ANALYSIS OF TRANSMEMBRANE DOMAINS 1 & 4 OF THE HUMAN ANGIOTENSIN II AT\textsubscript{1} RECEPTOR BY CYSTEINE-SCANNING MUTAGENESIS.

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Running title: SCAM method to study the ligand-binding pocket of the AT\textsubscript{1} receptor

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The octapeptide hormone angiotensin II (AngII) exerts a wide variety of cardiovascular effects through the activation of the AT\textsubscript{1} receptor, which belongs to the G protein-coupled receptor (GPCR) superfamily. Like other GPCRs, the AT\textsubscript{1} receptor possesses seven transmembrane domains that provide structural support for the formation of the ligand-binding pocket. Here, we investigated the role of the first and fourth transmembrane domains (TMDs) in the formation of the binding pocket of the human AT\textsubscript{1} receptor using the substituted-cysteine accessibility method. Each residue within the F28\textsuperscript{(1.32)}-I53\textsuperscript{(1.57)} fragment of TMD1 and L143\textsuperscript{(4.40)}-F170\textsuperscript{(4.67)} fragment of TMD4 was mutated, one at a time, to a cysteine. The resulting mutants receptors were expressed in COS-7 cells, which were subsequently treated with the charged sulphydryl-specific alkylating agent methanethiosulfonate ethylammonium (MTSEA). This treatment led to a significant reduction in the binding affinity of TMD1 mutants M30\textsuperscript{C(1.34)}-AT\textsubscript{1} and T33\textsuperscript{C(1.37)}-AT\textsubscript{1} and TMD4 mutant V169\textsuperscript{C(4.66)}-AT\textsubscript{1}. Although this reduction in binding of the TMD1 mutants was maintained when examined in a constitutively active receptor (N111G-AT\textsubscript{1}) background, we found that V169\textsuperscript{C(4.66)}-AT\textsubscript{1} remained unaffected when treated with MTSEA compared to untreated in this context. Moreover, the complete loss of binding observed for R167\textsuperscript{C(4.64)}-AT\textsubscript{1} was restored upon treatment with MTSEA. Our results suggest that the extracellular portion of TMD1, particularly residues M30\textsuperscript{(1.34)} and T33\textsuperscript{(1.37)}, as well as residues R167\textsuperscript{(4.64)} and V169\textsuperscript{(4.66)} at the junction of TMD4 and the second extracellular loop are important binding determinants within the AT\textsubscript{1} receptor binding pocket but that these TMDs undergo very little movement, if at all, during the activation process.

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 (1,2). Virtually all the known physiological effects of AngII are produced through the activation of the AT\textsubscript{1} receptor, which belongs to the G protein-coupled receptor (GPCR) superfamily (3,4). The AT\textsubscript{1} receptor belongs to the rhodopsin-like family A of G-protein-coupled
receptors (GPCRs), which have a seven-transmembrane-helix structure, an extracellular N-terminal tail, an intracellular C-terminal domain, three extracellular and three intracellular loops.

The seven transmembrane domains of GPCRs constitute structural support for signal transduction. Like other family A GPCRs such as rhodopsin and adrenergic receptors, the AT₁ receptor undergoes spontaneous isomerization between its inactive state and its active state (5). Movement of TMD helices through translational or rotational displacement is believed to be essential to achieve the active state (6-8). These conformational changes would sustain GTP/GDP exchange on specific guanine nucleotide binding proteins (G proteins) leading to activation of intracellular signaling cascades (5). For the AT₁ receptor, it has been proposed that TMD3, TMD5, TMD6, and TMD7 may participate in the activation process by providing a network of interactions throughout the AngII-binding pocket (9). The dynamics of this network would require that, following agonist binding, novel or existing interactions between the TMDs would either be created or broken respectively.

Based on homology with the recent high-resolution structures of the β₁ adrenergic, β₂ adrenergic and A₂A adenosine receptors (10-12) it was expected that the binding site of the AT₁ receptor would be formed between its seven, mostly hydrophobic transmembrane domains and would be accessible to charged water soluble agonists, like AngII. For this receptor, the binding site would thus be contained within a hydrophilic crevice, the binding pocket, extending from the extracellular surface of the receptor to the transmembrane portions. Since all crystallized structures to date suggest that TMD1 and TMD4 are somewhat removed from the binding pocket (13), these TMDs have not yet received extensive consideration as contributors to the formation of the ligand binding pocket. Nonetheless, experiments have revealed the proximity of TMD4 residues to the ligand-binding pocket in the β₁ and β₂ adrenergic receptors as well as the dopamine D2 receptor (14,15). Moreover, 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 (16). 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 (17-19).

The substituted-cysteine accessibility method (SCAM) (20-22) is an ingenious approach for systematically identifying residues in a transmembrane domain (TMD) that contribute to the binding pocket of a GPCR. MTS reagents react 10⁹ faster with ionized thiolates (S⁻) than with un-ionized thiols (SH) (23) and ionization of cysteine occurs to a significant extent only in the aqueous medium (24). Thus, in TMDs, the sulfhydryl of a cysteine residue, which is introduced by mutagenesis one at a time, facing toward the binding pocket should react much faster with charged sulfhydryl-specific reagents such as positively charged MTSethylammonium (MTSEA) than sulfhydryls facing towards the interior of the protein or the lipid bilayer (25). We use two criteria for identifying an engineered cysteine as forming the surface of the binding-site crevice: (i) the reaction with an MTS reagent alters binding irreversibly; (ii) this reaction is retarded by the presence of ligand. We previously used this approach to identify the residues in TMD2, TMD3, TMD5, TMD6 and TMD7 that form the surface of the binding-site pocket in the wild-type AT₁ receptor and in the constitutively active N111G-AT₁ receptor (26-30). Here, we report the application of SCAM to probe both TMD1 and TMD4 in the wild-type and constitutively active AT₁ receptor backgrounds.
EXPERIMENTAL PROCEDURES

