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Molecular Structure of the β-Adrenergic Receptor

Robert G. L. Shorr,*,‡ Darrell R. McCaslin,§ Mark W. Strohsacker,‡ Gary Alianell,∥ Richard Rebar,‡ Jeffrey M. Stadel,‡ and Stanley T. Crooke‡

Department of Molecular Pharmacology, Smith Kline and French Laboratories, Philadelphia, Pennsylvania 19101, Department of Anatomy, Duke University Medical Center, Durham, North Carolina 27710, and Beckman Instruments, King of Prussia, Pennsylvania 19406

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ABSTRACT: The β-adrenergic receptor from several tissues has been purified to homogeneity or photoaffinity radiolabeled and its subunit molecular weight determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. In this study we have examined the oligomeric structure of nondenatured β_1- and β_2-adrenergic receptor proteins, as solubilized with the detergent digitonin. Model systems used were frog and turkey red blood cell as well as rat, rabbit, and bovine lung plasma membrane preparations. To correct for the effects of detergent binding, sedimentation equilibrium analysis in various solvents, as adapted for the air-driven ultracentrifuge, was used. With this approach an estimate of 6 g of digitonin/g of protein binding was determined, corresponding to a ratio of 180 mol of digitonin/mol of protein. Protein molecular weights estimated by this method were 43 500 for the turkey red blood cell β_1 receptor and 54 000 for the frog red blood cell β_2 receptor. Molecular weights of 60 000–65 000 were estimated for β_1 and β_2 receptors present in mammalian lungs. These values agree with estimates of subunit molecular weight obtained by SDS gel electrophoresis of purified or photoradiolabeled preparations and suggest β-adrenergic receptors to be digitonin solubilized from the membrane as single polypeptide chains.

The β-adrenergic receptor recognizes epinephrine and norepinephrine and modulates the production of cyclic AMP by the enzyme adenylate cyclase. On the basis of pharmacological interactions, β receptors can be divided into two subtypes termed β_1 and β_2 (Lands et al., 1967). Both receptor subtypes stimulate adenylate cyclase activity. An early step in the mechanism by which this is believed to occur is the formation of a complex between receptor and a GTP binding regulatory component of the enzyme (Stadel et al., 1980). While the subunit molecular weight of the β-adrenergic receptor has been determined in several model systems (Shorr et al., 1981, 1982a,b; Benovic et al., 1983; Stiles et al., 1983), the nature

‡Smith Kline and French Laboratories.
§Duke University Medical Center.
∥Beckman Instruments.

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of a receptor oligomeric complex in the membrane or in detergent solution has not been determined. This is of considerable importance since the guanosine triphosphate (GTP)[1] binding regulatory protein has been purified to homogeneity (Hanski et al., 1981) and itself shown to be a complex of heterologous promoters (Hanski et al., 1982). The relationship between receptor subunits and these components then needs to be defined in biochemical terms, in order to understand the mechanism by which receptors modulate adenylate cyclase activity. In addition, it is important to begin to understand the molecular differences between β_1 and β_2 receptors. A principal step in this direction is the determination of molecular size and oligomeric structure of the β-adrenergic receptor proteins.

Experimental Procedures

Materials

Digitonin and deuterium oxide were from Gallard Schlessinger, [^{125}I]iodocyanopindolol (2200 Ci/mmol) and [^{3}H]dihydroalprenolol (34 Ci/mmol) were from New England Nuclear. Reagents for electrophoresis and Bradford protein assay were from Bio-Rad Laboratories. Protein standards, phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), and lactalbumin (M_r 14 400), were from Pharmacia. Prestained standards were from BRL. All protease inhibitors were from Sigma Chemical Co. An air-driven ultracentrifuge modified for use at 4 °C and speeds of 2000–25 000 rpm was purchased from Beckman Instruments. Deuterium [^{18}O]oxide ($D_2{}^{18}O$) was obtained from Stohler Isotope Chemicals. Whole turkey blood was obtained from Featherdown Farms. Frog red blood cell plasma membranes were a generous gift from Dr. Marc Caron (Howard Hughes Medical Institute, Duke University, Durham, NC). Rat and rabbit as well as bovine lungs were obtained from Hazelton Dutchland Co. Whatman glass-fiber filters (GF/c) for particulate assays were obtained from A. H. Thomas Co. Reagents and standards for amino acid analysis were from Pierce Chemical Co. Alprenolol was obtained from Dr. Carl Kaiser (SKF Medicinal Chemistry) and immobilized on agarose as described (Caron et al., 1980).

