Testing and characterizing enzymes and membrane-bound carrier proteins acting on amphipathic ligands in the presence of bilayer membrane material and soluble binding protein

Application to the uptake of oleate into isolated cells

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1. A multiphasic modelling approach [Heirwegh, Meuwissen, Vermeir & De Smedt (1988) *Biochem. J.* **254**, 101–108] is applied to systems containing poorly water-soluble amphipathic reactants, membrane material, soluble binding protein and acceptor protein (enzyme or membrane-bound carrier protein). 2. The field of application is constrained by the assumptions (i) that the amount of acceptor-bound substrate is small compared with the total amount and (ii) that all preceding chemical reactions and steps of mass transport are rapid compared with the chemical change monitored. 3. Initial-rate formulae for systems in which an acceptor interacts with unbound or protein-bound ligand are given. The saturation curves are near-hyperbolic or sigmoidal, depending both (i) on the form of ligand (unbound or protein-bound) acted upon by the acceptor and (ii) on whether the assays are performed at constant concentration of soluble binding protein C_p or at constant substrate/binding-site molar ratio R_s . 4. Several diagnostic features permit unequivocal distinction between acceptor action on unbound or protein-bound substrate. In the former case, saturation curves, run at the same constant concentration of one of several binding proteins of increasing binding affinity, will show progressively increasing inhibition, the shape changing from near-hyperbolic at $K_m' < K_1'$ to sigmoidal at $K_m' > K_1'$. K_m' is the effective Michaelis constant of the acceptor and K_1' the effective dissociation constant of the binding sites of the soluble protein (for the sites with the higher binding affinity, if several classes of binding site are present on the protein). Alternatively, the maximum velocity obtained at constant $R_s \leq 1$ should increase hyperbolically with $R_s/(1 - R_s)$ for a binding protein with a single class of binding site. The formula that applies when the binding protein contains two classes of independent binding site is also available. When the acceptor acts on protein-bound ligand, the maximum velocity obtained at constant binding-protein concentration, C_p , increases hyperbolically with C_p . 5. Application of these and additional criteria to initial-rate data on the uptake of oleate into isolated cells supports a mechanism of carrier-mediated uptake of the unbound ligand and allows one to clarify some observations that hitherto had been poorly explained. 6. The influence of soluble binding protein on the reaction and substrate specificities of ligand/acceptor interaction is also discussed. 7. In its present state, data treatment for 'double binding-protein systems' generally requires separate determination of the binding parameters of the soluble binding protein. Possible designs for direct application of data-fitting procedures are briefly discussed. 8. Details about the units used and about the derivation of ligand distribution functions, kinetic formulae and their properties, and formulae for interconversion of parameter values to various concentration scales have been deposited as Supplementary Publication SUP 50169 (16 pages) at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1992) **281**, 5.

INTRODUCTION

In enzyme-kinetic studies involving poorly water-soluble natural or xenobiotic amphipathic substrates performed in the presence of membrane material and soluble binding protein, a number of problems have to be faced. Amphipaths tend to aggregate [1], partition into membrane lipid [1–6] and are sequestered by binding protein [7–11]. Results obtained for such systems are often evaluated only qualitatively or with mathematical models that have been worked out only partially. This leads to considerable loss of information, often to ill-founded conclusions, e.g. with regard to the question whether unbound ligand or a protein/ligand complex interacts with an acceptor protein.

On the basis of the assumptions proposed [5,6], formulae have been derived for 'double binding-protein systems'. Attention will be paid to the problem of distinguishing between enzymic attack on either unbound or protein-bound ligand, to the role of substrate-binding protein in determining enzymic substrate specificity, and to data analysis. The consequences of the kinetic models for the interpretation of experimental results will be

illustrated by analysing literature data related to the mechanism of uptake into cells of natural fatty acids.

THEORY

Results obtained with biochemical systems containing amphipathic ligand(s) in the presence of membrane material and/or substrate-binding protein are usually interpreted with the following implicit assumptions: (i) the aqueous media inside and outside the membrane vesicles are dilute homogeneous solutions of identical composition; (ii) the phospholipid bilayers are dilute homogeneous lipidic solutions; (iii) the membrane partition coefficient P_A^m of any ligand, A, is constant. These assumptions, which have already been discussed in some detail [5,6], will be adopted. It is further assumed that the amount of substrate bound to an acceptor (enzyme or membrane-bound carrier protein) [12] is small compared with the total amount of ligand. In general, at $v/V_{\max} \geq 0.1$, the error on the estimated reaction rate is less than 1% when the ratio of the enzyme concentration over the Michaelis constant is less than 0.01 (K. P. M. Heirwegh, unpublished work). Depending on the other parameters, the

condition may be considerably less restrictive, as illustrated by the data in Figs. 1 and 3 (below). For a discussion of the influence of substrate depletion by binding to the acceptor on the kinetic properties of the present models, see the Supplementary Publication.

