and active representative structures, while blue circles mean the intermediate-only pockets P6 and P9. B The alignment of current allosteric modulators on their sites with intermediate structure and pockets P2, P4, and P8. C The overlap of P9 with current allosteric GPCR modulators in crystal structures. In (B) and (C), cyan cartoons depict the intermediate AT1 receptor and orange sticks show corresponding pockets defined by Fpocket. Sticks in other colors show the allosteric modulators. AT1R: angiotensin II type 1 receptor.

inhibits macrostate 7) obviously promoted Gq and Gi signals but inhibited G12 and β-arrestin 2 signals. In contrast, D2.50N showed relatively weaker Gq and Gi signals compared with G12 and β-arrestin 2 signals (Fig. 7F and Supplementary Note 17). Facilitating macrostate 5 and 6, Y7.53I also promoted Gq but inhibited β-arrestin 2. Thus, micro-switches facilitating macrostate 6 and restraining macrostate 7 (N3.35A, Y7.53I) led to the Gq activation and β-arrestin 2 inhibition, whereas the D2.50N mutation, boosting macrostate 7 and reducing macrostate 6, preferred β-arrestin 2 rather than Gq signal. This is in line with our hypothesis that macrostate 6 is biased to Gq pathway, whereas macrostate 7 is for β-arrestin 2 (Supplementary Note 16). As for Gi, it can be inferred that the intermediate macrostate 1 (inhibited by Y7.53I and D2.50N, facilitated by N3.35A) may play a critical role in the activation toward Gi since Y7.53I and D2.50N suppressed Gi signal but N3.35A increased it. Conversely, G12 activation may be related to the macrostate 5 (facilitated by Y7.53I, inhibited by N3.35A and D2.50N) because Y7.53I stimulated the G12 signal, while N3.35A and D2.50N repressed G12 activation. The effect of mutations can be confirmed by recent structural information. For instance, Y7.53I quenches polar interactions with R3.50 and Y5.58, which releases intracellular TM3 and TM5. The flexible TM3 can move downward and increase the interaction of ICL2 with transducers, while TM5 might extend toward G protein. Accordingly, the increased ICL2 interaction and extended TM5 are properties of GPCR-Gq/11 complex compared with GPCR-Gi/o complex60–62. Thus, Y7.53I promotes Gq but inhibits Gi signals in the AT1 receptor.

In summary, we observed hints for each biased signal pathway in our MD simulations for the apo AT1 receptor and confirmed them by site-directed mutation experiments. During biased signaling, different ligands stabilize distinct receptor conformers, reflecting the biased signaling of the AT1 receptor via a conformational selection mechanism. Taken together, tICA identified a transition pathway based on global movements, and the active and intermediate conformations provided indications of biased signaling.

Identification of a cryptic allosteric site in the intermediate state. We also used a pocket prediction algorithm to identify potential sites on macrostates and guide the allosteric drug design. Using Fpocket, we identified and clustered nine pockets on the hidden intermediate state (Fig. 3A) and several pockets in other states. Fpocket defines alpha spheres by Voronoi tessellation and regards the alpha spheres that are concentrated on clefts or cavities and with the ability to bind small molecules as the pocket63. In Fig. 8A and Supplementary Note 18, the positions of the pockets are depicted and the overlapping pockets between the intermediate state and other states are also highlighted. Most of the detected pockets in the intermediate state corresponded to pockets in the inactive and active states, except for P6 and P9, which were the cryptic pockets only in the intermediate state.

