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DETERMINATION OF THE BINDING CONSTANT OF LIGAND TO PROTEIN BY THERMAL INACTIVATION TECHNIQUE

EFFECT OF TWO BINDING SITES

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Summary

Theoretical considerations on the determining the binding constants (π) of ligands to proteins were carried out. Whereas for a one-subunit protein the relationship between thermal inactivation rates and ligand concentration there is a simple linear function, for a protein with two subunits, a second-order relationship is derived. If the theory for one-subunit proteins is applied to multi-subunit proteins, the derived values of π tend to be lower than the real binding constants. A method of determining the ligand binding constant for a two-subunit protein is described.

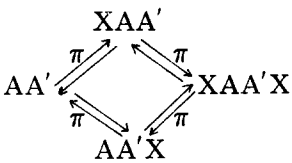
Introduction

The protection of enzymes by ligands against thermal inactivation can be used in determining the ligand binding constants. Theoretical considerations on this kind of determination have been published by several authors [1—6]. In all cases the basic assumption was the same: one protecting ligand is bound to one protein molecule. However, because most enzymes are composed of subunits and thus have several binding sites, the theory formed for one-ligand binding is not necessarily valid generally. Citri et al. [7] suggested a Hill-equation-type equation for the multi-ligand case. Its strict assumptions make, however, its applications to most cases of multi-ligand binding impossible. The case of protection in which two different ligands are bound to the protein, was considered by Südi [3] and Baykov and Avaeva [6].

In this paper the protection of a two-subunit enzyme against thermal inactivation is examined, and the binding constants derived by using the simple theory are proved to be apparently incorrect.

Theory

Let us assume that on a two-subunit enzyme each subunit has a binding site for a ligand and that no co-operativity exists in the ligand binding (neither positive nor negative). The case is described by Scheme 1, from which Eqn. 1 follows:



Scheme 1

$$\pi = \frac{[AA'][X]}{[AA'X]} = \frac{[AA'][X]}{[XAA']} = \frac{[AA'X][X]}{[XAA'X]} \tag{1}$$

where AA′ is the protein with two subunits, X is the ligand and π is the binding constant. Eqns. 2 and 3 are derived for the rate of inactivation, v, and from these, Eqns. 4 and 5 are further derived:

$$v = k_0[AA'] + 2k_1\frac{x}{\pi}[AA'] + k_2\left(\frac{x}{\pi}\right)^2[AA'] \tag{2}$$

where $x = [X]$ and k_i represents the reaction velocity constant of the inactivation of the complex $(AA')X_i$.

$$v = k([AA'] + 2\frac{x}{\pi}[AA'] + \left(\frac{x}{\pi}\right)^2[AA']) \tag{3}$$

where k is the apparent velocity constant of inactivation for the total enzyme.

$$k = \frac{k_0 + 2k_1\frac{x}{\pi} + k_2\left(\frac{x}{\pi}\right)^2}{\left(1 + \frac{x}{\pi}\right)^2} \tag{4}$$

$$k_0 - k = \frac{2(k_0 - k_1)\frac{x}{\pi} + (k_0 - k_2)\left(\frac{x}{\pi}\right)^2}{\left(1 + \frac{x}{\pi}\right)^2} = \frac{2M\frac{x}{\pi} + N\left(\frac{x}{\pi}\right)^2}{\left(1 + \frac{x}{\pi}\right)^2} \tag{5}$$

where $M = k_0 - k_1$, and $N = k_0 - k_2$

A consideration based on a similar principle gives the equation (6) for a four-subunit enzyme:

$$k = \frac{k_0 + 4k_1\frac{x}{\pi} + 6k_2\left(\frac{x}{\pi}\right)^2 + 4k_3\left(\frac{x}{\pi}\right)^3 + k_4\left(\frac{x}{\pi}\right)^4}{\left(1 + \frac{x}{\pi}\right)^4} \tag{6}$$

and generally:

$$k = \left[\sum_{i=0}^{n} \binom{n}{i} k_i \left(\frac{x}{\pi}\right)^i\right] \Big/ \left(1 + \frac{x}{\pi}\right)^n \tag{7}$$

