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The Three-State Model: A Minimal Allosteric Description of Homotropic and Heterotropic Effects in the Binding of Ligands to Hemoglobin

(oxygen binding/affinity states/effector substances)

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ABSTRACT **The effect of hydrogen ion, chloride, and 2,3-diphosphoglycerate upon the oxygen binding of hemoglobin may be satisfactorily described by an allosteric model employing three affinity states. The allosteric two-state model does not provide even an approximate description except under defined extreme conditions. In particular, the two-state model cannot provide a correct interpretation of recently reported electron spin resonance and nuclear magnetic resonance data.**

Precise measurements of the oxygenation of hemoglobin in the presence of varying amounts of H^+, Cl^-, and 2,3-diphosphoglycerate have recently been reported (1, 2). It was found that upon addition of each of these "effector substances" the Adair constant measuring the affinity of hemoglobin for the first O_2 molecule bound (K_1) decreased, while the Adair constant measuring the affinity of hemoglobin for the fourth O_2 molecule bound (K_4) remained essentially constant. It was pointed out (3) that these results are inconsistent with the predictions of the two-state allosteric model (4) but that they may be consistent with an allosteric description allowing for more than two affinity states. We present here a generalization of the allosteric model which successfully accounts for the reported heterotropic effects in terms of three affinity states, designated R, T, and S in descending order of affinity for oxygen. It will be shown that the lowest affinity state S cannot be regarded as a second-order perturbation of state T, and that in the general case, an "effective two-state" description of hemoglobin is not physically meaningful. Reported experimental observations relating to the conformation of hemoglobin are considered in the context of an allosteric model requiring three or more states, and it will be shown that these observations cannot distinguish between the alternative allosteric (4) and sequential (5, 6) descriptions of structure-function relationships in hemoglobin.

We begin by postulating that all three affinity states may bind H^+ (H) and Cl^- (C) in various quantities and with various affinities. Furthermore, the state S may bind one molecule of 2,3-diphosphoglycerate (D) per hemoglobin tetramer. It is assumed that anion binding takes place as follows (7).

$$-\mathrm{N} + \mathrm{H}^+ \rightleftarrows -\mathrm{NH}^+$$

$$-\mathrm{NH}^+ + \mathrm{A}^- \rightleftarrows -\mathrm{NH}^+\mathrm{A}^-$$

Therefore, to a first approximation the total number of anions bound will not exceed the number of protons bound. This approximation is not essential to the model but permits some simplification of expressions to be subsequently derived. The following expressions then describe all allowed equilibria in the absence of oxygen.

$$[RH_iC_j] = k^R_{ij}[R][H]^i[C]^j \qquad [1a]$$
$$(k^R_{ij} = 0 \text{ if } i < j)$$

$$[TH_iC_j] = k^T_{ij}\,[T][H]^i[C]^j \qquad [1b]$$
$$(k^T_{ij} = 0 \text{ if } i < j)$$

$$[SH_iC_jD_k] = k^S_{ijk}[S][H]^i[C]^j[D]^k \qquad [1c]$$
$$(k^S_{ijk} = 0 \text{ if } k > 1 \text{ or } i < j + k)$$

The fraction of hemoglobin existing in each of the three affinity states in the absence of oxygen may be determined from two allosteric equilibrium "constants" which vary with the concentrations of H, C, and D.

$$L(H,C) = \frac{[\text{total } T]}{[\text{total } R]} = \frac{\sum_{i=0}\sum_{j=0}[TH_iC_j]}{\sum_{i=0}\sum_{j=0}[RH_iC_j]} \qquad [2a]$$

$$M(H,C,D) = \frac{[\text{total } S]}{[\text{total } R]} = \frac{\sum_{i=0}\sum_{j=0}\sum_{k=0}[SH_iC_jD_k]}{\sum_{i=0}\sum_{j=0}[RH_iC_j]} \qquad [2b]$$

