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CALCULATION OF THE BIMOLECULAR RATE CONSTANT FOR "IRREVERSIBLE" ENZYME INHIBITORS

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SUMMARY

A method is developed that can be used to calculate the rate constant for the association of an irreversible inhibitor with the active site of an enzyme. An example of this type inhibition (the *p*-chloromercuribenzoate-inhibition of the ATP-phosphohydrolase activity of myosin, EC 3.6.1.3) is used to illustrate the procedure. The bimolecular rate constant for the *p*-chloromercuribenzoate–myosin reaction is calculated from an independent experiment and the values obtained by the two methods are shown to be essentially the same.

The calculation of the bimolecular rate constant for single-step processes is a well-known, straightforward application of chemical kinetics for most chemical reactions. In most cases the constant can be obtained directly from measurements of the initial rates obtained at known initial concentrations of the two reactants involved. Alternatively, the differential equation for the reaction can be written down, integrated, and the bimolecular rate constant obtained from the analysis of the time-dependence of the reactant concentrations.

Since the concentrations of the reactants in bimolecular reactions between enzymes and irreversible inhibitors usually can not be measured directly, the direct measurement of the time-dependent concentration of the reactants, or of the initial rates, is often not possible. However, it is still possible to exploit the usual methods of chemical kinetics to obtain the bimolecular rate constant for reactions between enzymes and irreversible inhibitors.

There are many inhibitors of enzyme-catalyzed reactions that may be correctly classified as essentially irreversible. The irreversible organophosphorus inhibitors such as diisopropylphosphofluoridate constitute one group of irreversible enzyme inhibitors and the irreversible –SH-binding inhibitors such as PCMB constitute another such group. These types of enzyme inhibitors play an important role in the study of enzyme mechanisms. Consequently, the characterization of the rate constants for their reactions with enzymes is of the utmost importance.

In many cases in which irreversible inhibitors are bound to the active site of an

Abbreviation: PCMB, *p*-chloromercuribenzoate.

enzyme, the rate of the reaction of the inhibitor with the enzyme is, at the low inhibitor concentrations used, relatively slow compared to the rate of the substrate combination. Thus, the rate of the inhibition reaction described in Eqn. 1 is slow compared to the rate of association of a substrate with the enzyme.

$$I + E \xrightarrow{k_0} EI \tag{1}$$

The differential equation for the reaction described in Eqn. 1 is,

$$-(\dot{I}) = -(\dot{E}) = k_0(E)\,(I) \tag{2}$$

where, under all conditions the initial rates, $(\dot{I})_0$ and $(\dot{E})_0$, are equal to $-k_0(E_0)(I_0)$.

If a large quantity of substrate is added to the mixture containing the inhibitor and the free enzyme after different amounts of EI have been formed, the free enzyme concentration will be rapidly reduced to a very low value. Thus, it would be expected, and it is commonly observed, that the rate of a reaction between the active site of an enzyme and an inhibitor is reduced when the substrate is present.

If an irreversible inhibitor is incubated with an enzyme for a short time, t, the enzyme will be distributed in two forms: the free enzyme and the EI complex. The free enzyme concentration will be $(E_0)-(EI)$, and the concentration of the EI complex will be $(I_0)-(I)$, where (E_0) is the total enzyme concentration and (I_0) is the total inhibitor concentration, both added initially. Substitution into Eqn. 2 gives:

$$-(\dot{E}) = -(\dot{I}) = k_0[(E_0) - (I_0) + (I)]\,(I) \tag{3}$$

If it is assumed that the free enzyme concentration in Eqn. 2 is approximately equal to the initial concentration of the enzyme–substrate complex formed immediately after addition of a large quantity of substrate to the mixture in which the enzyme–inhibitor reaction is occurring, it will be possible to estimate the concentration of the free enzyme at any time of substrate addition, t, from Eqn. 4:

$$(E)_t \quad (\text{before } S \text{ added}) = (ES)_t \quad (\text{after } S \text{ added}) = v_t/k_2 \tag{4}$$

where k_2 is the rate constant for the breakdown of the ES complex to free enzyme and products, and v_t is the activity of the enzyme remaining at time t. Since it is not always possible to measure the rate at time t, it is often necessary to extrapolate a plot of the measured rate at times after t, during which the inhibitor is displacing the substrate from the active site, back to t.