In most cases the analysis of protection of individual enzymes has been carried out by using the expressions $1/(k_0 - k) = f(1/x)$ [3,5], $(k_0 - k)/x = f(x)$ [4,6] or $(k_0 - k) = f(k_0 - k)/x)$ [5]. These give a straight line ina one-subunit case, whereas a second-order plot is derived in a two-subunit case. In the following considerations the general form of second-order equations was used:

$$AX^2 + 2BXY + CY^2 + 2DX + 2EY + F = 0 \tag{8}$$

The tangent of the curve at point (X_0, Y_0) is then

$$(Y - Y_0) = -\frac{AX_0 + BY_0 + D}{BX_0 + CY_0 + E}(X - X_0) \tag{9}$$

The asymptotes of a hyperbola are parallel with the pair of lines: $AX^2 + 2BXY + CY^2 = 0$ and the intercept of the asymptotes then coincides with that of the lines $AX + BY + D = 0$ and $BX + CY + E = 0$.

(a) Double reciprocal plot,

$$\frac{1}{k_0 - k} = f\left(\frac{1}{x}\right)$$

By setting $\frac{1}{k_0 - k} = Y$ and $\frac{1}{x} = X$ eqn. (5) gives:

$$\pi^2 X^2 - 2M\pi XY + 2\pi X - NY + 1 = 0 \tag{10}$$

This represents an hyperbola, with an intercept on the Y-axis at $Y = 1/N$ and with an asymptote:

$$Y = \frac{\pi}{2M} X + \frac{4M - N}{4M^2} \tag{11}$$

(b) $(k_0 - k) = f\left(\frac{k_0 - k}{x}\right)$.

By setting $k_0 - k = Y$ and $\frac{k_0 - k}{x} = X$

the following equation is derived:

$$\pi^2 X^2 + 2\pi XY + Y^2 - 2M\pi X - NY = 0 \tag{12}$$

This represents a parabola with intercepts on X-axis at $X = 2M/\pi$ and on Y-axis at $Y = N$. The slope of the axis of the parabola is $-\pi$. The slope of the tangent at point $((2M + N)/4\pi, (2M + N)/4)$, where $x = \pi$, is $N\pi/2M$.

(c) $\frac{k_0 - k}{x} = f(k)$

By setting $\frac{k_0 - k}{x} = Y$ Eqn. 4 becomes:

$$k^2 - 2\pi k Y + \pi^2 Y^2 - (k_0 + k_2)k + 2\pi k_1 Y + k_0 k_2 = 0 \tag{13}$$

This represents a parabola with an axis slope of $-1/\pi$.

(d) $\frac{x}{k_0 - k} = f(x)$

Also this expression gives a straight line in the one-subunit case according to the following equation:

$$\frac{x}{k_0 - k} = \frac{1}{k_0 - k_1} x + \frac{\pi}{k_0 - k_1} \tag{14}$$

In the two-subunit case Eqn. (5) becomes

$$X^2 - NXY + 2\pi X - 2M\pi Y + \pi^2 = 0 \tag{15}$$

where $X = x$ and $Y = x/(k_0 - k)$. This represents an hyperbola with an intercept on Y-axis at $Y = \pi/2M$ and with an asymptote

