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Mechanism of Cooperative Oxygen Binding to Hemoglobin

(spin-labeled triphosphate/concerted transition model/hemoglobin Chesapeake)

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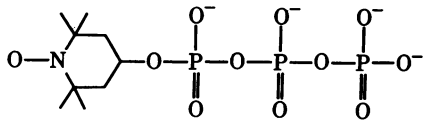
ABSTRACT **Evidence is presented that a generalized concerted transition model provides a quantitative understanding of (*a*) the molecular species that are present in solutions of partially liganded hemoglobin and (*b*) the macromolecular mechanism of cooperativity. Model parameters for hemoglobin A and for hemoglobin Chesapeake were determined from studies of the binding of spin-label triphosphates to ligand-free and partially liganded hemoglobin solutions, and to the hybrids $\alpha_1{}^{+CN}\beta_2$ and $\alpha_2\beta_2{}^{+CN}$. This model is the same as that proposed originally by Monod, Wyman, and Changeux [*J. Mol. Biol.* (1965) 12, 88] for hemoglobin, except that the α-subunits are treated as nonequivalent to the β-subunits.**

In a recent study (1), we have shown that the binding of a spin-label triphosphate to hemoglobin can be understood in terms of a generalized concerted transition model. This model is essentially the same as that first introduced by Monod, Wyman, and Changeux (2), except that the α- and β-subunits are treated as nonequivalent. We have further studied this problem using a second spin-label triphosphate, as well as a hemoglobin mutant (Chesapeake). The purpose of this paper is to present these new results, and to indicate the remarkably large body of information on hemoglobin that is accounted for by this generalized concerted transition model.

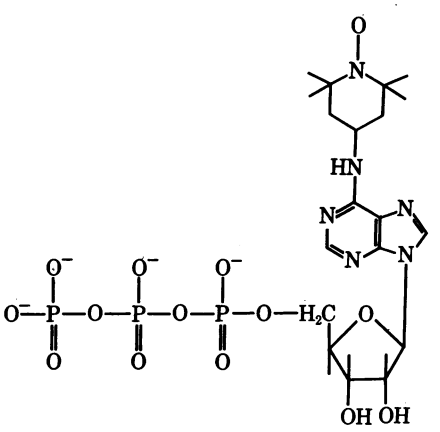
MATERIALS AND METHODS

Hemoglobin. Hemoglobin A was isolated (1) from blood samples obtained through the courtesy of Dr. Christina Harbury of the Veteran's Administration Hospital in Palo Alto. Hemoglobin Chesapeake was a gift of Dr. Samuel Charache. All hemoglobin solutions were rendered (1) phosphate-free. In all experiments, a hemoglobin concentration of about 3×10^{-4} M (tetramer) was used.

Spin Labels. 1-Oxyl-2,2,6,6-tetramethylpiperidine-4-triphosphate (I) and N^6-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) adenosine triphosphate (II) were used in these studies. The synthesis and hemoglobin-binding properties of SL (for Spin-Label)-TP (I) have been reported (1). SL-ATP (II) was a gift of Mr. D. Brutlag*.



(I)



(II)

Abbreviations: Hb, hemoglobin; SL, spin-label.

* Atkinson, M., Brutlag, D. & Kornberg, A., to be published.

Binding Studies. The affinity of SL-TP for ligand-free hemoglobin Chesapeake was measured (at $\bar{Y} = 0$) by a paramagnetic resonance technique (1); a value of 15 was used for η. The binding of SL-ATP to ligand-free hemoglobin A was studied by equilibrium dialysis, where SL-ATP concentrations were determined by assay for phosphate (3).

In this study, carbon monoxide was used as a heme ligand in order to avoid possible small effects, due to spin relaxation, of paramagnetic oxygen on signal amplitudes.

EXPERIMENTAL RESULTS

The dissociation constant of the ligand-free (HbA)-(SL-ATP) complex was $K_0 = 2.7 \pm 0.2 \times 10^{-5}$ M, with a binding stoichiometry of 1.0 ± 0.05 mol of SL-ATP per mol of the ligand-free HbA tetramer. This dissociation constant and stoichiometry are essentially the same as those (1) measured for the binding of SL-TP to ligand-free HbA ($K_0 = 2.5 \pm 0.5 \times 10^{-5}$ M).

