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Free Energy Changes and Components Implicit in the MWC Allosteric Model for the Cooperative Oxygen Binding of Hemoglobin

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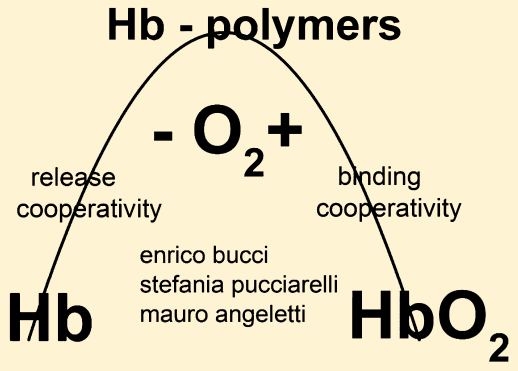
ABSTRACT: Hill's plots of oxygen binding isotherms reveal the presence of a transition between two different oxygen affinities at the beginning and end of the isotherm. They correspond to the two conformations anticipated by the MWC model, namely, the T and R conformations at the beginning and end of oxygen binding, when the lower affinity of the T form develops into the higher affinity of the R form. The difference between the binding Gibbs free energy changes of the two affinities (ΔG_L) is the free energy of binding cooperativity. Notably, ΔG_L is positive in favor of the T form, which moves to a higher energy level upon oxygen release. Osmotic stress reveals a higher volume/surface ratio of deoxyhemoglobin, with a positive ΔG_W also in favor of the T form. An increasing protein concentration shifts the isotherms to the right, indicating the formation of intermediate polymeric forms. The enthalpy of the intermediates shows a strong absorption of heat at the third oxygenation step because of polymer formation with quinary, and higher-order, structures. The disassembly of intermediate polymers releases energy with a negative ΔG that compensates and allows the positive values of ΔG_L. High-energy polymers are the barrier preventing the relaxation of the T and R conformations into one another. The MWC allosteric model is the best justification of oxygen binding cooperativity.



To explain the cooperativity of oxygen binding isotherms, Monod et al.[1] proposed the presence of two forms of hemoglobin (Hb) with low (T form) and high (R form) oxygen affinity. In the absence of oxygen, the allosteric equilibrium constant Lo = To/Ro is in favor of To, while with an increasing level of Hb saturation, Ro becomes the favored species.

Consistent with the model, linear extrapolation of the very first and very last saturation data of Hill's plot isotherms defines the presence of two hyperbolic bindings for the first and last oxygen bound to Hb.[2] They have a higher and lower P_{50} reflecting a lower and higher oxygen affinity, respectively. It is easy to reconcile the initial low affinity (K_T) with deoxyhemoglobin in the T form and the final high affinity (K_R) with oxyhemoglobin in the R form. The overall binding affinity of the isotherm is defined by the value of PO_2 at 50% saturation of the isotherm (P_{50}), or the value of P_m when available.

In terms of the thermodynamic implications of the MCW allosteric model, as mentioned above, Hill's plots of cooperative isotherms clearly indicate the presence of two different hyperbolic oxygen affinities of hemoglobin with lower (K_T) and higher (K_R) oxygen affinities.

The difference between the respective binding free energy changes, ΔG_T and ΔG_R, is

$$\Delta G_L = \Delta G_T - \Delta G_R \quad (1)$$

where ΔG_L defines the free energy change of cooperativity.

The lower affinity of the initial isotherm implies that $\Delta G_T > \Delta G_R$. Thus, in the difference, ΔG_L has a positive value, indicating that during deoxygenation the system in the T form gains a ΔG_L amount of free energy, so that the T form is at a higher energy level than the R form.

Implied in eq 1 is a scalar L_L defined as

$$L_L = \exp\left(\frac{\Delta G_L}{RT}\right) \quad (2)$$

which is equivalent to the allosteric constant, Lo, of the Monod model.[1] As implied in eq 2, we have

$$K_R = L_L K_T \quad (3)$$

as in the model.[1,2]

It should be stressed that the evolving equilibrium between the T and R forms during the binding event implies a barrier of

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energy that prevents their mutual collapse into a single conformational system.

