Henderson, R., & Unwin, P. N. T. (1975) *Nature (London) 257*, 28-32.
Henselman, R. A., & Cusanovich, M. A. (1974) *Biochemistry 13*, 5199-5203.
Jan, L. Y., & Revel, J. P. (1974) *J. Cell Biol. 62*, 257-273.
Kimura, K., Mason, T., & Khorana, G. (1982) *J. Biol. Chem. 257*, 2859-2867.
Levy, H. B., & Sober, H. A. (1960) *Proc. Soc. Exp. Biol. Med. 103*, 250-258.
MacKenzie, D., & Molday, R. S. (1982) *J. Biol. Chem. 257*, 7100-7105.
McCaslin, D., & Tanford, C. (1981) *Biochemistry 20*, 5207-5212.
Michel-Villaz, M., Saibil, H. R., & Chabre, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4405-4408.
Molday, R. S., & Molday, L. L. (1979) *J. Biol. Chem. 254*, 4653-4660.
Pober, J. S., & Stryer, L. (1975) *J. Mol. Biol. 95*, 477-481.
Röhlich, P. (1976) *Nature (London) 263*, 789-791.
Sale, G. J., Towner, P., & Akatar, M. (1978) *Biochem. J. 175*, 421-430.
Shichi, H. (1981) *Curr. Top. Membr. Transp. 15*, 273-289.
Smith, H. G., Stubbs, G. W., & Litman, B. J. (1975) *Exp. Eye Res. 20*, 211-217.
Steineman, A., & Stryer, L. (1973) *Biochemistry 12*, 1499-1502.

Purification and Characterization of the Mammalian β_2-Adrenergic Receptor†

Charles J. Homcy,* Stanley G. Rockson, Janice Countaway, and David A. Egan

ABSTRACT: Conditions for the effective solubilization and assay of the mammalian β-adrenergic receptor from canine lung were determined. The detergent to protein ratio and the absolute detergent concentration were key factors in releasing the membrane-bound receptor in high yield and in permitting detection of the solubilized receptor by a direct binding assay. Scatchard analysis of equilibrium binding utilizing [^{3}H]dihydroalprenolol showed a single class of binding sites with an affinity (K_D = 1.3 nM) identical with that of the membrane-bound receptor. Stereospecificity and agonist affinity were comparable and characteristic of the β_2 subtype. New affinity supports consisting of alprenolol, acebutolol, or nadolol, linked to agarose via different-length spacer arms and substituted in the 1-6 mM range, were synthesized. By comparison with values obtained from direct ^{14}C-labeled ligand incorporation, radioimmunoassay indicated that only 10-20% of the resin-immobilized ligand was available for binding. Each of the three affinity supports adsorbed the receptor, but biospecific elution was consistently achieved only from the resin substituted with acebutolol, the ligand that exhibited the lowest intrinsic affinity (K_D = 170 nM) for the receptor. Purified receptor from canine lung eluted from an AcA34 column equilibrated in 0.1% digitonin, with a Stokes radius of 49 Å. The sedimentation coefficient of the receptor–digitonin complex was measured at 7.1 S by utilizing 5-20% sucrose gradients. The entire yield of purified receptor from an entire lung was then subjected to sodium dodecyl sulfate gel electrophoresis under reducing conditions. A major band of 52 000-53 000 daltons was visualized, and coincident binding activity was detected with the use of the photoaffinity label [^{3}H]acebutolol azide, indicating that this subunit contains the hormone-binding site.

The pharmacologic properties of the β-adrenergic receptor have been extensively investigated. Over the past several years, several groups have had success in solubilizing and assaying β-receptor activity in solution (Caron & Lefkowitz, 1976; Vauquelin et al., 1977; Kleinstein & Glossman, 1978; Schocken et al., 1980). Other investigators (Haga et al., 1977; Strauss et al., 1979) have attempted to label the receptor prior to solubilization because they were unable to measure specific binding in the presence of detergent. Two groups (Limbird & Lefkowitz, 1977, 1978; Vauquelin et al., 1979) have explored the molecular characteristics of the receptor solubilized from either turkey or frog erythrocyte. Shorr et al. (1981) have recently reported on several of the biophysical parameters of a purified receptor preparation from frog erythrocyte. However, less progress has been achieved in characterizing the mammalian β-adrenergic receptor. In this paper, we detail the optimal conditions that permit the solubilization and characterization of the β-adrenergic receptor from canine lung. A new affinity purification procedure is described that employs a precursor of the β antagonist, acebutolol, and several of the physical properties of the highly purified receptor are reported.

Experimental Procedures

Materials

(−)- and (+)-alprenolol tartrate were the kind gifts of Ayerst and Hassle. [^{14}C]Alprenolol was donated by Hassle, as well. (+)- and (−)-propranolol were generously provided by ICI, Ltd., England. Acebutololamine and the ^{14}C-labeled derivative were the generous gifts of Dr. Wooldridge of May and Baker, Ltd. (±)-Nadolol and the ^{14}C-labeled derivative were kindly provided by Dr. Bruce Migdalof of Squibb. (−)-Isoproterenol, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, poly(ethylene glycol) 8000, bovine γ-globulin, bovine serum albumin (fatty acid free), Tris-HCl,[1] lysozyme, and sodium periodate were obtained from Sigma. 3-Iminobis(propylamine) was purchased from Pfaltz and Bauer. ϵ-Aminocaproic acid, succinic anhydride, *N*-acetylhomocysteine

† From the Cellular and Molecular Research Laboratory, Cardiac Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114. *Received July 27, 1982.* Supported in part by American Heart Association Established Investigatorship 80-148 (C.J.H.), AHA Grant in Aid 79-1016, AHA Multidisciplinary Training Grant HL-07208, Program Project Grant HL-19259-07, and a grant from R. J. Reynolds Industries, Inc.

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0006-2960/83/0422-0660$01.50/0

thiolactone, iodoacetamide, adipic dihydrazide, 1,6-diaminohexane, and sodium cyanohydridoborate of the highest grade possible were purchased from Aldrich.

Several preparations of digitonin of the highest grade were tried (cf. Results) and were purchased from Sigma (80% pure), Kodak (reagent grade), and Fisher (Fisher reagent grade). Agarose (Sepharose 4B) and Sephadex G-50 were obtained from Pharmacia. AcA34 was purchased from LKB. CNBr was obtained from Kodak. Protosol, Aquasol II, and [^{3}H]-dihydroalprenolol ([^{3}H]DHA) were purchased from New England Nuclear (28–49 Ci/mmol). Whatman GF/C filters were obtained from Scientific Products. Hydrofluor was obtained from National Diagnostics Laboratory.

Lubrol-PX was purchased from Sigma. Nonidet P40 was kindly provided by Dr. S. Wrenn of the University of Pennsylvania. Brij 35, 56, and 58 were provided as gifts by ICI America. Deoxycholate was obtained from Sigma. Bovine serum albumin (fatty acid free), rabbit γ-globulin, aldolase, cytochrome *c*, ribonuclease, myoglobin, ovalbumin, trypsinogen, β-lactoglobulin, and thyroglobulin (employed as standards for gel filtration, sucrose gradient centrifugation, or $NaDodSO_4$ gel electrophoresis) were obtained from Sigma or Pharmacia. Recrystallized $NaDodSO_4$ (electrophoresis grade), Coomassie blue, and bromophenol blue were purchased from Aldrich.

