Separation of rabbit mammary-gland prolactin receptors by ion-exchange chromatography, h.p.l.c.–gel filtration and ultracentrifugation

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Rabbit mammary-gland prolactin (Prl) receptors in the microsomal fraction were solubilized in 7.5 mM-Chaps or 1% Triton X-100 and analysed by ion-exchange chromatography using DEAE-Bio-Gel A. Prl receptors in the presence of 7.5 mM-Chaps were separated into two different fractions (Fr. A and B), both of which showed identical specificity of binding to peptide hormones as those in the Chaps or Triton extract. oPrl and human growth hormone (hGH) bound to the same site, but other non-lactogenic hormones (follicle-stimulating hormone, oGH, luteinizing hormone and insulin) failed to bind to the Prl receptors. The dissociation constant (K_d) for Prl binding to the receptors in Fr. A was about 50% of those in Fr. B, suggesting that the rabbit mammary gland contains two types of Prl receptors, one with a high, and one with a low, K_d for Prl binding. A decrease in the concentration of Chaps in the column buffer to 4 mM caused aggregation of the receptors in Fr. A. H.p.l.c.–gel filtration, using Shim pack 150 and 300 columns connected in series, separated the receptor as a protein with an M_r of 74000 ± 4900 (mean $\pm$ s.d.) in the presence of 5 mM-Chaps, or of 36800 ± 2100 in the presence of 7.5 mM-Chaps. Sucrose-gradient-centrifugation analysis showed that the Prl–receptor complexes in the presence of 5 mM-Chaps were sedimented between γ -globulin and bovine serum albumin (5.56 ± 0.22 S). As the Chaps concentration was increased to 7.5 mM, a further peak of the Prl–receptor complexes (4.01 ± 0.23 S) appeared below ovalbumin. The present data suggest that the binding subunit causes the monomeric subunit to aggregate with itself or with another specific associated protein of similar M_r .

INTRODUCTION

Prolactin (Prl) receptors are intrinsic membrane proteins. They have been solubilized by detergents with retention of binding activity (Shiu & Friesen, 1974; Liscia & Vonderhaar, 1982), and some of their chemico-physical properties have been determined (Carr & Jaffe, 1982; Liscia *et al.*, 1982; Gavish *et al.*, 1983; Haeuptle *et al.*, 1983; Necessary *et al.*, 1984; Katoh *et al.*, 1984; Sakai *et al.*, 1985). At present the native structure of Prl receptors is unknown. Bifunctional cross-linking reagents that attach labelled hormones covalently to the receptors have been used to study the molecular nature of the Prl receptors (Haeuptle *et al.*, 1983; Hughes *et al.*, 1983; Yamada & Donner, 1984, 1985; Bonifacino & Dufau, 1984; Kelly *et al.*, 1985). The electrical heterogeneity of Prl receptors has been reported (Sasaki *et al.*, 1982; Waters *et al.*, 1984).

We intended to examine the native structure of the detergent-solubilized Prl receptors. Prl receptors have been solubilized by using the non-ionic detergent Triton X-100 (Shiu & Friesen, 1974). In the present experiments, the zwitterionic detergent Chaps was also utilized to solubilize the membrane Prl receptors. Chaps did not

affect the molecule characteristics of labelled Prl and the binding of Prl to the soluble receptors (Liscia & Vonderhaar, 1982). The neutral charge of Chaps allows it to be utilized for ion-exchange chromatography (Hjelmeländ, 1980), and the solubilized Prl receptors were analysed by this technique. We examined the properties of hormonal specificity and Prl binding for the ion-exchange-fractionated Prl receptors. In the present study we further examined the chemico-physical properties of the Prl receptors by ion-exchange chromatography, h.p.l.c.–gel filtration and sucrose-gradient ultracentrifugation, with two different concentrations of Chaps. The present study was undertaken as an initial step towards future study of Prl-receptor structure.

MATERIALS AND METHODS

Hormones and chemicals

The hormones used (oPrl, NIH-P-S15, 30.5 i.u./mg; hGH, NIH-HS2160E, 1.7 i.u./mg; oFSH, NIH-FSH-S14; oLH, NIH-LH-S24; oGH, NIH-GH-S12) were gifts from the National Institute of Health, Bethesda, MD, U.S.A. Porcine insulin was a gift from Novo Industri A/S

Abbreviations used: (o)Prl, (ovine) prolactin; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]propanesulphonic acid; hGH and oGH, human and ovine growth hormone; Fr., fraction; (o)FSH, (ovine) follicle-stimulating hormone; (o)LH, (ovine) luteinizing hormone; PMSF, phenylmethanesulphonyl fluoride; BSA, bovine serum albumin; PEG, poly(ethylene glycol); ANS, 8-anilinonaphthalene-1-sulphonic acid.