Field of application of the partial-equilibrium assumption

For treatment of kinetic phenomena we have further assumed that all steps of mass transport, such as release of ligand from a phospholipid bilayer or from dissolved binding protein, are rapid compared with the process monitored (partial-equilibrium hypothesis) [5,6]. Although the exit rate constant of ligand present in membrane material is determined primarily by P_A^m , to which it is inversely related [13], the partial-equilibrium assumption seems to be met *in vitro* for a number of enzymes acting on substrates with even fairly high hydrophobicities. For example, the rate constants of release of long-chain fatty acids (P_A^m , 10^8 – 10^9) from unilamellar dimyristoyl phosphatidylcholine vesicles vary between 0.5 s^{-1} for stearate and 35 s^{-1} for myristate [14]. The rate constants for solvation of these fatty acids, bound to serum albumin, are about 100-fold smaller, but, as shown for stearate, may still be large enough to sustain microsomal synthesis of stearoyl-CoA. Similarly, conversion of chlorodinitrobenzene (P_A^m , 5×10^4) and of sulphobromophthalein (P_A^m , 10^6) by glutathione *S*-transferases [15] and of 17α -hydroxyprogesterone and progesterone by vesicle-reconstituted cytochrome *P*-450_{C21} [16] was not limited by their exit rates from egg phosphatidylcholine vesicles. Also, the rate of release of haem from bilayer vesicles is rapid and independent of the presence of haem-binding protein [17,18]. Thus, for membrane systems containing ligands with hydrophobicities such as indicated above, a state of partial equilibrium is likely to apply. In such cases, the membrane material provides storage space for readily available substrate. At equal local substrate concentration in the aqueous phase, kinetic systems containing membrane material can maintain the steady-state regime for longer periods of time than membrane-free systems. Binding protein is likely to fulfil a similar role.

For solutes such as phospholipids and cholesterol that are retained more strongly in bilayer vesicles than long-chain fatty acids [19], the partial-equilibrium assumption may not hold. It may also become invalidated in solvents of high viscosity [20,21].

General considerations

The present treatment considers 'double binding-protein systems' composed of (i) an acceptor which may be soluble or membrane-bound and acts either on unbound or protein-bound ligand and (ii) binding protein that is located in the aqueous phase and possesses one or two classes of binding site. Initial-rate measurements of reversible single-substrate/single-product conversion are considered in detail. The formulae also apply to initial-rate measurements of carrier-mediated uptake of ligands into cells. These fairly simple models are often sufficient for explaining the experimental data and are useful as a starting point for discussing more complicated cases. General statements to be made about the shape and other properties of the saturation curves characteristic of the systems considered are supported by algebraic analysis (see the Supplementary Publication).

For reasons already discussed [5], the local concentration of a component *J* present in a homogeneous phase *i* is expressed as the molar fraction x_J^i . Depending on the phase in which the reaction proceeds, the superscript *i* is 'aq' (aqueous phase) or 'm' (membrane-lipid phase). Concentrations referred to the system as a whole (called total concentrations) are the molar ratios n_J/N^{aq} where n_J is the total amount of *J* present in the system and N^{aq} the total amount of substance present in the aqueous phase.

Action on unbound substrate

Reversible enzymic conversion of unbound substrate in phase *i*, catalysed by a membrane-embedded enzyme, has been described previously [5]:

A and *Q* are the substrate and product, and *E*, *AE* and *QE* are uncomplexed enzyme and its substrate and product complex respectively. For reaction mixtures containing binding protein with two classes of independent binding site, the following parametric equations conveniently describe the relative initial reaction rate, *r*, obtained in the absence of product, as a function of the total concentration *A* (i.e. bound plus unbound substrate):

$$r = \frac{v}{V_{\text{max}}} = \frac{u}{K_m^{\text{app}} + u}$$

$$\text{with } K_m^{\text{app}} = K_m' \cdot F_A \quad (2)$$

$$A = u + C_1 \cdot \frac{u}{K_1^{\text{app}} + u} + C_2 \cdot \frac{u}{K_2^{\text{app}} + u}$$

$$\text{with } K_1^{\text{app}} = K_1' \cdot F_A$$

$$K_2^{\text{app}} = K_2' \cdot F_A \quad (3)$$

v and V_{max} are the initial and maximum forward reaction rates respectively. C_1 and C_2 are the site concentrations in class 1 and 2 of the soluble protein respectively (total site concentration, $C = C_1 + C_2$). The terms at the right-hand side of eqn. (3), taken from left to right, represent the concentration of unbound substrate and of substrate bound to the binding sites of class 1 and 2 of the soluble protein. The auxiliary variable, *u*, which is equal to the total concentration of unbound substrate (i.e. unbound substrate present in the aqueous and lipidic phases), is given by the product $x_A^{\text{aq}} \cdot F_A$ where x_A^{aq} is the local concentration in the aqueous phase of unbound *A* and F_A is the distribution function of *A* [5]. For a non-ionizing exchangeable solute *A*, F_A is given by:

$$F_A = 1 + P_A^m \cdot M + \sum (P_A^{ij} \cdot L_j) \quad (4)$$

where the membrane partition coefficients, P_A^m and P_A^{ij} , refer to the lipidic phase, *m*, containing the embedded enzyme, and any additional lipidic phase, *j* (*j* = 1, 2, ..., *k* - 1, *k*) respectively, and *M* and L_j are the corresponding membrane lipid concentrations.

K_m^{app} is the apparent Michaelis constant. K_1^{app} and K_2^{app} are apparent dissociation constants. Depending on the nature of the binding site of the enzyme, the effective Michaelis constant, K_m' , can take various forms [5]:

$$K_m' = K_m \quad \text{aqueous-faced hydrophilic site} \quad (5)$$

$$K_m' = K_m / P_A^m \quad \text{lipid-faced and mixed sites} \quad (6)$$

$$K_m' = K_m / P_A^{\text{bs}} \quad \text{aqueous-faced amphipathic site} \quad (7)$$

