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Title

Architecture of Allosteric Structure. Rate Equations, Rate Constants, and Equilibrium Constants for Reaction of: Hb_4 with O_2 and $(HbO_2)_4$ with Dithionate, in the Presence of 2,3-Bisphosphoglycerate

Sub-Title

Determination of the Equilibrium Constant for the O_2 -Mediated Change between T State and R State Conformations

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Organization of Manuscript

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Consecutive Equivalent First Order Reactions

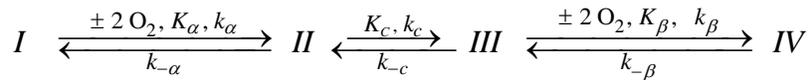
Special Considerations for Models of Human Hemoglobin

Key Words. Cooperative subunit assemblies; ordered reaction sequence, reaction rates, bisphosphoglycerate, conformation changes, biophysics, kinetics, red blood cells, human hemoglobin

Abbreviations. Abbreviations and conventions concerning representation of hemoglobin structure are stated in Knowles and Magde, "Essay Four".

Abstract

Three unknown quantities are all that is required to describe the O₂-equilibrium binding curve for fractional saturation of human hemoglobin in red blood cells, under standard conditions: K_α , the O₂-binding constant of equivalent α -chains; K_c , the equilibrium constant for the T \rightarrow R conformation change; K_β , the O₂-binding constant of equivalent β -chains. The model for formulation of the equation of state is a 3-stage ordered sequence of reactions.



The values of K_α , K_c , and K_β were established by determination of rate constants for the oxygenation reaction and the dithionite-mediated de-oxygenation reaction. The rate law for the forward reaction in the presence of excess O₂ yields k_α , k_c , k_{-c} , and k_β . The same rate law yields $k_{-\beta}$, k_{-c} , k_c , and $k_{-\alpha}$ for the dithionite-mediated de-oxygenation reaction. The rate constants for binding O₂ are pseudo-first-order. The rate constants for release of O₂ are first-order. Reactions involving O₂,

$I \rightarrow II$, $II \rightarrow I$, $III \rightarrow IV$, and $IV \rightarrow III$ are 2-step ordered sequences of equivalent subunits. Progress curves for a 2-step ordered sequence of equivalent chains collapse to a first order reaction.

$$K_\alpha = k_\alpha / k_{-\alpha} = 8.53 \times 10^3 \text{ L/mol. } K_\beta = k_\beta / k_{-\beta} = 2.38 \times 10^5 \text{ L/mol.}$$

Progress curves for both oxygenation and dithionite-mediated de-oxygenation reactions return $K_c = k_c / k_{-c}$. K_c is 0.0580 for the oxygenation reaction and 0.0358 for the dithionite-mediated de-oxygenation reaction. The corresponding values from the O₂-equilibrium binding curve are: $K_\alpha = 15.09 \times 10^3 \text{ L/mol}$, $K_\beta = 3.94 \times 10^5 \text{ L/mol}$, and $K_c = 0.02602$. Values of K_α , K_c , and K_β determined from rate constants of progress curves for oxygenation and dithionite-mediated de-oxygenation reactions are close to values of K_α , K_c , and K_β determined by analysis of the O₂-equilibrium binding curves for whole blood, by the Perutz/Adair equation.

INTRODUCTION

Previous communications in this series of papers (Knowles, linked ms 1, Knowles & Magde, linked mss 2 & 3) established a model accounting for the O₂-equilibrium binding curve of the binary complex of human Hb₄ and BPG, as it exists in rbc. The model presented in Figure (1) accounts for the sequence of reactions taking place in rbc at the constant pH of 7.4. Changes in rbc pH-values occurring in the pulmonary and systemic circulations are not addressed. pH-values decrease in the systemic circulations and rise in the pulmonary circulation. BPG, is represented as either a bullet or an open circle with a central dot, □ . A bullet indicates a BPG molecule bound to Tstate conformations, species I and II. An open circle with a central dot indicates a BPG molecule bound to Rstate conformations, species III and IV. ΔG° = -33.7kJ/ mol at 25°C for binding of BPG with E-free R(α*, β*) to yield species I, T(α* • β*) (DeBrun & Janssen, 1973). Conversion of the Tstate back to the Rstate will require an input of - 33.7 kJ/mol (Knowles & Magde, linked ms 3). ΔG° = -10.5 kJ/mol at 37°C for binding of BPG with an Rstate conformations, yielding Rstate species III and IV ,(Knowles & Magde, linked ms 3).

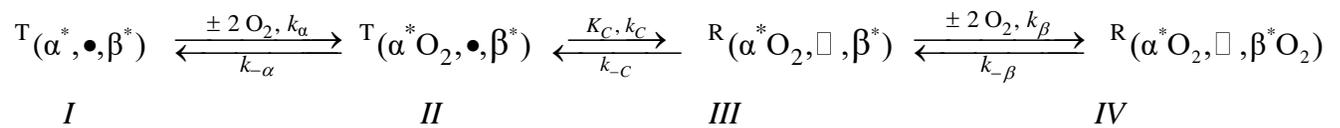


Figure 1. Symbolic Representation of the Sequence of Reactions in the Conversion of Hb₄/BPG to (HbO₂)₄/BPG in rbc. Rate constants are defined as being the value obtained with hypothetical monomeric α- and β-chains. Upper left T or R assigns conformational state. Upper right * denotes equivalent O₂-binding. A bullet, •, represents a molecule of BPG bound to Tstate conformations. □ represents a molecule of BPG bound to Rstate conformations.

The first stage in oxygenation of Hb₄/BPG, conversion of species I to species II consists of a sequence of two reactions in each of which O₂ binds to Tstate heme moieties of each α-chain, reacting equivalently. Conformational states, indicated by left superscripts, T or R, apply to either a single subunit or all subunits within parentheses.

Equivalent reactivity, indicated by a right superscript asterisk, *, is defined in Figure 2 for Tstate α -chains.

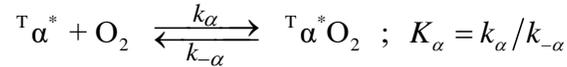
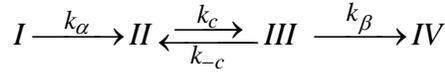


Figure 2. Equivalent binding in multi-subunit protein assemblies is defined as the equilibrium constant, K_α , for an isolated α -chain, the properties of which are not modified by removing the α -subunit from the entire assembly. A similar statement can be made for an isolated β -chain.

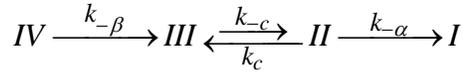
The equilibrium constant for the first Tstate α -chain to bind O_2 is $(2 k_\alpha / k_{-\alpha}) = 2K_\alpha$.