Methods

Membrane Preparation. Lungs were excised from pentobarbital-anesthetized dogs and immediately placed in 1 mM ice-cold $KHCO_3$, 1 mM $MgCl_2$, and 0.25 M sucrose, pH 8.5 (buffer A). Hilar vessels, bronchi, and pleura were discarded, and the parenchyma was minced in 5 volumes of buffer A. The mixture was then homogenized in a large Polytron tissue disruptor at setting 8 for 3 s and then at setting 6 for 10 s. The homogenate was diluted 2–3-fold and filtered through two layers of Japanese silk screen. This was spun at 2000 rpm in a Sorvall RC-2 centrifuge (SS34 rotor) for 10 min. The supernatant was then respun at 18 000 rpm for 20 min. The pellet consisted of two layers. The fluffy white upper layer was removed, respun, and resuspended in 100 mM Tris, 5 mM EGTA, and 1 mM $MgCl_2$, pH 7.4 (buffer B), and stored in liquid N_2 routinely for 4–6-week periods.

Receptor Assay—Particulate. One hundred microliters of membrane in buffer B, 25 μL of buffer or a competing unlabeled ligand, and 25 μL of ^{3}H-labeled ligand were incubated for 30 min at 22 °C. Separation of bound and free ligand was performed by vacuum filtration with Whatman GF/C filters.

Solubilization of Receptors. Solubilization was initiated by adding the digitonin solution to the membrane pellet, followed by gentle homogenization (three strokes) in a Dounce homogenizer. The mixture was gently rotated at room temperature for 1 h at 22 °C. Centrifugation was then routinely carried out at 40000*g* for 30 min at 4 °C in a Sorvall RC-2 centrifuge. When the supernatant was spun at 100000*g* for 1 h (Beckman ultracentrifuge), no additional precipitation of receptor activity occurred. Solubilized receptor was assayed by incubating 400 μL of supernatant with 50 μL of ^{3}H-labeled ligand and 50 μL of either a competing ligand or buffer. Specific [^{3}H]DHA binding was defined as that binding inhibited by 100 μM (−)-isoproterenol or 1 μM (−)-propranolol.

Following a 1-h incubation at 22 °C, the tubes were placed on ice, and 1.0 mL of an ice-cold solution of bovine γ-globulin (1 mg/mL) and 1.0 mL of an ice-cold solution of 25% poly(ethylene glycol) 8000 (PEG) in buffer B were added. The tubes were vortexed after each addition and then allowed to stand on ice for 10 min. These solutions were then poured onto Whatman GF/C filters under vacuum and washed with three 3-mL aliquots of 8% PEG in buffer B. Multiple detergents (cf. Materials) were tried, and specific binding was assessed both by the above technique and by separation on small G-50 columns as employed by Caron & Lefkowitz (1976) and Vauquelin et al. (1977). No differences were found. Specific receptor binding was detected only with digitonin. The filters were added to Hydrofluor (10 mL) and vortexed for 20 s prior to liquid scintillation counting at an efficiency of 33%.

Saturation binding studies were analyzed by the method of Scatchard (1949). Dissociation constants were calculated from competitive inhibition studies by the method of Cheng & Prusoff (1973).

Synthesis of Bromoalprenolol. (−)-Alprenolol tartrate (30.0 mg), to which 1.1×10^6 cpm of [^{14}C]alprenolol was added, was extracted into ethyl acetate (3.0 mL) after first being dissolved in H_2O (3.0 mL) that was made basic (pH 12) by the addition of 2 N NaOH. The extraction was repeated 3 times, and the extracts were pooled and then dried by rotoevaporation. The residue was then redissolved in H_2O (10 mL) by bringing the pH to 5.5 with 0.5 N HCl. The solution was then cooled on ice, and 30.0 mg of *N*-bromosuccinimide was added and the reaction allowed to proceed for 48–72 h at 4 °C with shaking. The product was extracted back into ethyl acetate after the pH had been brought to 12 with 2 N NaOH. The ethyl acetate phase was then dried by rotoevaporation after drying over $MgSO_4$. This is a modification of the method of Vauquelin et al. (1977). The product was evaluated by thin-layer chromatography (TLC) that employed a toluene–2-propanol–NH_4OH (85:14:1) system in which alprenolol migrated with an R_f of 0.43 and bromoalprenolol with an R_f of 0.25 (with a solvent front of 10 cm). Both by visual inspection on fluorescent TLC plates and by quantitative analysis (Galbraith Laboratories), the reaction had reached completion.

Alprenolol–Agarose. Fifty milliliters of washed Sepharose 4B was activated as described by Cuatrecasas (1970) by employing CNBr (11.1 g) and then reacted with 13.1 g of 3-iminobis(propylamine) overnight at 4 °C with gentle shaking. The resin was then washed sequentially with 1 L of 0.3 M $NaHCO_3$, pH 8.3, 1 L of 1 M NaCl, and 1 L of distilled H_2O. Five milliliters of the resin was then equilibrated in 0.3 M $NaHCO_3$, degassed under vacuum, and reacted with 100 μmol of *N*-acetylhomocysteine thiolactone under N_2 at 22 °C. After 1 h, 100 μmol of dithiothreitol was added. Fifteen minutes later, the resin was filtered and rapidly washed with distilled H_2O. The packed resin was added to a vial that contained 30.0 mg of bromoalprenolol (dried under N_2). Five milliliters of 0.3 M $NaHCO_3$, pH 8.3, previously degassed, was added, and N_2 was then bubbled through the mixture. The vial was sealed under N_2 and allowed to react for 72 h at 4 °C.

Nadolol–Agarose. Nadolol (1.1 mmol) was dissolved in ice-cold H_2O and the pH brought to 7.0. $NaIO_4$ (1.0 mmol) was added and the reaction allowed to proceed for 1 h in the dark on ice.

This solution was then added to either of two agarose–amine resins, the syntheses of which will be described. (A) 3-Iminobis(propylamine)–agarose (10 mL) was suspended in 0.2 M potassium borate, pH 9.5. Succinic anhydride, 6.0 mmol, was added in portions over a 15-min period, and the pH was maintained at 9.0–9.5 by the addition of 8 N NaOH. The reaction was allowed to proceed for 4 h and was judged to be complete by a negative ninhydrin test. This resin was then washed in 0.1 M NaOH. Adipic dihydrazide was then coupled as described below. (B) Fifty milliliters of CNBr-activated agarose was reacted with 13.1 g of ϵ-aminocaproic acid. Ten

milliliters of the resulting resin was reacted in H_2O, pH 4.75, with adipic dihydrazide (2 mmol) in the presence of 0.4 M 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide hydrochloride. The pH was maintained at 4.75 for 2 h by the addition of 2 N HCl.

The oxidized nadolol was coupled to the hydrazide terminus through the formation of a Schiff base, which was reduced in the presence of $NaCNBH_3$ (Borch et al., 1971). Ten milliliters of either resin was incubated with 1 mmol of $NaIO_4$-oxidized nadolol in a 0.3 M sodium phosphate buffer, pH 6.5, in the presence of 1 mmol $NaCNBH_3$. The reaction was allowed to proceed for 16 h at 4 °C. The resin was thoroughly washed with 2 M NaCl–distilled H_2O and resuspended in the appropriate buffer.

