Global Allostery Model of Hemoglobin: Modulation of O₂-Affinity, Cooperativity, and Bohr Effect by Heterotropic Allosteric Effectors*

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Running Title: Global Allostery Model of Hemoglobin
SUMMARY

The O2-equilibria of human adult hemoglobin have been measured in a wide range of solution conditions in the presence and absence of various allosteric effectors in order to determine how far hemoglobin can modulate its O2-affinity. The O2-affinity, cooperative behavior, and the Bohr effect of hemoglobin are modulated principally by tertiary-structural changes, which are induced by its interactions with heterotropic allosteric effectors. In their absence, hemoglobin is a high-affinity, moderately cooperative O2-carrier of limited functional flexibility, the behaviors of which are regulated by the homotropic, O2-linked T/R quaternary-structural transition of the Monod-Wyman-Changeux/Perutz model. However, the interactions with allosteric effectors provide such “inert” hemoglobin unprecedented magnitudes of functional diversities not only of physiological relevance but also of extreme nature, by which hemoglobin can behave energetically beyond what can be explained by the Monod-Wyman-Changeux/Perutz model. Thus the heterotropic effector-linked tertiary structural changes, rather than the homotropic ligation-linked T/R quaternary structural transition, are energetically more significant and primarily responsible for modulation of functions of hemoglobin.

Hemoglobin (Hb) has played a pivotal role in the understanding of the mechanisms of allosteric enzymes. Monod et al. (1) designated Hb an honorary enzyme, since it used the same molecule (O2) for signaling as well as regulation. With the advent of detailed molecular structure at atomic levels, the question of enzyme activity became one of molecular mechanism. In the case of an allosteric enzyme, there is a need to
assume at least two possible structures, customarily labeled T and R (1), and to regulate ligand-affinity in each structure. The first question of *signaling* is straightforward, for it involves alternate packing of interfaces, for example. Monod’s original proposal (1) to assign deoxy and oxy Hbs to the T and R states acquired a structural foundation, as crystallographic studies (2) revealed that the three-dimensional molecular structures of deoxy and ligated Hbs. The hemoglobin molecule, a hetero-tetramer, consists of two $\alpha$- and two $\beta$-subunits, each of which contains one O$_2$-binding heme group. These four subunits are paired as two dimers, $\alpha_1\beta_1$ and $\alpha_2\beta_2$. The structural studies showed that deoxy and ligated Hbs have two different modes of packing of the two dimers (the quaternary structures) with no major changes in the gross conformation of each of the subunits (the tertiary structures). Thus, Perutz (3) assigned “deoxy” and “oxy” Hbs to T and R quaternary states, which exhibited low and high O$_2$-affinity, respectively.

The second question concerning *regulation* is the deeper one, and for Hb has proved remarkably elusive. The essence of the question is to find a way in the low-affinity T (deoxy) state to store free energy that is made available to bind ligands in the high-affinity R (oxy) state. The MWC/Perutz stereochemical model (1,3), in which the O$_2$-affinity of Hb is regulated primarily by the T/R quaternary transition, approximates well the behaviors of Hb under physiological conditions. Therefore, Perutz (3) made the first proposal that salt bridges (formation/dissolution) are the key to regulation. Difficulty in establishing the centrality of the salt bridges led Perutz to propose that the affinity was lowered by storing energy as strain at the heme moiety, perhaps because of the spin radius of the high-spin iron. However, spin energy and heme strains store insufficient energy to account for this cooperativity. Hopfield (4) proposed a distributed strain model
in which small strains throughout the protein stored the energy difference. Molecular
modeling led Gelin et al. (5) to propose the movement of the proximal histidine pulling
on the F-helix was a locus of strain energy storage. Related proposals based upon
resonance Raman data were put forth by Nagai et al. (6) and by Friedman et al. (7).
Nonetheless, no definitive experiment has successfully ascribed to any structural feature
the amount of energy sufficient to account for the difference in the O2-affinity between T
(deoxy) and R (oxy) structures of Hb.