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(Bagsvaerd, Denmark). DEAE-Bio-Gel A was from Bio-Rad (Richmond, CA, U.S.A.). Chaps, PMSF and bromocryptine mesylate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Triton X-100 ['polyoxyethylene (10) octylphenyl ether'] was from Wako Pure Chemical Industries, Osaka, Japan. All other chemicals were of reagent grade.

Solubilization of the crude microsomes by Chaps

Lactating New Zealand rabbits were pretreated with bromocryptine mesylate for 36 h (Djiane *et al.*, 1977). The mammary glands were removed and homogenized in 0.25 M-sucrose. The microsomes (microsomal fraction), collected by differential centrifugation (100 000 *g*, pellet), were suspended in 25 mM-Tris/HCl (pH 7.4)/10 mM-MgCl₂/1 mM-PMSF (TMP buffer) to give a protein concentration of 10 mg/ml and treated with 1 mM-Chaps for 30 min at room temperature with stirring. The suspensions were centrifuged at 100 000 *g* for 70 min at 4 °C and the supernatants were discarded. Microsomes were resuspended in TMP buffer containing 7.5 mM-Chaps and treated again according to the same procedure as that described above. After centrifugation, the turbid layer at the top of the supernatants was carefully removed and the remaining supernatants were collected and stored at -80 °C until use. This fraction is referred to as the 'Chaps extract'. The microsomes were solubilized with 1% (w/v) Triton X-100 by the same procedure as that described above; this fraction is referred to as the 'Triton extract'. The amount of insoluble or soluble protein was determined by the method of Lowry *et al.* (1951), with BSA as standard.

Hormone binding to receptors

A 5 µg portion of Prl of hGH was iodinated by lactoperoxidase/H₂O₂ (Sakai *et al.*, 1975). The specific radioactivity of the labelled hormones was 70–85 µCi/µg. For the binding assay (Sakai *et al.*, 1985), tubes were incubated with 30 000 c.p.m. of ¹²⁵I-Prl in the presence or absence of excess (1 µg) unlabelled Prl for 16 h at room temperature. The total reaction volume and the final Chaps concentration were adjusted to 0.5 ml with TMP buffer containing 0.1% BSA (binding buffer) and to 5 mM respectively. After incubation, hormone-receptor complexes were precipitated by adding 0.5 ml of cold 0.1% γ-globulin in 0.1 M-phosphate buffer, pH 7.4, and 1 ml of a 32% (w/v) solution PEG 6000. Tubes were vortex-mixed for 10 s and centrifuged at 2000 *g* for 15 min. For the microsome Prl receptors, tubes were incubated in the same manner as for the soluble receptors, but Chaps was excluded. After incubation, the reaction mixture was diluted with 3 ml of binding buffer and centrifuged. Radioactivity in the precipitants was measured in an Aloka automatic γ-radiation spectrometer. Specific binding of ¹²⁵I-Prl was calculated as the difference between the radioactivity bound in the presence and absence of excess unlabelled Prl. For Scatchard (1945) analysis, the Prl receptors were incubated with 20 000 c.p.m. of ¹²⁵I-Prl or ¹²⁵I-hGH in the presence of 0, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2.4 and 3.2 ng of unlabelled Prl or hGH respectively. Each determination was in triplicate. The amounts of non-specific binding of ¹²⁵I-Prl or ¹²⁵I-hGH did not vary greatly and were 3.0–5.5% of total radioactivity added for the insoluble or soluble receptors.

Ion-exchange chromatography

Prl receptors were analysed by ion-exchange chromatography on DEAE-Bio-Gel A. The Chaps extracts were diluted with an equal volume of 7.5 mM-Chaps and approx. 30 mg of protein was passed (at 4 °C) over a column (1.3 cm × 15 cm) that had been equilibrated with 12.5 mM-Tris/HCl (pH 7.2)/7.5 mM-Chaps (TC buffer). The flow rate was approx. 20 ml/h. The column was washed with TC buffer until protein was no longer detected in the column effluent. The Prl receptors were eluted with a linear two-component gradient prepared from 160 ml of TC buffer and 160 ml of TC buffer containing 200 mM-NaCl. The Triton extracts were diluted with an equal volume of 7.5 mM-Chaps and analysed by the same procedure as that used for the Chaps extract. A small portion (50 or 100 µl) in each fraction (4.5 ml per tube) was used for Prl-binding assay described above, and Prl-binding activity was determined in duplicate.