Acebutolol–Agarose. Succinic anhydride was coupled to 3-iminobis(propylamine)–agarose as described. Five milliliters of this resin was then suspended in 5 mL of H_2O. Acebutololamine (0.7 mmol) and carbodiimide (1.0 mmol) were added, and the pH was titrated and maintained at 4.75 with 2 N HCl over the next hour. A second aliquot of carbodiimide was then added and the pH maintained at 4.75 for a second hour. The resin was then thoroughly washed as described above.

Washing Procedure. We adopted the policy of washing each resin in dioxane–H_2O (1:1) prior to use because we found that further unreacted ligand was removed even after extensive washing with salt solutions had been carried out.

Radioimmunoassay. A double antibody-precipitation technique was employed. A 100-μL aliquot of anti-acebutolol antiserum (1:1 dilution), 50 μL of 8 nM [^{3}H]DHA, and 50 μL of either ligand or resin at various dilutions were incubated for 2 h at 22 °C to ensure that equilibrium had been achieved. Antibody-bound ligand was precipitated by the addition of goat anti-rabbit γ-globulins as previously described (Rockson et al., 1980).

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951) or for the purified receptor preparation by automated amino acid analysis (Durrum analyzer) following dialysis against distilled H_2O, followed by lyophilization and hydrolysis in constant boiling 6 N HCl in evacuated tubes.

Preparation of Digitonin Solutions. The ability to dissolve digitonin and to maintain it in solution at 4 °C was partly related to the source and purity of the digitonin. Two techniques were employed to prepare stable solutions. The first method employed ultracentrifugation to remove any particulate material from a 1% digitonin–H_2O mixture. The supernatant was then lyophilized. The resulting powder would easily dissolve to form clear solutions even at a 1.2% concentration. The second technique involved heating the digitonin solution to 90–95 °C. A precipitate formed in digitonin solutions of greater than 0.1% when stored at 4 °C for more than 36 h.

Iodination of Standards. These were carried out as previously described according to the method of Marchalonis (1969).

Gel Filtration Chromatography. A 1 m × 2.0 cm or a 1 m × 2.5 cm Kontes column was packed with AcA34 preequilibrated with buffer B and 0.1% digitonin at 4 °C. The column was eluted with 4 column volumes prior to use. Fractions (160) were collected over a 10-h period under a constant pressure head of 1 m and a flow rate of 40 mL/h.

Sucrose Gradient Centrifugation. (*A*) *Method I.* Aliquots (0.5 mL) of purified receptor preincubated with 4.0 nM [^{3}H]DHA, with or without 1.0 μM propranolol, were applied to a 10.0-mL (5–20%) sucrose gradient in buffer B and 0.1% digitonin with standards in the same tube and spun for 15 h at 2–3 °C in an SW-41 rotor at 38 000 rpm. Sixty fractions were collected. The positions of the standards were determined by absorbance at 280 nm and, also, for hemoglobin and myoglobin at 550 and 580 nm, respectively. In certain cases, the standards had been iodinated and their positions determined by γ counting. Samples (0.2 mL) were then collected by puncturing the nitrocellulose tubes from below with an 18-gauge hooded needle and by using a Hoefer density-gradient fractionator. An identical aliquot of each fraction (50%) was then removed for liquid scintillation counting. This method provided excellent resolution and was routinely employed in the experiments detailed below.

(*B*) *Method II.* Aliquots (1.0 mL) of purified receptor were applied to a 30-mL (5–20%) sucrose gradient in buffer B and 0.1% digitonin with the indicated standards and spun for 36 h at 22 °C at 27 000 rpm in an SW-27 rotor. Direct-binding assays were performed on each fraction collected. However, the peaks were broad, and a relatively long time was required for significant movement of the protein peaks.

Photoaffinity Labeling. Acebutololamine was custom labeled by tritium exchange by ICN (Chemical and Radioisotope Division, Irvine, CA) to a specific activity of 8 Ci/mmol. The starting material was then converted to the azide, and its purity and identity were confirmed by thin-layer chromatography as previously described (Wrenn & Homcy, 1980). One picomole of purified receptor was incubated with [^{3}H]acebutolol azide (100 nM) for 2 h at 22 °C and then photolyzed for a 90-s interval followed by a 30-s interval. The photolysis chamber consisted of concentric glass cylinders, was cooled by circulating water at 4 °C in the inner jacket, and contained the receptor preparation in the outer jacket. The light source was a 450-W Hanovia lamp at a distance (radius) of 3 cm. Following photolysis, the preparation was dialyzed in 6 L of 10 mM Tris, pH 7.4, which was changed 3 times over a 24-h period. The material was then lyophilized in preparation for $NaDodSO_4$ gel electrophoresis.

$NaDodSO_4$ Gel Electrophoresis. A vertical-slab apparatus (Bethesda Research Lab) was employed essentially as described by Laemmli (1970). A 10% running gel and a 3% stacking gel were employed. The running buffer included 25 mM Tris, 192 mM glycine, and 0.1% $NaDodSO_4$, pH 8.3. The gel dimensions were 16 cm × 13 cm × 1.5 mm. Lyophilized samples were brought up in 50–100 μL of a solution including 10% (w/v) $NaDodSO_4$, 25% mercaptoethanol, 50% glycerol, 0.25 M Tris, pH 6.8, and 0.05% of the tracking dye bromophenol blue and heated in boiling water for 3 min. Electrophoresis was then carried out for 18 h at 45 mA. The gel was removed and placed directly in a fixing solution consisting of 11.4% (w/v) trichloroacetic acid and 30% (w/v) methanol for 1 h, allowed to stain for 18 h in 0.27% (w/v) Coomassie blue, 50% methanol, and 0.22% glycerol, and then progressively destained in a solution of 10% acetic acid and 30% methanol. Thereafter, the gel was transferred to a preserving solution containing 20% glycerol prior to drying on a Bio-Rad gel drier (Model 224).

Receptor preparations previously photolyzed with [^{3}H]-acebutolol azide were run in a similar manner. However, after destaining, these gels were cut into 3 mm wide sections and incubated with Protosol (New England Nuclear) for 1 h at 65 °C. Glacial acetic acid (100 μL) was added, followed by the addition of Aquasol II (10 mL). The counting efficiency was 30%. ^{14}C-Labeled standards were run in parallel tracks.

The apparent molecular weight of both the stained and radiolabeled receptors was determined by comparing their

Table I: Effect of Varying Digitonin/Protein Ratios and Digitonin Concentrations on Receptor Solubilization[a]

total protein (mg)	total digitonin (mg)	D/P[b] (mg/mg)	D[c] (%)	total vol (mL)	soluble protein (mg/mL)	total soluble protein (mg)	total binding (cpm)	nonspecific binding (cpm)	specific binding	solubilized receptor concn (fmol/mg)	total solubilized receptors (fmol)
						Procedure A[d]					
18.3	8.6	0.47	0.14	6	0.68	4.08	179 ± 7	126 ± 2	43	8.3	34
18.3	25.9	1.41	0.42	6	0.94	5.64	813 ± 42	174 ± 3	639	73.0	412
18.3	51.7	2.83	0.84	6	1.03	6.18	555 ± 16	98 ± 1	457	48.0	297
18.3	86.0	4.71	1.40	6	1.09	6.54	457 ± 12	111 ± 5	346	34.0	222
						Procedure B[e]					
21.6	21.6	1.0	0.42	5	0.57	2.85	303 ± 16	130 ± 9	173	33.0	94
21.6	32.4	1.5	0.65	5	1.19	5.95	519 ± 33	277 ± 22	242	22.0	131
21.6	32.4	1.5	0.32	10	0.69	6.90	710 ± 57	164 ± 29	546	85.0	587

[a] Binding activity was measured at a single [^{3}H]DHA concentration of 4.0 nM. [b] D/P = digitonin/protein ratio. [c] D = digitonin. [d] A constant amount of membrane protein (18.3 mg) was solubilized as described under Methods with varying amounts of detergent. The specific activity of the membrane preparation used in these experiments was 55 fmol/mg of membrane protein, calculated at a [^{3}H]DHA concentration of 4 nM. [e] A constant amount of membrane protein was solubilized at a D/P of either 1.0 or 1.5. Furthermore, at a D/P ratio of 1.5, two digitonin concentrations of either 0.65 or 0.32 (twice the volume) were employed.