Materials - Bovine serum albumin, bacitracin, and soybean trypsin inhibitor were from Sigma-Aldrich Canada Ltd. (Oakville, ON). The sulphydryl-specific alkylating reagent MTSEA (CH₂SO₂-SCH₂CH₂NH₂⁺) was purchased from Toronto Research Chemicals Inc. (Toronto, ON). The cDNA clone for the human AT₁ receptor subcloned in the mammalian expression vector pcDNA3 was kindly provided by Dr. Sylvain Meloche (University of Montréal). Lipofectamine™ 2000 and culture medium were obtained from Invitrogen Canada Inc. (Burlington, ON). ¹²⁵I-[Sar¹,Ile⁸]AngII (specific radioactivity ~1000 Ci/mmol) was prepared with Iodo-GEN® (Perbio Science, Erembodegem, Belgium) as reported previously (31).

Numbering of residues in TMDs - Residues in TMD1 and TMD4 of the human AT₁ receptor were given two numbering schemes. First, residues were numbered according to their position in the human AT₁ receptor sequence. Second, residues were also indexed according to their position relative to the most conserved residue in the TMD in which it is located (32). By definition, the most conserved residue was assigned the position index "50," e.g., in TMD4, Trp153 is the most conserved residue and was designated W153(4,50), whereas the downstream residue was designated L154(4,51) and the upstream residue was designated I152(4,49). This indexing simplifies the identification of aligned residues in different GPCRs.

Oligodeoxynucleotide site-directed mutagenesis - Site-directed mutagenesis was performed on the wild-type AT₁ receptor with the overlap PCR method (Expand high fidelity PCR system; Roche Applied Science, Indianapolis, IN). Briefly, forward and reverse oligonucleotides were constructed to introduce cysteine mutations between F28(1,32)-153 (1,57) for TMD1 and between L143(4,40) and F170(4,67) for TMD4, as well as to substitute residue R167(4,64) for either Lys, His, Asp, Glu or Ile. PCR products were subcloned into the HindIII-XbaI sites of the mammalian expression vector pcDNA3.1. Site-directed mutations were then confirmed by automated DNA sequencing by aligning the AT₁ sequence with multiAlin (33).

Cell Culture and Transfections - COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine and 10% (v/v) fetal bovine serum. The cells were seeded into 100-mm culture dishes at a density of 2 x 10⁶ cells/dish. When the cells reached ~90% confluency, they were transfected with 4 µg of plasmid DNA and 15 µl of Lipofectamine™ 2000 according to the manufacturer. 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 MTSEA treatment or binding assays.

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 MgCl₂), centrifuged at 2500 x g for 15 min at 4 °C, and resuspended in binding buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 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 ¹²⁵I-[Sar¹,Ile⁸]AngII in a final volume of 500 µl. Non-specific binding was determined in the presence of 1 µM unlabeled [Sar¹,Ile⁸]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 γ counting. Results are presented as means ± S.D. Binding data (B_max and K_d) were analyzed with Prism version 5.0 for Windows (GraphPad Software, San Diego CA), using a one-site binding hyperbola nonlinear regression analysis.
Treatment with MTSEA - The MTSEA treatment was performed according to the procedure of Javitch et al. (1994), with minor modifications. Two days after transfection, cells grown in 12-well plates were washed with PBS and incubated for 3 min at room temperature with freshly prepared MTSEA at the desired concentrations (typically from 0.5 mM to 6 mM) in a final volume of 200 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 nM [Sar^1,Ile^8]AngII for 90 min at room temperature. After washing with ice-cold PBS, the cells were lysed with 0.1 N NaOH, and the radioactivity was evaluated by γ counting. The percentage of fractional binding inhibition was calculated as (1 - (specific binding after the MTSEA treatment/specific binding without the treatment)) x 100.

Protection against MTSEA reaction by [Sar^1,Ile^8]AngII - Transfected cells grown in 12-well plates were washed once with PBS and incubated in the presence or absence of 100 nM [Sar^1,Ile^8]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 nM [Sar^1,Ile^8]AngII. The percentage of protection was calculated as [(inhibition in the absence of [Sar^1,Ile^8]AngII) - (inhibition in the presence of [Sar^1,Ile^8]AngII)]/(inhibition in the absence of [Sar^1,Ile^8]AngII) x 100.

RESULTS
Binding properties of mutant AT₁ receptors bearing Cysteines in TMD1 and TMD4 - To identify the residues in TMD1 and TMD4 that face the binding pocket of the AT₁ receptor, we mutated 26 consecutive residues between F28^1(1.32) and I53^1(1.57) of TMD1 and 28 consecutive residues between L143^3(4.40) and F170^4(4.67) of TMD4 to cysteine, one at a time. Each mutant receptor was transiently expressed in COS-7 cells. To assess the conservation of global conformation of these receptors after such substitution, pharmacological parameters describing the equilibrium binding of the radiolabeled ligand [Sar^1,Ile^8]AngII such as K_d and B_max were determined (Tables 1 and 2). Most mutant receptors exhibited high binding affinity for [Sar^1,Ile^8]AngII, similar to that of the wild-type AT₁ receptor with the exception of mutant 1H65C(4.62), which showed a fivefold reduction in binding. TMD1 mutants F28C(1.32)-AT₁, V29C(1.33)-AT₁, I31C(1.35)-AT₁, P32C(1.36)-AT₁, L35C(1.39)-AT₁, I38C(1.42)-AT₁ and G45C(1.49)-AT₁, and TMD4 mutants A163C(4.60)-AT₁, R167C(4.64)-AT₁, and F170C(4.67)-AT₁ did not display any detectable binding activity and were therefore not used for SCAM analysis.

