Biochimica et Biophysica Acta, 628 (1980) 220—227

BBA 29186

KINETICS OF THE HORMONE-RECEPTOR INTERACTION

COMPETITION EXPERIMENTS WITH SLOWLY EQUILIBRATING LIGANDS

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(Received June 8th, 1979)

Key words: Hormone-receptor interaction; Competition; Slowly-equilibrating system; (Kinetics)

Summary

Kinetics of formation of protein-tracer complex in the presence of competitor were calculated. The parameters were chosen so that they should realistically describe the in vitro association of steroid hormones with their receptors. Time necessary for equilibration depends on four rate constants in addition to initial concentrations and may be more than 1000 min. Competitors forming complexes with the protein that dissociate faster than the protein-tracer complex have relative binding affinities apparently decreasing with incubation time. Conversely, relative binding affinity apparently increases with time if the protein-competitor complex dissociates more slowly than the protein-tracer complex. Moreover, lack of equilibration is not easily detected. It is suggested that kinetic analyses, more detailed than usual, precede competition experiments.

Introduction

It has been shown earlier that saturation analysis in slowly equilibrating systems may result in erroneous estimations of dissociation constants for protein-ligand complexes [1]. The relevance of kinetic parameters should be emphasized also in competition studies [2,3]. Competition patterns are widely investigated in the hope of gaining insight into the nature of protein-ligand interactions [4—7]. They are also used in studies on structural requirements of drug action [3,8—12,22,36—37] or for receptor characterization [13—21]. In

experiments of this type concentration of protein-radioligand (tracer) complex is measured after equilibration of the binding molecule with the tracer in the presence of the competitor. In a number of cases of interest the above complexes are of high stability with half lives comparable to or higher than 'time of equilibration'. This means that the relevance of competition data can only be judged if we assess also the influence of kinetics on so called equilibrium measurements. The considerations and deductions presented in this paper are valid for any system with similar competition mechanisms. Still they can find application mainly in the study of hormone-receptor interactions. The examples are taken from the field of steroid receptor research.

Results

1. Time course of competition

Consider the following system of reactions

$$L_1 + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B_1 \qquad \text{(I)}$$

$$L_2 + P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} B_2 \qquad \text{(II)}$$

where L_1 stands for radiolabelled ligand, L_2 for unlabelled competitor, P for the binding protein, B_1 and B_2 for the bound form of the tracer and the competitor, respectively. This model corresponds to the assumption of one homogeneous binding site and purely competitive displacement of the tracer. Non-specific binding is neglected.

Let the concentration $[B_1]$ be measurable. It can also be calculated as a solution to the following system of differential equations

$$\frac{d[P(t)]}{dt} = -(k_1L_1 + k_2L_2 + k_{-2})[P(t)] + (k_{-1} - k_{-2})[B_1(t)] + k_{-2}P_0 \qquad (1)$$

$$\frac{d[B_1(t)]}{dt} = k_1L_1[P(t)] - k_{-1}[B_1(t)]$$

The initial conditions are:

$$[P(0)] = P_0; \quad [B_1(0) = 0 \qquad (2)$$

We made use of the stoichiometric constraint:

$$[B_1(t)] + [B_2(t)] + [P(t)] = P_0$$

Protein concentration is usually very low in receptor studies. If $P_0 << k_{-1}/k_1$ and $P_0 << k_{-2}/k_2$ as it was suggested [23], both reactions can be taken as pseudo-first order, i.e. $[L_1]$ and $[L_2]$ concentrations taken as constants. Now the apparent rate constants can be introduced $k_1' = k_1[L_1]$ and $k_2' = k_2[L_2]$. With this simplification the solution to system (1) is

$$[P(t)] = p_0 + p_1\, e^{r_1 t} + p_2\, e^{r_2 t} \qquad (3)$$

$$[B_1(t)] = b_0 + b_1\, e^{r_1 t} + b_2\, e^{r_2 t} \qquad (4)$$

where

$$r_1 = \frac{-(k'_1 + k_{-1} + k'_2 + k_{-2}) + \sqrt{(k'_1 + k_{-1} + k'_2 + k_{-2})^2 - 4(k_{-1}k'_2 + k_{-1}k_{-2} + k'_1k_{-2})}}{2}$$