H.p.l.c.-gel filtration

Prl receptors were analysed by h.p.l.c.-gel filtration at room temperature. Mobile buffer consisted of 25 mM Tris/HCl (pH 7.4)/150 mM-NaCl/5 mM (or 7.5 mM)-Chaps. The flow rate and operating pressure were 0.6 ml/min and 2.96 kPa (60 kgf/cm²) respectively. A 200 µl sample was introduced to shim pack 150 and 300 columns connected in series (0.75 cm × 100 cm, 50 cm each; Shimazu Seisakusho, Kyoto, Japan) and fractions (300 µl/tube) were collected. A 100 µl sample was employed for the determination of total binding or non-specific binding using the Prl-binding assay described above. The column was calibrated with marker proteins (Sigma) and oPrl: glutamate dehydrogenase (*M_r* 312 000), pyruvate kinase (237 000), γ-globulin (160 000), BSA (68 000), ovalbumin (45 000), Prl (23 000) and soybean trypsin inhibitor (20 000). The void volume of the column was determined by the appearance of Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden). The relationship between the retention time and logarithm of *M_r* was used to obtain the *M_r* of the Prl receptor.

Sucrose-gradient centrifugation

Analytical gradient centrifugation of the solubilized ¹²⁵I-Prl-receptor complexes through 5 ml of 5–22.5% (w/w)-sucrose gradients was used to estimate the sedimentation coefficient. Sucrose was dissolved in 25 mM-Tris/HCl (pH 7.4)/150 mM-NaCl/5 mM- (or 7.5 mM)-Chaps. To prepare the sucrose gradients, 1 ml each of a series of sucrose solutions of 20, 16.25, 12.5, 8.75 and 5% (w/w) was successively pipetted into a polyallomer tube (5.3 ml, 1.3 × 5.1 cm) placed in liquid N₂ by the method of Luthe (1983). Gradients were stored at -80 °C and thawed at least 20 h before use at 4 °C. Gradients were calibrated by using catalase (11.2–11.3 S), γ-globulin (6.6–7.3 S), BSA (4.3–4.5 S), ovalbumin (3.6–3.8 S), bovine pancreatic chymotrypsinogen A (2.54 S) and Prl (2.17–2.19 S). These values were obtained from CRC Handbook of Biochemistry [(1970) 2nd edn., Chemical Rubber Co., Cleveland, OH]. A 200 µl portion of the binding mixture was layered on to the gradients. The gradients were centrifuged at 50 000 rev./min (204 040 *g_{av}*) for 17 h at 5 °C by using a RPS-50 rotor in an 55P-72 ultracentrifuge (Hitachi Koki Co., Tokyo, Japan). After centrifugation, a 145 µl

fraction was collected from the bottom of the tube by using an automatic fractionator (DGF-1; Hitachi). The PEG precipitation assay described above was then carried out on a 100 μ l portion. The remainder of the sample was subjected to refractometry (ATC-1 instru-

ment; Atago Seisakusho, Tokyo, Japan) to ascertain the sucrose concentration at each separated zone. The sedimentation coefficient (S) was calculated by the method of McEwen (1967).

Determination of critical micelle concentration of Chaps

Chaps was dissolved in TMP buffer and the critical micelle concentration of Chaps was determined at room temperature.

Iodine method. Assay mixtures (2.0 ml) contained 0.23 mM-iodine and various concentrations of Chaps. The absorbance of each sample was measured at 360 nm (Ross & Oliver, 1959).

ANS method. Assay mixtures (3 ml) contained 10 μ M-ANS and various concentrations of Chaps. The fluorescence intensity of ANS was measured at an excitation wavelength of 392 nm and an emission wavelength of 465 nm (Ortner *et al.*, 1979).

RESULTS

Characterization of Prl receptors separated by ion-exchange chromatography

Prl receptors in the microsome fraction were solubilized with 7.5 mM-Chaps or 1% Triton X-100. Nearly 40 or 60% of Prl receptors in this fraction were solubilized by Chaps or Triton X-100 ($n = 5$) respectively. As shown in Fig. 1, Prl receptors in the Chaps extract, microsomes and 7.5 mM-Chaps-treated microsomes were subjected to Scatchard (1949) analysis using the inhibition of 125 I-Prl binding by increasing amounts of unlabelled Prl. The dissociation constant (K_d) for the Chaps-solubilized Prl receptors was 3.6×10^{-11} M, representing a potency 1.8 times less than that for the microsome Prl receptors. After

Fig. 1. Scatchard (1945) plots of binding of Prl by Prl receptors in microsomes, Chaps extract and 7.5 mM-Chaps-treated microsomes

Prl receptors were incubated with 125 I-Prl in the presence of various amounts of unlabelled Prl as described in the Materials and methods section. Bound and bound/free values were plotted as described by Scatchard (1945). Each tube contained the following amounts of protein: 50 μ g of microsome ($\circ$), 10 μ g of the Chaps extract ($\bullet$) or 60 μ g of microsome treated with 7.5 mM-Chaps ($\square$).