The equilibrium constant for the second Tstate α -chain O_2 binding reaction is $(k_\alpha / (2 k_{-\alpha})) = K_\alpha / 2$. The rate law for any 2-step ordered sequence of equivalent first order or pseudo first order reactions collapses to a first-order rate equation (Appendix A). Similar comments apply to the third stage, conversion of species III to species IV, consisting of a sequence of two reactions in which O_2 binds to Rstate β -chain heme moieties, reacting equivalently. The 1st-step for oxygenation is carried out under conditions where pseudo-first-order kinetics are observed. The 2nd-step, species II going to species III, accounts for an endothermic change in the conformation of the globin moiety, from Tstate to Rstate. The endothermic conformation change is coupled to (i) exothermic conversion of Tstate T($\alpha^*O_2\bullet$) chains to Rstate R($\alpha^*O_2\circ$) chains and (ii) exothermic formation of a binary complex, BPG/(Rstate conformation). The 2nd-step is reversible and does not involve an O_2 -binding reaction.

Under pseudo first-order conditions the kinetic model for the oxygenation sequence is comprised of 4 unknown quantities: k_α , k_c , k_{-c} , and k_β . In this instance species I is T(Hb₄/BPG) and species IV is R((HbO₂)₄/BPG). The kinetic model for the dithionite mediated deoxygenation sequence is comprised of 4 unknown quantities: $k_{-\beta}$, k_{-c} , k_c , and $k_{-\alpha}$. These kinetic models are summarized in Figure 3.



Oxygenation Sequence



Dithionate-Mediated DeOxygenation Sequence

Figure 3. Kinetic Models for the Oxygenation Sequence and the Dithionite Mediated Deoxygenation Sequence

This communication seeks to confirm the model presented in Figure (1) in terms of rate constants:

$$K_\alpha = k_\alpha / k_{-\alpha}; \quad K_\beta = k_\beta / k_{-\beta}.$$

$$K_C = \text{either } k_C / k_{-C}, \text{ for oxygenation or } k_{-C} / k_C, \text{ for deoxygenation.}$$

Values of K_α , K_β , and K_C , determined by thermodynamic and kinetic methods are expected to be in agreement if the model presented in Figures (1) and (3) is to be considered as a description of the reversible sequence of events accompanying the conversion of Hb_4 to $(\text{HbO}_2)_4$ in the presence of BPG, under standard conditions, in human red blood cells.

MATERIALS AND METHODS

Preparation of the Hb_4 /DPG Binary Complex. Red blood cells, collected by centrifugation of freshly drawn blood, were washed three times by: (i) suspension in 0.1 M NaCl and (ii) sedimentation by centrifugation. Washed rbc's were lysed by suspension in three volumes of 0.05 M BisTris, pH 7.4. The pH of the stock solution of BisTris was adjusted with HCl. Red blood cells were resealed by addition of solid NaCl to a final concentration of 0.1 M NaCl. Resealed rbc's were collected by centrifugation at 10,000 rpm for 1 hr. The supernatant solution is the binary complex of Hb_4 with DPG. The cell-free supernatant solution was used in experiments to establish the progress curves for the reaction of Hb_4 /DPG with excess O_2 and the progress curves for reaction of $(\text{HbO}_2)_4$ /DPG with dithionite.

Kinetics of Reaction. Stopped flow observations of progress curves were carried out with a Durrum-Gibson stopped flow instrument manufactured by Dionex, Sunnyvale, CA, Model D-110. The stopped flow spectrophotometer was fitted with stainless steel drive and stopping syringes (Knowles, 1979). Temperature controlled circulating water was pumped directly into the jacketed observation chamber and drained through the main water bath containing the syringe block. Photomultiplier voltage was monitored by a Picoscope ADC 212 Virtual Oscilloscope. Triggering for data collection preceded onset of mixing. Voltages were transferred into Excel for conversion to absorbance at a known time, A_t . Progress curves, A_t , at corresponding values of time, t , were transferred to MatLab files for determination of the best-fitting values of rate constants. Figures are composed in EXCEL. Reaction of Hb₄/BPG with O₂ was observed at 560 nm. Reaction of (HbO₂)₄/BPG with dithionite was observed at 578 nm.

Results

Rate Laws. The model on which the Perutz-Adair equation is based, described in Figures 1 and 3 predicts that progress curves for the binding of O₂ by Hb₄/BPG will obey a 3-stage ordered sequence. The rate law describing the progress curve for reaction of Hb₄/BPG with excess O₂ yields pseudo first-order values:

$$k'_\alpha \text{ and } k'_\beta, \text{ where } k_\alpha = k'_\alpha / [\text{O}_2] \text{ and } k_\beta = k'_\beta / [\text{O}_2]; \text{ and } K_C = k_c / k_{-c}.$$

Similarly, the progress curve for the dissociation of O₂ in the presence of excess dithionite will appear to be a 3-stage ordered sequence. The progress curves for reaction of (HbO₂)₄/BPG with dithionite yields first-order rate constants for release of O₂ from both α - and β -chains, directly.

$$k_{-\beta}; k_{-\alpha}; \text{ and } K_C = k_c / k_{-c}.$$

Insofar as these rate laws describe a pseudo-first-order equation and a first order equation, it is not necessary to capture 100% of the progress curve in order to

determine the values of k'_α , k'_β , $k_{-\beta}$, $k_{-\alpha}$, k_C and k_{-C} . Proof that an ordered sequence of equivalent first order reactions or pseudo first order reactions simplify to a first order expression is given in Appendix A. Bateman equations describe the time-dependence of the concentration of intermediate species. Separate Bateman equations exist for species *II* and *III*. These equations contribute to the definition of fractional saturation, $F_t = A_t / A_\infty$, the value of F_t being calculated from the progress curve at A_t and A_∞ . Progress curves are presented as absorbance at time = t, A_t , versus time, t.

Formulation of Equations of State for: (i) the Rate of the O₂ Binding Reaction of Hb₄/BPG and O₂ and (ii) the Rate of O₂ Release from (HbO₂)₄/BPG. The predicted time dependence of the concentration of species *I*, *II* and *III* are given in Figure 4.

Time dependence of species *IV*, $[IV]_t$, is defined as follows:

$$[IV]_t = [I]_0 - [I]_t - [II]_t - [III]_t.$$

$$[I]_t = [I]_0 \exp(-k_1 t)$$

$$[II]_t = \frac{k_1 [I]_0}{k_1^2 - k_1 p + k_2 k_3} \left(\frac{(x_2 - k_1)(k_{-2} + k_3 - x_1) \exp(-x_1 t)}{(x_1 - x_2)} \right. \\ \left. + \frac{(k_1 - x_1)(k_{-2} + k_3 - x_2) \exp(-x_2 t)}{(x_1 - x_2)} \right. \\ \left. + (k_{-2} + k_3 - k_1) \exp(-x_1 t) \right)$$

$$[III]_t = \frac{k_1 k_2 [I]_0}{k_1^2 - k_1 p + k_2 k_3} \left(\frac{(x_2 - k_1) \exp(-x_1 t)}{(x_1 - x_2)} \right. \\ \left. + \frac{(k_1 - x_1) \exp(-x_2 t)}{(x_1 - x_2)} + \exp(-k_1 t) \right)$$

$$\text{where: } p = k_{-2} + k_3 + k_2, \quad q = \sqrt{p^2 - 4k_2 k_3}, \\ x_1 = 0.5(p + q), \quad x_2 = 0.5(p - q)$$

Figure 4. Rate Equation for the Time Dependence of $[I]_t$. Bateman Equations for the Time Dependence of Concentration of Intermediate Species $[II]_t$ and $[III]_t$.

