The octapeptide hormone angiotensin II (AngII) is the active component of the renin-angiotensin system. It exerts a wide variety of physiological effects, including vascular contraction, aldosterone secretion, neuronal activation, and cardiovascular cell growth and proliferation (1). Virtually all the known physiological effects of AngII are produced through the activation of the AT₁ receptor, which belongs to the G protein-coupled receptor (GPCR) superfamily (2, 3). GPCRs all possess seven transmembrane domains (TMD), which provide structural support for signal transduction. The AT₁ receptor interacts with the G protein Gq/11, which activates a phospholipase C, which in turn generates inositol 1,4,5-trisphosphate and diacylglycerol from the cleavage of phosphatidylinositol 4,5-bisphosphate (4, 5). Inositol 1,4,5-trisphosphate causes the release of Ca²⁺ from an intracellular store, whereas diacylglycerol activates protein kinase C.

Like other GPCRs, the AT₁ receptor undergoes spontaneous isomerization between its inactive state (favored in the absence of agonist) and its active state (induced or stabilized by the agonist) (6). The rotation or translation of TMD helices caused by rigid body movement occurs during the activation of GPCRs (7–9). It has been proposed that TMD3, TMD5, TMD6, and TMD7 may participate in the activation process of the AT₁ receptor by providing a network of interactions through the AngII-binding pocket (10). The dynamics of this network are thought to be modified following agonist binding, thereby forcing the receptor to form new interactions between the TMDs.

Based on homology with the high resolution structure of rhodopsin, the archetypal GPCR (11), it was expected that the binding site of the AT₁ receptor would involve the seven, mostly hydrophobic TMDs and would be accessible to charged water-soluble ligands, such as AngII. For this receptor, the binding site would thus be contained within a water-accessible crevice, the binding pocket, extending from the extracellular surface of the receptor to the transmembrane portion. Using a photoaffinity labeling approach, we directly identified ligand contact points within the second extracellular loop and the seventh TMD of the AT₁ receptor (12–14). Interestingly, numerous mutagenesis studies have provided the basis for a model in which an interaction between Asn¹¹¹ in TMD3 and Tyr²⁹² in TMD7 maintains the AT₁ receptor in the inactive conformation. The agonist AngII would disrupt this interaction and promote the active conformational state (15). In support of this model, it was further shown that substitution of Asn¹¹¹ for a residue of smaller size (Ala or Gly) confers constitutive activity on the AT₁ receptor (16–18).

The substituted-cysteine accessibility method (SCAM) (19–21) is an ingenious approach for systematically identifying the residues in a TMD that contribute to the binding site pocket of the AT₁ receptor by providing a network of interactions through the AngII-binding pocket.
Ligand-binding Pocket of AT\(_1\) Receptor

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin, bacitracin, and soybean trypsin inhibitor were from Sigma. The sulfhydryl-specific alkylating reagent methanethiosulfonate-ethylammonium (MTSEA) was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). The cDNA clone for the human AT\(_1\) receptor subcloned in the mammalian expression vector pcDNA3 was kindly provided by Dr. Sylvain Mecloze (Université de Montréal). Lipofectamine™ 2000 and culture medium were obtained from Invitrogen. [\(^{125}\)I]-[\(^{3}\)H]AngII (specific radioactivity ~1500 Ci/ mmol) was prepared with Iodo-GEN® (Perbio Science, Erembodegem, Belgium) according to the method of Fraker and Speck (23) and as reported previously (24).

**Numbering of Residues in TMD3**—Residues in TMD3 of the human AT\(_1\) receptor were given two numbering schemes. First, residues were numbered according to their positions in the human AT\(_1\) receptor sequence. Second, residues were also indexed according to their relative position to the most conserved residue in the TMD in which it is located (25). By definition, the most conserved residue was assigned the position index 50, e.g., in TMD3, Arg125 was the most conserved residue and was designated Arg\(_{125}^{50}\); whereas the upstream residue was designated Arg\(_{126}^{50}\) and the downstream residue was designated Tyr\(_{127}^{50}\).