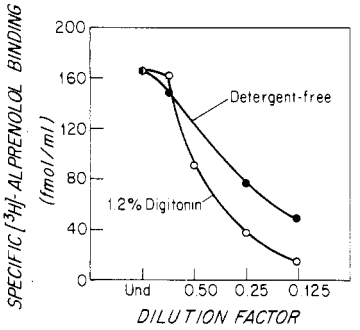

FIGURE 1: Pulmonary membranes were solubilized in 1.2% digitonin at a digitonin/protein ratio of 1.5. Thereafter, the supernatant was diluted with 1.2% digitonin buffer or with detergent-free buffer to progressively lower digitonin concentrations. Specific [^{3}H]DHA binding was determined as described under Methods. Masking of receptor binding following dilution in the 1.2% digitonin buffer is apparent when compared with dilution in a detergent-free buffer.

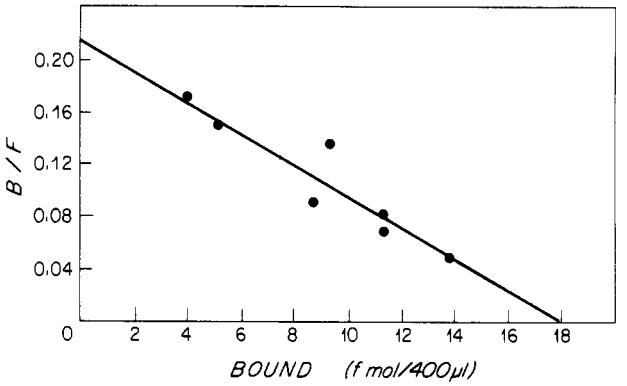

FIGURE 2: Scatchard analysis of an equilibrium binding assay was performed by employing 400 µL of a 0.3% solubilized digitonin–membrane preparation (0.20 mg/mL) and 50 µL of [^{3}H]DHA in final concentrations ranging from 0.35 to 3.0 nM. The K_D was 0.9 nM, and the receptor concentration was 196 fmol/mg, r = 0.93.

relative mobilities to those of the protein standards. R_f vs. log molecular weight was plotted. In each case, the r value was at least 0.98.

Results

The conditions promoting the best yield during detergent solubilization were investigated. These included the digitonin to membrane protein ratio, percent digitonin solution, and the final volume. Total protein, total receptor, receptor per milligram of protein, and yield were quantitated. The results of two such experiments are shown in Table I. A ratio approaching 1.5 mg of detergent/mg of protein appeared optimal in terms of specific binding (cpm), receptors solubilized per milligram of membrane protein, and total receptor yield. Effective solubilization and detection of receptors were achieved with detergent concentrations of 0.3–0.4% when the detergent/protein ratio was maintained at 1.5/1. The specific activity of the solubilized receptor (pmol/mg of protein) was equal to or greater than that of the starting membrane preparation. Solubilization under optimal conditions released approximately 50% of the receptor pool. Parallel experiments also indicated that higher digitonin concentrations, typically above 1.0%, masked specific [^{3}H]DHA binding. Figure 1 demonstrates that the dilution of a receptor preparation solubilized in 1.2% digitonin in a detergent-free buffer to progressively lower digitonin concentrations results in an apparent increase in specific [^{3}H]DHA binding when compared to dilution in 1.2% digitonin–buffer. In contrast, when an initial digitonin concentration of 0.3% was employed for solubilization, a parallel decrease in specific binding occurred upon dilution to lower detergent concentrations.

Characteristics of Solubilized Receptor Preparation: Comparison with the Particulate Form. Equilibrium binding experiments employing pulmonary membranes prepared and assayed as described under Methods were analyzed by the method of Scatchard (1949). Typically, these membranes possess 150–250 fmol of receptor/mg of membrane protein although certain preparations have assayed only in the 75–100 fmol/mg range. Little variability has been observed in terms of the affinity with preparations demonstrating an average K_D for (−)-alprenolol of 1.3 nM (SEM = 0.5, N = 7). (−)-Propranolol was 100-fold more potent than the (+) isomer. Agonist affinity for (−)-isoproterenol is in the range of 200 nM.

Parallel studies of the solubilized lung receptor from the same membrane preparation were also undertaken. Scatchard analysis (Figure 2) indicated a receptor K_D of 1.3 nM (SEM = 0.35, N = 5), an affinity identical with the particulate form. Solubilized receptor concentrations typically ranged from 150 to 300 fmol/mg and generally reflected that of the membrane preparation. Stereospecificity and agonist affinity were identical with those seen for the particulate receptor (Figure 3). Acebutolol has an affinity similar to that previously observed in the rat reticulocyte (Wrenn & Homcy, 1980), which is 100-fold lower than that for either alprenolol or propranolol. The rank order of potency of isoproterenol >

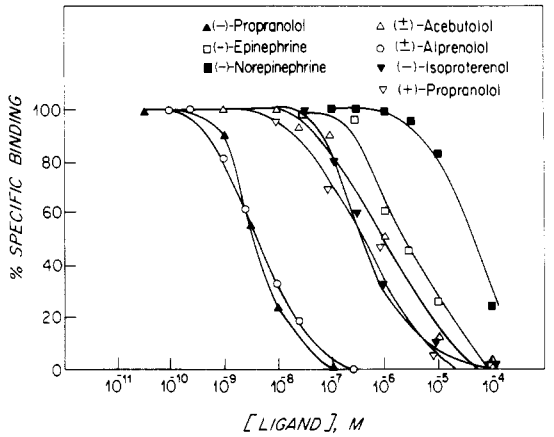

FIGURE 3: Competitive inhibition curves with the solubilized β receptor employing [^{3}H]DHA in a final concentration of 2.5 nM.

Table II: Purification of the β Receptor[a]

	protein (mg)	receptor concn (pmol/mg)	total receptors (pmols)	% yield	purification (x-fold)
starting particulate	946	0.226	214		
1st affinity resin	2.65	30.3	80.3	37	134
gel filtration	nd	nd	60.9	29	nd
2nd affinity resin	0.002 4	10 800	25.9	12	47 788

[a] Purification scheme for the β receptor from an entire dog lung. This protocol was carried out 3 times, yielding similar results. The receptor concentration was determined by saturation binding studies utilizing [^{3}H]dihydroalprenolol at each step except the last where the indicated value represents the receptor concentrations calculated at a [^{3}H]dihydroalprenolol concentration of 4 nM.

epinephrine > norepinephrine was similar in the membrane and soluble preparations and characteristic of the β_2 subtype.