K_m characterizes the interaction of the more hydrophilic part of the amphipath [5]. Eqn. (7) applies to substances of relatively low molecular mass, in particular to homologous series $L-[\text{CH}_2]_n-\text{H}$ [1]. P_A^{bs} is the incremental site-binding partition coefficient [5]. For enzymes and binding proteins that are present in the aqueous phase, a relationship similar to either eqn. (5) or (7) applies to each class of binding site. For the first binding class of a binding protein one has

$$K_1' = K_1 \quad \text{hydrophilic site} \quad (8)$$

$$K_1' = K_1 / P_1^{\text{bs}} \quad \text{amphipathic site} \quad (9)$$

Fig. 1. Enzymic action on unbound substrate at constant concentration of a substrate-binding protein with one or two classes of binding site: simulated saturation curves for single-substrate enzymic conversion in the absence of product

Duplicate values of the relative velocity, $v/V_{\max}$, were calculated at intervals of 0.006 R_s units using the concentration balance without (eqn. 3) or with insertion of a term for enzyme-bound substrate. Each pair of values agreed to better than 1%, except for two data points near R_s zero of the two curves at the left-hand side and three data points in the curve at the right-hand side. These points have been omitted. The calculations were based on the following data: concentration of enzymic sites, $0.4 \mu\text{M}$ ($K_m^{\text{app.}} = 1 \mu\text{M}$); soluble binding protein at site concentration, C , $100 \mu\text{M}$. —, Identical sites ($K_1^{\text{app.}}$); ----, two classes of independent binding site, each at concentration $C/2$, with dissociation constant $K_1^{\text{app.}}$ and $K_2^{\text{app.}} = 10 \times K_1^{\text{app.}}$ respectively. The values against the curves in the Figure represent $K_1^{\text{app.}}$ (μM).

Fig. 2. Enzymic action on unbound substrate at constant concentration of a substrate-binding protein with a single class of binding site

The lines delineate the regions of sigmoidal and hyperbolic saturation at a given concentration of soluble binding sites, C . The graphs are independent of the concentration units used. Calculations were based on C being $1 \mu\text{M}$ (A), $10 \mu\text{M}$ (B), $100 \mu\text{M}$ (C) or limitingly high (D). K_m' and K_1' are in micromolar units.

with similar relationships between K_2' and K_2 for the second binding class. As for K_m [5], K_1 and K_2 characterize the interaction of the more hydrophilic part of the amphipath.

Fig. 3. Enzymic action on unbound substrate at constant concentration of a substrate-binding protein with two classes of binding site: simulated saturation curves for single-substrate enzymic conversion in the absence of product

Relative velocities, $v/V_{\max}$, calculated at R_s values situated at 0.006 unit intervals using the concentration balance without (eqn. 3) or with insertion of a term for enzyme-bound substrate agreed to within less than 0.65%. The calculations were based on the following data: concentration of enzymic sites, $2 \mu\text{M}$ ($K_m^{\text{app.}} = 1 \mu\text{M}$); soluble binding protein at total site concentration, C , $100 \mu\text{M}$; two classes of independent binding site, each at concentration $C/2$, with dissociation constants $K_1^{\text{app.}}$ ($1 \mu\text{M}$) and $K_2^{\text{app.}}$ respectively. The values in the Figure against the curves represent $K_2^{\text{app.}}$.

Many properties of the saturation curves depend largely on affinity ratios such as $K_m^{\text{app.}}/K_1^{\text{app.}}$. These can be replaced by ratios of effective constants K_m'/K_1' , since the distribution function F_A cancels out. Further, when the binding sites on both the acceptor, E, and soluble binding protein, C, are hydrophobic, as is likely for reactions with amphipaths, the ratios will, to a first approximation, be constant for homologues of relatively low molecular mass. For example, for $K_m^{\text{app.}}/K_1^{\text{app.}}$ one has:

$$\frac{K_m^{\text{app.}}}{K_1^{\text{app.}}} = \frac{K_m'}{K_1'} = \frac{K_m}{P_A} \cdot \frac{P_1^{\text{bs}}}{K_1} \approx \frac{K_m}{K_1} = \text{constant} \quad (10)$$

since P_1^{bs} and P_A are generally comparable [1]. Affinity ratios are also useful for discussing the interplay of carrier-mediated ligand transport through membranes and consecutive enzymic reaction [22].

Saturation curves at constant concentration of binding protein.

The saturation curves $r = f(A)$ are monotonically increasing towards a horizontal asymptote at $r = 1$. Thus, for the observed maximum velocity $V_{\max}^{\text{obs.}}$ one has:

$$V_{\max}^{\text{obs.}} = V_{\max} \quad (11)$$

The curves can be hyperbolic or sigmoidal (Fig. 1).

For systems containing a soluble binding protein with a single class of binding site, curve behaviour depends on the conditions

$$K_m'/K_1' < 1 + K_1^{\text{app.}}/C \quad \text{near-hyperbolic} \quad (12)$$

$$> 1 + K_1^{\text{app.}}/C \quad \text{sigmoidal} \quad (13)$$

The correction term, $K_1^{\text{app.}}/C$, amounts to less than 10% when, e.g., $K_1^{\text{app.}}$ is $\leq 1 \mu\text{M}$ and the site concentration, C , is $\geq 10 \mu\text{M}$ (Fig. 2). Under these conditions the shape of the curves is determined primarily by the relative binding affinity, K_m'/K_1' , not by the affinity of the enzyme nor by that of the binding protein alone. When the substrate is bound less avidly to the enzyme than to the binding protein ($K_m' > K_1'$), saturation is sigmoidal and vice versa. In view of eqn. (10), the shape of the saturation curves is approximately independent of the hydro-

Fig. 4. Enzymic action on unbound substrate at constant substrate/binding-protein ratio in the presence of a binding protein with two classes of binding site

Saturation and double-reciprocal plots of the initial reaction rate, v , against the total substrate concentration, A , were calculated at A intervals of $0.006 \mu\text{M}$. Duplicate values of v calculated using the concentration balance without (eqn. 14) or with insertion of a term for enzyme-bound substrate agreed to within 1%. The calculations were based on the following data: $V_{\text{max}} = 1$; concentration of enzymic sites, $0.01 \mu\text{M}$ ($K_m^{\text{app}} = 1 \mu\text{M}$); binding protein with two classes of binding site ($n_1 = n_2 = 3$) with $K_1^{\text{app}} = 1 \mu\text{M}$ and K_2^{app} equal to K_1^{app} (—) or to $30 \times K_1^{\text{app}}$ (---); ligand/binding-site ratio, $R_s = 1$. The slope of the double-reciprocal plots at high $1/A$, i.e. low substrate concentration, is equal to $V_{\text{max}}/K_m^{\text{app}}$.