It should be possible to use experimental data involving v_t and t to obtain the bimolecular rate constant for the irreversible combination of an inhibitor with the active site of an enzyme. The expected relationship between v_t and t can be obtained directly from Eqn. 2 and 4. Rearranging slightly, we obtain:

$$t = \int_{(I_0)}^{(I)} \frac{\mathrm{d}(I)}{\{k_0[(I_0) - (E_0)] - k_0(I)\}\,(I)} \tag{5}$$

Eqn. 5 can be integrated to give:

$$t = \frac{1}{k_0 [(E_0) - (I_0)]} \ln \left\{ \frac{(E_0) - (I_0) + (I)}{(I)} \frac{(I_0)}{(E_0)} \right\} \quad (6)$$

Recalling that $(E_0) - (I_0) + (I) = v_t/k_2$ we may write:

$$t = \frac{1}{k_0 [(E_0) - (I_0)]} \ln \left\{ \frac{v_t}{v_t + k_2 [(I_0) - (E_0)]} \frac{(I_0)}{(E_0)} \right\} \quad (7)$$

Eqn. 7 illustrates that a plot of $\ln \frac{v_t}{v_t + k_2 [(I_0) - (E_0)]}$ *versus* t should be linear with a slope equal to $\frac{1}{k_0 [(E_0) - (I_0)]}$ and an intercept equal to $(E_0)/(I_0)$ on the ln axis. Since k_2 and (E_0) are generally known from kinetic studies conducted in the absence of the inhibitor, and since (I_0) should be known from the amount of inhibitor added initially, it is possible to calculate k_0, the bimolecular rate constant, from the slopes of these plots.

Fig. 1 is a linear plot of the suggested ln function of v_t *versus* t for a theoretical

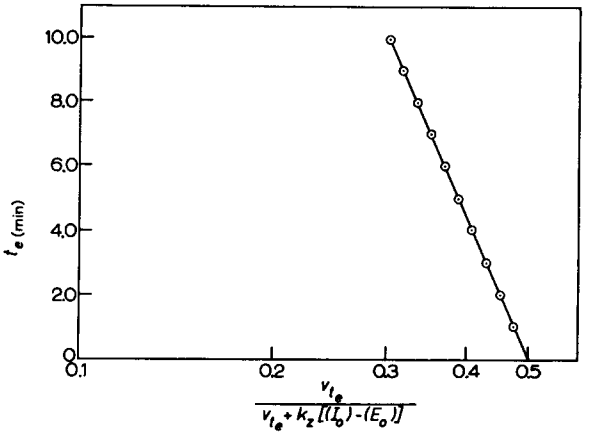

Fig. 1. Theoretical plot of the log function of v_t suggested in Eqn. 7 *versus* t for an enzyme that is irreversibly inhibited by an appropriate reactant. (E_0) is $1.0 \cdot 10^{-7}$ M, (I_0) is $2.0 \cdot 10^{-7}$ M, k_2 is 100 min^{-1}, and k_0, obtained from the slope $\frac{2.303}{k_0 [(E_0) - (I_0)]}$, is $5 \cdot 10^5$ M^{-1} min^{-1}.

case in which (I_0) is $2.0 \cdot 10^{-7}$ M, (E_0) is $1.0 \cdot 10^{-7}$ M, and k_2 is 100 min^{-1}. The value of k_0, obtained from the slope of this plot is:

$$k_0 = \frac{2.303}{\text{slope} \times [(E_0) - (I_0)]} = 5.0 \cdot 10^5 \text{ M}^{-1} \text{ min}^{-1} \quad (8)$$

GILMOUR AND GILLERT[1] have recently reported data that describe the time course of the reaction of PCMB with myosin. In these experiments the total amount of PCMB added was 6.3 moles/10^5 g myosin and the total myosin concentration was

$2.4 \cdot 10^{-2}$ g/l. However, this example of an irreversible inhibitor–enzyme interaction is complicated by the fact that about 60% of the PCMB-binding sites bind PCMB very rapidly and without inhibition before the remaining 1.9 moles PCMB per 10^5 g myosin react at all. Then the remaining 1.9 moles PCMB per 10^5 g myosin react with the myosin, leaving 1.2 moles PCMB binding sites per 10^5 g myosin unreacted (because an excess of PCMB was not added initially).

Since the molecular weight of myosin is not settled, several values between $4.2 \cdot 10^5$ g/mole and $6.2 \cdot 10^5$ g/mole were used to obtain the molar concentration of the myosin. The lower values below $4.8 \cdot 10^5$ g/mole did not fit the data reported by GILMOUR AND GILLERT very well because the log term in Eqn. 7 became negative even after appropriate corrections for the expected inactivation of myosin in the absence of PCMB were made. The molecular weight range between $4.8 \cdot 10^5$ and $6.2 \cdot 10^5$ g/mole gave indistinguishable results for the purposes of the type plot suggested in this paper, and for purposes of calculating k_0 in Eqn. 1.