By substitution of relations **1a-c** into **2a-b** and partial expansion of the indicated sums, the following is obtained:

$$L(H,C) = L_0\frac{\{1 + \sum_{i=1}k^T_{i0}[H]^i + \sum_{i=1}\sum_{j=1}k^T_{ij}[H]^i[C]^j\}}{\{1 + \sum_{i=1}k^R_{i0}[H]^i + \sum_{i=1}\sum_{j=1}k^R_{ij}[H]^i[C]^j\}} \qquad [3a]$$

and

$$M(H,C,D) = M_1(H,C) + M_2(H,C)\,[D] \qquad [3b]$$

where

$$M_1(H,C) \equiv M_0\frac{\{1 + \sum_{i=1}k^S_{i00}[H]^i + \sum_{i=1}\sum_{j=1}k^S_{ij0}[H]^i[C]^j\}}{\{1 + \sum_{i=1}k^R_{i0}[H]^i + \sum_{i=1}\sum_{j=1}k^R_{ij}[H]^i[C]^j\}}$$

$$M_2(H,C) \equiv M_0\frac{\{\sum_{i=1}k^S_{i01}[H]^i + \sum_{i=1}\sum_{j=1}k^S_{ij1}[H]^i[C]^j\}}{\{1 + \sum_{i=1}k^R_{i0}[H]^i + \sum_{i=1}\sum_{j=1}k^R_{ij}[H]^i[C]^j\}}$$

$L_0 \equiv [T]/[R]$ and $M_0 \equiv [S]/[R]$.

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Denoting the partial pressure of O_2 by p and the oxygen affinities of states R, T, and S by the association constants k_R, k_T, and k_S, respectively, the fractional oxygen saturation y is given by

$$y(p,H,C,D) = \frac{k_Rp(1 + k_Rp)^3 + L(H,C)k_Tp(1 + k_Tp)^3 + M(H,C,D)k_Sp(1 + k_Sp)^3}{(1 + k_Rp)^4 + L(H,C)(1 + k_Tp)^4 + M(H,C,D)(1 + k_Sp)^4} \quad [4]$$

The oxygen data cited (1, 2) consists of eight sets of p,y value pairs measured under the conditions listed in Table 1. We shall assume that under the conditions of data set 1,

$$\sum_{i=1} k_{i0}^T[H]^i \ll 1 \text{ and } \sum_{i=1} k_{i0}^R[H]^i \ll 1$$

resulting in the following simplifications:

$$L(8 \times 10^{-10}, 0) \approx L_0 \quad [5a]$$

$$M(8 \times 10^{-10}, 0, 0) \approx M_0 \quad [5b]$$

$$y(p, 8 \times 10^{-10}, 0, 0) \approx \frac{k_Rp(1 + k_Rp)^3 + L_0k_Tp(1 + k_Tp)^3 + M_0k_Sp(1 + k_Sp)^3}{(1 + k_Rp)^4 + L_0(1 + k_Tp)^4 + M_0(1 + k_Sp)^4}. \quad [6]$$

Eq. **6** contains five undetermined parameters (k_R, k_T, k_S, L_0, and M_0) and it is apparent that no determination of unique best-fit parameter values can be made by fitting this equation to data set 1 (Table 1); some further simplification is required. If it is further assumed that $L_0 \gg 1 \gg M_0$, then by virtue of the condition that $k_R > k_T > k_S$, Eq. **6** reduces to

$$y(p, 8 \times 10^{-10}, 0, 0) \approx \frac{k_Rp(1 + k_Rp)^3 + L_0k_Tp(1 + k_Tp)^3}{(1 + k_Rp)^4 + L_0(1 + k_Tp)^4} \quad [7]$$

which is just the well-known two-state allosteric saturation equation (4). Eq. **7** was least-squares fitted to data set 1 and a set of best-fit values for the three parameters k_R, k_T, and L_0 was obtained. The form of Eq. **4** applicable under the conditions of data set 2 is

$$y(p, 8 \times 10^{-10}, 0, 0.002) \approx \frac{k_Rp(1 + k_Rp)^3 + L_0k_Tp(1 + k_Tp)^3 + 0.002\,M_2k_Sp(1 + k_Sp)^3}{(1 + k_Rp)^4 + L_0(1 + k_Tp)^4 + 0.002\,M_2(1 + k_Sp)^4} \quad [8]$$

Fixing the values of k_R, k_T, and L_0 at the values obtained by fitting Eq. **7** to data set 1, Eq. **8** was fitted to data set 2 to obtain best-fit values of the two variable parameters k_S and M_2.

