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Analysis of ligand binding process using binding capacity concept✪

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Binding capacity is the homotropic second derivative of the binding potential with respect to the chemical potential of the ligand. It provides a measure of steepness of the binding isotherm and represents the extent of cooperativity. In the present study, the shape of the binding capacity curve for various systems was investigated and the relation between binding capacity and the extent of cooperativity examined. In this regard, a novel linear graphical method was introduced for binding data analysis. The stoichiometry of binding and the extent of cooperativity can be determined by this method. This method has been successfully applied to various systems such as binding of oxygen to hemoglobin, warfarin to human serum albumin and dodecyltrimethylammonium bromide to α-amylase.

Current discussions of biological mechanisms rely increasingly on ideas generated in the theory of macromolecular binding. The concept of biological control requires a wild variety of responses, often dramatic, to the concentration of one or more small molecular species (Schellman, 1975). So one of the main problems facing biochemists and biophysical chemists is analyzing and interpreting binding data. The binding data can be obtained by experimental techniques such as equilibrium dialysis, spectrophotometry, microcalorimetry, etc. The experimental data can be presented in different ways, such as Klotz (Klotz *et al.*, 1975) and Scatchard plots (Scatchard, 1949), and their interpretation can be carried out following schemes. One of the most popular concepts introduced recently is the bind-

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Abbreviations: DTAB, dodecyltrimethylammonium bromide; HSB, human serum albumin.

ing capacity concept (θ). It is the homotropic second derivative of the binding potential with respect to the chemical potential of the ligand (μ_i) and provides a measure of the steepness of the binding isotherm (Cera *et al.*, 1988). It represents the change in the number of moles of ligand per mole of macromolecule (v) that accompanies a change in the chemical potential of that ligand. The heat capacity and the compressibility define analogous concepts with respect to temperature and pressure.

By considering the ideal behavior ($\mu_i = \mu^0{}_i + RT \ln [L]_f$), binding capacity is equal to:

$$\theta = (\frac{\partial v}{\partial \mu_i})T, P, \mu_{j\neq i} = (\frac{\partial v}{RT\partial \ln[L]_f})T, P, \mu_{j\neq i} \quad (1)$$

Where R, T and $[L]_f$ are gas constant, absolute temperature and free concentration of the ligand, respectively. This parameter has been determined experimentally for several systems such as binding of oxygen to hemoglobin (Cera *et al.*, 1988). However, it can be estimated by calculating the steepness of the binding isotherm. In the present study, the binding capacity curve for various binding systems was evaluated and a novel linear graphical method has been introduced for binding data analysis.

EVALUATION OF BINDING CAPACITY CURVES

System with single binding set

For such a system, all of the binding sites can be related to a single category (set). The binding data of this system can be analyzed on the basis of empirical Hill equation (Hill, 1910):

$$v = \frac{g(K_H[L]_f)^{n_H}}{1 + (K_H[L]_f)^{n_H}} \quad (2)$$

This can be written in the logarithmic form as follows,

$$\ln(\frac{v}{g - v}) = n_H + \ln K_H + n_H \ln[L]_f \quad (3)$$

Where g, K_H and n_H are the number of ligand-binding sites, Hill binding constant and Hill coefficient, respectively.

Using equation 1 and chain rule in derivative process, the binding capacity is equal to:

$$\theta = \frac{n_H v(g - v)}{gRT} \quad (4)$$

And by operation of maximum criterion, it can be shown that:

$$v_{max} = \frac{g}{2} \quad (5)$$

Hence, the shape of the binding capacity curve is Gaussian with a maximum at half saturation. However, the steepness of the curves increases with increasing n_H (extent of cooperativity).

Figures 1 and 2 show the binding isotherms and binding capacity curves for three hypothetical systems, respectively. Obviously, the steepness of the curves increases with increasing n_H.

