EVALUATION OF PARTIAL RATE CONSTANTS OF COMPETITIVE INHIBITION BY ANALYSIS OF NONLINEAR KINETICS

Gabriel SCHREIBER, Asher GOPHER and Menachem GUTMAN
Biochemistry, G. S. Wise Faculty of Life Sciences, Tel-Aviv University, Israel

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1. Introduction

The rate of binding of small ligands to proteins is usually measured by rapid reaction techniques and the detection relies on changes in physical properties of either enzyme or ligand. As physical changes are not specific, and small in magnitude, substrate concentrations of highly purified proteins are recommended. The same kind of information can be obtained using a methodology which avoids the need of high concentration of pure enzyme and, in some cases, of sophisticated instrumentation with high time resolution. The observed parameter in this method is the change in the rate of catalysis which is induced by the addition of a ligand reacting with a site which is already saturated by the substrate. The higher is the substrate concentration, the smaller is the free site concentration and the slower is the rate of the reaction with the ligand. The change in the rate of catalysis with time is subjected to mathematical presentation leading to equation suitable for calculating the rates of association and dissociation of the ligand.

The example given below, the competitive inhibition of succinate dehydrogenase by malonate, demonstrates the advantages of the technique; the rate constants of association and dissociation of malonate are calculated using a catalytic amount of unpurified, membrane bound enzyme. The detection of the progress of the reaction is the usual method for monitoring the enzymic activity and finally, the rate constant of association 35×10^3 M^{-1} min^{-1} is measured over 1–3 min observation periods with no need of stopped flow instrumentation.

Abbreviations: PMS, phenazine methosulphate; DCPIP, dichlorophenol indophenol

2. Materials and methods

2.1. *ETP_H*

These were prepared according to [1] using beef heart mitochondria [2].

2.2. *Succinate dehydrogenase*

This was activated by incubating ETP_H (10 mg/ml) with 1 mM malonate (30 min, 30°C), the ETP_H were diluted, spun down and resuspended to 0.2 mg/ml in 0.18 M sucrose, 50 mM Tris acetate, 5 mM $MgSO_4$ (pH 7.4) at 5°C.

2.3. *Enzymic activity*

This was initiated by addition of succinate (15–50 mM) together with PMS (1 mM) and DCPIP (50 μM) (all precooled to 5°C), and reduction for 30–50 s of DCPIP measured at 600 nm after initiation of the reaction. Malonate (0.5–8 mM) was added using a stirring rod. The monitoring of the reaction was followed until linear rate of catalysis was established (V_∞), (4–5 min, at 5°C).

2.4. *Kinetic analysis*

This was carried by calculating the slope of the observed lines at various time points after addition of malonate and drawing log ($V_t - V_\infty$) versus time.

3. Theory, results and discussion

The kinetic equations for nonlinear catalysis can be written for various mechanisms. In this case we shall consider interaction of competitive inhibitor (I) with enzyme (E) and substrate (S). Given that other substrates (such as oxidant in our case) are saturating, all steps following formation of ES can be encompassed by k_{cat}. The equations to be considered are:

$$EI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E + I \qquad (1)$$

$$E + S \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (2)$$

We shall consider a case where the inhibitor, I, is added to the enzyme during catalysis. As the concentration of free enzyme is very small ($S >> K_M$), the reaction between E and I is going to be rather slow. Thus, during the time of this slow reaction many ES complexes will follow their normal catalytic cycle.

As EI concentration increases with time, the rate of catalysis will slow down. This will be observed as decelerated catalysis (see fig.1). Regarding the saturating concentrations of both ligands, we can employ steady state approximation for evaluating the kinetic equations describing the decelerated catalysis. Using the notations in eq. (1) and eq. (2):

$$\frac{dE}{dt} = (EI)\,k_1 - (E)\,(I)\,k_{-1}$$

$$+ (ES)\,(k_2 + k_{cat}) - (E)\,(S)\,k_{-2} = 0 \qquad (3)$$

From this equation we express (ES) as a function of (E), (S), (I) and EI):

$$(ES) = \frac{(E)\,(I)\cdot k_{-1} + (E)\,(S)k_{-2} + (EI)k_1}{k_2 + k_{cat}} \qquad (4)$$

