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Isolation of an angiotensin II-binding protein from liver

(angiotensin II receptor/crosslinking/affinity chromatography/immunoaffinity chromatography/dithiothreitol)

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ABSTRACT **A protein that specifically binds angiotensin II has been isolated in nearly homogeneous form by two independent approaches after solubilization from rabbit liver particles by treatment with digitonin. The protein purified by either of these methods resembles in size the single radioactive macromolecular component made by using disuccinimidyl suberate to crosslink radioiodinated angiotensin II with its receptor in the solubilized extract. In the first technique, angiotensin II as an affinity ligand specifically extracted the protein from a preparation that had been freed of angiotensin-degrading activity. In the second approach, the angiotensin II–protein complex was specifically precipitated by anti-angiotensin II antibodies and staphylococcal protein A-Sepharose. The protein could be eluted from the affinity column with angiotensin II or 4 M $MgCl_2$. The angiotensin II–protein complex dissociated in the presence of sulfhydryl-containing reagents, and these could therefore be used to elute it from either the chemical or the immunoaffinity-based matrix. This effect of sulfhydryl-containing reagents and the paradoxical observation that the isolated protein after denaturation exhibited a slower electrophoretic mobility in its reduced form than in its unreduced form suggest that the binding configuration of this protein may be sensitive to reduction.**

Angiotensin II is the biologically active component of the renin-angiotensin system (1, 2). Several mechanisms may contribute to its role as an important vasopressor agent. Among these are a contractive effect on blood vessels (1), a stimulation of cells of the adrenal zona glomerulosa to elaborate aldosterone (3–6), and a direct action on the central nervous system (7). These and other effects of angiotensin II are thought to be mediated by specific receptors on the surface of its target cells (8). Such receptors have been detected and their binding properties have been characterized in particulate fractions from various cell types (reviewed in ref. 9). In addition, they have recently been solubilized in active form from bovine adrenal (10) and rabbit hepatic (11) membranes. However, despite the fact that it is thought to mediate directly and thus determine the specificity of action of angiotensin II, the responsible receptor is the only component of the renin–angiotensin system that has not been purified and characterized in the isolated state. It has therefore not yet been possible to address directly certain important questions such as whether or not a chemically identical receptor molecule is responsible for the action of angiotensin II on different cell types.

As a result of the development of inhibitors of angiotensin-converting enzyme (12, 13), it has already become apparent that suppression of the renin–angiotensin system represents an important approach to antihypertensive therapy (reviewed in ref. 14). However, this enzyme is a dipeptidyl carboxypeptidase capable of acting on many peptide substrates in addition to angiotensin I (reviewed in ref. 15). Therefore, even biochemically specific inhibitors of its activity are unlikely to be physiologically specific with respect to the renin–angiotensin system. Since angiotensin II is the effector molecule of the system, an antagonist directed at its receptor might be expected to represent the desired physiologically specific inhibitor. The development of such an agent should be facilitated by characterization of the isolated receptor. We have chosen liver as the organ from which to purify this molecule because the required fresh tissue (11) can be easily obtained in large amounts and because isolated hepatocytes exhibit angiotensin-dependent changes in measurable biochemical parameters (16, 17), such as stimulation of phosphorylase activity.