With the values of k_R, k_T, and k_S fixed in the above manner, Eq. **4** was simultaneously fitted to data sets 3 and 4 to obtain best-fit values of L, M_1, and M_2 at pH 7.4, 0.005 M Cl^-, and then simultaneously fitted to data sets 5–8 to obtain best-fit values of the same three parameters for pH 7.4, 0.1 M Cl^-. The best-fit parameter values obtained by fitting to all eight data sets are listed in Table 2, and saturation curves calculated using the appropriate set of best-fit parameter values are plotted together with the experimental data in Figs. 1 and 2.

It should be stressed that the primary purpose of fitting Eq. **4** and its variants to the several data sets is not the estimation of parameter values as such, but rather to demonstrate that no less than three discrete affinity states must be invoked to account for heterotropic effects on the basis of the allosteric model. The relative magnitudes of the three intrinsic oxygen association constants, k_R, k_S, and k_T, indicate that the difference between the free energies of oxygen binding to state S and state T is approximately as great as the difference between the free energies of oxygen binding to state T and state R. Therefore, it would be entirely inappropriate to claim that heterotropic effects may be accounted for in a two-state context by invoking second-order perturbations of the low affinity state. Clearly the oxygen affinity of state S is no more a perturbation of that of T than the affinity of T is a perturbation of that of R.

One may ask whether it is permissible to treat an arbitrary solution of hemoglobin in the presence of allosteric effectors (including proton) as if it were effectively two-state, that is, containing one high-affinity state and one low-affinity "virtual state" which is really some sort of average of the two or more low-affinity states required by a more detailed analysis. Indeed, it is possible to fit a two-state equation of the form of Eq. **7** to each of the eight data sets plotted in Figs. 1 and 2, yielding a best-fit value of k_R which is essentially constant and best-fit values of L and k_T(apparent) which vary from set to set. According to the "effective two-state" approach, it might be argued that since k_T(apparent) represents an average over two or more affinity states whose relative abundances vary with effector concentration, the value of k_T(apparent) would likewise be expected to vary with effector concentration, as is observed.

TABLE 1. *Experimental conditions under which oxygenation data were obtained*

Data set no.	pH	Buffer	Total Cl^-(M)	2,3-diphosphoglycerate (mM)	Ref. no.
1	9.1	Glycine-NaOH	~0	0	1
2	9.1	Glycine-NaOH	~0	2.0	1
3	7.4	Bis-tris·HCl*	~0.005	0	1
4	7.4	Bis-tris·HCl*	~0.005	2.0	1
5	7.4	Bis-tris·HCl*	0.1	0	2
6	7.4	Bis-tris·HCl*	0.1	0.2	2
7	7.4	Bis-tris·HCl*	0.1	0.5	2
8	7.4	Bis-tris·HCl*	0.1	2.0	2

* 0.05 M.

T = 25°; total Hb concentration = 1.5×10^{-5} M (tetramer).

TABLE 2. *Best-fit parameter values in the three-state allosteric model*

Data sets	pH	Cl^-	L	M_1	M_2
1–2	9.1	0	488	$\ll 1$	4.2×10^5
3–4	7.4	0.005	1217	1495	5.13×10^9
5–8	7.4	0.1	7165	2.0×10^5	4.97×10^9

k_R = 3.78 $torr^{-1}$; k_T = 0.181 $torr^{-1}$; k_S = 0.0135 $torr^{-1}$

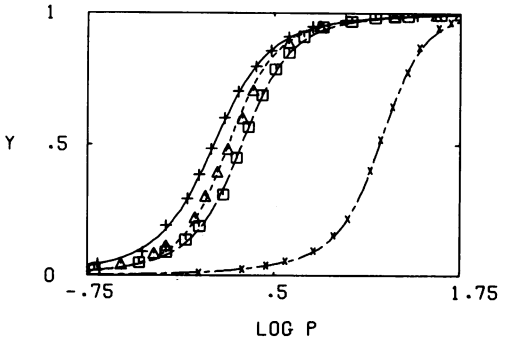

FIG. 1. Fractional saturation y versus log p. Data points are from ref 1, curves are calculated from Eq 4 using the appropriate set of best-fit parameters given in Table 2. +——+, data set 1; Δ- - -Δ, data set 2; □— —□, data set 3; ×— - —×, data set 4.

