Review Letter

LIGAND PARTITIONING INTO MEMBRANES: ITS SIGNIFICANCE IN DETERMINING K_M AND K_s VALUES FOR CYTOCHROME *P*-450 AND OTHER MEMBRANE BOUND RECEPTORS AND ENZYMES

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1. Introduction

Models presented for the interaction of ligands with receptors in membranes are often complex. These models have been proposed because the kinetic and equilibrium data seem to demand them. Cytochrome *P*-450–substrate interactions are a case in point. Spectral measurements have demonstrated that the apparent dissociation constant for cyclohexane binding is a function of cytochrome *P*-450 concentration [1]. The rate of benzpyrene hydroxylation catalysed by cytochrome *P*-450 is non-linear with enzyme concentration except at very low enzyme concentrations [2]. Imipramine metabolism studies give K_M values that are a function of protein concentration [3].

These, and other data on ligand–receptor interactions, are determined from analytical equations derived for single-phase systems. However, any membrane in suspension is a biphasic system, the phases being the lipid bilayer of the membrane, and the aqueous suspending medium, and so these expressions are not directly applicable. By examining the biphasic nature of the system, we here derive kinetic and equilibrium expressions for an accurate analysis of ligand–receptor interactions in membranes, and suggest suitable methods for determining true dissociation and kinetic constants. This involves considering the distribution of substrate between the two phases, and then how this distribution affects binding and kinetic expressions in terms of the experimental variables used and measured in such work.

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2. General formulation

2.1. *Substrate distribution*

Define S as total moles of free substrate, S_{aq} as the number of moles in the aqueous phase, S_{lip} as the number of moles in the lipid phase; V as the total volume, v_{aq} as volume of the aqueous phase, and v_{lip} as the volume of the lipid phase, and (X) is the concentration of X.

All unbound substrate will partition between the two phases, such that,

$$S = S_{lip} + S_{aq} \tag{1}$$

This distribution will be controlled by the partition coefficient, defined as,

$$K_p = \frac{(S)_{lip}}{(S)_{aq}} \tag{2}$$

Now as $V = v_{aq} + v_{lip}$

then $(S)V = (S)_{lip}\, v_{lip} + (S)_{aq}\, v_{aq}$

and when the relative volume of lipid is small,

$V \simeq v_{aq}$

$$\text{then } (S) = (S)_{lip}\,\frac{\nu_{lip}}{V} + (S)_{aq} \tag{3}$$

Substituting from (2) gives,

$$(S) = K_p\,(S)_{aq}\,\frac{\nu_{lip}}{V} + (S)_{aq}$$

$$\text{and } (S) = (S)_{lip}\,\frac{\nu_{lip}}{V} + \frac{1}{K_p}\,(S)_{lip}$$

$$\text{Thus } (S)_{aq} = \frac{(S)}{K_p\,\frac{\nu_{lip}}{V} + 1} \tag{4a}$$

$$\text{and } (S)_{lip} = \frac{(S)}{\frac{\nu_{lip}}{V} + \frac{1}{K_p}} \tag{4b}$$

2.2. *Interaction of ligand and receptors*

In a single phase system, that contains a receptor E

$$E + S \rightleftharpoons ES$$

the binding of the ligand is defined by

$$K_s = \frac{(E)(S)}{(ES)}$$

As $(E_o) = (E) + (ES)$, where (E_o) is intial receptor concentration, substitution gives

$$(ES) = \frac{(E_o)\,(S)}{K_s + (S)} \tag{5}$$

Often what is known experimentally is not (S), the equilibrium ligand concentration, but (S_o), the initial (or total) ligand concentration.

$$\text{and } (S_o) = (S) + (ES) + \sum_i (PS)_i$$

where $\sum_j (PS)_i$ = concentration of ligand receptors other than the one being considered,

$$\text{thus } (ES) = \frac{(E_o)\,\{(S_o) - (ES) - \sum_i (PS)_i\}}{K_s + \{(S_o) - (ES) - \sum_i (PS)_i\}} \tag{6}$$

Under experimental conditions when $(S_o) >> (ES) + \sum_i (PS)_i$ this simplifies to,

$$(ES) = \frac{(E_o)\,(S_o)}{K_s + (S_o)} \tag{7}$$

In a membrane suspension, however, this equation does not hold using the value (S_o), even when the condition,

$$(S_o) >> (ES) + \sum_i (PS)_i \text{ is met.}$$

The ligand concentration relevant to the binding equation is that exposed to the receptor active site, not the total added to the system. For a lipid faced binding site it is $(S_o)_{lip}$, the ligand concentration in the lipid that is relevant, and for an aqueous faced binding site it is $(S_o)_{aq}$, the ligand concentration in the suspending medium, as these are the concentrations equilibrating with the binding site. Further, as the receptor density in the membrane is constant, it is not possible to change its concentration without changing the relative lipid volume.

