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Research Article

Allosteric Structure of Effector-free Human Hemoglobin

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ABSTRACT

The O_2-affinity of human hemoglobin, free of E-molecules, was measured in 0.050 M Bis-Tris, pH 7.0 with HCl, 20°C. A Hill plot of O_2-equilibrium binding data reveals an initial slope of 2 and fails to demonstrate an upward inflection. Four O_2-binding reactions account for these results. The tetrameric hemoglobin structure in an E-free supporting electrolyte can be described as two cooperative dimeric subunits: ($^{T,1}\alpha$, $^{R,2}\beta$) and ($^{T,2}\alpha$, $^{R,1}\beta$). Subunits of human Hb_4 are α- and β-chains. Within the hemoglobin tetramer: (i) Parenthetical inclusions describe the composition of cooperative dimeric subunits; (ii) Superscripts in the upper left identify the position of the subunit in the tetramer, and conformational state; R for the high affinity state and T for the low affinity state. Equilibrium constants of these four steps are related to intrinsic O_2-binding constants for α and β chains, K_α and K_β, respectively, by statistical factors: The equation of state contains these four unknown quantities.

Keywords: E-molecules; Cooperative mechanisms; Affinity; Electrolyte

INTRODUCTION

O_2-Binding constants are not expected to be identical for each of the dimeric cooperative subunits: ($^1\alpha,^2\beta$) and ($^2\alpha,^1\beta$). The first cooperative subunit, ($^{T,1}\alpha$, $^{R,2}\beta$), binds O_2 while bound to an O_2-free cooperative dimer, ($^{T,2}\alpha,^{R,1}\beta$). The second cooperative dimer, ($^{T,2}\alpha$, $^{R,1}\beta$), binds O_2 while bound to $R(^1\alpha O_2,^2\beta O_2)$. The best fitting values obtained in fitting O_2-binding data in an E-free supporting electrolyte to the equation of state, in units of L/μmol are: K_1=0.2913; K_2=1.112; K_3=0.4912; K_4=0.3185. A plot of predicted values of F *versus* observed values of F is linear: F_{PRE}=0.9995 F_{OBS}+0.0007 with a correlation coefficient of 0.9995. In the instance of each cooperative dimer, K_α exceeds K_β. An equilibrium constant describing structural changes such as K_Δ=[^{R}state]/[^{T}state] does not appear in E-free supporting electrolytes [1].

$$F=((K_1[O_2](1+K_2[O_2](2+K_3[O_2](3+4K_4[O_2])))))/((4(1+K_1[O_2](1+K_2[O_2](1+K_3[O_2](1+K_4[O_2]))))))$$

O_2-Binding constants are not expected to be identical for each of the dimeric cooperative subunits: ($^1\alpha,^2\beta$) and ($^2\alpha,^1\beta$). The first cooperative subunit, ($^{T,1}\alpha$, $^{R,2}\beta$), binds O_2 while bound to an O_2-free cooperative dimer, ($^{T,2}\alpha,^{R,1}\beta$). The second cooperative dimer, ($^{T,2}\alpha$, $^{R,1}\beta$), binds O_2 while bound to $^R(^1\alpha O_2,^2\beta O_2)$. The best fitting values obtained in fitting O_2-binding data in an E-free supporting electrolyte to the equation of state, in units of L/μmol are: K_1=0.2913; K_2=1.112; K_3=0.4912; K_4=0.3185. A plot of predicted values of F *versus* observed values of F is linear: FPRE=0.9995 F_{OBS}+0.0007 with a correlation coefficient of 0.9995. In the instance of each cooperative dimer, K_α exceeds K_β. An equilibrium constant describing structural changes such as K_Δ=[^{R}state]/[^{T}state] does not appear in E-free supporting electrolytes [2].

