A method for determining kinetic parameters at high enzyme concentrations

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A graphical method is described which allows determination of kinetic parameters when substrate, inhibitor or activator concentrations must be in the vicinity of the enzyme concentration and a significant fraction of ligand is bound. Velocity is measured at several ligand:enzyme ratios at two or more enzyme concentrations. Results are obtained in terms of free and bound ligand corresponding to particular velocities. The relationship between velocity and bound and free ligand may then be analysed by any desired plotting technique. Preknowledge of the reaction mechanism or experimental determination of $V_{\max}$ is not required. The relationship between ligand bound and enzyme activity need not be linear and the method is equally suitable for analysing co-operative as well as simple kinetics. Application of the method is demonstrated by analysis of the inhibition of fructose 1,6-bisphosphatase by AMP.

When an enzyme binds substrate or effector (activator or inhibitor) with a sufficiently high affinity, the interaction must be studied at reactant concentrations such that ligand (substrate or effector) concentration is of the same order of magnitude as enzyme concentration. Under such conditions bound ligand is not negligible compared with total ligand, so that the commonly utilized assumption, that total ligand is equivalent to free ligand, may not be employed. The relationship between reaction velocity and ligand concentration under these conditions is more complex (Strauss & Goldstein, 1943; Henderson, 1972). Several graphical methods for determining kinetic parameters with tight-binding substrates or inhibitors have been proposed (Dixon, 1972; Henderson, 1973; Cha, 1976; Gaines, 1979) and the relative efficacy of several such methods has recently been evaluated (Greco & Hakala, 1979). Each method requires foreknowledge of the reaction mechanism and applies only to the mechanisms for which relationships were presented by the authors.

One of us showed earlier (Halfman & Nishida, 1972) that protein–ligand interactions may be indirectly quantified from measurements of any physical-chemical property which changes as a consequence of binding. The method has been used to quantify the binding of a variety of long chain alkyl compounds to a number of different proteins from binding-induced changes in intrinsic protein fluorescence (Halfman & Nishida, 1972) and from the effect of ligand binding on the hydrogen ion equilibria of the protein (Halfman & Steinhart,

1971). Results obtained in the latter cited studies were demonstrated to agree with results obtained by other established techniques. Bound and free ligand concentrations were determined from experimental data obtained at two or more protein concentrations, plotted as the measured response to binding on the ordinate and ratio of total ligand:total protein on the abscissa.

Enzyme activity is a direct consequence of substrate binding and likewise an influence on activity is a direct consequence of effector binding. We demonstrate in this report that the method cited above may also be used to quantify the binding of substrate (or effector) to enzyme. A hypothetical example is presented to illustrate the principle of the method, and the inhibition of fructose 1,6-bisphosphatase by AMP is analysed as an example of its application.

Materials and methods

Fructose 1,6-bisphosphatase was isolated from pig kidneys as described previously (Colombo & Marcus, 1973), except that a Whatman P11 phosphocellulose column (2 cm × 20 cm) was used instead of the Cellex P (BioRad) column (1.9 cm × 40 cm). Fructose 1,6-bisphosphatase activity was measured at 4°C by the formation of inorganic phosphate from fructose-1,6-bisphosphate. The assay mixture (1 ml) contained 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer (pH 7.5), 5.2 mM-MgSO₄, 0.11 mM-EDTA and AMP when indicated. The

enzyme was preincubated for 10 min at 4°C in this solution and the reaction was started by the addition of fructose-1,6-bisphosphate to a final concn. of 5 mM. Incubation was for 10 min at 4°C and the reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid. An aliquot of the supernatant fluid obtained after centrifugation was used to determine liberated inorganic phosphate in a final volume of 5 ml (Marcus, 1967). Enzyme dilutions were made in 50 mM-Tris/HCl (pH 8.0), containing 2 mM-MgSO₄ and 0.1 mM-EDTA.

A Wang model 2200 minicomputer was used to solve complex calculations and the associated drum plotter was employed for drawing figures.

Description and application of the method

The method is best described by analysing a hypothetical example. Consider an enzyme preparation at a concentration $E_t = 10^{-6}$ M and with K_m for substrate 10^{-8} M. Suppose K_m is unknown and is to be determined. E_t may also be determined, but, for the sake of brevity, we assume E_t is known. The method for measuring reaction velocity is such that E_t cannot be much below 10^{-8} M in order to obtain reasonably precise data. When E_t is not considerably less than K_m , a significant fraction of the total substrate, S_t , is bound. The relationship between bound substrate, [ES], and total reactant concentrations during the initial velocity time period in such cases has been shown (Strauss & Goldstein, 1943) to be:

$$[ES] = \frac{1}{2} \{ E_t + S_t + K_m - \sqrt{(E_t + S_t + K_m)^2 - 4E_t S_t} \} \quad (1)$$

and when $[ES]/E_t = v/V_{\max}$, it follows that:

$$v = \frac{V_{\max}}{2} \left\{ 1 + \frac{1}{E_t/K_m} \cdot \left(\frac{S_t}{K_m} + 1 \right) - \sqrt{\left[1 + \frac{1}{E_t/K_m} \left(\frac{S_t}{K_m} + 1 \right) \right]^2 - 4 \frac{S_t}{K_m} \cdot \frac{1}{E_t/K_m}} \right\} \quad (2)$$

or

$$v = \frac{V_{\max}}{2} \left\{ 1 + \frac{S_t}{E_t} + \frac{1}{E_t/K_m} - \sqrt{\left(1 + \frac{S_t}{E_t} + \frac{1}{E_t/K_m} \right)^2 - 4 \frac{S_t}{E_t}} \right\} \quad (3)$$