MATERIALS AND METHODS

Materials. Disuccinimidyl suberate and staphylococcal protein A-Sepharose were from Pierce and Sigma, respectively. Standard protein molecular weight markers and materials for gel electrophoresis were Bio-Rad products. Rabbit anti-angiotensin II antiserum prepared according to Gocke *et al.* (18) was generously provided by Jean Sealey and Joan Gerten-Banes. Brij 99 was from Emulsion Engineering (Sanford, FL). EN^{3}HANCE, Na^{125}I, and mono[^{125}I]iodinated angiotensin II (^{125}I-angiotensin II) (1880 μCi/μg; 1 Ci = 37 GBq) were purchased from New England Nuclear. The latter was subjected to gel filtration through Sephadex G-50 equilibrated and developed in 20 mM Tris·HCl, pH 7.4, containing 5 mM Na_2EDTA and 0.5% Brij 99 (TEB buffer) to remove a trace contaminant, which appeared in the void volume. The purified radioiodinated angiotensin II (which was used as a tracer for the affinity chromatography on angiotensin II-Sepharose 4B) migrated as a single component in two TLC systems [*tert*-butyl alcohol/3% ammonia (3:1, vol/vol) and *n*-butyl alcohol/acetic acid/water (25:4:10, vol/vol)], and these were used to detect angiotensin II-degrading activity. For all other experiments the commercial ^{125}I-angiotensin II was used without further purification. Sephadex G-50, SE-Sephadex C-50, Sepharose 6B, and cyanogen bromide-activated Sepharose 4B were from Pharmacia. For preparation of the angiotensin II affinity column, 10 g of the cyanogen bromide-activated 4B were allowed to react with 67 mg of angiotensin II (Bachem Fine Chemicals, Torrance, CA) according to the manufacturer's instructions, and 99% of the ligand was bound as determined by including a tracer amount of the radioiodinated derivative.

Assays and Partial Purification of Binding Protein. Protein-bound ^{125}I-angiotensin II was determined as radioactivity not adsorbed onto a dextran/charcoal mixture and important results were confirmed by measurement of radioactivity in the void volume after gel filtration through a column of Sephadex G-50 in TEB buffer as described elsewhere (11). The homogenization of fresh rabbit liver, isolation of particles sedimenting between 1000 and 100,000 × *g*, solubilization of binding activity with 1% digitonin, fractionation between 45% and 60% saturation with $(NH_4)_2SO_4$, and gel fil-

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Abbreviation: PCMS, *p*-chloromercuriphenylsulfonic acid.

tration through Sepharose 6B have also been previously described (11). These steps yield approximately 400 mg of protein from 150 g of liver containing a total of about 18 g of protein, 8.6 g of which are in the initial particulate fraction. There is an apparent 300-fold increase in specific binding per unit protein, much of which is due to removal of angiotensin II-degrading activity (11). At this stage of purification such activity is still present in the preparation but can be suppressed by a "cocktail" of protease inhibitors as described (11). The residual degrading activity, assayed by incubating the preparation with ^{125}I-angiotensin II (in 20 mM Tris·HCl, pH 7.4/4.5 mM Na_2EDTA/158 mM NaCl/0.25% Brij 99 for 60 min at 22°C) and analyzing the product by TLC (11), has been eliminated by addition of the following two steps. First, the concentrated Sepharose eluate obtained by the published (11) procedure (8–10 ml) is passed through a column of SE-Sephadex C-50 equilibrated with TEB buffer and the unadsorbed fraction containing all the binding activity is collected. Second, this SE-Sephadex fraction is adjusted to 12 mg of protein per ml by addition of buffer and maintained for 20 min at 48°C with constant stirring in a water bath prior to rapid cooling on ice. Although this heat step destroys 20–40% of the binding activity, it is critical for complete removal of degrading activities. The heated fraction is inactivated by freezing and thawing but retains its full binding activity for at least 2 weeks if stored at 0–4°C. This partially purified fraction was used in all experiments described in this paper. Various preparations of it bound 1–2 pmol of angiotensin II per mg of protein.

