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Molecular Characterization of the Solubilized Atrial Natriuretic Factor Receptor from Bovine Adrenal Zona Glomerulosa

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SUMMARY

The atrial natriuretic factor (ANF) receptor has been solubilized from bovine adrenal zona glomerulosa membranes with the nonionic detergent octyl-β-D-glucoside. Mathematical analysis of competition binding curves with solubilized receptor revealed the presence of two classes of binding sites with pK of 10.4 (K_d = 40 pM) and 8.2 (k_d = 6000 pM), similar to the native receptor of intact membranes. The hydrodynamic properties of the ANF receptor were determined by prelabeling the membrane receptor with ^{125}I-ANF prior to solubilization. The solubilized ^{125}I-ANF-receptor complex eluted as a major peak with a Stokes radius of 50.8 Å from a Superose 6 steric exclusion column. A partial specific volume of 0.770 ml/g and a sedimentation coefficient ($s_{20,w}$) of 6.34 S were determined by sucrose density gradient centrifugation in H_2O and D_2O. These data were used to calculate a molecular weight of 158,000 and a frictional ratio of 1.25 for the labeled receptor-detergent complex. The amount of detergent bound to the receptor was estimated to be 0.45 g/g of protein, assuming a partial specific volume of 0.730 ml/g for the protein. Correction for the mass contributed by the bound detergent yielded a molecular weight of 109,000 for the receptor protein. Affinity cross-linking of ^{125}I-ANF to its binding sites in zona glomerulosa membranes and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography revealed that one single band with apparent M_r 130,000 was specifically labeled. These results indicate that the ANF receptor from bovine adrenal cortex is a membrane protein with a total molecular weight of 110,000–130,000 and suggest that the native protein contains only one single polypeptide chain.

ANF is a peptide hormone stored in specific granules of mammalian atrial cardiocytes (1, 2). Recent studies have shown that ANF is stored in the form of the 126-amino acid proANF and that proANF is secreted from rat cardiocytes in culture (3). The major circulating form of the hormone has been previously identified as a 28-amino acid peptide (4, 5) but the exact maturation pathway of the precursor molecule is not completely defined. ANF is released in response to atrial distension (6, 7) and modulates electrolyte excretion and renal hemodynamics, smooth muscle tone, renin-angiotensin system, and blood pressure homeostasis (for review, see Refs. 8–10). These effects are mediated through binding of ANF to specific receptors which have been identified in several target tissues such as kidney (11, 12), adrenal cortex (13), vascular smooth muscle cells (14, 15), and brain (16).

We have previously reported the pharmacological characteristics of the ANF receptor in bovine adrenal cortex from extensive structure-activity relationship studies (17). Very little is known, however, concerning the structure and the molecular properties of the receptor. Recently, several groups (18–25) have identified specific ANF-binding sites in plasma membranes of different tissues and studied the constituent polypeptide chains of the protein using chemical cross-linking techniques and gel electrophoresis in SDS. All of these investigators have identified a polypeptide of M_r 110,000–180,000, and some reports also described a smaller molecular species of M_r 60,000–70,000 (18, 20, 22, 23). Solubilization and characterization of the hydrodynamic properties of the ANF receptor system would represent an important step toward the comprehension of the chemical structure and the molecular mechanisms of the receptor. The ANF receptor previously has been solubilized from bovine adrenal cortex (20), human placenta (24), and rat glomeruli (26) but was not further characterized. We report here the solubilization and characterization of the ANF receptor from bovine adrenal cortex. The receptor was solubilized from zona glomerulosa membranes with the nonionic detergent octyl-β-D-glucoside. The hydrodynamic properties of the solubilized ^{125}I-ANF-receptor complex were estimated from steric exclusion HPLC and sucrose density gradient centrifugation

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ABBREVIATIONS: ANF, atrial natriuretic factor; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; ACTH, adrenocorticotropic hormone.

experiments. The results from these experiments were compared to those obtained by SDS-polyacrylamide gel electrophoresis to obtain information on the subunit structure of the receptor.

Experimental Procedures

Materials. Rat [Ser[99]-Tyr[126]]ANF was purchased from Institut Armand Frappier, Laval, Canada. Carrier-free Na^{125}I was from Amersham Corp. Bis(sulfosuccinimidyl) suberate and iodo-beads were from Pierce Chemical Co. Octyl-β-D-glucoside was from Calbiochem. Angiotensin II and [Arg[8]]-vasopressin were from Peninsula Laboratories. ACTH, bovine serum albumin (for use in binding assays), aprotinin, leupeptin, pepstatin A, bovine γ-globulins, and deuterium oxide (99.8% pure) were obtained from Sigma Chemical Co. Polyethylene glycol 8000 was from J. T. Baker Chemical Co. Thyroglobulin, catalase, aldolase, bovine serum albumin, chymotrypsinogen A, and ribonuclease A were obtained from Pharmacia Fine Chemicals. Electrophoresis reagents were from Bio-Rad Laboratories.