$$r_2 = \frac{-(k'_1 + k_{-1} + k'_2 + k_{-2}) - \sqrt{(k'_1 + k_{-1} + k'_2 + k_{-2})^2 - 4(k_{-1}k'_2 + k_{-1}k_{-2} + k'_1k_{-2})}}{2}$$

$$p_0 = \frac{P_0k_{-2}}{k'_2 + k_{-2} + (k'_1k_{-2}/k_{-1})}; \quad b_0 = \frac{P_0k'_1k_{-2}/k_{-1}}{k'_2 + k_{-2} + (k'_1k_{-2}/k_{-1})}$$

$$p_1 = \frac{k'_1(k_{-1} + r_1)(P_0 - p_0) + b_0(k_{-1} + r_1)(k_{-1} + r_2)}{k'_1[(k_{-1} + r_1) - (k_{-1} + r_2)]}; \quad b_1 = \frac{k'_1p_1}{k_{-1} + r_1}$$

$$p_2 = P_0 - p_0 - p_1; \quad b_2 = \frac{k'_1p_2}{k_{-1} + r_2}$$

The speed at which the equilibrium is approached depends in general on four rate constants. In the $t \to \infty$ limit i.e. in equilibrium the degree of competition is expressed

$$\frac{[B_1(\infty)]}{[B_1^0(\infty)]} = \frac{K_1L_1 + 1}{K_2L_2 + K_1L_1 + 1} \tag{5}$$

Here $[B_1^0(\infty)]$ is the equilibrium concentration of the bound tracer in the absence of competitor, K_1 and K_2 are the association equilibrium constants for reactions I and II, respectively. Fig. 1 shows $[B_1(t)]$ functions calculated