Preparation of Native ^{125}I-Angiotensin II–Protein Complex and Its Crosslinked Derivative. In a typical preparation, the partially purified fraction (3 mg) in 20 mM Tris·HCl, pH 7.4, containing 0.15 M NaCl, 4.5 mM Na_2EDTA, 0.1 mM *p*-chloromercuriphenylsulfonic acid (PCMS), 0.25% Brij 99, and 4 nM ^{125}I-angiotensin II (3.4×10^6 cpm) was incubated for 60 min at 22°C in a final volume of 300 μl. At the end of the incubation, the reaction mixture was subjected to gel filtration through a column (0.7×27 cm) of Sephadex G-50 equilibrated and developed with TEB buffer. When a larger incubation volume with more protein was used (Table 1, part B) the incubation mixture was concentrated 4-fold by using an Amicon PM 10 filter and was then subjected to gel filtration through a column (0.9×40 cm) of Sephadex G-50. To the void volume containing the native angiotensin II–protein complex (2 ml, 7.4×10^5 cpm) was added 20 μl of a freshly prepared solution containing 50 mM disuccinimidyl suberate in dimethyl sulfoxide. Crosslinking was allowed to proceed for 15 min at 4°C, at which time 2 ml of 9 M urea was added to dissociate the fraction of angiotensin II that had not been covalently bound. After 20 min at 22°C, the solution was concentrated 4-fold by ultrafiltration and then again subjected to gel filtration through Sephadex G-50 (0.9×40 cm). The void volume containing the crosslinked angiotensin II–protein complex was concentrated to approximately 0.8 ml and contained 9.5×10^4 cpm (i.e., 13% of that in the original native complex) which appeared to be covalently bound, since it did not dissociate after treatments (incubation with 4.5 M urea or at acid or alkaline pH values) previously shown to release radioiodinated angiotensin II from its complex with the binding protein (11). It may be added parenthetically that the presence of disuccinimidyl suberate did not affect binding by the heated fraction.

Affinity Chromatography on Angiotensin II–Sepharose 4B. In a typical experiment, 8 ml (265 mg of protein) of the heat-treated preparation in TEB buffer containing 0.1 mM PCMS and 0.15 M NaCl ("loading buffer") was loaded into a column (10-ml packed volume, corresponding to 3.0 g of dry gel) of angiotensin II–Sepharose that had been equilibrated in the same buffer. After the protein solution had flowed into it, the column was closed and kept for 90 min at 22°C. It was then transferred to a cold room (0–4°C) and washed first with 10 ml of chilled loading buffer and then with about 100 ml of this buffer from which PCMS was omitted until the effluent was free of material absorbing UV light at 280 nm. Elution was carried out at 22°C with 10 ml of loading buffer containing 5 mM angiotensin II and a tracer amount of the purified radioiodinated derivative. The column was closed for 30 min after this solution had flowed into it and elution was then continued with the loading buffer (approximately 20 ml) until there was no radioactivity in the effluent. The eluate containing radioactivity was concentrated by ultrafiltration through an Amicon PM 10 filter and then diluted with TEB buffer and reconcentrated several times to partially remove unbound angiotensin II. To remove the unbound angiotensin II completely, the concentrated fraction (0.5 ml) was subjected to gel filtration through a column (0.9×40 cm) of Sephadex G-50 in TEB buffer and the void volume containing bound angiotensin II was collected, concentrated, and subjected again to the same procedure. The final void volume (15–20 μg of protein) was once again concentrated by ultrafiltration.

In certain experiments elution was carried out similarly, save that 4 M $MgCl_2$ or 0.1 mM dithiothreitol was used as eluant in the place of angiotensin II. The concentrated $MgCl_2$ eluate (1 ml containing approximately 50 μg from 265 mg of the heated fraction) was dialyzed overnight against TEB buffer and further purified by gel filtration through a column (1×90 cm) of Sephadex G-200 that was equilibrated and developed with TEB buffer and monitored by absorbance at 280 nm. The fractions composing the major protein peak were combined and concentrated (1 ml, approximately 25 μg).

Gel Electrophoresis and Autoradiography. Electrophoresis of reduced, denatured proteins was carried out by using 10% acrylamide gels and the discontinuous buffer system described by Laemmli (19). Stacking gels were 5% acrylamide. Samples were heated at 100°C for 3 min in 12.5 mM Tris·HCl, pH 6.8, containing 20% (vol/vol) glycerol, 1% $NaDodSO_4$ (wt/vol), and 5% (vol/vol) 2-mercaptoethanol. For dissolving crosslinked angiotensin II–protein complex bound to protein A-Sepharose and precipitates obtained by treatment with 5% trichloroacetic acid, the concentration of $NaDodSO_4$ in the buffer was increased to 5% (wt/vol). Slabs were stained for protein by using 0.05% Coomassie blue and fluorographed with EN^{3}HANCE according to the manufacturer's instructions. They were then dried and exposed to Kodak X-Omat AR film.