Representative data on the binding of SL-ATP to solutions of HbA partially liganded with CO are summarized in Fig. 1. The quantity F used in this figure has been discussed (1), and is defined by the following equation.

$$F = \frac{(l_0 - l)}{c_0}\left(1 + \frac{K_0}{l}\right) \qquad (1)$$

Here l is the concentration of free (unbound) spin-label triphosphate, l_0 is the total concentration of spin-label triphosphate (bound plus free), c_0 is the total concentration of HbA, and K_0 is the above-defined dissociation constant of the ligand-free (HbA)–(spin-label triphosphate) complex. In all

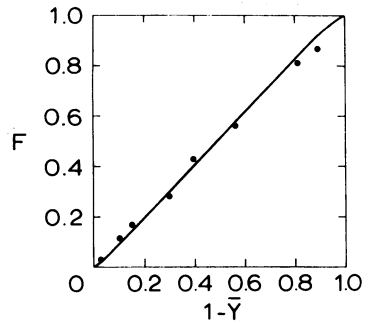


FIG. 1. A plot of the function F versus $1 - \bar{Y}$. F is a measure of the binding of the spin-label, SL-ATP, in hemoglobin A solutions partially liganded with CO. The *solid curve* is calculated from the generalized concerted transition model, with parameters given in the text and in Table 2. Here $c_0 = l_0$.

calculations, the value of K_0 is taken as 2.5×10^{-5} M for both spin-label phosphates. The values of l were determined directly from the paramagnetic resonance spectra (1). Note in Fig. 1 that for CO-ligation of HbA, the function F is very nearly equal to $1-\bar{Y}$, as was observed earlier for the binding of SL-TP to HbA. Here $\bar{Y}$ is the fractional saturation of the heme groups with CO, as determined optically.

The dissociation constant of the ligand-free (Hb Chesapeake)-(SL-TP) complex was $K_{Ch} = 7.2 \pm 0.5 \times 10^{-5}$ M, and the binding stoichiometry was 1.0 ± 0.05 mol of SL-TP per mol of ligand-free Hb Chesapeake tetramer. Representative data on the binding of SL-TP in solutions of Hb Chesapeake partially liganded with CO are given in Fig. 2. For reasons that will be clear later, the function F used in Fig. 2 is calculated from Eq. 1, with K_0 equal to 2.5×10^{-5} M. Values of the free label concentration, l, were determined (1) from the paramagnetic resonance spectra of partially liganded hemoglobin solutions, together with the value of $K_{Ch} = 7.2 \times 10^{-5}$ M, which was used to determine the concentration l at $\bar{Y} = 0$. Note that F deviates markedly from $1-\bar{Y}$ in Fig. 2.

In the present study, isosbestic points (see ref. 1) were found in the paramagnetic resonance spectra of both triphosphate spin-labels in solutions of partially liganded HbA and Hb Chesapeake.

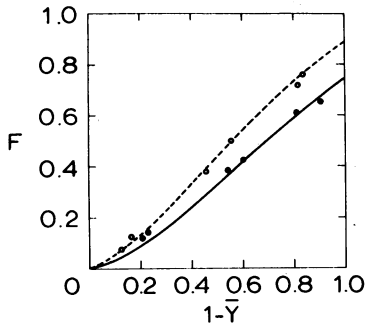


FIG. 2. Plots of the function F versus $1 - \bar{Y}$. F is a measure of the binding of the spin label, SL-TP, to solutions of hemoglobin Chesapeake partially liganded with CO. The *solid* and *dashed curves* are calculated from the generalized concerted transition model with the parameters given in the text and in Table 2. c_0/l_0 is 1 for ● and (——), and is 0.5 for O and (– – –).

TABLE 1. *The binding of SL-TP to hemoglobin*

Hemoglobin	Dissociation constant	$\left\{\frac{[T \text{ structure}]}{[R \text{ structure}]}\right\}$
A	2.5×10^{-5} *,†,‡	≫1
$\alpha_2{}^{+CN}\beta_2$	6.25×10^{-4} *,‡	0.67
$\alpha_2\beta_2{}^{+CN}$	3×10^{-5} *,‡	0.09
Chesapeake	7.2×10^{-5} *	0.53

* Measured by the paramagnetic resonance method.
† Measured by equilibrium dialysis.
‡ Ref. 1.

