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Determination of the seven apparent equilibrium constants for the binding of oxygen by hemoglobin from measured fractional saturations

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Abstract

Subunit dissociation has to be taken into account in the determination of the oxygen binding constants of hemoglobin, as described by Ackers and Halvorson in 1974. The seven apparent equilibrium constants for a particular set of conditions can be determined by using extrapolations to determine the fractional saturations Y_T of tetramer and Y_D of dimer from measured values of the fractional saturation Y of partially dissociated hemoglobin. Analytical methods are used to show that Y_T as a function of $[O_2]$ for tetramers can be calculated from Y of hemoglobin by linear extrapolation of measured Y values at high [heme] versus $[\text{heme}]^{-1/2}$ to $[\text{heme}]^{-1/2} = 0$. Y_D for dimers can be calculated from measured Y values by linear extrapolation of Y versus [heme] to [heme] = 0 if sufficiently low [heme] can be used. These extrapolations have been tested with numerical calculations of Y for a particular hemoglobin as a function of [heme] and $[O_2]$ by using the seven apparent equilibrium constants determined by Mills, Johnson, and Ackers in 1976. The proposed procedure also yields the apparent association constant K'' for 2TotD = TotT, where TotD is the sum of the dimers and TotT is the sum of the tetramers. This thermodynamic analysis of experimental data to determine the seven apparent equilibrium constants is independent of the model used to interpret the values of the thermodynamic parameters.

Keywords: Equilibrium constants for hemoglobin; Subunit dissociation of hemoglobin; Hemoglobin thermodynamics; Apparent equilibrium constants

1. Introduction

The interpretation of the fractional saturation Y of heme in hemoglobin by oxygen is complicated by the subunit dissociation. In 1974 Ackers and Halvorson [1] derived an analytic expression for the fractional saturation Y of hemoglobin when it is partially dissociated into dimers. As they point out, the equation that they derived, which involves equilibrium constants and concentrations of molecular oxygen and of heme in the system, is model independent and defines the phenomenological framework to which both experimental data and proposed models must conform. Because of the subunit dissociation, the two-component system (hemoglobin and molecular oxygen) involves nine reactants. The term reactants is used here rather than species because pH, $[Cl^-]$, and perhaps the concentrations of other species affect the binding of oxygen and have to be

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specified. When the pH and perhaps the free concentrations of other species are specified, the criterion of equilibrium is the transformed Gibbs energy G' and the equilibrium constants K' are referred to as apparent equilibrium constants to indicate that they are functions of pH, etc., as explained in the preceding article [2]. Since the number R' of independent equilibrium constant expressions is equal to the number N' of reactants minus the number C' of apparent components, seven independent reactions are required to discuss the chemical equilibria at specified pH, etc.; $R' = N' - C' = 9 - 2 = 7$ [3]. The choice of these seven reactions is not unique.

In studying oxygen binding by hemoglobin, it is convenient to consider $[O_2]$ as an independent variable. When $[O_2]$ is specified, the system at specified T, P, and pH does not go to the state with the minimum G'. In this case, it is necessary to define a further transformed Gibbs energy G'', as explained in the preceding article. When $[O_2]$ is specified, the hemoglobin-oxygen system becomes a one-component system. There are two reactants; one is made up of all the reactants containing tetramer and is referred to as TotT and the other is made up of all the reactants containing dimer and is referred to as TotD. The reaction is represented by

$$2\text{TotD} = \text{TotT} \tag{1}$$

The number R'' of independent reactions is equal to the number N'' of reactants minus the number C'' of apparent components. At specified $[O_2]$, there is a single reaction; $R'' = N'' - C'' = 2 - 1 = 1$.

This article provides calculations of the behavior of hemoglobin that has the seven apparent equilibrium constants determined by Mills, Johnson, and Ackers [4] at 21.5°C, 1 bar, pH 7.4, $[Cl^-] = 0.2$ M, and 0.2 M ionic strength. Binding experiments yield the fractional saturation Y of the heme in the solution, and, in principle, the determination of Y as a function of $[O_2]$ and [heme] could yield the seven equilibrium constants by nonlinear least squares treatment of all of the data together. However, there are several problems: (1) the cooperativity in the binding by the tetramer makes it difficult to determine the four constants involved, (2) the association of the dimers in the absence of molecular oxygen is strong and becomes much weaker as $[O_2]$ is increased, (3) the experimental Y values need to be tested to be sure that data have been obtained over sufficiently wide ranges of $[O_2]$ and [heme], and (4) the experimentally accessible range of hemoglobin concentrations is currently only about 0.04 μM to 5 mM [4,5].

The errors introduced by neglecting the dissociation of hemoglobin have been emphasized by Johnson and Lassiter [6]. As [heme] is increased, dimers make up a smaller fraction of the hemoglobin, but at 5 mM hemoglobin at pH 7.4, their effect on Y cannot be neglected. As [heme] is lowered to 0.04 μM, the dissociation of the tetramer increases, but it is not complete. This article is based on the concept that extrapolations can be used to determine both the fractional saturation Y_T of the tetramer and the fractional saturation Y_D of the dimer. This procedure also yields the equilibrium constant for the association of dimers to tetramers (Eq. (1)) at specified $[O_2]$.

2. Basic assumptions and calculations

It is assumed that hemoglobin is partially dissociated into dimers and that this reaction is at equilibrium. The tetramers and dimers are assumed to follow their respective Adair equations at specified T, P, pH, $[Cl^-]$, etc. The issue is "What procedures should be used for analyzing the experimental data with the objective of determining the seven equilibrium constants involved without assumptions about the model or information from other experimental methods?" A procedure is described here for the determination of Y_T, Y_D, and K'' at specified $[O_2]$ from measurements of Y as a function of [heme]. Repeating these calculations at a series of $[O_2]$ yields the seven apparent equilibrium constants for a particular T, P, pH, $[Cl^-]$, etc. This procedure is explored by calculating equilibrium concentrations of $D(O_2)_2$, DO_2, D, T, TO_2, $T(O_2)_2$, $T(O_2)_3$, and $T(O_2)_4$ using a specific set of the seven equilibrium constants over a wider range of [heme] and $[O_2]$ than is possible experimentally in order to provide a test of two types of extrapolations.

