Characterization of β -endorphin binding protein (receptor) from rat brain membranes

(isoelectric point/sedimentation constant/hydrodynamic properties/3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)

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ABSTRACT Rat brain membranes (RBM) bind β -endorphin with high affinity and specificity. We report herein the identification of a high molecular weight β -endorphin complex (receptor) in extracts of RBM preincubated with tritiated β -endorphin, by using the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Upon isoelectric focusing, this complex gives a single peak with an isoelectric point ($\pm$ SEM) of 4.50 ± 0.06 . Sucrose density gradient experiments in H_2O and $^2\text{H}_2\text{O}$ yield effective partial specific volume ($\bar{v}^* = 0.814 \text{ cm}^3/\text{g}$) and sedimentation constant $s_{20,w} = 15.6 \text{ S}$. Gel filtration yields an estimate of the hydrodynamic radius of 73 Å. The corresponding frictional ratio of 1.12 is consistent with an elliptical spheroid with an axial ratio of 3.1–3.2. The molecular mass of the complex is estimated to be 690,000 daltons. The $\bar{v}$ of the complex is greater than that of CHAPS or most globular proteins.

Solubilization of functional opiate receptors from brain or cultured neuroblastoma cells has been achieved with the use of a new class of detergent, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (1, 2). We have found that [$^3\text{H}_2$]Tyr 27 -labeled human β -endorphin (^3H - β $_h$ -EP) forms a complex with a component (receptor) of rat brain membrane (RBM) that survives solubilization with CHAPS and is sufficiently stable to permit physical characterization.

MATERIALS AND METHODS

β $_h$ -EP was prepared by solid-phase synthesis as described (3). ^3H - β $_h$ -EP was the product of catalytic reduction of a synthetic diiodotyrosine derivative of β $_h$ -EP (4). CHAPS was synthesized as described by Hjelmeland (5). Glycerol, bacitracin, and deuterium oxide were from Aldrich. Ampholines were from LKB and Pharmacia. Dithiothreitol, Hepes, and Tris were from Sigma. $^3\text{H}_2\text{O}$ was from Amersham. All other chemicals were of reagent grade.

RBM were prepared as described (6) and stored at -70°C . Ten milliliters of RBM in 50 mM Tris-HCl, pH 7.5/0.1% bacitracin/20% glycerol was incubated at room temperature for 1 hr with 1 nM of ^3H - β $_h$ -EP, chilled on ice for 10 min, and centrifuged at $15,000 \times g$ for 15 min. The pellet was resuspended in an equal volume of the same buffer; additions were made to give final concentrations as follows: dithiothreitol, 5 mM; Trasylol, 200 kallikrein inhibitor units/ml; phenylmethylsulfonyl fluoride, 0.1 mM; CHAPS, 10 mM. Insoluble material was removed by centrifugation at $100,000 \times g$ for 1 hr. The supernatant was applied to a $2.5 \times 30 \text{ cm}$ column of Sepharose 6B equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1% bacitracin and 1 mM CHAPS for isoelectric focusing experiments. For sucrose density gradient centrifugation, the eluant was 0.1 M KCl/10 mM Hepes/4 mM Tris-HCl/1 mM CHAPS

(pH 7.5). The columns were eluted at 4°C with a peristaltic pump at a flow rate of 31 ml/hr. Radioactivity in each fraction was determined by liquid scintillation counting. The fractions containing the radioactive peak were pooled, decreased to 1–2 ml in volume by ultrafiltration on a Amicon YM-10 membrane filter, and used immediately or stored at -70°C .

For isoelectric focusing, a 110-ml LKB isoelectric focusing column was used, with a 100-ml 5–50% sucrose gradient, 1% Ampholines, and 2 mM CHAPS. Electrode solutions were 0.15 M phosphoric acid and 0.15 M NaOH. The sample of solubilized complex was added to the mixing chamber after 2/3 of the gradient had entered the column. Power was maintained at 1 W for 24 hr, and the temperature was 4°C . The gradient was collected in 1-ml fractions, the pH was read at room temperature, and the radioactivity was determined by liquid scintillation counting.

For sucrose density gradient experiments, linear gradients (4.0 ml) were formed from 10–40% (wt/vol) sucrose in H_2O or $^2\text{H}_2\text{O}$ containing 0.1 M KCl, 1 mM CHAPS, 10 mM Hepes, and 4 mM Tris-HCl (pH 7.5). Soluble complex or standards (100 μl) were overlaid and centrifuged at 40,000 rpm in a Beckman SW 60 Ti rotor for 15 hr (H_2O) or 40 hr ($^2\text{H}_2\text{O}$). Fractions of each gradient were collected from the bottom of the tube through a glass capillary and the refractive index of every other fraction was measured immediately. Radioactivity was determined after addition of 0.4 ml of 10% NaDodSO $_4$. Protein of standards was estimated by Coomassie blue G-250 binding (7). The sedimentation constant ($s_{20,w}$) and effective $\bar{v}$ ($\bar{v}^*$) of the β -EP binding protein complex was determined by the method of Smigel and Fleischer (8).