Addition of 0.1 M NaCl to the supporting electrolyte results in an approximately 4-fold increase in the concentration of O_2 required for half saturation of hemoglobin: 1.9 μmol/L in 0.05 M Bis-Tris, pH 7, 20°C and 8.1 μmol/L in the presence of 0.10 M NaCl. O_2-Equilibrium binding data obtained in the presence of 0.10 M NaCl cannot be fitted by the equation of state described above for the E-free solution. O_2-Equilibrium binding data obtained in the presence of 0.100 M NaCl can, however, be fitted by the Perutz-Adair equation: K_α=5.3 × 104 L/mol; Kc=0.0325; K_β=1.8 × 106 L/mol [3,4].

Supplementing 0.050 M Bis-Tris, pH 7.0 with HCl with 0.100 M NaCl results in a supporting electrolyte that induces a T-state of relatively high affinity in Hb_4 compared to the T-state obtained in the binary complex of Hb_4/BPG. Hb_4 appears to respond to the presence of chloride ions in the central cavity. The mechanism of the response to chloride ions is attributed to neutralization of positively charged residues in the central cavity of Hb_4 by the relatively high concentration of chloride ions

It is of interest to consider the effect on the O_2-equilibrium binding properties of human rbc-lysates, brought about by quantitative removal of E-molecules. BPG, as well as many other E-molecules,

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stabilize a ^{T}state structural configuration (described as a conformation in earlier literature) of Hb_4 (Accession Code: UniProtKB-P69905 (HbA_Human)) resulting in markedly diminished affinity for O_2. Hb_4, in supporting electrolytes free of any E-molecules, is not in the structural configuration characteristic of Hb_4/BPG. Hb_4/BPG only exists *in vitro*, not being presented *in vivo* under normal conditions. E-free Hb_4 contains elements of structure which respond to the presence of BPG. Information concerning these incomplete unit(s) of allosteric structure is reflected in the O_2-equilibrium binding data obtained in E-free supporting electrolytes. O_2-Equilibrium binding data (Knowles)[1] and 19F-nmr data (Knowles)[2] identify cooperative dimeric subunits in the hemoglobin tetramer. Equation a, a model describing structural changes as E-free $\alpha^2\beta^2$ is converted to E-free $(\alpha O_2)^2(\beta O_2)^2$, by a sequence of four O_2-binding reactions, is supported by the experimental results described herein. This model can be viewed as a variant of the Adair model insofar as it is comprised of a sequence of O_2-binding reactions[3].

$$\underset{\text{I}}{(^{T,1}\alpha,^{R,2}\beta)(^{T,2}\alpha,^{R,1}\beta)} \xrightarrow[\pm O_2]{K_1 = 2K_\beta} \underset{\text{II}}{^{R}(^{1}\alpha,^{2}\beta O_2)(^{T,2}\alpha,^{R,1}\beta)} \xrightarrow[\pm O_2]{K_2 = K_\alpha} \underset{\text{III}}{^{R}(^{1}\alpha O_2,^{2}\beta O_2)(^{T,2}\alpha,^{R,1}\beta)}$$

$$\xrightarrow[\pm O_2]{K_3 = K_\beta} \underset{\text{IV}}{^{R}((^{1}\alpha O_2,^{2}\beta O_2)(^{2}\alpha,^{1}\beta O_2))} \xrightarrow[\pm O_2]{K_4 = K_\alpha/2} \underset{\text{V}}{^{R}((^{1}\alpha O_2,^{2}\beta O_2)(^{2}\alpha O_2,^{1}\beta O_2))} \quad \text{.....a}$$

Equation a is a model for the reaction sequence from E-free Hb_4, species I, to $(HbO_2)_4$, species V. Hemoglobin subunits are represented as α and β. Parenthetical inclusions describe cooperative dimeric subunits. Superscript integers to the upper left describe: the position of subunits in the hemoglobin tetramer and the conformational state, R or T

In this communication, an equation of state for E-free Hb_4 is presented for O_2-equilibrium binding curves and tested for its ability to account for equilibrium binding data. Fractional saturation, F, is defined in Equation (1), with integers representing the number of occupied O_2-binding sites on each of the species defined in Equation a.

$$F = \frac{\text{sites occupied by } O_2}{\text{total number of sites}} = \frac{[II] + 2\,[III] + 3\,[IV] + 4\,[V]}{4\,([I] + [II] + [III] + [IV] + [V])} \quad \text{.....(1)}$$