Iodination of ANF. ANF was radioiodinated by a solid-phase method using iodo-beads as the oxidizing agent (27). The monoiodinated peptide was separated from free iodine on a PRP-1 cartridge (Hamilton Co.) and purified by HPLC on a reverse phase Bio-Sil ODS-5S column (Bio-Rad). Elution was achieved at a flow rate of 1 ml/min with a 60-min linear gradient from 15 to 55% (v/v) acetonitrile in aqueous 0.1% trifluoroacetic acid. This method yielded ^{125}I-ANF specific activities of 2000 Ci/mmol as determined by self-displacement using a specific radioimmunoassay (28). This value is compatible with the theoretical value of pure monoiodinated ANF.

Preparation of membranes. Bovine adrenal glands were obtained shortly after slaughter and placed in cold phosphate-buffered saline. All subsequent operations were carried out at 4°. A 0.5-mm layer of cortex, corresponding to the zona glomerulosa, was dissected and placed in 20 volumes of ice-cold homogenization buffer (20 mM $NaHCO_3$, 10 mM EDTA, 10^{-5} M aprotinin, 10^{-5} M leupeptin, and 10^{-6} M pepstatin A). The tissue was briefly homogenized (three times for 10 sec, setting 6) with a Polytron homogenizer followed by homogenization with a Potter homogenizer. The homogenate was filtered through cheesecloth and centrifuged at 1,000 × *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 30,000 × *g* for 15 min. The resulting pellet was washed twice with 20 mM $NaHCO_3$, 1 mM EDTA, 10^{-6} M aprotinin, 10^{-6} M leupeptin, 10^{-7} M pepstatin A and resuspended by gentle homogenization in 2 ml of buffer (50 mM Tris-HCl, 250 mM sucrose, 0.1 mM EDTA, 1 mM $MgCl_2$, pH 7.4) per g of tissue. Membranes were frozen in liquid nitrogen and stored at −70° until use.

Solubilization procedure. All operations were carried out at 4°. Zona glomerulosa membranes were suspended in buffer A (50 mM KH_2PO_4, 100 mM NaCl, 20% glycerol, 0.1 mM EDTA, 10^{-6} M aprotinin, 10^{-6} M leupeptin, 10^{-7} M pepstatin A, pH 7.4) containing 40 mM octyl-β-D-glucoside to a protein concentration of 2 mg/ml. The suspension was agitated for 60 min at 4°, and insoluble material was removed by centrifugation at 30,000 × *g* for 30 min followed by passage through 0.22-μm Millipore filters. The clear yellowish filtrate constituted the solubilized fraction and was used for subsequent assays of ANF receptor-binding activity.

ANF binding assay. ^{125}I-ANF binding to solubilized fractions was assayed by a polyethylene glycol precipitation technique (29). Aliquots of solubilized fractions (70 μg of membrane protein) were incubated at 10° for 4 hr with 15 pM ^{125}I-ANF and varying concentrations of unlabeled ANF in a total volume of 1 ml of buffer B (50 mM KH_2PO_4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 10^{-6} M aprotinin, 10^{-6} M leupeptin, 10^{-7} M pepstatin A, 0.5% bovine serum albumin, pH 7.4). Bound ^{125}I-ANF was separated from free ligand by precipitation with 1 ml of 22% polyethylene glycol plus 0.2 ml of bovine γ-globulins (11 mg/ml) and filtration through Whatman GF/C filters pretreated with 1% polyethyleneimine. The filters were counted for radioactivity in an LKB 1272 CliniGamma counter with 81% efficiency. Competition curves were first analyzed according to a four-parameter logistic equation (30). Then, the curves were analyzed by nonlinear least squares curve fitting according to models for one or several classes of sites (31). Equilibrium constants K (i.e., $1/K_d$) for the binding of the ligand to the receptor sites are expressed as $-\log_{10}K_d$ or pK. The estimates for the density of receptor sites in pM are reported in fmol/mg of protein for convenience. A model involving two classes of binding sites was retained only when it statistically improved the goodness of fit.

Preparation of soluble hormone-receptor complex. Adrenal zona glomerulosa membranes (2 mg) were incubated with ^{125}I-ANF (50 pM) for 4 hr at 10° in 25 ml of buffer B. Nonspecific binding was determined in the presence of 10^{-7} M unlabeled ANF. The free ^{125}I-ANF was removed by centrifugation at 30,000 × *g* for 15 min and the membrane pellet was solubilized with 40 mM octyl-β-D-glucoside as described above. The solubilized material was applied to columns or to sucrose density gradients.