Methods

Preparation of Purified Plasma Membranes. Purified frog and turkey red blood cell plasma membranes were prepared as described earlier (Shorr et al., 1981, 1982a,b) except that buffers contained protease inhibitors at the following concentrations: soybean trypsin inhibitor, 10 μg/mL; benzamidine, 10^{-4} M; EDTA, 10 mM; bacitracin, 50 μg/mL; phenylmethanesulfonyl fluoride, 10^{-5} M. Bovine, rat, and rabbit lung plasma membranes were prepared by homogenization of lungs (25% in a Waring blender, in degassed, ice-cold 75 mM Tris-HCl, pH 7.2, 25 mM $MgCl_2$ with 50 mM EDTA, 10 μg/mL soybean trypsin inhibitor, 10^{-4} M benzamidine, 50 μg/mL leupeptin, 5 μg/mL chymostatin, and 10^{-5} M phenylmethanesulfonyl fluoride) followed by removal of large tissue fragments by filtering through two layers of cheesecloth. The homogenate was then transferred to a 2-L Parr nitrogen bomb and equilibrated at 4 °C with 800–1000 psi of purified nitrogen for 40 min. After release to atmospheric pressure and dilution with homogenization buffer to 4–6 L, large particulate material was removed by a low-speed centrifugation at 5000 rpm (20 min, 4 °C) in a Beckman J6M centrifuge. Plasma membranes were then collected by centrifugation of supernatant at 11 000 rpm for 45 min (4 °C) in a Beckman J2-21 centrifuge with JA-10 rotor. Pellets were washed twice with homogenization buffer, resuspended at a concentration of 5–10 mg/mL protein, frozen in liquid nitrogen, and stored at −70 °C until use. Protein concentrations were estimated by the method of Bradford (1976) with bovine serum albumin as standard.

Particulate Assays. Particulate preparations of frog and turkey red blood cell, rat, rabbit, and bovine lung plasma membranes were assayed for β-adrenergic receptor ligand binding activity with [^{125}I]iodocyanopindolol (30 pM) or [^{3}H]dihydroalprenolol (32 nM) (Shorr et al., 1981). Nonspecific binding was determined in the presence of 10^{-5} M alprenolol and bound ligand separated from free ligand by vacuum filtration over Whatman GF/c glass-fiber filters (Lefkowitz et al., 1974). Radioactivity was determined by using a Beckman GP-5500 gamma counter or LS 7500 liquid counter with HP/b counting fluor. Counting efficiencies were 87% for ^{125}I and 50% for ^{3}H.

Receptor Solubilization. Soluble receptor activity was prepared from plasma membrane preparations of bovine, rat, and rabbit lungs as well as frog and turkey red blood cells as described (Caron & Lefkowitz, 1976; Shorr et al., 1982a). Briefly, membranes were resuspended to a concentration of 7–10 mg of protein/mL with at least a 2-fold excess of detergent over protein concentration and dounce homogenized in 12–16 mM digitonin, 100 mM NaCl, and 10 mM Tris, pH 7.2, with protease inhibitors, including 25 mM EDTA. After the preparations were stirred on ice, insoluble material was removed by centrifugation (10 min) using a Beckman microfuge for small samples or a Beckman J2-21 centrifuge with JA-17 rotor for larger samples. Supernatants were stored on ice until use and pellets discarded.

Preparation of Purified β-Adrenergic Receptor. Purified receptors from turkey red blood cell plasma membranes were prepared as described (Shorr et al., 1982a). Briefly, membranes prepared from 4 L of whole turkey blood (1800 mL of packed cells) were first washed free of loosely bound protein by dounce homogenization and suspension in 700 mL of 1 mM digitonin, 100 mM NaCl, 10 mM Tris-HCl (pH 7.2), 25 mM EDTA, and protease inhibitors. Washed membranes were collected by centrifugation and resuspended by dounce homogenization in 700 mL of the same buffer as above except with 16 mM detergent. After the solution was stirred on ice for 1 h, insoluble material was removed by centrifugation and the extract loaded at 5 mL/min onto a 700-mL column of alprenolol–Sepharose at 30 °C. After extensive washing of the column with 1 mM digitonin, 100 mM NaCl, 10 mM Tris-HCl (pH 7.2), and 25 mM EDTA at 4 °C, receptor was eluted with 10^{-4} M alprenolol in the same buffer at 30 °C; 24-mL fractions were collected. Eluting ligand was then removed from an aliquot of each fraction by desalting on Sephadex G-50 prior to assay with [^{3}H]dihydroalprenolol (Shorr et al., 1981, 1982a).