Fractional saturation of Hb₄/BPG with O₂ at time, t, is defined as follows:

$$F_t = \frac{(2 [II]_t + 2 [III]_t + 4 [IV]_t)}{(4 ([I]_t + [II]_t + [III]_t + [IV]_t))} = \frac{F_{\text{NUMERATOR},t}}{F_{\text{DENOMINATOR},t}}$$

Substituting the analytical expression for [IV]_t into F_{DENOMINATOR,t} rearranging, combining like terms and carrying out summations leads to F_{DENOMINATOR,t} = 4 [I]₀. In F_{NUMERATOR,t} the term for species [IV]_t is replaced by its analytical expression resulting in F_{NUMERATOR,t} = 4 ([I]₀ - 4 [I]_t - 2 [II]_t - 2 [III]_t).

F_t, then, is defined as follows:

$$F_t = \frac{(4 ([I]_0 - 4 [I]_0 \exp(-k_1 t) - 2 [II]_t - 2 ([III]_t))}{(4 ([I]_0)}$$

The denominator divides into each term in the numerator

$$F_t = 1 - \exp(-k_1 t) - 0.5 ([II]_t / [I]_0) - 0.5 ([III]_t / [I]_0)$$

$$\text{Since } F_t = A_t / \Delta A_{\text{MAX}}$$

$$A_t = \Delta A_{\text{MAX}} (1 - \exp(-k_1 t) - 0.5 ([II]_t / [I]_0) - 0.5 ([III]_t / [I]_0))$$

Analytical expressions for [II]_t and [III]_t contain the term [I]₀. Substitution of analytical expressions for species [II]_t and [III]_t results in elimination of the term [I]₀. The rate law for F_t is presented in Figure 5.

$$\begin{aligned}
A_t = & \Delta A_{MAX} (1 - \exp(-k_1 t) \\
& - (0.5 k_1 / (k_1^2 - k_1(k_{-2} + k_3 + k_2) + k_2 k_3)) \\
& (((0.5(k_{-2} + k_3 + k_2 - ((k_{-2} + k_3 + k_2 - 4k_2 k_3)^{0.5})) - k_1) \\
& (k_{-2} + k_3 - 0.5(k_{-2} + k_3 + k_2 + ((k_{-2} + k_3 + k_2 - 4k_2 k_3)^{0.5}))) \\
& / ((k_{-2} + k_3 + k_2 - 4k_2 k_3)^{0.5})) \\
& \exp(-0.5(k_{-2} + k_3 + k_2 - ((k_{-2} + k_3 + k_2 - 4k_2 k_3)^{0.5}))t) \\
& + ((k_1 - 0.5(k_{-2} + k_3 + k_2 + ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5})) \\
& (k_2 + k_3 - 0.5(k_{-2} + k_3 + k_2 + ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5})) \\
& / ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5}) \\
& \exp(-0.5(k_{-2} + k_3 + k_2 + ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5})t) \\
& + (k_{-2} + k_3 - k_1) \exp(-k_1 t) \\
& - (0.5 k_1 k_2 / (k_1^2 - k_1(k_{-2} + k_3 + k_2) + k_2 k_3)) \\
& (((0.5(k_{-2} + k_3 + k_2 - ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5}) - k_1) \\
& / ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5})) \\
& \exp(-0.5(k_{-2} + k_3 + k_2 + ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5})t) \\
& + ((k_1 - (0.5(k_{-2} + k_3 + k_2 + ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5})) \\
& / ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5})) \\
& \exp(-0.5(k_{-2} + k_3 + k_2 - ((k_{-2} + k_3 + k_2)^2 - 4k_2 k_3)^{0.5})t) \\
& + \exp(-k_1 t)))
\end{aligned}$$

Figure 5. Rate Law for the Progress Curve of the Oxygenation Reaction of Hb₄/BPG. The equation, separated into sections representing concentration of species [I]_t, [II]_t, [III]_t, and [IV]_t is a single line of text:

$k_1 = k_\alpha$; $k_2 = k_C$; $k_{-2} = k_{-C}$; $k_3 = k_{\beta 3}$. The rate law also describes the dithionite-mediated deoxygenation reaction: $k_1 = k_{-\beta}$; $k_2 = k_{-C}$; $k_{-2} = k_C$; $k_3 = k_{-\alpha}$

Calculation of Rate Constants for the Oxygenation Reaction. A progress curve for the reaction of Hb₄/BPG with O₂ is illustrated in Figure (6). The progress curve was fitted by the rate law outlined in Figure 5. The magnitude of the absorbance excursion, ΔA_{MAX} , can be read: (i) directly from the progress curve or (ii) treated as an unknown and determined from curve fitting procedures. Procedure (ii) provides a more precise estimate of the value of ΔA_{MAX} . The value of ΔA_{MAX} would then be inserted into the curve-fitting algorithm prior to determination of the best fitting values for the pseudo-first-order rate constants: k_{α} , k_c , k_{-c} , k_{β} .

The progress curve of Figure (6) was compared to data simulated using the rate law, the best fitting values for the rate constants, and experimental values of t. Predicted absorbance excursions are plotted against observed absorbance excursions in Figure (7). The result is expected to be linear: $\Delta A_{PREDICTED} = \Delta A_{OBSERVED}$. The result for the best fitting straight line is: $\Delta A_{PREDICTED} = 0.9792 \Delta A_{OBSERVED} - 0.005$.

Calculation of rate constants for the progress curve illustrated in Figure (6) is described below. Pseudo-first-order rate constants, k'_{α} , k'_{β} , as well as absolute rate constants, k_c and k_{-c} , are returned by the curve fitting procedure.

$$k_{\alpha} = \frac{k'_{\alpha}}{[O_2]} = \frac{373.4/\text{sec}}{0.000142 \text{ (mol/L)}} = 2.629 \times 10^6 \frac{\text{L}}{\text{(mol sec)}}$$

$$k_c = 1,153/\text{sec}$$

$$k_{-c} = 19,710/\text{sec}$$

$$k_{\beta} = \frac{k'_{\beta}}{[O_2]} = \frac{24,310 / \text{sec}}{0.000142 \text{ (mol/L)}} = 1.71 \times 10^8 \frac{\text{L}}{\text{mol sec}}$$

