Identification of Angiotensin II-binding Domains in the Rat AT₂ Receptor with Photolabile Angiotensin Analogs*

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To identify binding domains between angiotensin II (AngII) and its type 2 receptor (AT₂), two different radiolabeled photoreactive analogs were prepared by replacing either the first or the last amino acid in the peptide with p-benzoyl-L-phenylalanine (Bpa). Digestion of photolabeled receptors with kallikrein revealed that the two photoreactive analogs label the amino-terminal part of the receptor within the first 182 amino acids. Digestion of 125I-[Bpa1]AngII-AT₂ receptor complex with endoproteinase Lys-C produced a glycoprotein of 80 kDa. Deglycosylation of this 80-kDa product decreased its apparent molecular mass to 46 kDa and further cleavage of this 46-kDa product with V8 protease decreased its molecular mass to 3.6 kDa, circumscribing the labeling site of 125I-[Bpa1]AngII within amino acids 3–30 of AT₂ receptor. Treatment of 125I-[Bpa8]AngII-AT₂ receptor complex with cyanogen bromide produced two major receptor fragments of 3.6 and 2.6 kDa. Cyanogen bromide hydrolysis of a mutant AT₂ receptor produced two major fragments of 12.6 kDa and 2.6 kDa defining the labeling site of 125I-[Bpa8]AngII within residues 129–138 of AT₂ receptor. Our results indicate that the amino-terminal tail of the AT₂ receptor interacts with the amino-terminal end of AngII, whereas the inner half of the third transmembrane domain of AT₂ receptor interacts with the carboxyl-terminal end of AngII.

The octapeptide angiotensin II (AngII) recognizes two distinct types of receptors on target cells: the type 1 receptor (AT₁) and the type 2 receptor (AT₂). The AT₁ receptor mediates all the known physiological actions of AngII including regulation of blood pressure and water and electrolyte balance (1). The functional roles of the AT₂ receptor are not well defined yet but recent studies suggest that it could act as a physiological antagonist of AT₁-mediated pressor effect and also regulate central nervous system functions related to locomotion and exploratory behavior (2, 3). Other studies also suggest that the AT₂ receptor inhibits cell proliferation and induces cell death (4, 5). AT₁ and AT₂ receptors have been cloned from several species. They are members of the G protein-coupled receptor superfamily, which is characterized by seven putative transmembrane helices. AT₁ and AT₂ receptors display a low degree (33%) of amino acid sequence similarity (6–9).

The elucidation of primary structures of numerous G protein-coupled receptors has prompted investigators to look for and identify domains in receptors directly involved in ligand binding. Most of this work was done on members of the β-adrenergic receptor family which bind bioamines (≤0.2 kDa) in the outer third of the plasma membrane between transmembrane helices. AT₁ and AT₂ receptors display a low degree (33%) of amino acid sequence similarity (6–9).