THE GENERALIZED CONCERTED TRANSITION MODEL

The generalized concerted transition model [generalized Monod, Wyman, Changeux model (2)] has been discussed (1). This model contains the following parameters. The allosteric equilibrium constant, L, is defined by the equation

$$L = [T]/[R] \qquad (2)$$

where $[T]$ and $[R]$ are the concentrations of the two protein structures of hemoglobin; here, the T and R structures are identified with the ligand-free and fully liganded structures of hemoglobin as determined by x-ray diffraction (4–6). In addition to the constant L, the model is based on the parameters $K_T{}^{\alpha}$, $K_T{}^{\beta}$, $K_R{}^{\alpha}$, and $K_R{}^{\beta}$, which are the α- and β-subunit oxygen dissociation constants in the T and R states. The following values of these parameters were found to account for our experimental results for HbA: $L = 3{,}000$; $K_T{}^{\alpha} = 24.2$, $K_T{}^{\beta} = 32.8$, $K_R{}^{\alpha} = 0.36$, $K_R{}^{\beta} = 0.18$ torr of oxygen†. These constants were determined as described (1), except for slight refinements to improve agreement between the model and experiment.

The above parameters for HbA, together with K_0, account for (*a*) the oxygen-binding curve of "stripped" (phosphate-free) human hemoglobin; (*b*) the oxygen-binding curve of hemoglobin in the presence of an equimolar concentration of SL-TP; (*c*) the relative dissociation constants of SL-TP complexes with HbA and the two hybrids $\alpha_2{}^{+CN}\beta_2$ and $\alpha_2\beta_2{}^{+CN}$; and (*d*) the binding of spin-label phosphates in solutions of HbA partially liganded with CO. The experimental and calculated dependence of F on $1-\bar{Y}$ are compared in Fig. 1, where F is measured with SL-ATP, and in ref. 1, where F is measured with SL-TP. In the present model, F is equal to the fraction of the hemoglobin molecules in the T state. This model is consistent with the observation of isosbestic points in the paramagnetic resonance spectra of SL-TP and SL-ATP in solutions of HbA partially liganded with CO (1).

The following parameters of the generalized concerted transition model were found to account for our experimental results for Hb Chesapeake: $L = 0.53$; $K_T{}^{\alpha} = 0.36$, $K_T{}^{\beta} = 32.8$, $K_R{}^{\alpha} = 0.36$, $K_R{}^{\beta} = 0.18$ torr of oxygen. These parameters, together with the value of the dissociation constant K_0 for HbA (we assume that the T structure is identical for HbA and Hb Chesapeake) account for (*a*) the SL-TP dissociation constant, K_{Ch}; (*b*) the low cooperativity for oxygen binding

† These parameters differ slightly from those given in ref. 1. The changes do not affect any of the calculated results or conclusions presented in ref. 1.

[calculated Hill's constant, $n = 1$ for stripped Hb Chesapeake; observed $n = 1.3$ (11)]; (*c*) the high oxgen affinity [observed $p_{50} = 0.28$ (11), calculated $p_{50} = 0.28$]; and (*d*) binding of SL-TP in solutions of Hb Chesapeake partially liganded with CO. The experimental and theoretical dependence of F on $1-\bar{Y}$ for Hb Chesapeake are compared in Fig. 2.

The various dissociation constants discussed above are summarized in Table 1. Table 2 gives sets of model parameters equivalent to those used above, where

$$c_\alpha = K_R^\alpha/K_T^\alpha \quad (3)$$

$$c_\beta = K_R^\beta/K_T^\beta \quad (4)$$

$$\gamma = K_R^\alpha/K_R^\beta \quad (5)$$

These parameters are convenient for theoretical calculations (1).

The parameters used here were determined by first setting K_R^α and K_R^β approximately equal to the oxygen affinities of isolated α- and β-chains, respectively (1,7). Values of c_α and c_β were then calculated from a value of L (1) that was selected to give, with K_R^α and K_R^β, an oxygen-uptake curve in agreement with that obtained for stripped HbA. The values of L, K_R^α, and K_R^β were then systematically varied until they gave the best fit to the experimental data for HbA, Hb Chesapeake, $\alpha_2{}^{+CN}\beta_2$, and $\alpha_2\beta_2{}^{+CN}$. The set of parameters presented in Table 2 represent what is likely to be a unique set that best fits all of the experimental data.

A comparison of experimental and calculated (with this model and the parameters listed in Table 2) oxygen-binding data is given in Table 3.

DISCUSSION

As discussed above, the generalized concerted transition model is remarkably effective in accounting for the binding of oxygen (or carbon monoxide), and the binding of spin-label triphosphates, as well as their linked interaction in HbA and in Hb Chesapeake. In addition to this agreement between model and experiment, there are further reasons for suspecting that the model is an authentic representation of the mechanism of cooperativity and the structures of hemoglobin present in solution. These reasons are enumerated below.

