Identification of Distinct Conformations of the Angiotensin-II Type 1 Receptor Associated with the G\textsubscript{q/11} Protein Pathway and the \(\beta\)-Arrestin Pathway Using Molecular Dynamics Simulations\(^*\)

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Background: The N111G and D74N mutations bias the AT\(_1\) receptor for the G\textsubscript{q/11} and \(\beta\)-arrestin pathways, respectively.

Results: Structural rearrangements of the AT\(_1\) receptor are induced by the N111G mutation and AngII.

Conclusion: Activation of the G\textsubscript{q/11} and \(\beta\)-arrestin pathways is associated with a decreased and increased stability, respectively, of the ground state of the receptor.

Significance: Distinct conformations of AT\(_1\) receptor are associated with distinct pathways.

Biased signaling represents the ability of G protein-coupled receptors to engage distinct pathways with various efficacies depending on the ligand used or on mutations in the receptor. The angiotensin-II type 1 (AT\(_1\)) receptor, a prototypical class A G protein-coupled receptor, can activate various effectors upon stimulation with the endogenous ligand angiotensin-II (AngII), including the G\textsubscript{q/11} protein and \(\beta\)-arrestins. It is believed that the activation of those two pathways can be associated with distinct conformations of the AT\(_1\) receptor. To verify this hypothesis, microseconds of molecular dynamics simulations were computed to explore the conformational landscape sampled by the WT-AT\(_1\) receptor, the N111G-AT\(_1\), receptor (constitutively active and biased for the G\textsubscript{q/11} pathway), and the D74N-AT\(_1\) receptor (biased for the \(\beta\)-arrestin1 and -2 pathways) in their apo-forms and in complex with AngII. The molecular dynamics simulations of the AngII-WT-AT\(_1\), N111G-AT\(_1\), and AngII-N111G-AT\(_1\) receptors revealed specific structural rearrangements compared with the initial and ground state of the receptor. Simulations of the D74N-AT\(_1\) receptor revealed that the mutation stabilizes the receptor in the initial ground state. The presence of AngII further stabilized the ground state of the D74N-AT\(_1\) receptor. The biased agonist [Sar\(^1\),Ile\(^4\),Ile\(^8\)]-AngII also showed a preference for the ground state of the WT-AT\(_1\) receptor compared with AngII. These results suggest that activation of the G\textsubscript{q/11} pathway is associated with a specific conformational transition stabilized by the agonist, whereas the activation of the \(\beta\)-arrestin pathway is linked to the stabilization of the ground state of the receptor.

The angiotensin-II type 1 (AT\(_1\))\(^2\) receptor, a class A GPCR, and its cognate ligand, the octapeptide hormone angiotensin II (AngII), are part of the renin-angiotensin-aldosterone system, responsible for controlling blood pressure and water retention via vascular smooth muscle contraction. The AT\(_1\) receptor can also activate steroidogenesis in the adrenal gland, neurosecretion, neuronal activity, cell growth, and proliferation (1). The AT\(_1\) receptor is classically known to signal through the G\textsubscript{q/11} pathway, but as is the case with many GPCRs, it also activates other pathways, including \(\beta\)-arrestins, G\textsubscript{12/13} proteins, and the epidermal growth factor receptor (2–5). This ability to activate different pathways can be biased by certain ligands (5, 6) or by mutating key amino acids in the receptor (4, 7), a phenomenon known as functional selectivity or biased signaling. Of particular interest is a region within the receptor identified as the major H-bond network (MHN). It is composed of several functionally important and conserved polar residues among class A GPCRs (8, 9). This region has also been identified as a sodium-binding site in some GPCRs (10, 11). Mutations within the MHN can impact the receptor’s ability to signal via certain pathways. The N111G mutation, known to increase the constitutive activity of the receptor on the G\textsubscript{q/11} pathway, impedes its phosphorylation by G protein-coupled receptor kinases and diminishes its coupling to \(\beta\)-arrestin1 after stimulation with AngII (7, 12, 13). However, the D74N mutation reduces signaling through the G\textsubscript{q/11} pathway but maintains wild-type level of signaling through the \(\beta\)-arrestin pathway when stimulated with AngII. The D74N-AT\(_1\) receptor shows increased \(\beta\)-arrestin signaling compared with the WT receptor when stimulated with the \(\beta\)-arrestin-biased agonist [Sar\(^1\),Ile\(^4\),Ile\(^8\)]AngII (4). These data suggest that the MHN is important to both the G\textsubscript{q/11} and the \(\beta\)-arrestin pathways and that mutations in this region of the receptor can tip the scale of functional selectivity in either direction. Although other regions are likely to play key roles in the biased signaling of the AT\(_1\) receptor, the characterization of the impact of the N111G and D74N mutations on the MHN and pHosphatidylcholine; MD, molecular dynamics; r.m.s.d., root mean square deviation; MHN, major H-bond network; SAS, solvent-accessible surface; PDB, Protein Data Bank; IP\(_3\), inositol monophosphate; BRET, bioluminescence resonance energy transfer.
the structure of the receptor in presence and in absence of AngII is of the utmost importance for understanding the underlying mechanisms of biased signaling of the AT1 receptor and GPCRs in general, which is poorly understood at the structural level.

In previous work, we showed that residues N111 and D74 are part of a relatively stable H-bond network in the ground state. The N111G mutation destabilized this H-bond network by removing the NH moiety involved in the internal stabilization of D74 carboxylate, thus favoring a reorientation of the side chain of D74 to form a new H-bond with residue N46. The importance of the Asp–Asn interaction for Gq signaling was confirmed in vitro (9). This study led us to postulate that the mutation of residue D74 to an asparagine could potentially stabilize the MHN and limit the reorganization of the MHN. Here, we further hypothesize that the destabilization and reorganization of the MHN (caused by the N111G mutation) and local structural changes of AT1 receptor are favoring Gq signaling, whereas the stabilization of the MHN (caused by the D74N mutation) is favoring β-arrestin signaling. To verify this hypothesis, we used microsecond time scale MD simulations of the WT-AT1 receptor, N111G-AT1 receptor, and D74N-AT1 receptor to explore their conformational landscape by looking at specific structural determinants. Furthermore, based on a previously developed model of the AT1 receptor in complex with AngII (14), we looked at how the presence of AngII in the binding pocket modified the conformational landscapes of the WT-AT1, D74N-AT1, and N111G-AT1 receptors and also how the β-arrestin-biased agonist [AB2]AngII (S18) favors Gq signaling. The simulations suggest that the N111G mutation destabilizes the ground state of the receptor. This destabilization favors conformational changes consistent with a transition from an inactive to an active state of the receptor engaged with a G protein. Conversely, we observe that the D74N mutation stabilizes the ground state, thus reducing the conformational landscape explored by the receptor. In accordance with our hypothesis, the presence of AngII in the WT-AT1 receptor favors the same conformational transitions as the N111G mutation. However, AngII further stabilizes the ground state of the β-arrestin-biased D74N-AT1 receptor. In the WT-AT1 receptor, the ligand S18 preferentially stabilized the ground state of the receptor compared with AngII.

