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Hydrodynamic Characterization of the Triton X-100 Solubilized Lactogenic Hormone Receptor of Rat Liver†

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ABSTRACT: Rat liver plasma membranes, prelabeled with radioactive human growth hormone, were extracted with Triton X-100. The solubilized lactogenic hormone receptor had a Stokes radius of 54.2 Å on Sepharose 6B chromatography. Numerical integration of the results of sedimentation experiments on the solubilized hormone–receptor–Triton X-100 complex in sucrose/H_2O and sucrose/^2H_2O density gradients produced an $s_{20,w} = 5.05 \times 10^{-13}$ S and a partial specific volume of 0.791 $cm^3\ g^{-1}$. From these data a molecular weight of 148 000 and frictional ratio of 1.40 for the hormone–receptor–Triton X-100 complex were calculated. Triton X-100 was calculated to comprise 32.4% of the complex, and thus, the hormone–receptor complex has a molecular weight of 99 800. These results indicate that the lactogenic hormone receptor, as would be expected of an integral membrane protein, has the capacity to bind a large amount of detergent.

The action of the lactogenic hormone prolactin is generally believed to be initiated by the binding of the hormone to a specific binding protein, known as the receptor, in the cell membrane. It is unknown, however, how the response of the cell is elicited by the binding of the hormone to its receptor on the cell surface. Specific lactogenic hormone binding sites have been described in a number of tissues in a wide variety of species (Posner et al., 1974a). The lactogenic hormone receptor in the female rat liver membrane has been characterized (Posner et al., 1974b; Herington et al., 1976), and its binding of hormone has been shown to be only slightly altered on solubilization with Triton X-100 (Bonifacino et al., 1981). The kinetics of binding of lactogenic hormones to rat liver membranes are similar to those found in another target tissue for the hormone, the rabbit mammary gland (Shiu & Friesen, 1974a).

We report here the hydrodynamic characterization of the lactogenic hormone receptor–detergent complex from rat liver membranes. Gel filtration and sedimentation velocity determination in sucrose/H_2O and sucrose/^2H_2O gradients were used to determine the size and weight of the receptor–detergent complex and to estimate the amount of detergent bound.

Experimental Procedures

Materials. ^{125}I for iodination of human growth hormone and ^3H_2O were obtained from Amersham Corp. Human growth hormone (hGH; HS2243E)[1] and ovine prolactin (oPRL; PS-14) were gifts from the NIAMDD, NIH. Triton X-100, toluene, and PPO–POPOP were purchased from RPI International. Sepharose 6B, Sephadex G-50, Blue Dextran 2000, and protein standards for the calibration of columns were obtained from Pharmacia Fine Chemicals. The proteins used as standards for the density gradient sedimentation experiments were purchased from Sigma, and ^2H_2O was obtained from Aldrich. Sprague-Dawley rats were obtained from King Animal Labs.

Preparation of Plasma Membranes. Rat liver membranes were prepared according to the method of Costlow & Gallagher (1977). In brief, tissue was excised and then homogenized in 10 volumes of 1 mM sodium bicarbonate and 0.5 mM calcium chloride, pH 7.0 (buffer A) at 4 °C. The homogenate was then diluted to 1 g of tissue/100 volumes of buffer A and filtered through cheesecloth. The homogenate was centrifuged at 900*g* for 20 min at 4 °C, and the resulting supernatant was then centrifuged at 20000*g* for 20 min at 4 °C. The pellet was resuspended in 1 volume of 25 mM sodium phosphate, 10 mM magnesium chloride, and 0.1% BSA, pH 7.0, and frozen until used.

Preparation of ^{125}I-hGH. Iodinated hGH was prepared by the lactoperoxidase method of Thorell & Johannson (1971) and purified by chromatography on Sephadex G-50. The specific activity, determined as described by Shiu & Friesen (1974a), ranged from 90 to 120 Ci g^{-1}.