The relative values of the parameters for HbA and Hb Chesapeake are readily understood from a physical point of view if one simply assumes that the effect of the HbA → Hb Chesapeake mutation (α-92 Arg → Leu) (12) is to lock the α-subunits in their tertiary structure with a high affinity for oxygen. This is a plausible consequence of the amino-acid substitution, and makes understandable the fact that the model best accounts for the observed data when $K_T^\alpha = K_R^\alpha$ for Hb Chesapeake, K_T^α (Hb Chesapeake) = K_R^α(HbA), K_T^β (Hb Chesapeake) = K_T^β(HbA), and K_R^β(Hb Chesapeake) = K_R^β(HbA). The lower value for L for Hb Chesapeake then follows immediately from the assumption that the α-subunits of Hb Chesapeake are locked in their structure with a high affinity for oxygen. That is, one expects that the ratio of [T structure] to [R structure] (= 0.53) is roughly the same as this ratio for $\alpha_2{}^{+CN}\beta_2$ (= 0.67).

Another interesting feature of the present analysis concerns the paramagnetic resonance spectra of SL-ATP bound to HbA and to Hb Chesapeake‡. The concerted transition model requires that the resonance spectrum of SL-ATP bound to HbA and Hb Chesapeake be identical, since only one phosphate binding (or T) structure is allowed. The apparently lower affinity of Hb Chesapeake for SL-TP (and presumably SL-ATP) is accounted for in the model in terms of a decreased equilibrium constant L, such that in a solution of ligand-free Hb Chesapeake, only 35% of the tetramers are in the T state. We do find that the resonance spectra exhibited by SL-ATP bound to HbA and to Hb Chesapeake are essentially identical (Fig. 3). Since the resonance spectra of spin labels bound to proteins are very sensitive to small changes in

TABLE 2. *Model parameters used to fit experimental data*

Hemoglobin	L	K_R^α	γ	c_α	c_β
A	3000	0.36	2	0.0149	0.0055
$\alpha_2{}^{+CN}\beta_2$	3000	0.36	2	0.0149	0.0055
$\alpha_2\beta_2{}^{+CN}$	3000	0.36	2	0.0149	0.0055
Chesapeake	0.53	0.36	2	1	0.0055

In all calculations, $c_0 = 3 \times 10^{-4}$, $\gamma = K_R^\alpha/K_R^\beta$, $c_\alpha = K_R^\alpha/K_T^\alpha$, and $c_\beta = K_R^\beta/K_T^\beta$. For $\alpha_2{}^{+CN}\beta_2$ and $\alpha_2\beta_2{}^{+CN}$, when the ferrous subunits are ligand-free, $[T]/[R]$ is equal to $Lc_\alpha{}^2$ and $Lc_\beta{}^2$, respectively. L refers to stripped hemoglobin in 0.1 M Cl^-, and increases when phosphate is added (see ref. 1).

TABLE 3. *Comparison of experimental and calculated oxygen-binding data*

Hemoglobin	Experimental p_{50}	Experimental n	Calculated* p_{50}	Calculated* n	Ref.
A					
stripped	1.83	2.8	1.83	2.9	1
+equimolar SL-TP	3.13	2.3	3.17	2.4	1
$\alpha_2{}^{+CN}\beta_2$					
stripped	—	—	0.23	1.1	
+phosphate†	(1.8, 2.3)	1.1–1.3	1.12	1.6	9, 10
$\alpha_2\beta_2{}^{+CN}$					
stripped	—	—	0.38	1.0	
+phosphate†	(1.4, 2.3)	1.0	0.90	1.4	9, 10
Chesapeake					
stripped	0.28‡	1.4	0.28	1.0	11
+phosphate§	0.48	1.6	0.50	1.2	11

* Calculated with parameters listed in Table 2. These apply for bis-Tris buffer at pH 7.3, 0.1 M Cl^-, 13°C.

† For calculated data, $l_0 = 1.5 \times 10^{-3}$. For experimental data, pH = 7.15 and 7, temperature is 25.5 and 20°C and buffer is 0.06 M and 0.02 M phosphate¶.

‡ Conditions: 0.05 M bis-Tris (pH 7.2), 0.1 M Cl^- and 10°C. [Hb] $\cong 5 \times 10^{-5}$ M.

§ For calculated data, $l_0 = 6 \times 10^{-4}$. For experimental data, [diphosphoglycerate] = 2×10^{-4}. Other conditions as in above footnote.