Also in eq 1, ΔG_L is non-zero and positive, while the conformational equilibrium requires a zero balance of all free energy exchanges as in

$$\Delta G_{eql} = \Delta G_T - \Delta G_R + \Delta G_{corr} = \Delta G_L + \Delta G_{corr} = 0 \quad (4)$$

where ΔG_{corr} is the free energy that allows the relaxation of the R form into the higher energy level of the T form.

For the origin of ΔG_{corr}, a possible hypothesis is the presence of a third intermediate component(s) that upon formation absorbs heat and energy from the environment (positive ΔG_{on}) and then, by degrading, would release the absorbed energy (negative ΔG_{off}) to the system and/or the environment. The $\Delta G_{balance}$ would be the net sum of positive and negative ΔG_i values related to assembly and/or disassembly of the third component(s) as in

$$\Delta G_{balance} = \sum_i \Delta G_i \quad (5)$$

At equilibrium it should be $\Delta G_{balance} = 0$. A negative non-zero balance would be the amount (ΔG_{corr}) released to the system, not to the environment, to compensate for the positive ΔG_L in eq 4. A positive balance would invalidate the hypotheses formulated above.

It should be stressed that the third intermediate component(s) must be at higher energy level than the T and R forms, to degrade into the lower levels of the two forms. Thus, the third component(s) would also provide the barrier necessary to prevent the mutual relaxation of the T and R forms into a single conformational system.

The implication of intermediate third components also has been proposed by Ackers et al., Perrella et al., and Smith et al. on the basis of equilibrium, cryo-electrophoresis, and kinetic data.[3−5] Ackers concludes that the data (his words) "imply the existence (in the Hb system, author's note) of at least three molecular structures; while a degeneracy of multiple structure into only a few dominant free energy levels (the T and R forms, author's note) is frequently to be expected, the reverse situation is extremely unlikely".[3]

In this report, to explore the thermodynamics involved in the binding statistics of the MWC model, we monitored the response of oxygen binding isotherms to challenges of temperature, osmotic stress, and protein concentration. In our laboratories, temperature dependence has already been explored[6] and is used here as reference data.

MATERIALS

Human Hb was prepared as described previously.[6]

Sebacyl cross-linked Hb (DECA) was prepared as described previously.[7]

All reagents used in the various manipulations were reagent grade and obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

PROTOCOLS

Measurements of OD changes were performed using a Varian 14 DS recording spectrophotometer.

Oxygen binding isotherms were measured following the technique developed by Dolman and Gill, using a thin-layer Hb solution exposed to successive dilution of the initial PO_2 with nitrogen.[8]

The osmotic dependence of the isotherms was measured at osmotic pressures varying from 1.0 to 80 atm, using betaine concentrations of ≤5 M. Buffer osmolality was measured using a Wescor (Logan, UT) osmometer. The potein concentration was near 30 mg/mL in 0.2 M borate buffer at pH 9.0 and 30 °C.

The protein concentration dependence of the isotherms was measured at Hb concentrations varying from 5 to 50 mg/mL in 0.1 phosphate buffer at pH 7.4 and 37 °C.

NUMERICAL ANALYSES

Numerical analyses of the isotherms were based on the binding polynomial, P_W[2]

$$P_W = 1 + \beta_1 X + \beta_2 X^2 + \beta_3 X^3 + \beta_4 X^4 = 1 + \sum_i \beta_i X^i \quad (6)$$

from which Adair's sequential binding equation is obtained

$$Y = \left(\beta_1 X + 2\beta_2 X^2 + 3\beta_3 X^3 + 4\beta_4 X^4\right) \Big/ \left[4(1 + \beta_1 X + \beta_2 X^2 + \beta_3 X^3 + \beta_4 X^4)\right] = \frac{\delta(\ln P_W)}{4\delta(\ln X)} \quad (7)$$

In these equations, X is the partial pressure of oxygen (PO_2) and β_i values are Adair's binding parameters at subsequent binding steps (i = 1−4). Y is the fractional saturation with oxygen. The intrinsic binding constant at each step was computed from

$$K_i = \frac{i\beta_i}{(5 - i)\beta_{(i-1)}} \quad (8)$$

while the relative proportion, α, of each intermediate as a function of PO_2 was computed from

$$\alpha_i = \frac{\beta_i X^i}{P_W} \quad (9)$$

The median ligand activity was computed from

$$P_m = \frac{1}{\sqrt[4]{\beta_4}} = \beta_4^{-0.25} \quad (10)$$

Alternatively, the value of P_{50} and the extent of cooperativity were estimated from the midpoint of the isotherm, using the Hill equation

$$Y = \frac{PO_2{}^n}{P_{50}{}^n + PO_2{}^n} \quad (11)$$

and its logarithmic transformation for the Hill's plots. In the equation, Y is the fractional saturation of hemoglobin with oxygen and n is the cooperativity index. Because of the quasi-symmetric shape of the isotherm, P_m and P_{50} are practically identical.