Effect of Extracellularly Added MTSEA on Binding Properties of Mutant Receptors in the wild-type background - To verify whether the reporter cysteines introduced into either TMD1 or TMD4 were oriented toward the binding pocket, mutant receptors were treated with concentrations of MTSEA varying between 0.5 mM and 6 mM. As reported previously (26), the various concentrations of MTSEA had very little effect (no more than a 20% reduction at high MTSEA concentrations) on the binding properties of the wild-type AT₁ receptor, which contains 10 endogenous cysteines (Fig.1). For TMD1 mutants, figure 2 shows that MTSEA at 0.5 mM or higher concentration strongly inhibited ligand binding towards mutants M30C(1.34)-AT₁ (binding inhibition of 69%) and T33C(1.37)-AT₁ (binding inhibition of 48%). For TMD4 mutants, figure 3 shows that MTSEA at 0.5 mM or higher concentration inhibited ligand
binding towards mutant V169C\(^{125}\)AT\(_1\) (binding inhibition of 34\%). The binding properties of all other mutant receptors were not significantly affected by MTSEA treatment.

**Effect of Extracellularly Added MTSEA on Binding Properties of Mutant Receptors in the N111G background** - We made use of the N111G-AT\(_1\) receptor to assess and map the potentially altered accessibility of MTSEA to engineered cysteines in a constitutively active N111G-AT\(_1\) receptor background. We determined the pharmacological properties of the 26 cysteine-substituted mutant receptors for TMD1 (Table 3) and 28 cysteine-substituted mutant receptors for TMD4 (Table 4). Within the N111G-AT\(_1\) receptor background, most cysteine-substituted mutants conserved a high binding affinity for the competitive ligand \(^{125}\)I-[Sar\(^1\),Ile\(^8\)]AngII (Table 2). TMD1 mutants F28C\(^{(1.32)}\)-N111G-AT\(_1\), I31C\(^{(1.35)}\)-N111G-AT\(_1\), P32C\(^{(1.36)}\)-N111G-AT\(_1\), L35C\(^{(1.39)}\)-N111G-AT\(_1\), G45C\(^{(1.49)}\)-N111G-AT\(_1\), N46C\(^{(1.50)}\)-N111G-AT\(_1\) and TMD4 mutants P162C\(^{(4.59)}\)-N111G-AT\(_1\), A163C\(^{(4.60)}\)-N111G-AT\(_1\), R167C\(^{(4.64)}\)-N111G-AT\(_1\) and F170C\(^{(4.67)}\)-N111G-AT\(_1\) showed no detectable binding affinity and were not used for the SCAM analysis. It is however noteworthy that the N46C\(^{(1.50)}\)-N111GAT\(_1\) mutant receptor exhibited binding activity in the constitutively active state but not in the inactive ground state (Table 1 versus Table 3), while the P162C\(^{(4.59)}\)-AT\(_1\) mutant receptor exhibited binding activity in the ground state background only (Table 2 versus Table 4).

Mutant receptors were subsequently treated with increasing concentrations of MTSEA and assessed for binding with \(^{125}\)I-[Sar\(^1\),Ile\(^8\)]AngII. Like the wild-type receptor, the N111G-AT\(_1\) receptor was relatively insensitive to a 3-min treatment with MTSEA concentrations ranging from 0.5 mM to 2 mM, again indicating the relatively low contribution of the endogenous cysteines in the binding site pocket (Fig. 4). For TMD1 mutants in the N111G background, figure 4 shows that treatment with 0.5 mM MTSEA strongly inhibited the \(^125\)I-[Sar\(^1\),Ile\(^8\)]AngII binding properties of the M30C\(^{(1.34)}\)-N111G-AT\(_1\) mutant (binding inhibition of 64\%). At 2 mM MTSEA, the binding properties of T33C\(^{(1.37)}\)-N111G-AT\(_1\) were significantly inhibited (binding inhibition of 45\%). The L48C\(^{(1.52)}\)-AT\(_1\) mutant receptor, which displayed sensitivity to 2 mM MTSEA in the ground state did not demonstrate any sensitivity to MTSEA in the constitutively active state. For TMD4 mutants in the N111G background, all mutant receptors were insensitive to MTSEA treatment (Fig. 5). It is interesting to note that V169C\(^{125}\), which was sensitive to MTSEA in the inactive ground state receptor background, lost its sensitivity in the N111G receptor background.

**Contribution of position R167\(^{(4.64)}\) to AT\(_1\) receptor binding** - To assess the contribution of the positively charged side-chain of R167\(^{(4.64)}\) in \(^{125}\)I-[Sar\(^1\),Ile\(^8\)]AngII binding to the AT\(_1\) receptor, we tested the binding affinities of the R167H, R167K, R167D, R167E and R167I mutant receptors. We found that with the exception of the R167K mutant, all mutant receptors did not display any binding activity (Table 5). In view of the similarity of the structure of MTSEA (SCH\(_2\)CH\(_3\)NH\(^+\)) to that of the arginine side-chain, we treated both the R167C-AT\(_1\) and R167C-N111G-AT\(_1\) mutant receptors with 0.5 mM MTSEA before proceeding with the binding assay (Table 5). We found that binding affinities were restored by the MTSEA treatment for both mutants, although the ground state receptor still displayed a 6-fold reduction of binding when compared to the wild-type hAT\(_1\) receptor (Table 5).