The allosteric architecture of species I through V is assigned in Equation a, cooperative units of structure being enclosed within brackets, as in ($^1\alpha$ $^2\beta$) and ($^2\alpha$ $^1\beta$). Substitution of analytical expressions for species I through V, obtained from individual mass action expressions, into (1) leads to the equation of state, (2), relating F to $[O_2]$

$$[I] = [I] \qquad [II] = K_1[O_2]\,[I] \qquad [III] = K_1 K_2 [O_2]^2\,[I]$$

$$[IV] = K_1 K_2 K_3 [O_2]^3\,[I] \qquad [V] = K_1K_2K_3K_4\,[O_2]^4\,[I]$$

$$F = \frac{(K_1[O_2](1 + K_2[O_2](2 + K_3[O_2](3 + 4K_4[O_2]))))}{(4(1 + K_1[O_2](1 + K_2[O_2](1 + K_3[O_2](1 + K_4[O_2])))))} \quad \text{.....(2)}$$

Equation (2), based on binding constants intrinsic to α-chains and β-chains, Equation a, requires four variables to describe the O_2-equilibrium binding curve modeled in Equation a. Equation (2), presented in a form compressed by repeated algebraic factoring, is found to describe the E-free O_2-equilibrium binding curve. O_2-Equilibrium binding constants are expected to be related to intrinsic binding constants for a- and b-chains, K_α and K_β, respectively, by statistical factors: K_1=2 K_β, K^2=K_α, K_3=K_β, K_4=0.5 K_α. Statistical factors suggest that the equation of state can be expressed with only 2 unknown quantities: K_β and K_α.

Efforts to establish an E-Free supporting electrolyte demand an amine buffer. Amine buffers, such as Bis-Tris, require neutralization with an acid to obtain a pH-value of 7. The conjugate base of any acid that might be utilized to neutralize a solution of Bis-Tris is likely to be an E-molecule. Therefore, it is necessary to use as low a concentration of buffering amine as possible. Use of a strong acid ensures that un-dissociated acids will not be present in the supporting electrolyte. HCl was chosen to adjust the pH of 0.05 M Bis-Tris. The pK-value of Bis-Tris suggests that the concentration of chloride ion in solutions used to establish binding data was 0.012 M. O_2-Equilibrium binding curves in 0.025 M Bis-Tris, pH 7 and 0.05 M Bis-Tris, pH 7 are identical, establishing that chloride ion, at the concentrations used in these experiments, does not act as an E-molecule. A response to chloride ions requires concentrations as high as 0.10 M [5,6].

MATERIALS AND METHODS

Stock solutions of human hemoglobin were prepared from whole blood obtained from a single donor. These stock solutions of hemoglobin were subjected to buffer exchange operations on a column of Sephadex G-25 equilibrated witheither (i) 0.050 M Bis-Tris, pH 7.0 or (ii) 0.050 M Bis-Tris, 0.100 M NaCl, pH 7.0. The hemoglobin solutions were further diluted with either (i) 0.050 M Bis-Tris, pH 7.0 or (ii) 0.050 M Bis-Tris, 0.100 M NaCl, pH 7.0 to provide approximately 200 mL of a hemoglobin solution suitable for use in the gas-liquid equilibration apparatus [7]. The details of the procedure for purification of stock solutions of hemoglobin, generation of gas mixtures of known composition, equilibration of hemoglobin solutions with gas mixtures, and spectroscopic analysis of Hb_4 solutions partially saturated with O_2 have previously been described (Knowles and Gibson)[9]. Bis-Tris, obtained from Aldrich Chemical Company, was crystallized from ethanol. The heme concentration of samples of hemoglobin used for elaboration of O_2-binding curves was 300-400 μM. Binding data were fitted to the appropriate equation of state using XYCALC, Version 3.0. XYCALC determined best fitting values of non-linear parameters by an iterative procedure based on Nelder and Mead's downhill simplex method. These results have been confirmed by use of the curve fitting tool in MatLab [8,9].