Affinity Purification. The structure of each of these resins is detailed in Figure 4. On the basis of ^{14}C-labeled ligand incorporation, each of the affinity resins was substituted in the 1–6 mM range. However, quantitation by radioimmunoassay employing an antibody raised against acebutolol and that recognizes a broad range of β antagonists (Rockson et al., 1980) indicated a 5–10-fold lower substitution for each of the ligands, indicating that only 10–20% of the bound ligand was available to the macromolecule. This latter measurement may provide a better estimate of available ligand substitution in terms of the resin's utility as an affinity support. The capacity of each of these resins to adsorb solubilized receptor was assayed by incubating 5 mL of a 0.3% digitonin extract with 0.5–1.0 mL of each of the resins in a batchwise fashion for 1 h at 22 °C. The material was then centrifuged at 2000g in a desk-top rotor and the supernatant removed for assay. As a control, succinylated diaminobis(propylamine)–agarose was similarly treated and the supernatant assayed for receptor activity. The resins were then washed 3 times with 20 mL of 0.1% digitonin–buffer B. The UV absorbance at 254 and 279 nm in the third wash was typically less than 0.01 unit. Desorption with 3.0 mL of the eluting buffer containing 1 mM (−)-alprenolol was carried out by incubation at 22 °C for 2 h. Concentrations of 0.1% digitonin were maintained. Excess ligand from the desorbing solution was removed by dialysis in 4 L of digitonin–buffer B, changed every 8 h over a 24-h period prior to assay. Each of the three resins adsorbed 80–90% of receptor activity from the supernatant after a 1-h incubation period. In contrast, the control resin absorbed less than 5% of the solubilized receptor. However, elution of the receptor in high yield (60–80%) was consistently achieved only from the acebutolol resin. High ionic strength (3 M NaCl) and ethylene glycol in concentrations up to 30% did not promote further elution of receptor from any of the three resins. Prior experiments did indicate that receptor activity was stable to treatment with both of these agents.

The results of a large-scale multistep purification are seen in Table II. This entire procedure was carried out over a 3-day period. All steps except the affinity procedures were performed at 4 °C. Protein content of the purified receptor preparations was determined with a Durrum amino acid analyzer, sensitive to the 100-pmol range. The particulate preparation (946 mg) was solubilized in 473 mL of a 0.3% digitonin–buffer B solution, yielding a receptor concentration of 231 fmol/mL (268 fmol/mg of solubilized protein). The solubilized receptor preparation was incubated by gentle

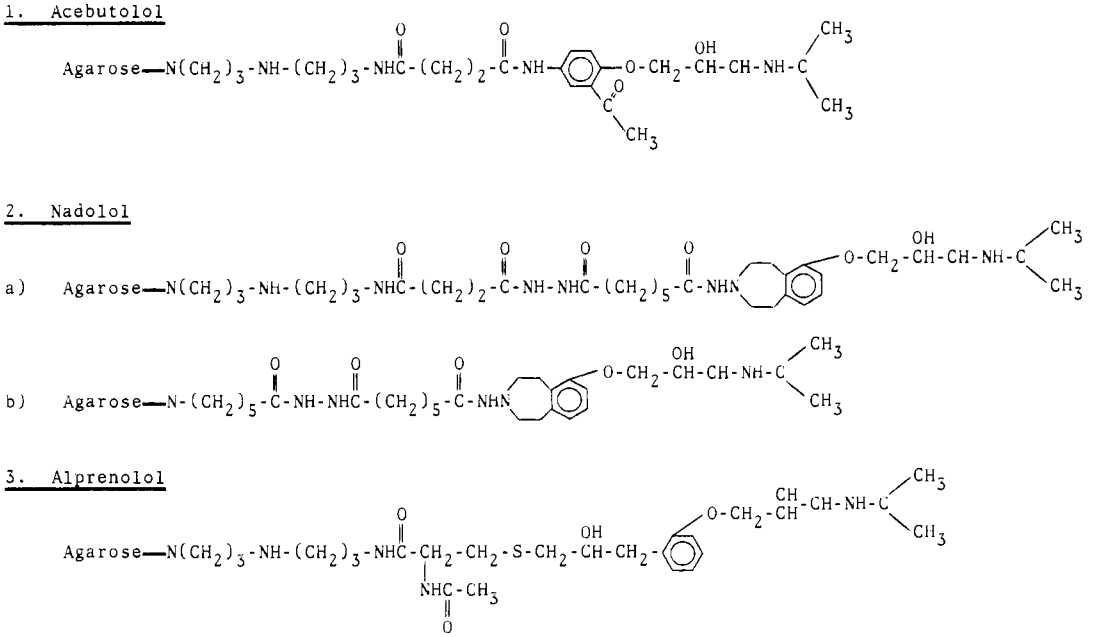

FIGURE 4: Structure of affinity resins whose syntheses are described under Methods.

Table III: Comparison of Affinities of Membrane-Bound, Solubilized, and Purified β_2-Adrenergic Receptor for Various Adrenergic Ligands[a]

	membrane	soluble	purified
(−)-alprenolol	1.3	1.3	
(−)-propranolol	6.8	1.4	2.2
(+)-propranolol	284	365	220
(−)-isoproterenol	130	146	104
(−)-epinephrine	2 500	1 000	2 100
(−)-norepinephrine	25 000	20 000	23 000

[a] Affinity constants (K_D) were determined for the particulate and solubilized preparations by Scatchard analysis. All other values were calculated from competitive inhibition studies utilizing the method of Cheng & Prusoff (1973). A K_D of 1.3 nM for [^{3}H]dihydroalprenolol was used in calculating the affinity constants for the purified receptor. In all of the competitive inhibition analyses, [^{3}H]dihydroalprenolol concentrations ranging from 2 to 4 nM were employed. The values indicated here are from a single experiment in which the same membrane preparation served as the source of solubilized and purified receptor and is representative of three separate experiments.

shaking with 40 mL of acebutolol–agarose for 2 h at room temperature, the supernatant was removed, and the resin was then washed 3 times with 200 mL of 0.1% digitonin–buffer B (total wash 600 mL). Elution was then carried out by incubation with 40 mL of 0.1% digitonin–buffer B containing 1 mM (−)-alprenolol for 2 h at room temperature.

The supernatant was then removed and the resin was eluted a second time; these fractions were then combined and concentrated to 8.0 mL in an Amicon stirred cell with a PM-10 membrane. This material was then applied to an AcA34 column (1 m × 2.0 cm) and eluted in 0.1% digitonin–buffer B. The fractions containing receptor activity were identified by direct-binding assay, combined, and reapplied to the acebutolol–agarose resin. The washing and elutions procedures were repeated, and the eluted receptor was then dialyzed extensively in digitonin–buffer B prior to assay. Portions of this material were employed in the binding experiments described below. Identically prepared receptor was also employed for determination of the Stokes radius and sedimentation velocity and for the NaDodSO$_4$ gel electrophoresis and photoaffinity labeling experiments.

The purified receptor demonstrated binding characteristics similar to both the particulate and solubilized preparations. Table III compares the dissociation constants for a variety of β agonists and antagonists for the starting particulate and the solubilized and purified β-receptor preparations. It is apparent that all of the appropriate binding properties including stereospecificity and the expected rank order of potency are maintained throughout the solubilization and purification procedures.

