Determining the Environment of the Ligand Binding Pocket of the Human Angiotensin II Type I (hAT₁) Receptor Using the Methionine Proximity Assay* [8]

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The peptide hormone angiotensin II (AngII) binds to the AT₁ (angiotensin type I) receptor within the transmembrane domains in an extended conformation, and its C-terminal residue interacts with transmembrane domain VII at Phe-293/Asn-294. The molecular environment of this binding pocket remains to be elucidated. The preferential binding of benzophenone photolabels to methionine residues in the target structure has enabled us to design an experimental approach called the methionine proximity assay, which is based on systematic mutagenesis and photolabeling to determine the molecular environment of this binding pocket. A series of 44 transmembrane domain III, VI, and VII X → Met mutants photolabeled either with 125I-[Sar₁,p'-benzoyl-L-Phe₇]AngII or with 125I-[Sar₁,p'-methoxy-p'-benzoyl-L-Phe₇]AngII were purified and digested with cyanogen bromide. Several mutants produced digestion patterns different from that observed with wild type human AT₁, indicating that they had a new receptor contact with position 8 of AngII. The following residues form this binding pocket: L112M and Y113M in transmembrane domain II; F249M, W253M, H256M, and T260M in TMD VI; and F293M, N294M, N295M, C296M, and L297M in TMD VII. Homology modeling and incorporation of these contacts allowed us to develop an evidence-based molecular model of interactions with human AT₁ that is very similar to the rhodopsin-retinal interaction.

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The octapeptide hormone angiotensin II (AngII) (1) is the active component of the renin-angiotensin system. Virtually all known physiological effects of AngII are produced through the activation of the hAT₁ receptor, which belongs to the class A rhodopsin-like family of the heptahelical G protein-coupled receptor (GPCR) superfamily (1, 2). Elucidating the stereochemistry of the ligand-receptor interaction is vital for understanding the mechanism of ligand binding, GPCR activation, and, eventually, rational drug design.

In the past, much effort was devoted to identifying the domains or individual residues of a given receptor that may interact with its ligand. Most experiments to address ligand-receptor interactions were performed with series of receptor mutants to identify specific residues critical to ligand binding (3–5). It is, however, speculative to deduce precise structures of ligand-receptor interactions through mutagenesis studies alone. More direct approaches have therefore been used to study ligand-receptor interactions. Among these is photoaffinity labeling, which allows covalent incorporation of the ligand within its binding site, presumably at the contact area of the photolabel in the receptor. This ligand-receptor contact can be identified by specific enzymatic or chemical digestion of the labeled receptor (6) or by mass spectrometry (7). The binding pockets within the transmembrane domains of several bioamine receptors have been identified using this kind of approach. The adenosine A₁ receptor (8) and the β₂ adrenergic receptor (9, 10) are typical examples. Peptidergic receptors such as hAT₁ and hAT₂ (11, 12), neurokinin receptors (13), and several other receptors from the secretin GPCR family B (14) have been also studied using this approach. We previously identified ligand-contact points within the second extracellular loop (ECL) and the seventh transmembrane domain (TMD) of the hAT₁ receptor (12, 15, 16). Although photoaffinity labeling has been widely used to study peptidergic GPCR binding pockets, generally only a single contact point between a given ligand and its cognate receptor has been identified. The resulting information does not, however, induce sufficient restrictions to generate credible GPCR structures in the ligand-bound state using homology modeling.

Labeling studies using benzophenone residues have identified many ligand-receptor contact points with a surprisingly high ratio of methionine contacts (17–19). Despite the fact that methionine represents a small proportion of the proteinogenic
The indexing simplifies the identification of aligned residues in different GPCRs.

**Oligodeoxynucleotide Site-directed Mutagenesis—**Site-directed mutagenesis was performed on the wt-hAT1 receptor using the overlap PCR method described elsewhere (24). Mutant receptors were cloned into HindIII-XbaI sites of the mammalian expression vector pcDNA3.1. Site-directed mutations were confirmed by manual and automated DNA sequencing.