Thus, if we assume a molecular weight of $4.8 \cdot 10^5$ g/mole the myosin concentration is $5.0 \cdot 10^{-8}$ M. On the basis of this molecular weight we find from GILMOUR AND GILLERT's paper that there are about 36 —SH groups per mole of myosin. However, 21 of these 36 PCMB-binding sites will react rapidly with the initial $1.5 \cdot 10^{-6}$ M PCMB with no inhibition. This will leave $4.6 \cdot 10^{-7}$ M PCMB to react with the remaining 15 —SH groups. If we assume that 1/15 of this PCMB will react at the active site of myosin, the effective PCMB concentration is $3.1 \cdot 10^{-8}$ M as far as the inhibition of the enzymic activity is concerned.

Similarly, if we assume a molecular weight of $6.2 \cdot 10^5$ g/mole, the myosin concentration is $3.9 \cdot 10^{-8}$ M. On the basis of this molecular weight there are about 46 –SH groups per mole of myosin. In this case 27 of the 46 PCMB-binding sites will react rapidly with the initial $1.5 \cdot 10^{-6}$ M PCMB, with no inhibition. This will still leave $4.6 \cdot 10^{-7}$ M PCMB to react with the remaining 19 –SH sites. If we now assume that 1/19 of this PCMB will react at the –SH group at the active site of myosin, the effective PCMB concentration is $2.4 \cdot 10^{-8}$ M.

Fig. 2 is the suggested log plot of the remaining ATPase activity of myosin *versus* the time of incubation with PCMB calculated from the data reported by

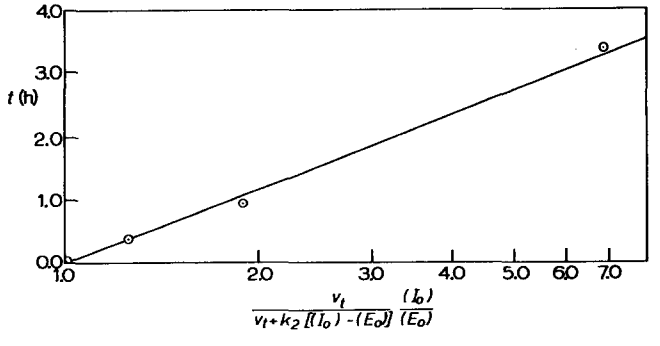

Fig. 2. Actual plot of the log function of v_t suggested in Eqn. 7 *versus* t for the irreversible inhibition of the ATPase activity of myosin by *p*-chloromercuribenzoate. (Data taken from the paper by GILMOUR AND GILLERT[1].) Slope: $\frac{2.303}{k_0[(E_0)-(I_0)]} = -4.7$; $k_0 = 3.6 \cdot 10^4$ M–SH^{-1} min^{-1}.

GILMOUR AND GILLERT[1], after appropriate corrections described in this text have been made for the initial, rapid, non-inhibitory reaction with PCMB. It can be seen that this plot is linear. The value of k_0 obtained directly from the slope is $3.6 \cdot 10^4$ M slow-binding –SH sites^{-1} min^{-1}.

The value of this rate constant can be estimated independently from measurements of the rate of PCMB-binding to the –SH groups of myosin obtained from the increase in absorbancy at 250 mμ. This data is also reported by GILMOUR AND GILLERT[1]. After the first 4.8 moles of PCMB per 10^5 g myosin have reacted with the fast-reacting PCMB-binding sites of myosin, an additional 1.2 moles of PCMB per 10^5 g myosin is added to the remaining 2.7 moles of PCMB-binding sites per 10^5 g myosin, the total myosin concentration being 0.2 g/l. The increase in A_{250} estimated from GILMOUR AND GILLERT's data is about 0.06 A_{250}/min. If we assume that 1/15 of this rate is due to the binding of PCMB at the active site of myosin (as before, the effective PCMB concentration is 1/15 the total PCMB concentration) and combine this result with the extinction coefficient for a single PCMB – –SH group interaction, the calculated rate of PCMB combination is $6 \cdot 10^{-7}$ M/min. Since the PCMB concentration is $2.4 \cdot 10^{-6}$ M, and the total remaining concentration of myosin –SH sites is $5.4 \cdot 10^{-6}$ M, k_0 can be obtained by conventional methods from

$$k_0 = \frac{\frac{\mathrm{d}\ (\text{PCMB})\ \text{at active site}}{\mathrm{d}t}}{(\text{PCMB}) \times (\text{slow myosin–SH sites})} \tag{9}$$

$$k_0 = \frac{6 \cdot 10^{-7}\ \text{M PCMB/min}}{2.4 \cdot 10^{-6}\ \text{M PCMB} \times 5.4 \cdot 10^{-6}\ \text{M–SH}} = 4.6 \cdot 10^4 \tag{10}$$

The value for k_0 obtained from Eqn. 10 clearly agrees quite well with the value ($3.6 \cdot 10^4$) obtained from Fig. 2.

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