RESULTS AND DISCUSSION

The affinity of Hb_4 for O_2 in 0.050 M Bis-Tris, pH 7.0 with HCl, 20.0°C

Equilibrium binding data are presented in Table 1, O_2 concentrations being expressed in units of μmoles/L. The concentration of O_2 in the liquid phase, required for half-saturation of Hb_4, is 1.90 μmol/L (corresponding to a partial pressure of 1.07 torr O_2 in the gas phase). The O_2-equilibrium binding curve is presented in Figure 1.

A hill plot of O_2 binding data is presented in Figure 2. The shape of the hill plot deviates from the expected sigmoid-shape in several respects: (i) the limiting n-value, as the concentration of O_2 approaches zero, is 2 rather than 1; (ii) an upward inflection is not observed; (iii) the n-value decreased from 2.0 to values approaching unity at concentrations of O_2 supporting values of F greater than 0.75.

The best fitting values obtained in fitting O_2-binding data in an E-free supporting electrolyte to the equation of state, in units of L/μmol are: K_1=0.2913; K^2=1.112; K_3=0.4912; K_4=0.3185. The correlation coefficient for fitting of binding data to the equation of state is 0.9995. Assigning equilibrium constants to

individual chains in the first cooperative subunit yields: K_β=0.146 and K_α=1.112. Equilibrium constants for the second cooperative subunit are K_β'=0.4912 and K_α'=0.637. A plot of predicted values of F *versus* observed values of F, (Figure 3), is linear, F_{PRE}=0.9995 F_{OBS}+0.0007, a straight line being F_{PRE}=F_{OBS}. The correlation coefficient is 0.9995. In each cooperative dimer, K_α exceeds K_β. Predicted values of F, F_{PRE}, were obtained using the best fitting values for the equilibrium constants together with Equation (2), and the experimentally determined values for concentration of O_2. The graph of F_{PRE} *versus* F_{OBS} is given in Figure 4.

The value of K_β, the first subunit to bind O_2, is small in comparison with the value of K_α=7.62. Binding of O_2 to the α-chain in each cooperative subunit is regulated by reaction of the β-chain with O_2. The value of $K_\alpha \backslash K_\beta$ for the second cooperative dimeric subunit is significantly less: 1.30, due to a decrease in the value of K_α and an increase in the value of K_β.

Affinity of Hb_4 for O_2 in 0.050 M Bis-Tris, 0.100 M NaCl, pH 7.0, 20.0°C

O_2-affinity of Hb_4 in 0.050 M Bis-Tris, pH 7.00, 20.0°C responds to the addition of NaCl to a final concentration of 0.100 M, binding data being presented in Table 2. The effect of addition of 0.100 M NaCl to 0.050 M Bis-Tris, pH 7.0, 20.0°C is: (i) to increase the O_2 concentration in the liquid phase at which Hb_4 is half-saturated with O_2 from 1.9 μmol/L to 8.1 μmol/L, and (ii) to change, dramatically, the shape of the Hill plot, (Figure 5). The equilibrium binding curve is markedly S-shaped. Fitting of O_2-equilibrium binding data to the Perutz/Adair equation yields: K_α=5.3 × 104 L/μmol; K_Δ=0.0325; K_β=1.8 × 105 L/μmol. The correlation coefficient for the curve fitting procedure is 0.9997 A graph of predicted values of F *versus* observed values of F should be linear: F_{PRE}=F_{OBS}. A graph of F_{PRE} *versus* F_{OBS} yields: F_{PRE}=0.995 F_{OBS}+0.0007, (Figure 6).

Table 1: Binding data for Hb_4 in 0.050 M BisTris, pH 7.0, 20.0°C.