**Experimental Procedures**

**Materials**—Desktop computers were used for the preparation and equilibration phase of the simulations. Production MD computations were made on the supercomputer Mammoth Parallèle II3 from the Université de Sherbrooke, managed by Calcul Québec and Compute Canada. All reagents were from Sigma unless otherwise indicated. Culture media, trypsin, FBS, penicillin, and streptomycin were from WISENT (St-Bruno, Quebec, Canada). Opti-MEM was from Invitrogen. Polyethylenimine (PEI) was from Polysciences (Warrington, PA). Coelenterazine 400A was from Gold Biotechnology (St. Louis, MO).

**Residue Numbering Scheme**—Residues of the AT1 receptor are given two numbering schemes. First, residues are numbered according to their positions in the AT1 receptor sequence. Second, residues are also indexed according to their position relative to the most conserved residue in the TMD where they are located. By definition, the most conserved residue is assigned the position index “50,” e.g. in TMD2, Asp74 is the most conserved residue and is designated D742.50, whereas the upstream residue is designated Asp732.49 and the downstream residue is designated L752.51. This indexing simplifies the identification of aligned residues in different GPCRs (17).

**Homology Modeling**—We used the I-TASSER server to generate multiple template homology structures of the AT1 receptor. The resulting five best structures provided in the output had near identical orientations of the side chains of the H-bond network. We selected the only structure that featured both known disulfides bonds, which had a high confidence score of 0.99 (18, 19). The backbone of the model is very similar to the crystal structure of the CXCR4 receptor (PDB code 3ODU), with a root mean square deviation (r.m.s.d.) distance of 0.90 Å between the positions of Ca atoms. Sequence alignment between AT1 and CXCR4 and superposition of the two structures (Fig. 3A) can be found in our previous work using this model (9). The homology model was also analyzed with ProCheck (20), and the Ramachandran plot indicated that over 97% of the residues were in the “most favored” and “additionally allowed” regions. The rest of the stereochemistry was also of high quality. The unstructured N- and C-terminal portions of the model were truncated by removing residues 1–14 and 319–359, respectively, to keep the simulation box as small as possible. This enables better performances for the MD simulations. AngII’s initial conformation and position inside the binding pocket were determined in a previous work (14). Models of the N111G-AT1 receptor and D74N-AT1 receptor were generated by replacing residue Asn111 or Asp74 by the corresponding residue using the mutagenesis feature in PyMOL.

**Molecular Dynamics Simulations**—The GROMACS software suite (21–24) was used to prepare and run the simulations. The AT1 receptor, N111G-AT1 receptor, and D74N-AT1 receptor models were inserted in a lipid bilayer consisting of 128 molecules of DOPC using the InflatableGRO approach (25). Simulation parameters were based on previous work (26–28). The membrane-receptor system was solvated with the SPC water model (29). Counter-ions were added at random positions, replacing water molecules, to keep the net charge of the system at 0. The f6g5z6 force field, modified to use the Berger parameters (30), was used for the calculations. Parameters for the DOPC molecules and the PDB file of the bilayer, developed by the Tieleman group (31–33), were obtained from Peter Tieleman’s website. A first equilibration phase was performed under conditions of constant pressure, temperature, and number of molecules (NPT) for 1 ns while gradually heating the system for the first 500 ps to reach the desired temperature of 310 K. During this first phase, the phosphate head group of the DOPC molecules was restrained. This was followed by a second equilibration in NPT conditions for 15 ns with the pressure set at 1 bar to allow for the removal of counter-ions and water. The last 10 ns of the simulation were used for analysis.

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Barostat with high-precision floating point format used as well as differences in imprecision of the calculations, which originate from the sin~gence in the multiple trajectories is the result of the inherent and velocities at the start of the production MD runs. Divergence in the multiple trajectories is the result of the inherent imprecision of the calculations, which originate from the single-precision floating point format used as well as differences in the order of addition of force caused by dynamic load balancing ((a + b) + c ≠ a + (b + c) due to rounding-off). The simulations were run in periodic boundary conditions at constant temperature (310 K) and pressure (1 bar) using the Nose-Hoover thermostat (37, 38) with $\tau_T = 0.2$ ps and the Parrinello-Rahman barostat with $\tau_P = 5$ ps, respectively. Simulation data were saved every 20 ps, for a total of 50,001 frames/µs. Stability of the systems was assessed by calculating the r.m.s.d. distance between the positions of Ca atoms of the TMDs during the simulations. In all trajectories, the r.m.s.d. converged to values between 2.5 and 3.5 Å independently of the receptor mutants, indicating that equilibrium was reached.

Trajectory Analysis—MD trajectory output from GROMACS was converted to PDB files for visual inspection with PyMOL (39) and to compressed XTC trajectory files for other analyses. Data regarding distances, dihedral angles, and solvent-accessible surface were performed, respectively, with the g_dist, g_angle, and g_sas tools within GROMACS. The r.m.s.d. for the region of TMD7 undergoing a conformational transition was calculated using the g_rms tool after performing a least squares fit on the backbone atoms of residues 288–301. Two-dimensional probability density functions describing the populations of substrates defined by the selected metrics were calculated using g_sham with a grid of 50 × 50 bins and nlevels = 200.

Constructs—The cDNA clone for the human AT<sub>1</sub> receptor was kindly provided by Dr. Sylvain Meloche (University of Montréal). The AT<sub>1</sub>-GFP10 construct was built by inserting the GFP10 sequence at the C terminus of the AT<sub>1</sub> construct, joined by the linker GSAGT, using the In-Fusion® PCR cloning system (Clontech) as recommended by the manufacturer. The RLuc-βarrestin1 and RLuc-βarrestin2 constructs were kindly provided by Dr. Michel Bouvier (University of Montréal). The N111G-AT<sub>1</sub>-GFP10 and D74N-AT<sub>1</sub>-GFP10 constructs were built using the QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. Briefly, forward and reverse oligonucleotides were constructed to introduce either the N111G or D74N mutation in the AT<sub>1</sub>-GFP10 receptor background. Site-directed mutations were then confirmed by automated DNA sequencing by aligning the AT<sub>1</sub> receptor sequence with MultAlin (40).

Cell Culture and Transfection—HEK293 cells were maintained in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified 5% CO₂ atmosphere. The day prior to transfection, cultured cells were washed with PBS at room temperature, trypsinized, and seeded at 150,000 cells/well in a 6-well plate. For transfection, 2 µg of the DNA construct containing the appropriate AT<sub>1</sub> receptor construct was added to 100 µl of Opti-MEM medium containing 6 µg of PEI, and the mixture was incubated for 20 min before being added to the cultured cells, as described previously (41). For β-arrestin recruitment assays, HEK293 cells (3 × 10⁶ cells) were transiently transfected with 8700 ng of AT<sub>1</sub>-GFP10 or mutant receptors and either 300 ng of RLuc-βarrestin1 or 300 ng of RLuc-βarrestin2 using linear PEI (1 mg/ml) (PEI/DNA ratio 4:1).

Inositol Phosphate Production—Inositol monophosphate (IP₁) production was determined using the IP-One assay (Cis-Bio Bioassays, Bedford, MA). Necessary dilutions of the agonist AngII were prepared in stimulation buffer (Hepes 10 mM, CaCl₂ 1 mM, MgCl₂ 0.5 mM, KCl 4.2 mM, NaCl 146 mM, glucose 5.5 mM, LiCl 50 mM, pH 7.4). 48 h after transfection, the cells were washed with phosphate-buffered saline (PBS) at room temperature. The cells were trypsinized and distributed at 20,000 cells/well (7 µl) in a white 384-well plate in stimulation buffer. Cells were stimulated at 37 °C for 30 min with increasing concentrations of AngII. Cells were then lysed with 3 µl of PI1-d2. After addition of 3 µl of anti-IP₁-cryptate antibody and cells were incubated for 1 h at room temperature under agitation. FRET signal was measured with a TECAN M1000 plate reader.