After completion of assays, fractions containing receptor activity were pooled and concentrated to 10 mL by Amicon ultrafiltration using a PM-30 membrane. Concentrated receptor was then desalted free of eluting alprenolol and further purified by size exclusion HPLC with digitonin-containing mobile phase (Shorr et al., 1982a,b). Typical yields of receptor activity were 500 pmol. To prepare material for amino acid analyses, an aliquot of purified receptor was radiolabeled with

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; [^{125}I]cyp, [^{125}I]cyanopindolol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; GTP, guanosine triphosphate; [^{125}I]pABC, (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

$Na^{125}I$ and Chloramine T (Shorr et al., 1982a), pooled with unlabeled receptor, and separated from residual contaminants by SDS–PAGE (Laemmli, 1970). Receptor bands were localized by autoradiography, excised from the gel, and electroeluted by using an Isco Model 1750 electroeluter (Isco Co.).

Purified β_2-adrenergic receptor from bovine lung plasma membranes was prepared essentially as described above for the turkey red blood cell. Material, after the size exclusion HPLC step, was utilized for the photoaffinity labeling experiments using (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol.

Photoaffinity Labeling and SDS–Polyacrylamide Gel Electrophoresis of β-Adrenergic Receptors. Plasma membrane preparations of β-adrenergic receptor were labeled with the photoaffinity probe (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol ([^{125}I]pABC) as described by (Lavin et al., 1982). After photolysis, membranes were pelleted and resuspended in 10% SDS, 5% mercaptoethanol, 12 mM Tris-HCl, pH 6.5, and 5% glycerol with a small amount of bromophenol blue. Residual insoluble material was removed by centrifugation, and aliquots of the photolabeled solubilized receptor were analyzed by SDS–polyacrylamide (10%) slab gel electrophoresis. Prestained standards of known molecular weight (BRL Labs, Bethesda, MD) were coelectrophoresed for estimates of subunit molecular size based on relative mobility (Weber & Osborn, 1975). Photoaffinity labeling with (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol of purified bovine lung plasma membrane β_2 receptors was performed as follows. A total of 10–20 pmol of receptor activity [estimated by assay with [^{3}H]dihydroalprenolol (Shorr et al., 1981)] was incubated with equimolar concentrations of (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol for 12–15 h at 0–4 °C. Samples were then desalted on columns of Sephadex G-50, photolyzed (Lavin et al., 1982), and subjected to size exclusion HPLC (Shorr et al., 1982b). Peaks of labeled receptor were pooled, concentrated by Amicon ultrafiltration, and analyzed by SDS–PAGE. Controls consisted of coincubations of receptor and (*p*-azido-*m*-[^{125}I]-iodobenzyl)carazolol in the presence of excess concentrations of isoproteronol or alprenolol.

Amino Acid Analysis of Purified Turkey Red Blood Cell β_1-Adrenergic Receptors. Amino acid analysis of purified turkey red blood cell β_1-adrenergic receptor was performed as follows: briefly, 20–30 pmol of receptor activity prepared by affinity chromatography, size exclusion chromatography, and preparative SDS–polyacrylamide gel electrophoresis was dialyzed extensively against 0.1% SDS and taken to dryness and hydrolyzed with 6 N HCl for 22 h at 110 °C in vacuo. Hydrolyzed amino acids were separated and detected on a Beckman 344 liquid chromatograph system with a Model 157 fluorescence detector and precolumn *o*-phthaldehyde derivatization of the hydrolysis residues. Precolumn reactions were performed automatically on a Waters WISP autosampler. Columns used were Altex 5-μm ultrasphere ODS (15 cm) at room temperature. Buffer A was composed of 25 mM sodium acetate, pH 5.9, and 2.5% tetrahydrofuran. Buffer B was 100% methanol. Gradients were from 20 to 80% B over 16 min. For proline determination, a Beckman Model 6300 analyzer with postcolumn ninhydrin detection was used. Composition data were utilized to determine a partial specific volume for the purified receptor protein according to the equation

$$v_p = n_i m_i / n_i v_i$$

where n_i is the number of residues for each individual amino acid and m_i and v_i are the molecular weight and partial specific volume of each amino acid, respectively. Residue numbers were approximated by using subunit molecular weights estimated by SDS–PAGE. Standard amino acid molecular weights and partial specific volumes of each residue were employed (Reynolds & McCaslin, 1985).

Centrifugation and Assay of Detergent-Solubilized and Purified Receptor Preparations. Sedimentation equilibrium gradients were established by centrifugation of 100-μL aliquots of detergent-solubilized membrane or purified receptor to which bovine serum albumin was added to a final concentration of 1%. Speeds were from 14 000 to 16 000 rpm for periods from 24 to 48 h depending on solvent density. After centrifugation, temperatures were determined by using a thermal couple (Fisher) and 10-μL fractions collected with a Gilson P-20 pipetman. After fractionation, receptor concentration was determined by dilution of samples to 500 μL with 1 mM digitonin, 100 mM NaCl, 10 mM Tris-HCl, pH 7.2, and 10 mM EDTA and assay with [^{125}I]iodocyanopindolol (30 pM). To determine nonspecific binding, fractions were collected, diluted, and assayed in the presence of [^{125}I]iodocyanopindolol and 3×10^{-5} M alprenolol. The difference in radioactivity between the two aliquots was defined as the specific binding component (>95% of the total bound radioactivity). Bound ligand was separated from free ligand by Sephadex G-50 chromatography. Total recoveries of activity in the assay fractions were estimated by assay of 10 μL of the initial starting materials diluted as described above. Samples were sedimented in triplicate or in replicates of six, and each gradient was analyzed separately as described.