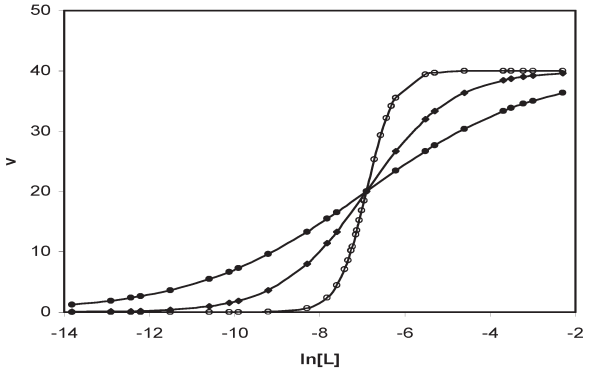

Figure 1. Binding isotherms for three hypothetical systems with g = 40, K_H = 10^3 M^{-1}, (○) n_H = 3, (■) n_H = 1 and (●) n_H = 0.5.

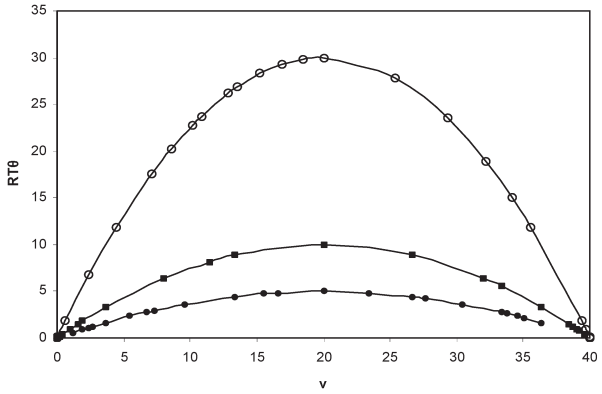

Figure 2. Binding capacity curves for three hypothetical systems with g = 40, K_H = 10^3 M^{-1}, (○) n_H = 3, (■) n_H = 1 and (●) n_H = 0.5.

System with two independent binding sets

All of the binding sites for such a system can be dividing into two categories, each of them related to a binding set. For independent binding sets, the occupation of the sites in one set has no effects on the binding process of the other set. For such a system, it can be written:

$$v = v_i + v_2 \tag{6}$$

Where v_1 and v_2 are the average number of bound ligand in the first and second binding sets, respectively.

Hill equation for such a system can be written as (Tanford, 1993):

$$v = \{\frac{g_1(K_{H1}[L]_f)^{n_{H1}}}{(1+(K_{H1}[L]_f)^{n_{H1}})}\} + \{\frac{g_2(K_{H2}[L]_f)^{n_{H2}}}{(1+(K_{H2}[L]_f)^{n_{H2}})}\} \tag{7}$$

Where g_1, K_{H1} and n_{H1} are the number of binding sites, binding constant and Hill coefficient for first binding set, and g_2, K_{H2} and n_{H2} are the corresponding parameters for the second binding set, respectively. By using equation (1), it can be written:

$$RT\theta = \{\frac{n_{H1}g_1(K_{H1}[L]_f)^{n_{H1}}}{(1+(K_{H1}[L]_f)^{n_{H1}})^2}\} + \{\frac{n_{H2}g_2(K_{H2}[L]_f)^{n_{H2}}}{(1+(K_{H2}[L]_f)^{n_{H2}})^2}\} \tag{8}$$

By taking the derivative of θ with respect to $\ln[L]_f$ and operation of maximum criterion, we have:

$$\{(n_{H1}^2 g_1 K_{H1}^{n_{H1}}[L]_f^{n_{H1}}) - n_{H1}^2 g_1 K_{H1}^{2n_{H1}}[L]_f^{2n_{H1}}\} + \{(n_{H2}^2 g_2 K_{H2}^{n_{H2}}[L]_f^{n_{H2}}) - n_{H2}^2 g_2 K_{H2}^{2n_{H2}}[L]_f^{2n_{H2}}\} = 0 \tag{9}$$

The exact solution of this rigorous statement is impossible, but interesting conclusions have been obtained by some simplifications. In the following, a method is proposed for a system with N independent binding sets.