Fitting Minimization. Isotherms were globally minimized, grouping together those exposed to various temperatures or osmotic stresses. This technique substantially reduced the correlation between the floating parameters, allowing estimations within a range close to 1% of the estimated value.

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RESULTS

Temperature Dependence. These data were previously reported by Bucci et al.[6] Isotherms were measured at seven temperatures between 20 and 37 °C. Numerical analyses were based on the binding polynomial

$$P_H = 1 + \sum_i \beta_{i,25} \times X^i \times \exp\left[\frac{\Delta H_i}{R}\left(\frac{1}{T} - \frac{1}{298.2}\right)\right] \quad i = 1{-}4 \tag{12}$$

where the reference parameter $\beta_{i,25}$ at 25 °C is corrected for the enthalpy at each binding step. The enthalpy of conformational changes per se was obtained by subtracting the enthalpy of the binding of oxygen to heme, estimated to be $\Delta H = -14$ kcal/heme. The corrected data are plotted in Figure 1. Notably, the

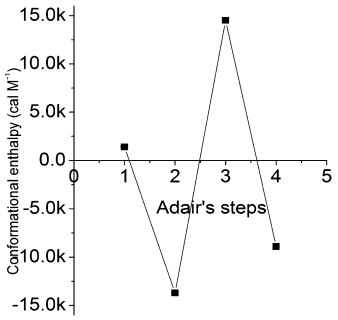

Figure 1. Conformational enthalpy of the intermediates of oxygenation of hemoglobin, in 0.1 M borate buffer at ph 9.0. Adapted from ref 6.

enthalpy of the third step of oxygenation shows a large heat absorption (ΔH = 14 kcal M^{-1}), while the second and fourth oxygenation steps show large heat releases (−13 and −8 kcal M^{-1}, respectively).

Osmotic Stress. The osmolite of choice was betaine (trimethyl glycine, molecular mass of 114.14 Da), which under the conditions of all experiments (0.2 M borate buffer at pH 9.0 and 30 °C) did not bind to HbA, as is shown by the linearity of the increases in osmolality produced by increasing concentrations of betaine in the presence of Hb (Figure 2).

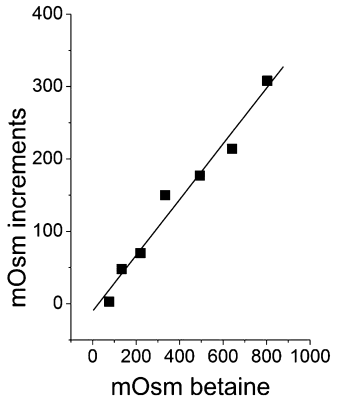

Figure 2. Increments of milliosmolality produced by the increasing milliosmolality of betaine over 240 mg/mL hemoglobin. Note the linearity with a slope of 0.99. In 0.2 M borate buffer at pH 9.0 and 30 °C.

Isotherms at three different osmotic pressures are shown in Figure 3. The presence of the osmolite right-shifts the

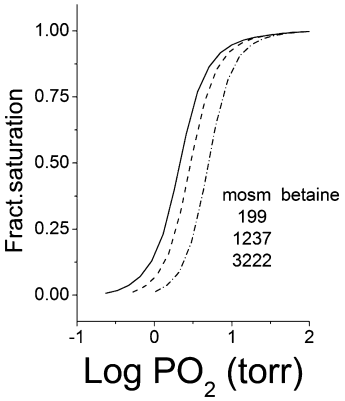

Figure 3. Increasing right displacements of binding isotherms produced by the increasing osmolality of betaine listed in the graphic, in 0.2 M borate buffer at pH 9.0 and 30 °C.

isotherms, increasing their P_{50} values, indicating that the free energy of binding of oxygen to Hb is increased by osmotic stress, as in

$$\Delta G_\Pi = \Delta G_0 + \Pi \Delta V \tag{13}$$

where ΔV is a volume change produced by the osmolite, Π = osm × RT is the osmotic pressure, and osm is the measured molality of the samples. The term $\Pi\Delta V$ is the free energy change, ΔG_W, of the osmotic stress that would increase ΔG_0 thereby reducing the oxygen affinity of Hb.

