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A new method for the determination of equilibrium constants through binding capacity measurements

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The recent discovery of the negligible contribution of the triply ligated species to the oxygenation process of human hemoglobin A_0 (S.J. Gill, E. Di Cera, M.L. Doyle, G.A. Bishop and C.H. Robert, Biochemistry 26 (1987) 3995) has pointed out the high precision of differential binding measurements. These measurements closely approximate the binding capacity (E. Di Cera, S.J. Gill and J. Wyman, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 449) of the system and can be used to calculate higher derivatives of the binding curve. We develop here a new method for the determination of equilibrium constants through binding capacity measurements by which the physical parameters expressing the optical properties of the system are eliminated in the data analysis.

1. Introduction

We have recently observed that the triply ligated species makes virtually no contribution to the oxygenation process of HbA_0 [1] under a variety of experimental conditions [1–5]. This observation has been made by high precision differential binding measurements by means of the thin-layer method [6]. We have also shown how the method allows measurements of the 'binding capacity' of the macromolecule [7]. This thermodynamic quantity expresses the change of the amount of ligand bound to the macromolecule due to a change in the chemical potential of the ligand, i.e., the derivative of the binding curve [7]. From a different standpoint, it has been shown that the derivative of the binding curve can be utilized to dissect the peculiar thermodynamic features of a binding system, provided one can measure it with sufficient accuracy [8]. With the availability of differential binding data [1–5] it is now possible to show how binding capacity measurements can be used to reduce the number of fitting parameters involved in the analysis of ligand-binding studies. The results are consistent with those obtained by means of other procedures that involve a larger number of parameters.

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2. Rationale

For a system where the degree of saturation of the macromolecule with a given ligand is linearly related to the optical signal of a spectrophotometer, the following equation applies (4)

$$A(x) = A(0) + [A(\infty) - A(0)] \cdot \theta(x) \qquad (1)$$

where $A(x)$ is the absorbance when the ligand activity is x, while $A(0)$ and $A(\infty)$ are the asymptotic values of A in the absence of ligand and under fully saturating conditions. The fractional saturation $\theta(x)$ is given by

$$\theta(x) = \frac{1}{t} \cdot \frac{\beta_1 x + 2\beta_2 x^2 + \ldots + t\beta_t x^t}{1 + \beta_1 x + \ldots + \beta_t x^t} \qquad (2)$$

where the βs are the overall equilibrium constants and t the total number of sites of the macromolecule. As one sees, eq. 1 contains the optical parameters $A(0)$ and $A(\infty)$, and the degree of saturation θ that is a function of the equilibrium constants. In the case of human hemoglobin, in its reaction with O_2 the number of equilibrium constants to be determined is four, and the total number of parameters involved in the fitting analysis is six. The apparatus of Imai et al. [9] is an example of experimental technique for the determination of $\theta(x)$ through eq. 1.

The thin-layer method [6] measures changes in the absorbance upon stepwise dilutions of the ligand. The underlying relation for the i-th dilution is [1]

$$A(x_i) - A(x_{i-1}) = \Delta A(x_i) = \Delta A_T \cdot [\theta(x_i) - \theta(x_{i-1})] \quad (3)$$

One sees, differently from eq. 1, that the difference $A(0) - A(\infty)$ can be bracketed into a single parameter ΔA_T, i.e., the total absorbance change observed upon complete saturation. This reduces the total number of parameters to be determined in the case of human hemoglobin to five. The thin-layer method thus requires one less parameter to be estimated in the fitting procedure and avoids the complications arising from the determination of the asymptotic optical readings $A(0)$ and $A(\infty)$ separately. Furthermore, eq. 3 is a measure of the derivative of the binding curve $d\theta/d\ln x$, the binding capacity of the system [7], since the Taylor expansion yields with very good approximation [2]

$$\Delta A(\xi_i) = \left(\frac{d\theta}{d \ln x}\right)_{\xi_i} \cdot \ln D \cdot \Delta A_T \quad (4)$$

where ξ_i is the geometric mean $\sqrt{x_i x_{i-1}}$ of the two partial pressures that define each dilution step i, and D the dilution factor [1]. A typical differential data set obtained by means of the thin-layer technique is shown in fig. 1, and compared to the the binding capacity according to eq. 4.