Steric exclusion HPLC. The Stokes radius of the solubilized ^{125}I-ANF-receptor complex was estimated by steric exclusion HPLC. Aliquots (250 μl) of the solubilized preparation were injected onto a Superose 6 (Pharmacia) column (10 × 300 mm) equilibrated with buffer C (50 mM KH_2PO_4, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, and 40 mM octyl-β-D-glucoside) at 4°. The column was eluted with buffer C at a flow rate of 0.5 ml/min and fractions of 0.5 ml were collected. The labeled hormone-receptor complex was determined by precipitation with polyethylene glycol and filtration through 1% polyethyleneimine-treated GF/C filters. The void volume (V_o) and the total volume (V_t) were determined with blue dextran and acetone, respectively, and the column was standardized with reference proteins of known Stokes radii. The elution volume of the reference proteins was determined from continuous monitoring of absorbance at 280 nm. The distribution coefficient, K_{av}, was calculated from the relation $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume (32).

Sucrose density gradient centrifugation. Linear gradients (4.9 ml) of 5–20% (w/v) sucrose in buffer C were prepared using a Beckman gradient former and stored at 4° for at least 4 hr before use. The gradients were overlayered with either 250 μl of sample or 250 μl of reference proteins. Centrifugation was carried out at 33,000 rpm at 4° in an SW 55 Ti rotor of a Beckman model L8-55M preparative ultracentrifuge for 15 hr. After centrifugation, fractions of 11 drops were collected from the bottom of each gradient. The ^{125}I-ANF-receptor complex was determined by precipitation with polyethylene glycol and filtration. Reference proteins were located by assay of the protein content in each fraction and used to calibrate the gradients. The fractional migration distance, y, of each protein was determined (33, 34).

Analysis of hydrodynamic data. The partial specific volume ($\bar{\nu}$) of the labeled receptor-detergent complex was estimated from data obtained by sedimentation experiments through sucrose gradients prepared in H_2O and D_2O and analyzed by the following equation (34):

$$\bar{\nu}_A = \frac{(1 - \bar{\nu}_B\rho_D{}^B) - a\,(1 - \bar{\nu}_B\rho_H{}^B)}{\rho_H{}^A\,(1 - \bar{\nu}_B\rho_D{}^B) - a\,\rho_D{}^A\,(1 - \bar{\nu}_B\rho_H{}^B)}$$

where $a = y_D{}^B y_H{}^A / y_H{}^B y_D{}^A$. Here A and B refer to the receptor-detergent complex and the reference protein, respectively, and the subscripts H and D refer to values measured in H_2O and D_2O. The reference protein aldolase was chosen for the computation of $\bar{\nu}$. The ρ values are specific for each y value and represent the average densities at 0.5 y. The sedimentation coefficient of the complex ($s_{20,w}$) was calculated from the equation (34):

$$s_{20,w}{}^A = \frac{y_A\,(1 - \bar{\nu}_A\rho_{20,w})(1 - \bar{\nu}_B\rho_{T,m}{}^B)}{y_B\,(1 - \bar{\nu}_B\rho_{20,w})(1 - \bar{\nu}_A\rho_{T,m}{}^A)} \cdot s_{20,w}{}^B$$

where $\rho_{20,w}$ is the density of water at 20°, and $\rho_{T,m}$ is the density at the average position of the molecule in the gradient. Aldolase was used as the reference protein.

As suggested by Siegel and Monty (35), the molecular weight of the

protein-detergent complex was calculated from the empirically determined Stokes radius (a) and sedimentation coefficient employing the following equation:

$$M = \frac{6\pi N \eta_{20,w}}{1 - \bar{\nu}\rho_{20,w}} a s_{20,w}$$

where N is Avogadro's number, $\eta_{20,w}$ is the viscosity of water at 20°, and $\rho_{20,w}$ is the density of water at 20°. The frictional ratio (f/f_o) of the complex was calculated from the equation (35):

$$f/fo = a\left(\frac{4\pi N}{3M\,(\bar{\nu} + (\delta/\rho))}\right)^{1/3}$$

where δ is the degree of hydration. An estimated hydration factor of 0.3 g/g was used for the protein component (36), whereas the hydration of the detergent was not taken into consideration. The amount of octyl-β-D-glucoside bound and the molecular weight of the protein component were calculated from the assumption that the partial specific volumes of the protein (0.730 ml/g) and detergent (0.859 ml/g)[3] components are additive.