Numerical analyses for estimating the ΔV_i values produced by the osmotic stress at each step of oxygen binding were based on the binding polynomial

$$P = 1 + \sum_i \beta_{i,o} \times X^i \times e^{\Pi \Delta V_i} \tag{14}$$

where ΔV_i values are the changes in volume at each step.

The overall volume change produced by the osmotic stress was also obtained from the slope of the plot of $\ln(P_{50})$ versus the measured osmolality of the buffer, using

$$\ln(P_{50,\Pi}) = \ln(P_{50,0}) + \text{osm} \times \Delta V_{ovr} \tag{15}$$

as shown in Figure 4.

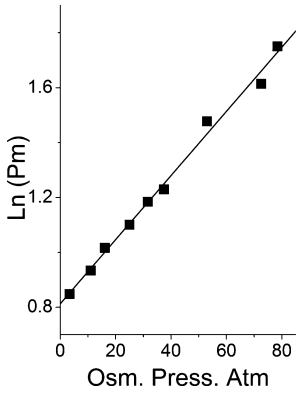

Figure 4. Dependence on osmotic pressure of the oxygen affinity [$\ln(P_m)$] of hemoglobin, in 0.2 M borate buffer at pH 9.0 and 30 °C.

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Table 1. Overall ΔV Values and Those at Individual Adair Steps (1−4)

ΔV_i (L M^{-1}), cumulative (i = 1−4)	ΔV_i (L M^{-1}), individual step (i = 1−4)
0.663	0.663
0.626	−0.037
1.126	0.500
1.127	0.001
overall ΔV (L M^{-1}) = 1.10	

The numerical data obtained from these experiments show a very good correspondence between the cumulative and overall displaced volumes [$\Delta V_{ovr} = \sum \Delta V_i$ = 1.2 L M^{-1} (Table 1)]. The step-by-step volume increases were not evenly distributed along the oxygen binding steps (Figure 5); they are evident only on the first and third steps.

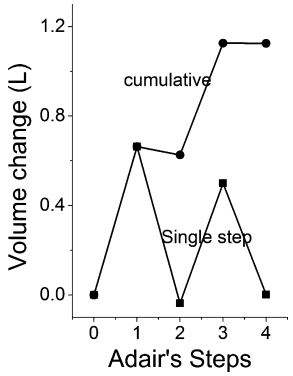

Figure 5. ΔV increments and cumulus with oxygenation, in 0.2 M borate buffer at pH 9.0 and 30 °C.

Colombo et al.[9] were the first to notice that addition of sugars not binding to Hb lowered its oxygen affinity. They interpreted this phenomenon as a solvation change involving 66 mol of water/mol of hemoglobin. The volume of 66 mol of water is near 1.2 L M^{-1}, consistent with our ΔV values.

Concentration Dependence. Experimental conditions were chosen for maximizing the effect of protein concentrations, similar to physiologic conditions: 0.1 M phosphate buffer at pH 7.4 and Hb concentrations between 5 and 50 mg/mL at 37 °C. Sebacyl cross-linked Hb (DECA) was chosen because the intramolecular cross-link eliminates dimer formation. DECA is characterized by a P_{50} near 30 mmHg and a cooperativity with n of >2.0, very similar to the P_{50} and cooperativity of normal blood.[7]

As shown in Figure 6, the oxygen affinity of DECA decreases with an increasing protein concentration, suggesting the association process of DECA tetramers into polymeric forms. The phenomenon can be described by

$$\Delta G_p = \Delta G_0 + K_{pol} C_{Hb} \quad (16)$$

where K_{pol} is a pseudoassociation constant for polymer formation because it refers to the total Hb concentration (C_{Hb}). The term $K_{pol}C_{Hb}$ is the free energy change (ΔG_C) of polymer formation that would increase ΔG_0, thereby reducing the oxygen affinity of Hb.

