Physicochemical Characterization of Photoaffinity-Labeled Angiotensin II Receptors


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Specific receptor sites for angiotensin II (All) were analyzed in the adrenal cortex and other target tissues including liver, anterior pituitary gland, and smooth muscle, after covalent labeling with the radioactive photoaffinity analog \(^{125}\text{I}[-\text{Sar}^1,(4-N_3)\text{Phe}^8]\) All. The photoreactive All derivative retained high affinity for adrenal receptors and full steroidalogenic activity in adrenal glomerulosa cells. In bovine adrenal cortex, covalent labeling of All receptors by the photoreactive analog was specifically inhibited by [Sar^1]AII with an IC\(_{50}\) of about 5 nM. The Mr of the covalent All-receptor complex during polyacrylamide gel electrophoresis of the labeled protein under reducing conditions was 58,000. Under non-reducing conditions, a minor band with Mr of 105,000 was also observed. Two labeled species were also found during gel permeation chromatography of the detergent-solubilized complex, with Mr of 64,000 and 86,000. The pl of the solubilized photolabeled complex was absorbed to wheat germ lectin Sepharose 6MB and could be eluted with N-acetylglucosamine. The Mr of specific All-binding sites in several target tissues, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, showed significant differences within and between species. The most striking differences were between rat adrenal cortex (79,000) and both rat liver (60,000) and bovine adrenal cortex (58,000). After enzymatic deglycosylation, the Mr of the major component present in the bovine and rat adrenal cortex decreased by 40% and 55% to 35,000 and 34,000, respectively, suggesting that variations in carbohydrate content contribute to the physical heterogeneity of All receptors in individual target tissues. (Molecular Endocrinology: 1: 147-153, 1987)

RESULTS

Photolabeling of All Receptors

In competitive binding studies, N\(_2\)[-Sar^1]All inhibited \(^{125}\text{I}\)All binding to bovine adrenal cortex particulate receptors with an IC\(_{50}\) of 0.7 nM, similar to the IC\(_{50}\) for [Sar^1]All of 1.3 nM and significantly more potent than the All IC\(_{50}\) of 5.6 nM (Fig. 1). In rat zona glomerulosa cells, the azido derivative was equivalent to All in its steroidalogenic potency and efficacy, as indicated by stimulation of aldosterone production in vitro (data not shown). In direct binding studies, the radiolabeled azido-hormone retained high affinity and specificity for particulate All receptors. During UV irradiation, up to 60% of the specifically bound hormone became covalently attached to adrenal membranes, as indicated by resistance to acid dissociation (Fig. 2). In the absence of photolysis,
Fig. 1. Displacement of 125I-AII Binding by All, [Sar1]AII, and N3-[Sar1]AII
Binding of 125I-AII to bovine adrenal cortical membranes (90 μg) was determined in the dark as in Materials and Methods in the presence of increasing amounts of All analogs. Data represent the mean ± SE of duplicate points and are typical of three similar experiments.

Fig. 2. Covalent Labeling of Bovine Adrenal All Receptors
Membranes were incubated with 125I-N3-[Sar1]AII in the presence or absence of 1 μM All. The membranes were then irradiated for varying periods of time, treated with acid as indicated, and washed by filtration to remove free hormone. Bars indicate the mean ± SE of triplicate determinations, and the data are representative of three individual experiments.

Chromatography and Electrofocusing
During gel permeation chromatography of photolabeled SDS- or Triton-solubilized bovine adrenal membranes, the bulk of the radioactivity eluted as a peak with Stokes’ radius corresponding to a protein of M, 64,000 ± 3,000 (mean ± SE, n = 13) (Fig. 4). A minor peak of M, 86,000 ± 2,000 (n = 0) was sometimes present, and represented up to 15% of the bound radioactivity. The elution profile of some preparations also showed a significant amount of radioactivity in the position of free All. This may have originated from degradation of the covalent hormone-receptor complex, or from the presence of 95–100% of the specifically bound ligand was dissociated by acid treatment. A brief wash with Tris buffer immediately before photolysis was found necessary to free the membranes of BSA and unbound 125I-N3-[Sar1] All, which otherwise resulted in high nonspecific levels of covalent attachment. Under these conditions, nonspecific labeling was usually less than 15% of the total bound radioactivity.