Binding Reaction. Membranes were resuspended by homogenization, and 180–300 μg of membrane protein was incubated with ^{125}I-hGH (~1.2×10^6 cpm) in 10 mM Tris, 10 mM $MgCl_2$, and 0.1% BSA, pH 7.4, for 20 h at 19 °C. The total volume was 1.5 mL. The extent of nonspecific binding was determined by adding an excess of unlabeled oPRL (6 μg) to an identical set of tubes. After incubation, the tubes were cooled on ice and 2 mL of 10 mM sodium phosphate, pH 7.0, was added. The tubes were then centrifuged at 20000*g* for 15 min at 0–5 °C, the supernatant was removed, and the pellets were counted. Total binding was generally between 25% and 35% of the counts added of which approximately 50% represented specifically bound hormone.

Solubilization. Solubilization was performed by adding 1 mL of 1% Triton X-100 in 10 mM Tris, pH 7.4 (made up in ^2H_2O when the material was to be applied to sucrose/^2H_2O

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[1] Abbreviations: hGH, human growth hormone; ^{125}I-hGH, ^{125}I-labeled hGH; oPRL, ovine prolactin; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; PPO–POPOP, 2,5-diphenyloxazole–1,4-bis(5-phenyloxazol-2-yl)benzene; cpm, counts per minute.

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gradients), to the membrane pellets. After homogenization and incubation for 30 min at 20 °C, the mixtures were centrifuged at 100000*g* for 1 h at 0–4 °C. The supernatant was removed and used for subsequent experiments. About 50% of the counts specifically bound to the membrane were solubilized by this procedure while a smaller proportion (about 30%) of the nonspecifically bound counts were solubilized.

Gel Filtration. Sepharose 6B chromatography was performed at 4 °C by using ascending flow at 23 mL h^{-1} in a 1.6 × 80 cm column equipped with flow adapters. The column was equilibrated and eluted with 0.1% Triton X-100 and 10 mM Tris, pH 7.4. The following proteins from Pharmacia were used to calibrate the column with respect to Stokes' radius by the method of Porath (1963): thyroglobulin, 85.0 Å; ferritin, 61.0 Å; catalase, 52.2 Å; aldolase, 48.1 Å; ovalbumin, 30.5 Å; ribonuclease, 16.4 Å. The void volume (V_0) was determined by using Blue Dextran 2000 and the total volume (V_t) with tritiated water. The distribution coefficient, K_d, was calculated from the elution volume (V_e) of a peak by

$$K_d = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

Density Gradient Centrifugation. Linear sucrose density gradients (4.6 mL) of 5–20% (w/w) sucrose in 0.1% Triton X-100 and 10 mM Tris, pH 7.4, were prepared in H_2O or ^2H_2O. Samples or standards with known sedimentation coefficients ($s_{20,w}$) and partial specific volumes ($\bar{\nu}$) in 0.2-mL aliquots were applied to the tops of the gradients. The standards used were the following: ribonuclease, 1.78 S, 0.703 $cm^3\ g^{-1}$ (Richards & Wycoff, 1971); carbonic anhydrase, 2.75 S, 0.731 $cm^3\ g^{-1}$ (Armstrong et al., 1966); lactate dehydrogenase, 6.95 S, 0.730 $cm^3\ g^{-1}$ (Schwert & Winer, 1963); aldolase, 7.70 S, 0.742 $cm^3\ g^{-1}$ (Sober, 1970; Taylor & Lowry, 1956); catalase, 11.3 S, 0.730 $cm^3\ g^{-1}$ (Sumner & Gralen, 1938). Centrifugation at 4 °C was carried out for 2 h at 55 000 rpm for sucrose/H_2O gradients and for 4 h at 45 000 rpm for sucrose/^2H_2O gradients in a VTi 65 rotor. The bottoms of the tubes were then pierced and fractions (~0.15 mL each) collected. The position of the protein standard was determined by measuring the A_{280}, and the position of ^{125}I-hGH was determined by measuring the radioactivity in a γ counter. The refractive index was used to derive the density and viscosity of the individual fractions of sucrose/H_2O gradients from standard tables while the density and viscosity of the individual fractions of the sucrose/^2H_2O gradients were derived as described by O'Brien et al. (1978).