Table 1
Calculated equilibrium composition (molar concentrations) and fractional saturation Y at 21.5°C, 1 bar, pH 7.4, $[Cl^-] = 0.2$ M, and ionic strength 0.2 M

$[O_2]$/M	5×10^{-6}	10^{-5}	2×10^{-5}
A. Hemoglobin at [heme] = 10^{-3} M			
$D(O_2)_2$	$4.169\,76\times10^{-6}$	$1.083\,62\times10^{-5}$	$1.477\,98\times10^{-5}$
DO_2	$1.022\,63\times10^{-6}$	$1.328\,78\times10^{-6}$	$9.061\,78\times10^{-7}$
D	$6.287\,29\times10^{-8}$	$4.084\,79\times10^{-8}$	$1.392\,84\times10^{-8}$
T	$1.831\,42\times10^{-4}$	$7.730\,39\times10^{-5}$	$8.987\,97\times10^{-6}$
TO_2	$4.026\,38\times10^{-5}$	$3.399\,05\times10^{-5}$	$7.904\,02\times10^{-6}$
$T(O_2)_2$	$2.458\,11\times10^{-6}$	$4.150\,24\times10^{-6}$	$1.930\,16\times10^{-6}$
$T(O_2)_3$	$4.976\,44\times10^{-6}$	$1.680\,43\times10^{-5}$	$1.563\,05\times10^{-5}$
$T(O_2)_4$	$1.653\,17\times10^{-5}$	$1.116\,48\times10^{-4}$	$2.076\,97\times10^{-4}$
B. Hemoglobin at [heme] = 10^{-5} M			
$D(O_2)_2$	$3.976\,24\times10^{-7}$	$9.698\,66\times10^{-7}$	$1.281\,17\times10^{-6}$
DO_2	$9.751\,65\times10^{-8}$	$1.189\,29\times10^{-7}$	$7.855\,12\times10^{-8}$
D	$5.995\,48\times10^{-9}$	$3.655\,98\times10^{-9}$	$1.207\,37\times10^{-9}$
T	$1.665\,37\times10^{-6}$	$6.192\,55\times10^{-7}$	6.75367×10^{-8}
TO_2	$3.661\,31\times10^{-7}$	$2.722\,86\times10^{-7}$	$5.939\,18\times10^{-8}$
$T(O_2)_2$	$2.235\,23\times10^{-8}$	$3.324\,62\times10^{-8}$	$1.450\,35\times10^{-8}$
$T(O_2)_3$	$4.525\,23\times10^{-8}$	$1.346\,14\times10^{-7}$	$1.174\,49\times10^{-7}$
$T(O_2)_4$	$1.503\,28\times10^{-7}$	$8.943\,74\times10^{-7}$	$1.560\,66\times10^{-6}$
C. Fractional saturation			
Y_T	0.127 577	0.552 791	0.918 279
Y([heme] = 10^{-3} M)	0.135 598	0.562 297	0.919 911
Y([heme] = 10^{-5} M)	0.204 067	0.637 877	0.932 426
Y_D	0.890 741	0.942 221	0.970 253

The equilibrium compositions are calculated by use of equcalc, a general equilibrium program written by Krambeck [7,8]. The calculation of the equilibrium composition requires a Newton–Raphson iteration to find the composition that minimizes the Gibbs energy (or transformed Gibbs energy) and satisfies the conservation equations for the system. These calculations are discussed in more detail in Appendix A and the basic programs in Mathematica [9] are given.

An example of the output of equcalcc is given in Table 1 in which the calculated equilibrium composition is given at [heme] $= 10^{-3}$ and 10^{-5} M for $[O_2] = 5\times10^{-6}$, 10^{-5}, and 2×10^{-5} M. This table indicates how Y depends on [heme], and this dependence is more thoroughly explored in this paper.

For the calculations reported here it is convenient to consider Y_T, Y_D, Y, and K'' to be defined by vector products (dot products). As described in Appendix A, vectors of $\underset{\sim}{c}$ equilibrium concentrations of the reactants $D(O_2)_2$, DO_2, D, T, TO_2, $T(O_2)_2$, $T(O_2)_3$, and $T(O_2)_4$ are calculated using equcalcc. The equilibrium concentration of molecular oxygen bound by the tetramer is given by the dot product

$$[\mathrm{TotO_2}]_T = \{0,0,0,0,1,2,3,4\} \cdot \underset{\sim}{c} \tag{2}$$

The equilibrium concentration of molecular oxygen bound by the dimer is given by the dot product

$$[\mathrm{TotO_2}]_D = \{2,1,0,0,0,0,0,0\} \cdot \underset{\sim}{c} \tag{3}$$

The total equilibrium concentration of tetramer is given by

$$[\mathrm{TotT}] = \{0,0,0,1,1,1,1,1\} \cdot \underset{\sim}{c} \tag{4}$$

The total equilibrium concentration of dimer is given by

$$[\mathrm{TotD}] = \{1,1,1,0,0,0,0,0\} \cdot \underset{\sim}{c} \tag{5}$$

The fractional saturation of tetramer is given by

$$Y_\mathrm{T} = \frac{[\mathrm{TotO_2}]_\mathrm{T}}{4[\mathrm{TotT}]} \tag{6}$$

The fractional saturation of dimer is given by

$$Y_\mathrm{D} = \frac{[\mathrm{TotO_2}]_\mathrm{D}}{2[\mathrm{TotD}]} \tag{7}$$

The concentration of heme is given by

$$[\mathrm{heme}] = 2[\mathrm{TotD}] + 4[\mathrm{TotT}] \tag{8}$$

The fractional saturation of hemoglobin is given by

$$\mathrm{Y} = \frac{[\mathrm{TotO_2}]_\mathrm{T} + [\mathrm{TotO_2}]_\mathrm{D}}{[\mathrm{heme}]} \tag{9}$$

The apparent equilibrium constant for the association of dimers to form tetramer (Eq. (1)) at specified $[O_2]$ is given by

$$K'' = \frac{[\mathrm{TotT}]}{[\mathrm{TotD}]^2} \tag{10}$$

Although K'' is considered to be dimensionless, the c° (standard concentration of 1 M) required here to make the right side dimensionless is omitted to simplify the later derivations and equations. The use of dot products avoids programming the polynomials that would otherwise be involved in these calculations.

