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THE RELATIONSHIP BETWEEN SUBSTRATE DISSOCIATION CONSTANTS DERIVED FROM TRANSPORT EXPERIMENTS AND FROM EQUILIBRIUM BINDING ASSAYS

IMPLICATIONS OF THE CONVENTIONAL CARRIER MODEL

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A kinetic analysis of substrate and inhibitor binding, based on the conventional carrier model, leads to the following conclusions. (1) The substrate constant derived from equilibrium binding studies is not a simple dissociation constant; rather, it is identical to the half-saturating substrate concentration for equilibrium exchange transport, which is a function of both the dissociation constant and the rate constants for carrier reorientation. In general, binding and transport constants are identical, assuming the same substrate distribution across the membrane in the two experiments. (2) Binding studies reveal only a single substrate site—even if the carrier is unsymmetrical, with different substrate affinities on the two sides of the membrane. (3) The binding constants for inhibitors are identical to the inhibition constants found in transport. (4) These rules, which apply to a carrier imbedded in the cell membrane or free in solution, offer a means of deciding whether an isolated carrier retains the properties of the intact system.

Introduction

The carrier-mediated transport of small molecules across biological membranes proceeds through three main steps: in the first, the substrate is recognized and bound by the carrier; in the second, the bound substrate is transferred across the membrane; and in the third, the substrate is released, leaving the carrier free. Elucidation of the transport mechanism requires, among other things, knowledge of the steps in which the substrate is bound and desorbed, and in particular, a clear separation of these steps from the movement of the free carrier and the carrier-substrate complex through the membrane. This is obviously true of active transport systems, where cellular energy is harnessed to produce an electrochemical gradient, and where, hypothetically, the driving force could be applied in two different ways; either it could induce an unsymmetrical change in the substrate's affinity on the inner and outer surfaces of the membrane, or it could alter the rate of translocation of the free carrier or carrier complex in one direction. In studies of facilitated transport systems, information about the binding step is required in order to decide the nature of the rate-limiting steps in transport, as well as the number and specificity of substrate sites.

Clearly, a true measure of the substrate dissociation constant is wanted. A difficulty, which is widely recognized, is that the half-saturating concentration in transport is not a true equilibrium constant, but instead is a function of both the substrate's affinity for the carrier site, and of rates of movement of the carrier across the membrane; how the rate constants enter into the expression

for the apparent dissociation constant depends on which steps are rate-limiting, as the kinetic analysis of the conventional carrier model shows [1–10]. In consequence, the interpretation of the experimental parameters for substrate transport is uncertain. For example, a change in the substrate half-saturation constant, observed upon energization of an active transport system, could result from a change either in the substrate's affinity, or in the rate of carrier transfer through the membrane; and without further information, it would not be possible to decide which mechanism operates.

Because of these uncertainties, another experimental method is needed, one giving the dissociation constant by itself. It is tempting to assume, as is often done, that a technique such as equilibrium dialysis would provide a solution to the problem, avoiding the complications inherent in transport asays; for if the addition of the substrate to the carrier is detected and measured directly, under equilibrium conditions, it seems reasonable, in the absence of a detailed analysis, to assume that the true equilibrium constant could be calculated (see, for example, Refs. 11–13). But this assumption is wrong. In theory, the two kinds of experiment (transport and binding) should give the same half-saturation constants. That they are identical is proven by the following kinetic treatment of the binding of substrates or inhibitors to the carrier. The carrier is assumed to be of the conventional type, isomerizing between two conformations: one conformation accepts substrate molecules from the external medium, the other, from the cell interior.

Kinetic theory

1. *Transport*

General equations for the transport scheme in Fig. 1 have already been derived [10]. If $[S_o]$ is the concentration of labeled substrate outside, $[S_i]$ the concentration of unlabeled substrate inside, the rate of entry can be written as:

$$v = \frac{\frac{\bar{V}_{S_o}[S_o]}{\bar{K}_{S_o}} + \frac{\bar{\bar{V}}_S[S_i][S_o]}{\bar{K}_{S_o}\bar{K}^S_{S_i}}}{1 + \frac{[S_i]}{\bar{K}_{S_i}} + \frac{[S_o]}{\bar{K}_{S_o}} + \frac{[S_i][S_o]}{\bar{K}_{S_o}\bar{K}^S_{S_i}}} \qquad (1)$$