Determinations of Protein. Protein concentrations were measured by the method of Bradford (20) with bovine serum albumin as the standard.

RESULTS

Reversible Sulfhydryl-Dependent Dissociation of Angiotensin II from Its Complex with the Partially Purified Binding Protein. Radioiodinated angiotensin II bound by the heated fraction was released by treatment with sulfhydryl reagents (Fig. 1). Release was dose-dependent between 0.05 and 0.1 mM dithiothreitol or 0.1 and 0.5 mM 2-mercaptoethanol. Full binding activity was restored to the protein preparation that had been treated with sulfhydryl reagents after the sulfhydryl compound was separated by gel filtration, dialysis, or both. Occasionally it was necessary to add an oxidizing agent, dithionitrobenzoic acid, to the incubation mixture to get back full binding activity, probably because the sulfhydryl reagent had been incompletely removed. This effect of sulfhydryl compounds may be related to their previously reported (21) inhibitory action on the binding of angiotensin II by adrenal membranes. Also of possible interest in this context is the puzzling complete dependence of binding by the heated fraction on the presence of 0.1 mM PCMS. This re-

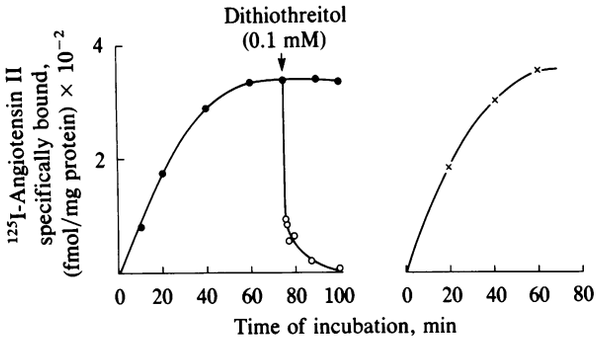

FIG. 1. Reversible sulfhydryl-dependent dissociation of angiotensin II from partially purified binding protein. The heated fraction (10 mg) was incubated at 22°C with 20 nM angiotensin II (containing 4.36×10^6 cpm of ^{125}I-angiotensin II), 20 mM Tris·HCl at pH 7.4, 4.5 mM Na_2EDTA, 0.15 M NaCl, 0.1 mM PCMS, and 0.25% Brij 99 in a final volume of 1.5 ml. At the indicated times 75-μl aliquots were withdrawn and assayed by the charcoal/dextran method (●). At 75 min, dithiothreitol was added (final concentration 0.1 mM) to a part (900 μl) of the reaction mixture and bound angiotensin II (○) was assayed similarly. At 110 min, 400 μl of the mixture containing dithiothreitol was passed through a Sephadex G-50 column (0.7 × 27 cm) equilibrated and developed with TEB buffer. The material eluting in the void volume was collected and dialyzed against 2 liters of the same buffer to remove completely any remaining unbound ^{125}I-angiotensin II and dithiothreitol. The dialyzed preparation was concentrated to approximately 200 μl (2.5 mg) and an aliquot (100 μl) was incubated with ^{125}I-angiotensin II as indicated above (20 nM angiotensin II, containing 8.72×10^5 cpm in a final volume of 300 μl). A 75-μl aliquot of the incubation mixture was withdrawn and assayed as indicated above at indicated times (×). Specific binding was calculated as the difference between total binding and nonspecific binding determined in the presence of 10 μM unlabeled angiotensin II. The fraction of nonspecific binding was always less than 15%.