The Stokes radius and sedimentation velocity ($s_{20,w}$) of this highly purified receptor preparation were then measured. For the gel filtration experiments, the resin AcA34 provided better resolution of the protein standards in the general molecular weight (M_r) range of 50 000–600 000 than did Sepharose 6B. The receptor eluted as a discrete peak just prior to the peak of the standard aldolase, which is a globular protein of M_r 160 000. Excellent recovery of applied receptor was obtained. The receptors consistently eluted with a calculated Stokes radius of 49.2 ● 0.7 Å (n = 4). Centrifugation through a sucrose gradient was carried out to determine the sedimentation velocity ($s_{20,w}$) of the purified receptor protein. The receptor–digitonin complex consistently migrated with a sedimentation velocity of 7.1 S (Figure 5). With the use of the relevant equations,[2] the calculated M_r of the entire receptor–digitonin complex was 150 000, and the frictional ratio was calculated to be 1.4.

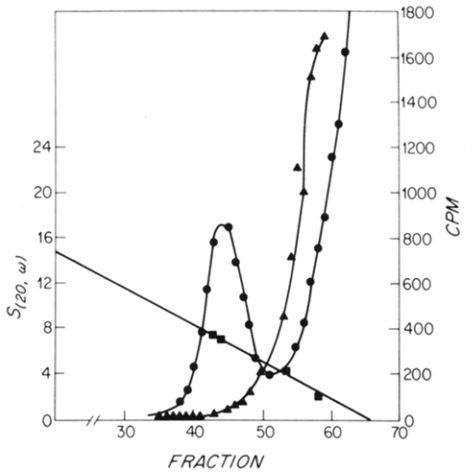

FIGURE 5: Sucrose gradient centrifugation was performed as described in the text (method 1). The standards (■) were run in the same tube as the receptor and included ferritin (fraction 8, not shown) 17.8 S, aldolase 7.6 S, rabbit γ-globulin 7.0 S, hemoglobin 4.1 S, and myoglobin 2.0 S. Similar results were obtained from three such experiments with an average $s_{20,w}$ of 7.1 ± 0.2. According to method II, the receptor sedimented with an s value just smaller than that of aldolase. Receptor was incubated with 4 nM [^{3}H]DHA in the absence (●) or presence (▲) of 1 μM (−)-propranolol.

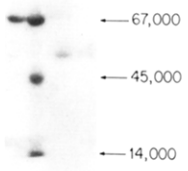

FIGURE 6: NaDodSO$_4$ gel of purified β receptor. Standards for M_r determination were run separately and in combinations, and included albumin 67 000, heavy chain of γ-globulin 52 000, ovalbumin 45 000, and ribonuclease 14 000. For visual comparison, the first track (left to right) includes albumin and the second track albumin, ovalbumin, and ribonuclease. 90% of the purified receptor preparation (3.0 μg) was applied to the third track, and 10% was applied to the last track. A major band was detected with a calculated M_r = 52 000 on the basis of its relative mobility. Refer to Methods for further details. This staining pattern was detected in three separate experiments.

As a last step in the characterization scheme, the purified receptor was sized by NaDodSO$_4$ gel electrophoresis following reduction as described under Methods. Three micrograms of protein was applied to a 3% stacking gel and a 10% separating gel and electrophoresed for 16 h at room temperature at 45 mA. A major band was consistently visualized with an M_r

[2] Molecular weight was calculated according to

$$M_r = [6\pi N\eta/(1 - \bar{v}\rho)]as_{20,w}$$

where N is Avogadro's number, η is the viscosity of water at 20 °C, ρ is the density of water at 20 °C, a is Stokes radius, $\bar{v}$ is the partial specific volume, and $s_{20,w}$ is the sedimentation coefficient. The frictional ratio was calculated according to

$$f/f_0 = a[4\pi N/(3M_r\bar{v})]^{1/3}$$

where a is Stokes radius, N is Avogadro's number, $\bar{v}$ is the partial specific volume, and M_r is the molecular weight.

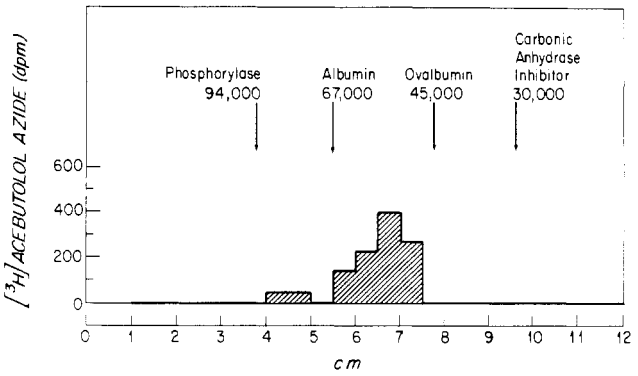

FIGURE 7: Purified receptor (1.0 pmol) was photolyzed with [^{3}H]-acebutolol azide (100 nM) in the presence or absence of 1.0 μM (−)-propranolol or 100 μM (−)-isoproterenol. The material was then treated as described under Methods and run parallel to the gel described in Figure 6. The gel was sliced into 3-mm sections and counted as described in the text. In the presence of 1 μM (−)-propranolol or 100 μM (−)-isoproterenol, the observed peak of binding activity was abolished with a background radioactivity of approximately 100 dpm uniformly distributed throughout the gel. The peak of specific radiolabeling was consistently detected in the molecular weight range 50000–55000 (three experiments). Refer to the text for further details.

of 52000–53000 (on three separate runs) (Figure 6).

In order to demonstrate that this band was coincident with receptor binding activity, it was necessary to employ [^{3}H]-acebutolol azide (100 nM) as a photoaffinity probe to label the purified receptor binding site. One picomole of the highly purified receptor was incubated with this concentration of [^{3}H]acebutolol azide for 2 h at 22 °C in the dark in the absence or presence of 1 μM (−)-propranolol or 100 μM (−)-isoproterenol and then exposed to long-wave UV light for 2 min. A gel of the same dimensions as used in the direct staining of the receptor subunit was cut and counted. Figure 7 demonstrates a peak of activity at a calculated M_r of 50000–55000. Similar results were obtained in three separate experiments. This binding was not apparent in samples in which 1 μM (−)-propranolol or 100 μM (−)-isoproterenol was present during photolysis.

Discussion

Although extensive progress has been made in the characterization of the β receptor isolated from either the frog or turkey erythrocyte, less work has been reported with the mammalian receptor. These investigations were aimed at the purification and characterization of the mammalian β_2 receptor of canine lung. Our first goal was to determine the optimal conditions for solubilization as well as to develop a reliable, simple assay for its detection. As reported by others, only in the presence of the detergent digitonin, a plant sterol, was direct assay of the soluble species possible. As reported by Haga et al. (1977), prelabeling of the receptor prior to solubilization allows specific binding to be detected in detergents such as Lubrol. However, the amount of specific binding is low, and such a procedure does not lend itself to the detection of the receptor during additional purification steps. Solubilization of the canine lung β_2 receptor was most efficiently achieved at detergent/protein ratios of 1.5 at 22 °C. At detergent concentrations above 0.3–0.4%, masking of receptor binding occurred; the mechanism of this effect may involve a decrease in the effective concentration of free [^{3}H]dihydroalprenolol at high digitonin concentrations.