‡ This spectral comparison is more informative for SL-ATP than for SL-TP, because the component of the resonance spectrum due to SL-ATP bound to HbA or Hb Chesapeake is relatively "strongly immobilized" (8) and is, therefore, well resolved from the spectral component due to SL-ATP free in solution. The resonance spectrum of SL-TP bound to HbA or Hb Chesapeake is relatively "weakly-immobilized," however, and is not clearly resolved from the component due to free SL-TP (see ref. 1).

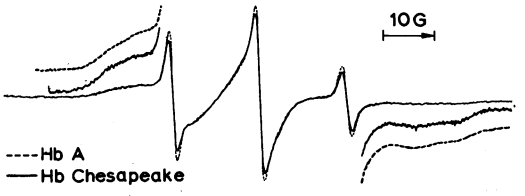


FIG. 3. The paramagnetic resonance spectra of the spin label SL-ATP in solutions of ligand-free hemoglobin A (- - -) and in solutions of ligand-free hemoglobin Chesapeake (——). Portions of the spectra shown at higher gains arise from the spin label bound to the hemoglobin. For HbA, $c_0/l_0 = 2$, and for Hb Chesapeake, $c_0/l_0 = 5$.

the protein structure (8), this result demonstrates that the HbA tetramer that binds SL-ATP and the Hb Chesapeake tetramer that binds SL-ATP must have very nearly the same structure at the triphosphate-binding site. This binding site must be identical, or nearly identical, to the 2,3-diphosphoglycerate-binding site (1), which probably lies along the dyad axis of symmetry of the hemoglobin molecule (13), and probably involves the α-amino groups of valines NA1 (1) of the β-subunits (11, 14–16). Since the model-building studies of Perutz (17) suggest that the structure of the diphosphoglycerate-binding site is closely related to the quaternary structure of hemoglobin, the structure of the SL-ATP binding site must be dependent on, and sensitive to, the quaternary structure of the hemoglobin molecule. Therefore, the striking similarity of the spectra due to SL-ATP bound to HbA and to Hb Chesapeake suggests (*a*) that the quaternary structures of HbA and Hb Chesapeake are very similar and can probably be taken to represent a single phosphate-binding or T structure, and (*b*) that the lower affinity of SL-TP for Hb Chesapeake relative to HbA is due, not to a change in the structure of the binding site, but to a decreased effective concentration of binding sites, i.e., a decreased equilibrium constant L.

The isosbestic points observed in the resonance spectra of both spin labels in solutions of partially-liganded HbA and Hb Chesapeake are also in accord with a single $R \rightleftharpoons T$ structural transition.

It is also of interest to consider the order of oxygenation of the α- and β-subunits. According to the present model, oxygenation of stripped HbA yields partial saturations of the α- and β-subunits, $\bar{Y}_\alpha$ and $\bar{Y}_\beta$, that are nearly equal to one another and to $\bar{Y}$, the average saturation of heme. (Phosphate has only a small effect on the relative values of $\bar{Y}_\alpha$ and $\bar{Y}_\beta$ for HbA). In the case of Hb Chesapeake, $\bar{Y}_\alpha$ and $\bar{Y}_\beta$ are distinctly different and more sensitive to phosphate concentration (see Fig. 4). The calculated values of $\bar{Y}_\alpha$ and $\bar{Y}_\beta$ for Hb Chesapeake account for the observations of Davis *et al.* (18) that at $\bar{Y} = 0.25$, the nuclear resonance spectra of the α-heme protons indicate that $(1 - \bar{Y}_\alpha)/(1 - \bar{Y}_\beta) = 0.60$. The model data in Fig. 4 give a value of this ratio that is equal to 0.66§. Davis *et al.* also report that in HbA, the α- and β-subunits oxygenate at the same rate $[(1 - \bar{Y}_\alpha)/(1 - \bar{Y}_\beta) = 1]$, which is also in accord with the calculated subunit saturations shown in Fig. 4.

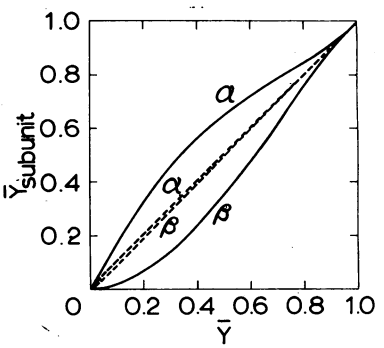


FIG. 4. Fractional α- and β-subunit saturations $\bar{Y}_\alpha$ and $\bar{Y}_\beta$ in HbA (- - -) and in Hb Chesapeake (——), as a function of average heme-saturation $\bar{Y}$, calculated with the parameters of the generalized concerted transition model given in the text and in Table 2. $c_0/l_0 = 0.2$.