3. Derivation of the dependence of the fractional saturation Y of heme on [heme] at specified $[O_2]$

The equation for the fractional saturation Y of hemoglobin (tetramer in equilibrium with dimer) was derived correctly by Ackers and Halvorson [1] in 1974 and has frequently been used to calculate the dependence of Y on these two independent variables. However, in investigating these dependencies, I have found that this equation can be written in a simpler way that can be used to derive linear limiting forms at high and low [heme]. This derivation is based on writing Y as the sum of the contributions by the dimer and the tetramer:

$$Y = f_\mathrm{D} Y_\mathrm{D} + f_\mathrm{T} Y_\mathrm{T} \tag{11}$$

where $f_\mathrm{D} = 2[\mathrm{TotD}]/[\mathrm{heme}]$ is the fraction of the heme in the dimer and $f_\mathrm{T} = 4[\mathrm{TotT}]/[\mathrm{heme}]$ is the fraction of the heme in the tetramer. The concentration of heme in the solution is given by

$$[\mathrm{heme}] = 2[\mathrm{TotD}] + 4[\mathrm{TotT}] = 2[\mathrm{TotD}] + 4K''[\mathrm{TotD}]^2 \tag{12}$$

where Eq. (10) has been used in writing the last form. Applying the quadratic formula shows that the equilibrium concentration of TotD is given by

$$[\mathrm{TotD}] = \frac{-2 + (4 + 16K''[\mathrm{heme}])^{1/2}}{8K''} \tag{13}$$

Since $f_T = 1 - f_D$, Eq. (11) can be written

$$Y = Y_T + f_D(Y_D - Y_T) \tag{14}$$

The fraction of heme in the dimer is given by

$$f_D = \frac{2[\mathrm{TotD}]}{2[\mathrm{TotD}] + 4[\mathrm{TotT}]} = \frac{1}{1 + 2K''[\mathrm{TotD}]} \tag{15}$$

Substituting Eq. (13) into Eq. (15) yields

$$f_D = \frac{2}{1 + (1 + 4K''[\mathrm{heme}])^{1/2}} \tag{16}$$

Substituting this equation into Eq. (14) yields

$$Y = Y_T + \frac{2(Y_D - Y_T)}{1 + (1 + 4K''[\mathrm{heme}])^{1/2}} \tag{17}$$

This equation is general and shows how the fractional saturation depends on [heme] at specified $[O_2]$. Eq. (17) shows that at specified $[O_2]$, the fractional saturation of heme is a function only of [heme] in a way that is determined by three parameters Y_T, Y_D, and K''. This equation is the same as that derived by Ackers and Halvorson [1], although it has a rather different form.

Eq. (17) suggests the strategy of determining Y_T, Y_D, and K'' at specified $[O_2]$ by varying [heme] and using extrapolations to determine Y_T and Y_D. The experimental problem is that there are limits on [heme] that are accessible experimentally at both high and low concentrations. If it is indeed possible to extrapolate measured values of Y to [heme] = ∞, Eq. (17) indicates that Y_T would be obtained. If it is indeed possible to extrapolate measured values of Y to [heme] = 0, Eq. (17) indicates that Y_D would be obtained. Because of the experimental limits on [heme], it is important to be sure that these extrapolations are linear in the range of experimentally accessible [heme]. The values of the limiting slopes are also of interest because they provide a way to determine K''. Note that if the slope of a plot is large, the determination of the intercept will be more uncertain.

4. Derivation of the limiting form of Eq. (17) at high [heme]

As is evident from Eq. (17), the question as to whether [heme] is high or low depends on whether [heme] > $1/4\ K''$ or [heme] < $1/4\ K''$. Of course, this criterion depends on $[O_2]$. In considering plots of Y versus some function of [heme], [heme] = $1/4\ K''$ will be used to divide the dependence of Y on [heme] into high-heme and low-heme regions. If $4\ K'[\mathrm{heme}] \gg 1$, Eq. (17) reduces to

$$Y = Y_T + \frac{(Y_D - Y_T)}{(K'')^{1/2}[\mathrm{heme}]^{1/2}} \tag{18}$$

Thus a plot of Y versus $[\mathrm{heme}]^{-1/2}$ at a specified $[O_2]$ must approach linearity as [heme] is increased. The intercept of the limiting slope of a plot of Y versus $[\mathrm{heme}]^{-1/2}$ at $[\mathrm{heme}]^{-1/2} = 0$ is Y_T, and the limiting slope

is $(Y_D - Y_T)/(K'')^{1/2}$. This slope is determined by two factors, $(Y_D - Y_T)$ and K''. The slope will be low at high $[O_2]$ because $Y_D - Y_T$ is small. The slope will be low at low $[O_2]$ because K'' is large.

Once Y_T has been determined at a series of $[O_2]$ by use of by use of extrapolations of this type, K'_{41}, K'_{42}, K'_{43}, and K'_{44} can be calculated by the method of nonlinear least squares.