Fig. 5. Enzymic action on unbound substrate at constant substrate/binding-site ratio, R_s

The apparent maximum rate, $V_{\text{max}}^{\text{obs}}$, is plotted as a function of R_s . —, Soluble binding protein with a single class of binding site; ---, soluble binding protein with two classes of binding site, $n_1 = n_2 = 3$, $K_2' = 10 \times K_1'$; true maximum rate, $V_{\text{max}} = 1$. K_m' varied between 2 nM and $20 \mu\text{M}$. The values shown against curves in the Figure are affinity ratios, K_1'/K_m' . Identical results were obtained both from the complete theoretical models (simulation) and from the derived formulae for $V_{\text{max}}^{\text{obs}}$. The values of $V_{\text{max}}^{\text{obs}}$ are independent of whether a term for enzyme-bound substrate is entered into the concentration balance (eqn. 14).

phobicity of the substrates. Allosterism reported in the literature may, in some instances, have been due to binding protein added to the system at constant concentration [23] or present endogenously.

Fig. 6. Enzymic action on a ligand-binding-protein complex, AC, at constant concentration of soluble binding protein

The relative velocity, $r = v/V_{\text{max}}$, for single-substrate conversion of AC in the absence of product was calculated as a function of the total ligand concentration, A , at $0.1 \mu\text{M}$ intervals, starting at r equal to 0.1 for the uppermost curve. Duplicate r values calculated using the concentration balance without (eqn. 19) or with insertion of a term for enzyme-bound substrate were identical to within 1%. The calculations were based on the following data: concentration of enzymic binding sites, $0.01 \mu\text{M}$ ($K_m = 1 \mu\text{M}$); concentration C_p of the soluble binding protein, $5 \mu\text{M}$; the values against the curves in the Figure represent the apparent dissociation constants, K_1^{app} (in μM). The substrate concentration range was chosen in such a way that the curve at $K_1^{\text{app}} = 0.1 \mu\text{M}$ practically reached saturation ($V_{\text{max}}^{\text{obs}}/V_{\text{max}} = 0.83$).

At a fixed R_s value, partial replacement of the sites on the soluble binding protein (K_1^{app}) by sites that bind the substrate more weakly (K_2^{app}) results in increased saturation of the enzyme, because of weaker competition by the binding protein (Fig. 1). The effect on saturation behaviour is minimal when the dissociation constant, K_2^{app} , of the more weakly binding sites is increased beyond $20 \times K_1^{\text{app}}$ (Fig. 3). Then, near R_s equal to 0, where the substrate is sequestered predominantly by the sites with the lower dissociation constant (K_1^{app}), the linear dividing line between sigmoidal and near-hyperbolic saturation is still at K_m'/K_1' equal to 1. Formulae for the conditions, similar to eqns. (12) and (13), that apply when the substrate-binding protein contains two classes of independent binding site, are given in the Supplementary Publication.

Saturation curves at constant substrate/binding-site ratio, R_s . Eqn. (3) becomes:

$$A = u + \alpha_1 \frac{A}{R_s} \cdot \frac{u}{(K_1^{\text{app}} + u)} + \alpha_2 \frac{A}{R_s} \cdot \frac{u}{(K_2^{\text{app}} + u)} \quad (14)$$

with $R_s = A/C$, $\alpha_1 = C_1/C$, $\alpha_2 = C_2/C$ constants. The substrate/binding-site ratio, R_s , is kept constant experimentally. The fractions of binding site, α_1 and α_2 , that are available in binding class 1 and 2 respectively, are characteristic parameters of the soluble binding protein. Eqns. (2) and (14) now describe the initial-rate measurements. The saturation curves [$r = f(A)$] are always near-hyperbolic (Fig. 4) with a horizontal asymptote $V_{\text{max}}^{\text{obs}}$ depending on R_s (Fig. 5).

For a soluble binding protein with a single class of binding site one has:

$$V_{\text{max}}^{\text{obs}} = V_{\text{max}} \quad R_s \geq 1$$

$$V_{\text{max}}^{\text{obs}} = V_{\text{max}} \cdot \frac{R_s}{(K_m'/K_1') \cdot (1 - R_s) + R_s} < V_{\text{max}} \quad R_s < 1 \quad (15)$$

At $R_s < 1$, a plot of $1/V_{\max}^{\text{obs.}}$, obtained at several values of R_s , against $(1-R_s)/R_s$ is linear, yielding $(K_m'/K_1')/V_{\max.}$ as the tangent and $1/V_{\max.}$ as the y -intercept. The saturation curves are hyperbolic in the neighbourhood of the origin:

$$v_h = V_{\max.} \cdot a \cdot \frac{A}{a \cdot K_m^{\text{app.}} + A}$$

$$\text{with } a = \frac{R_s \cdot [(K_m'/K_1') - 1]}{R_s \cdot [(K_m'/K_1') - 1] - (K_m'/K_1')} \quad (16)$$

as observed [24–26]. Independently of the particular set of values of R_s , $K_m^{\text{app.}}$ and $K_1^{\text{app.}}$, the initial slope of the limiting rectangular hyperbola is equal to $V_{\max.}/K_m^{\text{app.}}$. This is expected, since near infinite dilution of the soluble binding protein, dissociation of the ligand–protein complex should be complete, so that the systems would behave as if soluble binding protein were absent. $V_{\max.}/K_m^{\text{app.}}$ is an important parameter in characterizing intracellular transport and metabolism [27,28]. Note that the intercept at the y -axis of a plot of $1/v$ against $1/A$ (Fig. 3) generally differs from $1/V_{\max.}^{\text{obs.}}$ and from $1/V_{\max.}$.