In numerical analyses, Adair's parameter β_3 was so low that it was difficult to estimate. Therefore, for the concentration dependence data, we were unable to produce data for individual steps.

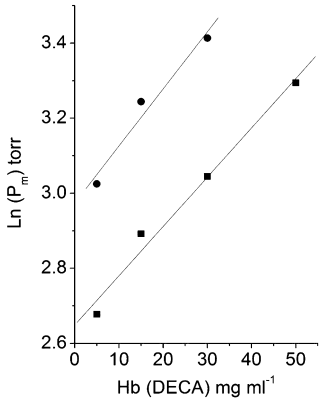

Figure 6. Concentration dependence of the oxygen affinity [ln(P_{50})] of DECA (sebacyl cross-linked hemoglobin[7]) in the presence (●) and absence of 3.0 M betaine (■), in 0.1 M phosphate buffer at pH 7.4 and 37 °C.

The negative slope (ΔHs) of the van't Hoff plots at 5 and 50 mg/mL hemoglobin remained constant with ΔH values of −6.1 and −6.5 kcal M^{-1}, respectively; only the intercept on the ordinates was higher at the higher Hb concentration. In the presence of 50 mg/mL Hb, addition of 3 M betaine further increased the ordinate intercept of the sloping line and the enthalpy slightly decreased to −8.2 kcal M^{-1}. Figure 7 shows the quasi-parallel upward displacement of the van't Hoff lines under the three conditions.

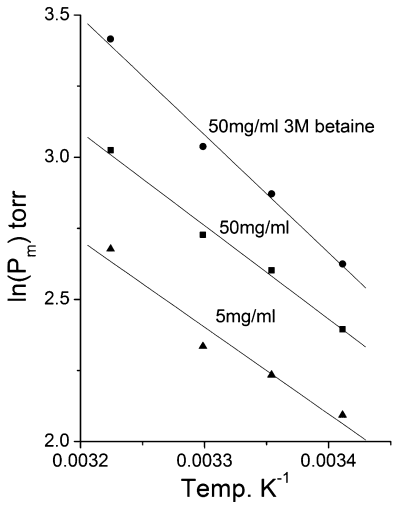

Figure 7. van't Hoff plots for DECA[7] at 5 mg/mL (▲), 50 mg/mL (■), and 50 mg/mL with 3 M betaine (●), in 0.1 M phosphate buffer at pH 7.4.

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Table 2. Osmotic Data for Hb in 0.2 M Borate Buffer at pH 9.0 and 30 °C[a]

Π (atm)	β_i (torr^{BI}) (i = 1−4)	K_i (mM^{-1}) (i = 1−4)	ΔG_i (cal M^{-1}) (i = 1−4)
11	0.236	190	−7171
	0.094	855	−8059
	0.051	2620	−8720
	0.023	5810	−9189
16	0.207	166	−7094
	0.077	798	−8019
	0.045	2823	−8764
	0.017	4866	−9085
25	0.144	115	−6880
	0.056	834	−8045
	0.026	2242	−8628
	0.012	5945	−9203
31.5	0.122	98	−6782
	0.061	990	−8193
	0.019	1567	−8369
	0.009	725	−9181

[a]The four rows at each Π value correspond to the four Adair steps (1−4) from top to bottom, respectively.

DISCUSSION

Osmotic Stress. On the basis of eq 13, as mentioned above, the right shift of the isotherm in response to increasing Π reveals the presence of a term

$$\Delta G_W = RT \ln(\Pi \Delta V) \quad (17)$$

that decreases the oxygen affinity of the system.

ΔG_W is the free energy change of the osmotic stress that, as proposed by Timasheff,[10] is the expression of an increased solvent preferential exclusion from the protein surface that increases in size. In hemoglobin, it is consistent with the increased hydrophobicity of the oxyhemoglobin surface, reported by Chothia.[11]

The ΔV_i data listed in Table 1 are positive, in favor of the deoxy T structure. Therefore, ΔG_W adds energy to the system in the T form contributing to the free energy of cooperativity ΔG_L. Assuming that the free energy changes ΔG_1 and ΔG_4 listed in Table 2 correspond to ΔG_T and ΔG_R, respectively, a comparison between ΔG_w, computed with eq 17, and ΔG_L, computed with eq 1, is shown in Table 3.