5. Derivation of the limiting form of Eq. (17) at low [heme]

If 4 K''[heme] $\ll 1$, the square root term in Eq. (17) can be rewritten using

$$(1+x)^{1/2} \approx 1 + x/2 \quad (x \ll 1) \tag{19}$$

to obtain

$$Y = Y_T + \frac{(Y_P - Y_T)}{1 + K''[\text{heme}]} \tag{20}$$

Since 4 K''[heme] $\ll 1$ is satisfied, we can use

$$\frac{1}{1+x} \approx 1 - x \quad (x \ll 1) \tag{21}$$

to obtain

$$Y = Y_D - (Y_D - Y_T)\,K''[\text{heme}] \tag{22}$$

Thus as [heme] $\rightarrow 0$, Y becomes a linear function of [heme], and Y approaches Y_D. The condition that 4 K''[heme] $\ll 1$ is hard to satisfy experimentally because low concentrations of heme have to be used. There is a steep slope at low $[O_2]$ because $(Y_D - Y_T)$ and K'' are both large. At high $[O_2]$ the slope will be smaller because $(Y_D - Y_T)$ and K'' are smaller. The determination of Y_D at a series of $[O_2]$ yields K'_{21} and K'_{22}.

Once Y_T and Y_D have been determined by extrapolation, the slope of each plot at specified $[O_2]$ yields K''. It is also possible to calculate K'' from each measured Y value by use of Eq. (17) written in the form

$$\frac{\left[\dfrac{2(Y_D - Y_T)}{Y - Y_T} - 1\right]^2 - 1}{4[\text{heme}]} = K'' \tag{23}$$

The optimum use of this equation needs to be investigated. The determination of K'' at a series of $[O_2]$ and knowledge of Y_T and Y_D as a function of $[O_2]$ makes it possible to calculate ${}^{o}K'_2$, the apparent association constant for the reaction 2D = T at the specified T, P, pH, $[Cl^-]$, ionic strength, etc.

6. Derivation of the slope of the plot of Y versus [heme] or [heme]$^{-1/2}$ at any value of [heme]

Another way to investigate the slopes of these plots is simply to take the derivative of Eq. (17) with respect to [heme]. This yields the slope of a plot of Y against [heme] at specified $[O_2]$:

$$\frac{dY}{d[\text{heme}]} = \frac{-4\,K''(Y_D - Y_T)}{\left(1 + (1 + 4\,K''[\text{heme}])^{1/2}\right)^2 (1 + 4\,K''[\text{heme}])^{1/2}} \tag{24}$$

When 4 K'' [heme] $\ll 1$, this reduces to the slope indicated by Eq. (22), as expected. When [heme] $\rightarrow \infty$,

$dY/d[\text{heme}]$ approaches zero. It is also of interest to consider Eq. (24) when $[\text{heme}] = 1/4\ K''$, which separates the high and low heme regions mentioned above. At this [heme],

$$\frac{dY}{d[\text{heme}]} = \frac{-4\ K''(Y_D - Y_T)}{\{1 + 2^{1/2}\}^2 2^{1/2}} = \frac{-K''(Y_D - Y_T)}{2.0607} \tag{25}$$

Thus when $[\text{heme}] = 1/4\ K''$, the slope is about half of the limiting slope at low [heme].

The derivative of Y with respect to $[\text{heme}]^{-1/2}$ at specified $[O_2]$ is

$$\frac{dY}{d[\text{heme}]^{-1/2}} = \frac{8\ K''(Y_D - Y_T)[\text{heme}]^{3/2}}{\{1 + (1 + 4\ K''[\text{heme}])^{1/2}\}^2 (1 + 4\ K''[\text{heme}])^{1/2}} \tag{26}$$

When $4\ K''[\text{heme}] \gg 1$, this reduces to the slope indicated by Eq. (18), as expected. When $4\ K''[\text{heme}] \ll 1$,

$$\frac{dY}{d[\text{heme}]^{-1/2}} = 2\ K''(Y_D - Y_T)[\text{heme}]^{3/2} \tag{27}$$

Thus the slope of a plot of Y versus $[\text{heme}]^{-1/2}$ approaches zero as $[\text{heme}] \to 0$. Of course, as $[\text{heme}] \to 0$, $Y \to Y_D$. It is also of interest to calculate the slope of this plot at the condition $[\text{heme}] = 1/4\ K''$, which separates the two regions mentioned above. Substituting this relation in Eq. (26) yields

$$\frac{dY}{d[\text{heme}]^{-1/2}} = \frac{Y_D - Y_T}{\{1 + 2^{1/2}\}^2 2^{1/2} (K'')^{1/2}} = \frac{Y_D - Y_T}{8.2426 (K'')^{1/2}} \tag{28}$$

Thus when $[\text{heme}] = 1/4\ K''$, the slope has been reduced by a factor of 8.2426 on its way to zero as $[\text{heme}] \to 0$.

7. Calculations and recommended procedures for analyzing experimental data

As explained in Section 2, the calculation of the equilibrium concentrations at specified $[O_2]$ and [heme] for a given set of the seven apparent equilibrium constants make it possible to calculate the fractional saturation Y of hemoglobin, but it also makes possible the calculation of Y_T, Y_D, and K'', which are functions of $[O_2]$ that do not depend on [heme]. By calculating the equilibrium concentrations at $[\text{heme}] = 2 \times 10^{-2}$ M and $[O_2] = 10^{-4}$, 2×10^{-5}, 10^{-5}, 5×10^{-6}, 10^{-6}, 10^{-7}, and 10^{-8} M, it is therefore possible to calculate Y_T, Y_D, and K'' at these $[O_2]$ by using Eqs. (2)–(10). These values are summarized in Table 2. Note that the apparent association

Table 2
Values of properties of the hemoglobin–oxygen system at 21.5°C, 1 bar, pH 7.4, $[Cl^-] = 0.2$ M, and 0.2 M ionic strength that are independent of [heme] but are functions of $[O_2]$