Calculations. Sedimentation equilibrium yields a value for the reduced molecular weight expressed as $M_p(1 - \phi\rho)$. In the absence of preferential interactions with solvent components [i.e., binding of detergent; this value is a function of the protein molecular weight (M_p) and the solvent density (ρ) in grams per cubic centimeter]. The term ϕ contains only the partial specific volume of the protein (v_p) in cubic centimeters per gram. When detergent is bound, ϕ also contains terms relating to the amount of bound detergent (δ) in grams per gram and the detergent partial specific volume (v_d) in cubic centimeters per gram (Reynolds & McCaslin, 1985; Tanford et al., 1974; Casa & Eisenberg, 1964). A plot of ln C vs. r^2 should be a single straight line for a homogeneous, molecular species (detergent–protein complex) where C is protein concentration and r is a function of radial distance. The slope of this plot yields the reduced molecular weight according to $M_p(1 - \phi\rho) = (2RT/\omega^2)/(\mathrm{d} \ln C/\mathrm{d}r^2)$, where R is the gas constant (8.3144×10^7 ergs deg^{-1} mol^{-1}), T is the temperature in kelvin, ω^2 is the squared radial velocity in radians per second, r is the radial distance from the center of rotation in centimeters, and C can be any measurement that is directly proportional to the protein concentration, in this case, the amount of bound radioligand. Each gradient is analyzed separately since minor fluctuations in total loading concentrations can yield shifts in the positions of the lines along the ln C axis. This does not, however, affect the calculated slopes that yield the reduced molecular weights. The determination of the reduced molecular weight at several densities permits a determination of the protein molecular weight and an estimation of the amount of bound detergent (Reynolds & McCaslin, 1985). For density variation we used isotopically substituted water, D_2O and $D_2{}^{18}O$. The use of these for density variation is unlikely to change preferential hydration significantly as might occur with high concentrations of small molecules such as sucrose (Reynolds & McCaslin, 1985).

Exchange of receptor into D_2O or $D_2{}^{18}O$ was accomplished by lyophilization of 100 μL of detergent-solubilized or purified

receptor containing bovine serum albumin at a final concentration of 1% and resuspension into 100 μL of D_2O or $D_2{}^{18}O$. The reduced molecular weight in solvents of varied density can in principle yield both the molecular weight of the protein moiety of the protein–detergent complex and the amount of detergent associated with the complex (Reynolds & McCaslin, 1985; Tanford et al., 1974). Since the v of digitonin (0.7478 cm^3/g) is nearly the same as that of most proteins, the range of densities accessible by use of isotopically enriched water is not sufficient to provide an unequivocal determination of the oligomeric state of the protein. An alternative interpretation of the reduced molecular weight then is $M_c(1 - v_c)$ where M_c is the molecular weight of the protein–detergent complex and v_c is its partial specific volume. If the effects of deuteration can be neglected within the limits of experimental error, a plot of reduced molecular weight as a function of δ approximates a straight line, the intercept on the ρ axis is $1/v_c$ of the complex, and the intercept on the $M_p(1 - \phi\rho)$ axis is the molecular weight of the detergent–protein particle. Moreover, for a complex composed only of detergent and protein

$$M_c = M_p(1 + \delta) \quad (1)$$

$$v_D = (v_p + v_c)/(1 + \delta) \quad (2)$$

Thus, the two intercepts provide solutions of these two equations for δ and M_p (Reynolds & Tanford, 1985). This approach imposes another restriction on the possible regression lines through the data as a function of ρ, since v_c must lie between the v_D and v_p. If the molecular weight of the polypeptide chain is known from other measurements (amino acid composition and gel electrophoresis), then this method provides a relatively rapid means for ascertaining the state of oligomerization in a detergent solution.

Results

Both detergent-solubilized and purified turkey red blood cell β-adrenergic receptors were centrifuged, fractionated, and receptor activity assayed with [^{125}I]iodocyanopindolol. Under these conditions gradients of ligand binding activity were established that on conversion to a plot of ln activity vs. r^2 resulted in a line whose slope is proportional to $M_p(1 - \phi\rho)$ (see Methods). These experiments were repeated at several speeds and run times to ensure that equilibrium had been reached and did not alter with detergent concentration in the sample. No differences were observed between digitonin-solubilized or purified receptor.