The localization of ligand-binding domains in the G protein-coupled receptor family has been mostly studied using approaches such as, site-directed mutagenesis, deletion analysis, and construction of chimeric receptors (17). Since these mutations may affect hormone binding indirectly by altering the conformation of a receptor or its expression at the plasma membrane, a more direct approach for the identification of the AT₂ receptor ligand-binding domains should be envisaged. We previously reported the covalent labeling of the AT₂ receptor with the photoreactive AngII analog [Bpa8]AngII (18–20). In the present study, another high affinity photoreactive analog was prepared by replacing the amino-terminal end of AngII with Bpa. These two photoreactive AngII analogs were used to label the AT₂ receptor of PC-12 cells. The peptide-binding domains of the receptor were identified with each ligand after targeted enzymatic and chemical fragmentation.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, bacitracin, soybean trypsin inhibitor, and cyanogen bromide (CNBr) were from Sigma. L-158,809 and PD 123319 were generous gifts from Merck and Parke-Davis Warner-Lambert, respectively. Glycopeptidase-F (PNGase-F) (EC 3.5.1.52), V8 protease (EC 3.4.21.19), endoproteinase Lys-C (endo Lys-C) (EC 3.4.21.50), and tissue kallikrein (EC 3.4.21.35) were from Boehringer Mannheim. The cDNA clone of the rat AT₂ receptor subcloned in the mammalian expression vector pcDNA1 was kindly provided by Dr. K. J. Catt (National Institutes of Health, Bethesda, MD). Lipofectamine and
culture media were obtained from Life Technologies, Inc. [Bpa1]AngII and [Bpa8]AngII were synthesized in our laboratories by the solid phase method and purified by high performance liquid chromatography as described (21). 125I-AngII, [Bpa1]AngII, and [Bpa8]AngII (specific radioactivities ~1000 Ci/mmol) were prepared with IODO-GEN as described previously (22). Briefly, 50 μg of peptide solution (0.2 mM) was incubated with 5 μg of IODO-GEN (Pierce Chemical Co.), 1 μM Na2HPO4 (2200 mM), 10 μM of acetic acid (2 M), and 30 μl of water for 30 min at room temperature. The labeled peptides were purified by high performance liquid chromatography on a C-18 column (10 μm) (Alltech Associates Inc.; number 290004) with a 20–40% acetonitrile gradient. The specific radioactivity of labeled peptides was determined by self-displacement and saturation binding analysis. Site-directed Mutagenesis—pcDNA1 containing the rat AT2 receptor cDNA clone was digested with HindIII and XhoI endonucleases and cloned into M13mp18 also digested with HindIII and XbaI. The codon change in the rat AT2 cDNA was made by site-directed mutagenesis using an in vitro mutagenesis kit (Sculptor, Amersham). One oligonucleotide was constructed to induce a mutation at methionine 116. The mutagenic primer is listed (altered nucleotide is underlined): methionine 116 to leucine (ratAT1M16L): 5-GGCCTTTGGACCGTGTGCGTGAATGGTGT-3. After confirmation of site-directed mutation by DNA sequencing, the ratAT1M16L gene was excised from the M13mp18RF form by digestion with HindIII and XhoI and subcloned into the multiple cloning site of pcDNA3 that had been digested by these same restriction endonucleases. Transfection of COS-7 Cells—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM t-glutamine and 10% (v/v) fetal bovine serum. Cells were seeded into 75-cm2 culture flasks at a seeding density of 4-5 x 104 cells per flask. After 2 days, the cultures were split at a ratio of 1:3 and cultured in DMEM with 10% (v/v) fetal bovine serum and 50 IU/ml penicillin, 60 μg/ml streptomycin, and 2 mM t-glutamine. Cells were kept in culture at 37 °C, in a CO2 incubator, and the medium was changed daily or every other day depending on the state of confluence. Binding Assays—Cell membrane preparation and binding assays were performed as described previously (15, 20). Photoaffinity Labeling—Transfectant COS-7 cells and PC-12 cells (∼1 x 107) were incubated in 4 ml of binding medium containing the photoreactive radioligands (6 nM), in the presence of L-158,809 (1 μM) (an AT2 selective non peptide analog). After 45 min at room temperature, cells were washed with 20 ml of ice-cold binding medium (without bovine serum albumin) and irradiated for 60 min at 0 °C under filtered UV light (365 nm) (mercury vapor lamp serial number JC-Par-38). Photoreactive radioligands (6 nM), in the presence of L-158,809 (1 μM) were added to a final concentration of 100 μg/ml. Samples were incubated at room temperature, in the dark, for 24–48 h. Reactions were terminated by adding 500 μl of water. Samples were lyophilized, resuspended in denaturing buffer, and analyzed by SDS-PAGE. Analysis of Products of Proteolysis and Chemical Cleavage—The products of proteolysis and chemical cleavage were analyzed by SDS-PAGE using 16.5% acrylamide Tris-Tricine gels (Bio-Rad) followed by autoradiography on x-ray films (Kodak XAR-5). 14C-Labeled low molecular weight standards (Life Technologies, Inc.) were used to determine apparent molecular masses. Running conditions, fixation, and coloration of gels were performed according to the manufacturer’s instructions.