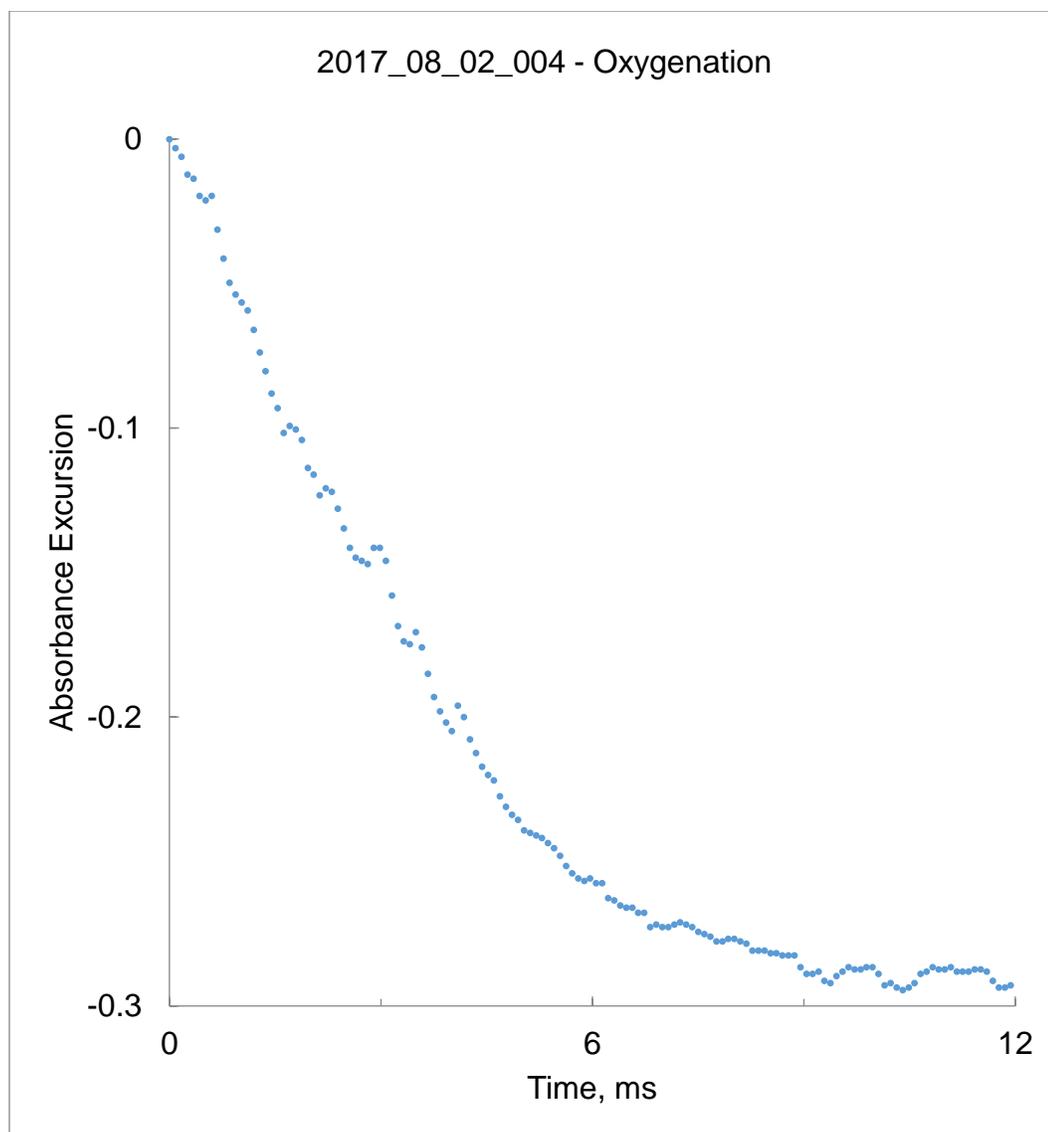


Figure 6. Progress Curve for Reaction # 2017_08_02_004: Hb₄/BPG with Dioxygen at 21°C. The best fitting values for k_1 , k_2 , k_3 , and k_4 (k'_α ; k_c ; k_{-c} ; k'_β) are: 373.4/sec; 1,153/sec; 19,710/sec; 24,310/sec. The correlation coefficient for observed and predicted values of ΔA at time, t , is calculated from best fitting values and the rate law, given in Figure (7), 0.9957.

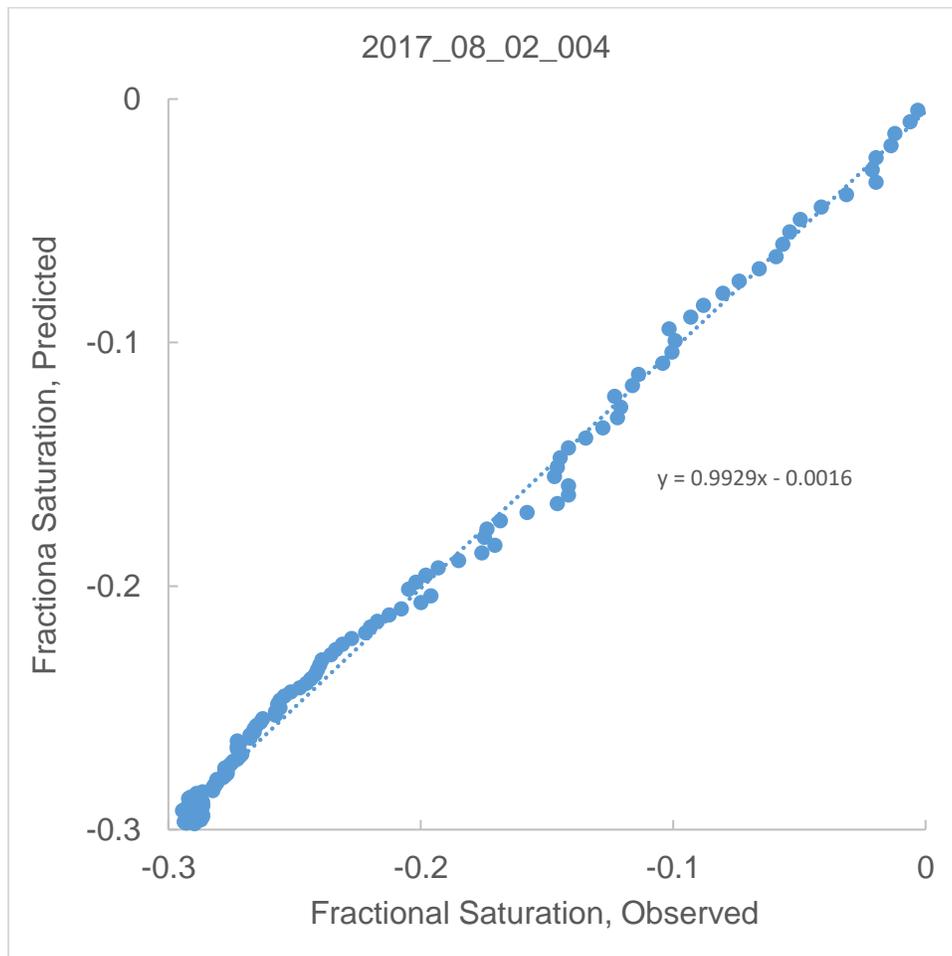


Figure 7. Comparison of Observed and Predicted Values of ΔA_t For Oxygenation of Hb₄/BPG. The observed values of ΔA_t are shown in Figure 6. Predicted values of absorbance are calculated using the best fitting values for k_α , k_c , k_{-c} , k_β .