Similar experiments were performed after samples had been exchanged into D_2O or $D_2{}^{18}O$, although centrifugation was for extended periods to ensure equilibrium in the higher density solvents. $M_p(1 - \phi\rho)$ was then determined as described under Methods and a plot constructed of $M_p(1 - \phi\rho)$ as a function of solvent density (Figure 1). A line fitted to these data must intercept the x axis at a value not less than $1/v_D$ and not greater than $1/v_p$. This range is particularly narrow in this case since, as we will show below, there is only a small difference in the partial specific volumes of digitonin and receptor. The x intercept of this line is $1/v_c$ where v_c is the partial specific volume of the receptor digitonin complex (Reynolds & McCaslin, 1985). This value determined for receptors in turkey red blood cells was 0.733 (Figure 1, top panel) and for other receptors was fixed at this value providing an additional restricting point in analyzing the sedimentation equilibrium data.

To determine the partial specific volume, of the receptor, amino acid analysis of purified turkey red blood cell preparations was performed. This was achieved after purified receptor protein was obtained by digitonin solubilization, affinity

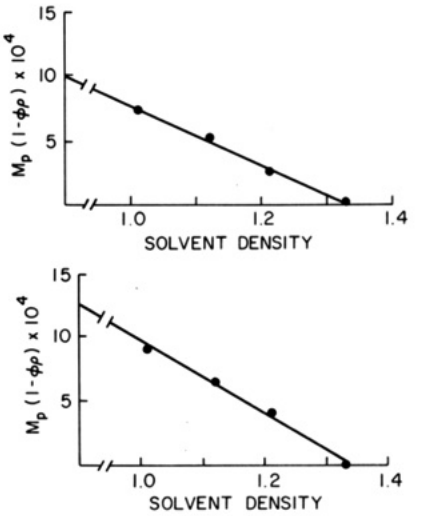

FIGURE 1: Calculation of protein molecular weight for the turkey red blood cell and frog red blood cell β-adrenergic receptor. (Top panel) $M_p(1 - \phi\rho)$ calculated for several runs (N = minimum 12) in differing solvents and plotted as a function of solvent density (g/mL). As described in the text, the ρ intercept (solvent–density) reflects the inverse of the partial specific volume of the receptor–digitonin complex. (Bottom panel) As described in the top panel except that the receptor was solubilized from preparations of frog red blood cell plasma membranes.

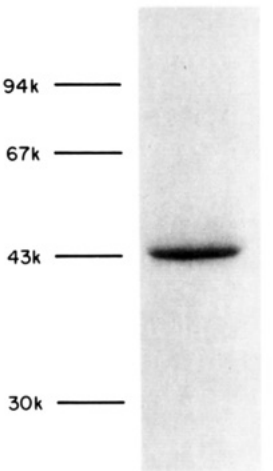

FIGURE 2: Analytical SDS gel electrophoresis of purified turkey red blood cell β-adrenergic receptor. Purified turkey red blood cell β-adrenergic receptor was obtained by affinity chromatography, size exclusion HPLC, and preparative SDS–PAGE. For determination of amino acid composition only the M_r 45 000 form of this receptor protein was used. In the gel shown 115 pmol (5 μg) of receptor was fractionated on a 10% SDS gel (Laemmli, 1970) and stained with Coomassie R250 (1% dye, 25% ethanol, and 10% acetic acid) and destained with 7% acetic acid. The mobility of protein standards (Pharmacia) is shown to the left of the figure.

chromatography, size exclusion HPLC, and SDS gel electrophoresis. Purity was confirmed by reelectrophoresing an aliquot of the receptor protein and staining with Coomassie R250 (Reissner et al., 1975). A typical preparation is shown in Figure 2. Acid hydrolysis and amino acid analysis of these preparations resulted in an estimate of residue content sufficient to determine v_p. This is summarized in Table I, where v_p was calculated to be 0.7364 g/mL. For digitonin v_D was calculated on the basis of known structure as 0.7478 g/mL (in water). From eq 2 then one can calculate δ, which is the amount of detergent bound per gram of protein. With this

$$\delta = (v_p - v_c)/(v_c - v_d)$$

value, we can solve for protein molecular weight using eq 1 [$M_p = M_c/(1 + \delta)$]. The value M_c is $M(1 - \phi\rho)$ at theo-

Table I: Estimate of Amino Acid Content of the Purified Turkey Red Blood Cell β_1-Adrenergic Receptor[a]

residue	apparent mol %	residue	apparent mol %
Asx	9	Tyr	3.5
Glx	12	Met	0.7
Ser	5.5	Val	7
His	3	Phe	4
Gly	11	Ile	5
Thr	5	Leu	9
Arg	5	Lys	6
Ala	9	Pro	5