Among the nine pockets, P1 was located at the center of the AT1 receptor and spanned from top to bottom. It revealed a large cavity inside the AT1 receptor that partly overlapped with the orthosteric site for AngII. Nevertheless, P1 was too large for the design of small-molecule modulators. In addition, the P2–P6 pockets laid on the membrane side, whereas the intracellular P7–P9 pockets were in the proximity of the transducer site (Fig. 8A). The volume of P2–P9 pockets could fit possible allosteric modulator sites, and P2, P4, P8, and P9 matched previous allosteric drug sites as well (Fig. 8B, C). In particular, P2 was mostly identical to the LY2119620 pocket in the M3 muscarinic acetylcholine receptor (PDB 4MQT)64. P8 also partially overlapped with the allosteric agonist AP8 pocket in free fatty acid receptor 1 (PDB 5TZY)65. In addition, BPTU interacted with P2Y1 receptor (PDB 4XNV)66 in the vicinity of P4, confirming the accuracy of our site prediction. P9 overlapped with three allosteric sites observed in GPCR-modulator structures as an intermediate-only potential pocket. The sites were as follows: the CCR2-RA-[R] site in CCR2 (PDB 5T1A), the vercironin site in CCR9 (PDB SLWE), and the Cmp-15PA site in β2AR (PDB 5X7D), and were not confined to the intermediate state67–69. Thus, P9 is a common allosteric site in the intermediate state of the AT1 receptor. However, since P2, P4, P8, and P9 emerged as the allosteric sites in other class A GPCR-modulator structures, the sites shown in Fig. 8A are highly likely allosteric sites.

Since P9 was excluded because of its universality, P6 remained the only cryptic allosteric site in the intermediate state. As P6 has never been reported in previous GPCRs and allosteric modulator structures, our findings indicate that it may be an allosteric site for drug design. We also conducted molecular screening on P6 using a GPCR allosteric compound library and found a binding pose highly overlapping with P6 from the best-scored ligand (Supplementary Note 19), further suggesting the targetable potential of P6.

Mutagenesis and FLAsH-BRET assay confirm the predicted cryptic binding site. Next, we confirmed that P6 was an allosteric
site firstly using clustered alanine-scanning mutagenesis. Three groups of mutations (Fig. 9A, B) were independently introduced to directly investigate the effect of allosteric perturbations of P6 on the transducer activity of the AT1 receptor. The cluster 1 mutations included P1.48A, L1.52A, and T1.57A, located on the intracellular side of TM1. The cluster 2 mutations were N7.49A, P7.50A, and Y7.53A on TM7. The cluster 3 mutations (K8.49A, P8.50A, K8.51A, Y8.53A, and P8.54A) are on the top of H8. Since the AT1 receptor acts as a model system for biased signaling, we further investigated whether these cluster mutations had effects on AngII-induced Gq activation as well as on β-arrestin 2 recruitment using BRET assays upon similar expression (Supplementary Note 20). Cluster 2 and 3 mutations completely depleted the two activation pathways of the AT1 receptor, whereas cluster 1 mutations decreased both two $E_{\text{max}}$ by 22–34% (Fig. 9C, D, Supplementary Note 20). Thus, the direct perturbation at P6 influenced the G protein and β-arrestin pocket.

To further determine the potential of P6 on our intermediate state to regulate the binding of downstream transducers to the G protein pocket, we applied the AlloSigMA algorithm, which evaluates allosteric effects using the Structure-Based Statistical Mechanical Model of Allostery (SB-MM). AlloSigMA analysis indicated that ligand binding on P6 influenced the G protein pocket of the receptor and that stable/bulkly or unstable/tiny mutations on several P6 residues changed the dynamics of the G protein pocket (Supplementary Note 21). Thus, we designed bulky mutations (G1.49L, F7.55W, and P8.54W, Fig. 10A) and tiny mutations (P1.48A, N7.49A, Y7.53A, and P8.50A, Fig. 10B) in the AT1 receptor and used BRET assays to test the regulation ability of P6 on both G protein and β-arrestin pathways. Under similar protein levels (Supplementary Note 22), point mutations disturbed both Gq and β-arrestin 2 activities (Figs. 10C, D, and Supplementary Note 22). In all mutations, although the P8.54W mutant retained the same $E_{\text{max}}$ for Gq as the WT AT1 receptor, the higher EC50 value suggested a weaker effect of AngII. Thus, point mutations guided by AlloSigMA suggested the existence of P6.