We have measured O2-binding curves of Hb in a wide range of solution
conditions in the presence and absence of various heterotropic allosteric effectors. These
measurements have been intended to probe the following questions: [i] how far Hb can
modulate its O2-affinity, [ii] how its cooperativity and its Bohr effect are modulated, and
[iii] by what mechanism such modulation of the O2-affinity is carried out. We have
demonstrated that the O2-affinity, the cooperativity, and the Bohr effect of Hb are
modulated in hitherto unimaginable extents by the interactions of the allosteric effectors
with Hb, especially in their interactions with oxy Hb. This has led us to an inevitable
conclusion that the substantial amount of the free energy of cooperativity/allostery must
be stored in the binding of the various effectors or in the tertiary structural constraints,
imposed by the interactions with the effectors. We show that the observed diverse
behaviors of Hb are energetically beyond what can be explained by the MWC/Perutz T/R
quaternary-transition model (3).

**EXPERIMENTAL PROCEDURES**

*Materials* - Human blood samples was purchased from the local blood bank
branch of the American Red Cross, from which Hb was purified according to the method
of Drabkin (8) and stripped of organic phosphates by the method of Berman et al. (9). Des-(β146 His, β145 Tyr)-Hb, or des-His-Tyr-Hb, was prepared by the digestion of Hb with carboxypeptidase A (Sigma Chemical, St. Louis, Missouri) at a ratio of enzyme/Hb (weight to weight) of 0.1 for 4 hr at 30°C in 0.05 M Tris-HCl buffer, pH 8.0 (10). The digestion was terminated by the addition of 1 mM hydrocinnamic acid. The so-treated Hb preparation was separated from released amino acids by molecular sieve through a column of Sephadex G-25 equilibrated with 10 mM bis-Tris propane buffer, pH 7.4.

**Oxygen Equilibrium Measurements** - Oxygen equilibria were measured by an improved version of Imai’s automatic polarography-spectrophotometry method (11-14) with the following modification. Absorbance was monitored using a computer-controlled Olis-Cary 118 spectrophotometer (Olis, Bogart, Georgia). Oxygen concentrations were monitored with a low zero-current, rapid-response electrode (O2 sensors, Gladwyne, Pennsylvania), using a custom-made amplifier (Biomedical Instrumentation Shop, University of Pennsylvania Medical Center, Pennsylvania). The signal was then digitized using a 12-bit A/D converter. Absorption spectra of oxy Hb and deoxy Hb in the visible region were independent of pH and the heterotropic effectors used. Therefore, absorbance changes at 560 nm were proportional only to the degrees of O2 saturation of Hb. The degrees of fractional O2-saturation (the Y values) were computed from the changes in absorbance at 560 nm by getting the absorbance values at the fully deoxy and oxy states by extrapolation with precautions previously described (13,14). The O2-equilibrium data were expressed as Hill plots of log [Y/(1-Y)] versus log pO2. The O2-equilibrium measurements were carried out in 0.1 M HEPES buffer (pH 6.6~9.0) at 15°C. The concentrations of reactants used were 60 µM (heme) Hb, 0.1M Cl⁻, 10mM BZF, 2mM
DPG, and 2mM IHP. We had previously shown that the effects of the tetramer-dimer
dissociation of Hb on O\textsubscript{2}-equilibrium parameters were minimally affected at [Hb] ≥ 60
µM (12). Oxygen-binding curves at each of 168 different solution conditions were
measured in triplicates in a cycle of the deoxygenation-followed-by-re-oxygenation
processes in order to assure the viability of the Hb preparation and the measuring
conditions. The formation of met Hb after each measurement was less than 3 %.