Calculation of Rate Constants for the Deoxygenation Reaction with Dithionite.

The progress curve for reaction of $(\text{HbO}_2)_4/\text{BPG}$ with 0.00611 M dithionite at 35.4°C , is illustrated in Figure 8. The progress curve was fitted to the kinetic model for the deoxygenation reaction, Figure 3. The best fitting values for $k_{-\beta}$, k_{-c} , k_c , and $k_{-\alpha}$, are: 718.7/sec; 1,143/sec; 41/sec; 309.2/sec, respectively. The correlation coefficient for the curve fitting procedure was 0.9994

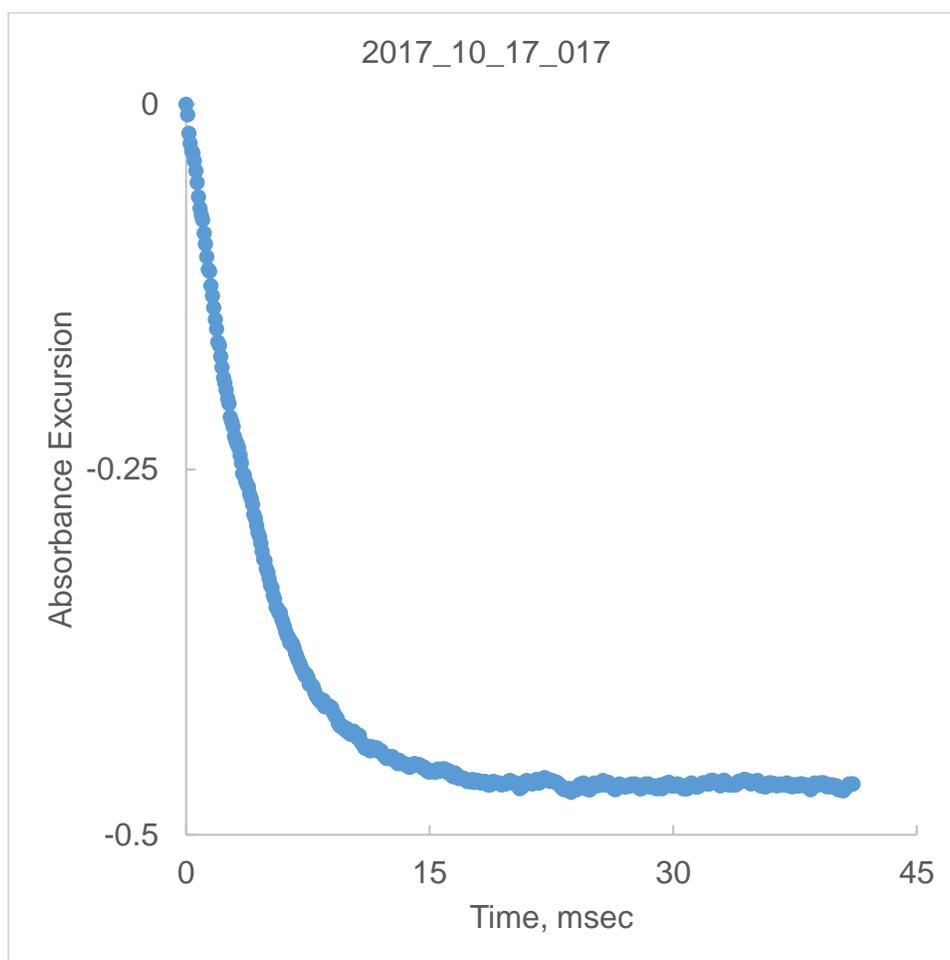


Figure 8. Progress Curve for Reaction # 2017_10_17_017: Reaction of $(\text{HbO}_2)_4/\text{BPG}$ with 0.0611 M Dithionite at 35.5°C , 0.05 M BisTris, 0.050 KCl, pH 7.4 34.5°C . The correlation coefficient for observed and predicted values of ΔA at time, t , was calculated from best-fitting values of observed rates the rate law, at experimentally observed values of t . 0.9995.

The predicted progress curve, ΔA_t , is compared with the observed progress curve, Figure 9. The progress curve, comprised of 483 data points over 40 msec, was predicted using the equation of state for the rate law, Figure (7) and best fitting values for the four rate constants. The result is expected to be linear:

$\Delta A_{\text{PREDICTED}} = \Delta A_{\text{OBSERVED}}$. The result for the best fitting straight line is:

$\Delta A_{\text{PREDICTED}} = 0.9981 \Delta A_{\text{OBSERVED}} - 0.0008$, a close approximation of a straight line.