This indexing simplified the identification of aligned residues in different GPCRs.

**Oligodeoxynucleotide Site-directed Mutagenesis**—Site-directed mutagenesis was performed on the wild-type AT\(_1\) receptor with the Oligo-direct PCR method (Expand high fidelity PCR system; Roche Diagnostics). Briefly, forward and reverse oligonucleotides were constructed to introduce cysteine mutations between Ile\(_{106}^{109}\) and Thr\(_{120}^{123}\). PCR products were subcloned into the HindIII-XbaI sites of the mammalian expression vector pcDNA3.1. Site-directed mutations were then confirmed by manual and automated DNA sequencing.

**Cell Culture and Transfections**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium containing 2 mM L-glutamine and 10% fetal bovine serum. The cells were seeded into 100-mm culture dishes at a density of ~2 × 10\(^5\) cells/dish. When the cells reached ~90% confluency, they were transfected with 4 μg of plasmid DNA and 15 μl of Lipofectamine™ 2000. After 24 h, transfected cells were trypsinized, distributed into 12-well plates, and grown for an additional 24 h in complete Dulbecco’s modified Eagle’s medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin before the MTSEA treatment and binding assay.

**Binding Experiments**—COS-7 cells were grown for 36 h after transfection in 100-mm culture dishes, washed once with PBS, and subjected to one freeze-thaw cycle. Broken cells were then gently scraped into washing buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl\(_2\), 0.1% bovine serum albumin, 0.01% bacitracin, 0.01% soybean trypsin inhibitor). Saturation binding experiments were done by incubating broken cells (20–40 μg of protein) for 1 h at room temperature with increasing concentrations of [\(^{125}\)I]-[\(^{3}\)H]AngII in a final volume of 500 μl. Non-specific binding was determined in the presence of 1 μM unlabeled [\(^{3}\)H]AngII. Bound radioactivity was separated from free ligand by filtration through GF/C filters presoaked for at least 3 h in binding buffer. Receptor-bound radioactivity was evaluated by \(\gamma\) counting.

**Treatment with MTSEA**—The MTSEA treatment was performed according to the procedure of Javitch et al. (19), with minor modifications. Two days after transfection, the cells, which were grown in 12-well plates, were washed with PBS and incubated for 3 min at room temperature with freshly prepared MTSEA at the desired concentration (typically from 0.5 to 6 mM) in a final volume of 2 μl. The reaction was stopped by washing the cells with ice-cold PBS. Intact cells were then incubated in binding medium (Dulbecco’s modified Eagle’s medium, 25 mM HEPES, 0.1% bovine serum albumin, pH 7.4) containing 0.05 mM [\(^{125}\)I]-[\(^{3}\)H]AngII for 90 min at room temperature. After washing with ice-cold PBS, the cells were lysed with 0.1 N NaOH, and the receptor-bound radioactivity was evaluated by \(\gamma\) counting. The percentage of fractional binding inhibition was calculated as (1 – (specific binding after the MTSEA treatment/specific binding without the treatment)) × 100.

**Protection against MTSEA Reaction by [\(^{3}\)H]AngII**—Transfected cells grown in 12-well plates were washed once with PBS and incubated in the presence or absence of 100 nM [\(^{3}\)H]AngII for 1 h at 16 °C (to avoid internalization of receptors). The cells were washed to remove excess ligand and then treated with MTSEA. The cells were washed three times with ice-cold PBS and once with an acidic buffer (150 mM NaCl, 50 mM acetic acid, pH 3.0) to dissociate bound ligand. They were then incubated for 3 h at 16 °C in binding medium (Dulbecco’s modified Eagle’s medium, 25 mM HEPES, 0.1% bovine serum albumin, pH 7.4) containing 0.05 mM [\(^{125}\)I]-[\(^{3}\)H]AngII. The percentage of protection was calculated as (inhibition in the absence of [\(^{3}\)H]AngII) – (inhibition in the presence of [\(^{3}\)H]AngII) inhibition in the absence of [\(^{3}\)H]AngII] × 100.

