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Review Article

Equation of State for O_2-Binding by Hemoglobin in Human Red Blood Cells

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ABSTRACT

O_2-Equilibrium binding data of hemoglobin in whole blood under standard conditions was fitted to an equation of state comprised of three unknown quantities: K_α, the equilibrium constant for binding O_2 by equivalent low affinity α-chains; K_Δ, a dimensionless equilibrium constant describing the change between low- and high-affinity structures of hemoglobin, ^{T}state and ^{R}state; K_β, the equilibrium constant for binding O_2 by equivalent high affinity β-chains. Values of the unknown quantities at pH 7.4 and 37°C are: K_α=15,090 L/mol; K_Δ=0.0260; K_β=393,900 L/mol. A graph of predicted versus observed values of fractional saturation, F, is linear: F_{PRE}=0.9998 F_{OBS}=0.0005, R^2=0.9997. The Perutz/Adair equation of state is defined as such insofar as all aspects of the stereo chemical are imposed on the earlier sequential binding model of Adair. The Perutz/Adair equation of state is general, describing: (i) the CO equilibrium binding curve of whole blood under standard conditions, $K_\alpha=4.27 \times 10^6$ L/mol, K_Δ=0.05741, and $K_\beta=99.1 \times 10^6$ L/mol; (ii) the O_2-equilibrium binding curve of purified hemoglobin in 0.100 M NaCl, 0.050 M BisTris, pH 7, 20°C, $K_\alpha=5.34 \times 10^4$ L/mol, K_Δ=0.03252, and $K_\beta=1.81 \times 10^6$ L/mol.

Keywords: Hemoglobin, O_2-Binding, Red blood cells, Stereo chemical model

KEY POINTS

- In the presence of stoichiometric concentrations of 2,3-Bisphosphoglycerate (BPG), O_2-free human hemoglobin experiences enhanced proximal strain in α-subunits and steric hindrance at the surface of the β-chains
- In the presence of stoichiometric concentrations of BPG, O_2-free human hemoglobin exhibits chain heterogeneity: α-chains reacting with O_2 before β-chains react with O_2
- α-Chains exhibit low affinity equivalent O_2-binding. β-Chains exhibit high-affinity equivalent O_2-binding.
- A structural change, ^{T}state to ^{R}state, occurs after equivalent O_2-binding by α-chains and before equivalent binding by β-chains
- An equation of state for the O_2-equiibrium binding curve is comprised of only three unknown quantities: an equilibrium constant for O_2-binding by equivalent α-chains, K_α; an equilibrium constant for O_2-binding by equivalent β-chains, K_β; an equilibrium constant for the ^{T}state to ^{R}state change, K_Δ.

INTRODUCTION

Values for K_α, K_Δ and K_β address only one of three separate aspects of allosteric architecture in human hemoglobin *in vivo*, the other two being: (i) exothermic binding of Hb_4 with BPG, converting ^{R}state to ^{T}state; and (ii) changes in pH of the interior of red blood cells (manifestation of the Bohr Effect). The Perutz/Adair equation defines the value of the equilibrium constant for the ^{T}state → ^{R}state structure change. The value of K_Δ accounts for the ability of red blood cells to release O_2 efficiently and provides a basis for high rates of resting metabolism accounting for warm blood. Upon release of O_2 from β-chains the ^{R}state structure collapses to the ^{T}state structure. The Perutz/Adair equation does not describe O_2-equilibrium binding data under all conditions. O_2-binding data obtained with purified human hemoglobin in 0.050 M potassium phosphate over a range of temperatures from 4°C to 30°C: does not, for example, demonstrate equivalent binding by β-chains (Knowles, unpublished results).

