Thermodynamic analysis of the interaction of prolactin with its receptor in the rabbit mammary-gland microsomes

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The interaction of prolactin (PRL) with its membrane receptor depends markedly on temperature. Thermodynamic parameters for this reaction have been evaluated from data for time-course kinetics and equilibrium binding at multiple temperatures between 19 and 31 °C. The free-energy change with temperature and the van't Hoff plot were found to be linear. These suggest that there are minimal structure changes at the PRL–receptor contact site over this temperature range. The positive signs of the entropy and enthalpy of reaction, and of the entropy of activation ($\Delta S^\ddagger$) for association, indicate that the hydrophobic bonding is the most significant force involved in PRL–receptor formation. The $\Delta S^\ddagger$ for dissociation was negative, and the enthalpy of activation for dissociation was about 20.3 kJ·mol⁻¹ larger than that for association, indicating that the PRL–receptor complex is further stabilized by contributions of hydrogen bonds and van der Waals contacts after the initial interaction. The free energy of activation for dissociation, at 25 °C, was about 2.5-fold larger than that for association. This would cause slow dissociation of PRL from its receptor.

INTRODUCTION

Prolactin (PRL) mediates its diverse physiological actions by interacting with a specific receptor located on the plasma membrane of the target tissue. PRL receptors have a hormonal specificity and a high affinity for the binding of lactogenic hormones (Nagasawa *et al.*, 1979). However, little information is available about the chemical nature of PRL–receptor interaction and its corresponding transition state. The present experiments undertaken here were designed to clarify thermodynamically the chemical interaction between PRL and its receptor.

We have shown that the inclusion of a hydrophobic-bond-breaking agent decreases greatly the association rate of PRL to the receptor, whereas the addition of a hydrogen-bond-breaking agent greatly accelerates the rate of PRL dissociation from the receptor (Sakai & Suzuki, 1989). We reasoned that, if hydrophobic effects play an important role in the formation of the PRL–receptor complex, it is possible to demonstrate this by thermodynamic analysis of the reaction. It has been shown that the reaction of hormone with its receptor is complex and involves more than one step (Andersen & Ebner, 1979; Waelbroeck *et al.*, 1979; Haro & Talamantes, 1985; Sanborn *et al.*, 1987). Ross & Subramanian (1981) presented a conceptual model of protein association consisting of two steps: the mutual penetration of hydration layers, causing disordering of the solvent, followed by further short-range interactions. If the PRL–receptor complex is stabilized after the initial interaction, it is expected that heat is required for the dissociation of PRL more than for the association.

In the present study the PRL binding assay using microsome PRL receptor and ¹²⁵I-PRL was carried out at multiple temperatures, and the effects of temperature on the association and on the dissociation rate constants of PRL were evaluated by thermodynamic analysis.

MATERIALS AND METHODS

PRL binding assay

Ovine PRL (NIADDK-oPRL-17; 30 i.u./mg) was kindly supplied by NIADDK, Bethesda, MD, U.S.A. PRL was