The relations above can be used to compute differences between ΔAs, thus approximating the second derivative of the binding curve $d^2\theta/d\ln x^2$. This process can be repeated as many times as the precision of the data can stand. The calculation of higher derivatives yields linkage relations of higher order of particular thermodynamic significance [7]. These derivatives also have an immediate practical application. Consider the expression

$$\Delta A(x_{i+1}) - \Delta A(x_i) = \Delta A_T \cdot [\theta(x_{i+1}) - 2\theta(x_i) + \theta(x_{i-1})] \quad (5)$$

which by Taylor's expansion can be seen to approximate the derivative $d^2\theta/d\ln x^2$. In the case of human hemoglobin, eq. 5 contains five parameters like eq. 3. If we divide eq. 5 by the sum $\Delta A(x_{i+1}) + \Delta A(x_i)$ we obtain at the point x_i

$$R(x_i) = \frac{\Delta A(x_{i+1}) - \Delta A(x_i)}{\Delta A(x_{i+1}) + \Delta A(x_i)} = \frac{\theta(x_{i+1}) - 2\theta(x_i) + \theta(x_{i-1})}{\theta(x_{i+1}) - \theta(x_{i-1})} \quad (6)$$

and the number of fitting parameters is reduced to the equilibrium constants which define θ.

The ratio in eq. 6 can be computed from binding capacity measurements, but its form is such that the points obtained for intermediate values of the ligand activity will incorrectly receive less weight in the analysis. This is because the denominator can be seen as a weighting factor of the numerator. The correct weighting factor for each point is [10]

$$w(x_i) = 1 \Big/ \left\{ \left(\frac{\partial R(x_i)}{\partial \Delta A(x_i)} \right)^2 \cdot (\delta \Delta A(x_i))^2 + \left(\frac{\partial R(x_i)}{\partial \Delta A(x_{i+1})} \right)^2 \cdot (\delta \Delta A(x_{i+1}))^2 \right\} \quad (7)$$

where the δs are the standard deviations of the differential measurements. Since the error is constant and uniformly distributed among the differential absorbance readings [1], one can multiply w by δ^2 to obtain

$$\bar{w}(x_i) = \frac{1}{4} \cdot \frac{(\Delta A(x_i) + \Delta A(x_{i+1}))^4}{\Delta A(x_i)^2 + \Delta A(x_{i+1})^2} \quad (8)$$

Hence the relation

$$\bar{\sigma}^2 = \sum \bar{w}(x_i) \cdot [F(x_i) - R(x_i)]^2, \quad (9)$$

where $\bar{\sigma}^2$ is the variance times δ^2 and F the fitting function, gives the correct expression to be minimized by nonlinear least-squares.

3. Materials and methods

The O_2-binding measurements were performed by means of the thin-layer method [6] as described in detail elsewhere [1]. The α- and β-chains from HbA_0 were a gift from Dr. Lawrence Parkhurst and were isolated according to the procedure of Geraci et al. [11]. Horse Mb was from Sigma. The solution conditions of all experiments were: 200 μM heme, 0.1 M phosphate, pH 7.5, 25°C [4]. Human HbA_0 was prepared according to the method reported by Williams and Tsay [12]. The experimental conditions were: 2 mM heme, 0.1 M Hepes, 0.1 M NaCl, 20 mM IHP, 1 mM Na_2EDTA, pH 7.0, 25°C. All fitting parameters were estimated by nonlinear least-squares minimization as described elsewhere [1]. Confidence intervals were determined at the 67% confidence cut-off by F-testing [10]. A Hewlett-Packard 9000/300 computer was employed in all analyses.