**Synthesis and Radioiodination of Photoligands—**Boc1-[Sar1,Bpa8]AngII was prepared according to Bosse et al. (22). [Sar1,p-MeO-Bpa8]AngII: p-methoxy-p-phenylalanine benzophenone was prepared according to Horner (25). Photobromination, resin alkylation, and peptide syntheses were carried out as described previously (22). The peptides were purified by reverse phase chromatography, which also permitted the separation of diastereomer peptides. Peptide purity as assessed by high performance liquid chromatography was at least 95%. The correct stereochemistry was assigned through comparison by high performance liquid chromatography with [Sar1,i-Bpa8]AngII and [Sar1,p-Bpa8]AngII made with Boc-i-Bpa and Boc-p-Bpa. Purified peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Tofspec2, Micromass). All 125I-AngII peptides (~1500 Ci/mmol) were prepared using Iodogen® (Perbio Science, Erembodegem, Belgium) as described by Fraker and Speck (26), except that an acetic acid buffer (pH 5.6) was used. The radiolabeled peptides were subjected to high performance liquid chromatography on a C-18 column (Waters) with a 20–40% acetonitrile gradient in 0.05% aqueous trifluoroacetic acid. The specific radioactivity of the radiolabeled peptides was determined by self-displacement and saturation binding analysis.

**Cell Cultures and Transfection of COS-7 Cells—**COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a 5% CO2 atmosphere. Cells were transfected at ~70% confluency with FuGENE 6 transfection reagent as per the manufacturer's instructions. Thirty-six hours after the initiation of transfection, the cells were washed once with phosphate-buffered saline (137 mM NaCl, 0.9 mM MgCl2, 3.5 mM KCl, 0.9 mA CaCl2, 8.7 mM Na2HPO4, and 3.5 mM NaHPO4) and immediately stored at –80 °C until used.

**Binding Studies and Photoaffinity Labeling—**Transfected COS-7 cells were thawed for 1 min at 37 °C. The broken cells were then gently scraped, resuspended in 10 ml of washing buffer (25 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM MgCl2), and centrifuged (500 g for 10 min at 4 °C). The pellet was dispersed in binding buffer (25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM MgCl2, and 0.1% (w/v) bovine serum albumin). For binding studies, the binding cell suspension was solubilized for 30 min at room temperature in the presence of 0.1 nM 125I-[Sar1,He8]AngII (1,500 Ci/mmol) with increasing concentrations of test peptide (15 concentration points in duplicate from 10−10 to 10−5 μM with half-log increases. Bound radioactivity was separated from free ligand by filtration at 4 °C through GF/C filters (Whatman)) to determine binding affinity. Receptor-bound radioactivity was evaluated by γ-counting. Results are presented as means ± S.D. Binding data were analyzed with the Kell program (Biosoft, Ferguson, MO), which uses a weighted nonlinear curve-fitting routine. Maximal binding capacities were determined by approximation using the formula (B/TIC)0 (27) from the displacement studies. For photolabeling studies, the broken cell suspension (1 mg of protein) was incubated for 90 min at room temperature in the presence of 3 nM 125I-[Sar1,Bpa8]AngII or 125I-[Sar1,p-MeO-Bpa8]AngII. After centrifugation at 500 × g, the pelleted broken cells were washed once and resuspended in 0.5 ml of ice-cold washing buffer and then irradiated for 60 min on ice under filtered (Raymaster black light filters, catalog number 5873, Gates and Co. Inc., Franklin Square, NY.) UV light (365 nm) (100 watt mercury vapor lamp, serial number JC-Par-38, Westinghouse). After centrifugation, the broken cell suspension was solubilized for 30 min at 4 °C in modified radioligand precipitation assay buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, and 1% (v/v) Nonidet P-40 supplemented with a protease inhibitor mixture (Complete EDTA-free) (Roche Diagnostics). The cell lysate was centrifuged (15,000 × g for 90 min at 4 °C) to remove insoluble material, and the supernatant was kept at –20 °C until used.

**Partial Purification of the Labeled Complex—**The solubilized photolabeled receptor complexes were diluted in an equal volume of 2× Laemmli buffer (120 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 200 mM dithiothreitol, and 0.05% (w/v) bromphenol blue) and incubated for 60 min at 37 °C. SDS-PAGE was performed as described previously (28) using a 7.5% polyacrylamide preparative gel. The gel
was then cut into slices, and the radioactive content was measured using a γ-counter. The labeled receptor was passively eluted from the gel slices into fresh electrophoresis buffer (25 mM Trizma Tris base (pH 8.3), 250 mM glycine, and 0.1% (w/v) SDS) for 3–4 days at 4 °C with gentle agitation as described by Blanton and Cohen (29). The eluate (−40 ml) was concentrated to a final volume of 0.100–0.250 ml using an Amicon-10 filter (Millipore) and stored at −20 °C.