Affinity labeling protocol. Affinity cross-linking of the ANF receptor was performed as described previously (18) with some modifications. Adrenal membranes (600 μg) were incubated for 12 hr at 10° with 10 pM ^{125}I-ANF in 30 ml of 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 5 mM $MnCl_2$, 0.5% bovine serum albumin, in the presence or absence of nonradioactive hormones. After incubation, membranes were washed, and the resulting pellet was resuspended in 50 mM KH_2PO_4 (pH 7.4) and incubated with 1 mM bis (sulfosuccinimidyl) suberate for 30 min at 4°. The reaction was stopped by addition of excess 1 M ammonium acetate and centrifugation at 30,000 × g for 15 min. The pellet was washed once and resuspended in sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and a trace of bromophenol blue) containing 5% 2-mercaptoethanol and heated at 100° for 5 min. Initially, membranes were incubated and washed in acidic buffer prior to electrophoresis, in order to remove noncovalently bound ^{125}I-ANF (18). However, we found that this acidic wash followed by heating at 100° resulted in a decrease in the labeling of an M_r 130,000 band and the appearance of other labeled bands, with a major one between 60,000 and 70,000 Da. For these reasons, this step was subsequently omitted.

SDS-polyacrylamide gel electrophoresis. Equivalent amounts of 200 μg of membrane proteins, processed as described above, were loaded on each lane of 1.5-mm-thick slab gel. Electrophoresis was carried out using the discontinuous buffer system of Laemmli (37) on a 7.5% acrylamide separating gel. Following electrophoresis, the gels were stained in Coomassie blue R-250, destained, and dried prior to autoradiography on Kodak X-Omat RP film with Dupont Lightning Plus intensifying screen.

Protein determination. Protein concentrations were determined by the method of Smith (38) using bovine serum albumin as standard. Octyl-β-D-glucoside does not interfere with this procedure.

Results

Solubilization of the ANF receptor. Incubation of bovine adrenal zona glomerulosa membranes with increasing concentrations of the nonionic detergent octyl-β-D-glucoside resulted in the solubilization of increasing amounts of membrane proteins and of ^{125}I-ANF binding activity (Fig. 1). At an optimal detergent concentration of 40 mM, 90% of the binding activity and 71% of the membrane proteins were released in a soluble form. Treatments with higher concentrations were less effective. Therefore, solubilization with 40 mM octyl-β-D-glucoside was selected for all subsequent studies. When solubilized fractions obtained by centrifugation at 30,000 × g and filtration through 0.22-μm Millipore filters were subjected to further centrifugation at 100,000 × g for 60 min, ^{125}I-ANF binding remained unchanged. The ^{125}I-ANF binding activity present in the solubilized fraction was stable to storage at 4° for at least 1 month. Competition binding curves of ANF for the binding of ^{125}I-ANF to the solubilized fraction were characterized by an ED_{50} of 120 pM and a slope factor of 0.83. Computer analysis of these curves according to a model for independent classes of sites reveals that the apparent heterogeneity suggested by the slope factor might be explained by the presence of two classes of binding sites with pK of 10.4 (K_d = 40 pM) and 8.2 (K_d = 6000 pM), respectively, and density of 368 and 3,177 fmol/mg of protein (Fig. 2). Nonspecific binding accounted for less than 5% of total ^{125}I-ANF binding. These characteristics are similar to those of the native receptor in intact membranes (13).

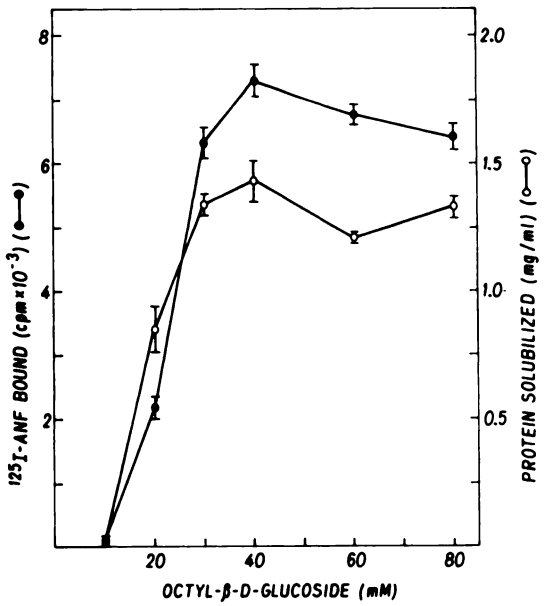

Fig. 1. Effect of octyl-β-D-glucoside concentration on the solubilization of ^{125}I-ANF binding activity and membrane proteins. Adrenocortical membranes (2 mg of protein/ml) were incubated for 60 min at 4° with the indicated concentrations of octyl-β-D-glucoside, and the resulting suspension was centrifuged at 30,000 × g for 30 min and filtered through a 0.22-μm filter. An aliquot of the filtrate (70 μg of membrane protein/ml) was incubated for 4 hr at 10° with 15 pM ^{125}I-ANF. Bound ligand was assessed by precipitation with polyethylene glycol. Specific ^{125}I-ANF binding was calculated from the difference between total binding and binding in the presence of 10^{-7} M ANF. Each data point is the mean ± standard error of triplicate determinations.