4. Results

The O_2-binding capacity of HbA_0 is shown in fig. 1 and the data transformed according to eq. 6

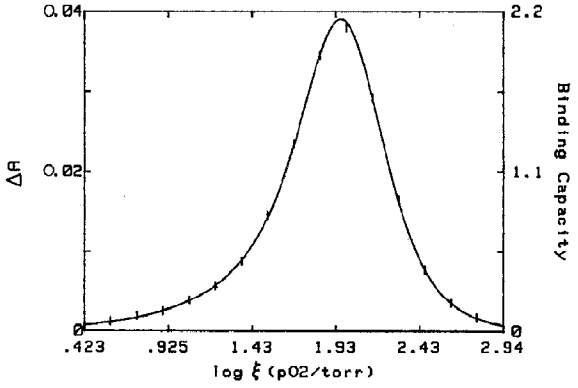

Fig. 1. Differential binding data for O_2 binding to HbA_0 under the experimental conditions described in the text. Bars are 8-times the standard error of the point. The curve depicts the binding capacity and was drawn according to eq. 4 with the parameter values given in table 1, as determined from fitting the data to eq. 3.

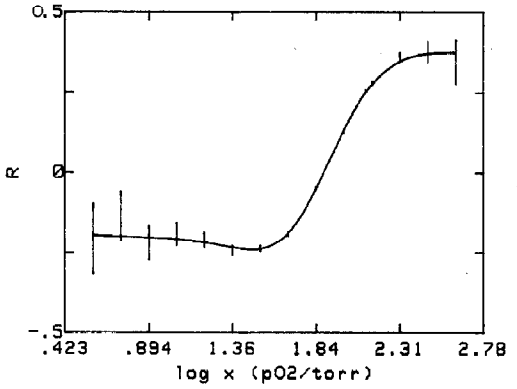

Fig. 2. Transformation of the binding capacity data shown in fig. 1 according to the method discussed in the text. Bars are 4-times the standard error of the point. Note how the errors are not uniform in this plot. The curve was drawn according to eq. 6 with the parameter values given in table 1.

are sketched in fig. 2. The O_2 equilibrium constants obtained from eq. 6 (see table 1) are consistent with those determined from eq. 3. The results obtained for simple systems such as the isolated chains and horse Mb are given in table 1 and are in agreement with the literature values [13–15]. In all cases the parameters estimated by the new method according to eq. 6 are in substantial agreement with those determined by fitting binding capacity data to eq. 3.

5. Discussion

The reduction of the number of parameters to be determined in a fitting procedure is a general concern. Very often the reduction is accomplished incorrectly by arbitrary extrapolations. In the case of data obtained by the apparatus of Imai et al. [9], for example, the optical parameters $A(0)$ and $A(\infty)$ are commonly fixed [16] in the fitting procedure, with the result that the equilibrium constants are biased [17] and fundamental properties of the ligation intermediates [1–5] are obscured. The thin-layer method [6] provides a meaningful reduction of the number of fitting parameters, since it collects data in a differential form, thus avoiding the determination of the end parameters $A(0)$ and $A(\infty)$ and any possible bias arising from

Table 1

Parameter values obtained from fitting the binding capacity data to eq. 3 (bold) and to eq. 6

The error intervals are at 67% confidence as determined by *F*-testing.

	β_1 ($torr^{-1}$)	β_2 ($torr^{-2}$)	β_3 ($torr^{-3}$)	β_4 ($torr^{-4}$)	ΔA_T	$\bar{\sigma}^2$
α-Chain	**1.4 ±0.1**	–	–	–	**0.0233±0.0004**	**0.00010**
	1.4 ±0.1	–	–	–	–	0.0062
β-Chain	**3.3 ±0.1**	–	–	–	**0.0212±0.0002**	**0.000043**
	3.3 ±0.1	–	–	–	–	0.0036
Horse Mb	**0.95 ±0.05**	–	–	–	**0.0247±0.0001**	**0.000027**
	0.96 ±0.08	–	–	–	–	0.0030
HbA_0	**0.019±0.002**	**0.00034±0.00008**	**0±5.5·10^{-9}**	**7.6·10^{-8}±3.2·10^{-9}**	**0.1972±0.0008**	**0.00012**
	0.018±0.003	0.00034±0.00012	0±5.4·10^{-9}	7.4·10^{-8} ±4.1·10^{-9}	–	0.0014

fixing them. The data collected in this way are a measure of the binding capacity [7] and can be used to dissect the fundamental thermodynamic features of the system. The method discussed here shows that by taking the ratio of binding capacity measurements one can reduce the number of fitting parameters even further by dropping the optical parameter ΔA_T and focusing the analysis on the equilibrium constants alone.

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