The present model not only accounts for various properties of HbA and Hb Chesapeake, but also for properties of the hybrids $\alpha_2{}^{+CN}\beta_2$ and $\alpha_2\beta_2{}^{+CN}$. The model accounts for the binding of SL-TP to these hybrids, relative to the binding to ligand-free HbA. Also this model accounts approximately for the oxygen-binding curves of these hybrids. Observed and calculated values of p_{50} for these hybrids are given in Table 3§¶. The present model is in qualitative agreement with the nuclear resonance (19), kinetic (20), and heme spin-label (21) work on these half-ferric hybrids, studies that show that the heme groups of the α-subunits are much more sensitive to the state of ligation of the β-subunits than are the β-hemes to ligation of α-subunits. This is because the model parameters obtained earlier (1) lead to the prediction that β-subunit ligation has a greater influence on the $R \rightleftharpoons T$ structural transition than does α-subunit ligation (i.e., $K_R{}^\alpha/K_T{}^\alpha > K_R{}^\beta/K_T{}^\beta$).

The present model also accounts quite nicely for the studies of Maeda and Ohnishi (22) of the kinetics of paramercuribenzoate reactions with the β-93 SH-groups of ligand-free hemoglobin, tetraoxyhemoglobin, and the two hybrids $\alpha_2{}^+\beta_2$ and $\alpha_2\beta_2{}^+$. Maeda and Ohnishi observed the following rate constants, respectively, for these species: 3.3×10^4, 1.5×10^6, 9.9×10^4, and 3.5×10^5 M^{-1} sec^{-1}. If we take 3.3×10^4 M^{-1} sec^{-1} and 1.5×10^6 M^{-1} sec^{-1} to be the rate constants for reactions with the T and R structures, then the parameters of our model (Table 2) lead to calculated rate constants for $\alpha_2{}^{+CN}\beta_2$ and $\alpha_2\beta_2{}^{+CN}$ that are equal to those observed experimentally, to well within the experimental error§. The calculation assumes that the ratio of the $T \rightleftharpoons R$ interconversion is rapid compared to the rate of the reaction with the mercurial.

The present work provides strong evidence for the validity of the generalized concerted transition model. The parameters derived for this model permit a calculation of the concentrations of the various molecular species present in partially liganded solutions; thus, this model can be tested by spectroscopic and other experiments in addition to those described here. Our description of Hb Chesapeake in terms of this model

§ In these calculations, we use a total triphosphate spin-label concentration of $l_0 = 1.5 \times 10^{-3}$ M. This value of l_0 gives a p_{50} for HbA (other parameters as in Table 2) approximately equal to the observed p_{50} of HbA in 0.1 M phosphate buffer.

¶ The actual differences between calculated and observed p_{50} values are estimated to be much smaller than they appear in Table 3, since the experimental values were obtained under pH and temperature conditions not strictly comparable to those to which the calculated values apply.

raises the possibility that the properties of a number of hemoglobin mutants may be understandable in terms of a small number of *physically plausible* changes in the model parameters. It is possible that there is a class of abnormal hemoglobins whose properties can be understood in terms of a locking of the α- or β-subunits in their structures with high or low oxygen affinity. For example, we have noted that the oxygen binding curve of hemoglobin Kansas (23, 24) can be accounted for approximately if the β-subunits are locked in their form with low oxygen affinity. In this case, ligand-free hemoglobin Kansas is expected to bind organic phosphates with the same binding constant as ligand-free hemoglobin A, whereas fully liganded Kansas is expected to bind organic phosphates with approximately 40% the stability constant of ligand-free hemoglobin A.

We are extremely grateful to Dr. Samuel Charache for his generous gift of hemoglobin Chespeake and to Dr. Chien Ho for his help in obtaining a sample of this mutant hemoglobin. We are also indebted to Douglas Brutlag for his gift of SL-ATP. R.T.O. is a National Institutes of Health predoctoral fellow. This work was supported by the Office of Naval Research under Grant N00014-67-A-0112-0045, and has benefited from facilities made available by the Advanced Research Projects Agency through the Center for Materials Research at Stanford University.

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