$$\text{i.e. } \frac{\nu_{lip}}{V} \;\alpha\; (E_o)$$

and defining k' as the receptor density coefficient,

$$\text{then } \frac{\nu_{lip}}{V} = k'\,(E_o) \tag{8}$$

Thus, substituting into eqs. (4a) and (4b), to obtain $(S)_{aq}$ and $(S)_{lip}$ values, and then into eq. (7), the equilibrium concentrations of ligand–receptor complexes can be defined as,

$$(ES) = \frac{(E_o)\,(S_o)}{K_s\,(K_p k'(E_o) + 1) + (S_o)} \tag{9a}$$

for an aqueous faced binding site

$$\text{and } (ES) = \frac{(E_o)\,(S_o)}{K_s(k'(E_o) + 1/K_p) + (S_o)} \tag{9b}$$

for a lipid faced binding site.

Under conditions when $(S_o) \gg (ES) + \sum_i (PS)_i$

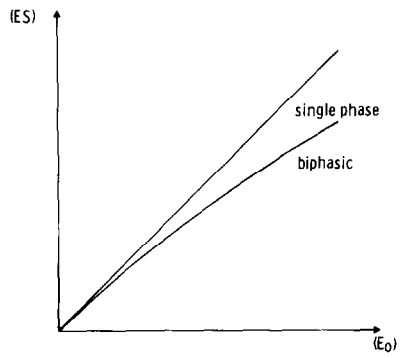

Fig.1. Variation of enzyme–substrate concentration with initial enzyme concentration for single phase and biphasic membrane systems.

the equilibrium concentrations of ES are more complex functions of receptor concentration, and a simple analysis of the binding behaviour cannot be carried out. For a further treatment see Appendix I.

2.3. *The dependence of (ES) on (E_o) in biphasic systems*

From eqs. (9a) and (9b), it can be seen that even for the simple model considered, (ES) does not vary linearly with (E_o) at constant substrate concentration. In fact plotting (ES) against (E_o) gives a hyperbolic curve (fig.1). This is in sharp contrast to the linear relationship obtained in single phase systems. As a consequence of this behaviour, receptor concentration can only be calculated from ligand–receptor concentration under strictly controlled conditions.

At low enzyme concentrations,

when $(K_p k'(E_o) + 1) \simeq 1$ for an aqueous faced binding site

$$(ES) = \frac{(E_o)(S_o)}{K_s + (S_o)}$$

and when $(k'(E_o) + 1/K_p) = 1/K_p$ for a lipid faced binding site,

$$(ES) = \frac{(E_o)(S_o)}{K_s/K_p + (S_o)}$$

Alternatively when the substrate concentration is high, such that

$(S_o) >> K_s(k_p k'(E_o) + 1)$ for an aqueous faced site, or

$(S_o) >> K_s(k'(E_o) + 1/K_p)$ for a lipid faced site, then

$(ES) \simeq (E_o)$ in both cases.

2.4. *The determination of true dissociation constants*

Writing eqs. (9a) and (9b) in the form,

$$(ES) = \frac{(E_o)(S_o)}{K_s^{app} + (S_o)}$$

where K_s^{app} is the apparent dissociation constant obtained in such a system,

then $K_s^{app} = K_s(1 + K_p k'(E_o))$ for an aqueous faced binding site,

and $K_s^{app} = K_s(1/K_p + k'(E_o))$ for a lipid faced binding site.

Then as $(E_o) \to 0$, $K_s^{app} \to K_s$ for an aqueous faced site, and $K_s^{app} \to K_s/K_p$ for a binding site exposed to the lipid bilayer. In both cases K_s^{app}/K_s varies linearly with enzyme concentration. Thus when the binding site is equilibrating with the aqueous compartment, the real dissociation constant can be obtained by determing K_s^{app} over a range of membrane concentrations and extrapolating to zero membrane concentration. For a receptor with a lipid faced active site, a similar analysis gives K_s/K_p, and K_s can be obtained only if K_p is known.

2.5. *Change of substrate and the deviation from single phase behaviour*

K_s^{app} is independent of substrate concentration for both environments considered. It is, however, a function of the partition coefficient and thus will vary with the nature of the substrate used. The variation of K_s^{app}/K_s with K_p is demonstrated in figs. 2(a) and (b).