Adair oxygenation parameters - Oxygen-binding data were analyzed by a non-
linear least square curve-fitting analysis according to the Adair scheme (15) using
Equation 1:

\[
Y = \frac{K_1p + 3K_1K_2p^2 + 3K_1K_2K_3p^3 + K_1K_2K_3K_4p^4}{1 + 4K_1p + 6K_1K_2p^2 + 4K_1K_2K_3p^3 + K_1K_2K_3K_4p^4},
\]

where Y and p are the degree of O\textsubscript{2}-saturation and the partial pressure of O\textsubscript{2}, respectively,
and K\textsubscript{1}, K\textsubscript{2}, K\textsubscript{3}, and K\textsubscript{4} are the intrinsic O\textsubscript{2}-equilibrium association constants at
oxygenation steps 1, 2, 3, and 4, respectively. Processed oxygenation data were arranged
in the form (log p, log(Y/(1-Y))) and fitted with the custom-made non-linear curve fitting
procedure in Origin Version 6.1 (Microcal, Northampton, Massachusetts), according to
Equation 1. Initial values for K\textsubscript{1} and K\textsubscript{4} could be readily obtained graphically from the
lower and upper asymptotes of the Hill plots, respectively, whereas initial estimates for
K\textsubscript{2} and K\textsubscript{3} were arbitrarily made from experimental data at ∼30 and ∼60% saturation,
respectively. Sets of fitted values for K\textsubscript{1}, K\textsubscript{2}, K\textsubscript{3}, and K\textsubscript{4} were obtained after the
convergence of successive iterations was achieved. Parameter, ΔH\textsuperscript{+}\textsubscript{average}, is the average
number of Bohr protons released (expressed as negative value) per binding site, which
are numerically calculated from averaging the number of Bohr protons released at each step of four Adair O₂-binding equilibria (14, 16).

Monod-Wyman-Changeux Allosteric Parameters - A non-linear least-squares regression curve-fitting analyses of O₂- equilibrium data were also performed according to the MWC allosteric model using Equation 2 (1).

\[
Y = \frac{L_0 K_T p(1+K_T p)^3 + K_R p(1+K_R p)^3}{L_0(1+K_T p)^4 + (1+K_R p)^4},
\]

where \( Y \) is the degree of saturation with O₂, p is the partial pressure of O₂, \( K_T \) and \( K_R \) are O₂-association equilibrium constants of T (deoxy) and R (oxy) states, respectively, and \( L_0 = \frac{[T_0]}{[R_0]} \) and \( L_4 = \frac{[T_4]}{[R_4]} = L_0(K_T/K_R)^4 \), allosteric equilibrium constants, where \([T_0]\), \([R_0]\), \([T_4]\), and \([R_4]\) stand for equilibrium molar concentrations of T (deoxy) and R (deoxy) conformers in the deoxy state and those of T (oxy) and R (oxy) conformers in the fully oxy state, respectively. Processed oxygenation data were arranged in the form (log p, log\([Y/(1-Y)]\)) and fitted with the custom-made non-linear curve fitting procedure in Origin Version 6.1 (Microcal, Notheamption, Massachusetts), according to Equation 2. Initial estimates for \( K_T \), and \( K_R \) could be readily obtained graphically from Hill plots, or fitted data sets according to Adair scheme. Namely, \( K_T \) and \( K_R \) can be estimated from the lower and upper asymptotes on the Hill plots, which can be approximated to \( K_1 \) and \( K_4 \), respectively. The \( L_0 \) values were estimated with an approximation of \( L_0 = (K_4 \times P_{50})^4 \) (16). Unique sets of fitted values for \( K_T \), \( K_R \), and \( L_0 \) were thus obtained after the convergence of successive iterations was achieved. Although Marden et al. (17) analyzed their O₂-binding data and concluded that \( K_R \) cannot be deduced from their O₂-binding equilibrium curves, our successive iterations using 10-fold increased or decreased initial values of \( K_T \),
KR, or L0 were found not to alter the values of KT, KR, and L0 at the convergence. This was perhaps due to our more extensive collection of accurate O2-binding data at higher saturation levels using the substantially improved instrumentation. The free energy of cooperativity ($\Delta G_0$) was calculated from $\Delta G_0 = -2.3RT \log (K_R/K_T)$.