Fig. 2. Ion-exchange chromatography of Prl receptors in the Chaps extract (a) and the Triton extract (b)

The Chaps ($\bullet$) or the Triton extract ($\bullet$, $\circ$) was diluted with an equal volume of 7.5 mM-Chaps and approx. 30 mg of protein was loaded on to a column. The Triton extract indicated by the $\circ$ symbol was obtained from microsomes previously treated with 7.5 mM-Chaps. In all, 70 fractions (4.5 ml/tube) were collected. Samples of volume 50 μ l were used for Prl-binding assay, and values with less than 500 c.p.m. of specific binding were not plotted. Horizontal bars indicate pools for Fr. A and B. The straight diagonal line with no points is the [NaCl] gradient.

Fig. 3. Competitive displacements of specific binding of labelled Prl by peptide hormones

Results in the upper or lower panels were obtained by using the Chaps extract (and its fractions) or the Triton extract (and its fractions) respectively. The amounts of protein used are indicated. The following amounts of unlabelled hormones were included: 0, 0.2, 0.4, 0.8, 1.6, and 3.6 ng of Prl (●) or hGH (○), and 0, 0.2, 1.2, and 10 ng of other hormones (■, FSH, LH and insulin; □, bovine GH). Specific binding of 3900–4100 c.p.m. was obtained by incubating with 20000 c.p.m. of labelled Prl. Results are expressed as percentages of specific binding of ^{125}I -Prl in the absence of the competitor hormones.

Table 1. Summary of dissociation constants for hGH and Prl bindings to Prl receptors in various fractions

Prl receptors in the Chaps or Triton extracts were separated into Fr. A and B by ion-exchange chromatography as shown in Fig. 2. Scatchard (1945) analysis, using the amounts of protein (μg) given in the parentheses, is described in the Materials and methods section. The maximum concentration of Triton X-100 in the reaction mixture was 0.013%, and the Prl binding was unaffected by this concentration. The values were from different preparations.

Prl receptors in:	$10^{11} \times \text{Dissociation constant (M)}$	
	^{125}I -Prl-Prl	^{125}I -hGH-hGH
Chaps extract	3.9–4.3 (10)	4.0–4.7 (7)
Fr. A	2.7–3.2 (9)	2.5–2.7 (7)
Fr. B	5.6–7.1 (18)	5.9–6.4 (11)
Triton extract	4.5–4.7 (17)	4.7–5.1 (9)
Fr. A	2.7–3.5 (24)	3.2–3.7 (24)
Fr. B	6.2–7.4 (21)	6.0–6.6 (15)

Fig. 4. Effect of the Chaps concentration on Prl receptors analysed by ion-exchange chromatography

The Chaps extract diluted with an equal volume of distilled water was analysed in the presence of 4 mM-Chaps (●). The fractions from 26 to 31 (indicated by an arrow) were combined, dialysed overnight against 20 vol. of 12.5 mM-Tris/HCl (pH 7.2)/7.5 mM-Chaps at 4 °C, and analysed in the presence of 7.5 mM-Chaps (○). Portions [50 μl (●) and 100 μl (○)] were used for the Prl-binding assay. Values representing less than 500 c.p.m. of the specific binding were not plotted in the Figure. The straight diagonal line shows the [NaCl] gradient.

treatment with 7.5 mM-Chaps, the K_d of the microsome Prl receptors increased from $6.5 \times 10^{-11} \text{ M}$ to $1.3 \times 10^{-10} \text{ M}$.

Prl receptors, solubilized in 7.5 mM-Chaps or 1% Triton X-100, were similarly separated into two different fractions (Fr. A and B) by ion-exchange chromatography (Fig. 2). Prl receptors in Fr. A and B were eluted by solvent concentrations of 50–55 mM and 120–130 mM-NaCl respectively. In the Chaps extract, the amount of Prl binding to the receptors in Fr. A was almost the same as that in Fr. B. In the Triton extract, the binding in Fr. A was slightly lower than that in Fr. B. The 7.5 mM-Chaps-treated microsomes were again solubilized in 1% Triton X-100, after which 20–30% of Prl receptors became soluble. Chromatographic analysis shows that the receptors in this Triton extract were eluted in the same position as was Fr. B, and no Prl-binding was detected at the position of Fr. A. The recovery of Prl receptors solubilized in 7.5 mM-Chaps was 72–86% ($n = 5$); Triton-solubilized Prl receptors showed 60–65% recovery ($n = 4$) after ion-exchange chromatography.