**Molecular Modeling**—All calculations were performed on a Silicon Graphics Octane2 work station. The theoretical structure of the hAT\(_1\) receptor was generated by homology modeling based on the crystal structure of bovine rhodopsin (Protein Data Bank code 1F88). A pairwise sequence alignment between the two primary structures was performed using the Homology program of Insight II (Accelrys Inc., San Diego, CAI). Once the structurally conserved regions (TMDs) were identified, the coordinates were transferred to the sequence of the hAT\(_1\) receptor. The structures of the loops of hAT\(_1\) were modeled using the loop generation program of Insight II. The potential energy of the model structure of hAT\(_1\) was minimized using the molecular modeling package of Insight II/Discover with a consistent valence force field (26). Two

| Residue | Kd (nM) | Bmax (fmol/mg) | Number |
|---------|---------|----------------|---------|
| WT (Cys\(_{121}^{50}\)) | 0.6 ± 0.3 | 1163 ± 663 | 11 |
| I103C | 0.8 ± 0.1 | 218 ± 90 | 3 |
| A104C | 1.0 ± 0.2 | 229 ± 98 | 3 |
| A106C | 0.8 ± 0.1 | 235 ± 55 | 3 |
| S127C | 0.3 ± 0.0 | 206 ± 62 | 4 |
| F110C | 1.1 ± 0.2 | 233 ± 33 | 4 |
| N111C | 1.5 ± 0.2 | 210 ± 90 | 3 |
| L112C | 1.1 ± 0.3 | 251 ± 177 | 4 |
| S113C | 3.4 ± 0.5 | 438 ± 84 | 3 |
| A114C | 0.7 ± 0.1 | 558 ± 153 | 3 |
| S115C | 1.3 ± 0.3 | 1339 ± 693 | 3 |
| V116C | 0.8 ± 0.0 | 866 ± 475 | 3 |
| F117C | 0.9 ± 0.6 | 911 ± 318 | 3 |
| L118C | 0.8 ± 0.1 | 1210 ± 569 | 3 |
| L119C | 0.7 ± 0.2 | 734 ± 197 | 3 |
| T120C | 0.5 ± 0.2 | 587 ± 112 | 3 |
| L122C | 0.5 ± 0.1 | 463 ± 150 | 4 |
| S123C | 0.6 ± 0.2 | 541 ± 130 | 3 |
| H124C | 0.8 ± 0.1 | 835 ± 144 | 3 |
| D125C | 0.9 ± 0.3 | 703 ± 62 | 4 |
| R126C | 0.7 ± 0.1 | 1030 ± 456 | 3 |
| Y127C | 0.4 ± 0.0 | 608 ± 279 | 3 |

*WT, wild type.*
disulfide bridges, Cys18–Cys274 and Cys101–Cys180, were used as distance restraints. A distance-dependent dielectric constant of 4 was used with a simple harmonic potential for bond length energy. No cross-term energies were included, and the peptide bonds were forced to planarity.

Data Analysis—Results are presented as means ± S.D. Binding data (B\text{max} and K\text{d}) were analyzed with the Kell program (Biosoft, Ferguson, MO), which uses a weighted nonlinear curve-fitting routine.

RESULTS

Binding Properties of Mutant Receptors Bearing Cysteines in TMD3—To identify the residues in TMD3 that face the binding site pocket of the AT\textsubscript{1} receptor, we mutated 24 consecutive residues between Ile\textsubscript{103}(3.27) and Tyr\textsubscript{127}(3.51) to cysteine, one at a time. Each mutant receptor was transiently expressed in COS-7 cells. To assess the conservation of the global conformation of these receptors after the substitutions, pharmacological parameters describing the equilibrium binding of the radiolabeled competitive ligand \[^{125}\text{I}-\text{[Sar}^{1},\text{Ile}^{8}]\text{AngII}\] such as K\text{d} and B\text{max} were determined (Table I). All the mutant receptors exhibited high binding affinity for \[^{125}\text{I}-\text{[Sar}^{1},\text{Ile}^{8}]\text{AngII}\] (similar to that of the wild-type AT\textsubscript{1} receptor) except for Y113C(3.37), which had a moderate 6-fold decrease in binding affinity. Mutants S105C(3.29), V108C(3.32), and S109C(3.33) did not demonstrate any detectable binding activity and were not used for the SCAM analysis. B\text{max} values for all detectable receptors ranged from 210 fmol/mg to 1.3 pmol/mg.