More complex formulae applying when the substrate-binding protein contains two classes of independent binding site are given in the Supplementary Paper. As for a system containing a soluble protein with a single class of binding site, the initial slope of the limiting rectangular hyperbola is equal to $V/K_m^{\text{app.}}$. Plots of $1/V_{\max.}^{\text{obs.}}$ against $(1-R_s)/R_s$ are linear only at relatively high values of the independent variable, i.e. relatively low R_s . For example, at $\alpha_1 = \alpha_2$, $K_2'/K_1' = 30$ and $K_1'/K_m' = 0.01$ – 10 , the curves are linear between $(1-R_s)/R_s$ equal to 1.3 and 9, corresponding to R_s values below 0.43.

Action on protein-bound ligand

Imagine an acceptor with aqueous-faced binding sites (E^{aq}) to interact with a ligand–binding–protein complex (1:1) present in the aqueous phase (AC)^{aq}. Single-substrate/single-product conversion is described by:

where AC and QC is the substrate and product, and E , ACE and QCE is uncomplexed enzyme and its substrate and product complex, respectively. In the absence of product, the relative initial rate, r , is given by:

$$r = \frac{v}{V_{\max.}} = \frac{x_{AC}^{\text{aq}}}{K_m + x_{AC}^{\text{aq}}} \quad (18)$$

where x_{AC}^{aq} is the concentration in the aqueous phase of ligand–protein complex.

Saturation curves at constant concentration of binding protein C_p . The concentration balance on ligand is:

$$A = x_{AC}^{\text{aq}} + K_1^{\text{app.}} \cdot \frac{x_{AC}^{\text{aq}}}{C_p - x_{AC}^{\text{aq}}}$$

$$\text{with } K_1^{\text{app.}} = K_1' \cdot F_A \quad (19)$$

The saturation curves, $v = f(A)$, are hyperbolic (Fig. 6) with a horizontal asymptote $V_{\max.}^{\text{obs.}}$ limited by C_p :

$$V_{\max.}^{\text{obs.}} = V_{\max.} \cdot \frac{C_p}{K_m + C_p} < V_{\max.} \quad (20)$$

A double-inverse plot of $V_{\max.}^{\text{obs.}}$ against C_p is linear, yielding $1/V_{\max.}$ as the y -intercept and $K_m/V_{\max.}$ as the slope. The

Fig. 7. Enzymic action on a ligand–binding–protein complex, AC , at constant ligand/binding-protein ratio

The relative reaction rate, $r = v/V_{\max.}$, for single-substrate conversion of AC in the absence of product is plotted as a function of the total ligand concentration, A , at $0.095 \mu\text{M}$ intervals. The calculations were based on the following data: total concentration of enzymic sites, E , $0.04 \mu\text{M}$; K_m , $1 \mu\text{M}$; ratio ligand/protein, 1. The values against the curves in the Figure are apparent dissociation constants, $K_1^{\text{app.}}$ (in μM). Duplicate r values calculated using the concentration balance without (eqn. 21) or with insertion of a term for enzyme-bound substrate were identical to within 1%, except for four data points near $A = 0$ for the curve where $K_1^{\text{app.}}$ is $0.01 \mu\text{M}$. These data points have been omitted.

saturation curves are hyperbolic in the neighbourhood of small substrate concentration.

Saturation curves at constant ligand/binding-protein ratio, A/C_p . One obtains:

$$A = x_{AC}^{\text{aq}} + K_1^{\text{app.}} \cdot \frac{x_{AC}^{\text{aq}}}{A/R_p - x_{AC}^{\text{aq}}}$$

$$\text{with } R_p = A/C_p = \text{constant} \quad (21)$$

The saturation curves $v = f(A)$ are monotonically increasing and sigmoidal with a horizontal asymptote at $r = 1$ (Fig. 7). Thus

$$V_{\max.}^{\text{obs.}} = V_{\max.} \quad (22)$$

The sigmoidal nature of the curves is easily understood, since, near infinite dilution, the ligand–binding–protein complex should be fully dissociated so that no complex is available for enzymic reaction. Unless measurements are extended to quite low ligand concentrations, the sigmoidal nature of the saturation curves may not be easily apparent when $K_1^{\text{app.}} \leq K_m$.