The binding specificity of hormones to Prl receptors in the Chaps extract, Fr. A and Fr. B was assessed by incubating ^{125}I -Prl with increasing amounts of peptide hormones (Fig. 3). The binding of ^{125}I -Prl to the receptors was clearly inhibited by the presence of unlabelled Prl and hGH in a dose-related manner, with the curves for hGH shifted to the left. None of the other hormones tested had any effect on the binding of ^{125}I -Prl to the receptor. Prl receptors in the Triton extract and its fractions also showed the same hormone-binding specificities (Fig. 3).

Fig. 5. H.p.l.c.-gel filtration of Prl receptors in the Chaps extract

A 200 μ l portion of the Chaps extract (approx. 0.3 mg of protein) was subjected to h.p.l.c.-gel filtration and eluted in the presence of 5 mM-(○) or 7.5 mM-Chaps (●). Fractions (300 μ l each) were collected and 100 μ l was used for the PrI-binding assay. Arrows indicate peaks of the following markers: 1, Blue Dextran 2000; 2, glutamate dehydrogenase; 3, pyruvate kinase; 4, γ -globulin; 5, BSA; 6, ovalbumin; 7, PrI; and 8, soybean trypsin inhibitor. The peak position of these markers, measured by a u.v. monitor, did not change in the presence of either 0, 5 or 7.5 mM-Chaps. The relationship between the retention time and logarithm of M_r (□, 2-8) in the Figure was used to obtain the M_r of the PrI receptor (○, ●).

The profiles of the dose-response curves and the hormonal specificity did not change greatly when either labelled PrI or hGH was used (results not shown).

The fractionated PrI receptors in the Chaps and the Triton extracts were analysed by Scatchard (1945) analysis. By using the amounts of protein in Table 1, nearly 20% of total radioactivity was found to be specifically bound to the PrI receptors. All the data could be resolved by straight-line plots, but the slope of the regression lines varied (results not shown). The K_d values for PrI and hGH binding to the various PrI receptor fractions are summarized in Table 1. The K_d varied with the fractions used; Fr. A contained PrI receptors with the lowest K_d , whereas Fr. B contained PrI receptors with the highest K_d . The receptors in the Chaps or the Triton extracts showed a K_d intermediate between those in Fr. A and B. No difference was found whether labelled PrI or hGH was used as the binding hormone.

Effect of Chaps on the solubilized PrI receptors

Different profiles of PrI receptors were obtained depending on the Chaps concentration of the column buffer. As shown in Fig. 4, in the presence of 4 mM-Chaps, most PrI receptors in the Chaps extract were eluted by 100–110 mM-NaCl. The peak fractions combined were

Fig. 6. Sucrose-gradient centrifugation of labelled PrI-receptor complexes

Prl receptors in the Chaps extract (50 μ g) were incubated with 150000 c.p.m. of 125 I-PrI in a reaction volume of 0.5 ml for 16 h. A 200 μ l portion of the binding mixture was applied on to sucrose gradients and centrifuged in the presence of 5 mM-(○) or 7.5 mM-(●) Chaps. Specific and non-specific bindings of labelled PrI per centrifuge tube were approx. 17500 c.p.m. and 3300 c.p.m. respectively. After centrifugation, a 145 μ l fraction was collected and the PEG precipitation assay was then carried out on a 100 μ l portion. The remainder was used for the determination of the sucrose concentration. Linear sucrose gradients were obtained from fractions of 1 ($R = 5.0$ cm, $Z_1 = 4.0\%$)–25 ($R = 8.6$ cm, $Z_2 = 16.8\%$). R is the distance from the centrifugal axis and Z_1 and Z_2 are the sucrose concentrations. This range was used to calculate the sedimentation coefficient using the Table for the particle density of 1.3 and $Z_0 = -15$ (McEwen, 1967). Arrows indicate the position of marker proteins: 1, 125 I-PrI; 2, chymotrypsinogen A; 3, ovalbumin; 4, BSA; 5, γ -globulin; and 6, catalase. The straight diagonal line shows the [sucrose] gradient.

again analysed in the presence of 7.5 mM-Chaps. The profile of the PrI-binding activity had two peaks, which were eluted by 50–55 mM- and 100–105 mM-NaCl respectively. The elution profile of PrI receptors in Fr. B was unaffected by the presence of either 4 mM- or 7.5 mM-Chaps (results not shown).