Table 3. Osmotic (ΔG_W) and Analytical (ΔG_L) Free Energy Changes for the Conformational Transition, with the Corresponding Scalars (L_W and L_L), and the Differences between Analytical and Osmotic Values

ATM	ΔG_W (kcal M^{-1})	ΔG_L (kcal M^{-1})	L_W	L_L	$\Delta G_L - \Delta G_W$ (cal M^{-1})
11	1.56	2.01	13	30	450
16	1.79	1.99	19	29	200
25	2.06	2.32	29	46	260
31.5	2.20	2.40	37	52	200
37.5	2.30	2.37	44	50	70
					average, 236

In Table 3, ΔG_L is always higher than ΔG_W by a few hundred kilocalories per molar. The difference is better expressed by the respective values of scalars L_W and L_L (eq 3). The differences are small and in the same direction. This may suggest that ΔG_W refers only to the solvation of Hb while ΔG_L includes the net sum of the positive and negative free energy changes resulting from the T ↔ R conformational rearrangements within the subunits and across their interfaces.[12] In other words, ΔG_W detects only the different solvation resulting from the conformational change included in ΔG_L.

The Third Components Are Polymeric. As shown in Figure 6, the P_{50} of DECA has a distinct dependence on protein concentration, implying the presence of a reversible associating system that modulates the oxygen binding isotherm, as shown by eq 16. It is a general phenomenon in hemoglobin systems, previously reported from our laboratory for human and bovine hemoglobins.[13]

In the experiments reported above, DECA is an intramolecularly cross-linked, nondissociable tetrameric Hb.[7] In the absence of dimers, the association process could be only a polymerization of tetrameric Hb molecules.

Detailed step analyses of the concentration-dependent isotherms are not available because, as mentioned above, the Adair's parameter of the third step, β_3, was so small that it was undefined, suggesting that the triligation fractional saturation of the isotherm results in the formation of components with very low oxygen affinities.

This phenomenon is concomitant with the strong heat absorption by the enthalpy of the third step of oxygenation, shown in Figure 1, with a ΔH_3 near 14 kcal M^{-1}.[6] It can be proposed that the triligation absorbs into the system the energy (ΔG_{on}) necessary for the formation of the stereochemistry of new polymeric forms with high energy levels and very low oxygen affinities.

The release of free energy by the negative enthalpies detected at the second and fourth steps (Figure 1), with a ΔH_2 near −13 kcal M^{-1} [6] and a ΔH_4 near −8 kcal M^{-1},[6] would result from the disassembly (ΔG_{off}) of unstable polymers relaxing into lower-energy, stable, tetrameric structures like the T and R forms.

Our data proposing the presence of unstable polymeric intermediates dovetail with Ackers' hypothesis of at least a third intermediate component degenerating into more stable forms.[3]

Energetic Relevance of the Intermediates of Oxygenation. Random-isodesmic aggregating polymers cannot be proposed because they would be at a lower energy level stabilizing intermediate forms, producing negative cooperativity, as described by Koshland et al.[14] In fact, the equilibrium contribution of high-energy components (the polymers) makes the average energy level of all of the intermediates higher than

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the levels of both the T and R forms. The high energy level of intermediates would be the barrier that prevents the T and R forms of Hb from relaxing into each other.

The high heat absorption at the third Adair's step (ΔH_3^6) implies the formation of quinary, and higher-order, polymeric forms. This hypothesis is supported by the fibers of HbS, which reveal the presence on the surface of tetrameric Hb of sites ready for the intermolecular contacts necessary for the self-assembly and formation of perfectly designed quinary and higher-order structures.[15] Similarly, HbS fibers also self-assemble into a very low oxygen affinity. It is very tantalizing to propose that the HbS fibers are the mutation-stabilized form of an intermediate polymer. The history of measured isotherms in our laboratories and the data of Campbell et al.[16] for fiber formation *in vitro* may support this proposition.