[a] Typically, 20–30 pmol of purified turkey red blood cell receptor prepared from plasma membranes by affinity chromatography size exclusion HPLC and SDS–PAGE was hydrolyzed with 6 N HCl, 110 °C, in vacuo and analyzed for amino acids. Residues were detected by precolumn *o*-phthaldehyde derivatization and reverse-phase separation using an Altex ODS ultrasphere column. Proline determinations were made by ion-exchange separation of amino acids and reaction with ninhydrin. Cysteine content was not determined. The results shown are representative of three separate runs with protein obtained from two distinct preparations. Standard errors were less than 10%.

retically zero density. This value is obtained by extrapolation of the data shown in Figure 1 and is the apparent size of the detergent–protein complex. As the receptor is resuspended in a water-containing buffer (the lowest solvent density is 1.0014 gm/mL) and our extrapolation is to a density of zero, each of the four data points shown in the figure was derived from a minimum of 12 determinations and three separate experiments to assure the validity of the extrapolation. Values determined for $M(1 - \phi\rho)$ in each separate experiment were also found to be highly reproducible (less than 10% variability). Consequently, the coefficient of correlation was >0.98, providing substantial confidence in the extrapolation. For the turkey β_1 receptor, M_c was calculated as 300 000. Solving the above equations then for receptor resulted in an estimate of approximately 6 g of digitonin/g of protein binding (180 mol/mol) and a protein molecular weight of 43 500. This value is similar to the size of the β receptor in this system determined by radiation target analysis (Shorr et al., 1984), protein purification and SDS–PAGE (Shorr et al., 1982a), or photoaffinity labeling and SDS–PAGE (Lavin et al., 1982).

A typical preparation of turkey receptor, photoaffinity labeled with (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol and fractionated by SDS–PAGE, is shown in Figure 3. The presence of two photolabeled bands in this species is characteristic of receptors from this source and has been reported elsewhere (Burgermeister et al., 1982; Shorr et al., 1982a; Lavin et al., 1982). Sedimentation equilibrium was then utilized to determine the molecular weight of receptor solubilized with digitonin from frog red blood cell, rat, rabbit, and bovine lung plasma membranes. These systems contain primarily β_2 (frog, rat, and bovine) and β_1 (rabbit) receptor populations, respectively (Dickinson et al., 1981). In these determinations, however, two assumptions were made. First, v_p is similar between species and subtypes, and second, detergent binding is also similar. A plot of $M_p(1 - \phi\rho)$ vs. solvent density for the frog red blood cell is shown in Figure 1 (bottom panel). Calculation of protein molecular weight from these data resulted in an estimate of 54 000. For the additional receptor systems, the data are summarized in Table II. Estimates of protein molecular weight in digitonin for the lung receptor systems range from 60 000 to 65 000 and, as was found for the turkey red blood cell, agree (where applicable) with sizes estimated for subunit molecular weight. This is also true for the frog red blood cell plasma membrane system where subunit size has been determined by radiation inactivation (Shorr et al., 1984) and purification (Shorr et al., 1982b) techniques.

