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On the determination of species fractions from ligand-binding data

Application to human hemoglobin

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A method outlined in a previous study (S.J. Gill, H.T. Gaud, J. Wyman and G. Barisas, Biophys. Chem. 8 (1978) 53) is applied for the determination of species fractions from ligand-binding data for the oxygen reaction with human hemoglobin. The results obtained by this alternative approach, which is based on the solution of a system of linear equations, are consistent with those obtained using nonlinear least-squares analysis.

1. Introduction

A critical step in the analysis of ligand-binding data is the evaluation of the species fractions of stoichiometric intermediates. These quantities reflect the cooperative nature of the binding phenomenon and provide a necessary basis for understanding the thermodynamic features of the system. In the case of oxygen binding to human hemoglobin an almost overwhelming set of experimental data has been collected over the past 20 years, and analysed according to nonlinear least-squares procedures [1–3]. The question arising at this point is whether the distribution of stoichiometric intermediates can be arrived at from experimental binding data through any alternative method, without using nonlinear least-squares procedures.

Alternative approaches have been devised by which some observable features of the experimental binding data can be exploited to derive the species fractions [4,5]. Gill et al. [5] showed how various properties of a ligand-binding curve provide simple linear equations in terms of the species fractions. This method is particularly attractive, since it takes into account the derivative of the binding curve, along with its asymptotic behavior and the median ligand activity. The solution of the set of equations is direct and gives the species fractions at any ligand activity. The availability of experimental binding data which yield accurate derivatives of the binding curve [3] has opened the way to practical application of this method.

In this paper we apply the procedure derived previously [5] to determine the species fractions for the oxygen-binding reaction with human hemoglobin directly from differential data obtained by means of the thin-layer technique [6].

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2. Theory

In this section we summarize the basic relations employed in the subsequent analysis of available data. Additional details of the approach can be found in a previous paper [5].

Consider a macromolecule M which contains t sites for ligand X, whose activity is x. The set of overall reactions between the unligated form of the macromolecule M_0 and j ($j = 0, 1 \ldots t$) ligand molecules X is described as follows

$$M_0 + jX = M_j \tag{1}$$

We associate to each of these $t+1$ reactions an overall equilibrium constant β such that

$$\beta_j = \frac{[M_j]}{[M_0]x^j} \tag{2}$$

The number $\bar{X}$ of ligands bound to the macromolecule is given by

$$\bar{X} = \frac{\beta_1 x + \ldots t\beta_t x^t}{1 + \beta_1 x + \ldots \beta_t x^t} = \frac{\sum j\beta_j x^j}{\sum \beta_j x^j} \tag{3}$$

The derivatives of $\bar{X}$ are obtained by differentiation of eq. 3. We are particularly interested in

$$\frac{d\bar{X}}{d \ln x} = \frac{\sum j^2\beta_j x^j \sum \beta_j x^j - \left(\sum j\beta_j x^j\right)^2}{\left(\sum \beta_j x^j\right)^2} \tag{4}$$

This is the binding capacity of the macromolecule and has recently been shown to be of particular relevance in a discussion of general concepts of linkage thermodynamics [7]. For our present purpose the binding capacity can be measured experimentally by means of a thin-layer technique [6] (see below).

We now wish to express $\bar{X}$, $d\bar{X}/d\ln x$ and other properties of the binding curve in terms of species fractions. The fraction α_j of the j-th ligated species is

$$\alpha_j = \frac{\beta_j x^j}{1 + \beta_1 x + \ldots \beta_t x^t} \tag{5}$$

The parameters α have the important conservation property

$$\sum \alpha_j = 1 \tag{6}$$

which shows that of the $t+1$ possible species fractions only t are independent.

Next we consider the i-th moment of the number of ligands bound

$$m_i = \sum j^i \alpha_j \tag{7}$$

It turns out that $\bar{X}$ as well as any derivatives of $\bar{X}$ may be expressed in terms of a linear combination of the moments, and hence of the species fractions, α. For example, $\bar{X}$ is equal to the first moment m_1, e.g.,

$$\bar{X} = \sum j\alpha_j = m_1 \tag{8}$$

The binding capacity is a function of both m_1 and m_2, e.g.,

$$\frac{d\bar{X}}{d \ln x} = \sum j^2\alpha_j - \left(\sum j\alpha_j\right)^2 = m_2 - m_1^2 \tag{9}$$

and so forth *.