β-Arrestin Recruitment in BRET-based Biosensor Assays—At 48 h post-transfection, cells were washed with PBS and resuspended in stimulation buffer. For the β-arrestin recruitment assays, the proximity of fusion protein RLuc-βarrestin to the reporter AT<sub>1</sub>-GFP10 is evaluated. Upon stimulation, RLuc-βarrestin is recruited to the AT<sub>1</sub>-GFP10 fusion protein, whereby the BRET signal is increased. Cells transfected with the appropriate constructs were stimulated with the indicated ligands in 96-well white plates (50,000 cells/well) for 8 min, and then coelenterazine 400A was added at a final concentration of 5 µM. All BRET signals were measured using a TECAN M1000 fluorescence reader (TECAN, Austria). The BRET ratio was calculated as the GFP10 emission over luminescence emission. Net BRET ratio was calculated by subtracting the BRET ratio upon maximal stimulation with the BRET ratio under basal conditions. All data were expressed as a percentage of maximal AngII response toward AT<sub>1</sub>-GFP10.

Results

Experimental Validation of the Biases Caused by the N111G and D74N Mutations—We verified that the WT-AT₁<sub>1</sub>, the N111G-AT₁<sub>1</sub>, and the D74N-AT₁<sub>1</sub> receptors can associate with β-arrestins (1 and 2) using the BRET2 assay. The N111G-AT₁<sub>1</sub>
receptor displayed a higher basal signal than the other two receptors (0.075 versus 0.045). After stimulation with AngII, the N111G-AT1 receptor showed poor BRET ratio increases of about 0.020 with both β-arrestins (Fig. 1, A and C). The D74N-AT1 receptor showed a BRET ratio increase of 0.060 with β-arrestin1 and a BRET ratio increase of 0.075 with β-arrestin2. The WT-AT1 receptor showed a BRET ratio increase of 0.075 for β-arrestin1 and a BRET ratio increase of 0.100 with β-arrestin2. We used the data to establish the maximum increase in β-arrestin recruitment relative to the WT-AT1 receptor after normalizing for each receptor’s level of expression (Bmax). Fig. 1, B and D, shows that stimulation of the D74N-AT1 receptor with AngII produced 74 and 77% of the signal of the WT-AT1 receptor for β-arrestin1 and a BRET ratio increase of 0.100 with β-arrestin2. The WT-AT1 receptor showed a BRET ratio increase of 0.075 for β-arrestin1 and a BRET ratio increase of 0.100 with β-arrestin2. The WT-AT1 receptor showed a BRET ratio increase of 0.075 for β-arrestin1 and a BRET ratio increase of 0.100 with β-arrestin2. We used the data to establish the maximum increase in β-arrestin recruitment relative to the WT-AT1 receptor after normalizing for each receptor’s level of expression (Bmax). Fig. 1, B and D, shows that stimulation of the D74N-AT1 receptor with AngII produced 74 and 77% of the signal of the WT-AT1 receptor for β-arrestin1 and a BRET ratio increase of 0.100 with β-arrestin2. The WT-AT1 receptor showed a BRET ratio increase of 0.075 for β-arrestin1 and a BRET ratio increase of 0.100 with β-arrestin2.

Inositol phosphate production assays confirmed the constitutive activity of the N111G-AT1 receptor and the poor activation of the Gq/11 pathway by the D74N-AT1 receptor (Fig. 1E). These results confirmed the bias of the N111G-AT1 receptor toward the Gq/11 pathway and the bias of the D74N-AT1 receptor toward the β-arrestin pathway.

**MD Simulations of the N111G Mutant Suggest That the Activation of the Gq/11 Pathway Is Linked to a Destabilization of the Helical Structure of TMD7 of the AT1 Receptor**—As described previously, we simulated the molecular dynamics of an experimentally validated homology model of the “resting” state of AT1 receptor (and mutants) embedded in a hydrated DOPC bilayer (9). Each system (WT-AT1, N111G-AT1, and D74N-AT1) was simulated for at least 1 μs of MD time carried out as 10 × 100-ns MD simulations. We also simulated each receptor

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**FIGURE 1.** Signaling properties of the WT-AT1 receptor and the biased mutants on the β-arrestins and inositol phosphate pathways. HEK293 cells were transfected with the indicated receptor, and their recruitment of β-arrestin1 (A), β-arrestin2 (C), and IP3 production (E) was assayed as described under “Experimental Procedures.” Each point represents the mean ± S.D. of duplicate determinations of a typical experiment, which is representative of at least three independent experiments. Bar graphs represent the mean ± S.E. for the maximum increase in β-arrestin1 (B) and β-arrestin2 (D) recruitment relative to the WT-AT1 receptor after normalizing for each receptor’s level of expression.
in the presence of AngII. We used ligand poses satisfying experimentally determined receptor contacts previously obtained by photoaffinity labeling as starting structure of the three complexes (14). In our previous MD simulation of the N111G receptor (9), we had observed a conformational change in TMD7, between residues I2887.39 and N2957.46. In fact, we had witnessed a transition from a helical to an extended configuration of the backbone. We thus monitored this region of the receptor in our most recent simulations to see whether it was consistent and reproducible.

By measuring the r.m.s.d. of the Cα atoms of residues I2887.39 to N2957.46 following a superposition of TMD7 in all frames of the MD simulations, it is possible to monitor the local structural change in the helix while ignoring rigid body movements. This analysis of the trajectories of the WT-AT1 receptor without ligand showed that residues I2887.39 through N2957.46 had a stable helical conformation. Indeed, a single population (with maximum probability (pmax) of 0.160 at r.m.s.d. = 0.040 nm) can be seen on the one-dimensional probability distribution function (Fig. 2). The D74N mutation showed higher probability (0.230) and narrower distribution around the same r.m.s.d., suggesting an increased stability of that region of TMD7 (Fig. 2A). The N111G mutation, as expected, showed a much broader r.m.s.d. distribution (reaching beyond 0.30 nm) with the appearance of new populations (pmax = 0.058), indicating a change in conformation of this region of TMD7 (Fig. 2B). An r.m.s.d. beyond 0.16 nm indicates a transition from an α-helical conformation toward the extended configuration (Fig. 3C). Below this threshold, intrahelical H-bonds are still present to stabilize a helical conformation. In the presence of AngII, the stability of the α-helical conformation of TMD7 was decreased for the WT-AT1 receptor as the population of low r.m.s.d. conformations was diminished (p = 0.023 at r.m.s.d. = 0.040 nm, pmax = 0.094 at r.m.s.d. = 0.096) to the favor of higher r.m.s.d. populations (r.m.s.d. > 0.30 nm) (Fig. 2). The presence of S18 in the WT-AT1 receptor caused a slight reduction, narrowing and rightward shift of the low r.m.s.d. population compared with the WT receptor (p = 0.053 at r.m.s.d. = 0.04 nm and pmax = 0.157 at r.m.s.d. = 0.056 nm) along with the appearance of higher r.m.s.d. populations (Fig. 2A). Similarly to its nonliganded state, the N111G-AT1 receptor in complex with AngII showed a wide distribution of populations (Fig. 2B). The AngII-D74N-AT1 receptor showed a higher and narrower population (pmax = 0.276) at r.m.s.d. = 0.04 nm compared with its nonliganded state, which suggests that the α-helical conformation was further stabilized by the presence of AngII (Fig. 2A). It thus appears that activation of the Gα11 pathway is associated with a loss of helical structure between residues 2887.39 and 2957.46 in TMD7, whereas maintaining the helical structure is associated with β-arrestin signaling.