Calculation of Sedimentation Constants. The sedimentation coefficient of a macromolecule can be calculated by

$$s_{20,w} = \frac{1 - \bar{\nu}\rho_{20,w}}{\omega^2 t \eta_{20,w}} \int_{r(0)}^{r(t)} \frac{\eta(r)\, dr}{r[1 - \bar{\nu}\rho(r)]} \quad (2)$$

where $\bar{\nu}$ is the partial specific volume of the macromolecule, ω is the angular velocity in radians per second, t is the sedimentation time in seconds, and $r(0)$ to $r(t)$ are the distances in centimeters from the rotor center through a gradient described by density $\rho(r)$ and viscosity $\eta(r)$. $\rho_{20,w}$ and $\eta_{20,w}$ are the density and viscosity of water at 20 °C (Tanford, 1961). Equation 2 was integrated by using Simpson's rule for various values of $\bar{\nu}$ as described by Smigel & Fleischer (1977). The values derived for $s_{20,w}$ for both the sucrose/H_2O and sucrose/^2H_2O gradients were then plotted vs. $\bar{\nu}$. The point where the two curves intersect defines the $s_{20,w}$ and $\bar{\nu}$ of the macromolecule.

Other Analytical Methods. Protein was determined by the method of Lowry et al. (1951) using BSA as the standard.

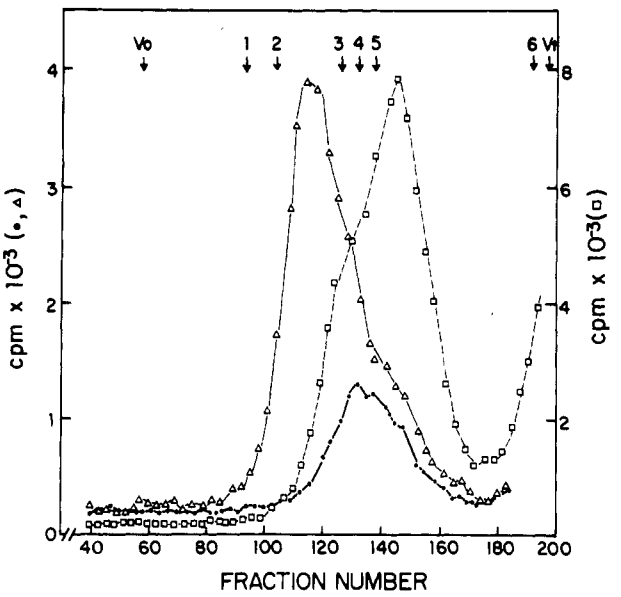

FIGURE 1: Sepharose 6B chromatography of Triton X-100 solubilized ^{125}I-hGH–receptor–detergent complex. The particulate membrane fraction was incubated with ^{125}I-hGH [total binding (△)] or ^{125}I-hGH plus excess unlabeled oPRL [nonspecific binding (●)] as described under Experimental Procedures. The pellets were resuspended in 1 mL of 1% Triton X-100 and 10 mM Tris, pH 7.4, and incubated at 20 °C for 30 min before centrifugation at 100000*g* for 1 h at 4 °C. The supernatants were applied to Sepharose 6B columns (1.6 × 80 cm) and eluted with 0.1% Triton X-100 and 10 mM Tris, pH 7.4, at a flow rate of 23 mL h^{-1}. Free ^{125}I-hGH (□) was also chromatographed. Arrows indicate the elution position of the standard proteins used for calibration: (1) thyroglobulin, (2) ferritin, (3) catalase, (4) aldolase, (5) ovalbumin, (6) ribonuclease.

Scintillation counting was performed in 5 mL of a mixture consisting of 2 L of toluene, 1 L of Triton X-100, and 126 mL of PPO–POPOP. All values represent the mean ± standard deviation of three determinations.