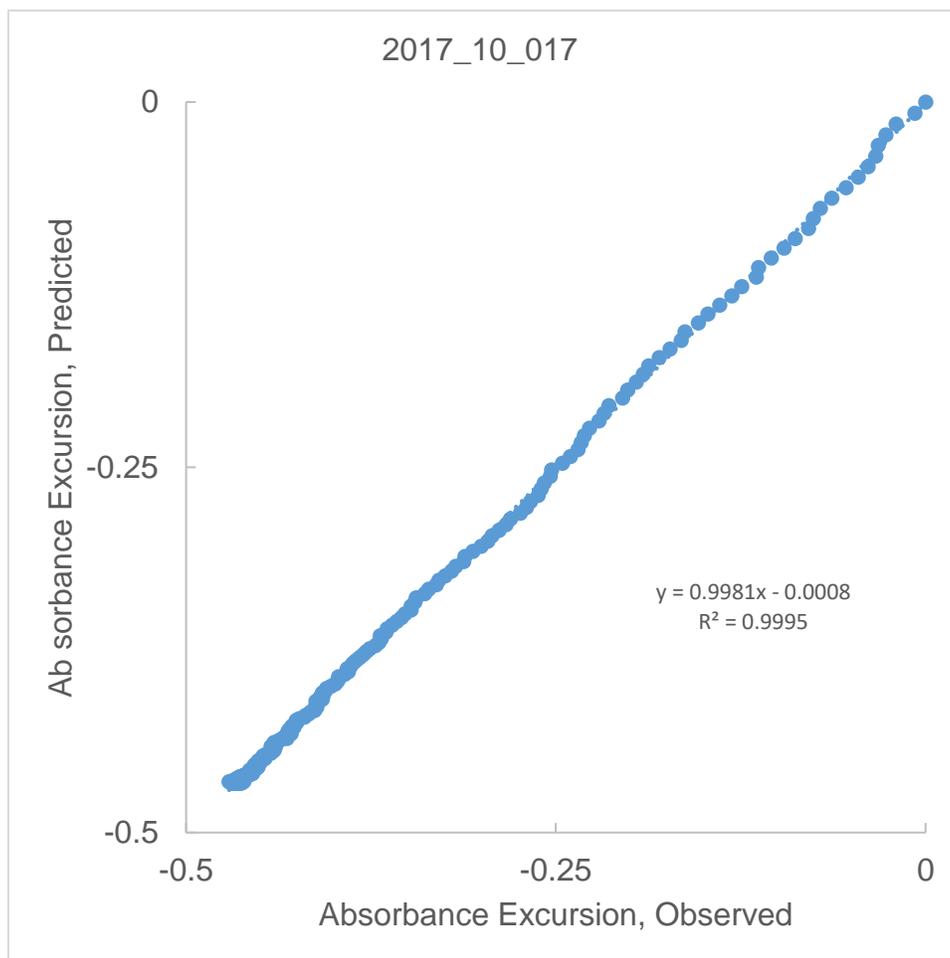


Figure 9. Progress curve (number 2017_10_17_017) of Observed and Predicted Values of Absorbance for Deoxygenation of $(\text{HbO}_2)_4/\text{BPG}$ with Dithionite. Conditions of reaction are: 0.0611 M sodium dithionite, 0.050 M BisTris, 0.050 M KCl, pH 7.4 with HCl, 34.5°C

Rate constants for the progress curve illustrated in Figure (8) returned by the curve fitting procedure are: $k_{-\beta} = 718.7 / \text{sec}$; $k_{-c} = 1143 / \text{sec}$; $k_c = 41 / \text{sec}$; $k_{-\alpha} = 309.2 / \text{sec}$.

Calculation of Equilibrium Constants. Calculation of equilibrium constants for the three mechanistic steps comprising the pathway from Hb_4/BPG to $(\text{HbO}_2)_4/\text{BPG}$, Figure (1) are summarized below, together with the values of equilibrium constants obtained by fitting Roughton's equilibrium binding data for whole blood, under standard conditions, to the Perutz/Adair equation. Data for the equilibrium constant for the conformation change, T-state to R-state, revealed by the progress curves for both the oxygenation reaction Figure (6) and the dithionite-mediated deoxygenation reaction, Figure 8, are also included.

$${}^T K_\alpha = \frac{k_\alpha}{k_{-\alpha}} = \frac{26.36 \times 10^5 \text{ L}/(\text{mol s})}{3.092 \times 10^2 / \text{s}} = 8,525 \text{ L/mol};$$

$${}^R K_\beta = \frac{k_\beta}{k_{-\beta}} = \frac{1.71 \times 10^8 \text{ L}/(\text{mol s})}{718.7 / \text{s}} = 2.38 \times 10^5 \text{ L/mol};$$

$$K_c = \frac{k_c}{k_{-c}} = \frac{1,153 / \text{sec}}{19,710 / \text{sec}} = 0.0585; \quad \text{for oxygenation reaction}$$

$$K_c = \frac{k_c}{k_{-c}} = \frac{41 / \text{sec}}{1,143 / \text{sec}} = 0.0358; \quad \text{for dithionite-mediated deoxygenation}$$

$$K_c^* = 0.02602 \quad K_\alpha^* = 15,090 \text{ L/mol} \quad K_\beta^* = 393,900 \text{ L/mol}$$

*, data from O_2 -equilibrium binding curve of wholeblood,
under standard conditions

The equilibrium constants determined by kinetic methods are close to but not identical to those determined by thermodynamic methods. The results, nevertheless, are probably within the errors inherent in both (i) the kinetic methods of analysis and (ii) uncertainty in the curve fitting operations of O_2 -equilibrium binding data to the Perutz-

Adair equation. Progress curves for oxygenation reactions were carried out at 21°C. Lowering the temperature at which an O₂-equilibrium binding curve of Hb₄, in the presence of E-molecules, is established results in an increase in the affinity for O₂. Progress curves for de-oxygenation reactions, in the presence of dithionite, were carried out at 34.5°C. The kinetic experiments return a lower value for K_α and a higher value for K_β than does the O₂-equilibrium binding curve. Further measurements of the oxygenation and de-oxygenation reactions with a thermally compliant cuvette assembly are justified.

Equilibrium constants determined by kinetic methods of analysis are, nevertheless, close to those determined by thermodynamic methods of analysis. Small variations in pH between the kinetic investigations reported here and the thermodynamic studies can easily explain the small discrepancies reported here. The model presented in Figures (1) and (3), then, is determined to be in agreement with both thermodynamic and kinetic descriptions of the binding of O₂ by human hemoglobin in the presence of BPG, under standard conditions.

DISCUSSION

Limitations on the Significance of Results Obtained Under Standard Conditions. Manifestation of the Bohr effect cannot be accommodated in experimental elaboration of O₂-equilibrium binding curves, under standard conditions. Acidification of the interior of rbc's *in vivo* occurs in response to trapping of CO₂ in rbc's by carbonic anhydrase, this process occurring continuously as rbc's pass through the capillary beds of the systemic circulation. Acidification of rbc's lowers the affinity of hemoglobin for O₂, facilitating release of O₂ from rbc's. Neutralization of acidification of the interior of rbc's follows release of CO₂ from whole blood into the lung. An increase in the pH of the interior of rbc's raises the affinity of hemoglobin for O₂, facilitating saturation of hemoglobin with O₂ in the lung. The pH-value of the interior of rbc's, then, undergoes a cyclic variation: (i) decreasing as rbc's pass through the systemic circulation and (ii) increasing as rbc's pass through the lungs. *This description of changes in pH-values within rbc's as they pass through the pulmonary and systemic circulations deserves more discussion.*

Conversion of $^T(\text{Hb}_4/\text{BPG})$ to $^R((\text{HbO}_2)_4/\text{BPG})$ in rbc's of whole blood, in the laboratory, is a sequence of five reactions: four O_2 -binding reactions and a single reversible change in the conformation of the four subunits of which the hemoglobin molecule is comprised. The equation of state describing dependence of F on $[\text{O}_2]$ is comprised of only three unknown quantities, due to equivalent reactions for both α - and β -chains. The conformation change is a reversible reaction, being first order in each direction. First-order rate constants can be determined from whatever part of a progress curve can be captured with the stopped-flow kinetics spectrophotometer. Values for K_α , K_c and K_β , determined by kinetic methods, confirm the values of K_α , K_c and K_β established by application of the Perutz/Adair equation to Roughton's data.

T State α -chains are the only reactive species present in $^T(\text{Hb}_4/\text{BPG})$. The T state \rightarrow R state conformation change, cannot occur until both $^T\alpha$ -chains have been converted to $^T(\alpha\text{O}_2)$ -chains. BPG inverts the order in which O_2 molecules react with the dissimilar pair of chains. In the absence of BPG, in an E-free supporting electrolyte, $^R\beta\text{O}_2$ -chains regulate the reactivity of α -chains (Knowles, linked ms 1). In the presence of BPG, α -chains regulate the reactivity of β -chains. T State α -chains maintain the conformation establishing steric hindrance to approach of O_2 to the distal surface of β -chain heme moieties. R State $\alpha^*\text{O}_2$ -chains maintain the conformation permitting unhindered binding of O_2 to the distal surface of β -chain heme moieties.