To characterize the membrane component(s) labeled by the azido-hormone, bovine adrenal particulate samples were incubated with 125I-N3-[Sar1]All in the presence of increasing amounts of All analogs. Analysis of the photolysis products by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis revealed progressive displacement of labeling (IC50 ~ 5 x 10^-9 M) from the major radioactive band of M, 55,000. Labeling experiments performed with the All antagonist analog, 125I-[Sar1,D-(4-N3)Phe8]AII (6), gave similar results (Fig. 3). Omission of 2-mercaptoethanol during sample preparation caused no apparent change in the major labeled band, but did result in the appearance of a minor (<5%) band of approximate M, 105,000 (data not shown).
Photoaffinity Labeled Angiotensin Receptors

Fig. 4. HPLC Gel Permeation Chromatography of \([^{125}I]N_3-[Sar^1]AII\)-Labeled Bovine Adrenal Membranes
The Triton-solubilized membranes were loaded onto Spherogel-TSK columns and eluted as described in Materials and Methods. The chromatogram shown is a representative profile of 13 separate experiments. Arrows indicate the elution positions of reference proteins.

Fig. 5. Wheat Germ Lectin Affinity Chromatography of Solubilized \([^{125}I]N_3-[Sar^1]AII\)-Labeled Bovine Adrenal Membranes
Photolabeled SDS-solubilized membranes were loaded onto 2 ml wheat germ lectin-Sepharose 6MB resin, the resin washed extensively, and specifically absorbed proteins eluted with the addition of N-acetylglucosamine at the indicated times. A typical chromatofocusing profile of the photolabeled, Triton-solubilized bovine adrenal membranes is shown in Fig. 6. The isoelectric point of the covalent hormone-receptor complex was 7.2 ± 0.1 (n = 6). This

experiments, indicating the presence of carbohydrate moieties on the AII-binding protein.

Wheat germ lectin Sepharose 6MB chromatography of SDS-solubilized, photolabeled adrenal membranes was performed in buffer containing 0.1% Triton X-100, after batch absorption to the resin. Under these conditions, 20–40% of the radioactive complex eluted in the initial wash, together with about 75% of the protein. Approximately one-half of the absorbed covalent hormone-receptor complex was then eluted with the specific sugar ligand for wheat germ lectin, N-acetylglucosamine (Fig. 5). Similar results were obtained in three
pl was consistent with the distribution of the complex over the pH range from 7.0 to 7.5 on autoradiography of two-dimensional gels (data not shown) and also with the fact that the complex absorbs to diethylaminoethyl resins at pH 8.0 (9).

**SDS-Polyacrylamide Gel Electrophoresis (PAGE) Analysis of All Receptors**

Membrane fractions from the adrenal cortex and liver of rat, rabbit, monkey, and cow, and also from the anterior pituitary, bladder, and mesenteric artery of the rat, were photolabeled with N₃-[Sar¹]AII and analyzed by SDS-PAGE followed by fluorography. As shown in Fig. 7, a single broad band was specifically labeled in all cases. However, the apparent molecular weights of these bands, as summarized in Tables 1 and 2, differed both between species for a given target tissue, and among different target tissues within a single species. A particularly striking difference was noted between the Mr of the All-receptor complex of the rat adrenal gland (79,000) and those of the rat liver (60,000) and bovine adrenal (58,000).

**Effects of Glycosidase Treatment**

To further characterize the nature of the All binding protein, photolabeled membranes were treated with the endoglycosidase, N-Glycanase, which cleaves N-linked saccharides. A marked decrease in Mr was evident in the glycosidase-treated adrenal cortical protein from cow (from 58,000 to 35,000) and rat (from 75,000 to 34,000), as shown in Fig. 8. These changes constitute reductions of 40% and 55% in Mr, respectively. The appearance of bands of intermediate size, presumably partial deglycosylation products, may have resulted from incomplete solubilization and/or denaturation before glycosidase treatment, rendering some linkages more accessible than others. Such intermediate bands were also observed after glycosidase treatment of labeled bovine adrenal membranes in the absence of SDS denaturation, which gave predominantly products of Mr 53,000 and 44,000, rather than 35,000 (data not shown).