$[O_2]$ μmol/L	F	$[O_2]$ μmol/L	F
0.303	0.0325	3.32	0.733
0.357	0.044	3.76	0.756
0.446	0.0675	5.32	0.826
0.553	0.0785	6.9	0.877
0.624	0.0945	8.14	0.903
0.731	0.153	9.42	0.913
0.946	0.207	10.6	0.918
1.18	0.291	11.8	0.929
1.46	0.372	12.8	0.935
1.75	0.447	15	0.941
2.05	0.526	17.3	0.948
2.36	0.573	21.3	0.957
2.66	0.635	25.9	0.965
3	0.687		

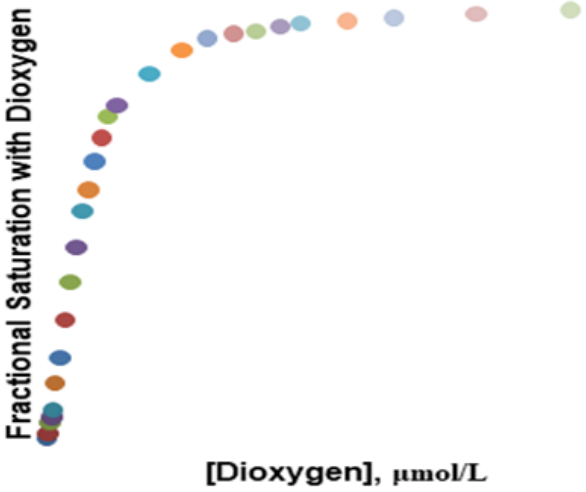

Figure 1: O_2 Equilibrium binding curve of E-free purified human hemoglobin. Data obtained in 0.050 M BisTris, pH 7.00 with HCl, 20.0°C.

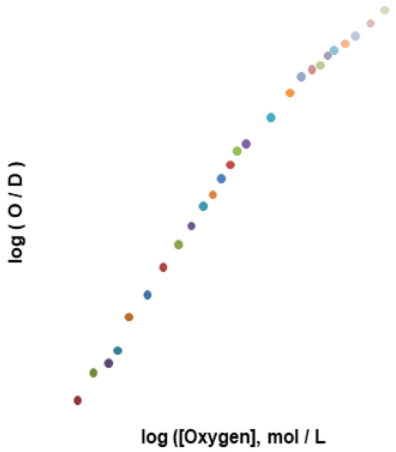

Figure 2: Hill plot of O_2 equilibrium binding data. The supporting electrolyte is 0.050 M BisTris, pH 7.00 with HCl, 20.0°C.

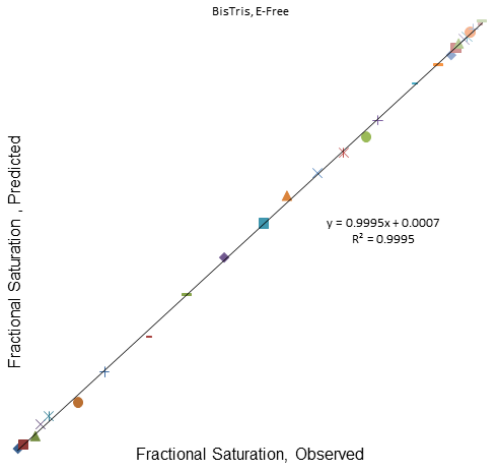

Figure 3: Evaluation of the ability of the equation of state to predict F of Hb_4 in E-free electrolytes. Comparison of observed values of F in 0.050 M BisTris, pH 7.0, 20.0°C with values of F predicted by the equation of state, Eq. (1.3) and the best fitting values of K.