When the binding site faces the lipid

$K_s^{app} \to K_s k'(E)_o$ as $K_p \to \infty$

i.e. for highly lipophilic compounds,

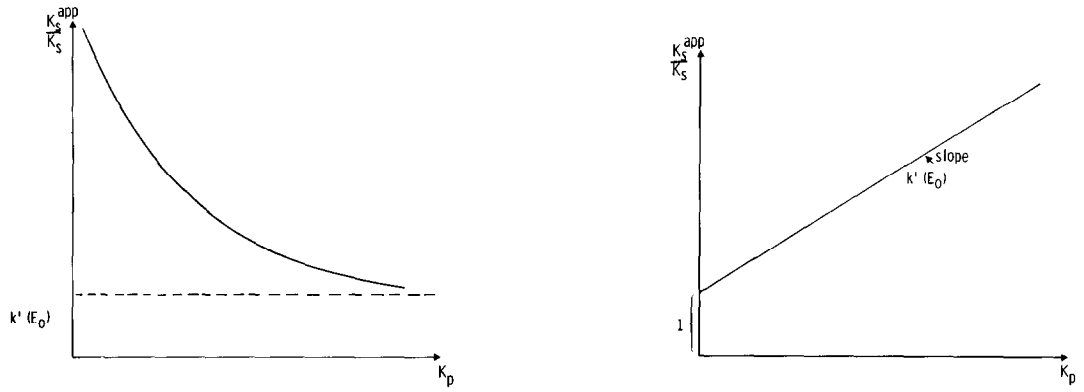

Fig.2. Change of substrate and the deviation from single phase behaviour. (a) Binding site in the aqueous phase. (b) Binding site in the lipid phase.

and $K_s^{app} \to \infty$ as $K_p \to 0$

i.e. for highly hydrophilic compounds.
When the binding site faces the aqueous phase,

$K_s^{app} \to \infty$ as $K_p \to \infty$

i.e. for highly lipophilic compounds,

and $K_s^{app} \to K_s$ as $K_p \to 0$

i.e. for highly hydrophilic compounds.

In this latter case, the variation of (ES) with (E) thus approaches that in single phase systems.

2.6. *Kinetics of membrane-bound enzymes*

For any system where,

$$E + S \underset{k-1}{\overset{k+1}{\rightleftharpoons}} ES \xrightarrow{k+2} E + \text{Products}$$

and where $\frac{d(ES)}{dt} = 0$, and in the absence of inhibition

then $\frac{d(P)}{dt} = \frac{k_{+2}\,(E_o)\,(S)}{\frac{k_{-1} + k_{+2}}{k_{+1}} + (S)}$

On writing $\frac{k_{-1} + k_{+2}}{k_{+1}} = K_M$

then the initial rate, $\frac{d(P)}{dt_{(t=0)}} = \frac{k_{+2}\,(E_o)\,(S_o)}{K_M + (S_o)}$ (10)

This equation is now of exactly the same shape as that used for binding. Again substitution can be made for (S_o) to obtain the equations for the two types of active site possible for membrane-bound receptors. If it is assumed that equilibration of substrate between the two phases is fast compared with the rate of reaction, exactly the same arguments and limitations apply as in the binding discussion, and we obtain for the initial rate,

$$\frac{d(P)}{dt_{(t=0)}} = \frac{k_{+2}\,(E_o)\,(S_o)}{K_M(K_p k'(E_o) + 1) + (S_o)} \qquad (11a)$$

for an active site facing the aqueous phase

and $$\frac{d(P)}{dt_{(t=0)}} = \frac{k_{+2}\,(E_o)\,(S_o)}{K_M(1/K_p + k'(E_o)) + (S_o)} \qquad (11b)$$

for an active site facing the lipid.

Thus, in the same way as the ligand–receptor concentration is not a linear function of (E_o), neither is the initial rate of reaction. In both environments of the active site, plotting $\frac{d(P)}{dt_{t=0}}$ against (E_o) gives a hyperbolic curve. Again this is in contrast to single phase systems where such a result requires a complex model. The limiting conditions for linearity of initial rate with enzyme concentration are the same, exchanging K_M for K_s. For substrate saturation in both cases,

$$\frac{d(P)}{dt} = k_{+2}\,(E_o)$$

and when the enzyme concentration is sufficiently low

$$\frac{d(P)}{dt_{(t=0)}} = \frac{k_{+2}\,(E_o)\,(S_o)}{K_M + (S_o)}$$

for an aqueous faced active site,

and $$\frac{d(P)}{dt_{(t=0)}} = \frac{k_{+2}\,(E_o)\,(S_o)}{K_M/K_p + (S_o)}$$

for a lipid faced active site.