RESULTS

Specificity of Photoreactive Analogs in Binding Experiments and Photoaffinity Labeling—Fig. 1 shows the primary structures of AngII and photoreactive AngII analogs used in this study. Asp4 and Phe8 were, respectively, replaced by Bpa to give [Bpa1]AngII and [Bpa8]AngII. In competitive binding assays, [Bpa4]AngII and [Bpa8]AngII exhibited high affinities for AT2 receptor of PC-12 cells with respective IC50 values of 1.07 ± 0.38 and 0.37 ± 0.21 nM, comparable to that of AngII (0.40 ± 0.10 nM) (mean ± S.D. of three experiments). In photoaffinity labeling experiments (Fig. 2), 125I-[Bpa1]AngII (lane 1) and 125I-[Bpa8]AngII (lane 4) specifically labeled the AT2 receptor which migrated as a glycoprotein of 140 kDa as described previously (19, 20). The labeling of the AT2 receptor by the two photoreactive analogs was completely abolished by PD123319 (10 μM) (an AT2 receptor selective ligand) (lanes 2 and 5) and by AngII (1 μM) (lanes 3 and 6) thereby confirming the specificity and the selectivity of the labeling. Although both photoreactive analogs successfully labeled the AT2 receptor, determination of covalent incorporation yields (calculated from the ratio of total radioactivity found in isolated bands to total specific binding observed before photolysis) revealed that 125I-[Bpa1]AngII, with a 10% yield of covalent labeling, was approximately six times more effective than 125I-[Bpa8]AngII (~60% yield of covalent incorporation). These differences are well illustrated in Fig. 2 where the intensity of 125I-[Bpa1]AngII labeling (lane 1) is clearly weaker than that of 125I-[Bpa8]AngII labeling (lane 4). It indicates that 125I-[Bpa1]AngII and 125I-[Bpa8]AngII interact distinctly with the AT2 receptor. 125I-[Bpa1]AngII and 125I-[Bpa8]AngII Label the Amino-
Tissue kallikrein is a serine protease that cleaves after phenylalanine-arginine or leucine-arginine amino acid combinations. The AT2 receptor contains only four of these amino acid combinations, including one located precisely halfway in the receptor molecule at arginine 182. The three other combinations are located in the carboxyl-terminal end of the molecule at arginines 330, 334, and 356 (Fig. 3). Kallikrein-treated $^{125}$I-[Bpa$_8$]AngII migrated with a molecular mass of 70 kDa (Fig. 4a, lane 1). This receptor fragment was the final digestion product since prolonged incubation with kallikrein (Fig. 4a, lanes 3 and 5) did not reveal any lower molecular weight fragment. As shown in Fig. 3, the AT$_2$ receptor is N-glycosylated exclusively in its amino-terminal extracellular tail (6, 7). The relatively high molecular mass of the 70-kDa digestion product and its glycoprotein-like migration behavior (broad band) suggest that it corresponds to the labeled 1–182 fragment of the AT2 receptor. To confirm the location of the 70-kDa receptor fragment, kallikrein-treated $^{125}$I-[Bpa$_8$]AngII-AT$_2$ complex was deglycosylated with PNGase-F. Under these conditions a labeled fragment of 18 kDa was produced (Fig. 4b, lane 5). This deglycosylation fragment exhibited about half the size of the nonkallikrein-treated deglycosylated AT$_2$ receptor (molecular mass of 35 kDa) (lane 3). Identical results were obtained with AT$_2$ receptor labeled with $^{125}$I-[Bpa$_1$]AngII. Together, these results show that $^{125}$I-[Bpa$_1$]AngII and $^{125}$I-[Bpa$_8$]AngII are labeling sites within the first 182 residues of the AT$_2$ receptor.

Mapping the $^{125}$I-[Bpa$_1$]AngII Binding Domain—After photolabeling with $^{125}$I-[Bpa$_1$]AngII, AT$_2$ receptor was partially purified and digested with endo Lys-C which cleaves on the carboxyl-terminal side of lysine residues. The digestion product migrated as a broad band of $\sim$80 kDa (Fig. 5a, lane 2). Again the high M$_r$ and the broadness of the band suggested a glycoprotein nature. After extraction of the endo Lys-C digestion product, treatment with PNGase-F resulted in a 4.6-kDa fragment which migrated as a sharp band suggesting that it was completely deglycosylated (Fig. 5c, lane 2). Interestingly, similar results were obtained with a simplified protocol where the photolabeled AT$_2$ receptor was simultaneously digested with endo Lys-C and PNGase-F (Fig. 5b, lane 2). Since as previously mentioned, the AT$_2$ receptor is N-glycosylated exclusively in its amino-terminal ectodomain, the cleavage probably occurred at one of two lysine residues, located at positions 38 and 42 (Fig. 3). Cleavage at these residues should produce either a 5.2-kDa fragment (including the photolabel of 1.3 kDa) that corresponds...
to the labeled 3–38 peptide or a 5.6-kDa fragment that corresponds to the labeled 3–42 peptide.