New Interhelical H-bonds Stabilize the Conformational Change in TMD7—In the resting state of the WT-AT1 receptor, an inter-helical H-bond between the side chains of S2526.47 and N2947.45 as well as H-bonds involving the side chains of N1113.35 and N2957.46 with the carboxylate of D742.50 were shown to stabilize the MHN (Fig. 3, B and E). However, analysis of the trajectories revealed that these inter-helical H-bonds were reorganized in the N111G-AT1 receptor. For example, the side chain of N2947.45 was re-oriented toward the side chain of D742.50 to form new H-bonds (Fig. 3, C and F). Concomitantly, the side chain of S2526.47 formed new stabilizing H-bonds with backbone atoms of residues 2917.42–2947.45, which underwent a conformational transition. Note that these new inter-TMD H-bonds replaced the intra-helical H-bonds in their α-helical state (Fig. 3, B and D). The re-orientation of residue D742.50 toward N461.50, previously observed in the N111G-AT1 receptor and identified as important for Gα11 signaling (9), was also observed in the trajectories (Fig. 3, C and F).

To verify the potential correlation between these switches in inter-TMD H-bonds and the conformational transition in TMD7, we generated two-dimensional probability distribution functions. In the first one (Fig. 4), we monitored the correlation between the conformational transition (r.m.s.d. of the backbone atoms of residues 2887.39–2957.46) and the formation of H-bonds between S2526.47 and TMD7 over all frames of the MD simulation. The formation of the H-bonds was monitored by measuring the distance between the Oγ atom of S2526.47 and the center-of-mass of the backbone amide hydrogens of N2947.45 and F2937.44 and carbonyl oxygen of A2917.42. In the second one (Fig. 5), we monitored the potential coupling between the conformational transition and the re-orientation of the side chain of N2947.45 as measured by the distance between the Nơ atom of N2947.45 and the Cγ atom of D742.50 over all frames of the MD simulations. The resulting two-dimensional probability distribution functions indicate that as the r.m.s.d. of the backbone atoms of residues 2887.39–2957.46 increases from around 0.05 nm in the resting state to beyond 0.30 nm (conformational transition in TMD7), the distance between the side chain of S2526.47 and the backbone atoms of residues 2917.42–2947.45 diminishes from about 0.54 to 0.36 nm (Fig. 4). As this occurs, the distance between the side chains of residues N2947.45 and D742.50 is also decreased from about 1.00 to 0.50 nm (Fig. 5). The conformational landscapes of the three systems activating the Gα11 pathway (AngII-WT-AT1, N111G-AT1, and AngII-N111G-AT1 receptors) all show a similar transition away from the initial resting conformation observed in the WT-AT1 receptor. However, the D74N-AT1 receptor, with and without AngII, is more stable than the WT-AT1 receptor without ligand in the initial resting state. Conformational landscapes generated for the S18-WT-AT1 receptor depict the same transition observed with the AngII-WT-AT1 receptor but also suggest that the initial resting state is favored by S18 compared with AngII as it has a similar relative population than in the WT-AT1 receptor without AngII (Figs. 4 and 5). These results further suggest a correlation between the conformational transition in TMD7 and the activation of the Gα11 pathway and provide explanations as to how this transition is stabilized by new inter-helical interactions.

Conformational Transition in TMD7 Is Associated with a Higher Probability of Opening the G Protein-binding Site—Crystal structures of GPCRs in complex with a Go-subunit (42–45) have revealed that the coupling involves an opening between TMD3 and TMD6 of the receptors that allows for the insertion of the C-terminal helix of the Go-subunit. Consequently, the coupling causes an increase in the interhelical distance between TMD3 and TMD6 at the cytosolic interface.
This has led to the hypothesis that when a GPCR couples to and activates G proteins, this is accompanied, among other potential conformational changes, by a relative displacement between TMD3 and TMD6. To unveil a potential link between the conformational transition in TMD7 and an increase in the interhelical distance between TMD3 and TMD6, we monitored the distance between the cytosolic ends of TMD3 (center-of-mass of the backbone atoms of residues S123 to Y127) and TMD6 (center-of-mass of the backbone atoms of residues I238 to I242) during the MD simulations. We then calculated the probability distribution functions between this distance and the r.m.s.d. of the backbone atoms of residues 288–295. All simulated systems sampled an open conformation as indicated by the increase of the distance between TMD3 and TMD6 from about 1.0 to 1.1 nm in the initial resting state to more than 1.5 nm. There is, however,
an interesting trend emerging from our analysis; it appears there is an increased probability of existence of the open state concurrent with the conformational transition in TMD7. Indeed, once the r.m.s.d. in TMD7 increases above a value of about 0.16 nm, the difference in probability between the open and closed states becomes less pronounced and favors the open state. This trend is particularly striking in the N111G-AT1 and AngII-N111G-AT1 receptors. Interestingly, the presence of the biased agonist SI8 in the WT-AT1 receptor increases the relative probability of finding the receptor with a stable helical TMD7 while decreasing the probability of the relative displacement between TM3 and TMD6 when compared with the AngII-WT-AT1 receptor. Therefore, the conformational transition in TMD7 seems to relieve structural constraints facilitating the opening between TMD3 and TMD6, but beyond this point, a higher r.m.s.d. between the backbone atoms of residues 2887.39–2957.46 is not associated with a larger probability of opening. In other words, although the conformational transi-
tion in TMD7 and the opening between TMD3 and TMD6 can occur independently, there appears to be a relationship between the loss of helical structure in TMD7 and the probability of opening the G protein-binding site between TMD3 and TMD6.

**Loss of α-Helical Conformation in TMD7 Is Associated with a Higher Probability of Opening the Hydrophobic Core Adjacent to the Binding Pocket**—We had previously identified a hydrophobic core between TMD3 and TMD7 (see Fig. 7 in Ref. 9). The stability of that hydrophobic core (probability of being formed during MD simulations) was correlated with the inability of an AT1 receptor mutant (N111W) to engage the Gq/11 pathway. Moreover, an MD simulation of the N111G-AT1 receptor showed that this hydrophobic core was destabilized and opened, effectively expanding the binding pocket (9). To substantiate this movement, we monitored the distance between TMD3 (backbone atoms of residues V1083.32 to L1123.36) and TMD7 (backbone atoms of residues 2887.39 to Y2927.43) in the region harboring the hydrophobic core in the different variations of the AT1 receptor. The two-dimensional probability distribution functions between the conformational transition from the r.m.s.d. of residues I2887.39 to N2957.46 of TMD7 and the distance between TMD3 and TMD7 in the hydrophobic core indicated that the distance between TMD3 and TMD7 can increase from about 1.1 nm in the ground state to reach 1.6 nm when the conformational transition in TMD7 is complete in the AngII-WT-AT1, N111G-AT1, AngII-N111G-AT1, and SI8-WT-AT1 receptors (Fig. 7). However, the calculations showed that the hydrophobic core has an increasing probability of staying closed in WT-AT1, D74N-AT1, and AngII-D74N-AT1 receptors (Fig. 7). Despite the conformational transition in TMD7 being observed in the SI8-WT-AT1 receptor, the initial resting conformation is more stable than with the AngII ligand and appears nearly as stable as the WT-

![FIGURE 4. Probability landscape generated by sorting frames of the MD simulations according to two measurements as follows: x axis, distance between the Oγ atom of residue S2526.47 and the center-of-mass of backbone atoms of A2917.42 (carbonyl O), F2937.44 (amine hydrogen) and N2947.45 (amine hydrogen); y axis, r.m.s.d. of the Cα atoms of residues 288–295 on TMD7.](image-url)
AngII receptor without ligand. These results suggest that the hydrophobic core is destabilized in Gq-active receptors and that this destabilization is correlated with the conformational transition in TMD7.