Following solubilization and purification, the receptor displayed all of the characteristics expected of the β_2-adrenergic receptor. It demonstrated saturability and retained its high affinity for (−)-alprenolol (1–2 nM), which was identical with the membrane-bound form. Stereospecificity was retained following solubilization, with a greater than 100-fold higher affinity for the (−) isomer of propranolol. The rank order of potency for agonists (isoproterenol > epinephrine > norepinephrine) was maintained following solubilization and purification and was consistent with a β_2-receptor subtype. U'Prichard et al. (1978) have previously reported that mammalian lung principally contains the β_2 subtype, as determined with a wide variety of subtype-selective agonists and antagonists in direct-binding assays. However, Minneman et al. (1979a,b) subsequently reported the presence of mixed populations of β receptors in most mammalian organs through the use of computer-based iterative procedures to analyze competitive binding studies employing subtype-selective ligands. As noted above, the membrane, solubilized, and purified receptor preparations from canine lung consistently demonstrated the binding characteristics of the β_2 subtype. Furthermore, in our purified receptor preparation a single principal subunit was consistently detected. However, the copurification of a small quantity of contaminating β_1 receptor cannot be excluded.

The development of effective affinity supports was the next major goal following the successful solubilization and assay of the receptor. Although all three affinity supports adsorbed receptor, biospecific desorption by competition with 1 mM (−)-alprenolol was most consistently achieved from the acebutolol resin. This ligand possesses an affinity for the β_2 receptor that is approximately 50–100-fold lower than that of alprenolol or nadolol, the latter compound being a congener of propranolol (C. Homcy, unpublished observations).

The specific activity of the receptor at the last purification step was greater than 10800 pmol/mg of protein, which approaches the specific activity of the homogeneous receptor that can be calculated to be 18000 pmol/mg if a single subunit of 52000 daltons is capable of binding ligand. It should also be emphasized that the specific activity of our purified receptor preparation is an underestimate in that the calculation was based on receptor binding at 4 nM [^{3}H]DHA, a ligand concentration that results in only 75% occupancy of the receptor pool. There appear to be differences in the calculated molecular parameters for the purified receptor from lung and those reported for the β receptor from other tissue sources. The nondenatured lung β_2 receptor in detergent solution has a functional Stokes radius of 49 Å when compared to standard globular proteins that do not bind detergent. This value differs from those in previously published papers of the β_2 receptor from frog erythrocyte. Two different sizes for this receptor have been reported under different elution conditions. Caron & Lefkowitz (1976) first reported an apparent molecular weight (as estimated by gel filtration) for the β-adrenergic receptor from frog erythrocyte of 130000–150000. They reported the same elution pattern whether chromatographed in buffer alone or in 0.1% digitonin. Later, Limbird & Lefkowitz (1977) reported a larger apparent size for the receptor chromatographed in 0.2% digitonin with a K_{av} approaching that of ferritin, a globular protein of 440000 daltons. The purified β receptor from canine lung consistently elutes with a K_{av} just larger than that of aldolase, a protein with a Stokes radius of 48 Å and a molecular weight of 160000. Furthermore, we also assessed a similarly purified receptor from guinea pig lung, and it elutes with an identical Stokes radius. More recently, Sfiorr et al. (1981) have reported a sedimentation velocity of 9 S for the frog erythrocyte receptor, which is greater than the value of 7.1 S measured for the lung β_2 receptor.

It is not possible to calculate the actual amount of digitonin bound per mole of receptor and, therefore, the actual molecular weight of the receptor protein. For a variety of other membrane proteins, including hormone receptors, detergent binding of 0.2–1.0 mg/mg of protein has been reported (Clarke, 1975; Neer, 1976; Schneider et al., 1980; Guellaen et al., 1979; Smigel & Fleischer, 1979; Siegel et al., 1981). It appears that such proteins can be anchored to a detergent micelle in solution. For digitonin, the micellar weight is 70 000 (Helenius & Simons, 1975). Other membrane proteins may bind lesser quantities of detergent that are associated with hydrophobic surface residues of the molecule. Most commonly, detergent binding is calculated by the method of Clarke (1975) whereby the sedimentation behavior of the protein in D_2O and H_2O is compared to those of standard globular proteins. The difference observed depends on the binding of detergent by the membrane protein. On the basis of this difference and the known partial specific volume ($\bar{v}$) of the detergent, one can calculate the $\bar{v}$ of the receptor. However, digitonin has a $\bar{v}$ similar to that of proteins (Steele et al., 1978), and thus the above method for calculating the $\bar{v}$ of the receptor protein becomes inapplicable. An alternative method will depend on the future availability or synthesis of [^{3}H]digitonin for determination of bound detergent by direct-binding studies as described by Clarke (1975). Until the exact determination of bound detergent can be made, it is not possible to be certain of the subunit composition of the receptor under nondenaturing conditions. However, certain possibilities are suggested. On the basis of its Stokes radius and sedimentation velocity, the M_r of the receptor–digitonin complex is in the range of 150 000. If this protein binds detergent in the ratio reported for a variety of membrane proteins as described above, then it is most likely that the receptor contains more than one M_r 52 000 subunit. However, such a determination must await the results of additional experiments as suggested above.

Despite the relatively low K_D and specific activity of the photoaffinity label, [^{3}H]acebutolol azide, the availability of pure receptor in relatively high concentration allowed this ligand to be employed to label the receptor's binding site and confirms that the purified subunit detected by staining was coincident with hormone binding activity. A principal peak of activity was identified in the region corresponding to the molecular weight range 50 000–55 000, although we did note that specific labeling was detected over a broader range than that corresponding to the stained band. This may have partly reflected differences in the preparation of the gel for liquid scintillation counting.

Shorr et al. (1981) iodinated a highly purified β-receptor preparation from frog erythrocyte in an attempt to identify the subunit composition. Following $NaDodSO_4$ electrophoresis, they were able to recover 7–8% of the applied binding activity coincident with a broad 58 000-dalton band. Lavin et al. (1981) have also reported that photoaffinity labeling experiments of the frog erythrocyte receptor indicate that the hormone binding subunit is in the range of 58 000 daltons. Again a broad band was detected ranging from about 45 000 to 65 000 daltons both by scintillation counting of gel slices and by autoradiography of a purified receptor preparation. More recently, photoaffinity labeling has demonstrated that the molecular weights of the hormone binding components differ in receptor subtypes from different species. Rashidbaigi & Ruoho (1981) utilizing (azidobenzyl)[^{125}I]pindolol identified two binding components of 45 000 and 48 000 daltons in the duck erythrocyte β receptor. Burgemeister et al. (1982) have reported, utilizing a similar probe, that the turkey erythrocyte β_1 receptor is composed of two principal hormone binding subunits of 40 000 and 50 000 daltons.

In summary, the β_2 receptor from canine lung has been purified approximately 50 000-fold by utilizing a new affinity support. The physical properties in detergent solution have been characterized and a principal subunit has been identified that is coincident with hormone binding activity.

Acknowledgments

We thank Dr. Edgar Haber for the opportunity to perform these experiments and for invaluable discussion. In addition, we acknowledge J. Ryan and D. Sylvestre for excellent technical assistance, R. Rubin for editorial assistance, and K. Taylor for assistance in the preparation of the manuscript. We also thank John Wechsler of Dr. Haber's laboratory for performing the amino acid analyses.

References

Borch, R. F., Bernstein, M. D., & Durst, D. H. (1971) *J. Am. Chem. Soc. 93*, 2897–2904.

Burgemeister, W., Hekman, M., & Helmreich, E. J. M. (1982) *J. Biol. Chem. 257*, 5306–5311.

Caron, M. G., & Lefkowitz, R. J. (1976) *J. Biol. Chem. 251*, 2374–2384.

Cheng, Y., & Prusoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099–3108.

Clarke, S. (1975) *J. Biol. Chem. 14*, 5459–5469.