$[O_2]$/M	Y_T	Y_D	K''	$(Y_D - Y_T)/(K'')^{1/2}$	$-(Y_D - Y_T)K''$	$[\text{heme}]_{div}$/M
10^{-4}	0.995 820	0.993 906	$9.425\,06 \times 10^5$	$-1.971\,26 \times 10^{-6}$	$1.803\,72 \times 10^3$	$2.652\,50 \times 10^{-7}$
2×10^{-5}	0.918 279	0.970 253	$9.824\,08 \times 10^5$	$5.243\,74 \times 10^{-5}$	$-5.105\,99 \times 10^4$	$2.544\,77 \times 10^{-7}$
10^{-5}	0.552 791	0.9422 21	$1.637\,08 \times 10^6$	$3.043\,65 \times 10^{-4}$	$-6.375\,30 \times 10^5$	$1.527\,11 \times 10^{-7}$
5×10^{-6}	0.127 577	0.890 741	$8.957\,00 \times 10^6$	$2.549\,98 \times 10^{-4}$	$-6.835\,66 \times 10^6$	$2.791\,11 \times 10^{-9}$
10^{-6}	0.011 072	0.619 669	$1.015\,06 \times 10^9$	$1.910\,22 \times 10^{-5}$	$-6.177\,65 \times 10^8$	$2.462\,90 \times 10^{-10}$
10^{-7}	0.001 097	0.139 942	$2.546\,40 \times 10^{10}$	$8.700\,96 \times 10^{-7}$	$-3.535\,56 \times 10^9$	$9.817\,77 \times 10^{-12}$
10^{-8}	0.000 110	0.016 005	$4.345\,35 \times 10^{10}$	$7.625\,37 \times 10^{-8}$	$-6.907\,14 \times 10^8$	$5.753\,28 \times 10^{-12}$

These properties are Y_T, Y_D, K'', the value of the limiting slopes, $(Y_D - Y_T)/(K'')^{1/2}$, of Y versus $[\text{heme}]^{-1/2}$ at high [heme], and the limiting slopes, $-(Y_D - Y_T)K''$, of Y versus [heme] at low [heme]. The last column gives [heme] dividing the high and low concentration regions described in the text.

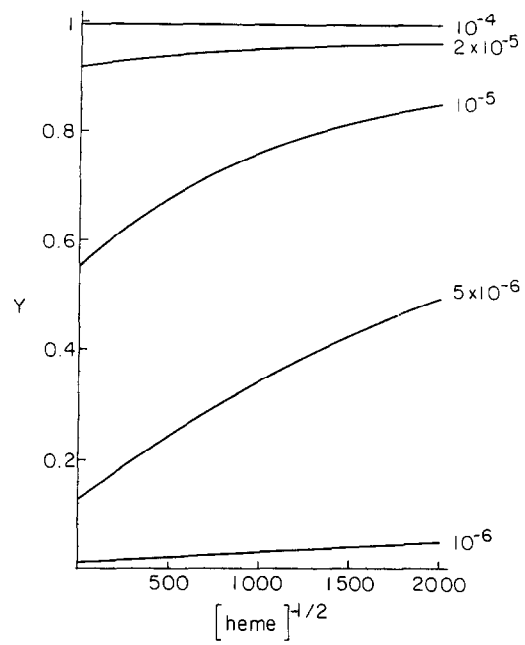

Fig. 1. Calculated plots of Y versus $[\text{heme}]^{-1/2}$ at five $[O_2]$, expressed as molar concentrations. Heme concentrations go as low as 0.25×10^{-6} M. The intercepts at $[\text{heme}]^{-1/2} = 0$ give Y_T. The limiting slopes are in agreement with Eq. (18). The limiting slope is given by $(Y_D - Y_T)/(K'')^{1/2}$, for which values are available in Table 2.

constants K'' of dimers to tetramers shows a positive cooperative effect; between $[O_2] = 5 \times 10^{-6}$ M and 10^{-6} M, K'' increases by a factor of about 100. This is due to the positive cooperative effect in the binding of oxygen by the tetramer.

The values of Y_T, Y_D, and K'' can be used to calculate Y at any desired [heme] by use of Eq. (17), and they have been used to calculate the plots shown in Figs. 1–4 by use of *Mathematica* [9]. Fig. 1 shows a plot of Y versus $[\text{heme}]^{-1/2}$ for $[O_2] = 10^{-4}$, 2×10^{-5}, 10^{-5}, 5×10^{-6}, and 10^{-6} M. The lowest heme concentration is this plot is 0.25×10^{-6} M. If lower heme concentrations had been used, all of the plots would have all levelled off at the Y_D values at these $[O_2]$. These plots show that the steepest initial slopes are in the neighborhood of $Y = 0.5$, as is indicated by Table 2. Fig. 2 shows the same plots down to a heme concentrations of 0.25×10^{-4} M. A $[O_2] = 10^{-5}$ M, the plot is slightly curved, but the figure shows that measurements at $[\text{heme}] > 10^{-5}$ M can be used to extrapolate to the correct value of Y_T at the intercept. The highest [heme] that has been used so far in the laboratory (0.02 M) is about 7 on the abscissa of this figure.

Fig. 3 shows a plot of Y versus [heme] for $[O_2] = 10^{-4}$, 2×10^{-5}, 10^{-5}, 5×10^{-6}, 10^{-6}, 10^{-7}, and 10^{-8} M. If these plots were followed to higher [heme], they would level off at the values of Y_T. Note that the limiting slopes at low [heme] become more and more negative as $[O_2]$ decreases until $[O_2] = 10^{-7}$ M. At 10^{-8} M the slope is not as negative as at 10^{-7} M (see Table 2). This figure shows that binding experiments have to be made at lower $[O_2]$ in order to be in the linear region. Fig. 4 shows a plot of Y versus $[O_2]$ up to 10^{-9} M. Calculations in this range of concentrations do indicate the region in which it is possible to make linear extrapolations to Y_D. The upper half of the binding curve for the dimer is easier to determine than the lower half. The direct determination of K'_{21} and K'_{22} from oxygen binding experiments will require very low [heme], which has not yet been achieved in oxygen binding experiments. Other hemoglobins may dissociate to dimers to a greater extent, so that this determination will be easier to make.