Osmotic stress offers a suggestion for the different shape of the R and T forms. A different solvation that involves 66 mol of water/mol of Hb implies a larger surface/volume ratio of the molecule with a larger osmotic shell for oxyhemoglobin. Conversely, the T form has a lower molecular surface/volume ratio. For the same mass, minimizing the surface gives to the T form a more compact and spherical shape, more spherical than that of the R structure with a larger surface/volume ratio.

The same reasoning can be applied to the hypothesis of Colombo et al.,[9] that 66 molecules of water bind directly on the surface of hemoglobin. ΔG_W would not distinguish between the equivalence of two hypotheses. The increased hydrophobicity of the system[11] would support the hydration shell proposal.

Distribution of the Intermediates. Adair's subsequent β_i parameters are statistical binding constants, which refer to all intermediates of ligation present along the evolving oxygen saturation of the isotherm. They describe the changing distribution of species increasingly saturated with oxygen, as shown in Figure 8. This is why we prefer to define the subsequent oxygenation steps as "Adair's steps".

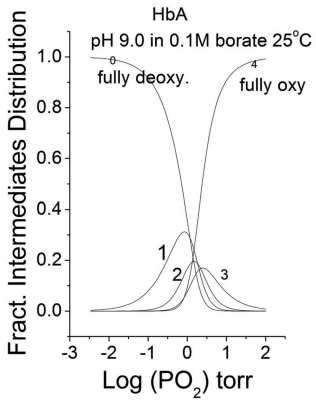

Figure 8. Distribution of liganded species (eq 9) as function of increasing PO_2, in 0.1 M borate buffer at pH 9.0 and 25 °C. The numbers reflect Adair's oxygenation steps.

Figure 5 shows the progress of ΔV_i values along four Adair's steps. The progress is not linear with the steps. The maxima at the first and third steps are probably due to the local prevalence of the most asymmetric species.

Similarly, for the enthalpies, as shown in Figure 1, the nonlinear progress would be due to the local prevalence of certain ligated species. The maximal heat absorption at the third Adair's step is reached when triligation makes the polymers the dominant species.

Comparisons of Figures 1, 5, and 8 support the hypothesis that there are large equilibrium conformational fluctuations among the intermediates of oxygenation, resulting in multiple forms of a single system.

Correction in Equation 4. The enthalpy values shown in Figure 1 are data taken from Bucci et al.[6] obtained in the absence of betaine. They are the free energies either absorbed (ΔH_3) or liberated (ΔH_2 and ΔH_4) from the environment upon the T ↔ R conformational changes. As discussed, they would correspond to formation and degradation of polymeric forms. The free energy balance between absorption and release can be estimated by

$$\Delta G_{\text{balance}} = \sum_{i=1}^{i=4} \Delta H_i^6 = -7.5 \text{ kcal M}^{-1} \quad (18)$$

where the residual −7.5 kcal M^{-1} is the energy not released to the environment, because it is delivered to and absorbed by the intermediates of Hb oxygenation. The average, equally distributed over four Adair's steps, is near −1.9 kcal M^{-1}, close to the ΔG_L values listed in Table 3.

Apparently, formation and degradation of polymeric components provide the ΔG_{corr} of −1.9 kcal M^{-1} necessary to have a ΔG_{eql} of 0 in eq 4 that justifies the higher energy level of the T form.

MCW Model Scenario. The energy necessary for the conformational change (ΔG_L = 2.0 kcal M^{-1}) includes the osmotic stress (ΔG_W = 1.6 kcal M^{-1}) and the net free energy change near 300 kcal M^{-1} of structural conformational changes, which, within the MWC model,[1] can be considered allosteric effectors specific for the T form that increase the energy level of the T form to higher ΔG_T values with a lower oxygen affinity.

Reversibility implies the release of a $-\Delta G_L$ free energy from the T form into the R structure. This decreases the energy level of the system and decreases the ΔG_R value to a higher oxygen affinity.

It appears that the MWC model implies an extra packet of ~2000 kcal M^{-1} that is in turn either absorbed or released by the system modulating its oxygen affinity. The energy of the packet is provided by the release into the system of the energy absorbed from the environment by the enthalpies of the intermediates.

The data suggest that the T and R forms of Hb are separated at the two ends of a reversible thermal itinerary across the higher energy levels of the intermediates.

