A Pitfall in the Interpretation of Data on Ligand–Protein Interaction

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Binding reactions are usually investigated by separation of free and bound labelled ligand. Underestimation of the amount bound leads to inaccurate estimation of the dissociation constant and to wrong conclusions on the validity of the model of binding. The activation of cyclic AMP-dependent protein kinase is treated as an example.

The reaction mechanism of a wide variety of biochemical events involves the reversible binding of two or more molecules. Such molecular interactions are of particular interest with respect to their fundamental role in biological regulation, e.g. activation or inhibition of enzymes, transport of molecules, molecule-storing reactions, hormone–receptor interaction, hybridization of polynucleotides, antigen–antibody reactions etc.

Experimentally, free ligand F is completely or partly labelled and combines with unlabelled receptor R to form the complex B . At equilibrium, the molecules of F and B are separated by one of a variety of techniques, e.g. precipitation, filtration on Millipore filters (Gilman, 1970; Walton & Garren, 1970). Very often complex B is measured and the concentrations of ligand F and receptor R are calculated from the known total concentration of ligand F and receptor R at the beginning of the reaction and from complex B .

The purpose of this report is to analyse the consequences of experimental underestimation of complex B on the graphical representations of binding data and on conclusions drawn from such representations for arguments in favour of specific models of interactions and of parameters such as the dissociation constant.

Methods and results

Two models of interaction will be considered: the classical model in which one molecule of ligand binds to one molecule of receptor:

and the proposed model for the activation of cyclic AMP-dependent protein kinase (Reimann, 1971; Tao, 1971; Gill & Garren, 1971):

or to generalize

The dissociation constant K_d is equal to the ratio k_{+1}/k_{-1} for both models.

The mass conservation during the reaction leads to:

$$[F] = [F]_i - [B] \quad (1)$$

$$[R] = [R]_i - [B] \quad (2)$$

where $[F]_i$ and $[R]_i$ are the initial concentrations of ligand and receptor and $[F]$ and $[R]$ the concentrations at equilibrium. Graphical representations of equilibrium data theoretically allow an easy discrimination between the two models (Swillens *et al.*, 1974).

Separation methods of bound and free ligand are based on the selective binding (e.g. on filter or precipitate) of the receptor molecules and thus of labelled molecules B , whereas free ligand has little or no affinity for this binding material. Two systematic errors may affect the experimental measurement of complex B , i.e. an underestimation because of a loss of complex B or an overestimation because of the contamination by free ligand. The latter error depends on the concentration of ligand present in the binding assay and should be eliminated by deducing the blank values. On the other hand, the loss of bound molecules during the separation cannot be directly detected and is thus considered in the following treatment.

Let us assume that the fraction of bound molecules lost is equal to α , i.e. $100\alpha\%$ of B molecules are not retained by the separation of complex B and ligand F . Since the total concentration of receptor remains constant during the assay, the parameter α is also constant. If $[X]$ refers to the measured concentration of the compound X , we have:

$$\alpha = \frac{[B] - [B']}{[B]} \quad (3)$$

Another parameter characterizing the loss of bound molecules may be defined as:

$$p = \frac{[B]}{[B]'} \quad (4)$$

It follows from eqns. (3) and (4) that:

$$\alpha = \frac{p-1}{p} \quad (5)$$

$$0 \leq \alpha \leq 1 \quad (6)$$

$$p \geq 1 \quad (7)$$

Eqns. (6) and (7) imply that the error only consists in an underestimation, i.e. the loss is proportional to the true concentration of bound molecules. Since $[F]$ and $[R]$ are computed by means of eqns. (1) and (2), the concentrations $[F]'$ and $[R]'$ will be higher if $[B]'$ is lower than the true value:

$$[F]' = [F]_t - [B]' \quad (8)$$

$$[R]' = [R]_t - [B]' \quad (9)$$

Model 1. The mass-action law is:

$$K_d = \frac{[F][R]}{[B]} \quad (10)$$

Thus K_d may be considered as the equilibrium concentration of ligand F when half the total concentration of receptor is occupied. By using eqns. (1), (2) and (4), the equilibrium concentrations $[F]$, $[R]$ and $[B]$ may be expressed in terms of the measured concentrations $[F]'$, $[R]'$ and $[B]'$, and thus eqn. (10) becomes:

$$K_d = \frac{[[F]' + (1-p)[B]'] [R]_t - [B]'}{[B]'} \quad (11)$$

where $[R]_t$ is the maximal concentration of bound complex with saturating concentration of ligand F , i.e. the whole amount of receptor is converted into the complex B . The Lineweaver-Burk plot (Lineweaver & Burk, 1934) is defined by the function $y(x)$

where $y = \frac{1}{[B]'}$ and $x = \frac{1}{[F]'}$.

Transformation of eqn. (11) leads to eqn. (12):

$$y^2[R]_t + xy[(1-p)[R]_t - K_d] - y - (1-p)x = 0 \quad (12)$$

which defines a hyperbola with an upward concavity. On the other hand, a Scatchard plot (Scatchard, 1949) is defined by the function $y(x)$ where

$y = \frac{[B]'}{[F]'}$ and $x = [B]'$. This representation is a hyperbola with a downward concavity:

$$xy(1-p) + y[K_d - (1-p)[R]_t] + x - [R]_t = 0 \quad (13)$$

This curve intersects the y -axis at the point:

$$y(0) = \frac{[R]_t}{K_d + (p-1)[R]_t}$$

The presence of a systematic error changes the linear relationship expected for model 1 into a non-linear

curve, which suggests positive co-operativity, as shown in Fig. 1.

For both representations, if the experimental curve is fitted by a straight line neglecting the curvature (which may be masked by the inaccuracy of experimental data), the numerical value of K_d deduced from this fitting may be much higher than the actual value. Depending on the experimental points chosen in the Scatchard plot, the slope of the line is lying between the slopes of the tangents of the hyperbola at the intersection of the curve with the axes. The computed K_d value cannot be less than the actual value and its maximal value is equal to $K_d \left(1 + \frac{\alpha[R]_t}{K_d}\right)^2$ (e.g. assuming $\alpha = 0.2$ and $K_d = 10^{-8}M$; if $[R]_t = 10^{-7}M$ we have $10^{-8}M \leq \text{computed } K_d \leq 9 \times 10^{-8}M$; if $[R]_t = 10^{-9}M$ then $10^{-8}M \leq \text{computed } K_d \leq 1.04 \times 10^{-8}M$).

Thus, with a low ratio $\frac{[R]_t}{K_d}$, an estimate of K_d could still be obtained.

Model 2. The mass-action law is:

$$K_d = \frac{[F][R]}{[B][C]} \quad (14)$$

In this case K_d is dimensionless.

If we suppose that the total concentrations of the two subunits of the molecule R are equal, then one molecule of C is released when one molecule of F binds to the receptor and thus:

$$[B] = [C] \quad (15)$$

The same theoretical treatment as for model 1 transforms the mass-action law (14) into the following one:

$$K_d = \frac{[[F]' + (1-p)[B]'] [R]_t - [B]'}{p[B]'^2} \quad (16)$$

As with model 1 eqn. (16) can be rearranged according to the representations of Lineweaver & Burk (1934) and Scatchard (1949) and the ' B^2/F versus B ' representation especially elaborated for this model (Swillens *et al.*, 1974):

$$\text{Lineweaver-Burk: } y = \frac{1}{[B]'} \quad x = \frac{1}{[F]'}$$

$$y^2[R]_t + xy(1-p)[R]_t - y - x[(1-p) + pK_d] = 0 \quad (17)$$

$$\text{Scatchard: } y = \frac{[B]'}{[F]'} \quad [x = [B]']$$

$$xy[(1-p) + pK_d] - y[R]_t(1-p) + x - [R]_t = 0 \quad (18)$$

$$'B^2/F \text{ versus } B': y = \frac{[B]'^2}{[F]'} \quad x = [B]'$$

$$xy[(1-p) + pK_d] + x^2 - y[R]_t(1-p) - x[R]_t = 0 \quad (19)$$

This last representation would be linear for model 2 if there were no systematic error, and becomes hyperbolic in the presence of error.