The stereo chemical model describing steps involved in conversion of Hb_4/BPG to $(HbO_2)_4$/BPG occurs in three stages [1,2]. In the first stage of the overall sequence of five discrete steps, both ^{T}state α-chains undergo identical and equivalent O_2-binding reactions, remaining in the unchanged ^{T}state. In the first stage, β-chains are precluded from reaction with O_2 by steric hindrance at the distal surface of equivalent β-chain heme moieties. The second stage in

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the sequence of five distinct reactions is a change in the structure of $((\alpha O_2)_2)\beta_2$/BPG, from ^{T}state to ^{R}state. The structure change (i) relaxes steric hindrance at the surface of the β-chains, opening a pathway for the third stage, and (ii) relaxes proximal strain in ^{T}state αO_2-chains, the conversion to ^{R}state αO_2-chains being accompanied by a negative free energy change. BPG binds to the ^{R}state structure. The third stage, consisting of a sequence of equivalent O_2-binding reactions by ^{R}state β-chains, is free of changes in structure at constant pH. Equation describes the three stages of reactions leading from Hb_4/BPG to $HbO_2)_4$/BPG under standard conditions of temperature, pH, and the presence of CO_2.

Sequence of reactions for conversion of Hb_4/BPG, species I, to $(Hb_4O_2)_4$/BPG, species VI, in three stages. K_1, K_2, K_4, and K_5 are equilibrium constants for O_2-binding reactions. K_Δ is the equilibrium constant for the ^{T}state to ^{R}state change. The change in structure occurs between species III and IV. Conformation state for each species is indicated by a left superscript T or R. DPG is represented as in T-state conformations and o in R-state conformations.

$$F = \frac{([II] + 2[III] + 2[IV] + 3[V] + 4[VI])}{(4([I] + [II] + [III] + [IV] + [V] + [VI]))} \quad \text{..... (1)}$$

F is fractional saturation of the binding sites of human hemoglobin with the sixth axial ligand: O_2 or CO. Analytical expressions relating the concentration of each of the species, II thru VI, to the equilibrium constants, K_1, K_2, K_Δ, K_4, and K_5, and concentration of O_2 are as follows:

$[I]=[I]\,[II]=K_1\,[O_2]\,[I]$

$[III]=K_1\,K_2\,[O_2]2\,[I]\,[IV]=K_1\,K_2\,K_\Delta\,[O_2]2\,[I]$

$[V]=K_1\,K_2\,K_\Delta\,K_4\,[O_2]3\,[I]\,[VI]=K_1\,K_2\,K_\Delta\,K_4\,K_5\,[O_2]4\,[I]$

O_2-binding sites of α-chains are assigned the property of being identical and equivalent. O_2-binding sites of β-chains are also assigned the property of being identical and equivalent. This permits redefinition of the value of equilibrium constants for O_2-binding sites: $K_1=2K_\alpha$; $K_2=K_\alpha/2$; $K_4=2K_\beta$; $K_5=K_\beta/2$. Four unknown quantities K_1, K_2, K_4, and K_5, are replaced by four expressions containing only two unknown quantities: K_α and K_β. Substitution of these statistical equivalents into the analytical expressions for species II thru VI, leads to the Perutz/Adair equation, Equation 2.

$$F = \frac{(2K_\alpha[O_2](1 + K_\alpha[O_2](1 + K_\Delta(1 + K_\beta[O_2](3 + 2K_\beta[O_2])))))}{(4(1 + K_\alpha[O_2](2 + K_\alpha[O_2](1 + K_\Delta(1 + K_\beta[O_2](2 + K_\beta[O_2])))))))} \quad \text{..... (2)}$$

Equation 2 expands the Adair equation to accommodate structural constraints inherent in the Perutz stereo chemical model. These steps serve to reduce the five unknown quantities of Equation 1, to three unknown quantities. These assumptions require that Equation (2) with three unknown quantities accurately predicts equilibrium binding curves for O_2. Results presented below show that Equation (2) describes equilibrium binding curves for (i) both O_2 and CO, in whole blood under standard conditions and for (ii) purified human hemoglobin in 0.050 M BisTris, 100 M NaCl, pH 7 with HCl, 20°C. Initial tests of Equation (2) were carried out with purified human hemoglobin in the presence in 0.050 M KPi. β-chains do not exhibit equivalent O_2-binding in the presence of 0.050 M KPi, pH 7.0, 20.0°C. Four unknown quantities are required to obtain fits of O_2-binding data with correlation coefficients of 0.999. Non-equivalent binding to β-chains in 0.050 M KPi, together with marked differences in the value of K_Δ, provides insight into the role of E-molecules in the manifestation of allosteric properties of enzymes demonstrating cooperative properties, in general [3-6].