System with N-independent binding sets

For such a system it can be written that

$$v = \sum_{i=1}^{N} v_i \text{ and } (\frac{\partial v}{\partial v_i}) = 1 \tag{10}$$

Where v_i is the average number of bound ligand per each macromolecule in the ith binding set. With respect to equation (1), the binding capacity of this system is equal to:

$$\theta = \frac{\partial v}{RT\partial \ln[L]_f} = \sum_{i=1}^{N} (\frac{\partial v_i}{RT\partial \ln[L]_f}) = \sum_{i=1}^{N} \theta_i \tag{11}$$

Where:

$$\theta_i = \frac{\partial v_i}{RT\partial \ln[L]_f} \quad (12)$$

The Hill equation for this system is:

$$v = \sum_{i=1}^{N} \frac{g_i (K_{Hi}[L]_f)^{n_{Hi}}}{1 + (K_{Hi}[L]_f)^{n_{Hi}}} \quad (13)$$

Where g_i, K_{Hi} and n_{Hi} are the number of binding sites, binding constant, and Hill coefficient for ith binding set, respectively. Following equation (13):

$$v_i = \frac{g_i (K_{Hi}[L]_f)^{n_{Hi}}}{1 + (K_{Hi}[L]_f)^{n_{Hi}}} \quad (14)$$

By using equations (1) and (13), it can be written:

$$\theta_i = \frac{n_{Hi} v_i (g_1 - v_i)}{g_i RT} \quad (15)$$

In respect to this equation and equation (10), it can be written:

$$\frac{\partial(RT\theta)}{\partial v} = \sum_{i=1}^{N} \left(\frac{1}{g_1}\right) \times \left(\frac{[\partial(n_{Hi} v_i (g_i - v_i))]}{\partial v_i}\right)\left(\frac{\partial v_i}{\partial v}\right) = \sum_{i=1}^{N} \frac{n_{Hi}(g_i - 2v_i)}{g_i} \quad (16)$$

This equation reveals the contribution of any set of binding sites in the overall binding capacity. The shape of the binding capacity curve depends on the relative values of K_{Hi}. In the extreme case when $K_{H\,(i-1)} >> K_{Hi}$, it can be assumed that the occupation of the ith binding sets does not occurr until the full occupation of the $(i-1)$th binding set has been reached.

In other words,

$$\begin{aligned} &v = v_1,\ \theta = \theta_1 && \text{if } v \le g_1 \\ &v = g_1 + v_2,\ \theta = \theta_2 && \text{if } g_1 < v < g_1 + g_2 \\ &v = g_1 + g_2 + \ldots + v_i,\ \theta = \theta_2 && \text{if } g_1 + g_2 + \ldots + g_{i-1} < v < g_1 + g_2 + \ldots + g_i \end{aligned} \quad (17)$$

By this assumption it can be written:

$$\begin{aligned} &\left(\frac{\partial\theta}{\partial v}\right) = \frac{n_{H1}(g_1 + 2v_1)}{RTg_1} && \text{if } 0 < v < g_1 \\ &\left(\frac{\partial\theta}{\partial v}\right) = \frac{n_{H2}(g_2 + 2v_2)}{RTg_2} && \text{if } g_1 < v < g_1 + g_2 \\ &\left(\frac{\partial\theta}{\partial v}\right) = \frac{n_{Hi}(g_i + 2v_i)}{RTg_i} && \text{if } g_1 + g_2 + \ldots g_{i-1} < v < g_1 + g_2 + \ldots g_i \end{aligned} \quad (18)$$

By application of maximum criterion, and using equations (16) and (18), it can be shown that:

$$\begin{aligned} &v_{\max,1} = \frac{g_1}{2} \\ &v_{\max,1} = g_1 + \frac{g_2}{2} \\ &v_{\max,i} = g_1 + g_2 + \ldots + \frac{g_1}{2} \end{aligned} \quad (19)$$

Consequently, the binding capacity curve consists of a series of consecutive maxima, the number of which should be equal to the number of the binding sets. The positions of the maxima determine the stoichiometry of each set. However, these curves overlap with each other generally and the extent of the overlap depends on the relative value of K_{Hi} (binding affinity of each set).