**CNBr Hydrolysis**—The partially purified photolabeled receptor (5,000–10,000 cpm) was diluted in a 3:5 mixture of 30% trifluoroacetic acid and CNBr dissolved in 100% acetonitrile to obtain a final concentration of 50 mM. Samples were incubated at room temperature in the dark for 16–18 h. One milliliter of water was added to terminate the reaction. The samples were lyophilized and resuspended in Laemmli buffer (1 X final), loaded at 2,500–3,500 cpm on 16.5% SDS-polyacrylamide Tri-Tricine gels (Bio-Rad), and revealed by autoradiography on x-ray films (Kodak Biomax® MS).14C-Labeled low molecular protein masses. Running conditions and fixation procedures were performed according to the manufacturer’s instructions.

**Inositol Phosphate Production**—COS-7 cells were seeded in six-well plates, transfected, and labeled for 24 h in serum-free, inositol-free Dulbecco’s modified Eagle’s medium containing 10 μCi/ml myo-[3H]-inositol (Amersham Biosciences). Cells were washed twice with phosphate-buffered saline containing 0.1% (w/v) dextrose and then incubated in stimulation buffer (Dulbecco’s modified Eagle’s medium containing 25 mM Hepes, 10 mM LiCl, and 0.1% bovine serum albumin, pH 7.4) for 30 min at 37 °C. Inositol phosphate production was induced with 100 nM AngII for 10 min at 37 °C in stimulation buffer. Incubations were terminated by the addition of ice-cold perchloric acid (5% (v/v)/final concentration). Water-soluble inositol phosphates were then extracted with an equal volume of a 1:1 (v/v) mixture of 1,2-tricloro-trifluoroethane and tri-n-octylamine. The samples were mixed vigorously and centrifuged at 2500 × g for 30 min. The upper phase containing the inositol phosphates was applied to an AG1-X8 resin column (Bio-Rad). The inositol phosphates were eluted sequentially by the addition of ammonium formate/formic acid solution of increasing ionic strength. Fractions containing inositol phosphates were collected and measured in a liquid scintillation counter.

**Molecular Modeling**—All calculations were performed on a Silicon Graphics Octane2 work station (Silicon Graphics Inc. Mountain View, CA). Molecular modeling of the hAT1 receptor and the complex [Sar²,Bpa⁸]AngII-hAT1, receptor was done with the INSIGHTII suite of programs (Homology, Discover, and Biopolymer; Accelrys, San Diego, CA). The molecular model of hAT1 (Swiss-Prot accession number P30558) was based on the bovine rhodopsin structure (Protein Data Bank code 1L9H) (30). The sequence alignment (supplemental data, available in the on-line version of this article) between the hAT1, receptor and the bovine rhodopsin used to identify and assign the structurally conserved regions had all the strictly conserved residues of family A members aligned: Asn-55-Asn-46(1.50), Asp-83-Asp-74(2.50), Arg-135-Arg-126(3.50), Trp-161-Trp-153(4.50), Pro-215-Pro-207(5.50), Pro-267-Pro-255(6.50), and Pro-503-Pro-299(7.50). The coordinates of the assigned structurally conserved regions were then transferred to the sequence of hAT1, followed by ECL2 and ECL3 generation with the data base in HOMOLOGY. The conserved disulfide bond between ECL1 and ECL2 was then added to hAT1, and the potential energy was minimized sequentially using Discover with a consistent valence force field (31). In the first step of the minimization, all of the heavy atoms were fixed and all atoms, except those of the TMD backbones, were free to move. In the final step, all of the atoms were unrestrained.