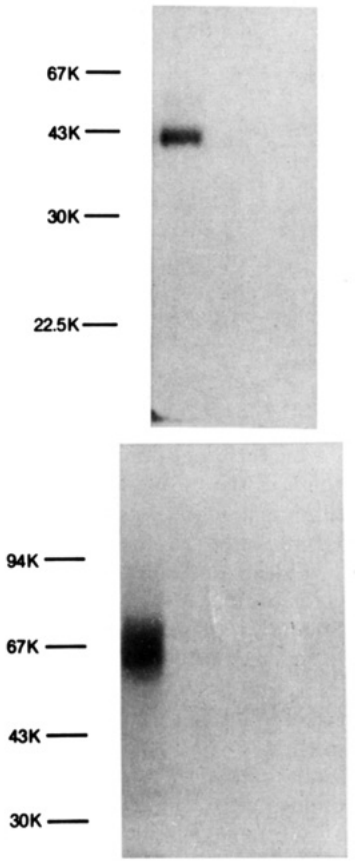

FIGURE 3: Polyacrylamide gel electrophoresis of (*p*-azido-*m*-[^{125}I]-iodobenzyl)carazolol turkey red blood cell labeled receptor. (Top panel) An aliquot of a turkey red blood cell plasma membrane preparation was labeled with (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol in the presence and absence of alprenolol as described under Methods. Samples were photolyzed, and covalently labeled receptor protein was solubilized with SDS, fractionated, and visualized by gel electrophoresis. To the left is shown the results obtained on labeling in the absence of a competing adrenergic agent. To the right, saturating concentrations of alprenolol were included to show specificity of the photolabel. Mobilities of molecular weight markers are shown to the far left of the figure. (Bottom panel) Polyacrylamide gel electrophoresis of (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol-labeled bovine lung β-adrenergic receptor. A total of 20 pmol of bovine lung plasma membrane β_2-adrenergic receptor purified by alprenolol–Sepharose affinity chromatography and size exclusion HPLC was incubated with equimolar concentrations of (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol (15 h at 0–4 °C). Samples were then photolyzed and desalted on Sephadex G-50 columns to remove excess label prior to chromatography on size exclusion HPLC columns as described (Shorr et al., 1982b). Peaks of labeled receptor were pooled and concentrated and aliquots subjected to electrophoresis as described under Methods. The left lane represents receptor treated with (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol alone. To the right is shown results obtained on coincubation with saturating concentrations of isoproteronol or alprenolol. Molecular weight standards are shown to the far left. Specifically labeled bands migrated with mobilities characteristic of proteins of M_r 63 000 and 65 000.

Photoaffinity labeling of bovine lung purified receptor preparations is shown in Figure 3 (bottom panel). In this case, two bands of M_r 63 000 and 65 000 are specifically labeled with the reagent (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol. Despite the presence in membranes of both β_1 and β_2 receptors [80% β_2–20% β_1 (Dickenson et al., 1981)], competition binding isotherms generated with purified receptor or crude detergent extracts, isoproteronol, epinephrine, and norepinephrine, demonstrated only β_2 receptor activity (data not shown). The presence of two photolabeled species in lung receptor preparations has been reported elsewhere (Benovic et al., 1984) and may reflect nonselective metal-dependent proteolysis (Benovic

Table II: Determination of β-Adrenergic Receptor Molecular Weight[a]

tissue source	bar[d] subtype	method: SDS–PAGE purified protein	SDS–PAGE photolabeled protein	radiation inactivation	sedimentation equilibrium
turkey RBC[b]	100% β_1	40K, 45K	40K, 45K	47K	43.5K
frog RBC	100% β_2	58K	58K	54K	54K
bovine lung	100% β_2	63K, 65K	63K, 65K	ND	63K
rat lung	100% β_2	65K	65K	ND	64K
rabbit lung	80/20% β_1/β_2	ND[c]	ND	ND	60K

[a] Molecular weight determinations for β-adrenergic receptors from several tissues are here compared. Subunit molecular sizes were determined by SDS–PAGE or radiation inactivation. Molecular weights in digitonin solution were determined by sedimentation equilibrium. Values for subunit size determined by SDS–PAGE of photolabeled and purified receptor in rat lung are taken from Benovic et al. (1984). Rabbit lung detergent extracts contained mixtures of β_1 and β_2 receptors in variable proportions. Values shown here are for membrane preparations. [b] RBC, red blood cell. [c] ND, not determined. [d] bar, β-adrenergic receptor.

et al., 1983). Nonetheless, examination of the molecular weight in digitonin of the bovine lung β_2 receptor by sedimentation equilibrium analysis yielded values consistent with those obtained for subunit size by SDS–PAGE.

For rabbit lung plasma membrane preparations, receptor populations are found to be 80% β_1 and 20% β_2 (Dickenson et al., 1980). Solubilization of receptor from this source led to a mixture, in solution, of the two receptor subtypes with varying ratios of β_1 and β_2 receptors (data not shown). Examination of receptor molecular weight in digitonin from this source by sedimentation equilibrium analyses also yielded values approximating M_r 60 000. A subunit size for the β receptor in rabbit lung plasma membrane preparations has not been reported. Collectively, however, these data suggest that both β_1 and β_2 receptors from mammalian and nonmammalian sources are solubilized with digitonin from plasma membranes as single polypeptide chains.

Discussion

β-Adrenergic receptors are characterized as plasma membrane bound proteins that require the use of detergents for solubilization from the membrane (Caron & Lefkowitz, 1976). Molecular sizes of detergent-solubilized plasma membrane bound proteins are frequently estimated by gel filtration, sucrose density gradient centrifugation, and polyacrylamide gel electrophoresis (Tanford & Reynolds, 1976). These methods frequently do not provide information on the molecular weight of the total *functional* unit.

Sedimentation equilibrium is a thermodynamically rigorous method that does not rely on empirical standardization against known proteins. Sedimentation equilibrium in solvents of varying density can directly yield information on the molecular weight of the functional protein unit and the amount of detergent which is bound to it (Edelstein & Schachman, 1967).