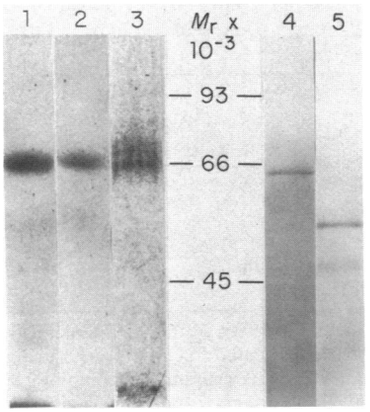

FIG. 2. Gel electrophoresis of crosslinked ^{125}I-angiotensin II–protein complex and of binding protein purified by using anti-angiotensin II antibodies. The first three lanes represent autoradiograms developed after exposure to film at −80°C for 3 days of aliquots containing 7000 cpm of crosslinked ^{125}I-angiotensin II–protein complex. Lane 1 contained the untreated complex, lane 2 the radioactivity precipitated by protein A-Sepharose after treatment with anti-angiotensin II antibodies, and lane 3 radioactivity removed from protein A-Sepharose by treatment with 3 M KSCN as described for Table 1. Lanes 4 and 5 were stained with Coomassie blue. The eluates they contained represent the total obtained from immune-specific protein A-Sepharose precipitate that had been obtained by using 53 mg of the heated fraction. The precipitate was first eluted with 0.1 mM dithiothreitol (lane 4) and then with 3 M KSCN (lane 5). The eluates were dialyzed against TEB buffer and their proteins were precipitated in the presence of 5% trichloroacetic acid and washed twice with diethyl ether. These eluates correspond to aliquots (1/5) of those of the native complex described for Table 1. All five samples were boiled in $NaDodSO_4$ buffer containing 5% 2-mercaptoethanol and subjected to slab gel electrophoresis. The positions of molecular weight markers (phosphorylase b, 93,000; bovine serum albumin, 66,000; ovalbumin, 45,000) are indicated between lanes 3 and 4.

quirement could be partially met by other organomercurials but not by mercuric chloride or a large number of protease inhibitors. There was no destruction of radioiodinated angiotensin II in either the presence or the absence of this compound. Furthermore, PCMS did not exhibit a protective effect on the binding activity. Indeed, a 2-hr preincubation at 22°C in its presence was associated with a 50% reduction in the binding of angiotensin II whereas the preparation was stable in its absence. The stimulatory effect of PCMS in the binding reaction is thus probably not mediated by its inhibition of a protease.

Purification of Binding Protein by Using Angiotensin II as an Immunoaffinity Ligand. One strategy that we used for purifying the putative receptor depends on the ability of anti-angiotensin II antibodies to recognize determinants of angiotensin II that do not interact with the binding protein. Purification of insulin receptors by using a similar strategy was reported by Heinrich *et al.* (22). The feasibility of this approach was first investigated by using radioiodinated angiotensin II that had been covalently crosslinked to the binding protein by treatment with disuccinimidyl suberate. Only a single protein in the relatively crude heated fraction was labeled by this procedure. This protein exhibited (Fig. 2, lane 1) an electrophoretic mobility in the reduced denatured state corresponding to a molecular weight of 66,000 ± 3000. Approximately 15% of this radioactive polypeptide formed an immune-specific complex with anti-angiotensin II antibodies that could be precipitated by reaction with protein A-Sepharose. The labeled protein was quantitatively released from the insoluble matrix in the presence of 3 M KSCN (Table 1; Fig. 2, lanes 2 and 3).