To allow ligand [Sar¹,Bpa⁸]AngII to be incorporated into the receptor, a cavity has to be generated. This was accomplished by a slight rotation (7°) of the α angle of Asn-231. The Bpa molecule was modeled using INSIGHTII BUILDER and was placed between TMD III, TMD VI, and TMD VII as suggested by the photolabeling results. A first minimization of the complex between hAT1 and Bpa (the coulombic terms were turned off) was performed using restraints (2 Å < d < 7 Å) between the Cα atoms of the photolabeled Met residues and the ketone oxygen of Bpa. The backbone atoms of hAT1 were held in their position during this step. An extended Ang-(1–7) peptide (Sar-Arg-Val-Tyr-Val-His-Pro) was then appended to the N-terminal of Bpa. A subsequent minimization step was performed until the maximum derivative was <0.1 kcal/mol. The complex was further refined by adding a second disulfide bond between Cys-18 and Cys-274 (3) and by relaxing the ECL and performing a final overall minimization step.

### RESULTS

**Site-directed Mutagenesis and Photoaffinity Labeling of hAT1**—To identify the receptor residues that participate in the ligand binding pocket of the C-terminal amino acid of AngII, 44 X → Met mutations were induced in TMD III, TMD VI, and TMD VII of wt-hAT1 (Fig. 2). Each mutant receptor was transiently expressed in COS-7 cells. Membranes containing wt-hAT1 or two selected mutants, F293M⁷,⁴⁴¹ and N294M⁷,⁴⁵¹,
were photolabeled with 3 nM $^{125}$I-[Sar$^1$,Bpa$^8$]AngII or $^{125}$I-[Sar$^1$,p-$\text{MeO}$-Bpa$^8$]AngII. They produced a broad band migrating diffusely between 75 and 180 kDa on SDS-polyacrylamide gels (Fig. 1B, lanes 1–3). Labeling was completely prevented when the experiments were carried out in the presence of 10 $\mu$M AngII (Fig. 1B, lanes 4–6) (22), confirming the specificity of the labeling.

Photolabeling of all ligand binding receptor mutants was performed in the same manner. Because both photoligands produced identical results, only those performed with $^{125}$I-[Sar$^1$,Bpa$^8$]AngII are shown in Figs. 3–5. Both fragments were identified in previous studies (12, 16). The following X $\rightarrow$ Met hAT1 mutants had exactly the same labeling pattern as wt-hAT1, indicating that the introduced Met residue did not participate in the labeling process: A106M(3.30), S107M(3.31), V108M(3.32), S109M(3.33), F110M(3.34), N111M(3.35), A114M(3.38), S115M(3.39), I245M(6.40), V246M(6.41), L247M(6.42), F248M(6.43), F249M(6.44), F250M(6.45), F251M(6.46), S252M(6.47), L254M(6.49), Q257M(6.52), I258M(6.53), F259M(6.54), P261M(6.56), L262M(6.57), V264M(6.59), and L265M(6.60) in TMD VI (selected results are shown in Figs. 3A and 4A).

**Digestion of the Labeled Receptor Mutants**—To identify the covalently modified regions, the labeled receptors were treated with CNBr and analyzed by SDS-PAGE. Autoradiography revealed a typical 7.2-kDa fragment from wt-hAT1 consisting of the C-terminal sequence (285–334 plus ligand) (Figs. 3–5), which comprises parts of TMD VII, the C-terminal tail, and the photoligand (Figs. 3–5). The typical 10-kDa fragment from incomplete digestion at position Met-334 was also present (285–359 plus ligand) (Figs. 3–5). Both fragments were identified in previous studies (12, 16). The following X $\rightarrow$ Met hAT1 mutants had exactly the same labeling pattern as wt-hAT1, indicating that the introduced Met residue did not participate in the labeling process: A106M(3.30), S107M(3.31), V108M(3.32), S109M(3.33), F110M(3.34), N111M(3.35), A114M(3.38), S115M(3.39), I245M(6.40), V246M(6.41), L247M(6.42), F248M(6.43), F249M(6.44), F250M(6.45), F251M(6.46), S252M(6.47), L254M(6.49), Q257M(6.52), I258M(6.53), F259M(6.54), P261M(6.56), L262M(6.57), V264M(6.59), and L265M(6.60) in TMD VI (selected results are shown in Figs. 3A and 4A).