**DISCUSSION**

In previous studies on the physical characteristics of photoaffinity labeled All receptors from canine adrenal gland and uterus (2), the presence of two binding species, of Mr 126,000 and 65,000 or 68,000, indicated that the All holoreceptor could be a dimer of two subunits of about Mr 60,000. The Mr values derived for the binding subunits of dog adrenal (65,000) and uterus (68,000) were similar, suggesting that the All receptor may be of comparable size in different target tissues. This possibility was supported by subsequent reports on the apparent molecular weights of All receptors from bovine adrenal [60,000 (5)] and rabbit liver [68,000 (10)]. However, during the present studies on the properties of covalent All-receptor complexes derived by photofinity labeling with ¹²⁵I-N₃-[Sar¹]AII, we observed several differences in the characteristics of All receptors from a number of target tissues. The C-terminal azido All derivative (3) employed in these studies has higher efficiency for labeling All receptors than N-terminal azidobenzoyl All derivatives (2) or chemical cross-linking methods (7, 8), and is the preferred reagent for the preparation of covalent All-receptor complexes.

The molecular weight of the photolabeled All receptor site was found to vary significantly with both the species and tissue of origin. The molecular weights of the bovine adrenal and rabbit liver sites were in agreement with those observed by Guillemette and Escher (5) and Sen et al. (10), respectively. However, the Mr of the rat adrenal All receptor (79,000) was significantly smaller than that (116,000) reported by Paglin and Jamieson.
Table 2. Molecular Weights of All-Binding Proteins from Rat Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mr \times 10^3</th>
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<tbody>
<tr>
<td>Adrenal Glomerulosa</td>
<td>79 ± 6 (6)</td>
</tr>
<tr>
<td>Liver</td>
<td>60 ± 7 (9)</td>
</tr>
<tr>
<td>Anterior Pituitary</td>
<td>92 ± 2 (2)</td>
</tr>
<tr>
<td>Mesenteric Artery</td>
<td>71 ± 2 (3)</td>
</tr>
<tr>
<td>Bladder</td>
<td>72 ± 3 (2)</td>
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Values indicate the mean ± se of data from the numbers of experiments given in parentheses.

Fig. 8. Effects of N-glycanase Digestion on Electrophoretic Properties of \[^{3}H\text{-}[\text{Sar}^1]\text{All]-Labeled Adrenal Receptors.} Bovine and rat adrenal membranes were photolabeled, solubilized, and denatured, then treated with N-glycanase and analyzed by SDS-PAGE. Lanes a and b, Bovine adrenal cortical membranes. Lanes c and d, Rat adrenal glomerulosa membranes. Lanes a and c, Controls. Lanes b and d, Membranes treated with N-glycanase.

This difference suggests that chemical cross-linking of the particulate hormone-receptor complex may lead to overestimation of the molecular weight of the hormone-binding moiety, presumably by the cross-linking of adjacent proteins to the receptor, or of the receptor subunits if its native form is dimeric (3). Although the major photoaffinity-labeled protein in the bovine adrenal cortex had a relative molecular weight of approximately 60,000 as determined by HPLC gel permeation chromatography and SDS-PAGE, minor species of higher molecular weight were also detected in the absence of reducing agents. These data, together with the results of cross-linking studies, suggest that the All hormone receptor is a dimer, or is closely associated with other membrane proteins.

The adrenal All-receptor complex behaved as a glycoprotein, and was of almost neutral pl value as indicated by its properties during chromatofocusing and ion exchange chromatography (9). The presence of carbohydrate residues was shown by both specific absorption of immobilized wheat germ lectin and by the effects of glycosidase digestion. The carbohydrate moieties contributed significantly to the apparent Mr of the receptor-binding protein. This was most evident in the rat adrenal, where treatment with N-glycanase reduced the estimated size of the labeled protein by more than 50%. It is noteworthy that some of the smaller degradation products appearing in Fig. 3 coincide with the N-glycanase products in Fig. 8, suggesting that glycosidase digestion may occur during the extraction of receptors from their tissues of origin.