LITERATURE REVIEW

The O_2-Equilibrium Binding Curve of Purified Human Hemoglobin in 0.050 M BisTris, 0.100 M NaCl, pH 7.00, 20.0°C. O_2-Equilibrium binding data obtained in 0.100 M NaCl, 0.050 M BisTris, pH 7 with HCl, 20.0°C was fitted to Equation 2. The best-fitting values of K_α, K_β, and K_Δ, together with statistical information on the curve-fitting procedure, are summarized in Table 1. Binding data for O_2 (Knowles, Architecture of Allosteric Structure. Equation 1 is well fitted by Equation 2, the value of R^2 being 0.9997.

Table 1: Curve fitting of the O_2 equilibrium binding curve of purified human Hb_4 in 0.050 M BisTris, 0.100 M NaCl, pH 7.00 with HCl, 20.0°C to the Perutz-Adair equation, Equation 2.

K_α	5.344×10^4 L/mol
K_Δ	0.03252
K_β	1.809×10^6 L/mol
R^2	0.9997
RMSE	0.005455

The first stage is characterized by a low value for K_α, 5.344×10^4 L/mol, relative to the value of K_β, 1.809×10^6 L/mol. The O_2-affinity expressed by the β-chains is greater than that of the α-chains by a factor of 33.85. α-Chains, nevertheless, are titrated with CO before β-chains (Knowles). These results support the assignment of distal side steric hindrance of β-chain heme moieties of Hb_4/BPG observed by Perutz. Purified human Hb_4/(Cl-1)n in 0.050 M BisTris, 0.100 M NaCl appears to behave as an ideal cooperative dimer, in which the O_2-affinity of the set of equivalent β-chains is elevated from a value significantly less than that of the first set of equivalent α-chains to a value almost 34-fold higher than that expressed by the first set of equivalent α-chains, upon binding of O_2. In the case of human Hb_4/BPG the cooperative mechanism: (i) relaxes proximal strain in ^{T}state αO_2-chains and (ii) provides unhindered access to the distal surface of the β-chain heme moiety to O_2. O_2-binding by the pair of equivalent ^{T}state α-chains both creates and enables the switching mechanism controlling access of pre-existing sterically blocked ^{R}state β-chains to O_2 molecules.

The ability of Equation 2 to predict observed data was tested by comparing predicted and observed values of F of hemoglobin with O_2. Predicted values of F, F_{PRE}, are plotted against the observed values of F, F_{OBS}, in Figure 1. This plot is expected to be linear, represented by the equation y=x, if the equation of state accurately describes the values of F_{OBS}. The best fitting straight line has the equation F_{PRE}=0.9998 F_{OBS}=-0.0005, quite close to y=x. Deviation of the best fitting straight line from y=x is not visible to the unaided eye.

The Perutz/Adair Equation 2 is found to accurately describe observed values of F_{OBS} with three unknown quantities. The general form of the Adair equation should be replaced by Equation 2. Gilbert Smithson Adair was correct in recognizing the conditions justifying the use of equivalent binding constants. The results obtained by the Perutz/Adair equation, constrained to equivalent binding by identical chains are subject to a level of interpretation

not possible with the results returned by curve fitting procedures using the general form of the Adair equation.

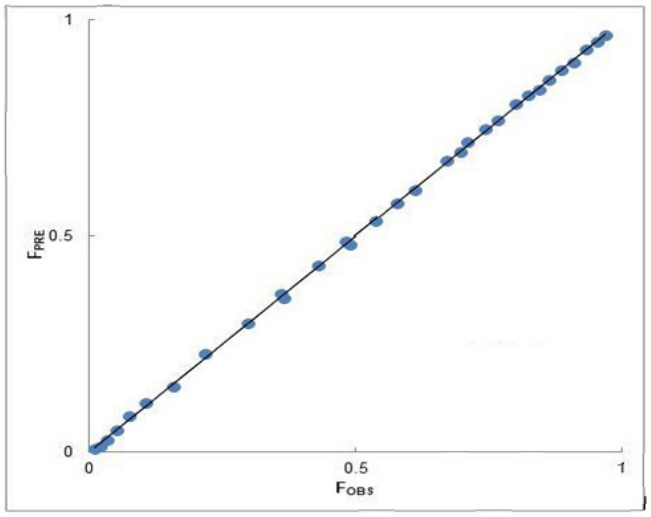

Figure 1: Observed and predicted values of F of the O_2-Equilibrium binding curve of purified Hb4 in 0.100 M NaCl , 0.05 M BisTris, pH 7.00 with HCl, 20.0°C. Predicted values of F, F_{OBS}, were calculated with Equation (2), using the best fitting values of K_α, K_β, and K_Δ, given in Table 1, and the corresponding O_2-values. y=0.9998x-0.00065, R^2=0.9997