The constants in Eqn. 1 are experimental parameters found in transport. The full expressions for these constants, in terms of the individual rate constants in Fig. 1, are listed in Table I: $\bar{V}_{S_o}$, the maximum rate of zero *trans* entry; $\bar{\bar{V}}_S$, the maximum rate of equilibrium exchange; $\bar{K}_{S_o}$, $\bar{K}_{S_i}$, the half-saturation constants for zero *trans* entry and zero *trans* exit, respectively; $\bar{K}^S_{S_i}$, the half-saturation constant for infinite *trans* exit.

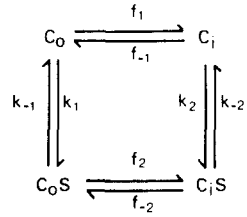

Fig. 1. Transport scheme for the conventional carrier model. The conformation of the carrier alternates between inward-facing and outward-facing forms, C_o and C_i; substrate molecules in the external or internal solutions, respectively, add to these two forms of the carrier.

Rate equations for particular transport experiments are found by substitution into Eqn. 1. In equilibrium exchange, the same substrate is present on both sides of the membrane, one pool labeled and the other not, and the unidirectional substrate flux is determined with the ratio of internal and external substrate concentrations equal to that attained in the final steady-state, where no net flux occurs: $[S_i] = \alpha[S_o]$. The constant, α, is equal to unity in facilitated transport systems (assuming that the substrate is a neutral molecule; of if it bears a net charge, that the membrane potential is zero). In active transport systems, where an electrochemical gradient is established, α is far from unity. Under the conditions of equilibrium exchange, Eqn. 1 was shown [10] to reduce to

$$v = \frac{\bar{\bar{V}}_S}{1 + \left(\bar{\bar{K}}_{S_o}/[S_o]\right)} \qquad (2)$$

$\bar{\bar{K}}_{S_o}$ is the half-saturating concentration of substrate in the external medium (see Table I). The corresponding concentration of the internal substrate is $\bar{\bar{K}}_{S_i}$, where $\bar{\bar{K}}_{S_i} = \alpha\bar{\bar{K}}_{S_o}$.

2. *Binding*

A general equation can be derived for the for-

TABLE I

EXPERIMENTAL PARAMETERS FOR TRANSPORT AND BINDING EXPRESSED IN TERMS OF INDIVIDUAL RATE CONSTANTS IN THE TRANSPORT SCHEME IN FIG. 1

Equilibrium constants for carrier-substrate complex formation are defined as follows: $K_{S_o} = k_{-1}/k_1$; $K_{S_i} = k_{-2}/k_2$. The symbol H stands for the following combination of constants: $H = k_{-1}f_{-2} + k_{-1}k_{-2} + k_{-2}f_2$.

Experimental parameter	General expression	Rapid dissociation [a]
α	$\frac{f_{-1}f_2k_1k_{-2}}{f_1f_{-2}k_{-1}k_2}$	
$\bar{V}_{S_o}$	$\frac{k_{-2}f_{-1}f_2C_t}{k_{-2}(f_2+f_{-1})+f_{-1}(f_2+f_{-2})}$	$\frac{f_{-1}f_2}{f_2+f_{-1}}$
$\bar{\bar{V}}_S$	$\frac{k_{-1}k_{-2}f_2f_{-2}C_t}{(f_2+f_{-2})H}$	$\frac{f_2f_{-2}}{f_2+f_{-2}}$
B_o	$\frac{f_{-1}(f_2+f_{-2}+k_{-2})}{k_{-2}(f_2+f_{-1})+f_{-1}(f_2+f_{-2})}$	$\frac{f_{-1}}{f_{-1}+f_2}$
B_i	$\frac{f_1(f_2+f_{-2}+k_{-1})}{k_{-1}(f_{-2}+f_1)+f_1(f_2+f_{-2})}$	$\frac{f_1}{f_1+f_{-2}}$
$\bar{K}_{S_o}$	$\frac{(f_1+f_{-1})H}{k_1\{k_{-2}(f_2+f_{-1})+f_{-1}(f_2+f_{-2})\}}$	$\frac{K_{S_o}(f_1+f_{-1})}{f_2+f_{-1}}$
$\bar{K}_{S_i}$	$\frac{(f_1+f_{-1})H}{k_2\{k_{-1}(f_{-2}+f_1)+f_1(f_2+f_{-2})\}}$	$\frac{K_{S_i}(f_1+f_{-1})}{f_{-2}+f_1}$
$\bar{K}^S_{S_i}$	$\frac{k_{-2}(f_{-1}+f_2)+f_{-1}(f_2+f_{-2})}{k_2(f_2+f_{-2})}$	$\frac{K_{S_i}(f_{-1}+f_2)}{f_2+f_{-2}}$
$\bar{\bar{K}}_{S_o}$	$\frac{K_{S_o}(1+(f_1/f_{-1}))}{1+(f_2/f_{-2})}$	$\frac{K_{S_o}(1+(f_1/f_{-1}))}{1+(f_2/f_{-2})}$
$\bar{K}_{I_o}$	$K_{I_o}(1+(f_1/f_{-1}))$	$K_{I_o}(1+(f_1/f_{-1}))$
$\bar{K}_{I_i}$	$K_{I_i}(1+(f_{-1}/f_1))$	$K_{I_i}(1+(f_{-1}/f_1))$