Equilibrium Constant for the Conformation Change. The equilibrium constant for the conformation change, $K_c = 0.02602$, is of enormous consequence. Following depletion of O_2 from equivalent R state (βO_2) chains, which possess very high affinity for O_2 , $^R\beta$ -chains revert to $^T\beta$ -chains, reimposing distal side steric hindrance to approach of O_2 to the distal surface of β -chains, effectively reducing the equilibrium binding constant of $^R\beta$ -chains from approximately 400,000 L/mol to less than 100 L/mol. This allows diffusive release of O_2 molecules from the rbc without the possibility of O_2 being recaptured by $^R\beta$ -chains. There is no use for high affinity $^R\beta$ -chains in the systemic circulation, The conformation change, R state \rightarrow T state, ensures the highest possible rate of diffusion of O_2 from rbc's. Diffusion of O_2 from rbc's is diminished so

long as R states persist in rbc's. The concentration of O_2 in the tissue irrigated by the systemic circulation can never rise so high as to donate O_2 to rbc's. The value of K_c may be the biophysical basis for high rates of resting metabolism in warm-blooded species.

Flash Photolysis. Progress curves following flash photolysis of CO derivatives of hemoglobin are often inconclusive. Fast rates of recombination with CO have been taken to support the 2-state model (Monod *et al.* 1965). The progress curve following photolysis of $R((HbCO)_4/BPG)$ in rbc lysates depend on the products of photolysis. Removing one CO molecule will generate two possible products: $R(((HbCO)_3(\alpha))/BPG)$ with a single $R\alpha$ -chain or $R(((HbCO)_3(\beta))/BPG)$ with a single $R\beta$ -chain. In the case of $R(((HbCO)_3(\alpha))/BPG)$, with a single $R\alpha$ -chain, one could observe something new: reaction of an $R\alpha$ -chain with CO. The R state $R(((HbCO)_3(\alpha))/BPG)$ tetramer would not relax to a T state molecule, the R state being stabilized by the large negative value of ΔG° released by binding of 3 molecules of CO. The progress curve following limited photolysis (less than 2%) of $R((HbCO)_4/BPG)$ in the presence of O_2 can be attributed to simultaneous binding of O_2 by $R\alpha$ -chains and $R\beta$ -chains, present in concentrations corresponding to the quantum yield of each chain. CO replacement reactions following O_2 -binding could then be monitored. The total time course following photolysis, then, would yield rate constants for O_2 binding to both α - and β -chains and CO replacement rates for O_2 -containing species.

Historical Perspective. Using continuous flow methods, Roughton and Hartridge carried out the first observations of progress curves for reaction of carbon monoxide with oxyHb (1923) and reaction of oxyHb with dithionite (1923a). Roughton also developed accurate methods for determination of equilibrium binding curves for both O_2 and CO, the first paper concerning O_2 equilibrium binding curves appearing in 1931 (Roughton & Forbes). Roughton attempted to account for his results in terms of the Adair model (1925), in which the conversion of Hb_4 to (HbO_2) was a sequence of four O_2 binding reactions. Each step is described by an O_2 -equilibrium binding constant: $K_1 = k_1/k_{-1}$; $K_2 = k_2/k_{-2}$; $K_3 = k_3/k_{-3}$; and $K_4 = k_4/k_{-4}$

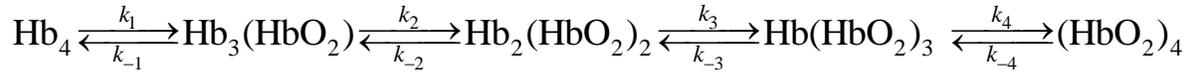


Figure (10). Adair's Model

By the time of the “Haemoglobin Symposium in Memory of Sir Joseph Barcroft in 1949” Roughton (1949) had established a well-defined program to describe the Adair model in physical chemical terms. The goal was to determine (i) the value of the four equilibrium constants in the equation of state defining fractional saturation of hemoglobin with O₂ and (ii) the value of rate constants of progress curves for both the oxygenation reaction, in the presence of excess O₂ and the deoxygenation reaction, in the presence of dithionite. If the four step Adair model was, in fact, an accurate description of the mechanism by which the oxygenation reactions occur, it should be possible to determine real values for the four equilibrium constants and the eight rate constants. Statements concerning the scope of these investigations appeared as early as 1949 (Roughton) and as late as 1972 (Roughton *et al.*).

Redefinition of Adair’s hemoglobin model permits a full and complete achievement of the goals of Roughton and Gibson. The Perutz-Adair equation arises by combining the elements of the Perutz structural (stereochemical) model, accommodating chain heterogeneity, while reducing the number of O₂ equilibrium constants in the sequential Adair equation from four to two: K_α and K_β. Reduction of the number of unknown quantities for O₂ equilibrium constants was realized by assigning equivalent O₂-equilibrium binding constants to both α-chains and both β-chains. In this manner, a sequence of four consecutive O₂ binding reactions requires only two unknown quantities, not counting the dimensionless equilibrium constant for the conformation change. The structural (stereochemical) aspect of the redefined model provides a mechanism whereby chain heterogeneity is established. Both equivalent α-chains react before both equivalent β-chains. Insofar as the 3-stage ordered-sequence is comprised of first-order or pseudo first-order reactions, rate constants are returned by any part of the progress curve captured by the kinetics spectrophotometer.

Our initial estimates of rate constants for the oxygenation and deoxygenation reactions confirm the equilibrium binding constants of the α- and β-chains returned by

the Perutz-Adair equation. This communication claims to have realized the goals of Roughton, fully describing the kinetic properties of each step in the sequential binding of four molecules of O₂ by hemoglobin tetramers in rbc's of whole blood under standard conditions of temperature and pH: 37°C and pH 7.4. This was realized using cell free preparations of human blood in which the stoichiometric ratio of hemoglobin tetramers to the naturally occurring E-molecule, BPG, is maintained intact. The progress curves for the reaction of Hb₄/BPG with O₂ can be formulated as a Bateman equation for an ordered sequence of reactions Figure (1), with the 1st and 3rd stages being pseudo 1st-order and the 2nd step being a reversible conformation change. The same equation describes the progress curve for the deoxygenation reaction of (HbO₂)₄/BPG with dithionite, the 1st and 3rd stages being 1st-order and the 2nd step being a reversible conformation change.

Evaluation of these results and conclusions of these four manuscripts will be judged by their ability to account for the larger aspects of respiratory physiology.

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References

Adair GS (1925). The Hemoglobin System. VI. The Oxygen Dissociation Curve of Hemoglobin. J Biol Chem 63, 529-5454.