The CO Equilibrium Binding Curve of Whole Blood in Isotonic Saline, pH 7.4, PCO_2=40 torr, 37°C (Data of Roughton 1970). The CO equilibrium binding curve of whole human blood was fitted to the Perutz-Adair equation. Roughton's data (1970) presented CO concentrations as torr, in the gas phase in equilibrium with the solution containing red blood cells. Units in torr were converted to the concentration of CO in the liquid phase in units of µmol/L at 37 degrees C: 1.000 torr=1.0356 µmol CO/L. The best-fitting values of K_α, K_β, and K_Δ, together with statistical information on the curve fitting procedure, are summarized in Table 2. Roughton's CO-equilibrium binding data is well fitted by Equation 2.

Table 2: Curve fitting of the CO-equilibrium binding curve of whole blood, pH 7.4, 37°C, under standard conditions, to the Perutz-Adair equation, Equation 2.

K_α	4.274×10^6 L/mol
K_Δ	0.05741
K_β	99.06×10^6 L/mol
R^2	0.9997
RMSE	0.00654

The first stage is, again, characterized by a low value for K_α, 4.274×10^6 L/mol, relative to the value of K_β, 99.06×106 L/mol. The O_2-affinity expressed by the β-chains is greater than that of the α-chains by a factor of 23.17. The α-chains, nevertheless, are titrated with CO before the β-chains. These results support the assignment of distal side steric hindrance of β-chain heme moieties in ^{T}state structures observed by Perutz. Whole blood, under standard conditions, behaves as an ideal cooperative dimer, in which the CO-affinity of equivalent β-chains is elevated from a value significantly less than that of the first set of equivalent α-chains to a value 23-fold higher than that expressed by equivalent ^{T}state α-chains. In the case of hemoglobin, within RBC's, the cooperative mechanism relies on control of steric hindrance at the distal surface of the β-chain heme moiety and this switch responds to CO-binding by the pair of equivalent T-state αO_2-chains. The results obtained with whole blood, under standard conditions, are similar to those obtained with purified hemoglobin in 0.050 M BisTris, 0.100 M NaCl, pH 7.00, 20.0°C, insofar as the pattern of returned values are concerned. The actual values are, of course, quite different, insofar as CO is more tightly bound than O_2. The value of K_Δ, however, is quite independent of the structure of the sixth axial ligand, being 0.05741 for binding of CO by whole blood and 0.03252 for binding of O_2 by purified hemoglobin in the presence of 0.100 M NaCl.

The ability of Equation 2 to describe observed data was tested by comparing predicted and observed values of fractional saturation of whole blood with CO. Predicted values of F, F_{PRE}, are plotted against the observed values of F, F_{OBS}, in Figure 2. Again, this plot is expected to be linear, (y=x), if the equation of state accurately predicts the values of F_{OBS}. The best fitting straight line has the equation

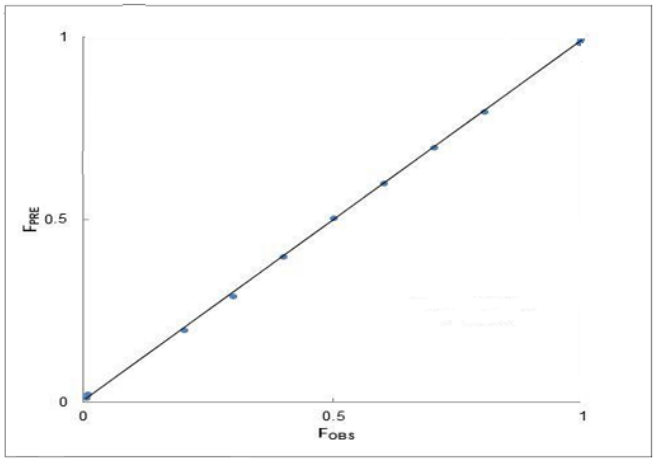

Figure 2: Observed and predicted values of fractional saturation of the CO- equilibrium binding curve of whole blood under standard conditions. Predicted values of F, F_{PRE}, were calculated using Equation (2) and the best fitting values of K_α, K_β, and K_Δ, given in Table 2, and the corresponding CO-values. y=0.9998x-0.00065, R^2=0.9997