Hydrodynamic properties of the solubilized ANF-receptor complex. To characterize the hydrodynamic properties of the solubilized ANF receptor, we prelabeled the membrane receptor with ^{125}I-ANF prior to solubilization with octyl-β-D-glucoside. Steric exclusion HPLC of the solubilized ^{125}I-ANF-receptor complex on a Superose 6 column in the presence of 40 mM octyl-β-D-glucoside is shown in Fig. 3. The column was calibrated with globular reference proteins and a linear empirical relationship was found between $(-\log K_{av})^{1/3}$ and Stokes radius. The elution profile consistently showed a major peak of radioactivity in a position corresponding to a mean Stokes radius of 50.8 ± 0.9 Å. A secondary peak of radioactivity was often seen before the major peak. This peak was typically variable both in elution time and size, in contrast with the major peak, and may represent an aggregated form of the

[3] C. Tanford, personal communication.

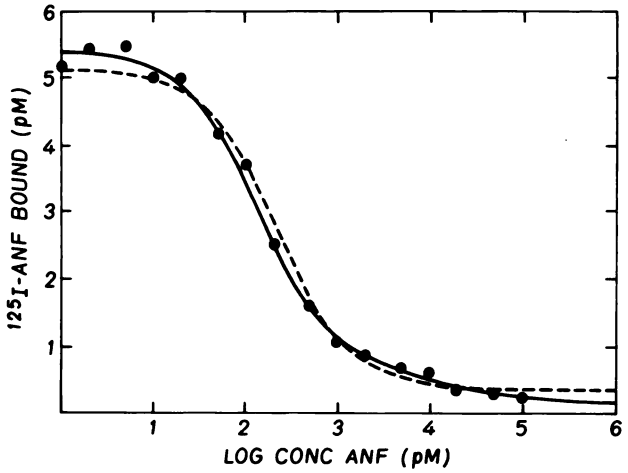

Fig. 2. Competition of ANF for binding of ^{125}I-ANF to solubilized adrenal zona glomerulosa membranes. Solubilized fraction (70 μg of membrane protein/ml) was incubated at 10° for 4 hr with 14.4 pM ^{125}I-ANF and varying concentrations of unlabeled ANF. Bound ^{125}I-ANF was determined by precipitation with polyethylene glycol and filtration. ●, the average of duplicate determinations of bound ^{125}I-ANF. Computer modeling indicated that the curve had a slope factor of 0.83 and was significantly better explained by a model for two classes of binding sites (—) than by a model for a single class of sites (– – –). The two populations of binding sites were characterized by pK values of 10.4 (K_d = 40 pM) and 8.2 (K_d = 6000 pM), respectively, and densities of 368 and 3177 fmol/mg of protein.

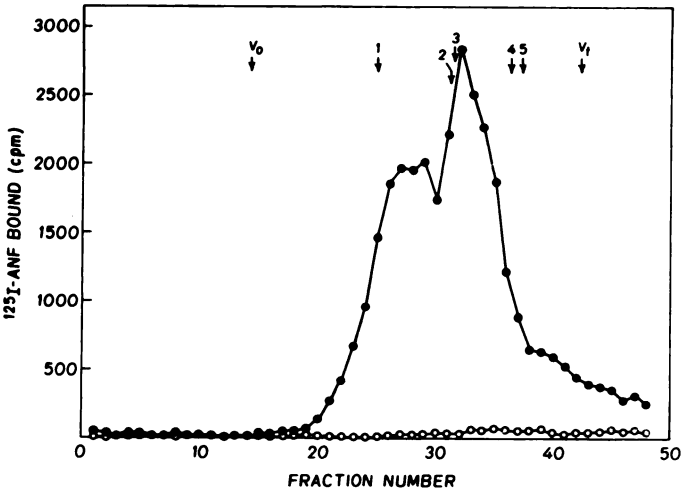

Fig. 3. Steric exclusion HPLC of solubilized ^{125}I-ANF-receptor complexes. Adrenal zona glomerulosa membranes were incubated with ^{125}I-ANF in the absence (●) or presence (○) of 10^{-7} M unlabeled ANF and solubilized with 40 mM octyl-β-D-glucoside (for details see Experimental Procedures). The solubilized preparation was then chromatographed on a Superose 6 column in buffer C at 4°. The solubilized ^{125}I-ANF-receptor complex was detected by polyethylene glycol precipitation and filtration. ↓, the elution position of reference proteins with known Stokes radii that were used to calibrate the column: *1*, thyroglobulin (85 Å); *2*, catalase (52.2 Å); *3*, aldolase (48.1 Å); *4*, chymotrypsinogen A (20.9 Å); and *5*, ribonuclease A (16.4 Å).

labeled ANF-receptor complex. We thus have considered only the major and constant peak in the calculation of the hydrodynamic properties of the receptor. No peak was detected when membranes were incubated in the presence of excess unlabeled ANF.