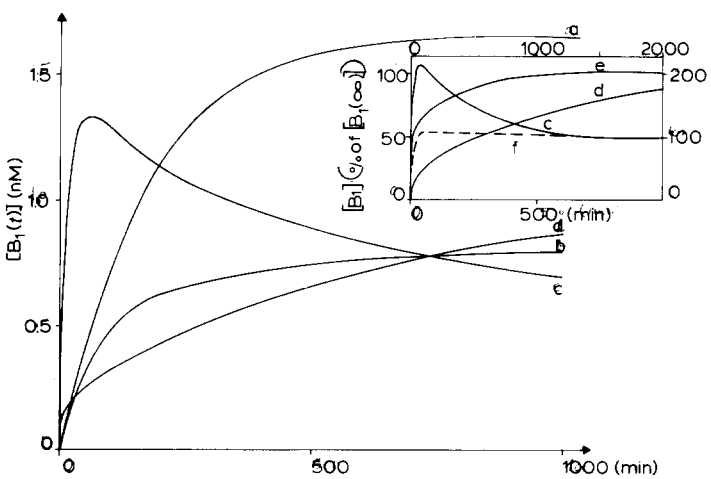


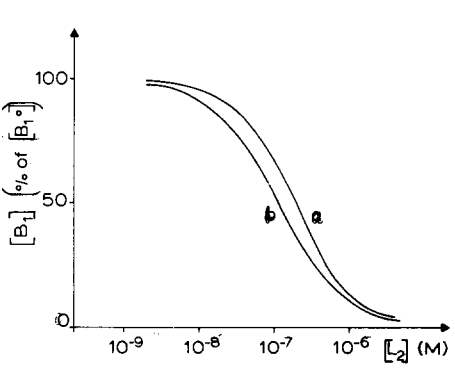
Fig. 1. Time course of the tracer-protein complex formation. The plots are calculated according to Eqn. 4. $k_1 = k_2 = 1 \cdot 10^5$ $M^{-1} \cdot min^{-1}$; $P_0 = 2 \cdot 10^{-9}$ M. Other parameters: curve a: $[L_1] = 4.8 \cdot 10^{-8}$ M, $[L_2] = 0$, $k_{-1} = 10^{-3}$ min^{-1}; curve b: $[L_1] = 4.8 \cdot 10^{-8}$ M, $[L_2] = 1 \cdot 10^{-7}$ M, $k_{-1} = 10^{-3}$ min^{-1}, $k_{-2} = 2 \cdot 10^{-3}$ min^{-1}; curve c: $[L_1] = 4.8 \cdot 10^{-7}$ M, $[L_2] = 1 \cdot 10^{-7}$ M, $k_{-1} = 10^{-2}$ min^{-1}, $k_{-2} = 10^{-3}$ min^{-1}; curve d: $[L_1] = 3 \cdot 10^{-7}$ M, $[L_2] = 3 \cdot 10^{-6}$ M, $k_{-1} = 10^{-3}$ min^{-1}, $k_{-2} = 10^{-2}$ min^{-1}. Inset: The amount of bound tracer is expressed as % of equilibrium value. Lower abscissa and left hand ordinate apply to curves d and e, upper abscissa and right hand ordinate apply to curves c and f. Parameters $k_1 = k_2 = 1 \cdot 10^5$ $M^{-1} \cdot min^{-1}$, $P_0 = 2 \cdot 10^{-9}$ M, and curve c: as curve c, main figure; curve d: as curve d, main figure; curve e: $[L_1] = 3 \cdot 10^{-6}$ M, $[L_2] = 3 \cdot 10^{-6}$ M, $k_{-1} = 10^{-3}$ min^{-1}, $k_{-2} = 10^{-2}$ min^{-1}; curve f: $[L_1] = 4.8 \cdot 10^{-7}$ M, $[L_2] = 5 \cdot 10^{-9}$ M, $k_{-1} = 10^{-2}$ min^{-1}, $k_{-2} = 10^{-3}$ min^{-1}.

according to Eqn. 4 for different sets of rate constants. The usual ligand concentrations in steroid binding and competition studies range from 10^{-10} to 10^{-6} M, reported association rate constants at low temperature for steroid hormone-receptor complex formation are 10^5 to 10^6 $M^{-1} \cdot min^{-1}$ [13,21,24,25, 27] and dissociation rate constants of steroid-receptor complexes are generally between 10^{-3} min^{-1} and 10^{-2} min^{-1} but much lower values occur also [13,21, 24—26]. It is seen that time required to approach equilibrium within, say, 10% depends on the actual rate constants and may be more than 10 h. If $k_{-1} > k_{-2}$ a significant overshoot is observed in $[B_1(t)]$ over equilibrium value. Equilibration time at a given set of rate constants is extremely dependent also on ligand concentrations. It indicates the pilot experiments exploring time of equilibration with one tracer and one competitor concentration do not provide sufficient information. However, rate of equilibration around the midpoint of the displacement curve (see below) depends mainly on k_{-1} and k_{-2}. Smaller association rate constant requires larger ligand concentration for half displacement and vice versa so that k_1' (or k_2') be the same.

2. Determination of the dissociation constant for the competitor-protein complex

Two different methods are common. In one of them the tracer concentration is fixed and its displacement by the competitor at various concentrations is registered. Data may be displayed as $[B_1]/[B_1^0] \times 100$ vs. log $[L_2]$ or $[B_1]^{-1}$ vs. $[L_2]$ plots [28]. K_2 can then be calculated from relative binding affinity of L_2 knowing K_1 [23].

In the other type of experiment the tracer concentration is varied and the competitor concentration is fixed. K_2 can now be evaluated from the slope of the Scatchard or from the intercept with the abscissa of the $1/[B_1]$ vs $1/[L_1]$ plots [29].



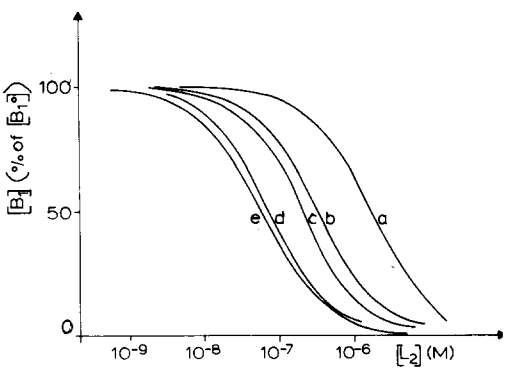


Fig. 2. Displacement curves for 'slow' tracer-'slow' competitor. The amount of bound tracer is expressed as % of that bound in the absence of competitor. Parameters: $P_0 = 2 \cdot 10^{-9}$ M, $k_1 = k_2 = 1 \cdot 10^5$ $M^{-1} \cdot min^{-1}$, $[L_1] = 4.8 \cdot 10^{-8}$ M, $k_{-1} = 10^{-3}$ min^{-1}, $k_{-2} = 2 \cdot 10^{-3}$ min^{-1}, Reaction time: 100 min, curve a; $t = \infty$, curve b.