F_{PRE}=0.9915 F_{OBS}+0.0052, quite close to y=x. The two lowest points deviate from the best fitting line.

The Perutz/Adair equation, Equation 2, with three unknown quantities, accurately and precisely describes Roughton's CO-equilibrium binding curve for whole blood, under standard conditions.

The O_2-Equilibrium Binding Curve of Whole Blood in Isotonic Saline, pH 7.4, p(CO_2)=40 torr, 37°C [7].

The O_2-equilibrium binding curve of whole blood, obtained under standard conditions, combining data collected by (1972) is presented in Figure 3. O_2 concentrations expressed in units of torr in the gas phase were converted to units of concentration in the liquid phase in equilibrium with the gas phase. The concentration of O_2 in the liquid phase in units of µmol O_2/L is equal to p(O_2) in units of torr multiplied by 1.00023. The binding curve was fitted to the Perutz-Adair equation. Best-fitting fitting values of K_α, K_β, and K_Δ, together with statistical information on the curve fitting procedure are summarized in Table 3.

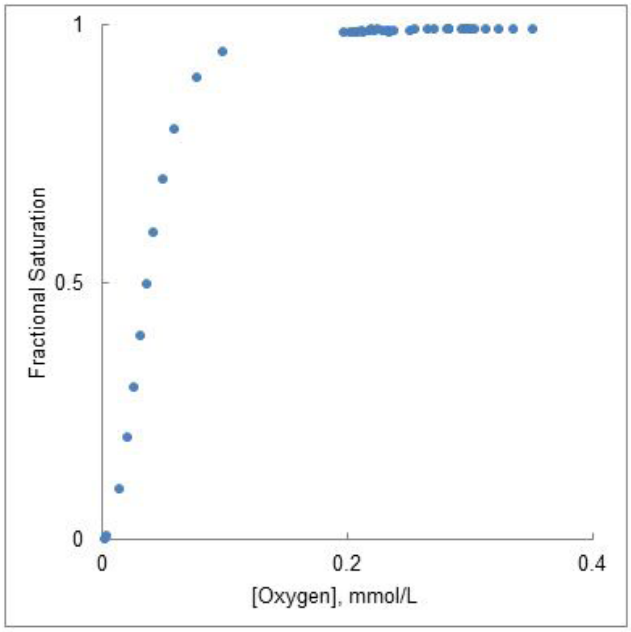

Figure 3: O_2-Equilibrium binding curve of whole human blood under standard conditions.

Table 3: Curve fitting of the O_2-equilibrium binding curve of whole blood, under standard condition, to Equation 2.

K_α	15.09×10^3 L/mol
K_Δ	0.02602
K_β	393.9×10^3 L/mol
R^2	0.9998
RMSE	0.003609

Again, the first stage is characterized by the low value for K_α of 15.09×10^3 L/mol, relative to the value of K_β, 393.9×10^3 L/mol. The O_2-affinity expressed by the β-chains is greater than that of the α-chains by a factor of 26.10. Whole blood, under standard conditions, appears to behave as an ideal cooperative dimer, in which the O_2-affinity of the second set of equivalent β-chains is elevated from a value significantly less than that of the first set of equivalent α-chains to a value 26-fold higher than that expressed by the first set of equivalent α-chains, upon binding of O_2 [8-16]. Results obtained with whole blood for O_2, under standard conditions, are similar to those obtained with (i) purified hemoglobin in 0.050 M BisTris, 0.100 M NaCl, pH 7.00, 20.0°C and (ii) whole blood for CO, under standard conditions, insofar as an identical pattern of returned values are concerned. The actual values are, of course, quite different, insofar as O_2 is bound much less tightly in whole blood, under standard conditions, than by either (i) purified hemoglobin in the presence of 0.100 M NaCl or (ii) CO in whole blood. The value of K_Δ is independent of the structure of the: (i) sixth axial ligand or (ii) composition of the supporting electrolyte. Direct comparison of the value of K_Δ with CO and O_2 in whole blood, shows only a 2.17-fold difference.