An analytical study of eqn. (19) showed that the

Fig. 1. Theoretical representations for models 1 and 2

The solid line and the analytic characteristic refer to the shape of the curve in the general case ($p = 1$, i.e. no artifact). Broken lines show the possible shapes of the curves when the bound complex is underestimated ($p > 1$).

curve intersected the x -axis at the points $x = 0$ and $x = [R]_i'$. If x_m is the abscissa of the maximum, we have:

$$K_d = \alpha \frac{[[R]_i' - x_m]^2}{x_m^2} \quad (20)$$

On the other hand, it follows from eqn. (18) that the curve of the Scatchard representation intersects the y -axis at the point:

$$y_0 = \frac{1}{p-1}$$

Table 1. Possible aspects of Lineweaver-Burk and Scatchard plots for model 2

The parameters α and p characterize the systematic underestimation of the bound complex B. The critical parameters α_c and p_c are defined by eqns. (22) and (23).

	Type of concavity in:		Conclusion
	Lineweaver-Burk plot	Scatchard plot	
α (or p) $>$ α_c (or p_c)	Upward	Downward	Positive co-operativity
α (or p) $= \alpha_c$ (or p_c)	No concavity	No concavity	Model 1 applies
α (or p) $<$ α_c (or p_c)	Downward	Upward	Negative co-operativity

and thus:

$$\alpha = \frac{1}{1 + y_0} \quad (21)$$

If the experiment is performed in such a way that the maximum in the 'B²/F versus B' plot and the intersection of the curve with the y-axis in the Scatchard plot could be accurately detected, then the direct determination of x_m and y_0 gives the numerical values of the unknown parameters K_d and α by using eqns. (20) and (21).

What is unexpected is the fact that eqns. (17) and (18) become linear when:

$$\frac{p}{p-1} = K_d$$

Thus, for a critical value of the parameter p or α , the Lineweaver-Burk and Scatchard plots are linear, which would suggest the validity of model 1 even though model 2 applies.

These critical values are:

$$p_c = \frac{1}{1 - K_d} \quad (22)$$

and thus:

$$\alpha_c = K_d \quad (23)$$

The shapes of the curve in the different representations are thus extremely affected by the presence of such a systematic error, as shown in Fig. 1.

Table 1 summarizes the possible aspects of Lineweaver-Burk and Scatchard plots for model 2 for different values of p or α . It has to be noted that the aspects of these two plots leading to model 1 or to the conclusion of a positive co-operativity can appear only if $K_d < 1$, because in this case $\alpha > \alpha_c = K_d$. Thus a downward concavity in the Scatchard plot requires that $K_d < 1$.

Discussion

This theoretical analysis shows the consequences of a systematic error in the determination of concentrations in a binding reaction. This error is related to an inaccurate separation of the components of the reaction. Whatever the model, the shape of the

curves in different graphical representations of equilibrium data may lead to erroneous conclusions as to which is the valid model. Moreover, it is necessary to observe caution when comparing data from different laboratories. Indeed, the conclusions drawn from the representations discussed in this paper may be contradictory if the experimental conditions were slightly different.

It is important to note that the representations are sensitive to a small but systematic underestimation of the concentration of B. This occurs because the concentration of free component is computed by means of eqn. (8) and is thus overestimated. Now, for enzymic reaction for instance, the underestimation of the complex (substrate-enzyme) concentration affects only the determination of the total amount of binding sites and not the concentration of free substrate, because it is assumed that substrate concentration is considerably higher than the enzyme concentration. It follows from this fact that the shapes of the curves are not modified in this case.

It has been stressed that, for cyclic AMP binding to protein kinase, this artifact may cause some misinterpretation in the validity of a hypothetical model.

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