$$Y = \frac{1}{N} X + \frac{2\pi(N - M)}{N^2} \tag{16}$$

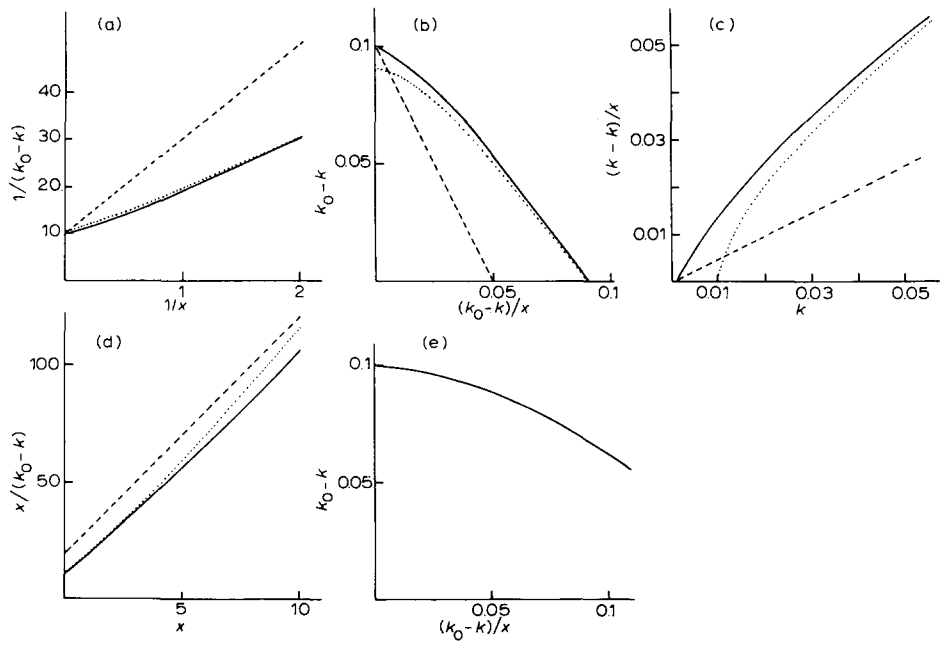


Fig. 1. Theoretical plots for differential thermal inactivation of an enzyme consisting of two subunits. The eaquations for each plot are discussed in the text. (a)—(d): The values of the parameters used in computations were as follows: $\pi = 2$ and $k_0 = 0.1$ in each curve and $k_1 = 0.01$ and $k_2 = 0.001$ (———); $k_1 = 0.01$ and $k_2 = 0.01$ (· · · · · ·); $k_1 = 0.05$ and $k_2 = 0.001$ (- - - - - -). (e) Curve $k_0 - k = f(k_0 - k)/x$ for a four-subunit case. The parameters were as follows: $\pi = 2$, $k_0 = 0.1$, $k_1 = 0.01$, $k_2 = k_3 = k_4 = 0.001$.

Fig. 1 presents theoretical curves for all four cases with three sets of k_i values. $k_0 = 0.1$, $k_1 = 0.01$, $k_2 = 0.001$ represents a case where the first ligand already causes a good protection which is further improved by the second. When $k_0 = 0.1$, $k_1 = 0.01$ and $k_2 = 0.01$ the second ligand does not increase the protection caused by the first, and when $k_0 = 0.1$, $k_1 = 0.05$ and $k_2 = 0.001$, a good protection is attained only with the second bound ligand. In all cases the curves greatly resemble a straight line; consequently it is in practice hard to distinguish between cases of one bound ligand and of two ligands. In addition, it is tempting to use straight lines (or a one-subunit theory) in interpreting the results. In practice the measurements are normally carried out with ligand concentrations near π, in which case the tangent at the point $x = \pi$ may represent such a straight line. If already the first bound ligand gives a good protection, the value of π^{app} derived from the tangent is in cases (a) — (d) $\pi^{app} = \pi/2$.

In case (a) the π^{app} based on the asymptote of the hyperbola is $\pi^{app} = 2/3\pi$, and in case (d), from the asymptote, $\pi^{app} \to 0$ if $M \to N$. Thus in all the cases presented the π^{app} value is lower than the real π. However, if a good protection is not attained before the second bound ligand, $\pi^{app} = \pi$. In the case of four subunits (e) the corresponding slope of the tangent gives $\pi^{app} = \pi/5$ with the k_i values used.

Application

Based on the graphs above, it is possible to try a solution of the real π, too. The measurements should be carried out in two regions of ligand concentrations: at very low concentrations, from 0 to $\pi/4$, and at high concentrations, above π. If the expression $x/(k_0 - k) = f(x)$ (case (d)) is used, the following values should be measured: the slope of the asymptote (α), the intercept of the curve and the Y-axis (β) and the slope of the tangent at point $x = 0$ (γ). Then:

$$\begin{cases} \alpha = \dfrac{1}{N} \\ \beta = \dfrac{\pi}{2M} \\ \gamma = \dfrac{4M - N}{4M^2} \end{cases} \tag{17}$$

The solution of π from the equation is:

$$\pi = \frac{\beta}{\gamma} + \sqrt{\frac{\beta^2}{\gamma^2} - \frac{\beta^2}{\alpha\gamma}} \tag{18}$$