Values of F_{PRE} are plotted against F_{OBS}, in Figure 4. The best fitting straight line has the equation F_{PRE}=0.9955 F_{OBS}=0.004, quite close to y=x.

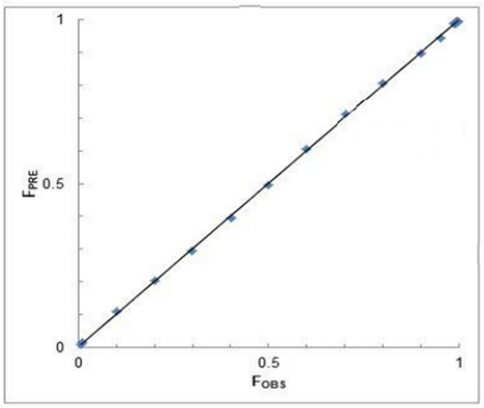

Figure 4: Observed and predicted values of F of the O_2-equilibrium binding curve of whole blood under standard conditions. Predicted values of F, F_{PRE}, were calculated with Equation (2), using the best fitting values of K_α, K_β, and K_Δ, given in Table 3, and the corresponding O_2-values. y=0.9998x-0.00065, R^2=0.9997

The Perutz-Adair equation, Equation 2, with three unknown quantities, accurately and precisely describes the Roughton/Severinghaus O_2-equilibrium binding curve for whole blood, under standard conditions.

DISCUSSION AND CONCLUSION

The Perutz/Adair equation fits equilibrium binding data of Hb_4/E for O_2 for a wide variety, but not all, E-molecules. The most important E-molecule for human hemoglobin is, of course, naturally occurring BPG. The Perutz/Adair equation is the simplest possible elaboration of the Adair equation, taking into consideration significant discoveries following Adair's publications. (i) Purple hexagonal crystals of horse Hb_4/BPG will shatter and dissolve if O_2 is allowed to diffuse into the crystals followed by formation of scarlet monoclinic needle crystals of (HbO_2)/BPG. This observation by Felix Haurowitz is a silent argument that the transition from ^{T}state to ^{R}state is structural rather than an insignificant conformational change. (ii) The subunits of which a hemoglobin molecule is comprised are not all identical. (iii) Recognition of the role of BPG and other E-molecules on the properties of human hemoglobin was unappreciated. (iv) Formulation of the stereochemical model introduced observations leading to formulation of this manuscript. (v) Demonstration of chain heterogeneity in O_2-equilibrium binding curves led to formulation. (vi) Imposition of equivalent binding on both the α-and β-chains reduces the number of unknown quantities required to describe O_2-equilibrium binding reactions from 4, in the original form of the Adair equation, to 3 in the Perutz-Adair equation, Equation 2. In this instance a mathematical advance demanded the acceptance of the chemical concept that a macromolecular structure with equivalent binding sites was rigid.

Introduction of the equilibrium constant for the inter-conversion of ^{T}state and ^{R}state structures introduced a third unknown quantity, a dimensionless equilibrium constant for the ^{T}state to ^{R}state change, K_Δ. Equation 2 permits, for the first time, meaningful comparisons of the values of K_α and K_β obtained from different species, as well as a meaningful interpretation of the value of K_Δ.