Similar operations were then performed with the native angiotensin II–protein complex that had been separated from the free octapeptide by passage through a column of Sephadex G-50. This complex was eluted from Sepharose 6B in the same position as the crosslinked product (Fig. 3), indicating that crosslinking did not produce a gross alteration of molecular weight. However, unlike radioactive material in the crosslinked derivative, that in the native complex was dissociated by treatment with 4.5 M urea and by incubation at pH 5.0 or 9.0. Sixty percent of the radioactivity in this complex was immunospecifically precipitated by anti-angiotensin II antibodies and protein A-Sepharose (Table I). Since we had found that sulfhydryl reagents dissociate the complex, we used dithiothreitol in the hope of specifically eluting the binding protein while leaving angiotensin II and the anti-angiotensin antibodies attached to the protein A-Sepharose. As expected, no radioactive protein was released from the crosslinked immunospecific protein A-Sepharose precipitate on treatment with dithiothreitol. However, as shown in Fig. 2, lane 4, treatment of the native immunospecific, Protein A-Sepharose precipitate with DTT released a single Coomassie blue-reactive component with an apparent molecular weight indistinguishable from that of the radioactive crosslinked product. Furthermore, this protein had been successfully separated from most of the radioiodinated angiotensin II and antibodies, each of which were eluted from the residual pellet (Table 2; Fig. 2, lane 5) by treatment with KSCN. However, unlike the relatively crude heated fraction from which it was obtained, the highly purified protein eluted with dithiothreitol failed to bind radioiodinated angiotensin II after removal of the sulfhydryl reagent.

Purification of Binding Protein by Affinity Chromatography with Angiotensin II as Ligand. This second strategy for purification depended critically on the complete absence of

Table 1. Immunoprecipitation by anti-angiotensin II antibodies of the ^{125}I-angiotensin II–protein complex and its crosslinked derivative

Complex	Serum	Protein A-Sepharose precipitate, %	Dithiothreitol eluate, %	KSCN eluate, %
Crosslinked	Nonimmune	<1	<1	<1
	Immune	15	<1	14
Native	Nonimmune	<1	<1	<1
	Immune	60	4	56

The crosslinked derivative of the angiotensin II–protein complex was prepared with the heated fraction as described in *Materials and Methods*. Aliquots of this crosslinked complex (1.5 mg of protein containing 4.6×10^4 cpm, corresponding to 15.9 fmol of angiotensin II in 0.4 ml of TEB buffer supplemented with 0.1 M NaCl) were incubated 18 hr on a roller at 4°C with 10 μl of either nonimmune or anti-angiotensin II antiserum. To precipitate immune complexes, 50 μl was added of a slurry containing protein A-Sepharose in TEB/NaCl buffer with a binding capacity of 10 mg of IgG per ml. Incubation was continued for 3 hr, after which the suspension was centrifuged for 5 min at 10,000 × *g*. The precipitate was washed repeatedly (three times) with 1-ml portions of TEB/NaCl until the supernatant fraction was free of radioactivity. The pellet was then extracted three times for 30 min with 1 ml of 0.1 mM dithiothreitol in the same buffer. This procedure was repeated with 3 M KSCN and the dithiothreitol and KSCN eluates were pooled, concentrated by ultrafiltration, and finally dialyzed against TEB.

The native angiotensin II–protein complex was prepared with the heated fraction (260 mg) under similar conditions as described in *Materials and Methods* except that the binding reaction was carried out with 40 nM angiotensin II obtained by diluting 0.39 nM ^{125}I-angiotensin II with the unlabeled octapeptide in a final volume of 11.25 ml. Aliquots of this native complex (130 mg containing 2×10^6 cpm, corresponding to 71 pmol of angiotensin II and diluted to 12 ml by TEB/NaCl) were incubated with 300 μl of nonimmune serum or anti-angiotensin II antiserum and then treated with 1.5 ml of the protein A-Sepharose slurry. Precipitates were processed and eluates were prepared and concentrated as described for the crosslinked complex except that washing and extraction volumes were each 100 ml and 3 ml, respectively.