To further define the $^{125}$I-[Bpa$^8$]AngII-binding domain, the 4.6-kDa fragment obtained after co-digestion with endo Lys-C and PNGase-F was submitted to digestion with V8 protease. Fig. 6, lane 3, shows that, under these conditions, the 4.6-kDa fragment was converted to a 3.6-kDa fragment. The only site of cleavage for V8 protease within amino acids 3–42 is after glutamate 30 (Fig. 3). Cleavage of the 4.6-kDa fragment at this site should produce either a labeled 2.6-kDa fragment (31–42 peptide + photolabel), a labeled 2.2-kDa fragment (31–38 peptide + photolabel), or a labeled 4.3-kDa fragment (3–30 peptide + photolabel) (Fig. 3). Based on the relatively high molecular mass of the digestion product (3.6 kDa), the 3–30 peptide is clearly a better candidate than the 31–42 or 31–38 peptides for the binding domain of $^{125}$I-[Bpa$^8$]AngII. This conclusion is further strengthened by experiments in which photolabeled-AT$_2$ receptor was co-digested with PNGase-F and V8 protease. Under these conditions, a 4.1-kDa fragment was obtained, locating the binding domain within the first 30 amino acids of the AT$_2$ receptor (results not shown). Together these results show that $^{125}$I-[Bpa$^8$]AngII is labeling a site within residues 3–30 of the extracellular amino-terminal tail of the AT$_2$ receptor (Fig. 10).

**Mapping the $^{125}$I-[Bpa$^8$]AngII-binding Domain—$^{125}$I-[Bpa$^8$]AngII-labeled AT$_2$ receptor.** A, partially purified photolabeled AT$_2$ receptor (24,000 cpm) was incubated in the absence (lane 1) or presence (lane 2) of endo Lys-C (2.5 µg) at 37 °C for 24 h. Samples were run on a 10% acrylamide separating gel followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of three separate experiments. B, photolabeled receptor (47,000 cpm) was incubated with PNGase-F (100 units/ml) at room temperature for 24 h. The sample was aliquoted in two fractions one of which received digestion buffer (lane 1) and the other received 1.8 µg of endo Lys-C (lane 2). Incubation was prolonged for 22 h at 37 °C. Samples were run on a 16.5% acrylamide Tris-Tricine separating gel followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of three separate experiments. C, photolabeled receptor (100,000 cpm) was incubated with endo Lys-C (1 µg) for 20 h at 37 °C. The sample was run on a 8% acrylamide acrylamide gel. The 80-kDa labeled receptor fragment was located and recovered by passive elution from gel slices. The receptor fragment (40,000 cpm) was incubated in the absence (lane 1) or presence (lane 2) of PNGase-F (40 units/ml) for 2 h at 37 °C. Samples were run on a 16.5% acrylamide Tris-Tricine separating gel followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of two separate experiments.

**FIG. 3. Two-dimensional representation of the primary structure of the rat AT$_2$ receptor and its potential sites of cleavage by specific proteases and CNBr.** The space after residue 182 indicates a tissue kallikrein recognition site; arrows indicate recognition sites for V8 protease; bold circles indicate recognition sites for endo Lys-C; closed circles indicate sites of hydrolysis for CNBr. Putative sites of N-glycosylation on asparagines 4, 13, 24, 29, and 34 are also indicated.