GRAPHICAL ANALYSIS OF THE BINDING CAPACITY DATA

In this part, we try to relate the concept of Hill coefficient to the binding capacity in order to extract a relationship between them.

The Hill coefficient is defined as the slope of the Hill graph,

$$n_H = \frac{d\ln(\frac{y}{1-y})}{d\ln[L]_f} = (\frac{1}{y(1-y)})\frac{dy}{d\ln[L]_f} \quad (20)$$

Where y is the fractional saturation of the macromolecule by the ligand which is defined as follows:

$$y = \frac{v}{g} \quad (21)$$

From the definition of binding capacity (Eqn. 1) the following equations can be written:

$$n_H = \frac{RT\theta}{gy(1-y)} \quad (22)$$

$$\theta = \frac{n_H v(1-y)}{RT} \quad (23)$$

Equation (23) can be rearranged to the following form:

$$\frac{RT\theta}{v} = n_H - \frac{n_H v}{g} \quad (24)$$

This equation suggests that the plot of $\frac{RT\theta}{v}$ *vs* v for a system with g identical and dependent binding sites, should be linear. The slope and the Y and X-intercepts are equal to $-\frac{n_H}{g}$, n_H and g, respectively.

Figure 3 shows the variation of $\frac{RT\theta}{v}$ *vs* v for three hypothetical systems with various cooperativity. The X-intercept for all of these curves is the same and equal to 40 (number of sites). The slope and the Y-intercept of these curves are different because of the different n_H values.

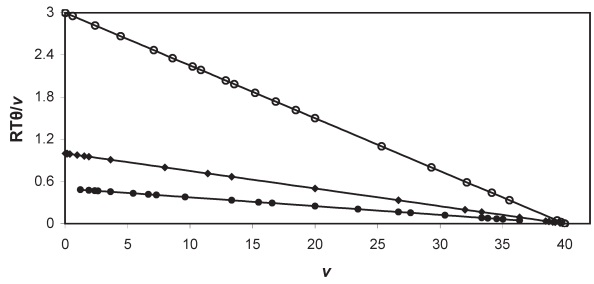

Figure 3. The variation of $\frac{RT\theta}{v}$ *versus* v for three hypothetical systems with g = 40, K_H=10^3 M^{-1}, (○) n_H = 3, (■) n_H = 1 and (●) n_H = 0.5.

This method can be used for any single and multi-independent binding set system with high variation in their binding affinity. In the following, the binding of oxygen to hemoglobin and warfarin to human serum albumin (HSA) as two known single binding set systems, and dodecyl trimethyl ammonium bromide, dodecyltrimethylammonium bromide (DTAB), to α-amylase as a two binding set system, have been analyzed by this method.

APPLICATIONS

Binding of oxygen to hemoglobin

The binding capacity data of this system have been measured previously (Cera *et al.*, 1988). Figure 4 shows the variation of $\frac{RT\theta}{v}$ *vs*

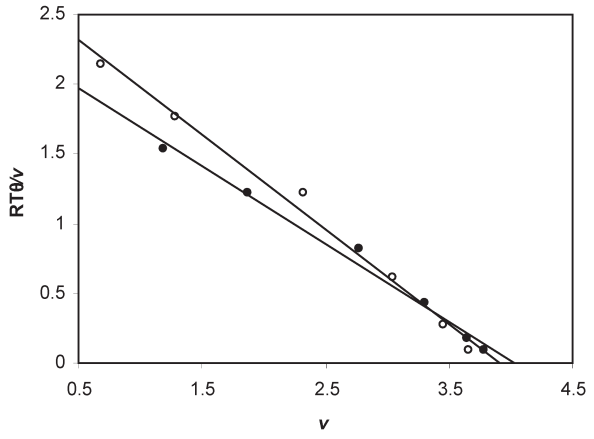

Figure 4. The variation of $\frac{RT\theta}{v}$ *vs* v for binding of oxygen to hemoglobin.