2.5. *The determination of true Michaelis constants*

The equations for initial rates (11a) and (11b) are exactly the same shape as those for binding, eqs. (9a) and (9b). Consequently the relationship of $K_M{}^{app}$, determined from single phase analysis, to K_M, is exactly the same as the relationship between $K_s{}^{app}$, and K_s. K_M or K_M/K_p can be determined from $K_M{}^{app}$ in the same way as K_s or K_s/K_p are determined from $K_s{}^{app}$.

3. Discussion

The analysis presented here supposes that two factors be considered when examining ligand–receptor interactions in membranes. These are the partitioning of the ligand between the aqueous phase and the membrane lipid, and the location of the receptor binding site with respect to the lipid environment. We know of no occasion when the consequences of these two factors have previously been considered, and suggest that reported estimates of binding and catalytic constants may be seriously in error. There is strong experimental evidence supporting the analysis derived and a few examples follow. Most of our evidence refers to cytochrome *P*-450–ligand interactions, since this is a system in which receptor binding of lipophilic compounds has been extensively studied. However, the consequences of the theory are perfectly general, and need to be considered whenever receptor–ligand interactions are examined in membranes.

Schuster and her associates [4], have studied the interaction of a substituted pleuromutilin with rat liver microsomes using both equilibrium dialysis and spectral techniques. They have obtained evidence that a large pool of the drug is in the membrane and its presence is a consequence of the membrane lipids, not the membrane proteins. It is suggested that this pool may be in equilibrium with a hydrophobic binding site on cytochrome *P*-450. Cohen and Mannering [5] have obtained evidence for such a site on cytochrome *P*-450 using alcohols to inhibit *p*-hydroxylation of aniline. The extent of inhibition increased with increasing hydrophobicity of the alcohol, consistent with the interaction taking place in a hydrophobic environment.

Stronger evidence for both conclusions has been obtained by Ibbetson and Freedman [6]. Using fluorescence techniques they have demonstrated that benzypyrene binding corresponded to a general dissolution of the hydrocarbon in the non-polar matrix of the membrane. When a first-order dissociation constant was assumed for this process a value of 1 μM was determined. This value is no higher than the determined values for benzpyrene hydroxylation [7,8]. This anomaly that the affinity of benzpyrene for the membrane is at least as high as that for the specific binding site of the enzyme, can be best explained if the enzyme active site is in the lipid environment. Using Ibbetson and Freedman's data it is possible to calculate a partition coefficient of approximately 4.39×10^3 for benzpyrene partitioning between lipid and aqueous phases (Appendix II). Since the experiments were carried out at low enzyme concentration, assuming $K_M{}^{app} = K_M/K_p$, a true K_M value of ~0.23 mM is obtained for hydroxylation of benzpyrene. The substrate has then of the order of a thousand fold greater affinity for the enzyme than it does for the membrane lipids.

Gillette has also provided data in support of this formulation, in that $K_M{}^{app}$ values for imipramine metabolism are a function of membrane concentration if values are estimated in a traditional way using total imipramine concentration [3]. They are, however, independent of membrane concentration if the aqueous concentrations of imipramine are used. This is expected as the lipid concentrations will be directly proportional to the aqueous concentration if the distribution of substrate is controlled by a partition coefficient.

Although the notion of a pool of substrate dissolved in the membrane has been postulated previously, this has generally been treated as controlled by a first-order binding constant, and some experimental evidence has been presented in favour of such an idea. Apart from the contradiction of postulating, on the one hand, a free pool of mobile substrate, and on the other hand, a direct lipid–substrate interaction at a molecular level, there is evidence against using a first order constant to describe the interaction and the evidence in favour of it is open to other interpretations. Blyth et al. [9] have found that sex steroid binding to rat liver microsomes is linear with concentration of the hormone, and that the postulated 'loose binding site' was not saturable, consistent with the distribution being controlled by a zero order partition coefficient. Schuster et al. [4] also found near linearity of 'binding' of pleuromutilin to rat liver microsomes and liposomes but did eventually saturate the postulated binding site. A likely explanation of their saturation is that with increasing ligand concentration, the ligand itself becomes a significant component of the lipid phase and modifies the solubility properties of the membrane. This is supported by the fact that changes in lipid composition can vastly modify the bulk properties of a membrane including solubility of substances in it. The failure to find a simple integral relationship of ligand to any lipid component at saturation is not consistent with the use of a first order constant to describe the interaction. A zero-order description is then quite consistent, with slight deviations at low concentrations, as concentration rather than activity terms are used, and 'substrate saturation' being a consequence of changing the physical properties of the bilayer, rather than saturating a binding site in it. It is also possible that appreciable concentrations of substrate in the bilayer may change the position of phase transition points of the membrane, and thus affect the activity of lipid-dependent proteins.