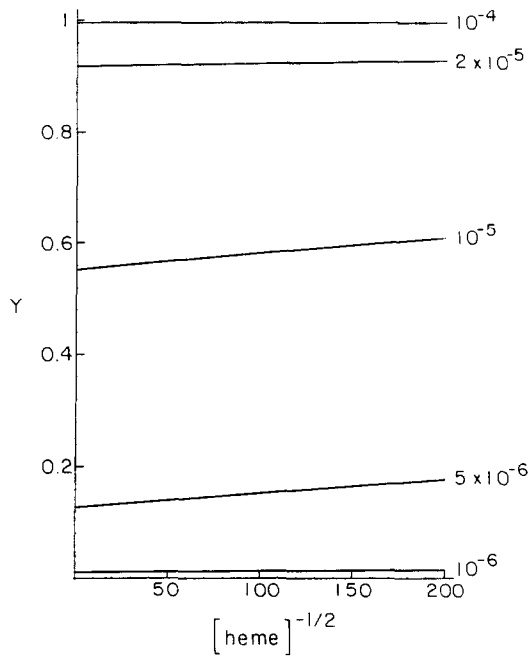

Fig. 2. Calculated plots of Y versus $[\text{heme}]^{-1/2}$ for heme concentrations as low as 0.25×10^{-4} M. These plots are not exactly linear, but approach linearity as the concentration of heme is raised.

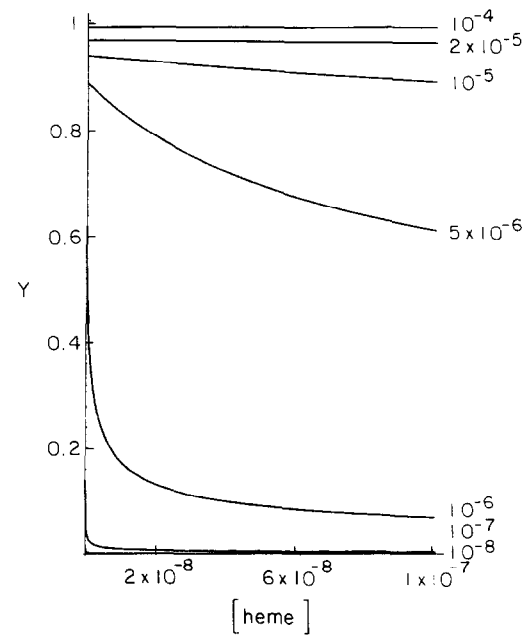

Fig. 3. Calculated plots of Y versus [heme] at heme concentrations below 10^{-7} M at seven $[O_2]$, expressed as molar concentrations. The intercepts give the values of Y_D, and the limiting slopes are in agreement with Eq. (22). The limiting slopes are so steep on this scale that the intercepts cannot be identified at the three lowest $[O_2]$.

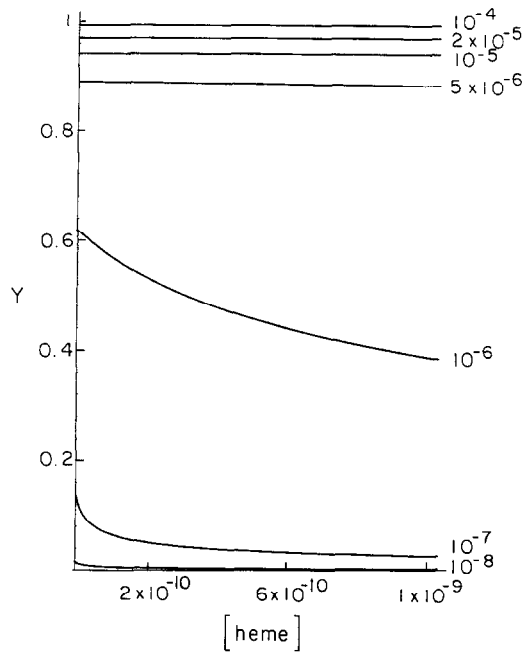

Fig. 4. Calculated plots of Y versus [heme] at heme concentrations below 10^{-9} M. The plots in this concentration range do show the limiting slopes and the values of Y_D at [heme] = 0.

On the basis of these calculations, the following procedure is recommended for the determination of the seven apparent equilibrium constants for hemoglobin at specified T, P, pH, [Cl^-], ionic strength, etc. from the fractional saturation Y measured over very wide ranges of [heme] and [O_2]: (1) To determine Y_T, plots should be made of Y versus $[\text{heme}]^{-1/2}$ at the highest heme concentrations used at a series of [O_2]. Treatment of Y_T as a function of [O_2] by nonlinear least squares yields K'_{41}, K'_{42}, K'_{43}, and K'_{44}. (2) To determine Y_D, plots should be made of Y versus [heme] at the lowest [heme] used at a series of [O_2]. Treatment of Y_D as a function of [O_2] by nonlinear least squares yields K'_{21}, and K'_{22}. It may be difficult to go to sufficiently low [heme], but this may be possible by using long absorption cells, multipath cells, or Fourier transform spectrometers. (3) Once Y_T and Y_D have been calculated, each measured Y value yields a value of K'' at a particular [O_2], as indicated by Eq. (23). The best range of Y values for making these calculations remains to be investigated. Values of K'' can also be calculated from slopes of the plots used in (1) and (2). The best values of K'' may be obtained from the slope of Y versus $[\text{heme}]^{-1/2}$ because these limiting slopes are most easily determined. Once the values of the oxygen association constants of T and D are known, each value of K'' yields a value of the apparent association constant ${}^{o}K'_2$ for 2D = T at the specified T, P, pH, [Cl^-], ionic strength, etc. in the absence of oxygen (see Eq. (23)). (4) A check on the values of the seven apparent equilibrium constants is that they can be used to calculate the shapes of all of the experimental plots, including their nonlinear regions.