Loss of α-Helical Conformation in TMD7 Increases the Accessibility of the Side Chain of Residue Y302<sup>7,53</sup> in the NPXXY Motif—Because the conserved NPXXY motif lies at the end of TMD7, we monitored how the conformational transition observed just above impacted the movement of the bottom portion of TMD7. We measured the distance between the NPXXY motif (taken as the center-of-mass of the backbone atoms of residues N298<sup>7,49</sup> to Y302<sup>7,53</sup>) and a fixed region in the middle of TMD3 (taken as the center-of-mass of the backbone atoms of residues N111<sup>3,35</sup> to S115<sup>3,39</sup>) to evaluate the movement of the NPXXY motif relative to the TM bundle of the receptor. The two-dimensional probability distribution functions between this distance and the r.m.s.d. of the Cα of residues I288<sup>7,39</sup> to N295<sup>7,46</sup> (Fig. 8) indicate that the basal distance in the WT-AT<sub>1</sub> receptor is about 1.5 nm, with a secondary population at about 1.75 nm. A population centered at \(d = 1.5\) nm was observed with the SI8-WT-AT<sub>1</sub> receptor but with a slightly narrower distribution and higher probability. However, conformations centered at \(d = 1.75\) nm display a much broader distribution in r.m.s.d. indicating the occurrence of the conformational transition in TMD7. Both the N111G mutation and the presence of AngII allow the distance between TMD3 and the NPXXY motif to increase beyond 2.0 nm, and the increase in distance appears facilitated by the increase in the r.m.s.d. of residues I288<sup>7,39</sup> to N295<sup>7,46</sup>. The D74N-AT<sub>1</sub> receptor with and without AngII shows an increased probability of sampling the initial resting state, and no significant secondary population at 1.75 nm is observed. The MD simulations were further analyzed to verify whether this movement could be associated with changes in the interactions formed by residues N298<sup>7,49</sup> or Y302<sup>7,53</sup> of the

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**FIGURE 5.** Probability landscape generated by sorting frames of the MD simulations according to two measurements as follows: \(x\) axis, distance between the Oγ atom of residue Ser<sup>252</sup> and the center-of-mass of backbone atoms of A291<sup>7,42</sup> (carbonyl O), F293<sup>7,44</sup> (amine hydrogen), and N294<sup>7,45</sup> (amine hydrogen); \(y\) axis, distance between the Cγ atom of N294<sup>7,45</sup> and the Cγ atom of D74<sup>2,50</sup> (or the mutated D74N).
Although the analysis did not identify new specific interactions, it did reveal a change in solvent accessibility for the side chain of residue Y302. Indeed, Y302 has a small solvent accessibility surface (SAS) in the initial resting state as it is buried between hydrophobic residues V49, A63, F66, L67, L70, L122, L123, L288, N295, and I242 (Fig. 9A). Fig. 10 shows that the most populated states have an SAS of 0.4 nm² in the WT-AT₁ receptor and the SI8-WT-AT₁ receptor. The SAS is reduced to 0.3 nm² for the D74N-AT₁ receptor and 0.2 nm² for the AngII-D74N-AT₁ receptor. In opposition, the SAS in the AngII-WT-AT₁ receptor has higher probabilities of reaching higher values. The most populated states are at SAS = 0.9 nm² for the N111G-AT₁ receptor with and without AngII. These results indicate that the conformational transition in TMD7 favors a movement of the NPXXY motif away from the TM bundle, which increases the solvent accessibility of Y302 (Fig. 9B).

β-Arrestin Bias Induced by the D74N Mutation and the SI8 Ligand Is Associated with a Restriction of the Conformational Landscape Explored by AT₁ Receptor—So far, our results focused on structural changes associated with Gq/11 signaling caused by either the presence of the endogenous agonist AngII or the N111G mutation. The MD simulations of the β-arrestin-biased D74N-AT₁ receptor with and without AngII revealed that it was conformationally more stable. Indeed both systems did not display significant conformational transitions as observed with the other systems. In fact, all the probability distribution functions calculated indicate that the D74N-AT₁ receptor explores a much smaller conformational landscape than the others. They also unveiled the existence of a highly populated state, which corresponds to the most probable and initial resting state of the nonliganded WT-AT₁ receptor. To understand the molecular mechanism for such stabilization, we monitored how the D74N mutation affects the MHN.

FIGURE 6. Probability landscape generated by sorting frames of the MD simulations according to two measurements as follows: x axis, distance between the center-of-mass of backbone atoms of residues S123 to Y127 (intracellular extremity of TMD3) and residues I288 to N295 (intracellular extremity of TMD6); y axis, r.m.s.d. of the Ca atoms of residues I288 to N295 on TMD7.
at position 74 allowed simultaneous interactions with the asparagines at position 111 and 46 (Fig. 3, D and G). It also caused residue N295 to be oriented slightly more toward TMD3 than TMD2 compared with the WT-AT1 receptor, allowing it to interact with the side chain of residue S115 (Fig. 3, D and G). This interaction tightened the helical conformation in this region of TMD7. By measuring the distance between the N6 atom of Asn295 and the Oy atom of S115, we observed that the tendency of N295 to be close to S115 was increased by the D74N mutation \( p_{\text{max}} = 0.210 \) at 0.28 nm when compared with the WT-AT1 receptor \( p = 0.082 \) at 0.28 nm and \( p_{\text{max}} = 0.198 \) at 0.39 nm. This tendency was further increased in the AngII-liganded D74N-AT1 receptor \( p_{\text{max}} = 0.327 \) at 0.28 nm (Fig. 11A). However, this was decreased by the presence of AngII in the WT-AT1 receptor \( p = 0.033 \) at 0.28 nm and \( p_{\text{max}} = 0.066 \) at 0.68 nm or by the N111G mutation \( p = 0.047 \) at 0.28 nm and \( p_{\text{max}} = 0.066 \) at 0.62 nm without AngII and \( p = 0.063 \) at 0.28 nm and \( p_{\text{max}} = 0.084 \) at 0.60 nm with AngII (Fig. 11B). The distance between S115 and N295 was also decreased, to a lesser extent, in the SI8-WT-AT1 receptor \( p_{\text{max}} = 0.147 \) at 0.41 nm (Fig. 11A). The results observed here with the SI8-WT-AT1 receptor are representative of what has been observed so far with the SI8 ligand in that it does not restrict the conformational landscape of the receptor as the D74N mutation does, but it shows a clear preference for the initial resting state compared with the AngII ligand. These results suggest that the D74N-AT1 receptor is more stable in the initial resting state than the WT-AT1 receptor and that the presence of AngII might further stabilize this conformation. Furthermore, the SI8 ligand is less destabilizing for the initial resting state of the WT-AT1 receptor than the AngII ligand.