iodinated by using lactoperoxidase and H₂O₂ (Sakai *et al.*, 1986), and the specific radioactivity of ¹²⁵I-PRL was about 2.2 MBq/μg. The binding buffer consisted of 25 mM-Tris/10 mM-MgCl₂/1 mM-phenylmethanesulphonyl fluoride/0.02% NaN₃/0.2% BSA, and was adjusted to pH 7.6 with HCl at the temperature at which it was used. Mammary glands from mid-lactating New Zealand White rabbits were used. Microsomes (75 μg of protein/tube) were incubated with ¹²⁵I-PRL (30 000 c.p.m.) in a total volume of 0.5 ml (Sakai *et al.*, 1986). For the Scatchard (1949) analysis, microsomes were incubated at 19, 23, 27 and 31 °C with ¹²⁵I-PRL in the presence of increasing concentrations of unlabelled PRL (0.2–3.6 ng). The temperature was maintained within ±0.1 °C. Incubation was continued for 20 h at 19 and 23 °C, or for 16 h at 27 and 31 °C. The association constant (K_a) of PRL binding was calculated from the slope of the Scatchard plot. To determine the association rate constant (k_{+1}), microsomes were incubated with ¹²⁵I-PRL at 19, 23, 27, and 31 °C. PRL binds to its receptor at a 1:1 ratio (Murakami *et al.*, 1988). Assuming that the reversible reaction obeys second-order kinetics, the k_{+1} was calculated from the slope of the association curve, according to the methods described by Shiu & Friesen (1974). To determine the dissociation rate constant (k_{-1}), microsomes were incubated at 19 °C for 20 h with ¹²⁵I-PRL in the presence or absence of excess unlabelled PRL (1 μg). The reaction mixture was diluted with 3 ml of binding buffer, centrifuged at 2300 g for 15 min at 19 °C, and the supernatant was discarded. Subsequently, 0.5 ml of binding buffer containing 1 μg of unlabelled PRL was added to each tube (Haro & Talamantes, 1986). The tubes were incubated at 19, 25, and 31 °C. The k_{-1} was calculated from the slope of the dissociation curve. When the reaction was terminated, the reaction mixture was diluted with 3 ml of binding buffer and tubes were centrifuged at 2300 g for 15 min at 4 °C. Radioactivity in the pellet was measured in a γ-radiation counter. The experiments were performed in triplicate, and each determination was repeated at least three times.

Thermodynamic analysis

The values for the K_a , k_{+1} , and k_{-1} , obtained at different temperatures, were used for the thermodynamic analysis. The

Abbreviations used: PRL, prolactin; assoc., association; dissoc., dissociation; ΔG , free energy of reaction; ΔH , enthalpy of reaction; ΔS , entropy of reaction; E_a , activation energy; $\Delta G^\ddagger$, free energy of activation; $\Delta H^\ddagger$, enthalpy of activation; $\Delta S^\ddagger$, entropy of activation; K_a , affinity constant; k_{+1} , association rate constant; k_{-1} , dissociation rate constant; r , correlation coefficient.

values for the K_a were used to generate the standard free energy (ΔG) of the PRL-receptor reaction using the equation:

$$\Delta G = -RT \ln K_a$$

and the values for ΔG was plotted as a function of temperature, T . R is the gas constant and T is the absolute temperature. The enthalpy of reaction (ΔH) was calculated from the van't Hoff plot. The entropy of reaction (ΔS) was calculated by the Gibbs function:

$$\Delta G = \Delta H - T\Delta S$$

The activation energy (E_a) was calculated by the Arrhenius equation, $\log k_{+1}$ (or k_{-1}) = $\log A - (E_a/2.303R)(1/T)$, where A is the frequency factor. The enthalpy of activation ($\Delta H^\ddagger$) was calculated by the equation:

$$\Delta H^\ddagger = E_a - RT$$

According to the transition-state theory, the free energy ($\Delta G^\ddagger$) and entropy of activation ($\Delta S^\ddagger$) were obtained from eqns. (1) and (2) respectively:

$$k_{+1} \text{ (or } k_{-1}) = (kT/h) e^{-\Delta G^\ddagger/RT} \quad (1)$$

$$k_{+1} \text{ (or } k_{-1}) = (kT/h) e^{\Delta S^\ddagger/R} e^{-\Delta H^\ddagger/RT} \quad (2)$$

where h and k are Planck's constant and the Boltzmann constant respectively. All thermodynamic data are standardized at 25 °C.

Statistics

A straight line was constructed by using the least-squares linear regression method. Statistical analysis was performed using Student's two-tailed t test. Differences were considered significant where the mean differed from the control at $P < 0.05$.

RESULTS

Free energy, enthalpy, entropy of PRL-receptor reaction

In the present experiments, the incubation temperatures were set at 19, 23, 27 or 31 °C, and the K_a and receptor concentration were determined by the Scatchard analysis (Fig. 1a). Linear Scatchard plotting allows us to determine exact values for the K_a and receptor concentration. The K_a varied markedly with temperature. The order of the K_a values was of 10^{10} M^{-1} . The receptor concentration was estimated to be $14.5 \pm 0.7 \text{ fmol/tube}$ (mean $\pm$ s.d.) at all temperatures examined. Non-specific binding of ^{125}I -PRL was always less than 1.5% of total radioactivity added. As shown in Fig. 1(b), a plot of $-\Delta G$ against the