Comparison with the results of the three-state model shows that in general the "effective two-state" approach is not meaningful. In Fig. 3 the fractional abundances of affinity states R, T, and S in the three-state model are plotted as functions of the oxygen saturation y at pH 7.4, 0.005 M Cl^-, stripped of 2,3-diphosphoglycerate (data set 3). It may be seen that the ratio of the abundance of S to that of T, which exceeds 1 for $y = 0$, decreases markedly with increasing y. Thus any property of the virtual low affinity state in an "effective two-state" model, including of course the oxygen binding constant k_T(apparent), must be *dependent* upon the degree of oxygen saturation. Since the allosteric model (4) postulates that the oxygen-binding constant of an affinity state is *independent* of the degree of oxygen saturation, it follows that one cannot attach physical significance to the ability of the two-state allosteric equation to fit arbitrarily selected saturation data. In most cases this ability is fortuitous and attributable to the flexibility conferred by three independently variable parameters. In this respect the allosteric two-state equation is comparable to the Hill equation (8), which accommodates most saturation data quite well with only two variable parameters, and to which no physical significance is attributed.

There are two special limiting cases in which a two-state allosteric model may possibly (but does not necessarily) retain significance. In the total absence of allosteric effector, the state S may be negligible in comparison with states T and R, and when hemoglobin is saturated with effector the state T may be negligible in comparison to states S and R. For example, Shulman *et al.* (9) have interpreted the oxygen-binding data of Roughton and Lyster (10) on human hemoglobin (pH 7.0, $T = 19°$) in 0.6 M phosphate buffer in terms of the two-state allosteric model. When corrections are made for the temperature difference (11), these data closely resemble those of data sets 6 and 7 (Table 1). Under these conditions the fractional abundance of state T is expected to be small but not negligible (about 0.1) throughout a large part of the saturation range (Fig. 4). Thus, a two-state description of ligand binding may possibly (but does not necessarily) suffice as a rough approximation at high phosphate concentrations. However, the two-state model has often been used unselectively to interpret data obtained under conditions such that the hemoglobin is neither totally stripped nor saturated with effector (12–15); under these conditions a two-state description is indisputably inapplicable.

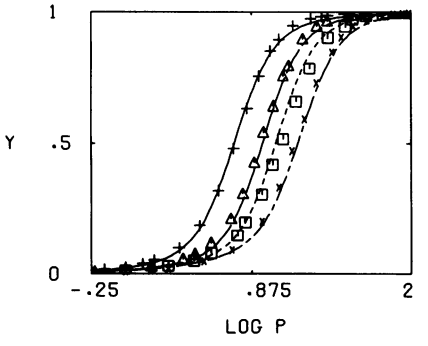

FIG. 2. Fractional saturation y versus log p. Data points are from ref 2, curves are calculated from Eq 4 using the appropriate set of best-fit parameters given in Table 2. +——+,data set 5; Δ—— ——Δ, data set 6; □- - -□, data set 7; ×—— - ——×, data set 8.

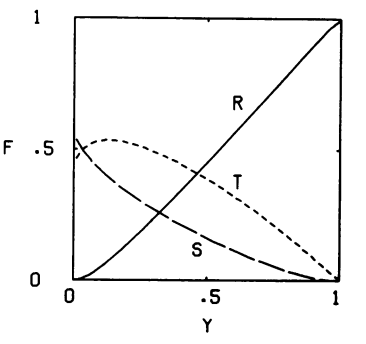

FIG. 3. Fractional abundance of affinity states f versus saturation y calculated for data set 3.