**Residue Phe-8 of AngII Is Inserted in the Hydrophobic Core of Gq-active Receptors**—The MD simulations of the WT-AT1, N111G-AT1, and D74N-AT1 receptors in complex with AngII

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**FIGURE 7.** Probability landscape generated by sorting frames of the MD simulations according to two measurements as follow: x axis, distance between the center-of-mass of backbone atoms of residues V108–L112 (region of the hydrophobic core on TMD3) and residues I288–Y292 (region of the hydrophobic core on TMD7); y axis, r.m.s.d. of the Cα atoms of residues I288–Y292 through N295 on TMD7.
FIGURE 8. Probability landscape generated by sorting frames of the MD simulations according to two measurements as follows: *x* axis, distance between the center-of-mass of the backbone atoms of residues N1113.35 to S1153.39 (middle of TMD3) and the center-of-mass of the backbone atoms of residues N2987.49 to Y3027.53 (NPXXY motif), *y* axis, r.m.s.d. of the Cα atoms of residues I2887.39 through N2957.46 on TMD7.

FIGURE 9. Snapshots from MD simulations showing different SAS of the Y3027.53 side chain from the NPXXY motif as it is surrounded by hydrophobic side chains. *A*, buried configuration, with SAS of 0.21 nm² for Y3027.53. *B*, a more exposed configuration, with SAS of 0.82 nm² for Y3027.53.
provided a lot of structural insights on how AngII can stabilize different states of the WT-AT₁ receptor and the two biased mutants. In all cases and in accordance with previous studies (14, 46–49), we have found that all AT₁-AngII complexes are rather dynamic. However, we noted distinct sectors of the AT₁ receptor suited to accommodate specific interactions with the side chains of the AngII ligand. For example, we noted that Arg² of AngII generally formed H-bonds with residues D2636.58 and D2817.32 (Fig. 12A) and that Val³ faced hydrophobic residues on ECL2 (Fig. 12B). Residue His⁶ in AngII was usually positioned between TMD1, -2, and -7 where it formed H-bonds with Y351.39 and sometimes Y2927.43. Residue His⁶ also interacted with W842.60 (Fig. 12C). Y351.39, Y2927.43, and W842.60 were also observed to interact with each other independently of the presence or absence of AngII. Residue R1674.64 of the AT₁ receptor could occasionally reach this binding area and form an H-bond with His⁶ in its neutral state. Residue Ile⁵ of AngII was above the usual position of His⁶ and surrounded by the side chains of residues I27¹.31, F28¹.32, I31¹.35, T88⁷.64, P285⁷.36, and I288⁷.39 (Fig. 12D). The C-terminal moiety of AngII was stabilized by an extensive network of polar residues and formed H-bonds with Y113³.37, K199⁵.42, N200⁵.43, H256⁶.51, Q257⁶.52, and T260⁶.55 (Fig. 12E). The side chains of residues Asp¹ and Tyr⁴ were much more mobile and interacted with various polar groups in the extracellular domains of the receptor as well as with water molecules (data not shown). The main difference in ligand binding that could be observed between Gq-active and Gq-inactive receptors was the propensity for the phenyl moiety of the Phe⁸ side chain of AngII to be inserted within the hydrophobic core when it was in the “open” configuration after the conformational transition in TMD7 had occurred (Fig. 12F). In such a case, the phenyl moiety of Phe⁸ contacted all residues of the hydrophobic core (F77².53, V108¹.32, L112³.36, I288⁷.39, A291⁷.41, and Y292⁷.42), as well as W253⁶.48

**FIGURE 10.** Probability landscape generated by sorting frames of the MD simulations according to two measurements as follows: x axis, distance between the center-of-mass of the backbone atoms of residues N111².35 to S115².39 (middle of TMD3) and the center-of-mass of the backbone atoms of residues N298⁷.48 to Y302⁷.53 (NPXXY motif); y axis, solvent-accessible surface of the side chain of residue Y302⁷.53.
and H256.5. When the hydrophobic core was closed, the phenyl moiety of Phe8 was adjacent to the hydrophobic core and made contacts mostly with residues among V108\textsuperscript{3.32}, L112\textsuperscript{3.36}, I288\textsuperscript{7.39}, W253\textsuperscript{6.48}, and H256\textsuperscript{6.51}, although not simultaneously (Fig. 12). The insertion in the hydrophobic core was not observed with the smaller Ile\textsuperscript{8} residue of the SI8 ligand. To monitor the insertion of Phe\textsuperscript{8} in the hydrophobic core, we measured the distance between the center-of-mass of the Phe\textsuperscript{8} side chain and the center-of-mass of the side chain of residues forming the hydrophobic core (F77\textsuperscript{2.53}, V108\textsuperscript{3.32}, L112\textsuperscript{3.36}, I288\textsuperscript{7.39}, A291\textsuperscript{7.41}, and Y292\textsuperscript{7.42}). The two-dimensional probability distribution functions showed that the distance between Phe\textsuperscript{8} and the hydrophobic core tends to decrease from about 0.74 to 0.40 nm as the hydrophobic core opens (Fig. 13, left) or the r.m.s.d. of TMD7 increases (Fig. 13, right). The landscape for the AngII-N111G-AT\textsubscript{1} receptor showed the two populations more distinctly, one with the hydrophobic core closed (d = 1.15 nm) and the Phe\textsuperscript{8} further from it (d = 0.74 nm), and one with the hydrophobic core open (d = 1.4 nm) and Phe\textsuperscript{8} inserted within it (d = 0.40 nm).
Distinct Conformations of AT₁ Receptor in Biased Signaling

Discussion

In this study, we used molecular dynamic simulations to unveil structural features that could explain the biased signaling properties of AT₁ receptor mutants (N111G-AT₁ receptor and D74N-AT₁ receptor) and of an AT₁ receptor ligand (SI8) observed experimentally. More precisely, we further confirmed and expanded our understanding of the molecular basis of N111G-AT₁’s biased signaling. Upon stimulation with its agonist AngII, this mutant efficiently activates the Gq pathway but βarrestin1 and -2 are recruited to a lesser extent than the WT-AT₁ receptor. Similarly, we established that the D74N-AT₁ receptor mutant efficiently recruits the βarrestin2 and -1 but does not activate the Gq/11 pathway (Fig. 1). These observations support the previous suggestions that the N111G-AT₁ receptor had limited coupling to βarrestin1 (12) and that the D74N-AT₁ receptor was biased for the βarrestin2 pathway and could not activate the Gq/11 pathway (4). However, we cannot overlook the increased basal BRET ratio of the N111G receptor, which could be indicative of a basal coupling between the N111G-AT₁ receptor and β-arrestins. It was shown that an increase in phosphorylation of the C-terminal tail of the AT₁ receptor is required for high affinity binding of β-arrestins and internalization (50, 51) and that the C-terminal tail of the N111G-AT₁ receptor, even after stimulation with AngII, is not phosphorylated beyond the basal level of the WT-AT₁ receptor (7). These observations support the notion that this basal coupling could be a transient, low affinity interaction caused by the increased opening of the G protein-binding site between TMD3 and TMD6, which is now known to be a common interface for the binding of β-arrestins and G proteins to the receptor (52, 53).