Two additional properties of the binding curve can be described. The first involves the median ligand activity x_m [8] which obeys the following integral equation

$$\int_0^t \ln(x/x_m)\, d\bar{X} = 0 \tag{10}$$

The median ligand activity is related to β_t by the familiar relation

$$\beta_t = (1/x_m)^t = \alpha_t/\alpha_0 x^t \tag{11}$$

The second is the limit of the function $d\bar{X}/dx$ for x which tends to zero **, this limit being equal to β_1, i.e.,

$$\lim_{x \to 0} \frac{1}{x} \frac{d\bar{X}}{d \ln x} = \beta_1 = \alpha_1/\alpha_0 x \tag{12}$$

* In general $d^{r-1}\bar{X}/(d \ln x)^{r-1} = m_r - m_{r-1}m_1$.

** A third relation is

$$\lim_{x \to \infty} \ln\left(\frac{\bar{X}}{1-\bar{X}} \frac{1}{x}\right) = \beta_t/\beta_{t-1} = \alpha_t/\alpha_{t-1}x$$

and gives another asymptotic property of the binding curve.

and is independent of the value of t.

Combination of eqs. 6–12 yields a system of five independent and linear expressions in α. In the case of a tetrameric macromolecule such as human hemoglobin this is sufficient to determine all the species fractions. In the case of $t > 4$ higher derivatives of $\bar{X}$ or other properties of the binding curve would need to be taken into account [5].

The set which is particularly relevant for our experiments dealing with the tetrameric macromolecule hemoglobin is

$$\alpha_0 + \alpha_1 + \alpha_2 + \alpha_3 + \alpha_4 = 1 \tag{13}$$

$$\alpha_1 + 2\alpha_2 + 3\alpha_3 + 4\alpha_4 = m_1 = \bar{X} \tag{14}$$

$$\alpha_1 + 4\alpha_2 + 9\alpha_3 + 16\alpha_4 = m_2 = \frac{d\bar{X}}{d \ln x} + \bar{X}^2 \tag{15}$$

$$Q\alpha_0 - \alpha_4 = 0 \tag{16}$$

$$P\alpha_0 - \alpha_1 = 0 \tag{17}$$

where $Q = \beta_4 x^4$ and $P = \beta_1 x$. The solution of the system is readily found by application of Cramer's rule to be

$$\alpha_0 = (6 - 5m_1 + m_2)/2(3 + Q + P) \tag{18}$$

$$\alpha_1 = P(6 - 5m_1 + m_2)/2(3 + Q + P) \tag{19}$$

$$\alpha_2 = [6(2Q - P) + m_1(9 - 7Q + 8P) - m_2(3 - Q + 2P)]/2(3 + Q + P) \tag{20}$$

$$\alpha_3 = [2(P - 8Q) + m_1(12Q - 3P - 4) - m_2(2 - 2Q + P)]/2(3 + Q + P) \tag{21}$$

$$\alpha_4 = Q(6 - 5m_1 + m_2)/2(3 + Q + P) \tag{22}$$

Therefore, if experimental measurements of $\bar{X}$ and $d\bar{X}/d\ln x$ are available one can determine all the species fractions directly from the relations above.

3. Experimental measurement of the binding capacity $d\bar{X}/d\ln x$

Spectroscopic techniques are widely employed in the study of ligand-binding processes. In the case of human hemoglobin in its reaction with oxygen the function $\bar{X}$ is extracted from absorbance readings by the following relation

$$A(x_i) = A(0) + \Delta A_T \theta(x_i) \tag{23}$$

where $\theta(x_i)$ is the degree of saturation ($\bar{X}/4$) at a given oxygen activity x_i, $A(x_i)$ the corresponding absorbance, $A(0)$ the reading in the absence of oxygen ($\theta(0) = 0$), and ΔA_T the total absorbance change observed in fully saturating ($\theta(\infty) = 1$) the molecule and equal to $A(\infty) - A(0)$. Imai's apparatus [1] is an example of experimental technique for the determination of $\bar{X}$ through eq. 23.