**Protection Assay** - To confirm that reporter cysteines accessible to MTSEA are located within the binding pocket, receptor mutants were saturated with the competitive ligand [Sar\(^1\), Ile\(^8\)]AngII prior to MTSEA treatment. Cells were then washed with an acid buffer to dissociate bound ligand. The receptors were then assayed for binding with the radiolabeled competitive ligand. Figure 6 shows how pre-incubating with
the competitive ligand [Sar\textsuperscript{1}, Ile\textsuperscript{8}]AngII protected mutant receptors M30C\textsuperscript{(1.34)} and T33C\textsuperscript{(1.37)} from the inhibitory effect of MTSEA, in both the wild-type and N111G backgrounds, with protection levels ranging from 45% to 60%. However, we found that preincubation with [Sar\textsuperscript{1},Ile\textsuperscript{8}]Ang II weakly protected the MTSEA-sensitive V169C\textsuperscript{(4.66)} mutant receptor (13% inhibition).

**DISCUSSION**

The rationale of this study, which relied on SCAM analyses, was to gain insight into the orientation of both TMD1 and TMD4 of the hAT\textsubscript{1} receptor by identifying the residues accessible to MTSEA within the binding site pocket. Mapping these residues in the ground state receptor and the constitutively active N111G background allowed us to measure relative changes in the position of certain residues, thus providing valuable information with which to infer structural changes underlying the activation of the AT\textsubscript{1} receptor. The SCAM method is based on the reactivity of engineered cysteines to MTSEA, a reagent that reacts 10\textsuperscript{9} times faster with ionized cysteines than with the un-ionized thiol (23) and thus will covalently alkylate any cysteine located in a hydrophilic environment. Indeed lipid-exposed, buried, or disulfide-bonded cysteines are unlikely to ionize to a significant extent and hence are assumed to be unaffected by such modification induced by MTSEA.

In the initial projection map of rhodopsin (34), both TMD1 and TMD4 appeared as outliers of the binding pocket, with a large lipid-exposed surface and few polar residues (13). Also, all crystalized GPCR structures to date suggest that both these TMDs are somewhat removed from the binding pocket and thus have not been the subject of intense scrutiny. In spite of this, a previous report has indicated that important peptide ligand-binding determinants are located within the AT\textsubscript{1} receptor N-terminus, in particular adjacent to the top of TMD1 (35). For a number of class A GPCRs, many site-directed mutagenesis studies had indicated that several residues spanning positions 4.61 to 4.72 contribute to agonist and/or antagonist binding, suggesting a role for the extracellular portion of TMD4 in ligand binding (35-44). A recent study on rhodopsin using photoreactive chromophores has shown that position 4.58 of TMD4 is directly labeled following photoactivation, implying a role for TMD4 during rhodopsin activation (45).

In this study, a surprising number of mutants in which residues (V26C, I27C, F28C, V29C, I31C, P32C, L35C) were replaced with Cys, did not show any detectable binding towards [Sar\textsuperscript{1},Ile\textsuperscript{8}]Ang II. Although this result suggests that these positions are involved in ligand binding, such a binding mode in which simultaneous direct contact between all these residues and the Ang II ligand occurs is unlikely. Although a previous report (46) proposed that residue Asp\textsuperscript{1} of Ang II interacts with the N-terminal domain proximal to TMD1, a direct interaction between Asp\textsuperscript{1} and a residue of this segment has not yet been directly proven. Therefore, a more plausible explanation for the lack of ligand binding would be that this stretch of residues is not interacting directly with the ligand but instead are involved in proper receptor folding and assembly (47).

As reported previously, the insensitivity of the wild-type receptor to MTSEA suggests either that endogenous cysteines are not alkylated by MTSEA or that their alkylation does not affect the binding of the ligand (26). Our approach of adding the MTSEA reagent to whole adherent cells expressing the AT\textsubscript{1} receptor essentially exposed only the extracellular ligand-accessible side of the receptor to MTSEA. For both TMD1 and TMD4, the MTSEA-accessible residues that we identified with the SCAM approach are located at the top (M30C\textsuperscript{(1.34)}, T33C\textsuperscript{(1.37)} and V169C\textsuperscript{(4.66)}) portion of the TMDs. These results suggest that this portion of both TMDs is involved in the interaction with
the ligand and are part of the binding pocket in the ground state of the receptor. Indeed, by a mechanism that could be steric, electrostatic, or indirect, the alkylation of these residues with MTSEA hampered the binding of the ligand. We found that the competitive ligand [Sar\(^1\), Ile\(^8\)]AngII protected mutants M30C\(^{(1.34)}\) and T33C\(^{(1.37)}\) from the effect of MTSEA and mutant V169C\(^{(4.66)}\) was very weakly protected (Fig 6). We conclude that V169\(^{(4.66)}\) is likely located at the margin of the binding pocket such that the presence of [Sar\(^1\), Ile\(^8\)]Ang II in the binding pocket cannot block the access of MTSEA, resulting in weak protection.