MD simulations (exploring the micro-second time scale) of the WT-AT₁, N111G-AT₁, and D74N-AT₁ receptors showed distinct structural and dynamic features of the AT₁ receptor associated with signaling through the Gq/11 pathway and the β-arrestin pathway. These structural differences span the whole receptor from top to bottom, including the binding pocket and hydrophobic core in the more extracellular region, the arrangement of the major H-bond network, and the structure of TMD7 in the middle of the receptor and the G protein binding domain in the extracellular extremity of the receptor.

MD simulations of the constitutively active N111G-AT₁ receptor have further validated our previous results obtained with shorter MD simulations. Briefly, a re-arrangement in the MHN and a conformational transition from a helical to an extended state change in a section of TMD7 occurring concurrently with the opening of an hydrophobic core (above the MHN) were proposed to lead to the activation of the Gq/11 pathway (9). Such a transition is validated by experimental data showing that the WT-AT₁ receptor can be photobleached on residues 293-44 to 297-48 (46, 54), and such a pattern can only be rationalized if this segment is in an extended conformation rather than an α-helix. We now show that the loss of α-helical conformation in a portion of TMD7 (Fig. 2), which is also linked to an opening of the hydrophobic core (Fig. 7), is promoted by new H-bonds involving S252-47 and N294-45. Although the side chain of N294-45 forms an H-bond with the side chain of

That second population is not observed in simulations with the SI8 ligand despite the opening of the hydrophobic core and the increase in the r.m.s.d. of TMD7. These results suggest that the side chain of residue Phe8 of AngII can stabilize the open conformation of the hydrophobic core but that the side chain of residue Ile6 of SI8 cannot.
Distinct Conformations of AT<sub>1</sub> Receptor in Biased Signaling

S252<sup>6-47</sup> in the initial resting state (observed in the MD simulations of the WT-AT<sub>1</sub>, D74N-AT<sub>1</sub>, and AngII-D74N-AT<sub>1</sub> receptor), the conformational change in TMD7 allows the side chain of N294<sup>7-45</sup> to re-orient toward and form an H-bond with residue D74<sup>2-50</sup> and also allows the side chain of S252<sup>6-47</sup> to form H-bonds with the backbone atoms of residues A291<sup>7-44</sup>, F293<sup>7-44</sup>, and N294<sup>7-45</sup> that are no longer forming an α-helix (Figs. 3–5). The possible interactions of N294<sup>7-45</sup> with S252<sup>6-47</sup> or D74<sup>2-50</sup> have been tested experimentally through reciprocal mutations of the amino acids (55). Based on the lack of activity of the receptor with reciprocal mutations (N294S/S252N and N294D/D74N), the authors suggested that these residues do not interact in the WT-AT<sub>1</sub> receptor. Our MD simulations, however, clearly suggest (Fig. 3) that the side chains of the polar residues within the MHN do not form mutually exclusive interactions in pairs. This can explain why reciprocal mutations of side chains that do interact with each other (occasionally) might not rescue the impairment to G<sub>i/11</sub> signaling caused by the single mutations. The conformational change of TMD7 also appears associated with an increased probability of opening the G protein-binding site at the intracellular side of the receptor, between TMD3 and TMD6 (Fig. 6), which is coherent with the constitutively active nature of the N111G-AT<sub>1</sub> receptor on the G<sub>i/11</sub> pathway (Fig. 1). Residues from the conserved (E/D)RY motif at the intracellular extremity of TMD3 have been suggested to stabilize the inactive state of GPCRs either through a

FIGURE 13. Probability landscape generated by sorting frames of the MD simulations according to two measurements as follows: x axis, distance between the center-of-mass of the side chain of residue Phe<sup>8</sup> and the center-of-mass of the side chains of residues V108<sup>3-32</sup>, L112<sup>3-36</sup>, F77<sup>2-33</sup>, I288<sup>7-39</sup>, A291<sup>7-44</sup>, and Y292<sup>7-45</sup> of the hydrophobic core; y axis, graphs on the left, distance between the center-of-mass of backbone atoms of residues V108<sup>3-32</sup>–L112<sup>3-36</sup> (region of the hydrophobic core on TMD3) and residues 1288<sup>7-37</sup>–Y292<sup>7-45</sup> (region of the hydrophobic core on TMD7); y axis, graphs on the right, r.m.s.d. of the Cα atoms of residues I288<sup>7-39</sup> through N295<sup>7-46</sup> on TMD7.
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salt bridge, referred to as the “ionic lock,” between R3.50 and a conserved E6.39 at the intracellular end TMD6, or between R3.50 and (E/D)3.49 (56). The AT1 receptor, however, does not feature the conserved glutamate at position 6.39, which is instead an asparagine that did not interact significantly with R1263.50 in our MD simulations. We also monitored H-bonds formed between D1253.39 and R1263.50 and between residues of the DRY motif and other neighboring residues, but no interaction involving the DRY motif of AT1 was linked to the opening of the G protein-binding site. This is in line with the alternative role proposed to be played by these residues for the AT1 receptor and otherGPCRs (56–59). Indeed, they have been proposed to be rather involved in the direct interaction with the G protein rather than modulating the conformational stability of the receptor. For the highly conserved NPXXY motif, we observed that the conformational transition in TMD7 allowed increased movement of the bottom portion of the helix containing this motif. This was also associated with an increased solvent accessibility of the side chain of residue Y3027.53 (Figs. 8–10). This increased accessibility could be important for G protein signaling by the AT1 receptor. Indeed, as reported elsewhere, Tyr302 is important to activate the Gq/11 pathway but not for the internalization of the AT1 receptor (60–62).

MD simulations of the WT-AT1 receptor in presence of its ligand AngII indicated that AngII induces the same conformational changes as those caused by the N111G mutation (Figs. 4–8 and 10). Moreover, our data suggest that the phenyl moiety of residue Phe8 of AngII could insert in the hydrophobic core and stabilize its open configuration, although Ile8 of SI8 could not (Figs. 12A and 13). The ability to insert in the hydrophobic core and elicit the conformational change could be unique to the phenyl moiety, as mutation of this residue to a tyrosine or diphenylalanine are the only tolerated changes that do not lead to an AngII analog with an antagonist profile on the Gq/11 pathway. Indeed, substitution of Phe8 for other hydrophobic residues, such as alanine, leucine, or isoleucine, turns the ligand into an antagonist on the Gq/11 pathways (15, 63–65). However, a larger side chain such as (pentabromo)Phe-8 or a change of orientation from L-Phe-8 to D-Phe-8 also results in conferring an antagonistic nature on the Gq/11 pathway (66, 67). Whether the side chain of Phe8 is inserted in the hydrophobic core or not, it can interact with the side chain of H2566.51, which is consistent with previously proposed contact points between the ligand and receptor (5, 68). Furthermore, analysis of the trajectories has unveiled distinct sectors well suited to accommodate certain side chains and the C-terminal moiety of the ligand (Fig. 12). The side chain of residue Arg2 forms H-bonds with the side chains of residues D2636.58 and D2817.32, which is in agreement with previous reports (5, 69–71). However, in our receptor model, access to the other side chain suggested to interact with Arg2, D2787-29, is hampered by the N-terminal domain that is constrained in that area by the conserved disulfide bond between cysteines 18 and 274. The side chains of residues Val5 and Ile6 are both stabilized by hydrophobic clusters, formed by F170ECL2, I172ECL2, F179ECL2, and A181ECL2 for Val5 and I271.31, F2813.32, I311.35, T887.44, P2857.36, and L2887.39 for Ile6. The side chain of residue His6 forms H-bonds with the side chains of residues Y351.39, Y2927.43, and R1674.64 and also interacts with W842.60. The C-terminal moiety had a wide variety of partners with which to form H-bonds, within a polar sector formed of Y1133.37, K1996.42, N2005.43, H2566.51, Q2576.52, and T2606.55 (Fig 12). It was previously suggested that residue K1996.42 could form H-bonds with the C-terminal moiety of AngII, and this was also supported by docking experiments (68, 71–73). In a study in which residue R1677.64 was shown to be important for the binding of AngII, the authors suggested that the side chain of R167 interacts with the hydroxy group of Tyr4 (70). In our MD simulations, such an interaction only rarely occurred. Actually, we observe that the side chain Tyr4 is highly mobile and found more often to interact with water molecules and extracellular loops. This conformation is similar to some of the poses obtained recently through molecular docking of AngII and ab initio reconstruction of the peptide within the binding pocket of AT1 (71). Other reports have suggested that the hydroxyl moiety of Tyr4 could form an H-bond with the side chain of residue N1113.35, but we have not witnessed such an event in our simulations (74, 75).