Two TMD III mutants (L112M(3.36) and Y113M(3.37)) displayed new fragments besides those associated with TMD VII, namely two new bands each (Fig. 3, lanes 2 and 3), one at 64 kDa (91–134 plus ligand) and the other below the 3.4-kDa marker, suggesting ligand release. TMD VI mutant F249M(6.44) had a new band at 5.2 kDa (244–284 plus ligand) and weak bands at 7.2 and 10 kDa (Fig. 4, lane 3). Mutant H256M(6.51) had the TMD VII-associated bands together with a band below 3.4 kDa, suggesting ligand release (Fig. 4, lane 4), whereas mutant T260M(6.55) had a faint band.
that migrated below the 3.4-kDa band in addition to the TMD VII-associated fragments (Fig. 4, lane 5).

The TMD VII Met mutants had different profiles because of the non-Met labeling of residues Phe-293(7.44) and Asn-294(7.45) in wt-hAT₁ (16). Met labeling thus had to be identified through ligand release as shown for residues Phe-293(7.44) and Asn-294(7.45) (16). Mutants I290M(7.41) and A291M(7.42) produced a small fragment that migrated below the 3.4-kDa band in addition to the TMD loops of hAT₁ were identical to those of the template structure, and the bovine rhodopsin used to identify and assign the structurally conserved regions had all the strictly conserved residues of class A GPCR aligned. The lengths of all TMDs and loops of hAT₁ were identical to those of the template structure, with the exception of the extracellular loops between the transmembrane regions TM IV and TM V (ECL2) and TM VI and TM VII (ECL3). There were no gaps in any of the TMDs. Pro and Gly, which can induce kinks in the TMD, were found at the same positions in bovine rhodopsin and hAT₁, with the exception of Pro-285, which was at position 7.36 instead of 7.38 as in bovine rhodopsin. It was not possible to satisfy all the Bpa-Cβ constraints (2 Å < d < 7 Å) with a single structure. The exclusion of the faintly Met-labeled mutant T260M, however, allowed for a structure with all ketone-sulfur distances in the 8 Å range. The molecular model of the [Sar₁,Bpa₈]AngII-liganded hAT₁ receptor is presented in Fig. 7 and in the supplemental material, available in the on-line version of this article.

DISCUSSION

In the present study, a new photoaffinity scanning approach called MPA was used as a strategy to probe the molecular binding environment of the hAT₁ receptor. In wt-hAT₁, non-Met contact is made by [Sar₁,Bpa₈]AngII at positions Phe-293(7.44) and Asn-294(7.45) in TMD VII (16). Other non-Met contacts using the Bpa moiety have also been reported on other GPCRs (14, 32, 33) and are logically the consequence of the absence of Met residues in the binding locus environment. If a Met residue is introduced into the hAT₁ structure in sufficient proximity to the photoactive residue of the bound ligand, then part or all of the labeling should occur at this introduced Met residue because of the Met-selective nature of the photogenerated benzophenone radical. CNBr digestion of the covalent complex should thus generate a new fragment and produce a different SDS-PAGE profile. The main usefulness of the MPA approach is that receptor-ligand contacts can be directly and immediately determined without lengthy, multiple purification steps, protein digestions, or other manipulations.

Kage et al. (18) observed that when the ε-methyl group of the Met side chain is labeled by Bpa, CNBr hydrolysis releases the labeling ligand as a thiocyanomethyl derivative (Fig. 6) that can be detected by mass spectrometry (18, 34) or, as in our study, by SDS-PAGE (16, 35). This observation led to the confirmation of suspected contact sites in several peptidergic receptors (15, 16, 35). The Met-selective labeling mechanism of the benzophenone radical is based on the formation of a charge-transfer complex between the photogenerated radical and the sulfur atom in the thioether (20, 21). The following step, that is, insertion of the ketone radical into an adjacent C-H bond, can occur either in the γ-methylene or the ε-methyl group, with only the latter leading to ligand release upon CNBr cleavage (Fig. 6). Exclusive γ-labeling has been confirmed by mass spectrometry, which has shown that different rearrangements take place but that neither protein cleavage nor ligand release occur upon CNBr digestion (36). The cause of exclusive γ-labeling instead of the anticipated ε-labeling is probably due to the sterical relations of the non-covalent charge...