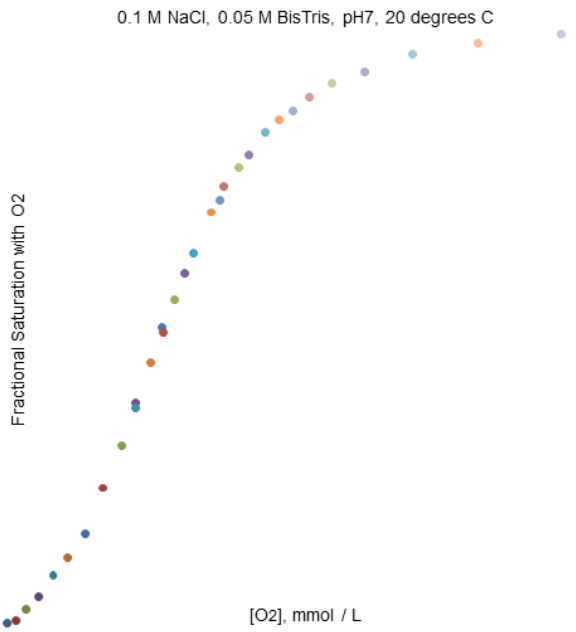

Figure 4: O_2 equilibrium binding curve of human hemoglobin in 0.050 M BisTris, 0.100 M NaCl, pH 7.0, 20.0°C. Half saturation was obtained at 8.1 µmol /L in the liquid phase.

Table 2: Binding data for Hb_4/(Cl-)n in 0.050 M BisTris, 0.100 M NaCl, pH 7.00 20.0°C.

$[O_2]$ µmol/L	F	$[O_2]$ µmol/L	F
0.829	0.012	10.4	0.675
1.33	0.029	10.8	0.695
1.95	0.051	11	0.717
2.64	0.084	11.7	0.747
3.34	0.114	12.2	0.768
4.42	0.151	13	0.805
5.08	0.227	13.7	0.826
5.99	0.297	14.4	0.839
6.65	0.366	15.2	0.863
6.7	0.356	16.3	0.884
7.41	0.431	17.9	0.903
7.97	0.488	20.2	0.931
8.03	0.48	23.4	0.95
8.59	0.535	27.5	0.964
9.08	0.575		

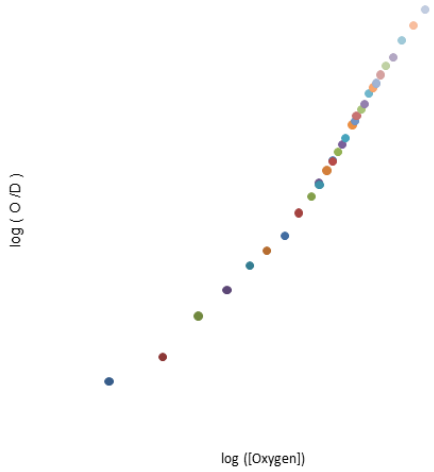

Figure 5: Hill plot of O_2 equilibrium binding data. The supporting electrolyte is 0.050 M BisTris, 0.100 M NaCl, pH 7.00 with HCl, 20.0°C.

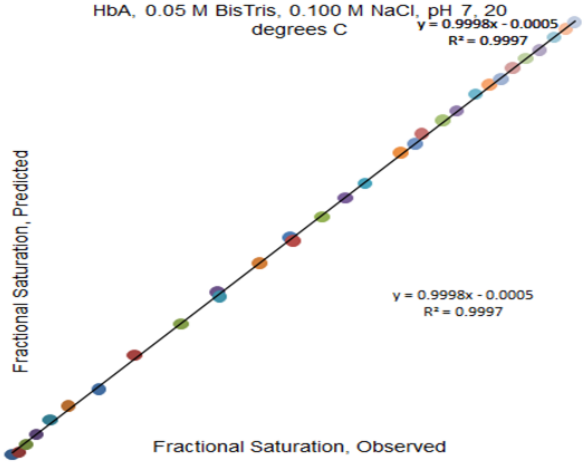

Figure 6: Observed and predicted values of F of the O_2 equilibrium binding curve of purified Hb_4 in 0.100 M NaCl, 0.05 M BisTris, pH 7.00 with HCl, 20.0°C. Predicted values of F, F_{PRE} , were calculated with the Perutz/Adair equation using the best fitting values of K_α, K_β, and K_Δ and the corresponding O_2-values.

The Hill plot of O_2-equilibrium binding date is similar to the majority of Hill plots obtained for human hemoglobin and described in the voluminous hemoglobin literature: (i) The n-value approaches unity as [O_2] approaches zero; (ii) The Hill plot demonstrates an inflection point with n-value of approximately 2.6: the n-value decreases beyond the inflection point. The concentration of O_2 required to attain F=0.5, however, is much lower than that required by BPG.