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059–3065.

Guellaen, G., Aggerbeck, M., & Hanoune, J. (1979) *J. Biol. Chem. 254*, 10761–10768.

Haga, T., Haga, K., & Gilman, A. G. (1977) *J. Biol. Chem. 252*, 5776–5782.

Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.

Kleinstein, J., & Glossman, H. (1978) *Naunyn-Schmiedebergs Arch. Pharmakol. 305*, 191–200.

Laemmli, V. K. (1970) *Nature (London) 227*, 680–685.

Lavin, T. M., Heald, S. L., Jeffs, P. W., Shorr, R. G. L., Lefkowitz, R. J., & Caron, M. G. (1981) *J. Biol. Chem. 256*, 11944–11950.

Limbird, L. E., & Lefkowitz, R. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 228–232.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marchalonis, J. J. (1969) *J. Biochem. (Tokyo) 113*, 299–305.

Minneman, K. P., Hegstrand, L. P., & Molinoff, P. B. (1979a) *Mol. Pharmacol. 16*, 34–46.

Minneman, K. P., Hedberg, A., & Molinoff, P. B. (1979b) *J. Pharmacol. Exp. Ther. 211*, 502–508.

Neer, E. J. (1976) *J. Biol. Chem. 251*, 5831–5834.

Rashidbaigi, A., & Ruoho, A. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1609–1613.

Rockson, S. G., Homcy, C. J., & Haber, E. (1980) *Circ. Res. 46*, 808–813.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Schneider, W. J., Goldstein, J. L., & Brown, M. S. (1980) *J. Biol. Chem. 255*, 11442–11447.

Schocken, D. D., Caron, M. G., & Lefkowitz, R. J. (1980) *J. Clin. Endocrinol. Metab. 50*, 1082–1088.

Shorr, R. G. L., Lefkowitz, R. J., & Caron, M. G. (1981) *J. Biol. Chem. 256*, 5820–5826.

Siegel, T. W., Ganguly, S., Jacobs, S., Rosen, O. M., & Rubin, C. S. (1981) *J. Biol. Chem. 256*, 9266–9273.

Smigel, M., & Fleischer, S. (1979) *J. Biol. Chem. 255*, 3689–3696.

Steele, J. C. H., Jr., Tanford, C., & Reynolds, J. A. (1978) *Methods Enzymol. 48*, 11-29.
Strauss, W. L., Ghai, G., Fraser, C. M., & Venter, C. J. (1979) *Arch. Biochem. Biophys. 196*, 566-573.
U'Prichard, D. C., Bylund, D. B., & Snyder, S. H. (1978) *J. Biol. Chem. 253*, 5090-5102.
Vauquelin, G., Geynet, P., Hanoune, J., & Strosberg, A. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3710-3714.
Vauquelin, G., Geynet, P., Hanoune, J., & Strosberg, A. D. (1979) *Eur. J. Biochem. 98*, 543-565.
Wrenn, S. M., Jr., & Homcy, C. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4449-4453.

Structure of Hen Phosvitin: A ^{31}P NMR, ^{1}H NMR, and Laser Photochemically Induced Dynamic Nuclear Polarization ^{1}H NMR Study†

Hans J. Vogel*

ABSTRACT: ^{1}H nuclear magnetic resonance (NMR) studies of hen egg yolk phosvitin (M_r = 35000) have confirmed earlier suggestions that the protein has a very flexible and open structure and is not compactly folded. More than half of a total 220 residues in this unusual protein are phosphoserine residues. ^{31}P NMR pH titration studies have shown that all of these are in the dianionic form at physiological pH and that they are exposed to solvent and can be protonated and deprotonated. The pK_as determined for several groups of resonances that could be resolved in the ^{31}P NMR spectra were all close to those observed for phosphoserine standards. The low Hill coefficient (n = 0.70) calculated for these titration curves indicates that the phosphoryl moieties influence each others titration behavior. Subsequent laser photochemically induced dynamic nuclear polarization (photo-CIDNP) ^{1}H NMR experiments suggest that the single tyrosine and tryptophan residues are both exposed to solvent. Surprisingly, all 11 histidine residues appear to be inaccessible because only very small CIDNP effects were observed for these residues. Since experiments with mixtures of aromatic amino acids indicated that the presence of tryptophan or tyrosine decreases the CIDNP intensities observed for histidine residues, CIDNP spectra for phosvitin were compared with those obtained for mixtures of aromatic amino acids resembling the composition of this protein. This demonstrated that the histidine residues are partially inaccessible in the intact protein, suggesting that the histidine residues may be protonated and hydrogen bonded to the phosphoryl moieties. This concept was further supported by the results of the ^{1}H NMR titration studies that indicated that all 11 histidines have an unusually high pK_a = 7.45 and a low Hill coefficient for titration of n = 0.80.

Both major egg yolk proteins phosvitin and lipovitellin are derived from a common precursor called vitellogenin. This protein is synthesized in the livers of all egg-laying animals. Its synthesis is under hormonal control; administration of estrogen has been reported to increase the rate of transcription (Tata, 1976; Ryffel, 1978). Vitellogenin undergoes extensive posttranslational modifications: phosphorylation, glycosylation, and attachment of lipid moieties. After synthesis and processing in the liver, vitellogenin is transported through the blood to the ovary where it is cleaved to yield the egg yolk proteins before they are deposited in the yolk (Tata, 1976; Ryffel, 1978). Each vitellogenin molecule gives rise to two phosvitin molecules (Deeley et al., 1975). Highly purified preparations of phosvitin all show some heterogeneity (Taborsky, 1974). This property appears to be mainly related to the existence of different vitellogenin genes (Wiley & Wallace, 1981). The extent of the posttranslational modifications appears to be constant at least in preparations of hen phosvitin (Taborsky, 1974). Phosvitin (M_r = 35000) has a very unusual amino acid composition (see Table I). More than half of the residues are phosphoserines, accompanied by a fairly large number of acidic amino acids. The basic amino acids that are present are insufficient to balance all the negative charges and thus the protein is a polyelectrolyte. Parts of the protein have been sequenced (Belitz, 1965). These studies have led to the surprising discovery that blocks of usually six but sometimes up to eight SerP[1] residues may occur in one stretch. Such a stretch is usually terminated by a basic residue. Moreover, these blocks of SerP residues are evenly spread throughout the

Table I: Amino Acid Composition of Hen Phosvitin[a]

residue	no. of residues/mol of protein	residue	no. of residues/mol of protein
Gly	7	Thr	5
Ala	7	Cys	0
Val	3	Met	1
Leu	3	Asx	13
Ile	2	Glx	13
Pro	3	His	11
Phe	2	Lys	17
Tyr	1	Arg	11
Trp	1	(P)	(112)
Ser	120	total	220

[a] The data are the average of the six independent determinations described by Taborsky (1974).

† From the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. *Received May 24, 1982; revised manuscript received October 27, 1982.* Supported by the Killam Memorial Scholarship Committee and the Alberta Heritage Foundation for Medical Research.

* Address correspondence to the author at the Department of Physical Chemistry 2, Chemical Center, Lund, S220-07, Sweden.

[1] Abbreviations: DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; SerP, phosphoserine residue; ThrP, phosphothreonine residue; CIDNP, chemically induced dynamic nuclear polarization; FMN, flavin mononucleotide; Trp, tryptophan; Tyr, tyrosine; His, histidine; CD, circular dichroism; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/83/0422-0668$01.50/0