The Chaps extracts were introduced to h.p.l.c.-gel-filtration columns (Fig. 5). Regardless of whether 5 mM- or 7.5 mM-Chaps was used, the PrI receptors could be resolved into single species. The position of the peak, however, differed clearly, depending on the Chaps concentration (tube 25 in 5 mM- or 32 in 7.5 mM-Chaps). The recovery was 30–40% ($n = 5$). PrI receptors in Fr. B, shown in Fig. 1, were not detectable after h.p.l.c.-gel filtration under the conditions used. The profiles represented an aggregated and a disaggregated form of PrI receptors in Fr. A. The M_r of PrI receptors was estimated to be 74000 ± 4900 (mean $\pm$ s.d., $n = 5$) in the presence of 5 mM-Chaps or 36800 ± 2100 ($n = 5$) in the presence of 7.5 mM-Chaps by comparison with the elution peaks of the marker proteins.

Labelled PrI-receptor complexes were analysed by sucrose-gradient centrifugation in the presence of 5 mM- or 7.5 mM-Chaps (Fig. 6). The sedimentation behaviour of the 125 I-PrI-receptor complexes was compared with

Fig. 7. Critical micelle concentration of Chaps

Chaps was dissolved in TMP buffer and the critical micelle concentration of Chaps was determined by iodine method (○) and the ANS method (●) as described in the Materials and methods section.

that of the marker proteins. The sedimentation coefficients of the marker proteins were as follows: catalase, 11.9 S; γ -globulin, 7.10 S; BSA, 4.77 S; ovalbumin, 3.56 S; chymotrypsinogen A, 2.52 S; and ^{125}I -Prl, 2.13 S. In the presence of 5 mM-Chaps, the PEG-precipitable radioactivity mostly sedimented between γ -globulin and BSA. The sedimentation coefficient of this complex was 5.56 ± 0.22 S ($n = 5$). The small peak coincided with that of free ^{125}I -Prl. In the presence of 7.5 mM-Chaps, the radioactivity of the main peak that appeared in the presence of 5 mM-Chaps decreased, and one more peak appeared below ovalbumin. The sedimentation coefficients of the first and second complexes were 5.39 ± 0.12 S and 4.01 ± 0.23 S ($n = 4$) respectively. The recovery of the Prl-receptor complexes before and after centrifugation was about 100%.

The absorbance of iodine and the fluorescence intensity of ANS were greatly increased by Chaps concentrations above 5 mM (Fig. 7). The critical micelle concentration of Chaps was estimated to be about 5.3–5.5 mM.

DISCUSSION

After solubilization of the microsome Prl receptors with Chaps or Triton X-100, the K_d for Prl binding by the solubilized receptors was decreased slightly (with an increased association constant; $K_a = 1/K_d$), consistent with previous findings (Shiu & Friesen, 1974; Haeuptle *et al.*, 1983; Katoh *et al.*, 1984; Waters *et al.*, 1984; Sakai *et al.*, 1985). The present data suggest that this is probably due to the selective solubilization of Prl receptors with a decreased K_d (Fig. 1). After solubilization by Chaps, Prl receptors with the increased K_d were left in the microsomes and solubilized by Triton X-100 (Fig. 2).

Chaps-solubilized Prl receptors showed a specificity for hormones identical with that shown by microsomal (Shiu

et al., 1973) or Triton-solubilized receptors (Shiu & Friesen, 1974). The solubilized Prl receptors were further separated by ion-exchange chromatography into two different fractions, both of which showed the same hormonal binding specificities. These fractionated receptors are characterized either by a high or by a low K_d for hormone binding. Nevertheless, they exhibit a high Prl-binding specificity and sensitivity, similar to those of the membrane Prl receptors. The affinity constant of Prl binding to the rabbit or rat mammary gland changed according to the stage in the animal's reproduction cycle (Perry & Jacobs, 1978; Guillaumot *et al.*, 1984). Two types of Prl-binding sites in the dispersed rabbit mammary cells (Suard *et al.*, 1979) and in the Chaps-solubilized hepatic membrane (Gavish *et al.*, 1983) have been shown by Scatchard (1945) analysis. We speculate that the ratio of Prl receptors with the high or low K_d changes during the oestrous cycle or lactation. Prl receptors internalize after the binding of Prl (Josefsberg *et al.*, 1979; Ferland *et al.*, 1984) and recycle through an energy-dependent uptake mechanism (Costlow & Hamble, 1982). Recently a rapid turnover (degradation) of the Prl receptors has been reported (Baxter, 1985).