Methionine Proximity Assay to Study the hAT₁ Receptor

![Figure 5](http://www.jbc.org/)

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Cells transfected with the appropriate receptor were assayed as described under "Experimental Procedures." Binding affinities (KD) and maximal binding capacities (B_max) are expressed as the means ± S.D. of the values obtained from n independent experiments. Mutants A104M (3.28), S105M (3.29), and D263M (5.58) did not have any detectable binding activity.

| Receptor   | Kd of α | Kd of β | B_max | Experiments (n) |
|------------|---------|---------|-------|----------------|
| WT         | 0.8 ± 0.2 | 0.9 ± 0.2 | 1121 ± 432 | 13 |
| A16OM (3.30) | 0.9 ± 0.1 | 1.0 ± 0.1 | 1275 ± 337 | 4 |
| S107M (3.31) | 0.7 ± 0.1 | 0.9 ± 0.1 | 1202 ± 408 | 3 |
| V108M (3.32) | 0.8 ± 0.1 | 1.0 ± 0.2 | 1123 ± 235 | 3 |
| S109M (3.33) | 0.9 ± 0.1 | 1.0 ± 0.1 | 989 ± 198 | 3 |
| F110M (3.34) | 0.9 ± 0.1 | 1.0 ± 0.2 | 1145 ± 374 | 3 |
| N111M (3.35) | 1.0 ± 0.2 | 1.1 ± 0.2 | 347 ± 72 | 4 |
| L112M (3.36) | 0.8 ± 0.2 | 0.8 ± 0.2 | 598 ± 75 | 5 |
| Y113M (3.37) | 1.0 ± 0.1 | 1.0 ± 0.1 | 365 ± 54 | 5 |
| A114M (3.38) | 1.0 ± 0.1 | 1.0 ± 0.2 | 541 ± 112 | 3 |
| S115M (3.39) | 1.0 ± 0.2 | 1.0 ± 0.1 | 371 ± 63 | 3 |
| V116M (3.40) | 0.6 ± 0.3 | 1.0 ± 0.3 | 401 ± 53 | 4 |
| F117M (3.41) | 0.8 ± 0.1 | 1.0 ± 0.1 | 404 ± 48 | 3 |
| L118M (3.42) | 0.8 ± 0.2 | 0.7 ± 0.2 | 847 ± 102 | 2 |
| L119M (3.43) | 0.9 ± 0.1 | 0.9 ± 0.2 | 1198 ± 402 | 3 |
| I245M (3.44) | 0.7 ± 0.2 | 0.9 ± 0.1 | 555 ± 89 | 3 |
| V246M (6.41) | 1.0 ± 0.1 | 1.0 ± 0.2 | 505 ± 52 | 4 |
| L247M (6.42) | 0.8 ± 0.2 | 0.9 ± 0.1 | 602 ± 90 | 4 |
| P248M (6.43) | 0.8 ± 0.2 | 0.8 ± 0.2 | 678 ± 65 | 3 |
| F249M (6.44) | 0.6 ± 0.2 | 1.0 ± 0.1 | 435 ± 89 | 5 |
| F250M (6.45) | 1.0 ± 0.2 | 1.1 ± 0.1 | 570 ± 111 | 3 |
| F251M (6.46) | 0.4 ± 0.2 | 1.3 ± 0.2 | 601 ± 146 | 4 |
| S252M (6.47) | 0.7 ± 0.1 | 0.8 ± 0.1 | 677 ± 100 | 3 |
| W253M (6.48) | 0.8 ± 0.1 | 0.8 ± 0.1 | 669 ± 78 | 5 |
| I254M (6.49) | 1.2 ± 0.2 | 1.4 ± 0.2 | 635 ± 98 | 3 |
| H256M (6.50) | 0.7 ± 0.1 | 0.8 ± 0.2 | 602 ± 86 | 5 |
| Q257M (6.51) | 0.6 ± 0.2 | 0.7 ± 0.2 | 524 ± 79 | 3 |
| P258M (6.52) | 0.8 ± 0.1 | 0.8 ± 0.1 | 1029 ± 336 | 3 |
| F259M (6.54) | 0.7 ± 0.1 | 0.7 ± 0.1 | 776 ± 63 | 3 |
| T260M (6.55) | 0.7 ± 0.3 | 0.4 ± 0.3 | 820 ± 114 | 3 |
| F261M (6.56) | 0.5 ± 0.1 | 0.5 ± 0.1 | 996 ± 223 | 3 |
| L262M (6.57) | 0.7 ± 0.1 | 0.7 ± 0.1 | 570 ± 132 | 3 |
| V264M (6.59) | 0.7 ± 0.2 | 0.7 ± 0.2 | 1109 ± 420 | 3 |
| L265M (6.60) | 0.7 ± 0.1 | 0.7 ± 0.1 | 1102 ± 327 | 3 |
| I290M (7.41) | 0.7 ± 0.2 | 0.8 ± 0.1 | 826 ± 187 | 6 |
| A291M (7.42) | 0.4 ± 0.3 | 0.4 ± 0.3 | 705 ± 231 | 6 |
| F293M (7.44) | 0.5 ± 0.1 | 0.5 ± 0.1 | 857 ± 219 | 7 |
| N294M (7.45) | 0.7 ± 0.1 | 0.7 ± 0.1 | 1022 ± 215 | 7 |
| N295M (7.46) | 0.7 ± 0.2 | 0.7 ± 0.1 | 610 ± 160 | 6 |
| C296M (7.47) | 0.7 ± 0.1 | 0.7 ± 0.1 | 639 ± 232 | 6 |
| L297M (7.48) | 0.7 ± 0.2 | 0.7 ± 0.2 | 623 ± 201 | 6 |
| L300M (7.51) | 0.7 ± 0.2 | 0.8 ± 0.1 | 598 ± 175 | 7 |