**FIG. 4. Kallikrein digestion of $^{125}$I-[Bpa$^8$]AngII-labeled AT$_2$ receptor.** A, photolabeled AT$_2$ receptor (50 µg of membrane protein) was solubilized and incubated in the absence (lanes 2, 4, and 6) or presence (lanes 1, 3, and 5) of tissue kallikrein (50 µg) at 37 °C for 1 h (lanes 1 and 2), 3 h (lanes 3 and 4), and 5 h (lanes 5 and 6). Samples were run on a 8% acrylamide separating gel followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of at least three separate experiments. B, $^{125}$I-[Bpa$^8$]AngII-labeled AT$_2$ receptor (50 µg of membrane protein) (lane 1) was incubated for 2 h at 37 °C in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of tissue kallikrein (50 µg) before digestion with PNGase-F (33 units/ml) for 2 h at 37 °C (lanes 3 and 5). Samples were run on a 12% acrylamide separating gel followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of three separate experiments. C, labeled receptor fragment was located and recovered by passive elution from gel slices. The receptor fragment (40,000 cpm) was incubated in the absence (lane 1) or presence (lane 2) of PNGase-F (40 units/ml) for 2 h at 37 °C. Samples were run on a 8% acrylamide acrylamide gel. The 80-kDa labeled receptor fragment was located and recovered by passive elution from gel slices. The receptor fragment (40,000 cpm) was incubated in the absence (lane 1) or presence (lane 2) of PNGase-F (40 units/ml) for 2 h at 37 °C. Samples were run on a 16.5% acrylamide Tris-Tricine separating gel followed by autoradiography. Protein standards of the indicated molecular masses (kDa) were run in parallel. These results are representative of two separate experiments.
AngII-photolabeled AT2 receptor was partially purified and digested with V8 protease. The patterns of fragmentation were clearly distinct from those obtained with 125I-[Bpa1]AngII-AT2 complex. Digestion with V8 protease produced 28- and 15.2-kDa fragments (Fig. 7, lane 2). Deglycosylation had no effect on the mobility of the 15.2-kDa fragment indicating that it was not a glycosylated fragment (result not shown). Knowing that the binding domain of 125I-[Bpa8]AngII is located within amino acids 1–182 (Fig. 4) and that the 15.2-kDa digestion product is not glycosylated, the fragment located between alanine 46 and glutamate 188 (estimated molecular mass of the peptide photolabel: 17.8 kDa) is the best candidate for the binding domain of 125I-[Bpa8]AngII (Fig. 3). Prolonged incubations in the presence of PNGase-F reduced the proportion of the 28-kDa fragment and increased the proportion of a fragment migrating close to the 15.2-kDa fragment, suggesting that the 28-kDa fragment is the glycosylated 31–188 peptide of AT2 receptor, containing a putative site of glycosylation at asparagine 34 (Fig. 3).

To further define the 125I-[Bpa8]AngII-binding domain, photolabeled AT2 receptor was submitted to hydrolysis with CNBr which cleaves specifically at the carboxyl-terminal side of methionine residues. Fig. 8, lane 2, shows that, under these conditions, two major digestion products of 3.6 and 2.6 kDa were obtained. These results are representative of at least three separate experiments.

Peptide-binding Domains of the AT2 Receptor

28-kDa fragment is the glycosylated 31–188 peptide of AT2 receptor, containing a putative site of glycosylation at asparagine 34 (Fig. 3).
binding assays, AngII exhibited a similar affinity for wild-type (IC\textsubscript{50} of 0.34 ± 0.03 nM; mean ± S.D. of three experiments) and mutant rAT\textsubscript{2}M116L (0.37 ± 0.17 nM) receptors expressed in COS-7 cells. [Bpa\textsuperscript{8}]AngII displayed also comparable binding affinities for the wild-type (0.31 ± 0.26 nM) and the mutant (0.23 ± 0.15 nM) receptors. The wild-type and the mutant rAT\textsubscript{2}M116L receptors were photolabeled with 125I-[Bpa\textsuperscript{8}]AngII, partially purified, and submitted to hydrolysis by CNBr. Fig. 9, lane 1, shows that CNBr hydrolysis of the wild-type AT\textsubscript{2} receptor produced the previously described 3.6- and 2.6-kDa fragments. CNBr hydrolysis of the rAT\textsubscript{2}M116L receptor still produced the 2.6-kDa fragment and a longer 12.6-kDa fragment (Fig. 9, lane 3). Under these conditions the only possibility is that the 2.6-kDa fragment is the 129–138 peptide located in the inner half of the third transmembrane domain of mutant AT\textsubscript{2} receptor. Indeed, if labeling had occurred between leucine 116 and methionine 128 in the mutant receptor, exclusively higher molecular mass labeled receptor fragments (13–15 kDa) would have been produced. The 12.6-kDa fragment produced by CNBr hydrolysis of rAT\textsubscript{2}M116L most probably corresponds to the mutant receptor 54–138 peptide (estimated molecular mass of the peptide + photolabel: 10.9 kDa). The incomplete CNBr hydrolysis of both native and mutant receptors suggest that cleavage at methionine 128 is impaired. This may result from reduced solubility of the protein in strong dissociating agents (like trifluoroacetic acid), sterical masking of the methionine residue by surrounding amino acids, or oxidation of the methionine residue occurring during protein manipulations and/or acid hydrolysis (25). Together these results show that 125I-[Bpa\textsuperscript{8}]AngII is labeling a site within residues 129–138 of AT\textsubscript{2} receptor (Fig. 10).