The FORTRAN program of Smigel (9) was rewritten for a small desktop computer in BASIC. To decrease the calculation time, a Simpson's rule numerical integration algorithm with adaptive step length (10) was substituted for that used by Smigel (9) and the viscosity and density gradients were interpolated with respect to radial distance by cubic splines (10). Buffer density was calculated from the $\bar{v}$ of its components, and buffer viscosity was measured in an Ostwald double-bulb viscometer at a number of temperatures. The $\bar{v}$ of CHAPS was determined with a mechanical oscillator densimeter.

Preparative and analytic gel filtration on Sepharose 6B was used to estimate the hydrodynamic radius of the complex. Blue dextran and $^3\text{H}_2\text{O}$ were used to mark the void volume (V_0) and internal volume (V_i), respectively. The elution volume (V_e) of monomeric proteins of known hydrodynamic radius (11) was used to construct a calibration curve from the relation $R_e = A + B \text{erf}^{-1}(1 - K_d)$, in which $K_d = (V_e - V_0)/(V_i - V_0)$ (12). All chromatography experiments were carried out at 4°C .

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Abbreviations: β -EP, β -endorphin (subscript h, human); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ^3H - β $_h$ -EP, [$^3\text{H}_2$]Tyr 27 -labeled human β -endorphin; RBM, rat brain membrane(s).

Table 1. Binding of ^3H - β_{h} -EP to RBM before and after CHAPS solubilization

^3H - β_{h} -EP, 25 pmol added	β_{h} -EP, pmol	V_0 fractions, cpm
Before CHAPS solubilization	0	1,203
	1,000 before	119
	1,000 after	1,651
After CHAPS solubilization	0	311
	1,000 after	231

Three and one-half milliliters of RBM (10 brains per 100 ml) was incubated with 10 mM CHAPS for 1 hr on ice and the soluble extract was clarified by centrifugation for 1 hr at $10,000 \times g$. ^3H - β_{h} -EP and β_{h} -EP were added as noted and incubated at room temperature for 1 hr. Formation of the high molecular weight complex was determined by gel filtration of 0.1-ml aliquots on Sephadex G-150 (8-ml column) equilibrated in 50 mM Tris-HCl (pH 7.5) buffer containing bacitracin at 0.1 mg/ml, 2 mM CHAPS, and 10% sucrose. Radioactivity in V_0 fractions was counted in 10 ml of scintillation fluid.

RESULTS AND DISCUSSION

^3H - β_{h} -EP formed a high molecular weight complex with a component from RBM that can be released into solutions by CHAPS (Table 1). A 40-fold excess of β_{h} -EP added before solubilization decreased the high molecular weight form of ^3H - β_{h} -EP by 90%, whereas the same excess had little effect when added after solubilization. After solubilization, formation of high molecular weight forms of ^3H - β_{h} -EP was greatly decreased. In similar experiments (not shown), no high molecular weight complex could be detected in Triton X-100 (1%) extracts of RBM preincubated with ^3H - β_{h} -EP.

The β_{h} -EP binding protein from rat brain is an acidic molecule. Five isoelectric focusing experiments gave a value ($\pm$ SEM) for the apparent isoelectric point of $\text{pI} = 4.50 \pm 0.06$ in pH 4–6, 3–5, or 3–10 gradients. Fig. 1 shows the result of one such experiment.

A gel filtration experiment showing the elution profile of a CHAPS extract of prelabeled complex prepared in the presence or absence of excess β_{h} -EP is shown in Fig. 2. A hydrodynamic radius of 73 Å for the ^3H - β_{h} -EP complex was obtained from the observed elution position as shown in Fig. 3. This value is in

Fig. 1. Isoelectric focusing of ^3H - β_{h} -EP complex. ^3H - β_{h} -EP was bound to RBM, extracted with 10 mM CHAPS, eluted from a Sepharose 6B column, loaded on a pH 4–6.5 gradient, and focused for 24 hr at 1 W at 40°C. The apparent isoelectric point of the complex is 4.47. The peak eluting with the anode solution is free ^3H - β_{h} -EP.

Fig. 2. Sepharose 6B chromatography of ^3H - β_{h} -EP complex. RBM were incubated with ^3H - β_{h} -EP (total binding, solid line) or ^3H - β_{h} -EP with excess β_{h} -EP (nonspecific binding, dashed line) and solubilized with 10 mM CHAPS as described. The column was equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1% bacitracin and 1 mM CHAPS and was eluted at a rate of 31 ml/hr. Arrow indicates the elution position of blue dextran and $^3\text{H}_2\text{O}$.

good agreement with that of Simonds *et al.* (1) for solubilized opiate receptor.