Ligand partition into the lipidic phase of membrane material

At fixed total concentration, ligand distribution into the lipidic compartment(s) decreases the response of an aqueous-faced catalytic site compared with a membrane-free system [5]. Also, since the local ligand concentrations in both the aqueous and lipidic phases decrease with increasing size of the partition compartment(s), it is necessary to extrapolate $K_m^{\text{app.}}$ to zero-membrane concentration ($F_A = 1$) for comparing Michaelis constants obtained for the same enzyme preparation assayed at different membrane-lipid concentrations or in different types of biological preparation [5]. Provided the experimental work is limited to a range of ligand concentrations for which P_A^m is constant, the increase of $K_m^{\text{app.}}$ with the membrane-lipid con-

centration should be linear for non-ionizing ligands [5]. This also applies when, for an ionizing ligand, only the neutral form partitions significantly into the membrane lipid (results not shown). The dependence of K_m^{app} on the membrane-lipid concentration explains a number of results that apparently are poorly understood [5,6]. For example, the increase with cell concentration of the K_m^{app} for initial uptake of oleate into hepatocytes, adipocytes and cardiac myocytes, observed by Sorrentino *et al.* [29], is apparently due entirely to extension of the partition compartment. Indeed, for each cell type we found that the available values of K_m^{app} [29] increase linearly with cell concentration, as is expected from variation of the distribution function, F_A , contained in K_m^{app} .

DISCUSSION

Enzymic substrate conversion is usually measured in the absence of ligand-binding protein, rendering data analysis fairly simple [5], provided ligand aggregation can be ruled out on addition of the concentrated donor solution to the incubation mixtures [30]. In principle, the same procedure can be applied to analysis of ligand uptake into isolated cells, preferably limiting the external ligand concentrations to the value required for obtaining a useful upper degree of saturation, say 90 % of the maximum response [31–33]. However, the presence in the incubation mixtures of soluble substrate-binding protein may be advantageous. Provided the acceptor competes efficiently with the binding protein ($K_m' \leq K_1'$), acceptor binding could reach a suitably high degree of saturation, while the presence of the soluble binding protein would avoid flooding the lipidic compartment of the membranes with ligand. Studies at constant ligand/protein ratio are particularly attractive because the saturation curves can be obtained by testing a number of dilutions prepared from a single concentrated well-characterized ligand/protein mixture. However, a word of caution should be added. On varying the binding-protein concentration along the saturation curves, substances interfering with the change monitored might, to varying extents, be sequestered and/or lipids modifying the binding parameters of the protein might be extracted from the membrane [34]. Assays at constant concentration of soluble protein present other advantages that will become clear below.

Type of mechanism

The shape of a saturation curve on its own has little diagnostic value for deciding whether the acceptor acts on unbound or protein-bound ligand. Finding hyperbolic saturation at low substrate concentrations in assays at constant ligand/binding-site ratio, R_s , if anything, rather supports the unbound ligand (Fig. 4), not the bound ligand to be the substrate, as has occasionally been argued. However, when the soluble protein is a protein that binds the ligand with relatively high affinity, the sigmoidicity expected when bound ligand is the substrate may be poorly evident (Fig. 7). In assays at constant concentration of soluble protein, an acceptor acting on unbound substrate only yields sigmoidal saturation curves when $K_1' < K_m'$ (Fig. 2). Sigmoidal saturation may further be due to allosterism or to kinetic mechanisms [35–38] other than those mentioned above. In contrast, simultaneous determination of the shapes and properties of several saturation curves is more useful.

The acceptor acts on unbound ligand. This hypothesis can be verified by establishing saturation curves at constant concentration of a few binding proteins with known relative binding affinities [39], e.g. various serum albumins [40,41]. Starting at relatively low binding affinity ($1/K_1'$), where the saturation curves will be near-hyperbolic ($K_m' < K_1'$), assays with soluble proteins of increasing affinity will demonstrate progressively

increasing inhibition of the response, the curves eventually becoming sigmoidal at $K_m' > K_1'$ (Fig. 1). Evidence of this kind shows that the microsomal enzymes which glucuronidate bilirubin-IX α [39] and testosterone [42] act on the unbound substrates. An alternative approach could consist in establishing saturation curves at several values of the ligand/binding-site ratio, R_s , lower than 1. Interaction of the acceptor with unbound ligand is fairly certain when the saturation curves are near-hyperbolic and $V_{max}^{obs.}$ increases hyperbolically with $R_s/(1 - R_s)$.

The acceptor acts on protein-bound ligand. This hypothesis can be tested by analysing saturation curves run at several constant protein concentrations, C_p . The curves should be near-hyperbolic, $V_{max}^{obs.}$ increasing hyperbolically with C_p . Confirmation may be sought by performing parallel binding assays in which in one set the ligand and in the other the binding protein is in radioactive form [43]. If protein-bound ligand interacts with the acceptor and ligand-free binding protein does not, equal quantities of both ligand and binding protein should be found in association with the acceptor protein, even away from equilibrium. If, in addition, ligand-free protein also binds to the target, the molar ratio of ligand and binding protein associated with the acceptor will be less than 1. Such a deficit does not prove, but only is compatible with, competition by ligand-free binding protein, C, since it may also arise from mechanisms more complex than those discussed in the present paper [44].

Specificity of ligand-acceptor interaction

The specificity of ligand transfer to freely dissolved or membrane-bound acceptors, E, is affected by the presence of binding protein, C. Only effects exerted at constant protein concentration are considered.

Action on unbound ligand. Enzymes showing both narrow reaction and substrate specificities are microsomal bilirubin UDP-glucuronyltransferase, which only acts on the endogenous substrate bilirubin-IX α and on closely related structural analogues [45], and mono-oxygenases involved in bile-acid biosynthesis [46,47]. In contrast, a number of mono-oxygenases [47,48] and UDP-glucuronyltransferases [48–50] show broad substrate specificities, permitting them to metabolize the large and ever-increasing numbers of xenobiotic compounds. The latter enzymes mainly recognize that restricted portion of their substrates where the enzymic action occurs. When the latter type of enzyme would be assayed with a series of homologous substrates at the same substrate concentration in the absence of soluble binding protein, the reaction rates might vary considerably, but aspecifically ($K_m' = K_m/P_A$), depending on the value of P_A ($P_A = P_A^m$ or P_A^{bs}), the apparent substrate specificity showing similar variation. In contrast, at fixed total concentration of soluble binding protein, the rates should largely be independent of the hydrophobicity of the substrates (eqn. 10). A binding protein, while permitting attack of the substrate by an enzyme of matched binding affinity ($K_m^{app} \approx K_1^{app}$), could render competition by an enzyme with higher K_m^{app} inefficient. Such a mechanism may explain the absence of a requirement for CoA in the microsomal esterification of retinol when it takes place in the presence of intracellular retinol-binding protein [51].