Results

Gel Filtration. Sepharose 6B chromatography of the ^{125}I-hGH–receptor–detergent complex revealed one major peak of radioactivity that had a K_d of 0.45 ± 0.03 (Figure 1). If the membranes were incubated with ^{125}I-hGH plus unlabeled oPRL, the Sepharose 6B profile of the solubilized material exhibited an absence of labeled hormone at that position. The small shoulder on the curve from incubations performed in the absence of unlabeled oPRL appears to be nonspecifically bound hormone, as it is in the same position as the radioactivity which remains with the membrane after incubation with ^{125}I-hGH plus unlabeled oPRL. Labeled hGH had a K_d of 0.62 ± 0.01 and was well separated from the position of the ^{125}I-hGH–receptor–detergent complex. The shoulder with a lower K_d is believed to represent aggregated hGH. The Stokes radius of the ^{125}I-hGH–receptor–detergent complex was determined to be 54.2 ± 3.7 Å from a plot of the $K_d^{1/3}$ of the standard proteins vs. their Stokes' radii as described by Porath (1963).

Sucrose Density Gradient. The sedimentation of the ^{125}I-hGH–receptor–detergent complex and proteins of known s values in sucrose/H_2O gradients is shown in Figure 2A, and the sedimentation in sucrose/^2H_2O gradients is presented in Figure 2B. As can be seen, the receptor-bound radioactivity sedimented near lactate dehydrogenase in sucrose/H_2O gradients but was closer to carbonic anhydrase in sucrose/^2H_2O gradients, suggesting that the complex has a higher partial specific volume than the protein standards. ^{125}I-hGH, on the other hand, apparently has a partial specific volume like the protein standards, as it sedimented in the same position relative

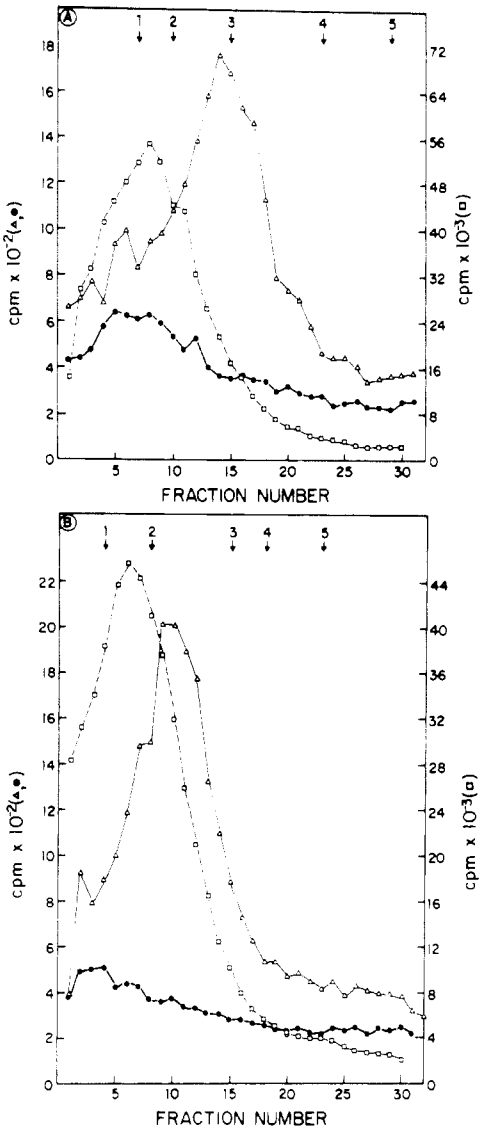

FIGURE 2: Sedimentation of Triton X-100 solubilized ^{125}I-hGH–receptor–detergent complex in (A) sucrose/H_2O and (B) sucrose/^2H_2O gradients containing Triton X-100. Sucrose/H_2O and sucrose/^2H_2O density gradients were prepared as described under Experimental Procedures and centrifuged for 2 h at 55000 rpm (sucrose/H_2O gradients) or for 4 h at 45000 rpm (sucrose/^2H_2O gradients) in a Beckman VTi65 rotor at 4 °C. (▲) Total binding; (●) nonspecific binding; (□) ^{125}I-hGH. Arrows indicate the position of standard proteins: (1) ribonuclease, (2) carbonic anhydrase, (3) lactate dehydrogenase, (4) aldolase, (5) catalase.