The determination of the binding capacity $d\bar{X}/d\ln x$ requires a different experimental approach. The thin-layer method [6] measures changes in the absorbance at any given oxygen partial pressure. The underlying relation is in this case

$$A(x_i) - A(x_{i-1}) = \Delta A(\xi_i) = \Delta A_T[\theta(x_i) - \theta(x_{i-1})] \tag{24}$$

Here ξ_i is an arbitrary value of the oxygen activity in the interval from x_i to x_{i-1}. In order to assess how accurately the finite differences in eq. 24 approximate the binding capacity $d\bar{X}/d\ln x$ we consider the Taylor expansion of $\theta(x_i) - \theta(x_{i-1})$ as follows

$$\theta(x_i) - \theta(x_{i-1}) = \frac{d\theta}{d \ln x}\ln(x_i/x_{i-1}) + \frac{1}{2}\frac{d^2\theta}{d \ln x^2}[\ln^2(x_i/\xi_i) - \ln^2(x_{i-1}/\xi_i)] + \frac{1}{6}\frac{d^3\theta}{d \ln x^3} \times [\ln^3(x_i/\xi_i) - \ln^3(x_{i-1}/\xi_i)] + \ldots \tag{25}$$

Here the derivatives must be calculated at ξ_i.

In the thin-layer technique the ligand activity x is changed by dilution steps according to the following relation

$$x_i = x_0 D^i \tag{26}$$

where x_0 denotes the initial ligand activity, x_i is x after the i-th dilution step, and D is the dilution factor, typically on the order of 0.7, which depends upon geometric features of the dilution valve [6]. Assume now that ξ_i is the geometric mean of the two partial pressures that define each step, i.e., let $\xi_i = \sqrt{x_i x_{i-1}}$ for step i; by virtue of

eq. 26 we can rewrite eq. 25 as follows

$$\Delta\theta(\xi_i) = \theta(x_i) - \theta(x_{i-1}) = \left(\frac{d\theta}{d \ln x}\right)_{x=\xi_i} \ln D + \frac{1}{24}\left(\frac{d^3\theta}{d \ln x^3}\right)_{x=\xi_i} \ln^3 D + \ldots \quad (27)$$

The second derivative cancels, as well as any even derivatives of θ, and the odd ones are to be calculated at $x = \xi_i$. Hence, we can write with very good approximation that

$$\Delta A(\xi_i) = \left(\frac{d\bar{X}}{d \ln x}\right)_{x=\xi_i} \ln D\Delta A_T/4 \quad (28)$$

which gives the relation for the binding capacity of human hemoglobin as a function of its optical properties, as measured by means of the thin-layer method.

A typical differential data set obtained by means of the thin-layer technique is shown in fig. 1 as a binding capacity plot. The curve is drawn according to eq. 28, with the Adair constants determined by nonlinear least-squares fitting of the data to eq. 24, and shows that the differential binding data nicely approximate the binding capacity of the macromolecule. The goodness of this approximation was also tested from simulated data with no error in the form of the ΔA_i using eq. 24, and then proceeding to fit using eq. 28. The standard error of the fit gives the systematic error due to the use of the 'incorrect' fitting function eq. 28 and was found to be only 2.2×10^{-5} per unit ΔA_T, a value 100-times smaller than the error typically found experimentally [2,3].

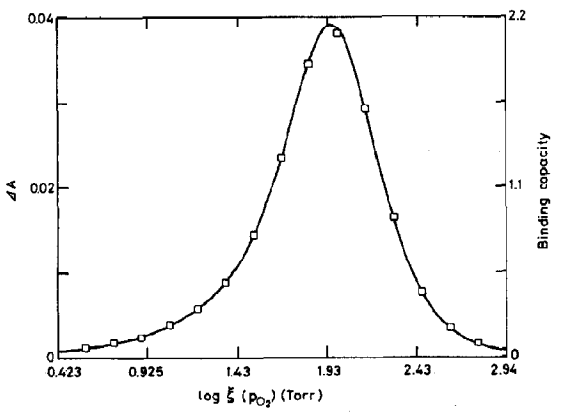

Fig. 1. Differential binding data plotted as absorbance changes (left) vs. the logarithm of oxygen partial pressure ξ_i (see text) at each step i. The data closely approximate the derivative of $\bar{X}$, i.e., the binding capacity [7] (right), as expressed by eq. 28. The continuous line depicts the binding capacity and was drawn from eq. 28 with the best-fit values of the β terms determined by nonlinear least-squares analysis of the data according to eq. 24: $\beta_1 = 0.019$ Torr^{-1}, $\beta_2 = 0.00034$ Torr^{-2}, $\beta_3 = 0$ Torr^{-3}, $\beta_4 = 7.57 \times 10^{-8}$ Torr^{-4}, $\Delta A_T = 0.1972$. Experimental conditions: 2 mM heme, 0.1 M Hepes, 1 mM Na_2EDTA, 0.1 M NaCl, 10 mM IHP, pH 7.0, 25°C. The dilution factor D was 0.6965.