To investigate the effects of mutations at the potential allosteric site on the AngII-induced AT1 receptor conformational change, we performed intramolecular FlAsH-BRET assays using our recently developed AT1 receptor conformational sensor, which was generated by incorporation of RLuc at the C-terminus and FlAsH motif (CCPGCC) into the ICL3 (referred to as AT1R conformation sensor)72,73. As expected, AngII stimulation on the AT1R conformation sensor decreased the BRET signal in a dose-dependent manner, suggesting an increase of the distance between the C-terminus and the FlAsH motif inserted at the ICL3 of the AT1 receptor (Fig. 10E, F). Notably, albeit with similar total and cell surface expression levels compared with the WT sensor, mutations at all seven sites led to the impairment of AngII-induced AT1 receptor conformational change, as revealed by the increased EC50 whereas G1.49L, F1.48A, N7.49A, Y7.53A, and P8.50A mutants also displayed decreased maximal response (Supplementary Note 22). These mutational effects on the downstream Gq signaling are consistent with the Gq BRET assay, suggesting that the AT1R conformation sensor can primarily reveal the conformational changes required for the AT1 receptor Gq signal. These data suggested that the potential allosteric site participated in AT1 receptor signal regulation through conformational modulation. Hence, considering the shared existence of P6 in the class A GPCR family, it is possible to develop general allosteric modulators targeting P6 and regulating GPCR activation.

**Discussion**

GPCRs have gained increasing attention as therapeutic targets for the treatment of sensory, circulation, and central nervous system disorders. Despite intensive research and the accumulation of considerable amounts of structural and functional data, it is still challenging to completely understand the mechanism of GPCR activation, elucidate the complex signaling machineries that can adopt a slew of distinct conformations to modulate multiple downstream signaling pathways (known as biased signaling), and design small-molecule allosteric modulators that can distinguish between closely related receptor subtypes1–22. As a quintessential model system for GPCR research, the AT1 receptor not only shows biased signaling by regulating its endogenous ligand AngII, but also shows great promise as a therapeutic target for treating hypertension. Recently, the antagonist-bound (inactive), AngII-bound, and biased agonist-bound (active) AT1 receptor structures have been determined23–25; however, the dynamic process of
receptor activation and its biased signaling through a conformational selection mechanism are still not fully understood.10,74

In the present study, we employed extensive MD simulations (300 µs) of the AT1 receptor and used the NEB method to define a pathway from the inactive to the active state. A free-energy landscape was identified and confirmed by MSM by referring to the common GPCR activation properties. MSM extracted representative conformations of the inactive, intermediate, and active states; consequently, a synergistic activation mechanism was proposed by comparing these conformations and the existence of the intermediate state. Then, tICA was applied to analyze the global movement, and it identified distinct active states, which provide hints for uncovering the biased signaling mechanism. The active AT1 receptor showed two distinct conformations, as determined by tICA, and thus supports the hypothesis that both the Gq-bound and β-arrestin 2-bound active conformations exist in the ensemble of the AT1 receptor. The two conformers are stabilized by Gq and β-arrestin 2 biased agonists, respectively,38, suggesting a population shift mechanism in the biased signaling80,81. In contrast, the balanced agonists equally stabilize the two conformers, and thus lead to no preference for the downstream proteins75. Therefore, the emergence of biased signaling can occur via a conformational selection mechanism. In other words, the regulation of GPCR activity is attributed to a generalized allosteric modulation42–84; hence, it is natural to design modulators targeting allosteric sites other than endogenous orthosteric sites85,86.

In the intermediate state, we discovered P6 as a site for allosteric regulation. Hidden between TM7 and H8, P6 was only observed during the upward movement of H8. Community analysis indicated that the impeding signal transmission between H8 and other parts impeded the formation of an active conformation (Supplementary Note 9). Mutagenesis experiments further confirmed that the residues around P6 transmit signals that modulate both G proteins and β-arrestins, and suggest that the allosteric perturbation from P6 has the potential to modulate the activity of the AT1 receptor. Several GPCRs currently lack features of endogenous ligands to initiate specific biased signaling. Thus, directly targeting intracellular TMs (such as P6) by allosteric modulators may provide a strategy to selectively regulate GPCRs.75,87 In particular, an unexploited allosteric site, P6 can be targeted in future allosteric drug designs for GPCRs. In order to fully realize P6 as an allosteric target, several GPCRs need to be structurally characterized in the apo state. Further structure-based drug design methodology and experimental investigation will be deployed to discover lead compounds. These studies are not only expected to provide insights into the activation mechanism of the AT1 receptor, but also offer versatile applications for GPCR biology, biophysics, and medicinal chemistry.