The results shown in Table 1 and Figures 1-3 represents the properties of Hb_4 tetramers free of the influence of E-molecules. The results shown in Table 2 and Figures 4-6 are representative of the properties of a Hb_4/E complex: Hb_4/(Cl-1)n.

Hb_4, in 0.050 M Bis-Tris, pH 7.0 with HCl, is, effectively, free of E-molecules. The O_2-equilibrium binding properties of Hb_4 differ markedly from the equilibrium binding properties of binary complexes of Hb_4 with E-molecules. In O_2-equilibrium binding experiments, E-free Hb_4 behaves as a pair of cooperative dimeric elements of allosteric structure. These elements of allosteric structure come into existence when a pair of identical αβ dimers reacts to form E-free Hb_4. The structural states assigned to each chain in Hb_4 are established by the results of O_2-equilibrium binding constants.

$$^{R}(^{1}\alpha,^{1}\beta)+^{R}(^{1}\alpha,^{1}\beta)\rightleftharpoons(^{T,1}\alpha,^{R,2}\beta)(^{T,2}\alpha,^{R,1}\beta)$$

The interface between the α- and β-subunits of physical dimers, $^{1}\alpha$, $^{1}\beta$ (MW=32 kD), remaining when an Hb_4 molecule dissociates into a pair of dimers, is not (cannot be) identical to the αβ interface formed when a pair of identical physical dimers associate to form tetrameric Hb_4 (MW=64 kD). Physical dimers are R-state and fail to demonstrate allosteric properties (Anderson et al., Edelstein et al., Edelstein and Gibson). The integral n-value of 2.0 for the Hill plot of E-free Hb_4 as F approaches zero, (Figure 3), defines a functional unit of co-operativity as being comprised of two O_2-binding sites. 19F-NMR experiments with E-free Hb_4 labeled at Cys-β93 by a disulfide linkage to the trifluoroethyl residue does not reveal chain heterogeneity while the same experiment in the presence of IHP demonstrates chain heterogeneity (Knowles). Strong binding of O_2 to a α-chain, following week binding of O_2 to a β-chain of E-free Hb_4 accounts for the absence of chain heterogeneity.

O_2-Binding constants are not necessarily identical for each of the dimeric cooperative subunits ($^{T,1}\alpha$, $^{R,2}\beta$) and ($^{T,2}\alpha$, $^{R,1}\beta$). The first cooperative subunit, ($^{T,1}\alpha$, $^{R,2}\beta$), binds O_2 while in combination with a second O_2-free cooperative dimer, ($^{T,2}\alpha$,$^{R,1}\beta$). The second cooperative dimer, ($^{T,2}\alpha$, $^{R,1}\beta$), binds O_2 while bound to an R-state oxy-dimer, R($^{1}\alpha O_2$,2βO_2). Clearly, ($^{T,2}\alpha$, $^{R,1}\beta$) and R($^{1}\alpha$O2, $^{2}\beta O_2$) are different structures. It is likely that may not be identical in each of the two cooperative subunits of E-free Hb_4.

The O_2-binding constants of β-and α-chains of the first cooperative dimeric subunit to bind O_2, in the E-free supporting electrolyte, in order of binding, are: K_β=1.45 × 105 L/mol; K_α=1.11 × 106 L/mol. They may be compared with the O_2-binding constants in the presence of the weakest of E-molecules, 0.100 M NaCl, in order of binding: K_α=5.34 × 104 L/mol; K_β=1.81 × 106 L/mol. E-molecule effects the values of both Kα and Kβ E-molecules reduce the affinity of β-chains below that of Tstate α-chains.

The first cooperative subunit of Hb_4 undergoes the following sequence of reactions:

The ^{T}state in α-chains is established by proximal strain in the imidazole bond to the ferrous atom of the heme moiety (Perutz 1970 Perutz 1987). The ^{T}state α-chain is converted to the ^{R}state α-chain upon reaction of the ^{R}state β-chain with O_2.