In each of the equilibrium binding experiments described above, the binary complex of human Hb_4 and an E-molecule, behaves in a fashion similar to that of a cooperative dimer comprised of

dissimilar subunits. The first subunit, or set of equivalent subunits, regulates the reactivity of the second subunit, or set of equivalent subunits. The ^{R}state to ^{T}state change induced by formation of the binary complex of human Hb_4 and BPG regulates O_2-affinity of α-chains by enhancing pre-existing proximal strain in the bond between an imidazole sidechain and the iron atom of the heme moiety, thereby reducing O_2-affinity of α-chains. The ^{T}state also regulates O_2-affinity of β-chains by blocking access of O_2 to the distal surface of the β-chain heme moiety. Binding of O_2 to β-chains requires (i) saturation of low affinity ^{T}state α-chains and (ii) over riding an endothermic ^{T}state to ^{R}state structure change. Reversal of the exothermic conformation change induced by the E-molecule, $\Delta G°$=-33.7 kJ/mol, requires an energy source. Energy sources are provided by conversion of ^{T}state αO_2-chains to ^{R}state αO_2-chains and exothermic binding of BPG to ^{R}state structures. The change in energy from the change in state for the pair of ^{T}state αO_2-chains can be estimated if it is assumed that ^{R}state O_2-affinity is similar to that expressed by β-chains, K_α increasing from 15.09 L/mmol to 393.9 L/mmol, for an increase in $\Delta G°$ of -16.8 kJ for the pair of α-subunits. This energy would be available to uncouple BPG from ^{T}state conformations sufficiently for the endothermic ^{T}state to ^{R}state change to take place. These numbers alone, closely account for the values of K_Δ.

The data for whole blood with O_2 and CO can be compared and used to confirm the validity of Haldane's Second Law. Haldane's Second Law requires that the equation of state for F of hemoglobin with either O_2 or CO be identical in form, the only difference being that the values of the equilibrium constants differ. Determined that equilibrium constants for CO exceed those for O_2 by a factor (M-factor) of 235.15 results given in Tables 2 and 3 return an M-factor of 283 for α-chains, in the T-state, and 252 for β-chains, in the R-state. Haldane's Second Law would appear to be substantiated by these results. The values of M are in good agreement but not as significant as is the fact that the Perutz/Adair equation fits both sets of data: CO-binding and O_2-binding. The Perutz-Adair equation can be modified and used to generate O_2-equilibrium binding curves in the presence of CO (unpublished data, Knowles). The Perutz-Adair equation, then, holds promise of further clarification of the functional properties of hemoglobin molecules. The Perutz/Adair equation of state, Equation 2, is incomplete. Standard conditions do not, in fact, replicate conditions experienced by red blood cells as they pass through the pulmonary circulation (increasing-pH values) and systemic circulation (decreasing pH-values). A full description of the equation of state must take into account the pH-dependence of F in red blood cells.

The 2-state model does not describe the equilibrium binding curves obtained with either O_2 or CO. The 2-state model does not resemble the model presented. This work cannot make a judgment about the ability of 2-state model that may well describe the properties of other enzymes that cannot, in turn, be accommodated by the Perutz/Adair equation. Other methods of investigation may provide additional insights. Analysis of progress curves for the reaction of $^{T}((Hb_4)$/BPG) with O_2 or reaction of $^{R}((HbO_2)_4$/BPG) with dithionite may either support or refute the model which underlies the Perutz/Adair equation.

The following elements of allosteric structure are implicit in the Perutz/Adair equation. Two cooperative dimeric subunits, ($T1_\alpha$, $R2_\beta$) and ($T2_\alpha$, $R1_\beta$), exist in Hb_4, in which β-chains regulate α-chains. The order of binding of O_2 to a cooperative dimer is first β, then α. An ^{R}state to ^{T}state structure change arises from binding of BPG to Hb_4, enhancing pre-existing proximal strain in α-chains, thereby reducing the affinity of α-chains for O_2 and introducing steric hindrance at the distal surface of β-chain heme moieties. BPG acts as a non-competitive inhibitor. The order in which O_2 binds to the subunits of Hb_4 is inverted by formation of the Hb_4/BPG binary complex. ^{T}state α-chains are identical and equivalent in the presence of BPG. Binding of O_2 to β-chains requires a structure change from ^{T}state to ^{R}state. The ^{R}state structure formed in response to oxygenation reactions of α-chains binds BPG. The significant free energy change required for the formation of an oxygenated ^{R}state from the oxygenated ^{T}state is provided by: (i) the very low value of K_Δ; (ii) conversion of ^{T}state αO_2-chains to ^{R}state αO_2-chains; (iii) binding of BPG by the ^{R}state structure. No structural changes occur upon binding of O_2 to ^{R}state structures. These elements of allosteric structure comprise the architecture of allosteric structure at constant pH.

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