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The apparent K_m is a misleading kinetic indicator

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When information concerning whether or not a ligand interacts with the same enzyme species as do the substrates, the variation of the Michaelis constant K_m (for each substrate) with ligand concentration is sometimes used as a diagnostic. It is shown that the Michaelis constant is of no particular value in this respect and may be misleading. Thus, depending on the mechanism, K_m may vary with ligand concentration even though the ligand interacts with species far removed in the mechanism from the substrate-binding steps, and it may stay constant in cases where the ligand competes directly for the free enzyme. In contrast, the slope of a double-reciprocal plot of the kinetic data ($= K_m/V_{max.}$) (or, equivalently, the ordinate intercept of a Hanes plot A/v versus A, where A is the substrate concentration) independently of the particular mechanism involved uniquely signifies whether or not such interaction occurs. The results clearly indicate that, for purposes other than communicating the substrate concentration yielding control of the enzymic activity, usage of K_m and its variation with ligand concentration should be avoided and interest instead focused on the slope, in accordance with the long-established rules of Cleland [Biochim. Biophys. Acta (1963) **67**, 188–196], for which the present analysis provides the formal framework.

INTRODUCTION

There are many enzymes for which, at least within certain ranges of substrate concentration, the initial steady-state velocity v in terms of the substrate concentration A may be described by the Michaelis–Menten equation:

$$v = \frac{V_{max.}A}{K_m + A} \quad (1)$$

The value of the kinetic parameters $V_{max.}$ and K_m may depend on other ligands or substrates involved in the reaction mechanism. The Michaelis constant K_m is often referred to as the 'apparent affinity constant'. Operationally, it is the value of A yielding half-maximal rate, and thus is of importance in establishing the substrate concentration range exhibiting control of the enzymic activity under the given circumstances.

In many cases it is desirable to examine, without a full-scale kinetic investigation of the enzyme, how a particular ligand binding to the enzyme influences the activity with particular reference to which steps or which enzyme intermediates are directly interacting with the ligand. For this purpose quite often the variation of K_m with ligand concentration is reported. For example, if it is found that K_m is constant when the moderator concentration is varied, the inference (sometimes explicitly stated) is that the ligand does not bind to the enzyme intermediates involved with the substrate, and, conversely, if a variation of K_m is observed, then the moderator is involved with the same intermediates as is the substrate. However, this is not necessarily true. Thus it is standard textbook material that, whereas in the case of a non-competitive inhibitor the apparent K_m is independent of inhibitor concentration, the same quantity in the case of a competitive inhibitor is a linear function of inhibitor concentration, and yet in both cases the inhibitor binds to the empty enzyme form. It thus seems that the properties of the apparent K_m and their interpretation depend on the particular mechanism involved.

Some 20 years ago Cleland [1] published a set of 'slope and intercept rules' that, independently of the mechanism involved, can be used to determine the type of enzyme intermediate directly interacting with a ligand. (For concreteness I shall here, following Cleland [1], refer to the slope and intercept of a double-reciprocal plot, v^{-1} versus A^{-1}. The same quantities are obtained experimentally as, respectively, the intercept and slope of a Hanes plot, A/v versus A.) The purpose of the present paper is to provide a formal framework for these rules, and to show that variation of the slope, or lack thereof, with ligand concentration uniquely signifies whether or not that ligand interacts with the same enzyme species as does the substrate. This is done in the following section by considering a few selected cases that together exhibit the necessary characteristic features. I use Cleland's nomenclature [2] throughout. A, B and C denote substrates, and products are denoted P and Q.

THEORY

Inhibitors

A ligand can influence the rate of a step in a kinetic mechanism only by binding to one or more intermediates, thus giving rise to new, ligand-bound, intermediates. In general, this will complicate the mechanism and its steady-state rate equation considerably.

However, in many cases the binding and release rates of ligands to and from enzyme intermediates would be expected to be large relative to the rates with which the substrates are processed by the enzyme. On general grounds we expect that small inorganic ions, effective in millimolar concentrations, are examples of this behaviour. In such cases the basic kinetic mechanism need not be altered at all, but the kinetically distinguishable intermediates in that mechanism should now be considered as pools, each consisting of several intermediates in internal rapid equilibrium at all times. Cha [3] has shown that for such a mechanism the rate equation, including the

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specific dependence on the ligand concentration, may be obtained as follows.