To further investigate the mechanism by which the AT\(_1\) receptor undergoes structural changes during the transition from its inactive to its active state, we took advantage of the constitutively active N111G-AT\(_1\) 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, at least in part, by the constitutively active receptor (5,48). Thus, within the structural background of the N111G-AT\(_1\) receptor, we verified the accessibility of both TMD1 and TMD4 residues to MTSEA and we compared the pattern obtained with that of the wild-type receptor. For TMD1, we found that Cys-substituted mutants M30C\(^{(1.34)}\)-N111G-AT\(_1\) and T33C\(^{(1.37)}\)-N111G-AT\(_1\) maintained their sensitivity to MTSEA in the constitutively active receptor background whereas for TMD4 we found that mutant V169\(^{(4.66)}\)-N111G-AT\(_1\) lost its sensitivity to MTSEA (Fig. 5). In the protection assay, the competitive ligand [Sar\(^1\),Ile\(^8\)]AngII offered effective protection to both sensitive mutants M30C\(^{(1.34)}\)-N111G-AT\(_1\) and T33C\(^{(1.37)}\)-N111G-AT\(_1\) against the alkylating effect of MTSEA (Fig. 6). For TMD4, the divergence in the sensitivity of Cys-substituted mutants in the wild-type background and in the N111G-AT\(_1\) receptor background for position V169\(^{(4.66)}\) suggests that the accessibility of this residue and its spatial proximity within the binding pocket was altered due to the single substitution of an Asparagine for a Glycine at position 111 in TMD3. It should be noted that position 4.66 is located at the junction between TMD4 and the 2nd extracellular loop (ECL2) of AT\(_1\). Interestingly, the ECL2 of rhodopsin has recently been shown to move away from the binding pocket following activation, highlighting a possible role for this ECL in the GPCR activation process (49). However, for TMD1, we detected no such divergence in sensitivities between both receptor backgrounds, which indicates no major structural changes of TMD1 during activation.

A peculiar observation was made regarding the R167C\(^{(4.64)}\)-AT\(_1\) mutant receptor. As noted we found that this mutant was unable to bind [Sar\(^1\),Ile\(^8\)]AngII either in the inactive or constitutively active state. But because the structure of MTSEA (SCH\(_2\)CH\(_2\)NH\(_3\)\(^+\)) is reminiscent of the Arg residue side-chain, we thought of testing whether we could rescue binding by treating cells expressing the mutant receptor with the charged MTSEA. Indeed, binding was restored partly (i.e. Kd of 5.45 nM) in the ‘wild-type’ background and completely in the constitutively active background state. To determine the contribution of R167\(^{(4.64)}\) in the ligand-binding of the AT\(_1\) receptor, we also replaced Arg with His, Lys, Glu, Asp and Ile and found that, except for the R167K mutant receptor, substitution at this position totally abolished the binding affinity to [Sar\(^1\),Ile\(^8\)]Ang II. These results suggest the loss of binding affinity of the R167C\(^{(4.64)}\) mutant receptor is due to the loss of an electrostatic charge that contributes to AT\(_1\) binding, either by direct interaction with the ligand or by an indirect effect on receptor conformation. While the His residue at position 167 may also possess a positive charge under physiological conditions, it may be the lack of side-chain extension into the binding pocket that would explain the loss of binding affinity for the R167H mutant receptor. Therefore, it is
reasonable to infer that residue R167(4.64) is located in the water-accessible binding pocket by its accessibility to MTSEA. In support of the importance of this position, one report suggested that the side chain of the R167(4.64) of TMD4 may be involved in an NH-aromatic interaction with the phenolic side chain of the Tyr4 residue of Ang II (50). However, the report that the replacement of Arg with Gln totally abolished the binding affinity excludes the potential involvement of NH–aromatic interaction (51). The presence of an ionic bond linking residue R167(4.64) with residue D263(6.58) of TMD6 was also proposed (9).

In conclusion, we have identified specific residues at the top of both TMD1 and TMD4 that participate in the formation of the ligand-binding pocket of the AT1 receptor. Our data comparing the ground state versus an activated state of the AT1 receptor imply that these TMDs undergo little or no movement during the AT1 activation process. Taken together with crystallographic data of other GPCRs as well as data using SCAM that has been obtained from our group on the movements of AT1’s other TMDs, the results presented here will provide a framework in which to describe the dynamics of AT1 activation.
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The abbreviations used are: AngII, angiotensinII; AT$_1$ receptor, Angiotensin II Type-1 receptor; GPCR, G protein-coupled receptor; TMD, transmembrane domain; SCAM, substituted-cysteine accessibility method; MTSEA, methanethiosulfonate-ethylammonium; DMEM, Dulbecco’s Modified Eagle’s Medium.
FIGURE LEGENDS

FIGURE 1. Schematic representation of the human AT$_1$ receptor. The numbers indicate the positions of cysteines and other residues in the receptor. The gray closed circles represent cysteine residues that are thought to be linked via disulfide bridges and the black closed circles represent cysteine residues whose side chains do not form a disulfide bridge. Mutated TMD1 residues are located between F28$^{(1.32)}$ and I53$^{(1.57)}$ inclusively while mutated TMD4 residues are located between L143$^{(4.41)}$ and F170$^{(4.66)}$ inclusively. Potential N-glycosylation sites (N$^4$, N$^{176}$, N$^{188}$) are indicated. Asn$^{111}$ in TMD3 is also shown in gray.

FIGURE 2. Effects of MTSEA on different mutant AT$_1$ receptors bearing a reporter cysteine in TMD1. Intact COS-7 cells transiently expressing wild-type (WT) or mutant AT$_1$ 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 $^{125}$I-[Sar$^1$,Ile$^8$]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. It was set at a value corresponding to binding inhibition 20% greater than the value for the N111G-AT$_1$ 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 black bars indicate mutant receptors for which binding activities were reduced after treatment with MTSEA. Each bar represents the means ± S.D. of data from at least three independent experiments.

FIGURE 3. Effects of MTSEA on different mutant AT$_1$ receptors bearing a reporter cysteine in TMD4. Intact COS-7 cells transiently expressing wild-type (WT) or mutant AT$_1$ 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 $^{125}$I-[Sar$^1$,Ile$^8$]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. It was set at a value corresponding to binding inhibition 20% greater than the value for the N111G-AT$_1$ 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 black bar indicates the mutant receptor for which binding activities were reduced after treatment with MTSEA. Each bar represents the means ± S.D. of data from at least three independent experiments.