DeBruin SH & Janssen LMH (1973). The Interaction of 2,3-Diphosphoglycerate with Human Hemoglobin. J Biol Chem 248, 2774-2777.

Knowles F (1979). Stainless-Steel Syringes for Stopped Flow Kinetics. *Anal Biochem* 93, 407-408.

Knowles F (1984). Application of ^{19}F NMR Spectroscopy to a Study of CO Binding to Human Hemoglobin Modified at Cys- β 93 with the S-Trifluoroethyl Residue. *Arch Biochem Biophys* 230, 327-334.

Monod J, Wyman J, Changeux J-P (1965). On the nature of allosteric transitions: a plausible model. *J Mol Biol* 12, 88-118.

Roughton FJW & Hartridge H (1923). The velocity with which carbon monoxide displaces oxygen from combination with haemoglobin. *Proc R Soc Lond B* 94, 336-367.

Roughton, FJW & Hartridge H (1923a). Kinetics of haemoglobin. II. Velocity with which oxygen dissociates from its combination with haemoglobin. *Proc R Soc Lond A* 104, 395-430.

Roughton FJW & Forbes WH (1931). The equilibrium between oxygen and haemoglobin. The oxygen dissociation curve of dilute blood solutions. *J. Physiol* 71, 229-260.

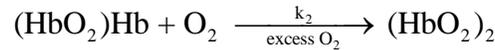
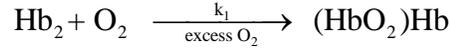
Roughton FJW (1949). The intermediate compound hypothesis in relation to the equilibria and kinetics of the reactions of hemoglobin with oxygen and carbon monoxide. In *Haemoglobin; a symposium based on conference held at Cambridge in June 1948 in memory of Sir Joseph Barcroft*, ed. FJW Roughton and JC Kendrew, 83-93. New York.

Roughton, FJW, DeLand EC, Kernohan JC, Severinghaus JW (1972). In *Oxygen affinity of hemoglobin and red cell acid base status* ed. Rorth M & P Astrup P, pp. 73-83. Copenhagen, Munksgaard.

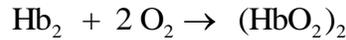
Appendix A

Consecutive Equivalent First Order Reactions

Consider a scheme of two consecutive pseudo-first-order reactions with stoichiometry given by



The hypothetical macromolecule, Hb₂, is defined to be dimeric, each constituent monomer containing a heme moiety capable of reversibly binding a molecule of either O₂ or CO. The dimeric model is intended to be a simplified model for human hemoglobin. In the presence of excess O₂, (HbO₂)(Hb) is produced as an unstable intermediate in the overall reaction given by



We can write the following rate equations, understanding that the rate constants, k_1 and k_2 , are pseudo first-order rate constants, the concentration of O₂ being much greater than the concentration of Hb₂.

$$\frac{d[\text{Hb}_2]}{dt} = -k_1 [\text{Hb}_2] \equiv -k_1' [\text{O}_2] [\text{Hb}_2]$$

$$\frac{d[(\text{HbO}_2)\text{Hb}]}{dt} = k_1 [\text{Hb}_2] - k_2 [(\text{HbO}_2)\text{Hb}] \equiv k_1' [\text{O}_2] [\text{Hb}_2] - k_2' [\text{O}_2] [(\text{HbO}_2)\text{Hb}]$$

$$\frac{d[(\text{HbO}_2)_2]}{dt} = k_2 [(\text{HbO}_2)\text{Hb}] \equiv k_2' [\text{O}_2] [(\text{HbO}_2)\text{Hb}]$$

The first of these three equations is solved by the method for a first-order equation. The equation of state for [Hb₂] is

$$[\text{Hb}_2] = [\text{Hb}_2]_0 \exp(-k_1 t)$$

Substituting the value for [Hb₂] into the second rate equation and rearranging, we obtain a first order linear differential equation.

$$\frac{d[(\text{HbO}_2)\text{Hb}]}{dt} + k_2 [(\text{HbO}_2)\text{Hb}] = k_1 [\text{Hb}_2]_0 \exp(-k_1 t)$$

If $[(\text{HbO}_2)\text{Hb}]_0 = 0$, the solution for $[(\text{HbO}_2)\text{Hb}]$ is

$$[(\text{HbO}_2)\text{Hb}] = [\text{Hb}_2]_0 \left(\frac{k_1}{k_2 - k_1} \right) (\exp(-k_1 t) - \exp(-k_2 t))$$

The solution for $[(\text{HbO}_2)_2]$ can be obtained directly from the mass conservation equations.

$$[\text{Hb}_2]_0 = [\text{Hb}_2] + [(\text{HbO}_2)\text{Hb}] + [(\text{HbO}_2)_2]$$

$$[(\text{HbO}_2)_2] = [\text{Hb}_2]_0 - [\text{Hb}_2] - [(\text{HbO}_2)\text{Hb}]$$

Substituting the equations of state for $[\text{Hb}_2]$ and $[(\text{HbO}_2)\text{Hb}]$ into the second mass conservation equation one obtains the equation of state for $[(\text{HbO}_2)_2]$.

$$[(\text{HbO}_2)_2] = [\text{Hb}_2]_0 \left(1 - \exp(-k_1 t) - \left(\frac{k_1}{k_2 - k_1} \right) (\exp(-k_1 t) - \exp(-k_2 t)) \right)$$

With the equations of state presented above, it is possible to simulate data for each of the concentrations of the reactants in the conversion of Hb_2 to $(\text{HbO}_2)_2$.

Special Considerations for Models of Human Hemoglobin

In the case of reaction of a dimeric hemoglobin molecule with, for example, O_2 , it would be normal to start with a solution free of O_2 . In this case, then, the intermediate, $(\text{HbO}_2)\text{Hb}$, and the end product, $(\text{HbO}_2)_2$, are absent at $t = 0$. The progress curve monitored at an appropriate wavelength in the O_2 difference spectrum. Such monitoring records the time dependence of all intermediates, simultaneously. The time course obtained by spectroscopic procedures, then, is directly proportional to fractional saturation of Hb_2 with O_2 . The equation of state for fractional saturation of Hb_2 binding sites with O_2 is

$$F = \frac{[(\text{HbO}_2)\text{Hb}] + 2 [(\text{HbO}_2)_2]}{2 [\text{Hb}_2]_0}$$

A rate law based on fractional saturation is readily obtained by substituting the individual rate laws for $(\text{HbO}_2)\text{Hb}$ and $(\text{HbO}_2)_2$ presented above.

$$F = \frac{[\text{Hb}_2]_0 (2 \exp(-k_2 t) - 2 \exp(-k_1 t)) + 2 [\text{Hb}_2]_0 (1 + \exp(-k_1 t) - 2 \exp(-k_2 t))}{2[\text{Hb}_2]_0}$$

Factoring out common terms and combining similar quantities, the rate law reduces to a first order equation.

$$F = 1 - \exp(-k_2 t)$$