Central to the allosteric model, whether two-state or n-state, is the postulate that alterations in oxygen affinity arise exclusively from transitions between alternate quaternary structures (conformational states) of the hemoglobin molecule (4). This postulate requires the existence of at least one distinguishable quaternary structure corresponding to each affinity state. The results of several electron spin resonance (12, 13) and nuclear magnetic resonance (14, 15) experiments have been cited as evidence that hemoglobin may exist in one of only two quaternary conformations, corresponding to those of deoxyhemoglobin and fully heme-liganded hemoglobin. These experiments were conducted under conditions such that at least three affinity states are required to provide an allosteric description of ligand binding (see above). Thus, the allosteric model can only be reconciled with the results of these electron spin resonance and nuclear magnetic resonance studies if it is assumed that large changes in oxygen affinity

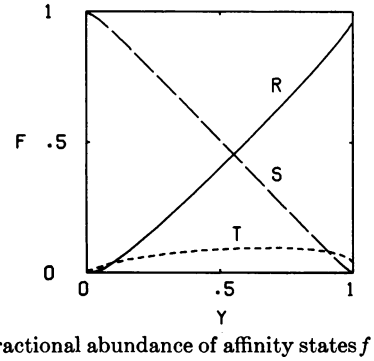

FIG. 4. Fractional abundance of affinity states f versus saturation y calculated for data set 6.

can be associated with what would otherwise be regarded as minor perturbations of a single quaternary structure (or spectrum associated with a given quaternary structure). For example, it might be argued that the small differences observed between the conformations of deoxyhemoglobin with and without bound 2,3-diphosphoglycerate (16) are sufficient to distinguish between affinity states S and T, respectively, in the three-state model. However, by adopting this view we are in effect admitting that substantial alterations in ligand affinity may be associated with conformational alterations which are too subtle to be resolved by any particular spectroscopic probe of structure. In particular, unresolved conformations must be invoked to interpret the reported electron spin resonance and nuclear magnetic resonance data (12–15) in the context of an allosteric description requiring three or more affinity states. It should be pointed out that unresolved conformations may also be invoked, with equal justification, to interpret the same data in the context of a multi-state sequential model.

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1. Tyuma, I., Kamigawara, Y. & Imai, K. (1973) *Biochim. Biophys. Acta* **310**, 317–320.
2. Tyuma, I., Imai, K. & Shimizu, K. (1973) *Biochemistry* **12**, 1491–1498.
3. Imai, K. (1973) *Biochemistry* **12**, 798–808.
4. Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118.
5. Koshland, D. E., Nemethy, G. & Filmer, D. (1966) *Biochemistry* **5**, 365–385.
6. Saroff, H. A. & Yap, W. T. (1972) *Biopolymers* **11**, 957–971.
7. Gurd, F. R. N. (1970) in *Physical Principles and Techniques of Protein Chemistry*, ed. Leach, S. J. (Academic Press, New York), Part B, chap. 15, Sec. II.A.6.
8. Antonini, E. & Brunori, M. (1971) in *Hemoglobin and Myoglobin in Their Reactions with Ligands* (North-Holland, Amsterdam), chap. 14, Sec. 1.2.
9. Shulman, R. G., Ogawa, S. & Hopfield, J. J. (1972) *Arch. Biochem. Biophys.* **151**, 68–74.
10. Roughton, F. J. W. & Lyster, R. L. J. (1965) *Hvalradets Skrifter* **48**, 185–198.
11. Imai, K. & Tyuma, I. (1973) *Biochem. Biophys. Res. Commun.* **51**, 52–58.
12. Ogata, R. T. & McConnell, H. M. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 325–336.
13. Ogata, R. T. & McConnell, H. M. (1972) *Biochemistry* **11**, 4792–4799.
14. Shulman, R. G., Ogawa, S. & Hopfield, J. J. (1971) *Cold Spring Harbor Symp. Quant. Biol.* **36**, 337–341.
15. Ogawa, S. & Shulman, R. G. (1972) *J. Mol. Biol.* **70**, 315–336.
16. Arnone, A. (1972) *Nature* **237**, 146–149.