1. The steady-state rate equation for the simple mechanism in terms of rate constants is found by the usual procedures.

2. In the resulting expression, any rate constant out of a pool containing several species in equilibrium consists of the sum of the apparent rate constants for each species in the pool. For any species, the apparent rate constant is obtained by multiplying its intrinsic conversion rate constant by the fractional concentration of that species in the pool, which depends on the ligand concentration and its associated dissociation constant. Thus, if the pool is $E \rightleftharpoons EL \rightleftharpoons EL_2$ in rapid equilibrium, and all species can react, with intrinsic rate constants $k_{(0)}$, $k_{(1)}$ and $k_{(2)}$ respectively, the apparent rate constant out of the pool is:

$$k_{app.} = \frac{k_{(0)} + 2k_{(1)} \cdot L/K_L + k_{(2)} \cdot (L/K_L)^2}{(1 + L/K_L)^2}$$

where K_L is the intrinsic (site) dissociation constant for L (equivalent and independent sites for L have been assumed).

In this way the dependence of the total steady-state rate on ligand concentration is obtained.

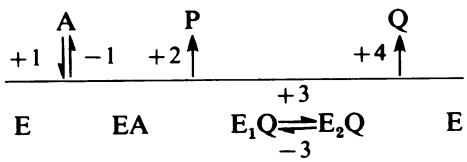
In a similar way the influence of the ligand on the kinetic parameters $V_{max.}$, K_m and R ($= K_m/V_{max.}$) is obtained. The quantity in question is first written in terms of apparent rate constants, and the ligand dependence is then found by inserting the rate-constant-dependence on the ligand concentration according to point 2 above. To make predictions concerning the interference of a ligand in the mechanism it is therefore sufficient to study the structure (in terms of rate constants) of the various quantities that can be easily determined experimentally, such as K_m, $V_{max.}$ and R for a number of mechanisms.

In the examples below only steady-state initial rates, i.e. in the absence of products, are considered. The full rate equations, as well as the rules for obtaining K_m and $V_{max.}$ therefrom, may be found in Segel [4], or they can be obtained by using the systematic approach described by Huang [5]. In all cases only the expressions, in terms of rate constants, of K_m, $V_{max.}$ and R are given. The slope of the plot of v^{-1} versus reciprocal substrate concentration X^{-1} is designated $R_{1/X}$. These are obtained from the full rate expressions when written in double-reciprocal form with the appropriate substrate as variable.

Example 1. Ordered Uni Bi with isomerization:

$$A \rightarrow P + Q$$

Mechanism:



$$V_{max.} = \frac{k_2 k_3 k_4 E_0}{k_{+3}(k_{+2} + k_{+4}) + k_{+2}(k_{+4} + k_{-3})} \quad (2)$$

$$K_m = \frac{k_{+3} k_{+4}(k_{-1} + k_{+2})}{k_{+1}[k_{+3}(k_{+2} + k_4) + k_{+2}(k_{+4} + k_{-3})]} \quad (3)$$

$$R_{1/A} = \frac{k_{-1} + k_{+2}}{k_{+1} k_{+2} E_0} \quad (4)$$

It is noted that K_m depends on all the rate constants in the mechanism. A ligand interacting with any one intermediate will therefore have an effect on K_m. If, for example, a dead-end inhibitor I interacts with the species E_1Q, yielding the pool $E_1Q \rightleftharpoons E_1QI$, the rate constant k_{+3} in eqns. (2) and (3) would be:

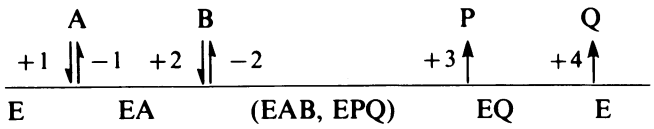
$$k_{+3} = \frac{k_{+3}^{(0)}}{1 + I/K_I} \quad (5)$$

and insertion of this in the equations would render both $V_{max.}$ and K_m dependent on I. But $R_{1/A}$ is simple: it depends only on rate constants characterizing the two intermediates E and EA with which the substrate is involved. If a change in ligand concentration results in a change in $R_{1/A}$, then one or more of the rate constants k_{-1}, k_{+1} and k_{+2} depend(s) on ligand concentration, and this in turn means that the ligand binds to one or both of the intermediates E and EA. It is easy to show that the same expression for $R_{1/A}$ would have been obtained if the isomerization $E_1Q \rightleftharpoons E_2Q$ were absent: any complexity in a Uni Bi mechanism subsequent to the state EA has no influence on the value of the slope $R_{1/A}$ (unless the intermediate EA isomerizes before the release of the first product P), but K_m becomes correspondingly more complex. This seems to be a general property (see below).