FIGURE 4. Effect of MTSEA on different mutant N111G-AT$_1$ receptors bearing a reporter cysteine in TMD1. Intact COS-7 cells transiently expressing the mutant N111G-AT$_1$ 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 $^{125}$I-[Sar$^1$,Ile$^8$]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. It was set at a value corresponding to binding inhibition 20% greater than the value for the N111G-AT$_1$ receptor. The white bars indicate mutant receptors for which binding
activities were not appreciably reduced when compared with that of the N111G-AT₁ receptor after treatment with MTSEA. Each bar represents the means ± S.D. of data from at least three independent experiments.

**FIGURE 5. Effect of MTSEA on different mutant N111G-AT₁ receptors bearing a reporter cysteine in TMD4.** Intact COS-7 cells transiently expressing the mutant N111G-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 ^125_I-[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. It was set at a value corresponding to binding inhibition 20% greater than the value for the N111G-AT₁ receptor. The white bars indicate mutant receptors for which binding activities were not appreciably reduced when compared with that of the N111G-AT₁ receptor after treatment with MTSEA. Each bar represents the means ± S.D. of data from at least three independent experiments.

**FIGURE 6. [Sar¹, Ile⁸]AngII protection of MTSEA-sensitive mutant receptors.** Intact COS-7 cells transiently expressing MTSEA-sensitive mutant AT₁ receptors were pre-incubated for 1 h at 16°C in the absence or presence of 100 nM [Sar¹, Ile⁸]AngII. The cells were then incubated for 3 min at 16°C in the continued absence or presence of [Sar¹, Ile⁸]AngII with optimal MTSEA concentrations to achieve maximal binding inhibition of each receptor. The MTSEA concentrations were as follows: 0.5 mM for M30C-AT₁, T33C-AT₁, N111G-M30C-AT₁, N111G-T33C-AT₁; and 2 mM for V169C-AT₁. The cells were then washed with ice-cold PBS and incubated for 3 h at 16°C with 0.05 nM ^125_I-[Sar¹, Ile⁸]AngII. Protection was calculated as described under “Experimental Procedures.” Each bar represents the means ± SD of data from at least three independent experiments.
TABLE 1: Binding Properties of AngII to TMD1 Cysteine-Substituted AT₁ Mutant Receptors

|        | K<sub>d</sub> (nM) | B<sub>max</sub> (fmol / mg) | n |
|--------|-------------------|-----------------------------|---|
| WT     | 0.53 ± 0.16       | 1279 ± 36                   | 6 |
| F28C   | ndb               | ndb                         | 3 |
| V29C   | ndb               | ndb                         | 3 |
| M30C   | 1.13 ± 0.30       | 247 ± 85                    | 3 |
| I31C   | ndb               | ndb                         | 3 |
| P32C   | ndb               | ndb                         | 3 |
| T33C   | 0.59 ± 0.22       | 721 ± 49                    | 3 |
| L34C   | 0.91 ± 0.59       | 974 ± 35                    | 3 |
| L35C   | ndb               | ndb                         | 3 |
| S36C   | 0.55 ± 0.22       | 1370 ± 53                   | 3 |
| I37C   | 0.75 ± 0.07       | 1397 ± 25                   | 3 |
| I38C   | ndb               | ndb                         | 3 |
| F39C   | 0.39 ± 0.18       | 783 ± 18                    | 3 |
| V40C   | 0.59 ± 0.43       | 927 ± 32                    | 3 |
| V41C   | 1.46 ± 1.11       | 1015 ± 30                   | 3 |
| G42C   | 0.75 ± 0.21       | 936 ± 12                    | 3 |
| I43C   | 0.47 ± 0.14       | 950 ± 33                    | 3 |
| F44C   | 0.68 ± 0.46       | 1139 ± 65                   | 3 |
| G45C   | ndb               | ndb                         | 3 |
| N46C   | 0.35 ± 0.14       | 992 ± 10                    | 3 |
| S47C   | 0.57 ± 0.03       | 1030 ± 35                   | 3 |
| L48C   | 1.80 ± 0.40       | 612 ± 17                    | 3 |
| V49C   | 0.41 ± 0.09       | 981 ± 29                    | 3 |
| V50C   | 1.26 ± 0.21       | 954 ± 89                    | 3 |
| I51C   | 0.51 ± 0.30       | 860 ± 15                    | 3 |
| V52C   | 0.35 ± 0.02       | 1345 ± 42                   | 3 |
| I53C   | 0.85 ± 0.49       | 867 ± 25                    | 3 |