8. Discussion

The primary reason for making these plots is that all of the seven equilibrium constants for partially dissociated hemoglobin can be evaluated from oxygen binding measurements that yield the slopes and intercepts of the linear regions. Plotting data as Y versus $[\text{heme}]^{-1/2}$ and [heme] shows where the linear regions are for a particular hemoglobin under a particular set of conditions. It is important to obtain data in the linear regions

because they are the regions where oxygenated forms of T and D are beginning to predominate. The set of 7 constants determines the shapes of these plots and not simply the linear regions, and so these plots provide a test as to whether the data really are fit with a particular set of seven equilibrium constants.

The cooperativity in the binding of oxygen by hemoglobin makes the determination of the Adair constants of the tetramer difficult in any case, but the subunit dissociation increases the difficulties because seven equilibrium constants have to be determined, rather than four. In going to lower $[O_2]$, the increase of K'' by approximately a factor of 10^5 makes the job even harder. Johnson and Lassiter [6] have pointed out that in the 1970s it was assumed that [heme] $= 5 \times 10^{-5}$ M was sufficient to avoid dissociation, and that more recently it has been assumed that [heme] $= 2 \times 10^{-3}$ M is sufficient. The calculations presented here emphasize the quantitative effects of subunit dissociation for one particular hemoglobin and suggest procedures for determining the seven apparent equilibrium constants involved.

A general equilibrium program has been used to calculate the compositions for various values of [heme] and $[O_2]$ for a particular human hemoglobin at a particular set of conditions. These calculations show that the oxygen binding properties of tetramers can be obtained by linear extrapolation of the Y values versus $[\text{heme}]^{-1/2}$ to $[\text{heme}]^{-1/2} = 0$ at a specified $[O_2]$. The oxygen binding properties of dimers can be obtained by the extrapolation of Y versus [heme] to [heme] $= 0$ at low [heme]. The apparent association constant K'' at specified $[O_2]$ can be determined in a number of ways and used to calculate ${}^{o}K_2'$, the apparent association constant for 2D = T in the absence of oxygen.

The current best values of ${}^{o}K_2'$, K_{21}' and K_{22}' are based on other types of experimental methods. However, in principle all seven apparent equilibrium constants can be obtained from measurements of Y. There is an experimental challenge to extend oxygen binding experiments to lower heme concentrations. Changing the type of hemoglobin or T, P, pH, $[Cl^-]$, etc. may make it easier or harder to carry out the recommended extrapolations. The effect of $[O_2]$ on K'' is striking and is undoubtedly affected by small changes in the structures of the α and β subunits.

There is a possibility that the association/dissociation of hemoglobin is more complicated than simply dissociation into dimers. At high concentrations $(\alpha\beta)_4$ might be formed, and at low concentrations α and β might be formed. The plots discussed here are based on the assumption that only dimers and tetramers are involved. Other types of association/dissociation would cause deviations in these plots.

If the seven apparent equilibrium constants can be measured at a series of temperatures at constant P, pH, $[Cl^-]$, ionic strength, etc., it is possible to calculate standard transformed enthalpies and entropies of reaction for the seven reactions and standard transformed enthalpies and entropies of formation of the various forms at the specified P, pH, $[Cl^-]$, etc., as discussed in the preceding article. All of these thermodynamic properties are model independent but provide physical quantities that models should explain. Further equilibrium constants and standard thermodynamic properties can be obtained by investigating the effects of pH, $[Cl^-]$, etc. on the seven equilibrium constants discussed here.

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Appendix A

These calculations have been made with a general equilibrium program that can be used for solution reactions with any number of reactants as independent variables. Krambeck [7,8] wrote a program in APL that yields the equilibrium composition for a multi-reaction gas system described in terms of a conservation matrix, a

vector (lnk) of standard Gibbs energies of formation of the reactants multiplied by $-(1/RT)$, and an initial composition vector $\underset{\sim}{c}_0$. This program calculates an approximate composition and improves it by a Newton–Raphson iteration. Recently, Krambeck has translated this program into equcalc in *Mathematica* [9], with a version equcalcc for solution reactions. A vector $\underset{\sim}{c}$ of equilibrium concentrations can be calculated using equcalcc and inputting the conservation matrix and standard Gibbs energies of formation of the reactants [2]. For the present calculations, it is more convenient to use the apparent stoichiometric number matrix for the seven reactions and a vector of the natural logarithms of the seven apparent equilibrium constants. In order to specify the system with the matrix of apparent stoichiometric numbers of a set of independent reactions (rather than a conservation matrix), the program eqrxc [10] uses NullSpace to calculate a conservation matrix for input in equcalcc and LinearSolve to calculate the natural logarithm of equilibrium constants of formation reactions for input to equcalcc. These programs are given below. One advantage of this approach is that it can be used for any system described by a set of independent reactions. In the calculations described here, the apparent stoichiometric number matrix $\underset{\sim}{\upsilon}''$ at specified $[O_2]$ is given by

$$\underset{\sim}{\upsilon}'' = \begin{array}{c} \begin{array}{ccccccccc} \text{Reaction} & 63 & 64 & 65 & 66 & 67 & 68 & 69 \\ & & \multicolumn{7}{c}{\text{Reactant}} \end{array} \\ \left[\begin{array}{ccccccc} D(O_2)_2 & 0 & 1 & 0 & 0 & 0 & 0 & 0 \\ D(O_2) & 1 & -1 & 0 & 0 & 0 & 0 & 0 \\ D & -1 & 0 & 0 & 0 & 0 & 0 & -2 \\ T & 0 & 0 & -1 & 0 & 0 & 0 & 1 \\ T(O_2) & 0 & 0 & 1 & -1 & 0 & 0 & 0 \\ T(O_2)_2 & 0 & 0 & 0 & 1 & -1 & 0 & 0 \\ T(O_2)_3 & 0 & 0 & 0 & 0 & 1 & -1 & 0 \\ T(O_2)_4 & 0 & 0 & 0 & 0 & 0 & 1 & 0 \end{array}\right] \end{array} \tag{A1}$$