This term is introduced into the detailed balance equation:

$$Et = E + EI + ES$$

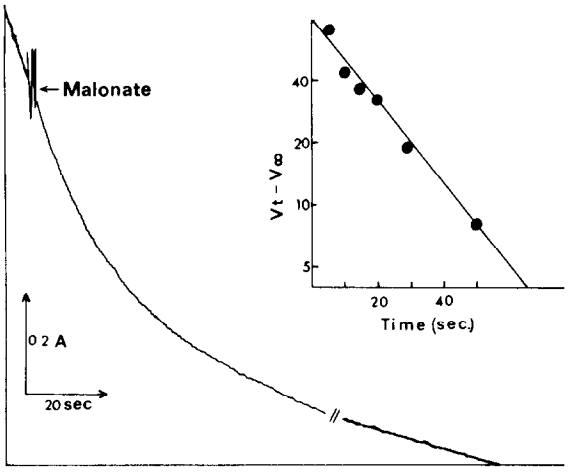

Fig.1. Time curve of the effect of malonate on the rate of succinate oxidation. Succinate dehydrogenase activity was measured as in section 2. The reaction was initiated by addition of PMS (1 mM) and DCPIP (50 μM) to 1.08 mg ETP_H in 30 mM succinate, 0.18 M sucrose, 50 mM Tris–acetate, 5 mM $MgSO_4$, 2 mM KCN, pH 7.4, at 5°C. The reaction was followed (at 600 nm) for 30 s then when indicated malonate was added to 1.5 mM and the monitoring of the reaction was continued till linear rate was observed. Insert: Semilogarithmic presentation of the curvature of the line.

and after rearrangement we can express (E) as function of (Et) and (EI):

$$(E) = \frac{(Et) - (EI)\left(1 - \dfrac{k_1}{k_2 + k_{cat}}\right)}{1 + \dfrac{(I)\cdot k_{-1}}{k_2 + k_{cat}} + \dfrac{(S)\cdot k_{-2}}{k_2 + k_{cat}}} \qquad (5)$$

the last term in the denominator is equal to $\frac{(S)}{K_m}$ and shall be used as such for the rest of the treatment.

The progress of the reaction is the increase in EI concentration, which is given by the differential equation:

$$\frac{d(EI)}{dt} = (E)\,(I)\,k_{-1} - (EI)\,k_1 \qquad (6)$$

In this equation we substitute (E) as given in eq. 5.

Consequently the differential equation becomes a function of (Et) (which is constant) and only one variable (EI). After rearrangement to isolate all terms associated with (EI) we obtain:

$$\frac{d(EI)}{dt} = \frac{(E_t)\,(I)\,k_{-1}}{1 + \dfrac{(I)\,k_{-1}}{k_2 + k_{cat}} + \dfrac{(S)}{K_m}}$$

$$+ (EI)\left(k_1 + \frac{(I)\,k_{-1}\left(1 - \dfrac{k_1}{k_2 + k_{cat}}\right)}{1 + \dfrac{(I)^{k_{-1}}}{k_2 + k_{cat}} + \dfrac{(S)}{K_m}}\right) \qquad (7)$$

This equation can be simplified by expressing it as $\frac{d(EI)}{dt} = A - B(EI)$ where A and B are the first and second terms in eq. (7). After integration of the simplified equations between limits $0 \rightarrow t$ we obtain the expression of (EI) at time t, $(EI)_t$, as a function of its initial concentration, $(EI)_o$, and t:

$$(EI)_t = \frac{A - [A - B(EI)_o]\,e^{-Bt}}{B} \qquad (8)$$

This expression, is inconvenient to work with. We can easily measure $\frac{d(EI)}{dt}$ but not the actual concentration of EI, thus we substitute (EI) from eq. (8) into eq. (6). After certain algebric steps we end with eq. (9):

$$\frac{d(EI)}{dt} = [A - B(EI)_o]e^{-Bt} \qquad (9)$$

The logarithmic form of eq. (9) is even simpler:

$$\ln \frac{d(EI)}{dt} = \ln[A - B(EI)_o] - Bt \qquad (10)$$

Drawing the logarithm of the change in the rate of catalysis $\frac{\Delta(EI)}{\Delta t}$ versus time will yield a straight line with a slope (observed rate constant of deceleration) $k_{obs} = B$. (see insert to fig.1). Writing the full expression of B we obtain:

$$k_{obs} = k_1 + \frac{(I)^{k_{-1}}\left(1 - \dfrac{k_1}{k_2 + k_{cat}}\right)}{1 + \dfrac{(I)^{k_{-1}}}{k_2 + k_{cat}} + \dfrac{(S)}{K_m}} \qquad (11)$$

In this, eq. (1) is multiplied in nominator by $(1 - \frac{k_1}{k_2 + k_{cat}})$. As the inhibitor dissociation is much slower than the sum of the rates of free enzyme formation both by dissociation of substrate plus the catalytic turnover, we can approximate:

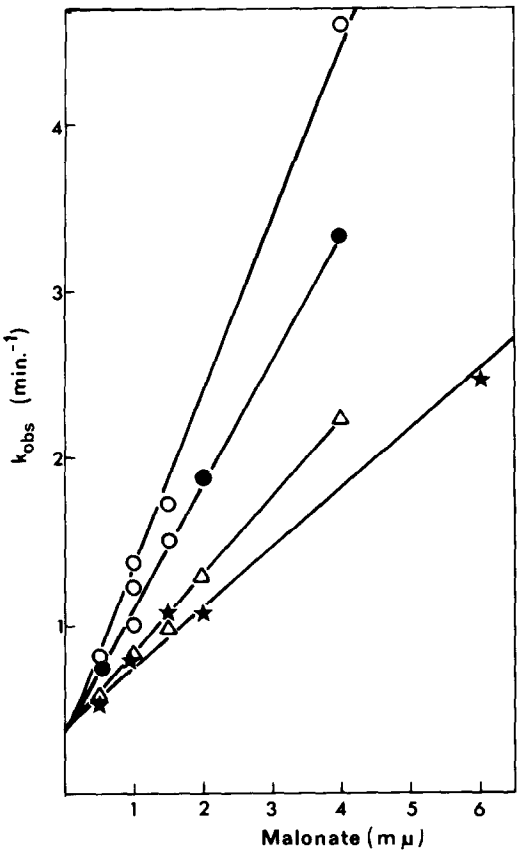

Fig.2. The dependence of the apparent rate of deceleration on malonate concentrations. The experiment described in fig.1 was repeated with the indicated succinate and malonate concentrations. The lines were analyzed as in insert to fig.1. The observed rate constants are drawn with respect to malonate concentrations. Succinate concentrations were: (○) 15 mM; (●) 20 mM; (△) 30 mM; (★) 50 mM.

Table 1
Calculation of the rate constants of association and dissociation of malonate from the competitive site of succinate dehydrogenase

Succinate (mM)	k_1 (min^{-1})	k'_{-1} [b] (M^{-1} min^{-1})	$(1 + \frac{(S)}{K_m})$ [a]	k_{-1} (M^{-1} min^{-1})
15	0.233	1084	34.3	37.6×10^3
20	0.341	755	45.4	34.3×10^3
30	0.336	472	67.6	32.9×10^3
50	0.479	306	112.0	34.3×10^3

[a] K_m = 450 μM (5°C), pH 7.4. A.G., M.G., unpublished results
[b] k'_{-1} was obtained from the slopes of the lines in fig.3

$$k'_{-1} = k_{-1}/1 + \frac{(S)}{K_m}, \text{ eq. (12)}$$

$k_1 = 0.347 \pm 0.101$ min^{-1}
$k_{-1} = (34.8 \pm 2)\ 10^3$ M^{-1} min^{-1}
$K_i = 10.1 \pm 3.4$ μM

Data taken from fig.2

$$1 >> \frac{k_1}{k_2 + k_{cat}}.$$

In the denominator, we find the ratio $\frac{(I)\,k_{-1}}{k_2 + k_{cat}}$. This is the ratio of the rate of conversion of E to EI versus the rate of E formation from ES both by dissociation and catalytic cycle. If this ratio is small, (and it can be made deliberately small by controlling the concentration of I added to the experiments) eq. (11) becomes a simple expression

$$k_{obs} = k_1 + k_{-1} \frac{(I)}{1 + \frac{(S)}{K_m}} \qquad (12)$$

The observed rate constant of deceleration is a linear function of inhibitor concentration. It intercepts the Y axis at k_1, the rate constant of EI dissociation, and the slope of the line is a function of S/K_m. As seen in fig.2, k_{obs} measured with different concentrations of succinate and malonate, intercept the Y axis at a single point $k_1 = 0.347 \pm 0.1$ min^{-1}. The rate of association, calculated from the slopes of the lines (table 1) $k_{-1} = (34.8 \pm 2)\ 10^3$ M^{-1} min^{-1}. The ratio $k_1/k_{-1} = 10$ μM is essentially identical to $K_i = 7 \pm 3$ μM as measured by Dixon plots.

The case we presented exemplifies the ease by which nonlinear kinetics can be used for measuring rate of reactions which otherwise call for sophisticated instrumentation and large supply of purified enzyme. Nonlinear rate equations can be written for other kinetic mechanisms, such as protein conformation in chloroplast's ATPase (M.G., Carmeli, Lifshitz and G.S., to be published).

References

[1] Hansen, M. and Smith, L. S. (1974) Biochim. Biophys. Acta 81, 214–224.
[2] Ringler, R. L., Minakami, S. and Singer, T. P. (1963) J. Biol. Chem. 238, 801–810.