The MPA approach enabled us to discover nine additional contacts for position 8 of AngII and thus to generate an improved liganded receptor model with a refined environment surrounding residue 8 of AngII (Fig. 7).

Most CNBr digestion profiles of labeled Met mutants indicate essentially ε-methyl labeling of the introduced Met residues with ensuing ligand release. The exclusive γ-methylene incorporation by mutants F249M and W253M suggests that the benzophenone moiety is in close proximity to the TMD VI backbone. Such a favorable environment is present when the hydrophobic benzophenone residue is embedded in the hydrophobic aromatic cluster motif(6.40)IVLFFFFSWL(6.49) of TMD VI as seen in Fig. 7.

Studies using the substituted cysteine accessibility method (SCAM) on hAT1 suggest that AngII inserts into a binding pocket where TMD III and TMD VII participate (35, 38). The present study identified TMD III, TMD VI, and TMD VII as part of the ligand binding pocket, which is also the case for bioamine GPCRs (39–41) and rhodopsin (42–44). This is further proof of a highly conserved feature among this very large family of receptors. Interestingly, in TMD III of hAT1, the experimentally determined contact points were Leu-112(3.36) and Tyr-113(3.37). In bovine rhodopsin, the corresponding resi-
dues Gly-121\(^{(3.36)}\) and Glu-122\(^{(3.37)}\) are retinal contact points (42, 43). Two additional hAT\(_1\) TMD III mutants (A104M\(^{(3.28)}\) and S105M\(^{(3.29)}\)) were compatible with this interpretation and were among the few X → Met mutants displaying impaired AngII-binding. In bovine rhodopsin, positions 3.28 and 3.29 also interact with retinal. We therefore hypothesized that the increased bulk of these Ala\(^{3.28}\)Met and Ser\(^{3.29}\)Met substitutions might cause sterical interference with normal ligand binding in hAT\(_1\). The hAT\(_1\) TMD VI mutants F249M\(^{(6.44)}\), W253M\(^{(6.48)}\), and H256M\(^{(6.51)}\) were MPA-positive and also corresponded perfectly to the retinal contact residues Phe-261\(^{(6.44)}\), Trp-265\(^{(6.48)}\), and Tyr-268\(^{(6.51)}\) of rhodopsin (43). TMD VII is the contact for the photolabeling analogue [Sar\(^1\),Bpa\(^8\)]AngII on residues Phe-293\(^{(7.44)}\) and Asn-294\(^{(7.45)}\) (16). In many MPA-positive mutants, concomitant labeling of TMD VII was also observed (e.g., Y113M\(^{(3.37)}\) and especially T260M\(^{(6.55)}\)). MPA on TMD VII could not be demonstrated through the formation of new fragments but solely through ligand release, which was evident in most TMD VII mutants. The ligand release profile of the TMD VII Met mutants indicated that there were continuous ligand contacts from Phe-293\(^{(7.44)}\) through Leu-297\(^{(7.48)}\), with no ligand release in the biologically relevant X → Met mutants flanking this sequence (Ile-290\(^{(7.41)}\), Ala-291\(^{(7.42)}\), and Leu-300\(^{(7.51)}\)). Continuous labeling through five residues in TMD VII implied that one and one-half helical turns with residues pointing away from the receptor core may intermittently contact the photoligand. This is in stark contrast to TMD VI, where a clearly defined internal TMD face emerged (Fig. 7). Recent results with SCAM analyses showed that TMD VII can move considerably in hAT\(_1\), depending on its activation status (35). Additionally, TMD VII is the only TMD with two Pro residues (Pro-285\(^{6.36}\) and Pro-299\(^{7.50}\)), which flank the target sequence in hAT\(_1\). Furthermore, in bovine rhodopsin this segment does not have a typical α-helical structure (43). It is therefore reasonable to argue that this part of the TMD structure is somewhat destabilized, permitting outward pointing residues to turn intermittently inwards. The photochemistry of benzophenone has the particular capacity of repeat