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SHORT COMMUNICATION

On the Evaluation of the Number of Binding Sites in Proteins From Steady State Fluorescence Measurements

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Abstract The number of binding sites for a given solute in a protein is a most relevant parameter. This number can be derived from fluorescence quenching data which provides the fraction of sites occupied at a given free solute concentration. Data are generally treated according to Scatchard´s or Ward´s equations. Lately, a double logarithmic plot of the data has been extensively used with this purpose. The present communication discus the validity of this procedure. It is concluded that this type of plot provides an evaluation of the stoichiometry (molecularity) of the binding process but not the number of equivalent binding sites per protein.

Keywords Intrinsic protein fluorescence · Solute binding · Number of binding sites

Introduction

Changes in the intrinsic fluorescence yield of proteins promoted by a quencher and/or in the substrate fluorescence with the protein concentration are widely employed to evaluate the substrate association to the macromolecule [1–3]. Also, this type of measurements has been employed to evaluate the microenvironment of the bound quencher [1–6] and the average distance between the donor and acceptor groups [7–22]. Furthermore, these techniques allow and evaluation of the number (and types) of binding sites present in the macromolecule [2, 23].

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The number (and types) of binding sites is one of the most relevant parameters regarding the protein/solute association and several treatments of the data have been proposed to allow for its evaluation. The complexity of the treatment and the accuracy of the data needed to derive meaningful results depend of the number of binding site types present in the considered system. In the following discussion, we will consider mostly data treatments aimed to determine the number of equivalent ideal sites that behaves according to Langmuir association, i.e., all the sites have the same affinity for the solute and this affinity is independent of the fraction of free sites.

Classical methods that employ fluorescence data to determine the number of equivalent binding sites per protein are the Scatchard representation [2], the continuous variation titration [24] and the procedure described by Ward following the proposal of Stinson and Hollbrook [25]. An alternative treatment of the data has been frequently employed in the last years and is based on a double logarithmic plot [3–6, 9–22]. The purpose of the present communication is to discuss the validity of this procedure and its pertinence in the evaluation of the number of equivalents sites present in each macromolecule.

Data Treatment and Discussion

Let as consider the quenching of the intrinsic fluorescence of a protein (P) by an additive (Q). Each protein molecule has **n** equivalent sites and, when the analytical concentrations of the protein and the quencher are P^o and Q^o, respectively, a fraction θ of the sites are occupied by the quencher. If binding of the solute does not introduce conformational changes in the protein, the value of θ can

be derived from the intensity of the intrinsic protein fluorescence:

$$\theta = (F^{\circ} - F)/(F^{\circ} - F^{-\infty}) \quad (1)$$

Where

F^{o} is the fluorescence in absence of quencher
F is the flurescence at a given quencher concentration
F^{∞} is the fluorescence from a protein "saturated" of quencher.

Although fluorescence quenching is widely employed to obtain θ values, other techniques, such as dialysis or light absorption measurements, can be employed to asses the fraction of the binding sites occupied by solute molecules.

The rates of the binding and desorption processes can be represented, under ideal conditions, by expressions (2) and (3)

$$\text{Adsorption Rate} = k_{+} n(1-\theta)\, P^{\circ}(Q^{\circ} - \theta\, n P^{\circ}) \quad (2)$$

$$\text{Desorption Rate} = k_{-}\, \theta\, n P^{\circ} \quad (3)$$

At equilibrium, both rates must be equal and the binding constant, defined as k_{+} / k_{-}, is given by:

$$K = \theta/((1-\theta)(Q^{\circ} - \theta\, n P^{\circ}) \quad (4)$$

Rearrangement of this equation leads to:

$$1/(1-\theta) = (K\, Q^{\circ})/\theta - n P^{\circ} K \quad (5)$$

If P^{o} is kept constant, a plot of the left hand side against $\mathbf{Q^{o}}$/ θ provides K (from the slope) and n (from the ordinate). In general, if the analytical concentration of the quencher and the protein are variable, Eq. 5 can be rearranged to:

$$1/[(1-\theta)P^{\circ}] = (K\, Q^{\circ})/\theta P^{\circ} - n\, K \quad (6)$$

and a plot of the left hand side against (Q^{o} / θ P^{o}) gives K from the slope and n (from the ordinate/slope). This procedure is an extension of Scatchard treatment and is similar to that given by Ward [1]

The log-log relationship is fundamentally different. Starting from the premise that the binding process can be represented by Eq. 7

$$P + M\, Q \leftrightarrow \text{binding} \quad (7)$$

where **m** represents the kinetic reaction order (molecularity in Q). The adsorption rate, when $Q \approx Q^{o}$, is given by Eq. 8:

$$\text{Adsorption Rate} = k_{+}(1-\theta) m\, P^{\circ}[Q^{\circ}]^{m} \quad (8)$$

and the desorption rate is:

$$\text{Desorption Rate} = k_{-}\theta\, m\, P^{\circ} \quad (9)$$

The equilibrium constant can be expressed as,

$$K = \theta/((1-\theta)[Q^{\circ}]^{m}) \quad (10)$$

This equation can be re-arranged to,

$$\theta/(1-\theta) = K[Q^{\circ}] \quad (11)$$

and, hence, if $F^{\circ} >> F^{\infty}$,

$$(F^{\circ} - F)/F = K[Q^{\circ}]^{m} \quad (12)$$

and,

$$\ln[(F^{\circ} - F)/F] = \ln K + m \ln[Q^{\circ}] \quad (13)$$

A plot of the left hand side of Eq. 13 as a function of ln [Q^{o}] gives, from the slope, the value of **m**. However, this parameter does not express the number of independent and equivalent sites **n**. It rather measures ***the number of Q molecules that interact simultaneously with each site***. As correctly stated by Chipman et al. [23], the slope of the plot gives the stoichiometry of the quencher/protein complex. This restrictive definition of **m** explains why, when this treatment is attempted, the value of **m** is always very close to one. It is suprising that in most recent works employing Eq. 13 the slope of the plot is wrongly equated to the **number (n)** of equivalent sites [3–6, 9–22].

Conclusion

In conclusion, we consider that expressions such as that given in Eq. 13 are useful to determine the stoichiometry of association processes (i.e. the number of molecules coordinated to a given ion or the number of solutes bound to a single site in a protein), but they cannot be employed to evaluate the number of equivalent sites in each protein.

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