By assuming that at the starting ligand activity x_0 the macromolecule is fully saturated * ($\theta(x_0) = 1$ and $A(x_0) = 0$), the following relation applies by virtue of eq. 25

$$\bar{X}(x_i) = 4\left(1 - \sum_{j=1}^{i} \Delta A(x_j)/\Delta A_T\right) \quad (29)$$

Eq. 29 shows that $\bar{X}$ can be derived from binding capacity data and can be used along with eq. 28 to solve the system of linear equations, eqs. 13–17, for the case of a tetrameric macromolecule.

4. Application to human hemoglobin

Experimental oxygen-binding data taken in the form of the ΔA parameters were transformed into $\bar{X}$ and $d\bar{X}/d\ln x$ data points and are listed in table 1. The binding capacity was calculated from the ΔA according to eq. 28. The $\bar{X}$ values were determined by means of eq. 29 with ΔA_T taken as the sum of all $\Delta A(x_i)$ plus a correction due to the systematic deviation **. The values of $\bar{X}$ at ξ_i were calculated by averaging the $\bar{X}$ values at x_i.

* This assumption holds good in the majority of cases for human hemoglobin. A typical experiment of oxygen binding is started with the ligand at atmospheric pressure where $\theta(x_0) > 0.998$.

** The systematic deviation is due to the fact that the sum of all ΔA terms gives ΔA_T only when $\theta(x_0) - \theta(x_N)$ (where N is the number of experimental points) is equal to unity. In practice the sum of all ΔA terms is about 2% less than the true ΔA_T. A good approximation of ΔA_T is thus obtained from the relation $\Delta A_T = 1.02 \times \Sigma\Delta A(x_i)$.

Table 1

Absorbance changes for oxygen binding to human hemoglobin under experimental conditions reported in the legend to fig. 1

The values of $\bar{X}$ and $d\bar{X}/d \ln x$ were calculated as described in the text.

Dilution step i	p_{O_2} (x_i) (Torr)	ΔA_i	$p_{O_2}\sqrt{1/D}$ (ξ_i) (Torr)	$\bar{X}_i$	$(d\bar{X}/d \ln x)_i$
1	418.4	0.0017	501	3.98	0.0954
2	291.4	0.0035	349	3.93	0.196
3	203.0	0.00775	243	3.82	0.435
4	141.4	0.0165	169	3.57	0.926
5	98.47	0.02925	118	3.11	1.64
6	68.58	0.038	82.2	2.42	2.13
7	47.77	0.0345	57.2	1.69	1.94
8	33.27	0.0235	39.9	1.1	1.32
9	23.17	0.0145	27.8	0.714	0.813
10	16.14	0.0088	19.3	0.478	0.494
11	11.24	0.00575	13.5	0.331	0.323
12	7.830	0.0039	9.38	0.233	0.219
13	5.453	0.0025	6.53	0.168	0.14
14	3.798	0.0019	4.55	0.123	0.107
15	2.645	0.00125	3.17	0.0911	0.0701

and x_{i-1}. The values of binding capacity and $\bar{X}$ are listed in table 1.

The value of the median oxygen partial pressure was determined by numerical integration of the binding curve $\bar{X}$ vs. the logarithm of oxygen partial pressure according to eq. 10, using the trapezoidal rule. The value of β_1 was calculated according to eq. 12 from four experimental points at the lowest partial pressures. The system of eqs. 13–17 was then solved at each value of oxygen partial pressure for the parameters α * and the resulting species fractions are plotted in fig. 2. The continuous lines were drawn from the best fit values of Adair constants determined by nonlinear least-squares analysis of the data [10]. The method correctly gives a negligible contribution of the triply ligated species [3,10], in agreement with nonlinear least-squares analysis.