Fig. 2. Rate constants of association (a) and of dissociation (b) of PRL at different temperatures

Incubation temperatures (°C) are shown on the Figure. Three of the six tubes were incubated in the presence of, and three in the absence of, 1 μg of unlabelled PRL. Six tubes from each set were taken out at each point. The mean values from three determinations were plotted. (a) The k_{+1} was calculated by the procedure of Shiu & Friesen (1974). $Q = D^2 - 4ab$; $\beta = D + \sqrt{Q}$; $\gamma = D - \sqrt{Q}$; $D = -(a+b+c)$; a = initial concentration of ^{125}I -PRL; b = receptor concentration (from the Scatchard analysis); $c = (a-x_e)(b-x_e)/x_e$; x_e = ^{125}I -PRL at equilibrium; x = ^{125}I -PRL bound at time t . The slope of the straight line yields the k_{+1} . (b) A_0 and A_t are the amounts of specific binding of ^{125}I -PRL at zero time and t respectively, after initiation of the dissociation reaction. The values of $\log(A_t/A_0)$ are plotted as a function of incubation time, t .

Fig. 1. Scatchard plots (a), temperature-dependent free-energy change (b), and van't Hoff plot of affinity constants (c)

Incubation temperatures (°C) are shown in the Figure. The K_a was obtained from Scatchard analysis. The calculated regression line is $\Delta G = -0.3472T + 50.27$ for (b) or $\ln K_a = -6066(1/T) + 41.83$ for (c).

temperature generated a straight regression line ($r < -0.99$). The value for ΔG is $-53.26 \pm 0.377 \text{ kJ} \cdot \text{mol}^{-1}$ (mean $\pm$ s.d., $n = 4$) at 25°C . The ΔH is determined from the slope of the van't Hoff plot ($r < -0.99$), which is shown in Fig. 1(c). The value for ΔH is $47.61 \pm 5.52 \text{ kJ} \cdot \text{mol}^{-1}$ and did not change at any temperature examined. The positive sign of ΔH indicates that the interaction of PRL with its receptor is endothermic. The value for ΔS is $336.6 \pm 11.13 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. Therefore the free-energy change can be expressed as a function of temperature:

$$\Delta G = 47.61 - 0.3366 T \text{ (kJ} \cdot \text{mol}^{-1}\text{)}$$

Free energy, enthalpy and entropy of activation, and activation energy

To clarify temperature-dependent changes in k_{+1} and k_{-1} the association (assoc.) and dissociation (dissoc.) of PRL were determined at various temperatures. The values for specific binding were transformed and were plotted against the incubation time (Fig. 2). For the association, Fig. 2(a) shows that the values generated a linear curve ($r > 0.96$). The k_{+1} changed as a function of the incubation temperature. A plot of $\log k_{+1}$ against $1/T$ generated a straight regression line ($r < -0.98$) (Fig. 3). The dissociation of PRL from the receptor changed in a temperature-dependent manner, as shown in Fig. 2(b). When incubation temperature was increased from 19 to 31°C , the amount of PRL specifically bound to the receptor decreased by 10 to 13 % during the first 4 h incubation period and, thereafter, the dissociation data obeyed first-order kinetics. In the presence of $2 \mu\text{g}$ of unlabelled PRL/ml, the k_{-1} was 1.5–1.8-fold greater than in its absence. However, the E_a did not change either in the presence or absence of unlabelled PRL. The two van't Hoff plot lines ran parallel in the presence and absence of unlabelled PRL (results

Fig. 3. van't Hoff plots for the rate constants of association (k_{+1} , ●) and of dissociation (k_{-1} , ○) of PRL

The values shown in Fig. 2 were plotted as a function of temperature ($1/T$). $\log k_{+1} = -2627(1/T) + 17.31$, and $\log k_{-1} = -3685(1/T) + 10.43$.

Table 1. Thermodynamic parameters for the association and dissociation of PRL

The values for the thermodynamic parameters were calculated from the data shown in Fig. 3 (results are means $\pm$ s.d., $n = 3$). Statistical significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (compared with the value for association).