Values in the table are expressed relative to radioactivity present in the starting material—i.e., 4.6×10^4 and 2.0×10^6 cpm, respectively, of crosslinked and native complexes.

peptidases that might degrade the angiotensin II used as affinity ligand, a requirement that was fulfilled by the heated fraction. The specific angiotensin-binding activity of the heated fraction failed to emerge in the flow-through after chromatography on Sepharose containing covalently attached angiotensin II. Three different eluants were found to remove from this column a protein that exhibited approximately the same molecular weight (65,000 ± 3000) as the chemically crosslinked angiotensin II–protein complex when examined by gel electrophoresis in the reduced, denatured state (Fig. 4). No such specific protein was eluted when the same experiment was carried out with a control column of cyanogen bromide-activated Sepharose 4B to which angiotensin II had not been attached. It was the only detectable protein when angiotensin II containing a tracer amount of the radioiodinated derivative was used as eluant (Fig. 4, lane 1). The bound radioiodinated angiotensin II in the eluate was not dissociated during repeated ultrafiltration through Amicon PM 10 membranes or gel filtration on Sephadex G-50 or by extensive dialysis at 4° or 22°C. Nor was it displaced when incubated overnight with 5 mM unlabeled angiotensin II at 22°C. However, it was released intact and identified by TLC after treatment with 0.5 M acetic acid or with 5% trichloroacetic acid.

An interesting property of the protein released by competitive displacement with angiotensin II is that it migrates considerably more rapidly in the unreduced state during electrophoresis through polyacrylamide gels containing $NaDodSO_4$ than it does after reduction (Fig. 4, lanes 5 and 6). This unusual characteristic is also exhibited by the protein containing covalently crosslinked ^{125}I-angiotensin II (not shown).

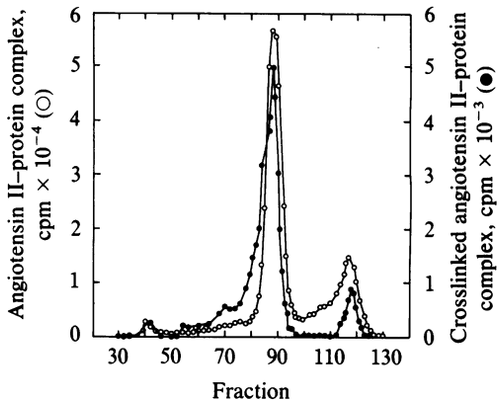

FIG. 3. Gel filtration of native and crosslinked angiotensin II–protein complex. The heated fraction (9.2 mg) was incubated with 4 nM ^{125}I-angiotensin II at 22°C for 60 min and the native angiotensin II–protein complex (1.3 ml, 2.6×10^6 cpm) was isolated. An aliquot (0.3 ml) was subjected to gel filtration at 4°C on a column (1.5 × 60 cpm) of Sepharose 6B that was equilibrated and developed with TEB buffer. Fractions (1 ml) were collected at a flow rate of 8 ml/hr and their radioactivities were determined (○). The remainder of the isolated complex (1.0 ml, 2.0×10^6 cpm) was treated with disuccinimidyl suberate and the crosslinked angiotensin II–protein complex (1.25 ml, 2.5×10^5 cpm) was isolated. An aliquot (0.3 ml, 6×10^4 cpm) was then subjected to gel filtration under conditions identical to those described above (●).

A protein of similar molecular weight was also eluted from the column with 4 M $MgCl_2$ or 0.1 mM dithiothreitol. In addition to the major protein component, gel electrophoresis of