Effect of Extracellularly Added MTSEA on the Binding Properties of Mutant Receptors—To verify whether the reporter cysteines introduced into the TMD3 of the AT\textsubscript{1} receptor were oriented toward the binding pocket, mutant receptors were treated with concentrations of MTSEA varying between 0.5 and 6 mM. We verified whether the wild-type AT\textsubscript{1} receptor, which contains 10 endogenous cysteines (Fig. 1), was sensitive to the MTSEA treatment. Fig. 2 shows that the various concentrations of MTSEA had very little effect (no more than a 20% reduction) on the binding properties of the wild-type AT\textsubscript{1} receptor, indicating that the endogenous cysteines made a relatively small contribution to the binding site pocket. A 3-min treatment with 0.5 mM MTSEA (Fig. 3A) strongly inhibited the binding properties of mutants A104C(3.28) and L112C(3.36), whereas it had only a minor effect on the binding properties of the other mutant receptors. At higher MTSEA concentrations, the binding properties of mutant N111C(3.35) were also slightly affected (Fig. 3B). Overall, the most reactive cysteines were those substituted for Ala\textsuperscript{104}(3.28) and Leu\textsuperscript{112}(3.36), whereas the cysteine substituted for Asn\textsuperscript{111}(3.35) was less reactive.

Altered Accessibility to TMD3 Reporter Cysteines in the Constitutively Active N111G-AT\textsubscript{1} Receptor—We made use of the constitutively active N111G-AT\textsubscript{1} receptor to assess and map the potentially altered accessibility of MTSEA to the engineered cysteines. We first determined the pharmacological properties of the 23 cysteine-substituted mutant receptors. Within the N111G-AT\textsubscript{1} receptor background, 20 cysteine-substituted mutants conserved a high binding affinity for the competitive ligand \[^{125}\text{I}-\text{[Sar}^{1},\text{Ile}^{8}]\text{AngII}\] (Table II), whereas one mutant (Y113C(3.37)-N111G-AT\textsubscript{1} receptor) displayed a moderate 5-fold decrease in binding affinity. The S105C(3.29)-N111G-AT\textsubscript{1} and V108C(3.32)-N111G-AT\textsubscript{1} receptors did not have any detectable binding activity and were not used for the SCAM analysis. Interestingly, the S109C(3.33) mutation that caused a drastic loss of binding affinity in the wild-type receptor background did not cause a significant loss of binding affinity (1.9 nM) when engineered in the N111G-AT\textsubscript{1} receptor background. B\text{max} values for all detectable receptors ranged from 311 fmol/mg to 1.8 pmol/mg (Table II).

Fig. 4 (as well as Fig. 2) show that, like the wild-type recep-
tor, the N111G-AT1 receptor was relatively insensitive to a 3-min treatment with MTSEA concentrations ranging from 0.5 to 2 mM, again indicating the relatively low contribution of the endogenous cysteines to the binding site pocket. Cysteine-substituted N111G-AT1 receptor mutants were also treated with increasing concentrations of MTSEA, and their binding properties were evaluated as indicated under “Experimental Procedures.” Each curve represents the means ± S.D. of data obtained from at least three independent experiments.

**Fig. 2.** MTSEA treatment of the wild-type (WT) AT1 receptor and sensitive reporter cysteine-bearing mutant receptors. Intact COS-7 cells transiently expressing wild-type, N111C, or A104C AT1 receptors were incubated for 3 min at room temperature with increasing concentrations of freshly prepared MTSEA (0.5–6 mM). The intact cells were then incubated for 90 min at room temperature with 0.05 nM 125I-[Sar1, Ile8]AngII, and their binding properties were evaluated as indicated under “Experimental Procedures.” Each curve represents the means ± S.D. of data obtained from at least three independent experiments.