	$\Delta G^\ddagger$ ($\text{kJ} \cdot \text{mol}^{-1}$)	$\Delta H^\ddagger$ ($\text{kJ} \cdot \text{mol}^{-1}$)	$\Delta S^\ddagger$ ($\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$)	E_a ($\text{kJ} \cdot \text{mol}^{-1}$)
Association	42.26 ± 0.08	47.78 ± 2.13	18.43 ± 7.23	50.29 ± 2.13
Dissociation	$105.0 \pm 1.60^{***}$	$68.07 \pm 4.94^*$	$-124.0 \pm 14.7^{**}$	$70.45 \pm 4.94^*$

not shown). A plot of $\log k_{-1}$ against $1/T$ generated a straight regression line ($r < -0.99$) (Fig. 3). The dissociation rate is more sensitive to temperature than is the association rate.

As shown in Fig. 3, the two linear van't Hoff plot lines allowed us to calculate activation energies for the association and dissociation of PRL individually. The values for the $\Delta G^\ddagger$, $\Delta H^\ddagger$, $\Delta S^\ddagger$ and E_a are listed in Table 1. We were able to confirm the relationship:

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$$

The temperature-dependent changes in the $\Delta G^\ddagger_{\text{assoc.}}$ and $\Delta G^\ddagger_{\text{dissoc.}}$ are:

$$\Delta G^\ddagger_{\text{assoc.}} = 47.78 - 0.0184T$$

and

$$\Delta G^\ddagger_{\text{dissoc.}} = 68.07 + 0.124T \text{ (kJ} \cdot \text{mol}^{-1}\text{)}.$$

The $\Delta G^\ddagger$ and the $\Delta H^\ddagger$ were both positive. The values for association were significantly different from those for dissociation. These differences are most obvious between $\Delta G^\ddagger_{\text{assoc.}}$ and $\Delta G^\ddagger_{\text{dissoc.}}$ or between $\Delta S^\ddagger_{\text{assoc.}}$ and $\Delta S^\ddagger_{\text{dissoc.}}$. The value for the $\Delta G^\ddagger_{\text{dissoc.}}$ at 25°C was 2.48-fold larger than that for the $\Delta G^\ddagger_{\text{assoc.}}$. The $\Delta S^\ddagger_{\text{assoc.}}$ had a positive sign, whereas the $\Delta S^\ddagger_{\text{dissoc.}}$ had a negative sign. The values for the $E_{a,\text{dissoc.}}$ and $\Delta H^\ddagger_{\text{dissoc.}}$ are about $20.3 \text{ kJ} \cdot \text{mol}^{-1}$ larger than those for association.

DISCUSSION

Values for the K_a , k_{+1} and k_{-1} were determined at multiple temperatures between 19 and 31°C , and these data were used in thermodynamic analysis. The van't Hoff plots and the plot of ΔG against temperature were all linear. Similar results were reported for the fat-cell-membrane insulin receptor (Cuatrecasas, 1971) and mouse liver PRL receptor (Haro & Talamantes, 1985). It appears that the interaction of PRL with mouse liver PRL receptor is much faster. Similar binding characters have been reported in mouse mammary-gland PRL receptor (Sakai *et al.*, 1975, 1978). The data suggest that the interaction of PRL with the rabbit mammary PRL receptor is different from that with mouse liver PRL receptor. The linear van't Hoff plot and free-energy change can be reasonably expected to give reliable values for ΔH and ΔG , and consequently for ΔS . The data suggest that there are minimal structural changes at the PRL–receptor contact site over this temperature range and no difference in the species of non-covalent forces contributing to the reaction. Changes in secondary and tertiary structure accompanying the association resulted in the curvilinear van't Hoff plot and free-energy change (Osborne *et al.*, 1976). Over a broader temperature range, curvilinear van't Hoff plots or free-energy changes have been reported for the follitropin receptor (Andersen *et al.*, 1983; Sanborn *et al.*, 1987), insulin receptor (Waelbroeck *et al.*, 1979), glucocorticoid receptor (Wolff *et al.*, 1978), and self-associating proteins (Osborne *et al.*, 1976; Formisano *et al.*, 1977; Ross & Subramanian, 1981). In the present study the temperature varied within a relatively narrow range, and the effects of temperature

on association and dissociation were evaluated separately. This approach may be valid in other systems where thermodynamic analysis has been applied, such as in protein-protein interaction.