The ^{T}state in the presence of chloride ions, Hb_4/(Cl-1)n, enhances pre-existing proximal strain in α-chains as well as establishing distal side steric hindrance to O_2-binding by the β-chain. Distal side steric hindrance of β-chain heme moieties is a unique characteristic of all binary complexes of Hb_4 and E-molecules, Hb_4/(Cl-1)n in this instance. The order of binding of O_2 to chains in E-free Hb_4 is: The order of binding of O_2 in Hb_4/(Cl-1)n is: α,α, β,β.

An alternative model for O_2-binding by Hb_4, expressed in terms of the statistical assigned equilibrium constants, yields close agreement of predicted and observed values with the four-step ordered sequence cited above.

The equation of state for the model in Equation b, Equation 3, can be expressed in terms of only two unknown quantities

The correlation coefficient for the equation of state, Equation (3), for the alternative model, Equation b, is 0.9992. These values are reasonably close to those presented for the model based on a sequence of four steps.

CONCLUSION

It is likely that a significant step in the evolution of the fully cooperative tetrameric hemoglobin molecule we know today occurred when ($^{1}\alpha$ $^{1}\beta$) dimers underwent mutations leading to formation of tetramers resulting in ($^{1}\alpha$ $^{2}\beta$) and ($^{2}\alpha$ $^{1}\beta$) interfaces potentially supporting cooperative properties. Such a step may precede (i) Development of an E-molecule contributing to proximal strain in ^{T}state α-chains, (ii) Establishing steric hindrance to O_2-binding by β-chains, and (iii) Sensitivity of O_2-binding to the pH of the supporting electrolyte (Bohr Effect).

E-free Hb_4 possesses a fundamental element of allosteric structure: identified as a pair of cooperative dimers that exist in Hb_4 and do not exist in the dimeric molecules obtained upon dissociation of Hb_4 into dimers. The O_2-affinity of E-free Hb_4 is too high to be useful as an O_2 transport protein, being half saturated with O_2 at less than 2 μmol/L. E-free $(HbO_2)_4$ generates an insufficient concentration gradient to move O_2 from rbcs to mitochondria by the process of diffusion. BPG is a non-competitive inhibitor of O_2 binding to Hb_4, the resulting Hb_4-inhibitor complex, Hb_4/BPG, possessing lower affinity for O_2, being half saturated with O_2 at approximately 60 μmol/L at pH 7.4 and 37°C. The role of Hb_4 as an O_2-transpsort protein is best managed as follows: (i) Hb_4 should possess a high O_2-affinity in the pulmonary circulation (the lung) and a low affinity for O_2 in the systemic circulation (muscles, kidney, etc.). E-Free Hb_4 possesses a pair of cooperative dimers: ($^{T1}\alpha$, $^{R2}\beta$) and ($^{T2}\alpha$, $^{R1}\beta$). These cooperative dimers are an underlying element of allosteric structure upon which other elements of allosteric structure are constructed. These other elements of allosteric structure can be summarized as follows: (i) Enhancement of proximal strain in α-chains; (ii) Introducing steric hindrance to O_2-binding by β-chains; (iii) A high affinity of ^{T}state structures for BPG and a low affinity of Rstate structures for BPG; (iv) Introduction of pH-sensitivity to O_2-binding (Bohr Effect): (v) Packaging these elements of structure in a manner supporting respiration in mammals.

The following linked manuscripts address the role of the BPG mediated R to T structural change, a second element of allosteric

structure diminishing the O_2-affinity of ^{T}state α-chains and regulating access of O_2 to ^{R}state β-chains. A third element of allosteric structure converts ^{T}state/BPG complexes to ^{R}state/BPG complexes in conjunction with conversion of Tstate αO_2 chains to ^{R}state αO_2-chains. These elements of allosteric structure by BPG can be shown to account for the characteristic shape of O_2-equilibrium binding curves of the binary complex of Hb_4 and BPG obtained under conditions of constant pH. A fourth element of allosteric structure, not addressed in experiments conducted at constant pH-values, concerns the rise in pH in the pulmonary circulation and the decrease in pH in the systemic circulation.

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