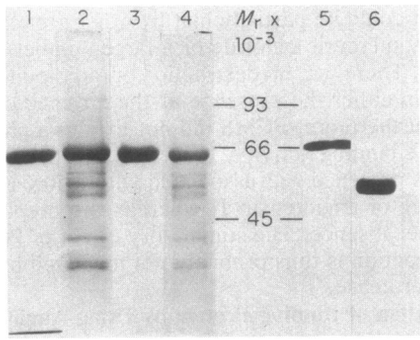

FIG. 4. Gel electrophoresis of proteins obtained by affinity chromatography on angiotensin II-Sepharose. Lanes 1–4 were stained with Coomassie blue and contained proteins that had been concentrated by precipitation in 5% trichloroacetic acid and washed with ether prior to analysis. Lanes 1, 3, and 4 represent the total eluates obtained from 50 mg, and lane 2 from 25 mg, of the heated fraction which was subjected to affinity chromatography. Lane 1 contained protein eluted by 5 mM angiotensin II. The eluate in lane 2 was obtained with 4 M $MgCl_2$. This material was further purified by gel filtration on Sephadex G-200 to yield the fraction analyzed in lane 3. Lane 4 contained the eluate obtained with 0.1 mM dithiothreitol. Lanes 5 and 6 show autoradiograms of a sample corresponding to the eluate obtained with angiotensin II (lane 1), which was radioiodinated exactly as described by Hunter (23) except that TEB buffer was used throughout the procedure. Lane 5 contained an aliquot (50,000 cpm) that was reduced and denatured prior to analysis, as were the proteins in lanes 1–4. The aliquot in lane 6, containing equivalent radioactivity, was boiled in denaturing buffer from which 2-mercaptoethanol had been omitted. Omission of 2-mercaptoethanol had no effect on the mobility of the marker proteins (not shown). The autoradiograms were developed after exposure at −80°C for 18 hr.

these eluates also revealed the presence of several smaller polypeptides (Fig. 4, lanes 2 and 4). These may represent degradation products of the larger biospecifically elutable protein, which could be resolved free of them by gel filtration on Sephadex G-200 (Fig. 4, lane 3), since they share with it the unusual property of sulfhydryl-dependent release from the matrix. Although these methods presumably eluted the binding protein without attached ligand, the concentrated eluates lacked binding activity, and such activity was not reconstituted when they were mixed with the inactive flow-through fraction.

DISCUSSION

Perhaps the most impressive of a large body of evidence indicating that the actions of angiotensin II are mediated by a specific surface receptor (reviewed in ref. 9) is the correlation between binding of the octapeptide to whole cells or membranes and a biological response such as increased elaboration of aldosterone by adrenal cortical cells (24) or increased activity of certain enzymes in hepatocytes (17). Our previous results (11) have indicated that specific binding of angiotensin II by extracts of rabbit liver, which can be quantitatively solubilized with digitonin, is accounted for by a single membrane-bound protein. The molecular weight of the responsible macromolecule was estimated as approximately 65,000–70,000 after radioiodinated angiotensin II had been crosslinked to it in a crude solubilized extract with disuccinimidyl suberate (11). Capponi and Catt (25) had estimated a similar size for the specific angiotensin II-binding molecule that they labeled in dog adrenal and uterine membranes by using a radioiodinated photoaffinity analog (26). In this communication we report two independent techniques, specifically based on recognition of angiotensin II, by which we have isolated a protein of this molecular weight in almost homogeneous form. We cannot estimate recovery of this polypeptide because its angiotensin-binding activity is greatly influenced by removal of peptidases in early stages of the purification procedure and is lost during the final step. Nonetheless, it is likely to correspond to a very minor fraction of total hepatic protein, since the maximal yield we have obtained from 150 g of liver containing 18 g of protein is only about 40 μg. Thus, even if the recovery is as low as 10%, the purification factor required to achieve homogeneity would still be almost 50,000. Although the evidence described above strongly suggests that this macromolecule is the one to which angiotensin II is specifically bound, the isolated protein itself fails to bind the octapeptide. The possibility has thus not been excluded that the presence of another molecule may also be required for binding to occur. In this context it is perhaps worth noting that Paglin and Jamieson (27) estimated a molecular weight of 116,000 for the labeled macromolecular component obtained when radioiodinated angiotensin II was allowed to react with intact rat adrenal membranes in the presence of disuccinimidyl suberate.

If antibodies can be generated against the protein we have purified, they should be useful in establishing whether it is truly the angiotensin receptor and in determining where it is localized and whether it is immunologically identical in different anatomic loci. The unexpected observation that treatment of this protein with sulfhydryl-containing reagents disrupts its noncovalent linkage with angiotensin II and paradoxically decreases its mobility during $NaDodSO_4$ gel electrophoresis could be of considerable interest. Thus, it seems conceivable that a specific configuration of this protein may be required for its biological activity and that identification of the requisite conformation might facilitate development of an antihypertensive receptor antagonist.

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