[a] The expressions have been simplified for the case where substrate dissociation (k_{-1} and k_{-2}) is very rapid compared with carrier reorientation (f_1, f_{-1}, f_2, and f_{-2}).

mation of the carrier-substrate complex under conditions where the substrate is accessible to the outward-facing conformation C_o, the inward-facing conformation C_i, or both. The derivation, based on the conventional carrier model (Fig. 1), and outlined in the Appendix, follows the schematic method of King and Altman [14].

The relationship between the concentration of bound substrate molecules $[S_b]$ and the concentration of free substrate, $[S_o]$ and $[S_i]$, is

$$[S_b] = \frac{\left(\frac{[S_o]B_o}{\bar{K}_{S_o}} + \frac{[S_i]B_i}{\bar{K}_{S_i}} + \frac{[S_o][S_i]}{\bar{K}_{S_o}\bar{K}^S_{S_i}}\right)C_t}{1 + \frac{[S_o]}{\bar{K}_{S_o}} + \frac{[S_i]}{\bar{K}_{S_i}} + \frac{[S_o][S_i]}{\bar{K}_{S_o}\bar{K}^S_{S_i}}} \quad (3)$$

C_t is equal to the total carrier concentration in all forms; and $[S_b]$ to the concentration of the carrier-substrate complex: $[S_b] = [C_oT] + [C_iT]$. The substrate half-saturation constants are identical to those already defined for transport [10]. The new experimental constant, B_o, is the fraction of the carrier to which substrate molecules are bound under conditions where there is a saturating concentration of substrate in the external solution, but no substrate inside ($[S_o] \to \infty$; $[S_i] = 0$). The other new constant, B_i, is the converse: the fraction of carrier complex with a saturating concentration of substrate inside, and none outside. The full expressions for these constants, in terms of individual rates in the conventional carrier scheme (Fig. 1), are given in Table I.

The similarity between Eqn. 3, for binding, and Eqn. 1, for transport, is significant; especially that the denominators are the same — this congruence,

by itself, implies that the predicted experimental half-saturating concentrations will be identical in the two kinds of experiment.

a. Substrates

Under the conditions of equilibrium exchange, the substrate is present in both compartments at concentrations attained in the final steady state ($[S_i] = \alpha[S_o]$; Eqn. 3 becomes

$$[S_b] = \frac{[S_o]\left(\frac{B_o}{\bar{K}_{S_o}} + \frac{\alpha B_i}{\bar{K}_{S_i}}\right)\left(1 + \frac{\alpha[S_o]/\bar{K}_{S_o}\tilde{K}^S_{S_i}}{B_o/\bar{K}_{S_o} + \alpha B_i/\bar{K}_{S_i}}\right)C_t}{1 + \frac{[S_o]}{\bar{K}_{S_o}} + \frac{\alpha[S_o]}{\bar{K}_{S_i}} + \frac{\alpha[S_o][S_o]}{\bar{K}_{S_o}\tilde{K}^S_{S_i}}} \quad (4)$$