A limitation on the accuracy to which real K_s and K_M values can be determined is imposed by the accuracy to which K_p values can be estimated. Schuster et al. [4], have pointed out that partitioning is very sensitive to lipid composition, particularly cholesterol levels. It is therefore important to measure partition coefficients in the direct environment of the binding site, rather than use a model system (e.g. octanol/H_2O) for estimating values. A further consideration is the possibility that saturation of a lipid facing enzyme may not be possible. If the saturation level of the lipid itself is lower than that of the enzyme, then the limiting substrate concentration accessible to the binding site may be too low to permit saturation of the site itself. This would lead to errors in determination of K_s and K_M values.

Of some significance, in relation to the theory derived are experimental techniques used for determining thermodynamic and kinetic parameters. Many of the binding constants are determined from 'bound' versus 'free' plots of various sorts, e.g. Scatchard plots. In terms of our analysis what is normally considered 'free' in such work is in fact S_{aq}, and the 'bound' component is $\{S_{lip} + ES\}$. If a binding site is directly exposed to the lipid environment then such values are not meaningful to the determination of binding constants, and the analysis of Scatchard plots and the like becomes extremely complex.

The experimental determination of enzyme levels from kinetic analysis, particularly when induced and non-induced levels of enzyme are compared, is also open to error. The constant k' is a measure of the enzyme or receptor density in the membrane and is a parameter that may change following induction of enzyme synthesis. Again the cytochrome *P*-450 complex has been considered in depth in this respect and a vast amount of literature has accumulated suggesting that enzyme density can be changed by administration of a number of drugs, steroids and carcinogens (for review see [10]). It is clear from eqs. (11a) and (11b) that in biphasic systems the departure from linearity of rate versus enzyme concentration plots is a function of the constant k'. Thus when attempting to estimate enzyme levels in induced and non-induced states, it is important to establish linearity for both membranes. An enzyme concentration at which this is true in one membrane, may not be a valid concentration for estimation in another membrane of different enzyme density.

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support, and the Medical Research Council for financial assistance.

Appendix I

Factors influencing the equilibrium concentration of ES when $(S_o) >> \sum_i (PS)_{-i} + (ES)$

The concentration of protein bound substrate is given by,

$$\sum_i (SP_i) = \sum_i (SP_i)_{aq} + \Sigma (SP_i)_{lip}$$

This can be expressed in terms of total protein concentrations,

$\sum_i (Pi)_{aq}$, $\sum_i (Pi)_{lip}$, such that

$$\sum_i (SP_i) = \sum_i \frac{(Pi)_{aq}\,(S)_{aq}}{Ka_i + (S)_{aq}} + \sum_i \frac{(Pi)_{lip}\,(S)_{aq}}{\frac{Kl_i}{K_p} + (S)_{aq}}$$

where Ka_i and Kl_i are dissociation constants for SPi_{aq} and SPi_{lip} respectively.

Under conditions when $(S_o) >> (E_o)$,

then $(S)_{aq}$ is a function of (S_o); $\frac{\nu_{lip}}{\nu_{aq}}$; $(Pa)_1, \ldots$.

$(Pa)_n$; $(Pl)_1 \ldots (Pl)_n$

and hence (ES) will be a function of (E_o), (S_o), $\frac{\nu_{lip}}{\nu_{aq}}$,

$(Pa)_1 \ldots (Pa)_n$ and $(Pl)_1 \ldots (Pl)_n$.

Appendix II

The determination of the partition coefficient of benzpyrene between the aqueous suspending medium and the membrane lipid

The calculations are based on data from Ibbetson and Freedman [6].

At a total benzpyrene concentration of 16 μM, and a membrane protein concentration of 0.32 mg/ml, 18 μmol of benzpyrene are bound/g protein. Thus in a volume of 1 litre, 5.76 μmol of benzpyrene are bound and 10.24 μmol are free. Assuming a phospholipid : protein ratio for smooth microsomes of 0.4 [11], then 0.128 g phospholipid contains 5.76 μmol of benzpyrene. Assuming a density of 1 g/ml for a phospholipid bilayer [12], then $(\text{benzpyrene})_{lip} \simeq$ 45 mM, and $(\text{benzpyrene})_{aq} \sim 10.24\ \mu$M. Thus $K_p \sim 4.39 \times 10^3$.

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