**Fig. 3.** Effects of MTSEA on different mutant AT1 receptors bearing a reporter cysteine in TMD3. Intact COS-7 cells transiently expressing wild-type (WT) or mutant AT1 receptors were incubated for 3 min at room temperature with freshly prepared 0.5 mM MTSEA (A) or 2 mM MTSEA (B). The intact cells were then incubated for 90 min at room temperature with 0.05 nM 125I-[Sar1, Ile8]AngII. The percentage of binding inhibition was calculated as indicated under “Experimental Procedures.” The vertical line represents an arbitrary threshold used to identify cysteine-sensitive mutants and was set at a value corresponding to binding inhibition 20% greater than the value for the wild-type AT1 receptor. The white bars indicate mutant receptors for which binding activities were not appreciably reduced when compared with the wild-type receptor after treatment with MTSEA. The gray and black bars indicate mutant receptors for which binding activities were slightly reduced (gray) or strongly reduced (black) after treatment with MTSEA. Each bar represents the means ± S.D. of data from at least three independent experiments.

FIG. 2. MTSEA treatment of the wild-type (WT) AT1 receptor and sensitive reporter cysteine-bearing mutant receptors. Intact COS-7 cells transiently expressing wild-type, N111C, or A104C AT1 receptors were incubated for 3 min at room temperature with increasing concentrations of freshly prepared MTSEA (0.5–6 mM). The intact cells were then incubated for 90 min at room temperature with 0.05 nM 125I-[Sar1, Ile8]AngII, and their binding properties were evaluated as indicated under “Experimental Procedures.” Each curve represents the means ± S.D. of data obtained from at least three independent experiments.

**Fig. 3.** Effects of MTSEA on different mutant AT1 receptors bearing a reporter cysteine in TMD3. Intact COS-7 cells transiently expressing wild-type (WT) or mutant AT1 receptors were incubated for 3 min at room temperature with freshly prepared 0.5 mM MTSEA (A) or 2 mM MTSEA (B). The intact cells were then incubated for 90 min at room temperature with 0.05 nM 125I-[Sar1, Ile8]AngII. The percentage of binding inhibition was calculated as indicated under “Experimental Procedures.” The vertical line represents an arbitrary threshold used to identify cysteine-sensitive mutants and was set at a value corresponding to binding inhibition 20% greater than the value for the wild-type AT1 receptor. The white bars indicate mutant receptors for which binding activities were not appreciably reduced when compared with the wild-type receptor after treatment with MTSEA. The gray and black bars indicate mutant receptors for which binding activities were slightly reduced (gray) or strongly reduced (black) after treatment with MTSEA. Each bar represents the means ± S.D. of data from at least three independent experiments.
tendent, S109C(3.33)-N111G-AT, had a low sensitivity to MTSEA treatment (Fig. 5B).

Protection against MTSEA Reaction by a Pretreatment with [Sar¹,Ile⁸]AngII—To confirm that reporter cysteines accessible to MTSEA are located within the binding pocket, receptor mutants were saturated with the competitive ligand [Sar¹,Ile⁸]AngII protected mutant receptors labeled competitive ligand. Fig. 6 shows how a preincubation with the competitive ligand [Sar¹,Ile⁸]AngII prior to the MTSEA treatment. The cells were then washed with an acid buffer to dissociate the bound ligand. The receptors were then assayed for binding with the radiolabeled competitive ligand. Interestingly, most of the MTSEA-accessible residues that we identified with the ligand-accessible side of the receptor to MTSEA. Interestingly, most of the MTSEA-accessible residues that we identified with the SCAM approach lie in the middle (N111C(3.35), L112C(3.36)) to the top portion of TMD3 (A104C(3.28)) (Fig. 7A). This suggests that the residues involved in the interaction with the ligand are mostly located within this interface. Thus, residue Ala¹⁰⁴(3.28) would delineate the top of the binding pocket, whereas residues Asn¹¹¹(3.35) and Leu¹¹²(3.36) would delineate the bottom of the water-accessible binding pocket of the receptor. By a mechanism that could be steric, electrostatic, or indirect, the alkylation of these residues with MTSEA would hamper the binding of the ligand. It is very likely that these two subsets of residues do not interact with the same portion of the ligand. Furthermore, it is assumed that water-accessible residues are in the binding site pocket if a competitive ligand protects them from the effect of MTSEA. The competitive ligand [Sar¹,Ile⁸]AngII protected all the residues tested, thus supporting the notion that these specific residues within TMD3 are located in the binding pocket.