The sedimentation profile of the solubilized ^{125}I-ANF-receptor complex after ultracentrifugation through a linear sucrose gradient in water containing 40 mM octyl-β-D-glucoside is shown in Fig. 4. Calibration of the gradients with reference proteins yielded a linear relationship between the fractional migration distance and $s_{20,w}$. The labeled receptor sedimented as a single peak in a position corresponding to a measured apparent sedimentation coefficient (s_H) of 6.23. In the presence of excess ANF, no peak of radioactivity was found. When centrifugation was carried out in sucrose gradients prepared in D_2O, the ^{125}I-ANF-receptor complex migrated more slowly with a mean apparent sedimentation coefficient (s_D) of 5.48. The higher value obtained in H_2O indicates that the partial specific volume of the labeled complex is larger than those of reference proteins due to the presence of bound detergent.

The individual values of s_H and s_D were combined to calculate the $\bar{\nu}$ value, the $s_{20,w}$, and the molecular weight of the labeled receptor-detergent complex (Table 1). The frictional ratio of the complex was estimated from the values of the Stokes radius, $\bar{\nu}$, M, and an estimated hydration of 0.3 g of H_2O/g of protein.

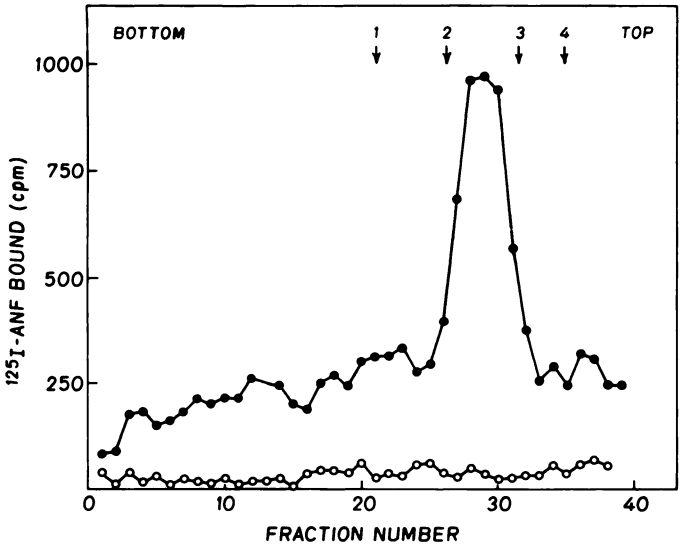

Fig. 4. Sucrose density gradient centrifugation of solubilized ^{125}I-ANF-receptor complexes. The solubilized ^{125}I-ANF-receptor complexes were prepared as described in Fig. 3 (with the exception that glycerol was absent from the solubilization buffer) and sedimented through a 5–20% sucrose density gradient in buffer C by centrifugation at 33,000 rpm at 4° in a Beckman SW 55 Ti rotor. Fractions were collected from the bottom and the ^{125}I-ANF-receptor complex (●, total; ○, nonspecific) was detected by precipitation with polyethylene glycol and filtration. ↓, the sedimentation position of the reference proteins used to calibrate the gradients: *1*, catalase ($s_{20,w}$, 11.30); *2*, aldolase ($s_{20,w}$, 7.35); *3*, bovine serum albumin ($s_{20,w}$, 4.41); and *4*, ribonuclease A ($s_{20,w}$, 2.00).

TABLE 1

Hydrodynamic properties of the solubilized ^{125}I-ANF-receptor complex

Physical parameter	Value[a]
Stokes radius, *a* (Å)	50.8 ± 0.9 (7)[b]
Partial specific volume, $\bar{\nu}$ (ml/g)	0.770 ± 0.009 (16)
Sedimentation coefficient, $s_{20,w}$ (S)	6.34 ± 0.17 (4)
Molecular weight, M_r	158,000
Frictional ratio, *f*/*fo*	1.25
Detergent bound (g/g of protein)	0.45 (0.66–0.22)[c]
Molecular weight of protein	109,000 (95,000–130,000)[c]

[a] The hydrodynamic parameters were analyzed as described in Experimental Procedures.

[b] The values given are the mean ± standard error for the number of determinations shown in parentheses.

[c] The values in parentheses are those obtained when the partial specific volume of the protein is assumed to be 0.710 and 0.750 ml/g, respectively.