(○) buffered case, (●) unbuffered case.

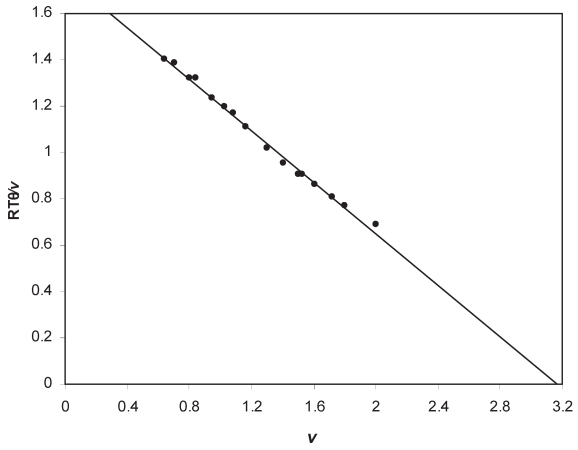

Figure 5. The variation of $\frac{RT\theta}{v}$ *vs* v for binding of warfarin to HSA, 67 mM phosphate buffer, pH 7.4, 37°C.

v for this system. The X-intercept is equal to 4, which represents the number of binding sites. The values of n_H calculated from the slope of the lines are 2.66 and 2.26 for buffered and unbuffered cases, respectively. The results are in good agreement with the previous results and confirm the validity of the proposed method.

Binding of warfarin to HSA

One of the most important specific drug-binding sites on HSA is the warfarin binding site. Warfarin is bound to a site known as warfarin-azapropazone binding area or site I of the HSA protein (Birkett *et al.,* 1980; Fehske *et al.*, 1981; 1982). The binding isotherm of this system is usually hyperbolic that is difficult to fit (Kruz, 1986). This makes the interpretation of the data difficult. The warfarin binding data at pH 7.40 in a 67 mM sodium phosphate buffer at 37°C have been measured previously by the equilibrium dialysis technique (Villamor & Zaton, 2001). Figure 5 shows the variation of $\frac{RT\theta}{v}$ *vs* v for this system. From the slope and intercepts of this curve the values of 3.17 and 1.76 are obtained for g and n_H, respectively.

Binding of dodecyl trimethylammonium bromide to α-amylase

The interaction of ionic surfactants with globular proteins has been studied extensively (Jones, 1975; 1992). It has been well established that such interactions frequently lead to the destruction of native structure of proteins and the formation of unfolded protein-surfactant complexes. Due to the importance of the denaturation studies which are capable of yielding information about the native state (Jones & Brass, 1991) understanding the mechanism of surfactant binding is very important. It is generally accepted that binding of ionic surfactant molecules to proteins initially involves the ionic binding of surfactants to the ionic sites of the protein. Further binding occurs by hydrophobic cooperative interactions (Goddard, 1993; Bordbar *et al.*, 1997). Due to this fact, for binding of ionic surfactants to globular proteins, two binding sets with a relatively high difference in their binding affinity exist (Jones & Brass, 1991; Bordbar *et al.*, 1996). A number of graphical and computer-assisted methods for resolution and characterization of binding sets was employed (Jones, 1988; 1992; Bord-

bar *et al.*, 1997) which are not comprehensive. In this part, unpublished binding data for the interaction of dodecyl trimethylammonium bromide with α-amylase have been analyzed on the basis of our proposed model. The binding data were measured using DTAB-selective membrane electrodes as a simple, fast and accurate method (Gharibi *et al.*, 1998). Figure 6 is the binding isotherms for interaction of