to the protein standards in both sucrose/H_2O and sucrose/^2H_2O gradients. When oPRL was included in the incubation of ^{125}I-hGH with the membranes prior to solubilization with Triton X-100, labeled material in the position associated with the ^{125}I-hGH–receptor–detergent complex was reduced. This means that the peak observed in the absence of unlabeled oPRL is due to specifically bound hormone. Figure 3 shows the $s_{20,w}$ values for the ^{125}I-hGH–receptor–detergent complex calculated as a function of the partial specific volume as described by Smigel & Fleischer (1977) (eq 2 under Experimental Procedures). The curves from the sucrose/H_2O and sucrose/^2H_2O gradients intersect at a point equivalent to a sedimentation coefficient of 5.05 ± 0.39 S and a partial specific volume of 0.791 ± 0.020 cm^3 g^{-1}.

Calculation of Molecular Weight and Detergent Bound to Receptor. The molar mass (M) of the ^{125}I-hGH–receptor–detergent complex was calculated by using the Stokes radius (54.2 Å), sedimentation coefficient (5.05 S), and partial

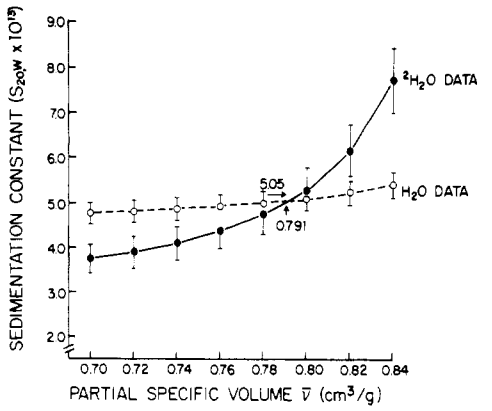

FIGURE 3: Determination of $s_{20,w}$ and $\bar{\nu}$ from sedimentation in sucrose/H_2O and sucrose/^2H_2O gradients. The curves were calculated by using eq 2 under Experimental Procedures for $\bar{\nu}$ from 0.70 to 0.84 cm^3 g^{-1}, with $\rho(r)$ and $\eta(r)$ being derived from the refraction index. Error bars represent ±1 SD from three separate experiments. Horizontal and vertical arrows indicate the values of $s_{20,w}$ and $\bar{\nu}$, respectively, consistent with the sedimentation of the ^{125}I-hGH–receptor–detergent complex in both sets of gradients.

specific volume (0.791 cm^3 g^{-1}) by the formula (Siegel & Monty, 1966)

$$M = \frac{6\pi\eta_{20,w}NR_s s_{20,w}}{1 - \bar{\nu}\rho_{20,w}} \quad (3)$$

where $\eta_{20,w}$ is the viscosity of the medium, N is Avogardo's number, R_s is Stokes' radius, $s_{20,w}$ is the sedimentation coefficient, $\bar{\nu}$ is the partial specific volume, and $\rho_{20,w}$ is the density of the media. A molecular weight of 148000 was obtained.