Cells transfected with the appropriate receptor were assayed as described in the Experimental Procedure. Binding affinities (K<sub>d</sub>) and maximal binding capacities (B<sub>max</sub>) are expressed as the means ± SD of values obtained in <i>n</i> independent experiments performed in duplicate. Nbd indicates no detectable binding.
|         | $K_d$ (nM) | $B_{\text{max}}$ (fmol / mg) | n  |
|---------|------------|-------------------------------|----|
| WT      | 0.80 ± 0.24| 1549 ± 529                    | 6  |
| F170C   | ndb        | ndb                           | 2  |
| V169C   | 1.97 ± 0.44| 237 ± 74                      | 2  |
| N168C   | 1.77 ± 0.13| 332 ± 9                       | 2  |
| R167C   | ndb        | ndb                           | 2  |
| H166C   | 0.58 ± 0.67| 295 ± 86                      | 2  |
| I165C   | 12.06 ± 1.65| 511 ± 227                   | 2  |
| I164C   | 1.07 ± 0.05| 354 ± 3                       | 2  |
| A163C   | ndb        | ndb                           | 2  |
| P162C   | 6.37 ± 1.66| 398 ± 276                     | 2  |
| L161C   | 0.35 ± 0.03| 520 ± 88                      | 2  |
| S160C   | 0.63 ± 0.17| 827 ± 272                     | 2  |
| A159C   | 0.27 ± 0.12| 659 ± 83                      | 2  |
| L158C   | 0.72 ± 0.06| 959 ± 179                     | 2  |
| G157C   | 0.42 ± 0.21| 551 ± 45                      | 2  |
| A156C   | 0.76 ± 0.22| 478 ± 152                     | 2  |
| L155C   | 0.81 ± 0.08| 929 ± 97                      | 2  |
| L154C   | 0.62 ± 0.07| 598 ± 313                     | 2  |
| W153C   | 1.05 ± 0.16| 1086 ± 26                     | 2  |
| I152C   | 0.70 ± 0.12| 984 ± 517                     | 2  |
| I151C   | 1.06 ± 0.02| 979 ± 155                     | 2  |
| I150C   | 0.82 ± 0.28| 244 ± 29                      | 2  |
| T148C   | 0.83 ± 0.06| 911 ± 11                      | 2  |
| V147C   | 0.76 ± 0.04| 1148 ± 326                    | 2  |
| K146C   | 0.92 ± 0.27| 721 ± 270                     | 2  |
| A145C   | 0.87 ± 0.13| 912 ± 87                      | 2  |
| V144C   | 0.91 ± 0.08| 1164 ± 269                    | 2  |
| L143C   | 0.76 ± 0.11| 1196 ± 165                    | 2  |

Cells transfected with the appropriate receptor were assayed as described in the Experimental Procedure. Binding affinities ($K_d$) and maximal binding capacities ($B_{\text{max}}$) are expressed as the means ± SD of values obtained in $n$ independent experiments performed in duplicate. Nbd indicates no detectable binding.
TABLE 3: Binding Properties of TMD1 cysteine-substituted N111G-AT₁ constitutively active receptors

|        | Kᵩ (nM) | Bₘₐₓ (fmol / mg) | n  |
|--------|---------|------------------|----|
| N111G  | 0.53 ± 0.16 | 1279 ± 36        | 6  |
| F28C   | ndb     | ndb              | 2  |
| V29C   | 1.82 ± 0.32 | 236 ± 65        | 2  |
| M30C   | 1.36 ± 0.17 | 204 ± 21        | 2  |
| I31C   | ndb     | ndb              | 2  |
| P32C   | ndb     | ndb              | 2  |
| T33C   | 1.27 ± 0.25 | 976 ± 26        | 2  |
| L34C   | 1.08 ± 0.42 | 1025 ± 85       | 2  |
| L35C   | ndb     | ndb              | 2  |
| S36C   | 0.94 ± 0.19 | 763 ± 34        | 2  |
| I37C   | 1.25 ± 0.07 | 853 ± 24        | 2  |
| I38C   | 1.04 ± 0.38 | 974 ± 57        | 2  |
| F39C   | 1.13 ± 0.22 | 448 ± 33        | 2  |
| V40C   | 1.29 ± 0.06 | 343 ± 28        | 2  |
| V41C   | 1.92 ± 0.26 | 469 ± 13        | 2  |
| G42C   | 2.14 ± 0.39 | 836 ± 22        | 2  |
| I43C   | 0.96 ± 0.12 | 754 ± 64        | 2  |
| F44C   | 1.07 ± 0.25 | 707 ± 17        | 2  |
| G45C   | ndb     | ndb              | 2  |
| N46C   | ndb     | ndb              | 2  |
| S47C   | 1.06 ± 0.21 | 363 ± 28        | 2  |
| L48C   | 1.19 ± 0.08 | 742 ± 16        | 2  |
| V49C   | 0.96 ± 0.22 | 379 ± 26        | 2  |
| V50C   | 1.02 ± 0.35 | 763 ± 21        | 2  |
| I51C   | 0.99 ± 0.37 | 681 ± 16        | 2  |
| V52C   | 1.13 ± 0.24 | 390 ± 15        | 2  |
| I53C   | 1.42 ± 0.15 | 452 ± 90        | 2  |

Cells transfected with the appropriate receptor were assayed as described in the Experimental Procedure. Binding affinities (Kᵩ) and maximal binding capacities (Bₘₐₓ) are expressed as the means ± SD of values obtained in n independent experiments performed in duplicate. Nbd indicates no detectable binding.
TABLE 4: Binding Properties of TMD4 cysteine-substituted N111G-AT1 constitutively active receptors

|        | K<sub>d</sub> (nM) | B<sub>max</sub> (fmol / mg) | n  |
|--------|-------------------|-------------------------|----|
| N111G  | 1.24 ± 0.25       | 1094 ± 28               | 5  |
| F170C  | ndb               | ndb                     | 2  |
| V169C  | 1.85 ± 0.15       | 287 ± 19                | 2  |
| N168C  | 2.71 ± 0.82       | 319 ± 58                | 2  |
| R167C  | ndb               | ndb                     | 2  |
| H166C  | 1.19 ± 0.15       | 226 ± 12                | 2  |
| I165C  | 4.97 ± 0.21       | 251 ± 68                | 3  |
| I164C  | 0.62 ± 0.04       | 244 ± 11                | 2  |
| A163C  | ndb               | ndb                     | 2  |
| P162C  | ndb               | ndb                     | 2  |
| L161C  | 1.27 ± 0.02       | 375 ± 76                | 2  |
| S160C  | 0.76 ± 0.12       | 629 ± 70                | 2  |
| A159C  | 1.22 ± 0.34       | 658 ± 82                | 2  |
| L158C  | 1.96 ± 0.02       | 1071 ± 80               | 2  |
| G157C  | 1.69 ± 0.05       | 1264 ± 38               | 2  |
| A156C  | 2.06 ± 0.24       | 1327 ± 16               | 2  |
| L155C  | 1.26 ± 0.13       | 537 ± 69                | 2  |
| L154C  | 1.45 ± 0.03       | 739 ± 23                | 2  |
| W153C  | 1.27 ± 0.2        | 505 ± 70                | 2  |
| I152C  | 1.26 ± 0.10       | 426 ± 12                | 3  |
| I151C  | 1.34 ± 0.19       | 1413 ± 19               | 3  |
| I150C  | 0.95 ± 0.36       | 782 ± 46                | 3  |
| T148C  | 1.19 ± 0.04       | 893 ± 34                | 3  |
| V147C  | 1.63 ± 0.22       | 1298 ± 59               | 3  |
| K146C  | 1.64 ± 0.18       | 653 ± 30                | 2  |
| A145C  | 1.18 ± 0.04       | 720 ± 59                | 2  |
| V144C  | 1.19 ± 0.24       | 1073 ± 90               | 3  |
| L143C  | 1.36 ± 0.16       | 827 ± 15                | 3  |