Eqn. (2) describes the relationship between initial reaction velocity (v = units of activity/ml of enzyme preparation) and total substrate and enzyme concentration relative to K_m . Reaction velocities which would be obtained with several different concentrations of the hypothetical enzyme preparation are represented in Fig. 1. The broken curve would be obtained if measurements could be made at sufficiently low enzyme concentration and would be independent of enzyme concentration ($E_t \ll K_m$). The lowest enzyme concentration employed (5×10^{-9} M, curve c) is seen not to be sufficiently low ($E_t =$

Fig. 1. Initial reaction velocities, v , predicted by eqn. (2) for a hypothetical enzyme preparation

$E_t = 10^{-6}$ M; $K_m = 10^{-8}$ M; v is in terms of units of activity/ml of enzyme preparation in the assay tube; $V_{\max}$ is assigned a value of 3.0 units/ml. The different curves result from different enzyme concentrations in the assay tube. For curves (a), (b), and (c), $E_t = 20$, 10, and 5 nM, respectively. The broken curve results when $E_t \ll K_m$.

$0.5 K_m$) to assume reliably that total substrate is equivalent to free substrate. Indeed, the observation that the relationship between reaction velocity and substrate concentration is dependent upon enzyme concentration demonstrates that the assumption is not valid.

Eqn. (3) describes the relationship between velocity and total substrate:enzyme ratio as a function of enzyme concentration relative to K_m . Bound and free substrate concentrations may be determined from data plotted in these terms, as illustrated in Fig. 2. In this particular case, velocity is related to the molar ratio of substrate bound to enzyme in a simple linear manner, i.e. $v/V_{\max} = [ES]/E_t$. Thus, any particular $[ES]/E_t$ corresponds to a particular velocity regardless of enzyme concentration. Also, any particular $[ES]/E_t$ is associated with a particular free substrate concentration $[S]$ regardless of enzyme concen-

Fig. 2. Initial velocity curves of Fig. 1, but with the abscissa in terms of S_t/E_t .

The broken curve results when $E_t \gg K_m$ and represents the stoichiometric relationship between v and S_t/E_t .

tration, as is evident from expressions based upon equilibrium considerations such as $[ES]/E_t = [S]/(K_m + [S])$, which applies in the case of this hypothetical example. Referring to Fig. 2, therefore, it is evident that at v_x there is a particular $([ES]/E_t)_x$ which is the same at each enzyme concentration represented by curves (a), (b) and (c). Since bound substrate is the difference between total and free, it follows that

$$([ES]/E_t)_x = (S_t/E_t)_b - [S]_x/E_{tb} = (S_t/E_t)_c - [S]_x/E_{tc} \quad (4)$$

where $(S_t/E_t)_b$ and $(S_t/E_t)_c$ are the total substrate:enzyme molar ratios at E_{tb} and E_{tc} , yielding $([ES]/E_t)_x$ and v_x . Thus:

$$[S]_x = \frac{(S_t/E_t)_c - (S_t/E_t)_b}{1/E_{tc} - 1/E_{tb}} \quad (5)$$

and

$$([ES]/E_t)_x = \frac{(S_t/E_t)_b - (S_t/E_t)_c \cdot E_{tc}/E_{tb}}{1 - E_{tc}/E_{tb}} \quad (6)$$

Eqns. (5) and (6) show how $[S]$ and $[ES]/E_t$, corresponding to particular values of v , may be determined from data obtained at two or more values of E_t and plotted in the manner of Fig. 2. The many corresponding values of v , $[S]$ and $[ES]/E_t$ so determined may then be analysed by any desired plotting technique.

For the sake of brevity the simplest possible case was analysed above to demonstrate the principle of the method. The method may, however, be applied to any enzyme-ligand interaction where any $[ES]/E_t$ is associated with a particular v and a particular $[S]$ regardless of enzyme concentration. It is not

necessary that velocity and ligand binding be related in a linear or even monotonic manner. There may be more than one $[ES]/E_t$ associated with a particular value of v , as might occur when there are two substrate binding sites and binding to the second site is weaker and inhibitory. The only case where the above requisite would not be realized is when reversible protein-protein subunit interactions occur which influence ligand binding.

The hypothetical example considered above illustrated the method for quantifying tight-binding substrates. The same analysis may be utilized for quantifying tight-binding inhibitors or activators. We used the method to analyse data on the inhibition of kidney fructose 1,6-bisphosphatase by AMP at 4°C.