Overall, the broader area explored in the probability landscapes by the Gq/11 active systems (AngII-WT-AT1 receptor, N111G-AT1 receptor, and AngII-N111G-AT1 receptor) support the notion of a destabilization of the initial resting state of the receptor model. In addition, no simulation of these systems has converged to one single stable state or conformation. It is important to keep in mind that the MD simulations are most likely and inherently biased from a conformational standpoint as they all start from the same initial resting state. Because of this limitation and the short time scale covered in the MD simulations, it is important to emphasize that we have sampled only a portion of the conformational landscape of the AT1 receptor. We are looking forward to using the different conformations reached in the current MD simulations as starting points for a multitude of other trajectories to explore more extensively the conformational landscape of the receptor and approach ergodicity to obtain a more accurate thermodynamic characterization of its different states. For example, it will be interesting to see whether trajectories where the conformational transition in TDM7 occurred will explore other accessible “active” states or revert back to the resting state. Furthermore, the addition of an intracellular effector in the simulations, such as a trimeric G protein, should allow for the stabilization of specific conformations sampled in the current MD simulations and therefore help in the discrimination of conformations of the intracellular portion compatible with the engagement of the effector. Nonetheless, the results presented here are in agreement with the activation model of the β2-adrenergic receptor by agonists proposed by Nygaard et al. (76) from a combination of solution state NMR (cHSQC of [ε-13C]methionines) and MD simulations. More specifically, they have used line shape analysis of assigned [ε-13C]methionines to estimate the dynamic nature of the receptors under different conditions. From these data, they suggest that agonist binding to this GPCR (in absence of an intracellular effector) is associated with a conformational heterogeneity and flexibility following the destabilization of the inactive state.
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Results obtained with the β-arrestin-biased D74N-AT₁ receptor indicate that there are no major conformational changes from the initial resting state unlike those observed for the N111G-AT₁ receptor. There is a difference in the configuration of the side chains at the MHN due to the D74N mutation allowing it to interact with both N461.50 and N1113.35 simultaneously, which appears to diminish movement within the MHN (Fig. 3D). This interaction also causes a reorientation of the side chain of residue N2957.46 toward S1153.39 of TMD3 (Fig. 4b), a β-arrestin biased ligand-receptor complex (77), but absent in the crystal structure of the unbiased 5-HT1B-ergotamine complex (PDB code 4iar). This suggests that this interaction might indeed be associated with the stabilization of a β-arrestin-biased conformation of the receptor.

The aforementioned reduction of movement caused by the increased interactions within the MHN of the AT₁ receptor was evident in the probability landscapes as the D74N-AT₁ receptor always displayed an increased probability of being in the initial resting state compared with the WT-AT₁ receptor, which indicates a conformation of lower energy (Figs. 2, 4–8, and 10). This stability was often enhanced by the presence of AngII in the binding pocket of the D74N-receptor (Figs. 2, 6–8, and 10). There was one of the 10 trajectories of the AngII-D74N-AT₁ receptor that displayed a slight kink in TMD7, causing an increase in r.m.s.d. that was visible as a small secondary population on the probability landscapes. We ran 10 more 100-ns MD simulations from the final frame of that trajectory, and because the kink disappeared in all of them, it was interpreted as a random event not linked to the activation of the β-arrestin pathway (data not shown). MD simulations with the β-arrestin-biased SI8 ligand in the WT-AT₁ receptor did not show such a drastic stabilization of the initial resting state as the D74N mutation, but the probability landscapes did display a preference for the initial resting state of the receptor when compared with MD simulations of the WT-AT₁ receptor in presence of AngII.

Therefore, the results suggest that the β-arrestin recruitment requires the receptor to be stabilized in the initial resting conformation. This resting state, however, does not preclude the opening of the intracellular G protein/β-arrestin binding site between TMD3 and TMD6 (Fig. 6). This is similar to what was observed in simulations of the β2-adrenergic receptor showing that the ligand-binding site, connector region (in the middle of the receptor), and G protein-binding site were only weakly coupled and could fluctuate between active and inactive conformations independently (78). Because of the limited duration of the MD simulations, the possibility that a rare conformational change not sampled yet could be linked to the activation of the β-arrestins pathway cannot be dismissed. Also, we focused our observations on specific conformational properties that we first identified with the N111G-AT₁ receptor and associated with the activation of the G_{q/11} pathway.

Interestingly, it was noted in the first ever released crystal structure of bovine rhodopsin that “H-VII is considerably elongated in the region from Ala2995 to Tyr3019 (79), which corresponds to residues A291.74 to L2971.48 of the AT₁ receptor. This is similar to the conformational transition we observed with the AT₁ receptor. Although this is not observed in other crystal structures of class A GPCRs, rhodopsin is the only known crystal structure that features a second proline residue in TMD7, other than in the NPXYX motif (79). So although it is believed that class A GPCRs should share common activation mechanisms due to the presence of some highly conserved residues (80–84), the conformational transition in TMD7 might require the presence of a second, nonconserved proline in the upper portion of TMD7.

In conclusion, MD simulations of the AT₁ receptor have revealed that the D74N mutation, biasing the receptor toward the β-arrestins pathways, stabilizes the initial and resting conformation through additional H-bonds formed within the MHN. Adding the AngII agonist to the D74N-AT₁ receptor further stabilizes the initial resting conformation. At the opposite, the N111G mutation, biasing the receptor toward the G_{q/11} pathway, destabilizes the initial conformation and favors various conformational changes, including the re-orientation of side chains and H-bonds formed within the MHN, the loss of the regular α-helical structure in a part of TMD7, the opening of a hydrophobic core toward the ligand-binding pocket, and the opening of the G protein-binding site at the cytosolic side of the receptor. Adding the unbiased agonist AngII to the WT-AT₁ receptor causes the same conformational changes and possibly stabilizes them through the insertion of the side chain of residue Phe8 within the hydrophobic core. Moreover, the biased agonist SI8 displays a preference for the stabilization of the initial resting state of the WT-AT₁ receptor.

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