Example 2. Ordered Bi Bi:

$$A + B \rightarrow P + Q$$

Mechanism:



$$V_{max.} = \frac{k_{+3} k_{+4} E_0}{k_{+3} + k_{+4}} \quad (6)$$

$$K_a = \frac{k_{+3} k_{+4}}{k_{+1}(k_{+3} + k_{+4})} \quad (7)$$

$$K_b = \frac{(k_{-2} + k_{+3}) k_{+4}}{k_{+2}(k_{+3} + k_{+4})} \quad (8)$$

$$K_{ia} = \frac{k_{-1}}{k_{+1}} \quad (9)$$

$$R_{1/A} = \frac{1}{k_{+1} E_0}\left(1 + \frac{K_{ia} K_b}{K_a} \cdot \frac{1}{B}\right)$$

$$R_{1/A}\ (\text{B saturating}) = \frac{1}{k_{+1} E_0} \quad (10)$$

$$R_{1/B}\ (\text{A saturating}) = \frac{k_{-2} + k_{+3}}{k_{+2} k_{+3} E_0} \quad (11)$$

Again, the Michaelis constants K_a and K_b both depend on a rate constant characterizing a step in which neither substrate is involved: if a ligand interferes with EQ, k_{+4} and hence both K_a and K_b will depend on ligand concentration, even though that ligand does not interact with any species involving the substrates A and B. The slopes, however, are simple: $R_{1/A}$, with B saturating,

depends only on k_{+1}, the substrate-binding rate constant, whereas $R_{1/B}$, corresponding to the substrate adding just before the release of the first product, when the other substrate, A, is saturating, contains only the rate constants k_{+2} and k_{-2}, characterizing binding and release of substrate B, and k_{+3}, characterizing the conversion of the enzyme–substrate complex obtained when B has been bound. It is noted that the simple forms of the slopes $R_{1/A}$ and $R_{1/B}$ are obtained only in the presence of saturating concentrations of the appropriate other substrate. Experimental determination of the slopes under these conditions automatically ensures that no difficulties are encountered even if the mechanism allows for random addition of A and B. In the general case, such a mechanism will give rise to non-linear double-reciprocal plots, but saturation with one substrate effectively excludes the pathway along which that substrate adds last, and the resulting mechanism is essentially a monosubstrate mechanism yielding linear double-reciprocal plots, and thus the slope R has the form as in example 1 above.

If the isomerization of the central complex EAB $\rightleftharpoons$ EPQ can be distinguished kinetically, with forward and reverse rate constants k_{+i} and k_{-i} respectively, eqns. (6)–(8) become considerably more complicated, while the slopes are:

$$R_{1/A}\ (\text{B saturating}) = \frac{1}{k_{+1}E_0} \tag{12}$$

$$R_{1/B}\ (\text{A saturating}) = \frac{k_{-2}(k_{+3}+k_{-i})+k_{+i}k_{+3}}{k_{+2}k_{+3}k_{+i}} \tag{13}$$

Here $R_{1/A}$ is the same as for the Ordered Bi Bi case without isomerization; the complexity in the mechanism is subsequent to EA (compare example 1). The slope $R_{1/B}$ is more complex, but it is noted that again it contains only rate constants characterizing intermediates with which substrate B is directly involved and, in addition, intermediates reversibly connected to them in the sense of Cleland [1]. Hence ligand influence on the slope $R_{1/A}$ and/or $R_{1/B}$ is again evidence that the ligand interacts with one or more of the 'initial' intermediates in the mechanism.

In the other extreme case, when the concentration of the central complex at steady state is zero and EA is converted into EQ by essentially simultaneous addition of B and release of P (a Theorell–Chance mechanism), the slopes are:

$$R_{1/A}\ (\text{B saturating}) = \frac{1}{k_{+1}E_0} \tag{14}$$

$$R_{1/B}\ (\text{A saturating}) = \frac{1}{k_{+2}E_0} \tag{15}$$

and a dependence on either slope on ligand concentration (i.e. k_{+1} or k_{+2} is a function of the ligand concentration) uniquely signifies binding of that ligand to E or EA, respectively.

These examples illustrate the general procedure that can be used for any mechanism. Thus, for a Ping Pong Bi Bi mechanism the slopes are

$$R_{1/A} = \frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}E_0} \tag{16}$$

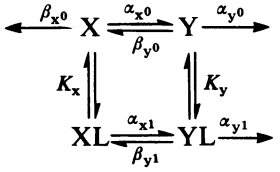
$$R_{1/B} = \frac{k_{-3}+k_{+4}}{k_{+3}k_{+4}E_0} \tag{17}$$

(k_{+2} and k_{+4} characterize product release from the two enzyme–substrate complexes). As expected for a Ping Pong mechanism in which the two 'free' enzyme forms E and F are separated by a product-release step, the two slopes $R_{1/A}$ and $R_{1/B}$ have the same form in terms of rate constants as found for a monosubstrate mechanism (example 1).