Action on protein-bound ligand. Various scenarios are conceivable. The protein, C, could force a substrate into a more or less reactive conformation, possibly permitting enzymic action on both unbound and protein-bound ligand [7]. Binding protein could also shield some part of a substrate from enzymic attack, thus increasing the reaction-specificity of the process. Finally, the binding protein, C, could single out certain ligands according to their respective binding affinities, an acceptor, E, further selecting those protein-ligand complexes, AC, that bear appropriate recognition sites on their protein moiety. Such double-selection

Fig. 8. Oleate uptake by isolated rat hepatocytes as a function of the total ligand concentration

○---○, Uptake at constant (0.15 mM) BSA concentration; ●—●, both oleate and BSA varied at a constant 1:1 molar ratio. Data are taken from [55] with the permission of the publishers (MTP Press).

processes, which should considerably enhance the selectivity of the overall process, could play a role in mechanisms involved in transferring to the nucleus substances that trigger specific processes at the level of the genome, e.g. aromatic hydrocarbons [52–54].

Carrier-facilitated uptake of ligands into isolated cells

Carrier-mediated initial-rate uptake of ligands can be treated by the same methods as those discussed above. Suppose that a membrane-embedded carrier protein acts on unbound ligand via an aqueous-faced binding site which may either be fully hydrophilic or amphipathic. For carriers involved in the uptake of amphipaths, the binding sites are likely to be amphipathic ($K_m' = K_m/P_A^{bs}$; eqn. 7). When binding protein with one or two classes of binding site is present in the external medium, the rate measurements are described by eqns. (2) and (3) (for assays at constant protein concentration) or by eqns. (2) and (14) (for assays at constant ligand/binding-protein ratio), provided the concentration of enzyme-bound substrate can be neglected in the concentration balances shown in eqns. (3) and (14). The principles discussed above will be illustrated by reinterpretation of kinetic data [55,56] on the uptake of natural fatty acids by stirred suspensions of isolated hepatocytes, adipocytes and cardiac myocytes. With a major exception [57], it is generally admitted that the uptake of natural fatty acids occurs mainly by a carrier-mediated process via the unbound ligands [29,55,58,59]. The latter hypothesis is strengthened considerably by the fact that a number of apparently obscure results can be interpreted satisfactorily by the partial-equilibrium models described above.

First consider whether the model equations are applicable to the kinetic data to be discussed [55,56]. Adopting for mammalian cells a protein content of 300–400 μg of protein/ 10^6 cells and taking 30 kDa as the average molecular mass of the protein subunits, the total subunit concentration would have been of the order of 3 μM in the incubation mixtures (0.33×10^6 cells/ml). Estimating that the protein present in the plasma-membrane fraction is at maximum 10% of the total cellular protein and further noting that the postulated fatty-acid carrier protein must itself represent only a fraction of the protein in the plasma membrane, the binding site concentration, E , of the carrier protein must have been well below 0.01 μM . Since evidence, discussed below, indicates that the K_m^{app} for the uptake process

is on the order of 1 μM , the restrictive relationship for application of our models, $E/K_m^{\text{app}} \leq 0.01$, appears to be met. We may then consider the following observations.

(a) At a constant oleic acid/BSA molar ratio, A/C_p , equal to 1, the initial rates increased hyperbolically with the ligand concentration to a maximum value $V_{\text{max}}^{\text{obs}}$. In contrast, at constant BSA concentration, the rates increased linearly, exceeded $V_{\text{max}}^{\text{obs}}$, and, apparently, never reached saturation (Fig. 8). (i) Assuming the existence of six oleic acid-binding sites on BSA ($K_1' = 0.25 \mu\text{M}$, $n_1 = 3$; $K_2' = 8.0 \mu\text{M}$, $n_2 = 3$) [60], the ligand/binding-site ratio, R_s , was 0.17 at constant ligand/protein ratio equal to 1. For the postulated carrier-mediated process it follows that $V_{\text{max}}^{\text{obs}}$ must have been considerably below the true maximum uptake rate, V_{max} (Fig. 5). The measurements at constant C_p apparently have been limited to a concentration region well below values permitting one to reach the true saturation level. (ii) Much of the existing confusion about the interpretation of the kinetic data stems from attempts to interpret the results in terms of changes of the unbound ligand concentration due to the soluble protein alone. As pointed out above, curve shape is determined jointly by the binding properties of both the acceptor and the substrate-binding protein. In particular, the linear increase of the uptake rates with total ligand concentration, at constant protein concentration, C_p , is entirely conceivable, implying compensation of the binding tendencies of the soluble protein and the membrane-embedded carrier (Figs. 1 and 3). Taking account of the fact that the affinity of the second class of oleic acid-binding sites is relatively small ($K_2'/K_1' \approx 30$), the value of K_m' should be comparable with the dissociation constant, K_1' , of the higher-affinity binding class.