The sources of positive enthalpy and entropy changes arise from ionic and hydrophobic interactions (Ross & Subramanian, 1981; Strazza *et al.*, 1985). The fact that the sign of $\Delta S^{\ddagger}_{\text{assoc.}}$ is positive is further evidence that the driving forces for the formation of the transition state are hydrophobic; a negative value would be expected if there were more hydrogen bonds in the transition state than in the reactants (Wolff *et al.*, 1978). It appears that the first event is most appropriately described in terms of the 'hydrophobically bound complex' (terminology of Ross & Subramanian, 1981). The chaotropic agents interfered in the binding of PRL to its receptor by decreasing the k_{+1} of PRL binding to the receptor in a concentration- and agent-dependent manner (Sakai & Suzuki, 1989). Hydrophobic bonding appears to be the most important force participating in the PRL-receptor complex-formation. Hydrophobicity is the major factor stabilizing the protein-protein association (Chothia & Janin, 1975).

The chemical modifications of PRL and the receptor (with specificity towards histidine, lysine, tryptophan or tyrosine residues) resulted in a loss of receptor- and PRL-binding activity respectively (Andersen & Ebner, 1979; Andersen *et al.*, 1979; De la Llosa *et al.*, 1985; Mahajan & Ebner, 1986a,b). These amino acids play an important role in interacting through non-covalent bonds both intra- and inter-molecularly. By sequence comparison among various mammalian PRLs, two highly conserved regions, presumably a portion of the receptor-binding region of PRL, show high hydrophilic properties with charged and hydroxy amino acids (Kato, 1988).

The rate of PRL dissociation from the receptor was more sensitive to temperature than was the association rate. Heat causes denaturation or unfolding of globular proteins by disrupting the hydrogen bonds between the paired amino acids. Because of the large contributions of $\Delta S^{\ddagger}_{\text{dissoc.}}$ to $\Delta G^{\ddagger}_{\text{dissoc.}}$, much heat would be required to form the transition state of the PRL-receptor complex before dissociation. The value of $\Delta S^{\ddagger}_{\text{dissoc.}}$ is negative, and the values for the $\Delta G^{\ddagger}_{\text{dissoc.}}$ and $\Delta H^{\ddagger}_{\text{dissoc.}}$ are significantly larger than those for association. The data indicate that the PRL-receptor complex is further stabilized by contributions of hydrogen bonds and probably van der Waals contacts after the initial interaction. This is also supported by our previous findings showing that the PRL-receptor complex is unstable in the presence of hydrogen-bond-breaking agents (Sakai & Suzuki, 1989). Mammary PRL receptors have a high specificity for the binding of hormones. The proper formation of hydrogen bonds and of van der Waals contacts requires complementarity of the surfaces involved (Chothia & Janin, 1975). It has been reported that the dissociation of PRL from the mammary receptor is a very slow reaction (Van der Gugten *et al.*, 1980; Kelly *et al.*, 1983). The turnover time for the PRL receptor in rat liver was 40–50 min (Baxter, 1985). Taken together, it appears that, in the situation *in vivo*, most PRL are internalized through the binding to the receptor.

Thermodynamic analysis suggests that the reaction of PRL with its receptor is complex and involves more than one step. At least two steps are involved in the PRL-receptor interaction, for example:

[PRL-receptor] is an initially interacting state, and ([PRL-receptor]) is a thermodynamically stable state. The same model has been reported in the mouse liver PRL receptor (Haro & Talamantes, 1985).

Thermodynamic analysis can be used to clarify the chemical nature of PRL-receptor interaction in considerable detail. At present, the three-dimensional structures of PRL and the receptor are unknown. No information is available concerning the changes in secondary and tertiary structures of these molecules accompanying the reaction. Extensive work, including elucidation of these structures, is clearly required to refine the modelling of the PRL-receptor interaction.

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