A value of 1.25 was obtained, which is within the range expected for globular proteins (39, 40). The molecular weight of the receptor-detergent complex was corrected for the mass contributed by the bound detergent using a calculated $\bar{\nu}$ of 0.770 ml/g for the complex, a $\bar{\nu}$ of 0.859 ml/g for octyl-β-D-glucoside, and an average $\bar{\nu}$ of 0.730 ml/g for the protein (39). This gave a molecular weight of 109,000 for the protein component alone and a level of octyl-β-D-glucoside binding of 0.45 g/g of protein. Estimates of the physicochemical parameters are summarized in Table 1.

Affinity labeling of the ANF receptor. Analysis of the constituent polypeptide chains of the receptor protein was performed by SDS-polyacrylamide gel electrophoresis following affinity cross-linking of ^{125}I-ANF to zona glomerulosa membranes. Autoradiograms of gels obtained under reducing conditions showed that a single band migrating at M_r 130,000 was labeled (Fig. 5).

The specificity of the labeling was assessed by incubating adrenal membranes with ^{125}I-ANF in the presence of various unlabeled hormones. The labeling of the band was reduced in a dose-dependent manner in the presence of unlabeled ANF with a significant inhibition observed at 10^{-11} M but was not affected by high concentrations of ACTH, angiotensin II, and vasopressin.

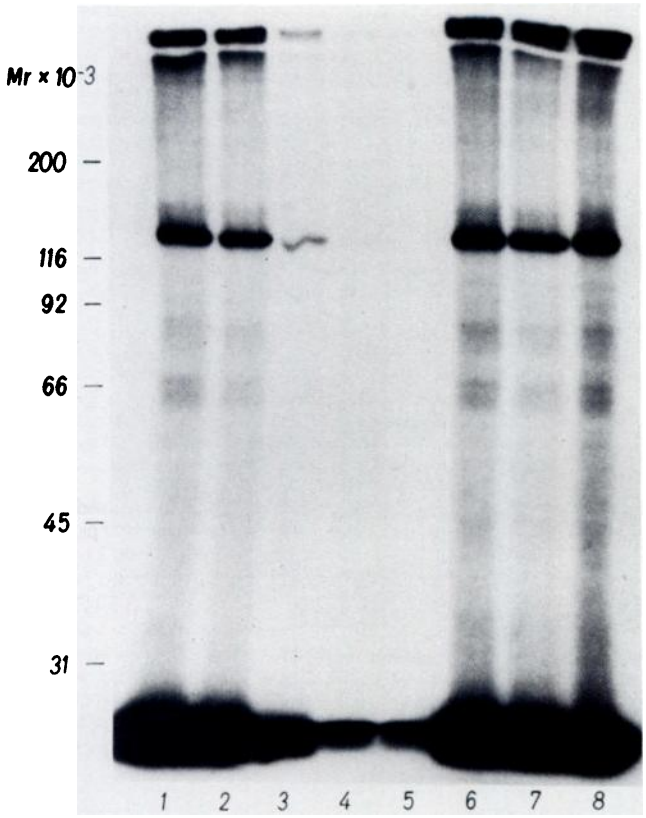

Fig. 5. Electrophoretic analysis of adrenal zona glomerulosa membranes cross-linked with ^{125}I-ANF in the presence of various unlabeled hormones. Adrenal membranes were incubated for 12 hr at 10° with 10 pM ^{125}I-ANF in the absence of any other peptide (*lane 1*) or in the presence of unlabeled ANF at 10^{-11} (*lane 2*), 10^{-10} (*lane 3*), 10^{-9} (*lane 4*), and 10^{-8} (*lane 5*) M, ACTH (*lane 6*), angiotensin II (*lane 7*), and vasopressin (*lane 8*), all three at a final concentration of 10^{-6} M. Cross-linking was performed with 1 mM bis (sulfosuccinimidyl) suberate for 30 min at 4°. The reaction was quenched and the washed membranes were solubilized by heating at 100° for 5 min in the sample buffer containing 5% 2-mercaptoethanol. Approximately 200 μg of proteins were subjected to SDS-gel electrophoresis on a 7.5% acrylamide gel. Shown is an autoradiogram of the fixed, dried gel after a 12-hr exposure. Molecular weight standards are: myosin (200,000), β-galactosidase (116,250), phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000).

Discussion

The ANF receptor was solubilized from bovine adrenocortical membranes with the detergent octyl-β-D-glucoside. This detergent was chosen for several reasons, including its ability to preserve the binding characteristics of the native receptor from intact membranes, its nonionic nature, and its high critical micellar concentration (25 mM) (41) which allows more efficient removal by dialysis. In addition, octyl-β-D-glucoside has been used successfully for the solubilization of other membrane receptors (42, 43). The soluble state of the receptor was confirmed by the usual experimental criteria for solubilization. The ^{125}I-ANF binding activity passed through 0.22-μm Millipore filters, and no decrease in binding was observed in the supernatant following further centrifugation at 100,000 × *g* for 60 min. The solubilized sites were not retained by glass fiber filters without polyethylene glycol precipitation. Finally, the ^{125}I-ANF-receptor complex was well partitioned between the void volume and the total volume of a Superose 6 steric exclusion column (Fig. 3).