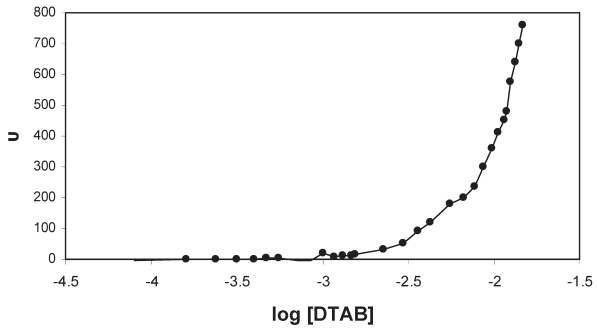

Figure 6. Binding isotherm for interaction of DTAB with α-amylase at 25°C, pH 9.70, and 10^{-4}M NaBr.

DTAB with α-amylase at 25°C, pH = 9.70 and 10^{-4} M NaBr.

The plot of $\frac{RT\theta}{\upsilon}$ *vs* υ for this system is shown in Fig. 7. This curve can be divided to two linear regions. The values of the X-inter-

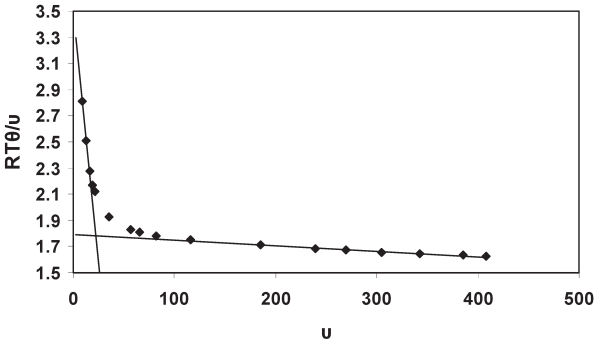

Figure 7. The plot of $\frac{RT\theta}{\upsilon}$ *vs* υ for interaction of DTAB with α-amylase at 25°C, pH 9.70, and 10^{-4} M NaBr.

cept of the first and second parts should be equal to g_1 and $g_1 + g_2$, respectively. The values of n_{H1} and n_{H2} can be determined from the slope of the lines. The values of 4.59, 1.79, 28.26 and 580.57, were estimated for n_{H1}, n_{H2}, g_1 and g_2, respectively.

CONCLUSION

The binding capacity curve consists of N consecutive Gaussian curves for a system of N independent binding sets. These curves overlap with each other and the extent of the overlap depends on the relative binding affinity of the sets. Experimental data relating the number of ligand-occupied sites on a macromolecule to ligand concentration can be analyzed by different methods. Hill's equation is usually helpful for evaluation of the cooperativity of ligand binding. Application of this equation (in graphical form) to a system characterized by occurrence of "cooperative interaction" allows determination of neither the microscopic binding constants of a ligand to particular subunits of a multi subunit protein nor the intrinsic number of the ligand-binding sites. The plot of $\frac{RT\theta}{\upsilon}$ *vs* υ provides a novel graphical method for determination of Hill coefficient (n_H) and the number of binding sites under the assumption of the presence of identical binding sites. Some advantages of this method are as follows:

- It is possible to measure the binding capacity data experimentally. This has already been done by means of the thin-layer method (Cera *et al.*, 1988). It is reasonable to analyze this kind of data directly; the proposed method is one of the best empirical methods for such an analysis.
- In some binding systems, the experimental determination of all regions of binding isotherms is impossible (especially the end regions). In this case, the determination of the stoichiometry of binding is erroneous. For such systems the binding capacity may be determined by calculating the steepness of the binding isotherms with respect to Eqn. 1. Analysis of the calculated binding capacity data determines the stoichiometry

of binding precisely. The other usual analyzing method such as Hill equation can not provide this information.

◆ Application of Hill and Scatchard plots for analysis of multi binding set systems is usually erroneous and not much informative (Bordbar *et al.*, 1996), while our proposed method can clearly determine the number of binding sets and their characteristics.

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