The labeled protein in rat adrenal glomerulosa was physically distinct from that in liver, suggesting the existence of at least two types of All receptors. These results may be relevant to the suggestion by Peach (11) that receptors in various All target tissues differ in their pharmacological properties. However, within any given tissue, only a single broad band was consistently labeled by the photoaffactive All derivative. The presence of lower molecular weight species in some gels was not reproducible, and was probably attributable to glycosidic or proteolytic digestion after photoaffinity labeling of the membranes receptor sites.

The broadness of the specifically labeled species observed by SDS-gel electrophoresis was consistent with the glycoprotein nature of the All receptor. In some instances the labeled band appeared to be a doublet, but whether this represented two species of All-binding sites, or was the product of minor receptor degradation, is not certain. It has been postulated that two types of angiotensin receptor are present in cardiac and vascular tissues (12). Whereas our observations neither support nor refute this possibility, it is interesting to note that the labeled bands from smooth muscle tissues, such as bladder and mesenteric artery, were particularly broad and diffuse.

The basis for the difference between the All-binding sites in rat liver and rat adrenal, and whether their physical properties are relevant to functional differences between the two receptors, is not yet clear. It is possible that there is more than one gene for the All receptor, and that these are differentially expressed in the various All target tissues. A more likely possibility is that different posttranslational modifications of a single gene product occur within individual target tissues. In particular, variation in the degree of glycosylation, which contributes substantially to the molecular weights of the All-binding sites, could be responsible for the differences among individual target tissues and between species. Thus, the rat adrenal receptor may be simply a more heavily glycosylated form of the corresponding liver receptor. This question is currently under investigation to clarify the role of carbohydrate components in the physical and functional properties of the All receptor.
MATERIALS AND METHODS

Synthesis and Purification of Peptides

The photoreactive All derivative N2(Sar)3All was prepared essentially as described (3) from [Sar-(4-NO2)Phe]3All. The nitro peptide was prepared by solid phase synthesis from commercially available amino acids, with the exception of (4-NO2)Phe, which was found to contain significant (>20%) contaminants that were not removable by recrystallization. This amino acid was therefore prepared by condensation of malonic acid ethyl ester with p-nitrobenzyl bromide (13). The L isomer was isolated in pure form from the resulting racemic mixture by acetylation, followed by treatment with Acylase I (Sigma, St. Louis, MO). After HF cleavage from the resin [Sar-(4-NO2)Phe]3All was purified by Sephaxdex G-25 gel filtration and counter-current distribution. The subsequent reactions to yield N2(Sar)3All proceeded quantitatively. Purity of all peptides was confirmed by TLC, HPLC, and amino acid analysis. The azido-All antagonist analog, [Sar-D-0-(4-NO2)Phe]3All, was the gift of Dr. E. Escher (University of Sherbrooke, Sherbrooke, Quebec, Canada) and [Sar]3All was the gift of Dr. M. C. Khotis (Cleveland Clinic, Cleveland, OH). Monomodiiodinated [35S]All analogs were prepared by a modified IODO-GEN (Pierce, Rockford, IL) method (14) (Meloy Laboratories, Springfield, VA) and had specific activities of about 1500 Ci/MMmol.