The physicochemical properties of the solubilized ANF receptor were determined after prelabeling the membrane receptor with ^{125}I-ANF. The dissociation at 4° of bound ^{125}I-ANF from particulate or solubilized membrane receptor occurs at a slow rate, allowing the characterization of hormone-receptor complex at low temperature. Results from steric exclusion HPLC and sedimentation velocity were used to calculate a molecular weight of 109,000 for the receptor protein alone and a value of 0.45 g of octyl-β-D-glucoside bound per g of protein. This extent of detergent binding indicates the presence of hydrophobic sites on the surface of the protein, which is to be expected for integral membrane proteins. The calculation of the frictional ratio, which reflects the molecular asymmetry of the protein-detergent complex, yielded a value of 1.25, compatible with a globular shape. Theoretical limitations associated with the determination of the hydrodynamic properties of detergent-solubilized proteins have been discussed (40, 44).

The subunit structure of detergent-solubilized proteins can be determined by comparing the polypeptide chain molecular weight obtained by gel electrophoresis in SDS with the molecular weight of the protein component of the complex, obtained by subtracting the contribution of bound detergent (44). Macromolecular complexes are known to dissociate into their subunits after treatment with SDS and reducing agents but retain their native structure after solubilization with mild nonionic detergents. The hydrodynamic molecular weight of 109,000 estimated for the ANF receptor protein is commensurate with the M_r 130,000 band obtained by gel electrophoresis. These data therefore indicate that the ANF receptor in detergent solution is most probably constituted of only one single polypeptide chain. Although the subunit structure of the receptor-detergent complex might differ from that of the receptor-lipid complex in the membrane, there is good reason to believe that the receptor protein is also a monomer in the membrane.

The identification by SDS-gel electrophoresis of a single

labeled band of M_r 130,000 is comparable to the findings of other groups who have described the labeling of a major polypeptide band of M_r 120,000–160,000 (19, 21, 24, 25). We have also previously reported the labeling by affinity cross-linking of an additional M_r 68,000 band together with other minor labeled bands (18). Similarly, other investigators have formerly reported the presence of a low molecular weight band between 60,000 and 70,000 Da in different tissues (20, 22, 23). These findings were interpreted in a number of ways. It has been reported that the ANF receptor might be composed of different components with M_r 60,000–70,000 and M_r 120,000 (18, 22), that the M_r 130,000 band is composed of disulfide-linked subunits with the binding site located on an M_r 70,000 component (20), that ANF binds to two proteins of M_r 66,000 and 180,000 which are intimately involved in the receptor structure and represent perhaps the precursor and mature form of the protein (23), and, finally, that the M_r 65,000 band results from the nonspecific cross-linking of bovine serum albumin present in the buffer (19). However, we have since observed that these low molecular weight bands were appearing only when the membrane proteins were washed in acidic buffer and heated at 100°. This wash step in acidic buffer was initially included in order to remove noncovalently bound radiolabeled hormone, but most probably resulted in the partial hydrolysis of the M_r 130,000 receptor band (not shown). Therefore, the results that we now report support the notion that the ANF receptor is constituted of a single polypeptide of M_r 110,000–130,000. However, additional studies will be required to confirm that this conclusion applies to other tissues.

Interestingly, the physicochemical characteristics of the ANF receptor obtained by hydrodynamic measurements and SDS-gel electrophoresis have certain similarities with the characteristics reported for the particulate form of guanylate cyclase from sea urchin spermatozoa (45). ANF has been shown to activate particulate guanylate cyclase in adrenal cortex (46, 47) and other target tissues (48), and a role for this enzyme and cyclic GMP in mediating some of the effects of ANF has been proposed. This suggests that the ANF receptor might correspond to guanylate cyclase acting as a specific receptor. This hypothesis would be supported by a recent report by Kuno *et al.* (25) on the co-purification of an ANF receptor and particulate guanylate cyclase from rat lung. Additional work is, however, needed to unambiguously prove that these two activities reside on the same macromolecule.

In summary, we report the first hydrodynamic characterization of the molecular structure of the ANF receptor. Our results indicate that the ANF receptor protein in adrenal cortex has a molecular weight of 110,000–130,000 and is most probably composed of a single polypeptide chain. This work should prove helpful toward understanding the structure and the mode of action of the ANF receptor.

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