Cells transfected with the appropriate receptor were assayed as described in the Experimental Procedure. Binding affinities (K<sub>d</sub>) and maximal binding capacities (B<sub>max</sub>) are expressed as the means ± SD of values obtained in n independent experiments performed in duplicate. Nbd indicates no detectable binding.
|                | $K_d$ (nM) | $B_{\text{max}}$ (fmol / mg) | n |
|----------------|------------|-----------------------------|---|
| WT             | 0.8 ± 0.38 | 1036 ± 214                  | 3 |
| N111G          | 0.53 ± 0.16 | 1279 ± 36                  | 3 |
| R167C          | ndb        | ndb                        | 5 |
| N111G-R167C    | ndb        | ndb                        | 3 |
| R167C (MTSEA)  | 5.45 ± 1.16 | 317 ± 68                   | 3 |
| N111G-R167C (MTSEA) | 1.04 ± 0.25 | 722 ± 54                | 3 |
| R167K          | 0.6 ± 0.21  | 563 ± 107                   | 3 |
| R167H          | ndb        | ndb                        | 3 |
| R167D          | ndb        | ndb                        | 3 |
| R167E          | ndb        | ndb                        | 3 |
| R167I          | ndb        | ndb                        | 3 |

Cells transfected with the appropriate receptor were assayed as described in the Experimental Procedure. Cells expressing the R167C$^{(4,64)}$-AT$_1$ and N111G-R167C$^{(4,64)}$-AT$_1$ mutant receptors were preincubated for 3 min at room temperature with freshly prepared 0.5 mM MTSEA in a final volume of 2 ml prior to the freeze-thaw cycle. Binding affinities ($K_d$) and maximal binding capacities ($B_{\text{max}}$) are expressed as the means ± S.D. of values obtained in $n$ independent experiments performed in triplicate. Nbd indicates no detectable binding.
Figure 2

TMD1 AT₁

A

MTSEA 0.5 mM

B

MTSEA 2 mM

% Binding Inhibition

% Binding Inhibition

WT
M30C
T33C
L34C
S36C
I37C
F39C
V40C
G42C
I43C
F44C
G45C
N46C
S47C
L48C
V49C
V60C
I51C
V52C
I53C

WT
M30C
T33C
L34C
S36C
I37C
F39C
V40C
G42C
I43C
F44C
G45C
N46C
S47C
L48C
V49C
V50C
I51C
V52C
I53C
Figure 3

TMD4 AT1

A

MTSEA 0.5 mM

V169C  
N168C  
H166C  
I165C  
I164C  
D162C  
L161C  
S160C  
A159C  
L158C  
G157C  
A156C  
L155C  
L154C  
W153C  
I152C  
I151C  
I150C  
T148C  
V147C  
K146C  
A145C  
V144C  
L143C  
WT

% Binding Inhibition

B

MTSEA 2 mM

V169C  
N168C  
H166C  
I165C  
I164C  
D162C  
L161C  
S160C  
A159C  
L158C  
G157C  
A156C  
L155C  
L154C  
W153C  
I152C  
I151C  
I150C  
T148C  
V147C  
K146C  
A145C  
V144C  
L143C  
WT

% Binding Inhibition
Figure 4

TMD1 N111G-AT1

A

MTSEA 0.5 mM

B

MTSEA 2 mM

% Binding Inhibition

N111G
M30C
T33C
L34C
S36C
I37C
I38C
F39C
V40C
V42C
I43C
F44C
S47C
L48C
V49C
V60C
I51C
V52C
I53C

% Binding Inhibition

N111G
M30C
T33C
L34C
S36C
I37C
I38C
F39C
V40C
V42C
I43C
F44C
S47C
L48C
V49C
V60C
I51C
V52C
I53C
Figure 5

TMD4 N111G-AT

A

MTSEA 0.5 mM

B

MTSEA 2 mM

% Binding Inhibition

V169C
N168C
H166C
I165C
I164C
L161C
S160C
A159C
L158C
G157C
A156C
L155C
L154C
W153C
I152C
I151C
I150C
T148C
V147C
K146C
A145C
V144C
L143C
N111G
Figure 6

![Bar chart showing protection percentages for different conditions. The x-axis represents different conditions: M30C, T33C, N111G-M30C, N111G-T33C, V169C. The y-axis represents protection in percent, ranging from 0 to 80. The chart shows varying protection levels across the conditions.](image-url)
Analysis of transmembrane domains 1 & 4 of the human angiotensin II AT1 receptor by cysteine-scanning mutagenesis
Liping Yan, Brian J. Holleran, Pierre Lavigne, Emanuel Escher, Gaetan Guillemette and Richard Leduc

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