Methods

MD simulation system setup. The inactive AT1 receptor structure in complex with an antagonist, ZD7155, and a co-crystallized apocytochrome b562RIL (PDB 5.58
4YAY), as well as the fully active structure complexed with a partial agonist, S1I8, and an additional 0.15 mol L
1 KCl were also solvated in the systems. After the preparation, the inactive (PDB 4YAY) and active (PDB 6DO1)
structures were set as the initial and end states, respectively. The Amber ff14SB potential was adapted for atom
interactions91. First, we conducted 4000 steepest descent cycles, followed by 7000 conjugate descent cycles. Second,
every atom encountered 1.5 × 10⁴ cycles of steepest descent and 1.5 × 10⁵ cycles of conjugate gradient
minimization next. With 10 kcal mol⁻¹ Å⁻² position restraint on proteins and lipids, the systems were gradually heated from 0 to 300 K in 300 ps
and equilibrated for 700 ps under canonical ensemble conditions. Then, the 12 replicas underwent 10 ps MD simulations with an integration step of 2.0 fs. Finally, we collected 150 independent repeat trajectories with random initial velocities. The total simulation timescale was 300 μs. During simulations, the Particle mesh Ewald method was applied to calculate the long-range electrostatic interactions, and a cutoff of 10 Å was used for short-range electrostatic and van der Waals interactions. The SHAKE algorithm was employed for covalent bonds containing hydrogen. A temperature of 300 K was controlled by Langevin thermostat, while the collision frequency was 1.0 ps⁻¹. The snapshots were written out every 200 ps.

Markov State Model construction. According to the activation parameters, an MSM was built using the PyEMMA protocol (http://www.emma-project.org/latest/). Through the implied timescale validation (Supplementary Note 5), we confirmed that the AT₁ receptor systems were Markovian and reliable with a 200 model state microstate with a lag time of 8 ns and a maximum k-means iteration number of 200. Then, the microstates were clustered into three macrostates via the FCCA+ algorithm, which was configured by a Chapman–Kolmogorov test. Using TPT, we measured the transition probability matrix of the MSMSMs and computed the mean first passage time for each activation and inactivation process96. Based on the mdtraj package, we extracted the structures close to the microstate cluster centers of each macrostate into the trajectories for the corresponding macrostates. Then, using three trajectories, we selected the representative conformation of each macrostate, Sₜ. As shown in Eq. (5), the conformation with the highest Sₜ among the trajectories was regarded as the most representative conformation of the macrostate. The dᵢ is the RMSD between the conformations i and j, while dᵢj is the standard deviation of dᵢ.

\[ Sₜ = \frac{dᵢ}{dᵢj} \]  

(5)

Measurement of receptor expression by ELISA. HEK293 cells were transiently transfected with Flag-tagged WT AT₁ receptors or mutants, Flag-tagged V2R receptor, or Flag-tagged AT₁R conformational sensor or sensor-based mutants in 24-well plates. After incubation at 37 °C for 48 h, the cells were fixed with DPBS containing 4% (w/v) formaldehyde for 5 min at room temperature. For whole-cell ELISA, the cells were incubated in blocking solution (5% BSA in DPBS) containing 0.2% Triton X-100 for 1 h at room temperature. For cell surface ELISA, the cells were incubated in blocking solution without Triton X-100. The cells were washed three times with DPBS and incubated overnight with an anti-Flag primary antibody (Sigma Aldrich, Cat F1804, 1:1000) at 4 °C, followed by incubation with a horseradish peroxidase-conjugated secondary anti-rabbit antibody (Thermo Fisher, Cat A-21235, 1:5000) for 1 h at 22 °C. After washing, tetramethyl benzidine solution was added and the reaction was stopped by adding an equal volume of 0.25 M HCl solution. The solution was incubated for 5 min. The optical density of each well was measured at 450 nm using the TECAN luminance counter (Infinite M200 Pro NanoQuant). The optical density was plotted against the respective amounts of respective plasmids to determine the relative expression levels of each receptor or mutants. The levels of the AT₁ receptor mutants were normalized to that of the WT receptor.

Site-directed mutagenesis. All AT₁ receptor mutants used in the present study were generated by site-directed mutagenesis. The successful introduction of the mutations in the polymerase chain reaction products was verified by DNA sequencing. Corresponding primer sequences were shown in Supplementary Data 2.