This equation may be simplified by substituting into it the following relationships, which are required by the principle of microscopic reversibility, and whose validity can be confirmed with the aid of the expressions for experimental parameters given in Table I:

$$\frac{B_o}{\bar{K}_{S_o}} + \frac{\alpha B_i}{\bar{K}_{S_i}} = \frac{1}{\bar{\bar{K}}_{S_o}} \quad (5)$$

$$\frac{1}{\bar{K}_{S_o}} + \frac{\alpha}{\bar{K}_{S_i}} = \frac{1}{\bar{\bar{K}}_{S_o}} + \frac{\alpha\bar{\bar{K}}_{S_o}}{\bar{K}_{S_o}} \quad (6)$$

Eqn. 4 yields:

$$[S_b] = \frac{C_t}{1 + \left(\bar{\bar{K}}_{S_o}/[S_o]\right)} \quad (7)$$

Eqn. 7 can be cast in the familiar form of the Scatchard plot:

$$\frac{[S_b]}{[S_o]} = \frac{C_t}{\bar{\bar{K}}_{S_o}} - \frac{S_b}{\bar{\bar{K}}_{S_o}} \quad (8)$$

The binding constant found under these conditions is seen to be identical to that for equilibrium exchange, $\bar{\bar{K}}_{S_o}$ (Eqn. 2) *.

b. Inhibitors

By definition, a competitive inhibitor is not transported across the membrane: if S in Fig. 1 represents an inhibitor, the rate constants for reorientation of the carrier complex, f_2 and f_{-2}, are equal to zero. When these values are inserted into the expressions for the experimental constants in Eqn. 3 (see Table I), B_o and B_i are found to be equal to unity, and $1/\tilde{K}^S_{S_i}$ to zero. In this case Eqn. 3, with the symbol I, for the inhibitor, replacing S, reduces to **

$$[I_b] = \frac{\left(\frac{[I_o]}{\bar{K}_{I_o}} + \frac{[I_i]}{\bar{K}_{I_i}}\right)C_t}{1 + \frac{[I_o]}{\bar{K}_{I_o}} + \frac{[I_i]}{\bar{K}_{I_i}}} \quad (9)$$

If the inhibitor is restricted to the external solution ($[I_i] = 0$), Eqn. 9 becomes

$$[I_b] = \frac{C_t}{1 + \left(\bar{K}_{I_o}/[I_o]\right)} \quad (10)$$

The binding constant in Eqn. 10, $\bar{K}_{I_o}$, is identical to that found in the inhibition of transport [10]. It differs from the true dissociation constant, K_{I_o}, by a factor that depends on the carrier partition between internal and external forms (see Table I).

With an inhibitor in both compartments at equal concentrations ($[I_o] = [I_i] = [I]$), the binding equation becomes

$$[I_b] = \frac{C_t}{1 + \left(\frac{1}{[I]}\left(\frac{1}{\bar{K}_{I_o}} + \frac{1}{\bar{K}_{I_i}}\right)\right)} \quad (11)$$

* It is instructive to compare these results with binding and transport equations for non-equilibrium conditions. With the substrate confined to the external solution, the binding equation becomes $[S_b] = B_o C_t/(1 + (\bar{K}_{S_o}/[S_o]))$, and the rate of substrate uptake is given by $v = \bar{V}_{S_o}/(1 + (\bar{K}_{S_o}/[S_o]))$. The two equations have the same denominator, and therefore the substrate half-saturation constants are the same. The numerator of the binding equation is $B_o C_t$, showing that only a fraction of the carrier, B_o, forms a complex with the substrate. Under equilibrium conditions, all the carrier, C_t, forms a complex (Eqn. 7). A non-transported substrate analog can form a complex with all the carrier, whether it is present on one side of the membrane or both (see Eqns. 9 and 10).

** The half-saturation constants for an inhibitor, $\bar{K}_{I_o}$ and $\bar{K}_{I_i}$, are found from the expressions for the substrate half-saturation constants in Table I, by substituting $f_2 = f_{-2} = 0$.

The experimental constant, $\left(\frac{1}{\bar{K}_{I_o}}+\frac{1}{\bar{K}_{I_i}}\right)^{-1}$, is identical to the competitive inhibition constant found under the same conditions in transport [10].