Besides providing the energy necessary for cooperativity, the high energy level of polymeric intermediates provides most of the barrier that prevents the T and R forms from relaxing into each other.

In this view, the MWC allosteric model best describes a system in which the evolving equilibrium between two conformations is regulated by intermediates all averaged by and included in the allosteric constant Lo.

APPENDIX

Initial and Final Parabolas. In eqs 13 and 16, ΔG_w and ΔG_C are mass-dependent extensive additive parameters that do not interfere with the intensive parameters of the system as shown in Figure 7. Thus, referring to ΔG_1 and ΔG_4 (Table 2) as ΔG_T and ΔG_R, respectively, and from eqs 13 and 16, for the hyperbolic binding of the T form, we would have

$$\Lambda G_T = \Delta G_{deoxy} = \Delta H_0 - T\Delta S_0 + K_{pol}C_{Hb} + \Delta G_L \quad (19)$$

where, according to eq1, the positive ΔG_L parameter specifically adds energy to the system to form the T structure and decrease its oxygen affinity.

With regard to the R form, reversibility implies an opposite negative ΔG_L in eq 1; therefore, for the R form, we would have

$$\Delta G_R = \Delta G_{oxy} = \Delta H_0 - T\Delta S_0 + K_{pol}C_{Hb} + (\Delta G_L{}^* - 1) \quad (20)$$

where a negative ΔG_L specifically adds its negativity to ΔG_R, decreasing the energy level of the system in the R form and further increasing its oxygen affinity.

It is as if there is a fundamental hyperbolic oxygen binding of Hb

$$\Delta G_0 = \Delta H_0 - T\Delta S_0 \quad (21)$$

modulated by protein concentration, osmotic stress, and structural changes.

Governing Equations. Fluctuations of the intermediates of oxygenation prevent their description with a universal equation. Only averages may be used. It is well-known that the Hill equation (eq 11) and its logarithmic transformation average in the scalar n all of the intermediates and are very useful for estimating the overall oxygen affinity (P_{50}) and presence of cooperativity ($n > 1.0$) in the isotherm. Most useful are the statistical Adair's parameters (eqs 6−10) for following the equilibrium distribution of the intermediates and computing P_m.

Simultaneous numerical analyses of groups of isotherms are useful for reducing the correlations among the floating parameters. Di Cera and Gill[17,18] have developed numerical procedures for obtaining Adair's parameters and oxygen saturation fractions from single isotherms.

The MWC binding model adequately describes the system and is useful for simulations. It cannot be used for numerical analyses because, as shown in eq 3, there is a correlation equal to 1.0 between the floating parameter Lo and either K_R or K_T (depending on the equation spelling). This kind of correlation cannot be reduced by simultaneous group analyses and prevents minimizations.

Significance for Biochemistry. Cooperativity is physiologically relevant. In fact, at the periphery, the acquired lower affinity helps the mass action of oxygen release, which corrects for the asymmetry of slow oxygen supply from red cells and fast mitochondrial consumption.[19,20] In the lungs, the acquired higher affinity assures a full saturation of Hb exposed to a modest PO_2.

It is tantalizing to present hemoglobin as a molecular machine that extracts oxygen from the lungs and expels it to the tissues under the pumping action of the assembly and disassembly of polymeric forms during the binding event. The MWC model best describes this mechanism with two switching conformations pumped by the allosteric constant Lo.

The thermodynamics of the T ↔ R conformational change finalize and justify the origin of the cooperativity of oxygen binding isotherms, after almost a century of search based on the binding statistics of nonunique allosteric models.

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DEDICATION

This paper is dedicated to the late Gary K. Ackers. We miss a friend. Science is missing one of its major contributors.

ABBREVIATIONS

ΔH, enthalpy; ΔG_C, free energy change for polymer formation; ΔG_{eql}, equilibrium free energy change of cooperativity; ΔG_L, free energy change of cooperativity; ΔG_{off}, free energy of polymer disassembly; ΔG_{on}, free energy of polymer formation; ΔG_R, free energy change of oxygen binding to the R form; ΔG_T, free energy change of oxygen binding to the T form; ΔG_W, free energy change of osmotic stress; DECA, sebacyl cross-linked hemoglobin; L_L, scalar of ΔG_L; L_W, scalar of ΔG_W.

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