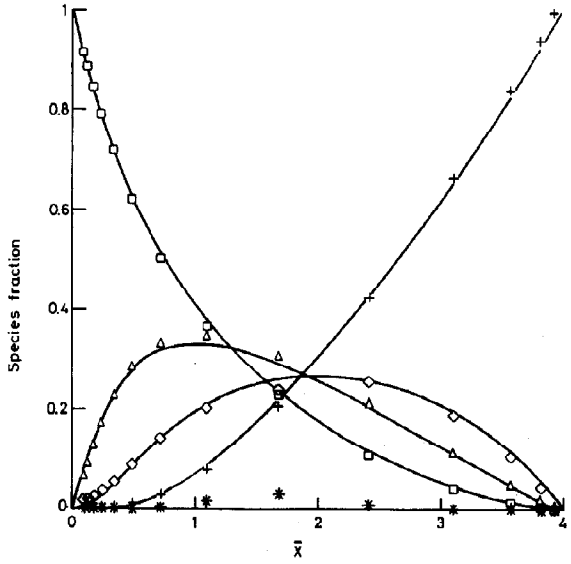

Fig. 2. Species fractions of the oxygenation intermediates of human hemoglobin under the experimental conditions reported in the legend to fig. 1. The species fractions were determined from solution of the system of eqs. 13–17: (■) α_0, (△) α_1, (◇) α_2, (*) α_3, (+) α_4. The continuous lines depict the species fractions calculated from nonlinear least-squares analysis of the data according to eq. 24, with the values of the β terms reported in the legend to fig. 1.

The consistency of the method has extensively been tested by fitting simulated data with known error and overall equilibrium constants. In all cases the distribution of intermediates was found to be consistent, within errors, with that expected. The degree of approximation of the method has been assessed by fitting simulated data with no

* The values of the parameters α were constrained to be positive, the physically meaningful case. Without the constraint α_3 was found to be slightly negative, particularly at very high saturation, as a consequence of the negligible population of the triply ligated species [3].

error. The systematic deviation, when present, was less than 2% on the determination of the species fraction values.

5. Conclusion

The method applied here allows determination of species fractions from experimental binding data in the form of binding capacity [7]. Binding capacity data [3] obtained by means of the thin-layer technique [6] are highly desirable, either because of the underlying thermodynamic relevance of this quantity [7], and because of the high precision and amount of information provided by a differential technique [9]. Another advantage of differential binding data is the possibility, investigated here, of solving the system of linear expressions, eqs. 13–17, with a minimum of analysis. The consistency of this simple and approximate procedure with respect to non-linear least-squares methods is attractive. We feel that this method may be best employed in conjunction with nonlinear least-squares procedures to check the validity of the conclusions drawn from the fitting analysis.

Appendix

In this appendix we correct some misprints of a previous paper [5]. When the Hill plot is used to construct the system of linear equations in the αs one has

$$\alpha_0 + \alpha_1 + \alpha_2 + \alpha_3 + \alpha_4 = 1$$
$$\alpha_1 + 2\alpha_2 + 3\alpha_3 + 4\alpha_4 = m_1 = 2$$
$$\alpha_1 + 4\alpha_2 + 9\alpha_3 + 16\alpha_4 = m_2 = n_{50} + 2$$
$$K_1 x_{50}\alpha_0 - \alpha_1 = 0$$
$$K_4 x_{50}\alpha_3 - \alpha_4 = 0$$

where K_1 and K_4 are the first and fourth intrinsic Adair constants, and x_{50} and n_{50} denote the ligand activity and Hill coefficient at half saturation. Solving for α_1, α_2, and α_3 yields

$$\alpha_1 = (1/2 + K_4 x_{50})n_{50}/D$$
$$\alpha_2 = [(1 + 3/K_1 x_{50} + 3K_4 x_{50} + 8K_4/K_1) - (1 + 3/2K_1 x_{50} + 3K_4 x_{50}/2 + 2K_4/K_1)n_{50}]/D$$
$$\alpha_3 = (1/2 + 1/K_1 x_{50})n_{50}/D$$

with $D = 1 + 3/K_1 x_{50} + 3K_4 x_{50} + 8K_4/K_1$. Consequently, the intrinsic constants K_2 and K_3 are given by

$$K_2 = \frac{\alpha_2}{\alpha_1 x_{50}} = [(1 + 3/K_1 x_{50} + 3K_4 x_{50} + 8K_4/K_1) - n_{50}(1 + 3/2K_1 x_{50} + 3K_4 x_{50}/2 + 2K_4/K_1)] \times [(1/2 + K_4 x_{50})n_{50}]^{-1}$$

$$K_3 = \frac{\alpha_3}{\alpha_2 x_{50}} = [(1/2 + 1/K_1 x_{50})n_{50}] \times [(1 + 3/K_1 x_{50} + 3K_4 x_{50} + 8K_4/K_1) - n_{50}(1 + 3/2K_1 x_{50} + 3K_4 x_{50}/2 + 2K_4/K_1)]^{-1}$$

since K_1 and K_4 can be measured from the Hill plot as intercepts of the lower and upper asymptotes with the $\ln x$ axis.

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