BRET measurement. β-arrestin 2 recruitment. HEK293 cells were transiently cotransfected with β-arrestin 2-RLuc and YFP-tagged WT or mutated AT₁ receptor. After 24 h, the cells were seeded on 96-well microplates and incubated for 24 h at 37 °C, after which they were washed twice with HBSS and stained with AngII at different concentrations. Luciferase substrate coelenterazine-h (Promega) was added at the concentration of 5 μM before light emissions were recorded using a Mithras LB940 microplate reader (Berthold Technologies). The BRET signal was determined by calculating the ratio of luminescence at 530/485 nm.

G protein activation. Gq (Gq-Rluc8, G83, Gq9-GFP10), Gi (Gi12-Rluc8, G83, Gi9-GFP10), and G12(G12-Rluc8, G83, Gi9-GFP10) BRET probes were from the TRUPTHET kit, which was a gift from Bryan Roth (Addgene kit #10000001663). HEK293 cells were transiently co-transfected with WT or mutated AT₁ receptor along with specific G protein BRET probes according to the experimental setting. After 24 h, the cells were seeded on 96-well microplates and incubated for an additional 24 h. For the constitutive activity measurement, cells transfected with varying amounts of WT or mutated AT₁ receptor, or VᵢR were washed twice with...
HBSS buffer and the BRET signal was directly recorded after the addition of 5 μM luciferase substrate coelenterazine 400a using a Mithras LB940 microplate reader. For the AngII-stimulated G protein activation, the cells were washed twice with HBSS and stimulated with AngII at different concentrations. BRET signal was subsequently measured after the addition of the luciferase substrate and was calculated as the ratio of light emission at 510/400 nm.

The data obtained in the G protein activation assay and β-arrestin recruitment assay were normalized as a percentage of the Emax of WT AT1 receptor (reference receptor) in each pathway. The normalized data were analyzed using the “Operational Model” in GraphPad Prism to determine the transduction coefficient log(τ/Ka), in which τ is the transducer ratio and Ka is an agonist-receptor dissociation constant99, of the WT AT1 receptor and mutants at each signaling pathway99,100.

The equation for the “Operational Model” was defined by the following parameters:

Equation Type - Explicit Equation: EY = a function of X and parameters

\[
Y = \frac{A_1 + (A_2 + K_a + a)X}{(A_1 + (A_2 + K_a + a)X)}
\]

Where X = basal + (1 + 2a) \( \log \frac{X}{X_{WT}} \) and Y = basal + (1 + 2a) \( \log \frac{X}{X_{WT}} \)

The SEM of the \( \Delta \)log(τ/Ka) of AT1 receptor mutants in each signaling pathway was determined by the Eq. (6):

\[
\Delta \log \left( \frac{\tau}{K_a} \right) = \log \left( \frac{\tau_{mutant}}{\tau_{WT}} \right)
\]

The SEM of the \( \Delta \)log(τ/Ka) was calculated according to Eq. (7):

\[
SEM_{\Delta \log_{\tau/K_a}} = \sqrt{SEM_{\log_{\tau}mutant}^2 + SEM_{\log_{\tau}WT}^2}
\]

Data availability

The data that support this study are available from the corresponding authors upon reasonable request. The activation parameter and tICA data generated in this study are provided in the Source Data. The initial structures for MD simulations were obtained from the RCSB PDB database (PDB 4YAY and 6DO1). Other GPCRs were also downloaded from the RCSB PDB database (https://www.rcsb.org/). Pocket prediction was accomplished by fpocket, see http://fpocket.sourceforge.net/. Other simulation analyses were based on AMBER suite, according to http://ambermd.org/. Source data are provided with this paper.

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**Author contributions**

S.L. and J.Z. conceived and supervised the project. S.L., J.Z., J.S., and X.H. designed the experiments and revised the manuscript. X.H., Z.Y. and S.Z. designed and performed the experiments, and drafted the manuscript. X.H. contributed to MD simulations and data analysis. Z.Y., S.Z., and J.W. acquired and analyzed BRET data. Z.C., A.R., D.N., and J.P. acquired data and revised the manuscript. All authors discussed the results and reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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