Fig. 3. Displacement curves for 'fast' tracer-'slow' competitor. Parameters: $P_0 = 2 \cdot 10^{-9}$ M, $k_1 = k_2 = 1 \cdot 10^5$ $M^{-1} \cdot min^{-1}$, $[L_1] = 4.8 \cdot 10^{-7}$ M, $k_{-1} = 10^{-2}$ min^{-1}, $k_{-2} = 10^{-3}$ min^{-1}. Reaction time: 10 min, curve a; 100 min, curve b; 200 min, curve c; 1000 min, curve d; and $t = \infty$, curve e.

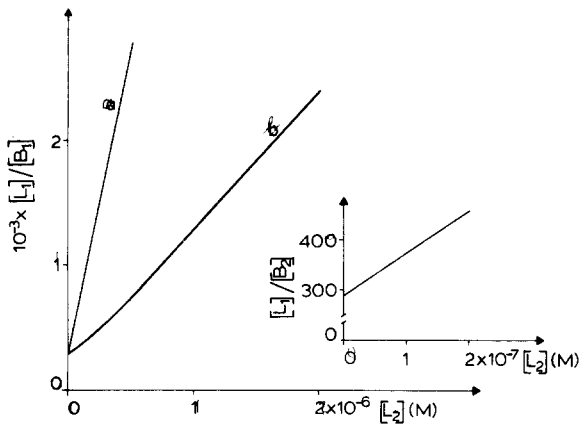


Fig. 4. Linearized competition plots for 'fast' tracer-'slow' competitor. Parameters: the same as Fig. 3 Reaction time: $t = \infty$, curve a; 100 min, curve b. The slight curvature of line b is observable only if com petitor concentrations span over several orders of magnitude. Inset: first part of line b.

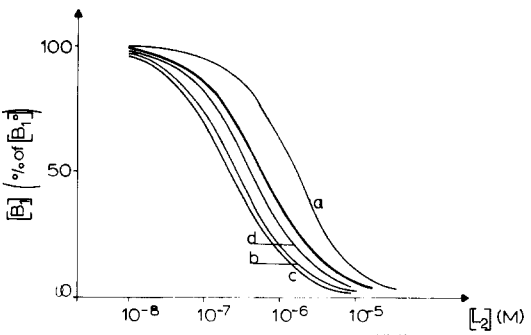


Fig. 5. Displacement curves for 'slow' tracer-'fast' competitor. Parameters: $P_0 = 2 \cdot 10^{-9}$ M, $k_1 = k_2 = 1 \cdot 10^5$ M $\cdot$ min^{-1}, $[L_1] = 4.8 \cdot 10^{-8}$ M, $k_{-1} = 10^{-3}$ min^{-1}, $k_{-2} = 10^{-2}$ min^{-1}. Reaction time: $t = \infty$, heavy line; 10 min, curve a; 100 min, curve b; 200 min, curve c; 1000 min, curve d.

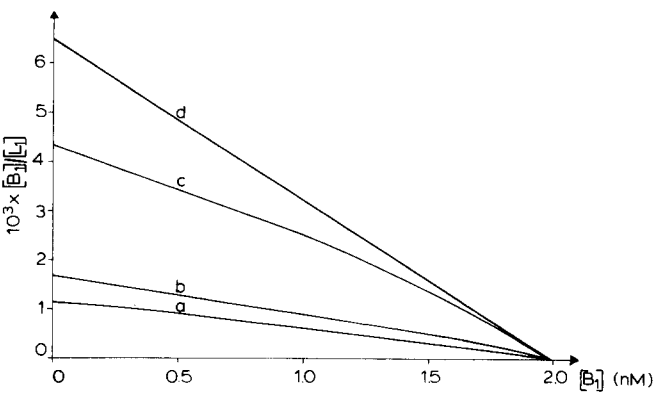


Fig. 6. Scatchard curves in the presence of competitor. Parameters: $P_0 = 2 \cdot 10^{-9}$ M, $k_1 = k_2 = 1 \cdot 10^5$ M^{-1} $\cdot$ min^{-1}, $[L_2] = 3 \cdot 10^{-6}$ M, $k_{-1} = 10^{-3}$ min^{-1}, $k_{-2} = 10^{-2}$ min^{-1}. Reaction time: 100 min, curve a 200 min, curve b; 1000 min, curve c; $t = \infty$, curve d.