(b) Sorrentino *et al.* [29] further observed that the apparent maximum rates of oleate uptake, $V_{\text{max}}^{\text{obs}}$, into hepatocytes, adipocytes and cardiac myocytes, increased with the oleate/albumin ratio, A/C_p . We have shown that, for each cell type, a plot of $1/V_{\text{max}}^{\text{obs}}$ (taken from [29]) against $(1 - R_s)/R_s$ yielded a linear relationship. This relationship, which was derived for systems containing substrate-binding protein with a single class of site (eqn. 15), also applies to the present assays. The assays were at $R_s \leq 0.33$ corresponding to $(1 - R_s)/R_s \geq 2$. By simulation it was shown that, under these circumstances, $1/V_{\text{max}}^{\text{obs}}$ increases linearly with $(1 - R_s)/R_s$ for K_m'/K_1' equal to 0.01–10 and $K_2'/K_1' \approx 30$.

(c) Finally, we showed by simulation that, for data such as presented in Fig. 8 for uptake at constant ligand/albumin ratio, with and without 66 μM added BSA, plots of $1/v$ against $1/A$ mimic competitive inhibition by added protein, as has been observed [61].

Interpretation of experiments in terms of total and local concentrations and data analysis

Usually, response values proportional to acceptor-bound ligand, e.g. initial-rate values, are obtained at several total-ligand concentrations. To reduce the problem of data analysis to that of acceptor interaction, the results are often interpreted in terms of calculated local substrate concentrations. This procedure presents several drawbacks. (i) The binding parameters of the added protein often have to be guessed. (ii) Even if known in some reference solvent, they might be different in the presence of membrane material, owing to competition or allosteric modification of the binding sites by some component extracted from the membrane, e.g. fatty acid [34]. (iii) Finally, such an analysis may suggest confirmation of a postulated mechanism of action, whereas, in reality, equally satisfactory fits of the data to alternative mathematical models are possible. This problem has been discussed in some detail with regard to establishing the nature and location of acceptor sites [5,6]. For unbiased assessment, the results should first be interpreted in terms of total

concentrations, as indicated in the present paper. Provided the experimental work is limited to a range of ligand concentrations for which P_A^m is constant [5], all formulae have the same mathematical structure in the presence ($F_A \geq 1$) and in the absence ($F_A = 1$) of membrane material. Then, at fixed value of the distribution function, F_A , interpretation of the results in terms of total substrate concentration and data treatment are no more difficult than for corresponding single-phased systems.

First consider acceptor action on a ligand-protein complex. A fairly simple approach could consist in running saturation curves at several values of C_p and obtaining preliminary estimates of K_m and V_{max} from a double-reciprocal plot of $V_{max}^{obs.}$ against C_p . Final estimates of the parameters could then be obtained by a non-linear fit of the model (eqns. 18 and 19) to the data with A and C_p as the independent variables.

Now consider reaction of acceptor sites with unbound substrate. Little information is available about the application of data-fitting procedures to obtaining the binding and/or kinetic parameters for systems containing more than one binding component. According to Bates *et al.* [62], fitting the double Michaelis-Menten equation (parameters: $V_{max,1}$, $K_{m,1}$, $V_{max,2}$ and $K_{m,2}$) to binding data is extremely ill-conditioned mathematically. This is, perhaps, not surprising, because over a range of relatively low substrate concentrations the near-hyperbolic curves closely approach rectangular hyperbolae, which permit the estimation of only two parameters. In a similar study, Hsu & Tseng [63] concluded that fairly large numbers of response values (50 data pairs used) obtained at equally spaced substrate concentrations are required before any confidence can be placed in the fitted parameters. In the present case the problem may be less exacting, because the concentrations of acceptor and substrate-binding protein can be varied independently. Also, when the curves are sigmoidal, additional mathematical information is provided by the inflection point.

In preliminary experiments, the model for enzymic conversion of unbound substrate (eqns. 2 and 3) was fitted to simulated data obtained at a single constant concentration of binding protein with one class of binding site. A series of 16–24 equally spaced substrate concentrations and the corresponding r values (range 0.1–0.9), provided with relative random error of 1–5%, were generated at C equal to 1–100 μM . $K_m^{app.}$ and $K_1^{app.}$ covered the range 1–10⁻³ μM . By using a non-linear model-fitting quasi-Newton algorithm [64], both the kinetic parameters of the enzyme, E , and the binding parameters of the binding protein, C , could be obtained in the region of sigmoidal saturation ($K_m^{app.} \geq K_1^{app.}$), but the algorithm often failed to converge. The procedure was most successful when the ratio $K_m^{app.}/K_1^{app.}$ was 1–100. Partition of the 24 data points over three or four saturation curves, each run at a different site concentration, C , might be worth exploring. Model-fitting (eqns. 2 and 14) to data obtained at constant substrate/binding-site ratio, R_s , might similarly be attempted by simultaneously fitting saturation curves obtained at several R_s values ($R_s < 1$). Preliminary estimates of V_{max} , $K_m^{app.}$ and K_m'/K_1' can be obtained as detailed above. K_m' is generally smaller than $K_m^{app.}$.

If a direct non-linear fitting procedure does not yield satisfactory results, the kinetic and binding parameters required for interpreting the properties of systems containing both an acceptor and a binding protein will have to be estimated separately. In doing so, due attention should be paid to the possible impact of extracted lipid on the binding parameters. For determination of the apparent Michaelis constant, $K_m^{app.}$, and maximum reaction rate, V_{max} , the critical problem of avoiding ligand aggregation on its introduction into the incubation mixtures may be solved by using ligand/phospholipid mixtures as the transfer vehicle [30]. Data treatment for the latter systems has been discussed [5].

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