Another possibility is to use the expression $1/(k_0 - k) = f(1/x)$, and to measure the slope of the asymptote (β), the intercept of the curve and the Y-axis (α) and the slope of the tangent at point $1/x = 1/\pi$ (γ). Then:

$$\begin{cases} \alpha = \dfrac{1}{N} \\ \beta = \dfrac{\pi}{2M} \\ \gamma = \dfrac{4N}{(2M+N)^2} \end{cases} \tag{19}$$

and

$$\pi = \left(\frac{2\beta^2}{\alpha\gamma} - \frac{\beta}{\alpha}\right) + \sqrt{\left(\frac{2\beta^2}{\alpha\gamma} - \frac{\beta}{\alpha}\right)^2 - \frac{\beta^2}{\alpha^2}} \tag{20}$$

Also other selections of three measured quantities (α, β and γ) can be used as starting points. The above methods appear, however, to be the best I have found. The parabolic expressions cannot be used because the quantity $(k_0-k)/x$ has a very marked deviation at low x-values. A double reciprocal plot sharply curves near $1/x = 0$, and the slope of its tangent at $1/x = 0$ thus tends to give erroneous results.

Example

The determination of the binding constant of oxalate to pantothenase is presented in Fig. 2. Pantothenase is a two-subunit enzyme well protected against heat inactivation by oxalate, and showing hyperbolic kinetics (v vs. s and v vs. $1/i$) [8]. The value of π, 2.36 mM, derived from Fig. 2 is still much

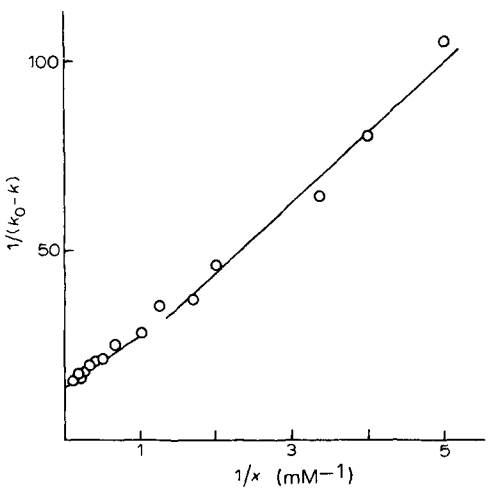


Fig. 2. Determination of the binding constant of oxalate to pantothenase. The assay was carried out as described elsewhere [8]. The reaction mixture contained 25 µl of pantothenase solution (180 µg/ml) containing 0.2% of bovine serum albumin, 25 µl of 100 mM potassium phosphate pH 7.00, 25 µl of potassium oxalate solution and 50 µl of 75 mM potassium [1-^{14}C]pantothenate. The inactivation mixture contained the three first components of the list. The duration of inactivation at 36°C was 20 min.

lower than the value of the inhibition constant by oxalate at 36°C, 6.3 mM. The inhibition constant was measured between 20 and 30°C; the derived ΔH of oxalate binding was −60kJ/mol. The inhibition constant at 36°C was calculated according to the van 't Hoff equation. π^{app} derived from Fig. 2 is 1.19 mM.

Discussion

It is very common that the binding constants derived with thermal inactivation technique are lower than the corresponding kinetic constants [5,7,9,10]. The application of the one-ligand theory to a multi-subunit enzyme is one source of such differences, but yet other causes may exist. A very possible cause, suggested by Citri [11], is for example the presence of intermediate stages during thermal denaturation, when the ligand may be bound to the intermediate stages with a different affinity.

According to the theory a difference between measured and real binding constants will appear when a considerable protection is given already by the first bound ligand. Such a situation is very possible because a major change in the conformation of the protein molecule occurs in thermal denaturation, and also the subunits are bound to each other with several bonds. If one subunit is stabilized by a ligand, the stabilization may be transmitted to the other subunits of the enzyme, too. This does not require any conformational change on ligand binding; the increase of rigidity of the subunit caused by the ligand and its transmission to other subunits is sufficient. So the protection with the first bound ligand is possible without any co-operativity in the binding of the ligands. Co-operativity would make the theoretical considerations above much more complicated.

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