Prl receptors in the microsomes were solubilized with 7.5 mM-Chaps. The behaviour of the soluble Prl receptors was greatly affected by Chaps at concentrations either below or above the critical micelle concentration in the presence of NaCl. The structure of the Prl receptor is probably stabilized by both hydrophobic and ionic bonds. Recently, Yamada & Donner (1985) have demonstrated that the Triton-solubilized hepatic Prl receptor is composed of monomeric components held together by non-covalent forces. Our data suggest that rabbit mammary gland contains two Prl-binding subunits. One species of the Prl-binding subunit is non-dissociable under the conditions used in the present study. The other species is dissociable by the presence of 7.5 mM-Chaps. This is consistent with the evidence obtained by ion-exchange chromatography, h.p.l.c.-gel filtration or ultracentrifugation. The M_r of the dissociable species was estimated to be 74000 ± 4900 or 36800 ± 2100 by h.p.l.c.-gel filtration in the presence of low or high Chaps concentrations respectively, suggesting that the binding subunit causes this monomeric subunit to aggregate with itself or with another specific associated protein with a similar M_r . The M_r of the non-dissociable Prl receptors could not be determined by h.p.l.c.-gel filtration, but ultracentrifugation analysis shows that its M_r appears to be close to that of the aggregated Prl receptors. Chaps is much more effective at breaking protein-protein bonds than is Triton X-100 and disaggregates proteins to the monomeric form (Hjelmeland, 1980). The M_r of this small subunit is almost identical in value with those reported by others (Borst & Sayare, 1982; Liscia & Vonderhaar, 1982; Liscia *et al.*, 1982; Haeuptle *et al.*, 1983; Hughes *et al.*, 1983; Yamada & Donner, 1984; Borst *et al.*, 1985; Katoh *et al.*, 1985; Kelly *et al.*, 1985). The hormone-receptor complex has an M_r of 97000–99800 as revealed by hydrodynamic analysis of hepatic Prl receptors (Jaffe, 1982; Rae-Venter & Dao, 1983), which estimated the M_r of the receptor alone to be 73000–77800. This agrees with our estimated M_r of the aggregated Prl receptor (74000). These M_r values, however, are considerably lower than the reported ones of 220000 (Shiu & Friesen, 1974), 200000 (Haeuptle *et al.*, 1983) and 133000 (Katoh *et al.*, 1984). In liver,

Chaps-solubilized Prl receptors did not interact with concanavalin A (Vonderhaar *et al.*, 1985), whereas Prl receptors solubilized with Triton X-100 contained specific associated proteins with which concanavalin A interacted (Bhattacharya & Vonderhaar, 1982). Triton X-100 is a mild detergent that does not inhibit protein-protein aggregation (Lang *et al.*, 1980; Pilch & Czech, 1980). This detergent forms a high- M_r micelle and binds to membrane proteins in considerable amounts (Clarke, 1975; Newman *et al.*, 1977; Smigel & Fleischer, 1977). The difference is probably due to the amount of detergent bound to proteins and to the existence of other associated proteins in the receptor molecule.

In conclusion, Prl receptors can be fractionated, by ion-exchange chromatography, into two different species both of which have high Prl binding specificity and sensitivity. The present data also suggest that the rabbit mammary gland contains the aggregated Prl-binding subunit held together by non-covalent forces and, probably, a non-dissociable binding subunit