The observed partial specific volume of the complex results from the contribution of the weight fraction of Triton X-100 (X_d) and its partial specific volume ($\bar{\nu}_d$) and the contribution of the weight fraction of protein (X_p) and the partial specific volume of the protein ($\bar{\nu}_p$) (Smigel & Fleischer, 1977):

$$\bar{\nu} = X_p\bar{\nu}_p + X_d\bar{\nu}_d$$

The partial specific volume of Triton X-100 above its critical micellar concentration is 0.908 cm^3 g^{-1} (Tanford et al., 1974). If we assume an average value for the partial specific volume of a typical nonglycosylated protein of 0.736 cm^3 g^{-1}, the weight fraction of Triton X-100 in the ^{125}I-hGH–receptor–detergent complex is 0.324 g of Triton X-100/g of complex. Therefore, the molecular weight of the ^{125}I-hGH–receptor complex is 99800, and based on a molecular weight of 22000 for hGH (Li & Dixon, 1971), the molecular weight of the receptor is 77800. We have also calculated, based on a molecular weight of 636 for Triton X-100 (Makino et al., 1973), that there is 76 mol of Triton X-100/mol of receptor.

The frictional ratio (f/f_0) of the ^{125}I-hGH–receptor–detergent complex was calculated to be 1.40 from the formula (Tanford, 1961)

$$f/f_0 = R_s\left[\frac{4\pi N}{3M(\bar{\nu} + \delta/\rho_{20,w})}\right]^{1/3}$$

where δ is the solvation factor and is assumed to be 0.2 g of solvent/g of protein (Tanford, 1961) and N is Avogadro's number.

Discussion

The lactogenic hormone receptor is an integral part of the plasma membrane, and its solubilization would be expected to involve the binding of detergent to the hydrophobic areas of the molecule (Helenius & Simons, 1975). Triton X-100 has often been used for the solubilization of membrane proteins since it does not generally lead to denaturation and loss of

activity. Using the analytical approach described by Smigel & Fleischer (1977), we were able to determine the molecular weight of the ^{125}I-hGH–receptor–detergent complex as well as the amount of detergent in the complex and thereby derive the molecular weight of the receptor itself.

The lactogenic hormone receptors in the female rat liver have been characterized by using ^{125}I-hGH as the labeled ligand (Posner et al., 1974b; Herington et al., 1976; Bonifacino et al., 1981). Its use in incubations containing Triton X-100 avoids the complications associated with the effect of Triton X-100 on oPRL, as labeled oPRL has been shown in previous studies to behave anomalously in Triton X-100 (Shiu & Friesen, 1974b; Carr & Jaffe, 1981).

The molecular weight for the ^{125}I-hGH–receptor–detergent complex determined in these studies, 148 000, is less than those suggested in previous reports of the molecular weight of lactogenic hormone receptors. Shiu & Friesen (1974b) reported a molecular weight of 220 000 for the receptor from the rabbit mammary gland on the basis of its elution from Sepharose 6B. This value is based on the assumption that the shape and partial specific volume of the receptor were like those of the protein standard used to calibrate the column. We (Carr & Jaffe, 1981) have previously reported a molecular weight of 170 000 for the female rat liver lactogenic hormone receptor on the basis of Ferguson plots of the mobility of the receptor during polyacrylamide disc gel electrophoresis in gels of different acrylamide concentration. However, the real parameter determined was the geometric mean radius which was converted to the molecular weight as described by Rodbard (1976) but with an assumed partial specific volume for the complex of 0.736 $cm^3\ g^{-1}$. If we use the partial specific volume for the complex of 0.791 $cm^3\ g^{-1}$, the value determined in these studies, the data from our previous study (Carr & Jaffe, 1981) can be recalculated to give a molecular weight of 159 000 which is within 7.5% of the value reported here.

The molecular weight of the lactogenic hormone receptor calculated from the data of this study is based on a number of assumptions which have been treated in detail by Smigel & Fleischer (1977). Probably the most tenuous of these is that the nondetergent component of the receptor–detergent complex has a partial specific volume of 0.736 $cm^3\ g^{-1}$. If the protein were glycosylated, the partial specific volume would be less than the assumed value and the actual amount of detergent bound would be higher than that reported here. The calculated molecular weight of the hormone–receptor complex and receptor itself would, therefore, be smaller than that given under Results. More precise estimates of the partial specific volume of the receptor are at present impossible due to the lack of significant quantities of homogeneous preparations.

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