Sucrose density gradients in H_2O and $^2\text{H}_2\text{O}$ gave estimates of the $\bar{v}$ (0.814) and sedimentation constant (15.6 S) as shown in Fig. 4. The sedimentation constant, hydrodynamic radius, and $\bar{v}$ of the complex may be used to calculate both the molecular mass (M^*), 690,000 daltons, and the frictional ratio, 1.12, as shown in Table 2. The frictional ratio is that of an oblate or prolate ellipsoid with axial ratio of 3.1–3.2. The complex ($\bar{v}^*$

Fig. 3. Determination of hydrodynamic radius of ^3H - β_{h} -EP complex. A 0.9×58 cm column of Sepharose 6B was eluted at 6 ml/hr. Blue dextran and $^3\text{H}_2\text{O}$ were used to mark V_0 and V_1 , respectively. Bovine serum albumin (36 Å) and RNase (18.5 Å) in 0.1 M KCl/50 mM Tris-HCl, pH 7.5, and transferrin (84 Å), bovine serum albumin (78 Å), lysozyme (27.5 Å), and human IgG (63 Å and 38 Å) in 0.1% Na-DodSO₄/50 mM Tris-HCl, pH 7.5, were used as standards. The linear regression of hydrodynamic radius (Å) vs. $\text{erf}^{-1}(1 - Kd)$ had a slope = 92.4, y-intercept = 7.91, and correlation $r^2 = 0.99$. The elution position of bovine serum albumin and RNase were checked on the larger 2.5×38 cm Sepharose 6B column, and the same calibration curve was used, as suggested by Tanford *et al.* (11). The Kd of ^3H - β_{h} -EP complex was the same in buffers with or without 0.1 M KCl. The hydrodynamic radius (indicated by arrow) estimated for the ^3H - β_{h} -EP complex is 73 Å.

FIG. 4. Sedimentation constant and $\bar{v}$ of ^3H - β -EP complex. Sucrose density gradients (10–40%, wt/vol) in H_2O or $^2\text{H}_2\text{O}$ containing 0.1 M KCl, 10 mM Hepes, 4 mM Tris-HCl, and 1 mM CHAPS (pH 7.5) were centrifuged for 15 or 40 hr, respectively, at 5°C. Fractions were collected and the radial position of the ^3H - β -EP complex and standard proteins was determined by liquid scintillation spectroscopy or protein assay. The sucrose gradient was determined by refractive index measurements. $s_{20,w}$ was calculated for a range of partial specific volumes as described by Smigel and Fleischer (8). Each point represents the mean of three experiments. The relative error of all points lays in the range 4–12%. The intersection of the curves gives the values of $\bar{v}$ (0.814) and sedimentation constant (15.6) that are consistent with both H_2O and $^2\text{H}_2\text{O}$ experiments. The relative error of $s_{20,w}$ for standard proteins (ovalbumin, catalase, thyroglobulin, and β -galactosidase) calculated in the same fashion ranged from 5% to 14%.

= 0.814) cannot be composed only of protein ($\bar{v}_p = 0.735$) and CHAPS ($\bar{v}_D = 0.802$). Either the binding protein has an unusually high specific volume or a third component of high specific volume is present in the complex. A protein with a specific volume ≥ 0.81 must be enriched in hydrophobic residues, whereas the most likely candidate for a third component of high specific volume is membrane lipid. The high specific volume of the β -EP binding protein complex thus indicates a highly hydrophobic character for the binding protein, either through composition or association with lipid. This is consistent with the observed sensitivity of opiate receptors to phospholipases and detergents.

It is not possible with the present data to estimate the relative proportion of proteins, detergent, and lipid (if any) in the intact complex. If we assume that the specific volume of the protein is similar to that of globular protein and use a specific volume

Table 2. CHAPS-solubilized ^3H - β -EP binding protein complex (receptor)

Properties	Values
pI	$4.50 \pm 0.06^\dagger$
$s_{20,w}$	15.6 S
$\bar{v}^*$	0.814
R	73 Å
M^{**}	690,000
$f/f_0^\S$	1.12
Axial ratio	3.1–3.2

† Mean $\pm$ SEM.

$$^\ddagger M^* = \frac{s_{20,w} \cdot 6\pi \eta_{20,w} \cdot N \cdot R_e}{1 - \bar{v}^* \rho_{20,w}}$$

$$^\S f/f_0 = R \left[\frac{4\pi N}{3M^*(\bar{v}^* + \delta)/\rho_{20,w}} \right], \text{ in which } \delta = 0.2 \text{ g of } \text{H}_2\text{O per g of complex.}$$

of 1.1 for lipid, then the lipid content must be in the range 5–22%, corresponding to a protein content of 0–78% and detergent content of 95–0%. An independent measurement of bound lipid or bound detergent is needed to determine the composition of the soluble complex (receptor).

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