activation (45), allowing the interception of an intermediate state by a preferred partner (Met selectivity). Labeling is limited to a short segment of TMD VII at a depth comparable with the contacts in TMD III and with the lowermost contacts in TMD VI (F249M\(^{(6.44)}\) and W253M\(^{(6.48)}\)) (Fig. 7). During the modeling process, a liganded hAT\(_1\) structure was sought where all experimentally determined contacts were within ≤8 Å of the Bpa ketone oxygen. In the resulting structure, a single Met contact (T260M\(^{(6.55)}\)) was not compatible with a reasonable action radius of the Bpa-Met interaction, and two other residues (H256M\(^{(6.51)}\) and Y113M\(^{(3.37)}\))
displayed borderline distances of ~9 Å. However, these two mutants, and especially T260M (6.55), displayed concomitant conduction. The Asn-294 residue is essential for receptor activation with the mutated residues. Considering the location of the residues close to the membrane surface, these contacts are very suggestive of a transient ligand-receptor structure during the ligand binding process. Of all the Met-mutated receptors, only a few mutants displayed altered binding properties. Ala-scan and SCAM analysis (Cys-scan) produced some binding-impaired mutants (35, 38). Our mutagenesis scheme (X → Met) was applied mainly in the TMD area, and mutants with impaired ligand binding were only observed at the extracellular membrane interface. Met is an unbranched pseudo-aliphatic amino acid and can thus adapt to a given hydrophobic environment with minimal steric hindrance. The MPA-negative mutants were assessed for receptor functionality. Nine of eleven mutants displayed AngII-stimulated inositol phosphate production, confirming biologically relevant receptor mutants. The remaining two mutants seemed to interfere with the activation mechanisms. N294M and L297M displayed normal AngII binding (Table I) but no inositol production. The Asn-294 residue is essential for receptor activation (16, 46) but not for peptide ligand binding. L297M is more enigmatic, because almost isosteric changes were introduced. This residue is immediately adjacent to the NPXY motif where a Tyr → Phe mutation also leads to AngII binding but a non-activable AT1 mutant (47, 48). This area seems to be exceptionally sensitive to small changes. In general, however, the X → Met mutagenesis strategy seems to be of little consequence either to receptor structure or function.

In conclusion, MPA made it possible to experimentally determine the binding environment of a given ligand residue. The MPA strategy can be applied to a large variety of receptor structures because X → Met mutagenesis appears to be generally of little biological consequence. The determination of these ligand contacts also allowed the construction of an evidence-based model of the hAT1 receptor and showed that the receptor structure and ligand binding environment were very similar to those of bovine rhodopsin.

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FIG. 7. Molecular model of the [Sar2, Bpa8] AngII-liganded hAT1 receptor. A, side view. The receptor backbone is shown in gray, Met-mutated residues are either blue (MPA-negative) or green (MPA-positive). The ligand is shown in yellow with a mesh surface of the benzoephene residue. For clarity, the ligand side chains are omitted except for the Bpa residue in position S. B, top view of the transmembrane section; color identification is the same as described for panel A. Only the position of the Bpa residue is shown.
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Determining the Environment of the Ligand Binding Pocket of the Human Angiotensin II Type I (hAT₁) Receptor Using the Methionine Proximity Assay

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