REFERENCES

- Baxter, R. C. (1985) *Endocrinology* (Baltimore) **117**, 650–655
- Bhattacharya, A. & Vonderhaar, B. K. (1982) *Arch. Biochem. Biophys.* **215**, 425–432
- Bonifacino, J. S. & Dufau, M. L. (1984) *J. Biol. Chem.* **259**, 4542–4549
- Borst, D. W. & Sayare, M. (1982) *Biochem. Biophys. Res. Commun.* **105**, 194–201
- Borst, D. W., Sayare, M. & Posner, B. I. (1985) *Mol. Cell. Endocrinol.* **39**, 125–130
- Carr, F. E. & Jaffe, R. C. (1982) *Mol. Cell. Endocrinol.* **25**, 317–326
- Clarke, S. (1975) *J. Biol. Chem.* **250**, 5459–5469
- Costlow, M. E. & Hamble, A. (1982) *J. Biol. Chem.* **257**, 9330–9334
- Djiane, J., Durand, P. & Kelly, P. A. (1977) *Endocrinology* (Baltimore) **100**, 1348–1356
- Ferland, L. H., Djiane, J., Houdebine, L.-M. & Kelly, P. A. (1984) *Endocrinology* (Baltimore) **115**, 1842–1849
- Gavish, M., Amit, T., Barkey, R. J. & Youdim, M. B. H. (1983) *FEBS Lett.* **154**, 81–86
- Guillaumot, P., Sabbagh, I., Bertrand, J. & Cohen, H. (1984) *Biochem. Biophys. Res. Commun.* **123**, 612–617
- Hauptle, M.-T., Aubert, M. L., Djiane, J. & Kraehenbuhl, J.-P. (1983) *J. Biol. Chem.* **258**, 305–314
- Hjelmeland, L. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6368–6370
- Hughes, J. P., Simpson, S. A. & Friesen, H. G. (1983) *Endocrinology* (Baltimore) **112**, 1980–1985
- Jaffe, R. C. (1982) *Biochemistry* **21**, 2936–2939
- Josefsberg, Z., Posner, B. I., Patel, B. & Bergeron, J. J. M. (1979) *J. Biol. Chem.* **254**, 209–214
- Katoh, M., Djiane, J., Leblanc, G. & Kelly, P. A. (1984) *Mol. Cell. Endocrinol.* **34**, 191–200
- Katoh, M., Djiane, J. & Kelly, P. A. (1985) *Endocrinology* (Baltimore) **116**, 2612–2620
- Kelly, P. A., Katoh, M., Djiane, J. & Sakai, S. (1985) *Methods Enzymol.* **109**, 156–170
- Lang, U., Kahn, C. R. & Chrambach, A. (1980) *Endocrinology* (Baltimore) **106**, 40–49
- Liscia, D. S. & Vonderhaar, B. K. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5930–5934
- Liscia, D. S., Alhadi, T. & Vonderhaar, B. K. (1982) *J. Biol. Chem.* **257**, 9401–9405
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Luthe, D. S. (1983) *Anal. Biochem.* **135**, 230–232
- McEwen, C. R. (1967) *Anal. Biochem.* **20**, 114–149
- Necessary, P. C., Humphrey, P. A., Mahajan, P. B. & Ebner, K. E. (1984) *J. Biol. Chem.* **259**, 6942–6946
- Newman, S. T., Rossi, G. & Metzger, H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 869–872
- Ortner, M. J., Sik, R. H., Chignell, C. F. & Sokoloski, E. A. (1979) *Mol. Pharmacol.* **15**, 179–188
- Perry, H. M., III & Jacobs, L. S. (1978) *J. Biol. Chem.* **253**, 1560–1564
- Pilch, P. F. & Czech, M. P. (1980) *J. Biol. Chem.* **255**, 1722–1731
- Rae-Venter, B. & Dao, T. L. (1983) *Arch. Biochem. Biophys.* **222**, 12–21
- Ross, S. & Oliver, J. P. (1959) *J. Phys. Chem.* **53**, 1671–1674
- Sakai, S., Kohmoto, K. & Johke, T. (1975) *Endocrinol. Jpn.* **22**, 379–387
- Sakai, S., Katoh, M., Berthon, P. & Kelly, P. A. (1985) *Biochem. J.* **224**, 911–922
- Sasaki, N., Tanaka, Y., Imai, Y., Tsushima, T. & Matsuzaki, F. (1982) *Biochem. J.* **203**, 653–662
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Shiu, R. P. C. & Friesen, H. G. (1974) *J. Biol. Chem.* **249**, 7902–7911
- Shiu, R. P. C., Kelly, P. A. & Friesen, H. G. (1973) *Science* **180**, 968–971
- Smigel, M. & Fleischer, S. (1977) *J. Biol. Chem.* **252**, 3689–3696
- Suard, Y. M. L., Kraehenbuhl, J.-P. & Aubert, M. L. (1979) *J. Biol. Chem.* **254**, 10466–10475
- Vonderhaar, B. K., Bhattacharya, A., Alhadi, T., Liscia, D. S., Andrew, E. M., Young, J. K., Ginsburg, E., Bhattacharjee, M. & Horn, T. M. (1985) *J. Dairy Sci.* **68**, 466–488
- Waters, M. J., Lusins, S. & Friesen, H. G. (1984) *Endocrinology* (Baltimore) **114**, 1–10
- Yamada, K. & Donner, D. B. (1984) *Biochem. J.* **220**, 361–369
- Yamada, K. & Donner, D. B. (1985) *Biochem. J.* **228**, 383–390

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