Efforts to determine the oligomeric structure of nondenatured β-adrenergic receptors in detergent have been hampered by the almost exclusive use of digitonin. This plant glycoside has, to date, been found to be the only detergent useful for the solubilization of receptor from plasma membrane preparations, in a state capable of recognizing ligands in postsolubilization assays. On sucrose density gradient centrifugation, however, β-adrenergic receptors solubilized with digitonin sediment with coefficients of 8.5–10 S (Shorr et al., 1981, 1982a). These values are consistent with a protein–degergent complex considerably larger than subunit sizes estimated by SDS–PAGE or radiation, but due to the similarities in partial specific volumes of detergent and proteins, corrections for detergent binding and determination of total protein molecular weight are not possible by this technique. We have, therefore, utilized sedimentation equilibrium as adapted to the air-driven ultracentrifuge (Bothwell et al., 1978; Pollet et al., 1979) to estimate the protein molecular weight of the nondenatured β-adrenergic receptor. We have also developed methods to prepare significant amounts of receptor protein and, on the basis of amino acid composition, determined the partial specific volume of the purified turkey red blood cell β_1-adrenergic receptor. In digitonin, the protein is estimated to bind 6 g of detergent/g of protein (180 mol/mol). This represents the first time that sufficient quantities of β_1-adrenergic receptors have been prepared to allow confirmation of purity by standard protein staining procedures. Furthermore, the amino acid analysis presented is the first such analysis for β-adrenergic receptors. Under these conditions, a detergent-corrected molecular size of M_r 43 500 was determined for the digitonin-solubilized turkey red blood cell β_1-adrenergic receptor and M_r 54 000 for the frog red blood cell β_2 receptor. Since these results agree with estimates of subunit molecular weight by SDS–PAGE, they suggest these receptors to have been solubilized from the membrane as single polypeptide chains. Nonetheless, both avian and amphibian model systems are β_1 and β_2 receptor-like in that they have been found to be anomalous in some of their pharmacological properties. We, therefore, examined additional β receptors obtained from mammalian lungs.

In applying sedimentation equilibrium analyses to these systems, assumptions were made that partial specific volumes and detergent binding ratios would be similar to those of the turkey red blood cell β receptor. Since partial specific volume is an additive function, this assumption is reasonable even though there are differences in subunit size and extent of glycosylation amongst receptor subtypes and tissue sources (Stiles et al., 1984). Similarly, large differences would be required (>2-fold) to change our estimates of subunit number in an oligomeric complex. As summarized in Table II, the molecular sizes of β_1 and β_2 receptors from mammalian lungs were remarkably similar and agreed with subunit size determinations. The molecular size estimated for the turkey red blood cell receptor, however, was considerably smaller. Although this apparent difference is not definitively explained, it may reflect differences in the extent of glycosylation of receptor species. It has recently been reported that treatment of photolabeled lung β-adrenergic receptors with Endo F, an endoglycosidase enzyme, decreased the molecular size of the lung receptor subunits estimated by SDS–PAGE to approximately M_r 49 000 (Stiles et al., 1984). No changes are observed in the molecular size of the turkey red blood cell receptor, however, on treatment with this enzyme under the same conditions (unpublished observations). Thus, the similarities or differences in protein structure are still not known between receptor subtypes. We can, however, conclude from the study presented here that both receptor subspecies from several tissues are solubilized as monomeric polypeptides with digi-

tonin. These results differ from radiation inactivation studies in which the size of a functional unit (enzyme activity or receptor binding site) can be measured. An appropriate example is the enzyme alkaline phosphatase known to be a homodimer of M_r 80 000 with two catalytic centers. On radiation inactivation analysis, this enzyme appears as a functional unit of M_r 40 000 (Shorr et al., 1983).

As reviewed elsewhere (Stiles et al., 1984), considerable evidence has been presented that suggests that receptor interactions with components of the GTP-binding regulatory protein are part of the initial steps in β-adrenergic agonist induced adenylate cyclase modulation. The mechanisms and stoichiometry of this interaction are not yet understood. In earlier reports, purified receptors prepared in digitonin from several sources were shown to contain sufficient information to modulate adenylate cyclase activity in reconstitution assays (Cerrione et al., 1983). Although reassociation of receptors to oligomeric complexes is possible in the reconstitution system studied, it is more likely that the receptor functions as a monomer. This is suggested by the relatively low concentrations of receptor (picomolar) employed in the reconstitution assays. Furthermore, the membrane vesicles are likely to result in an environment not dissimilar to that of digitonin, and digitonin-solubilized receptors from sources similar to those used in the present studies have been shown to behave as monomers in detergent. Given the differences in molecular weight and glycosylation previously reported (Stiles et al., 1984), if reassociation to oligomers were also required, differences in oligomeric structure might also be expected. Nonetheless, receptor solubilized and purified in digitonin has been determined to be a monomeric chain in detergent, is fully functional in its ligand binding properties (Shorr et al., 1981, 1982b), and can be reconstituted with adenylate cyclase (Cerrione et al., 1983). This suggests the possibility that β-adrenergic receptors are active in the membrane as a single polypeptide chain.

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