ACTIVATORS

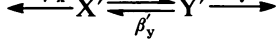
It is clear that the above considerations are not limited to inhibitors. Compounds that by binding to the enzyme activate the steps in which that intermediate is involved may be studied by these methods provided that they maintain internal binding equilibrium. If this is not the case, such compounds must appear specifically in the mechanism in a manner analogous to that of the substrates. Even in the equilibrium binding case, however, the effect of an activator (or a partial inhibitor) will in general be more complex than that of a dead-end inhibitor. This is because such a compound, since its presence provides an alternative pathway, must bind to at least two different intermediates, i.e. be present in several adjacent equilibrium pools in the mechanism, and thus must influence several consecutive steps (i.e. rate constants in both directions) in a mechanism. The effect of an activator thus cannot solely be an acceleration of a single step.

Consider the part of a general sequence with the arbitrary enzyme intermediates X and Y and an activator L binding to X, and consequently also to Y:



(S1)

which, in terms of equilibrium pools X′ and Y′, is simply:



(S2)

where α and β are forward and reverse first-order rate constants respectively. By using Cha's method [3] to find the apparent rate constants in scheme (S2) we obtain:

$$\beta'_x = \frac{\beta_{x0}}{1+L/K_x} \tag{18}$$

$$\alpha'_x = \frac{\alpha_{x0}+\alpha_{x1}\cdot L/K_x}{1+L/K_x} \tag{19}$$

$$\beta'_y = \frac{\beta_{y0}+\beta_{y1}\cdot L/K_y}{1+L/K_y} \tag{20}$$

$$\alpha'_y = \frac{\alpha_{y0}+\alpha_{y1}\cdot L/K_y}{1+L/K_y} \tag{21}$$

where L is the ligand concentration and K_x and K_y are dissociation constants defined in scheme (S1). By assumption (L is an activator), $\alpha_{x1} > \alpha_{x0}$. Consequently, $K_x \neq K_y$, because detailed balance at equilibrium requires:

$$\alpha_{x0}\beta_{y1} \cdot K_x = \beta_{y0}\alpha_{x1} \cdot K_y \quad (22)$$

Thus all the apparent rate constants in the sequence (S2) are affected by the ligand concentration L, whereas, in contrast, only two rate constants are affected if L is a dead-end inhibitor binding only to, say, X. In that case, the schemes (S1) and (S2) and eqns. (18)–(21) apply with $\alpha_{x1} = \alpha_{y1} = \beta_{y1} = 0$ and $K_y = \infty$, and the constraint eqn. (22) is absent. As a result, only β'_x and α'_x are functions of L.

DISCUSSION

The examples discussed under 'Inhibitors' in the Theory section may be said to form a 'basis set': more complex mechanisms can be divided into sequences, each of which belongs to one or the other class of examples discussed.

It is apparent from the examples presented that the Michaelis constants in general are complicated functions of the rate constants, some of which characterize steps far removed in the mechanism from those directly involving the substrates. In contrast, the slopes in double-reciprocal plots are always simpler and contain only rate constants characterizing (*a*) intermediates directly interacting with the appropriate substrate, (*b*) the complex of the enzyme with the substrate in question, or (*c*) intermediates reversibly connected to them, if any. Therefore variation with ligand concentration of K_m for a substrate does not in general yield information concerning the precise interaction of the ligand with the enzyme, whereas that of the slope does. In fact, whereas the slope in all cases depends on the 'initial' rate constants in a mechanism starting with the substrate-free enzyme form, the ordinate intercept (in double-reciprocal plots) characterizes the 'remaining' steps, as may be seen from the expression for $V_{max.}$ in the above examples.

If the details of the interaction of a ligand with an enzyme are desired, it is necessary to study the functional forms of slopes (and intercepts, if possible) in terms of ligand concentration. The general procedure was shown above with schemes (S1) and (S2). Such studies, using the above results for the case of Na^+ and K^+ influence on the kinetics of Na^+-dependent ATPase and $Na^+ + K^+$-dependent ATPase, have recently been published [6,7].

The results presented here clearly suggest that, although the Michaelis constant for a substrate is an important quantity as a measure of the substrate concentration yielding control of the enzymic activity, as a kinetic diagnostic it is of no particular value and may be misleading. In contrast, the slope is a valuable diagnostic, irrespective of the particular mechanism, and therefore, when ligand interaction is studied, its variation, rather than that of K_m, should be studied. The use of Cleland's 'slope rule' is thus not merely a question of convenience: it is essential if unequivocal conclusions concerning the ligand interaction with the enzyme are to be reached.

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