Membrane Preparations

Particulate fractions from bovine, rat, rabbit, and monkey adrenal cortex were prepared essentially as described previously (15), with the exception that all tissues were minced and homogenized in 20 mM NaHCO3 containing 0.1 mM phenylmethylsulfonfyl fluoride, 50 μg/ml soybean trypsin inhibitor, 20 mM Na molybdate, 0.01% dimethylsulfoxide, and 0.5 mM dithiothreitol, before centrifugal fractionation at 4°C. The 35,000 × g membrane pellets were then washed twice with 20 mM Tris-HCl, pH 7.4, containing 0.02% NaN3 (Tris buffer). Bovine adrenal cortical membranes were prepared in quantity essentially as described (3), with the exception that all tissues were minced and homogenized in 20 mM NaHCO3 containing 0.1 mM phenylmethylsulfonfyl fluoride, 50 μg/ml soybean trypsin inhibitor, 20 mM Na molybdate, 0.01% dimethylsulfoxide, and 0.5 mM dithiothreitol, before centrifugal fractionation at 4°C. The 35,000 × g membrane pellets were then washed twice with 20 mM Tris-HCl, pH 7.4, containing 0.02% NaN3 (Tris buffer). Bovine adrenal cortical membranes were prepared in quantity and used fresh or stored in aliquots at −70°C before use, the frozen membranes were thawed and washed with Tris buffer. All other membranes were freshly prepared and kept on ice before binding studies and photoaffinity labeling.

Radioligand Binding Assays

Receptor-binding assays were performed by a modified version of a previously described procedure (16). Briefly, 50–100 μg membranes were incubated with approximately 0.1 nM radiolabeled hormone and varying amounts of unlabeled hormone in a total volume of 300 μl containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5–10 mM MgCl2, 0.2% BSA, and 0.02% NaN3 (assay buffer). This buffer was used for binding studies and photoaffinity labeling. The assay buffer for rat, rabbit, and monkey adrenal glomerulosa, and rat anterior pituitary, bladder, and mesenteric artery, also contained 2 mM EGTA and 0.5 mM dithiothreitol. Incubations were performed at 22°C for 45 min, and were stopped by the addition of 2 ml ice-cold PBS, followed by rapid filtration through Whatman GF/C filters, presoaked in 0.2% BSA, to isolate receptor-bound radioactivity.

Protein Determinations

In particulate samples, protein was measured by the method of Lowry et al. (17) after hydrolysis in 0.1 N NaOH. Protein in solubilized samples was determined by the method of Bradford (18) using Bio-Rad (Richmond, CA) Protein Dye reagent and standards containing equivalent detergent concentrations. In both assays, crystalline BSA was used as the protein standard.
Lectin Affinity Chromatography

Aliquots of the SDS-solubilized photolabeled adrenal membranes (0.5 ml, 2 mg protein, and 150,000 cpm) were added to 2 ml wheat germ lectin Sepharose 6MB (Pharmacia, Piscataway, NJ) equilibrated in Tris buffer containing 100 mM NaCl, 0.1% Triton X-100, and 1 mM each of CaCl₂, MgCl₂, and MnCl₂. The resin was agitated gently overnight in a closed column, then washed with the equilibration buffer at 0.4 ml/min until no further protein was detected in the eluate (10-column volumes). Specifically bound proteins were then eluted from the lectin by the addition of 0.3 M and 1 M N-acetylglucosamine to the buffer, and 1-ml fractions were collected.

Chromatofocusing

The isoelectric point of the All-receptor complex was determined by analysis of Triton X-100-solubilized [3H-N3-Sar₁]All-labeled bovine adrenal membranes on a Mono-P-chromatofocusing column (Pharmacia). The detergent extract was diluted to 0.1% Triton with 75 mM Tris-HCl, pH 9.3. The Mono P column, attached to a Pharmacia FPLC unit, was equilibrated in 75 mM Tris-HCl, 0.1% Triton, pH 9.3. A 500-μl sample containing 250–500 μg protein was loaded onto the column and eluted with a 1:10 dilution of Polybuffer 96-HCl (Pharmacia), pH 6.0. The elution buffer flow rate was 0.25 ml/min, and 0.5-ml fractions were collected.

Glycosidase Treatment of Labeled Adrenal Membranes

Photoaffinity-labeled particulate samples (50–100 μg protein containing 8,000 to 20,000 cpm) were denatured and solubilized by boiling for 3 min in 0.5% SDS and 0.1 M 2-mercaptoethanol. Nonidet P-40 was added to 7.5-fold excess over the detergent concentration, and the samples were incubated overnight at 30 C. The treated protein was then boiled in the presence of 3% SDS and 1% 2-mercaptoethanol and 5% glycerol and analyzed by SDS-PAGE.

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