**DISCUSSION**

Our results indicate that, upon binding, the amino-terminal tail of the AT\textsubscript{2} receptor interacts with the amino-terminal end of AngII whereas the inner half of the third transmembrane domain of AT\textsubscript{1} receptor interacts with the carboxyl-terminal end of AngII. To our knowledge, this is the first study providing data on the binding domains of the AT\textsubscript{2} receptor. Our results and those of other groups suggest that there may be a common scheme for the binding domains of small peptide hormones in the G-protein-coupled receptor superfamily. For example, it has been shown that charged residues in the second and third extracellular loops of the AT\textsubscript{1} receptor are major docking points for the amino-terminal end (Asp\textsuperscript{5} and Arg\textsuperscript{5} residues, respectively) of AngII (13, 26). On the other hand, the hydrophobic nature of the phenyl group at position 8 in the AngII molecule (the carboxyl-terminal end of the peptide) suggests that its site of interaction with the AT\textsubscript{1} receptor is within the membrane. Actually, recent studies suggest that upon binding, the carboxy-terminal end of the AngII molecule occupies a space between helices III, V, VI, and VII of the AT\textsubscript{1} receptor (26, 27). Similarly, binding domains for the undecapeptide substance-P (SP) were identified in the first and second extracellular loops, the amino-terminal ectodomain and the outer half of the transmembrane helices II and VII of the NK-1 receptor (14, 15, 28).

In an elegant study using photoreactive analogs of SP, Li et al. (15) have shown that the amino-terminal side (the fourth position) of the 125I-[Tyr\textsuperscript{1},Bpa\textsuperscript{4}]SP peptide interacts with the extracellular amino-terminal tail of the NK-1 receptor while the COOH-terminal side (the ninth position) of the 125I-[Tyr\textsuperscript{1},Bpa\textsuperscript{4}]SP peptide interacts with the second extracellular loop. The authors proposed a model in which the carboxy-terminal end of the peptide positions itself between helices in the outer part of the plasma membrane whereas the amino-terminal portion of the peptide is stabilized by ectodomains of NK-1 receptor. Based on our results, it is tempting to propose that the AngII molecule binds the AT\textsubscript{2} receptor in a similar fashion, with the carboxy-terminal end sitting deep within the transmembrane domains and the amino-terminal end interacting with the ectodomains of the receptor. The location of the carboxy-terminal portion of AngII deep in the plasma membrane fits well with known pharmacological properties of AngII. The agonistic nature of AngII is conferred by its carboxy-terminal phenylalanine residue (29) and intrahelical amino acids are known to play a major role in G-protein coupled receptor activation upon direct interaction with ligands (10). Our results also suggest that the carboxy-terminal end of AngII interacts with the third transmembrane domain of the AT\textsubscript{2} receptor. Similarly, it has been shown that residues in the third transmembrane domain of the AT\textsubscript{1} receptor are required for high affinity binding (16, 26).
Our results also raise another interesting point. If the AngII molecule interacts at the same time with residues in the amino-terminal extracellular tail as well as residues deeply located in the third transmembrane domain, one could speculate that the ectodomain must lie near the outer membrane surface. The AngII molecule has an estimated length of about \(-30\) Å (probably less in solution) which is shorter than a transmembrane helix (\(-40\) Å). To account for such a requirement, the putative disulfide bridge located between the amino-terminal tail and the third extracellular loop of the \(\text{AT}_2\) receptor may play an important conformational role in bringing the amino-terminal tail in close proximity to the plasma membrane surface (Fig. 10).

In conclusion, we have identified two peptide-binding regions in the \(\text{AT}_2\) receptor with the use of highly potent and specific AngII photoreactive analogs. This approach also allowed the determination of the AngII molecule’s orientation in its binding pocket. Based on these results, we conclude that AngII interacts with an extracellular segment and a transmembrane helix of \(\text{AT}_2\) receptor. This interaction pattern, also found in other G-protein coupled receptors for small bioactive peptides, may correspond to a highly conserved feature among this very large family or receptors. By recovering large amounts of labeled receptor, it will be possible to sequence the fragments and pinpoint the precise interaction sites.

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