It is well established that the allosteric AMP inhibition of fructose 1,6-bisphosphatase exhibits positive co-operativity and is temperature-dependent in such a manner that inhibition increases approx. 20-fold as temperature decreases from 30 to 2°C (Taketa & Pogell, 1965; Colombo *et al.*, 1972). One of us previously studied the inhibition of kidney fructose 1,6-bisphosphatase by AMP at 3°C (Colombo *et al.*, 1972) and obtained k_4 (AMP concn. at 50% inhibition) = 8 μM and α (co-operativity index) = 1.44. Slower reaction velocities at low temperatures require the use of higher enzyme concentrations to obtain sufficiently precise data. The use of higher enzyme concentrations in conjunction with increased affinity for AMP at lower temperatures suggested that the parameters we obtained earlier at low temperatures might be inaccurate, since a significant fraction of the total AMP may have been bound. The latter possibility might also account for the differences between the parameters we obtained and those reported earlier by another group of investigators (Taketa & Pogell, 1965) at 2°C ($k_4 = 5.5 \mu\text{M}$, $\alpha = 2.3$) for the rat liver enzyme. It is important to note that the latter investigators used a sufficiently sensitive method for measuring fructose 1,6-bisphosphatase activity so that the enzyme concentration employed in their study was sufficiently low to be negligible [1/500 dilution of tissue in the assay tube with E_t approx. 8–16 μM in the tissue (Riou *et al.*, 1977)].

We re-investigated the problem by determining inhibition curves of pig kidney fructose 1,6-bisphosphatase by AMP at 4°C at different enzyme concentrations over a 10-fold range. A different degree of inhibition was observed in each case, indicating that a significant fraction of the inhibitor is bound at each enzyme concentration. Plots suggested by other investigators (Dixon, 1972; Henderson, 1973) are not appropriate for analysing the data because of the co-operative interaction of AMP with fructose 1,6-bisphosphatase. However, the results can be analysed by the method presented

Table 1. Calculation of bound and free inhibitor concentrations from data of Fig. 3

Eqn. (5) was used to calculate [I]; eqn. (6) was used to calculate [EI]/D. D is enzyme dilution, i.e., ml of enzyme preparation/ml of assay volume.

v	I _t /D (mM) from curve:			[I] (μM) calculated from:			[EI]/D (μM) calculated from:		
	(a)	(b)	(c)	(a,b)	(a,c)	(b,c)	(a,b)	(a,c)	(b,c)
1.00	0	0	0	—	—	—	—	—	—
0.90	0.030	0.080	0.180	1.67	1.67	1.67	13.3	13.3	13.3
0.80	0.055	0.130	0.285	2.50	2.56	2.58	30.0	29.4	26.7
0.70	0.075	0.180	0.385	3.50	3.44	3.42	40.0	40.6	43.3
0.60	0.100	0.230	0.490	4.33	4.33	4.33	56.7	56.7	56.7
0.50	0.120	0.280	0.600	5.33	5.33	5.33	66.7	66.7	66.7
0.40	0.145	0.340	0.725	6.50	6.44	6.42	80.0	80.6	83.3
0.30	0.175	0.410	0.880	7.83	7.83	7.83	96.7	96.7	96.7
0.20	0.215	0.525	1.150	10.33	10.39	10.42	112.0	111.0	108.0
0.10	0.275	0.725	1.630	15.00	15.06	15.08	125.0	124.0	122.0

Fig. 3. Inhibition of fructose 1,6-bisphosphatase activity by AMP

V_0 is the initial reaction velocity in the absence of AMP. For curves (a), (b), and (c), $D = 0.01$, 0.0025 and 0.001 respectively (D = enzyme dilution, i.e., volume of enzyme preparation per total assay volume). The solid line curves are determined by eqn. (7) with $k_i = 5.3 \mu\text{M}$, $E_t = D \times 138 \mu\text{M}$ and $\alpha = 2.05$. The broken curve is for $E_t \gg k_i$.

in this paper. Data are shown in Fig. 3 in terms of AMP concentration/ml of enzyme on the abscissa. From the curves, values for free, [I], and bound, [EI], AMP corresponding to particular velocities were calculated from eqns. (5) and (6), respectively, with the appropriate substitution of inhibitor for substrate. Results are presented in Table 1, which also serves to illustrate in greater detail the manner by which results are calculated. The computed stoichiometric curve (broken line, Fig. 3) gives $E_t = 138 \mu\text{M}$, which is in excellent agreement with that determined on the basis of the dry weight and an M_r of 140 000 for the purified enzyme (Marcus & Hubert, 1968). A Hill plot of the results yields a co-operativity index, α , of 2.05 and a 'midpoint k' ,

k_i , of $5.3 \mu\text{M}$. The relationship between velocity and inhibitor concentration described by the Hill plot is:

$$[EI]/E_t = 1 - v/V_0 = \frac{([I]/k_i)^\alpha}{1 + ([I]/k_i)^\alpha} \quad (7)$$

The solid line curves in Fig. 3 are determined by eqn. (7) with the above obtained values for the parameters E_t , k_i and α . Agreement with the experimental data is excellent. The parameters are also in good agreement with those reported for the rat liver enzyme (Taketa & Pogell, 1965).

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