The rows and columns are identified to show how the matrix is constructed; the equation numbers are from the preceding article. The kr matrix is calculated for the desired $[O_2]$ by using

$$\{K'_{21}[O_2], K'_{22}[O_2], K'_{41}[O_2], K'_{42}[O_2], K'_{43}[O_2], K'_{44}[O_2], {}^{\prime\prime}K_2\}$$
$$= \{3.253 \times 10^6[O_2], 8.155 \times 10^5[O_2], 4.397 \times 10^4[O_2], 1.221 \times 10^4[O_2],$$
$$4.049 \times 10^5[O_2], 6.644 \times 10^5[O_2], 4.633 \times 10^{10}\} \tag{A2}$$

Two checks can be applied to the calculated vector $\underset{\sim}{c}$ of equilibrium concentrations. The first check is that the equilibrium concentrations must account for the correct total concentration of heme. The test is based on

$$\underset{\sim}{A}\,\underset{\sim}{c} = \underset{\sim}{A}\,\underset{\sim}{c}_0 \tag{A3}$$

where A is the conservation matrix and $\underset{\sim}{c}_0$ is the initial concentration vector. The conservation matrix is {2,2,2,4,4,4,4,4} and $\underset{\sim}{c}_0$ is {0,0,0,[heme]/4,0,0,0,0}. The second check is that it should be possible to treat the calculated composition as experimental data and calculate the correct values of the seven apparent equilibrium constants from it. This is most conveniently done with

$$\left(\underset{\sim}{\upsilon}''\right)^T \ln \underset{\sim}{c} = \ln \underset{\sim}{K}_r \tag{A4}$$

where $\underset{\sim}{K}_r$ is the vector of apparent equilibrium constants at the specified $[O_2]$. The concentration vectors $\underset{\sim}{c}$ calculated here satisfy both of these tests.

The basic *Mathematica* [9] programs used are as follows:

```
eqrxc[nt_,lnkr_,no_]:=Module[{as,lnk},
(*   nt=transposed stoichiometric number matrix
lnkr= natural log of equilbrium constants of rxs (vector)
no=initial composition vector*)
(*Setup*)
lnk=LinearSolve[nt,lnkr];
as=NullSpace[nt];
equcalcc[as,lnk,no]
]

equcalcc[as_,lnk_,no_]:=Module[{l,x,b,ac,m,n,e,k},
(*   as=conservation matrix
lnk= natural logarithm of equilibrium constant of
     formation (vector)
no=initial composition vector *)
(*Setup*)
{m,n}=Dimensions[as];
b=as.no;
ac=as;
(*Initialize*)
l=LinearSolve[ as.Transpose[as],-as.(lnk+Log[n]) ];
(*Solve*)
Do[  e=b-ac.(x=E^(lnk+l.as) );
     If[(10^-10)>Max[ Abs[e] ], Break[] ];
     l=l+LinearSolve[ac.Transpose[ as*Table[x,{m}]],e]
     ,{k,100}];
     If[ k=100,Return["Algorithm Failed"] ];
     Return[x]
]
```

Notation (Note that all of these properties are dimensionless except for $\underset{\sim}{c}$.)

A conservation matrix (dimensionless)

$\underset{\sim}{c}$ concentration vector (M)

C' number of apparent components after the chemical potentials of one or more species have been specified (dimensionless)

C'' number of apparent components after the transformed chemical potentials of one or more reactants have been specified after specifying the chemical potentials of one or more species (dimensionless)

f_D fraction of the heme in dimer (dimensionless)

f_T fraction of the heme in tetramer (dimensionless)

N' number of reactants after the chemical potentials of one or more species have been specified (dimensionless)

N'' number of reactants after the transformed chemical potentials of one or more reactants have been specified after specifying the chemical potentials of one or more species (dimensionless)

K'_{4i} successive apparent equilibrium constants for the association of O_2 with the tetramer ($i = 1 - 4$) at specified T, P, pH, $[Cl^-]$, ionic strength, etc. (dimensionless)

K'_{2i} successive apparent equilibrium constants for the association of O_2 with the dimer ($i = 1 - 2$) at specified T, P, pH, $[Cl^-]$, ionic strength, etc. (dimensionless)

$^{o}K'_2$ apparent association constant for 2D = T at specified T, P, pH, $[Cl^-]$, etc. (dimensionless)

K'' apparent association constant for 2TotD = TotT at a specified $[O_2]$, T, P, pH, $[Cl^-]$, etc. (dimensionless)

$\underset{\sim}{K}_r$ vector of apparent equilibrium constants at specified $[O_2]$ (dimensionless)

R' number of independent reactions after the chemical potentials of one or more species have been specified (dimensionless)

R'' number of independent reactions after the transformed chemical potentials of one or more reactants have been specified after specifying the chemical potentials of one or more species (dimensionless)

Y fractional saturation of hemoglobin (equilibrium mixture of T and D) with O_2 (dimensionless)

Y_T fractional saturation of tetramer with O_2 (dimensionless)

Y_{D_n} fractional saturation of dimer with O_2 (dimensionless)

$\underset{\sim}{\nu}$ apparent stoichiometric number matrix ($N' \times R'$) (dimensionless)

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