**Table II**

| K<sub>d</sub> (nM) | B<sub>max</sub> (fmol/mg) | Number |
|------------------|----------------|--------|
| N111G (Cys¹²¹)  | 1.1 ± 0.2 | 1806 ± 643 | 3 |
| I103C            | 1.7 ± 0.7 | 363 ± 261  | 2 |
| A104C            | 1.6 ± 0.7 | 702 ± 219  | 2 |
| A106C            | 1.2 ± 0.3 | 547 ± 225  | 2 |
| S107C            | 1.3 ± 0.2 | 1617 ± 430 | 2 |
| S109C            | 1.9 ± 0.5 | 1039 ± 230 | 2 |
| F110C            | 1.0 ± 0.3 | 804 ± 302  | 2 |
| L112C            | 1.3 ± 0.0 | 425 ± 138  | 2 |
| Y113C            | 5.6 ± 0.8 | 311 ± 28   | 2 |
| A114C            | 0.9 ± 0.3 | 1036 ± 46  | 2 |
| S115C            | 1.0 ± 0.3 | 509 ± 165  | 2 |
| V116C            | 1.0 ± 0.0 | 825 ± 464  | 2 |
| F117C            | 0.9 ± 0.3 | 843 ± 328  | 2 |
| L118C            | 1.1 ± 0.1 | 1123 ± 1244| 2 |
| L119C            | 1.6 ± 0.1 | 1016 ± 178 | 2 |
| T120C            | 0.7 ± 0.1 | 960 ± 163  | 2 |
| L122C            | 1.2 ± 0.2 | 515 ± 18   | 2 |
| S123C            | 1.1 ± 0.0 | 558 ± 589  | 2 |
| I124C            | 1.1 ± 0.1 | 884 ± 117  | 2 |
| D125C            | 1.4 ± 0.1 | 640 ± 133  | 2 |
| R126C            | 0.6 ± 0.1 | 604 ± 566  | 2 |
| Y127C            | 1.3 ± 0.4 | 362 ± 74   | 2 |

**Fig. 4.** MTSEA treatment of the N111G-AT<sub>1</sub> receptor and sensitive reporter cysteine-bearing mutant N111G-AT<sub>1</sub> receptors. Intact COS-7 cells transiently expressing the N111G-AT<sub>1</sub>, Y113C-N111G-AT<sub>1</sub>, or V116C-N111G-AT<sub>1</sub> receptors were incubated for 3 min at room temperature with increasing concentrations of freshly prepared MTSEA (0.5–6 mM). The intact cells were then incubated for 90 min at room temperature with 0.05 nm [¹²⁵I]-[Sar¹,Ile⁸]AngII, and their binding properties were evaluated as indicated under “Experimental Procedures.” Each curve represents the means ± S.D. of data from at least three independent experiments.
Based on the x-ray high resolution crystal structure of bovine rhodopsin and the pattern of the residues accessible to MTSEA, a ground state AT₁ receptor model was obtained (Fig. 7, A, C, and D). The model predicts that MTSEA-sensitive Ala₁₀₄(3.28) faces TMD7, Asn₁₁₁(3.35) is close to TMD2, whereas Leu₁₁₂(3.36) faces TMD6 (Fig. 7, C and D). Each one of these residues lies on the same α-helix face and is accessible within the hydrophilic binding pocket. Furthermore, the model predicts that endogenous Cys₁₂₁(3.45) is located on the opposite lipid-exposed face of the helix. This prediction is consistent with our results showing a low sensitivity of the wild-type receptor to MTSEA (Fig. 2). Our finding that residues Ala₁⁰₄(3.28) and Leu₁₁₂(3.36) are located in the binding pocket of the AT₁ receptor is in accordance with the current models proposed for bovine rhodopsin and the dopamine D₂ receptor. Indeed, residues Glu₁¹³(3.28) and Gly₁₂₁(3.36) are thought to be contact points with retinal in the
crystal structure of bovine rhodopsin (11), whereas the SCAM approach was used to show that residues Phe 110(3.28) and Cys118(3.36) are located in the binding pocket of dopamine D2 receptor (27). In light of these results, this orientation of TMD3 in the ligand-binding pocket appears to be a common feature of class A GPCRs.