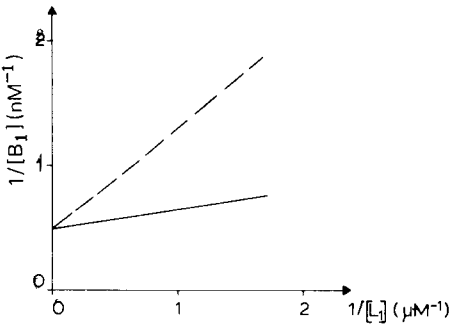


Fig. 7. Linearized competition plots for 'slow' tracer-'fast' competitor. Parameters: the same as in Fig. 6. Reaction time: $t = \infty$, solid line; 100 min, broken line.

Figs. 2—7 show how changes in 'equilibration time' influence the competition pattern.

It is obvious that none of the usual presentations reveal the system to be far from equilibrium. Deviations from linearity, where expected (Figs. 4, 6, 7), are too small to be detected experimentally. Competitor concentration required for 50% displacement with finite incubation time may deviate several-fold from equilibrium value (Figs. 2, 3, 5). The same is true for the slope (Fig. 6) or intercept with the abscissa (Figs. 4, 7) on the linearized displacement curves.

Difference of measured vs. true values can even be higher in real systems since dissociation rate constants for protein-ligand complexes as small as $4 \cdot 10^{-5}$—10^{-4} min^{-1} have also been reported [13,25,26,30 *] and 100—200 min at 0°C is quite usual for equilibration time [2,3,7,9,10,14—18,31,33].

It is striking that when both L_1 and L_2 are 'slow ligands' ($k_{-1} = 10^{-3}$ min^{-1}; $k_{-2} = 2 \cdot 10^{-3}$ min) equilibrium is reached earlier than in the case of one 'slow'-one 'fast' (k_{-1} or $k_{-2} = 10^{-2}$ min^{-1}) ligand. A 'fast' competitor competes better in the short term, whereas a 'slow' competitor displaces more and more tracer with time, after a very short initial interval (Fig. 5).

Discussion

It has been reported from several laboratories that competition patterns change with time [2,3,31] or that no equilibrium can be reached in competition experiments even after very long time [9,29]. Our deductions offer a simple explanation to these findings.

It was found also that sensitivity of radioimmunoassay depended strongly on incubation time, and a similar theoretical background was given to these results [32]. As the relative binding affinity of a given competitor may apparently increase ($k_{-1} > k_{-2}$) or decrease ($k_{-1} < k_{-2}$) with incubation time even relative affinities of the binding protein for different ligands can be misjudged if complete equilibration is not assured. So it can be explained that competition order of several steroids varied with temperature [33] the short incubation time did not allow equilibration at 0°C, whereas it proved sufficient at the higher temperature. Indeed, only slight variation was found in competition pattern

* Recalculated from half life of the complex.

with temperature in a similar system but applying longer incubation time at 0°C [34].

As to the absolute values of affinity constants one should bear in mind that the apparent association constant of the tracer is also strongly dependent on incubation time [1]. This questions the reliability of thermodynamic parameters deduced from competition experiments in slowly equilibrating systems with short 'equilibration' times.

Use of incubation times long beyond necessity may not be preferable, however, not only for inconvenience in experimentation but also because it raises the problem of protein inactivation [2,13,24,35] and, in complex systems, of ligand metabolism even at low temperatures [37—39]. It would be advisable therefore to assess dissociation rate constants, at least roughly, for all ligands involved in competition experiments. If it is impossible, we suggest that time necessary for equilibration be determined with high as well as low competitor and/or tracer concentrations.

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