Discussion

Several important points are brought out by the analysis. First, the dissociation constants found in transport and binding assays are identical, and therefore the latter can give no added information, beyond what may be derived from measurements of transport. This is true of experiments with intact cells, with membrane vesicles, or with the carrier in an artificial membrane. Second, asymmetry in the carrier structure or in the carrier mechanism cannot be demonstrated in binding studies under equilibrium conditions. As is well known, a transport mechanism, either facilitated or active, may be highly asymmetric, and no theoretical restraint keeps the equilibrium binding constants on the two sides of the membrane from differing by a large factor. The only restraint, imposed by the principle of microscopic reversibility, is that the equation for α (Table I) should be satisfied. But no matter what the actual substrate affinities on either side of the membrane, the Scatchard plot for such a system is bound to be linear (Eqns. 7 and 8). Similarly, the reciprocal plot for exchange transport is linear (Eqn. 2).

The application of these ideas to an isolated carrier molecule in solution should also be considered. If the carrier has not been modified during its purification, it should be free to undergo a normal conformational change between inward-facing and outward-facing forms, and the two forms would necessarily be exposed to the same substrate concentration, just as they are in equilibrium exchange. Therefore, even if the two conformational states have different affinities for the substrate, binding studies would give evidence of only a single substrate site. Any discrepancy between the affinity constants determined with the isolated carrier and the intact system would be interpreted as due either to a modification of the carrier structure, or to a perturbed equilibrium of the two carrier conformations in the new environment. These inferences suggest a useful application, namely a test of the degree to which the isolated carrier retains the properties, and presumably the structure, which it possessed in the cell membrane.

A final comment is that the available experimental results are in accord with the analysis. Rates of lactose transport, and binding of lactose to the carrier, were both determined in membrane vesicles derived from *Escherichia coli*, which retain a functional lactose transport system [12,13]. The experiments of interest were done on the non-energized system. First, the lactose half-saturation constants for equilibrium binding and for equilibrium exchange agreed closely [12]; and second, Scatchard plots of the binding data were linear over a wide range of lactose concentrations [13].

Appendix

To derive an equation for substrate binding, expressions are first written down for the steady-state concentrations of each of the four carrier species in the conventional carrier model (Fig. 1), following the schematic method of King and Altman [14]. The concentrations, as a fraction of the total amount of the carrier, C_t, are found to be:

$$[C_o]/C_t = (f_{-2}k_{-1}k_2[S_i]+f_{-1}H)/\sum \quad (12)$$

$$[C_i]/C_t = (f_2k_{-2}k_1[S_o]+f_1H)/\sum \quad (13)$$

$$[C_oS]/C_t = \{f_{-1}k_1[S_o](f_{-2}+k_{-2})+f_1k_2f_{-2}[S_i] + k_1k_2f_{-2}[S_o][S_i]\}/\sum \quad (14)$$

$$[C_iS]/C_t = \{k_2f_1[S_i](f_2+k_{-1})+f_{-1}k_1f_2[S_o] + k_1k_2f_2[S_o][S_i]\}/\sum \quad (15)$$

where $H = f_{-2}k_{-1}+k_{-1}k_{-2}+f_2k_{-2}$, and where Σ is the sum of all the terms in the numerators of Eqns. 12–15.

The concentration of substrate bound to the carrier, $[S_b]$, is equal to the concentration of the carrier-substrate complex:

$$[S_b] = [C_oS]+[C_iS] \quad (16)$$

Substituting the expressions for $[C_oS]$ and $[C_iS]$, from Eqns. 12–15, yields Eqn. 17.

$$[S_b] = \frac{[S_o]k_1f_{-1}(f_2+f_{-2}+k_{-2})+[S_i]k_2f_1(f_2+f_{-2}+k_{-1})+[S_o][S_i]k_1k_2(f_2+f_{-2})}{(f_1+f_{-1})H+[S_o]k_1\{k_{-2}(f_{-1}+f_2)+f_{-1}(f_2+f_{-2})\}+[S_i]k_2\{k_{-1}(f_1+f_{-2})+f_1(f_2+f_{-2})\}+[S_o][S_i]k_1k_2(f_2+f_{-2})} \quad (17)$$

When Eqn. 17 is divided by $H(f_1+f_{-1})$, and simplified by substituting in the experimental constants in Table I, it reduces to Eqn. 3.

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