To further investigate the mechanism by which the AT1 receptor undergoes structural changes during the transition from its inactive to its active state, we took advantage of the constitutively active N111G-AT1 receptor. It is believed that the isomerization of conformers toward the active state, which involves transmembrane movement, is stabilized by the binding of an agonist and would be mimicked in part by the constitutively active receptor (6, 28). Thus, within the structural background of the N111G-AT1 receptor, we verified the accessibility of TMD3 residues to MTSEA, and we compared the pattern obtained with that of the wild-type receptor. We found that Cys-substituted residues Ala104(3.28) and Leu1112(3.36) maintained their sensitivity to MTSEA in the N111G-AT1 receptor background (Fig. 5). Interestingly, in the N111G-AT1 receptor background (Fig. 7B), two additional Cys-substituted residues (Ala109(3.33) and Val116(3.40)) were found to be sensitive to the MTSEA treatment. In the protection assay, the competitive ligand [Sar1,Ile8]AngII offered effective protection to all the sensitive mutants (A104C(3.28), A109C(3.33), L112C(3.36), and V116C(3.40)) against the alkylating effect of MTSEA, suggesting that these residues are located in the binding pocket. The increased number of sensitive Cys-substituted residues in the N111G-AT1 receptor background (Fig. 7B) suggests that the accessibility of residues in TMD3 and their spatial proximity within the binding pocket were altered due to the single substitution of Asn111 for Gly in TMD3. The molecular model-
The highly conserved DRY sequence at the C-terminal end of TMD3 is generally considered to be a major structural determinant of GPCR function (32, 33). Previous studies on the β2 adrenergic receptor and the 5-hydroxytryptamine 2A receptor have shown that an ionic interaction between the arginine of the DRY motif in TMD3 and a residue close to the cytosolic extremity of TMD6 stabilizes the inactive conformation of these receptors (34, 35). Furthermore, disruption of this ionic interaction leads to a constitutively active 5-hydroxytryptamine 2A receptor (35). Our results with the constitutively active N111G-AT1 receptor point to an altered accessibility of certain residues of TMD3. This change is likely due to a rotation of TMD3 that modifies the environment of the DRY motif. In the ground state model, Arg1263.50 lies close to the cytosolic end of TMD6, whereas in the activated state model, it is oriented toward the center of the binding pocket. This new orientation of Arg1263.50 is compatible with a destabilization of the inactive conformation and therefore might contribute to generating the active state. Such a pattern of helical movement related to the activation of the AT1 receptor might be a general mechanism associated with the agonist-induced activation ofGPCRs.

In conclusion, our data comparing the ground state versus an activated state of the AT1 receptor strongly point toward a major counterclockwise rotation of TMD3 within the binding pocket. This rotation exposes residues Ala114, Ala109, Asn111, and Val116 of TMD3 to a water-accessible crevice. This movement of TMD3 upon activation of the AT1 receptor is reminiscent of the movement recently observed with rhodopsin (36), the AT1 receptor (22, 37), and the MC4 receptor (31) and might therefore be a structural feature common to numerous rhodopsin-like GPCRs.

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