**Proton NMR measurements** - The NMR experiments were made with a Bruker ARX-500 NMR spectrometer at 15°C and 29°C, using with 2mM (heme) Hb, 10mM BZF, 10mM IHP, 0.1 M Cl⁻, and 0.1M HEPES buffer, pH 7.0 in 90% H2O-10% D2O. The molar ratio of [effector]/[Hb] of 5 used for NMR is less than those used in the oxygenation experiments. However, actual degrees of saturation of Hb with effectors in NMR experiments were substantially more than those of the oxygenation experiments, since the saturation depends on the concentrations of the reactants rather than the molar ratio as long as [effector]>[Hb] or free effectors are available. The water signal was suppressed by using a jump-and-return pulse sequence (18). Proton chemical shifts were referred to internal sodium 3-(trimethylsilyl) propionate-2,2,3,3-d4 (TSP-d4).

**Probes of quaternary structures in solution** - In addition to exchangeable proton NMR spectroscopy in the hydrogen-bonded region (19-21), the quaternary state of Hb in solution was probed by the following techniques: (i) UV fine structure difference spectrophotometry (22,23) using a Hewlett-Packard 8452A diode-array spectrophotometer, (ii) UV-CD spectrophotometry (24) using an AVIV 62DS CD spectrophotometer, and (iii) spectrophotometric assay of the reactivity of the ($\beta$93Cys-SH groups (16,25-28).

**RESULTS**
Conditions for measurements of oxygen binding curves - In order to measure the reversible O₂-binding curves of Hb in the widest possible range of well-controlled conditions, we made extensive examinations of experimental conditions that are optimally suited. The HEPES buffer system was chosen among many buffers commonly used in Hb research (phosphate, Tris-Cl⁻, bis-Tris-Cl⁻, bis-Tris propane, HEPES, etc.) for the following reasons. The buffering capability of HEPES covers a reasonably wide range of physiological pH (pH 6.6 to 9.0). The HEPES buffers keep all the allosteric effectors used soluble in the entire pH range at 15°C. The HEPES buffers neither interact with the allosteric effectors used nor alter Hb irreversibly and/or non-specifically in the entire range of pH employed. The HEPES buffers exhibit no detectable allosteric effect on Hb at the concentration employed. Although the majority of previous Hb works had been carried out at higher temperatures (20, 25, or 37°C), we had chosen the measuring temperature at 15°C, which was an optimal compromise of competing requirements to obtain quantitatively full O₂-binding curves under our wide-ranged experimental conditions. Since we dealt with Hb at low-affinity (P₅₀ of up to 150 torr), nearly full oxygenation was feasible only at lower temperatures (≤15°C) even at atmospheric pressure of 100% O₂ (the maximal O₂ pressure used in our experiments of pO₂ = 760 torr minus saturated aqueous vapor pressure). On the other hand, deoxygenation was more readily accomplished at higher temperatures especially for high affinity states of Hb (P₅₀ = 0.1 to 0.5 torr). Furthermore, the formation of met Hb during measurements was greatly reduced at this temperature. The Cl⁻ concentration was fixed at 0.1 M (the physiological concentration). The concentrations of other allosteric effectors were set at 2 mM DPG, 2 mM IHP, and 10 mM for BZF. These concentrations were chosen to maintain the
effectors soluble in the entire pH range used at 15°C. Thus, [effector] was always in large excess over [Hb], except for [H⁺], which was varied from 10⁻⁹ M to 10⁻⁶.6 M.

_Hill plots of O₂-binding curves_ - Figures 1 and 2 illustrate Hill plots of the O₂-binding data in the absence and presence of 0.1 M Cl⁻, respectively. It is noted that the Hill plots in the absence of heterotropic allosteric effectors above pH 7.8 are fully superimposable with a fixed cooperativity of n_{max} = 2.5 (Fig.1A). However, as pH is decreased below pH 7.8, the Hill plots shift toward the right with slight downward-shifts of the lower and upper asymptotes without changes in the cooperativity. In the presence of other heterotropic allosteric effectors, these pH-dependent shifts of the Hill plots become more and more pronounced in the order of apparent allosteric potencies of H⁺ < Cl⁻ < DPG < BZF < IHP < BZF+DPG < BZF+IHP under the prescribed concentrations of the effectors (Figs. 1 and 2). The downward shift of the lower asymptotes approach and eventually go beyond an apparent minimal value of P_{50} = 100 torr (the lower dotted line) in the presence of the maximal allosteric effect (with BZF+IHP at pH 6.6) (Figs. 1F). The downward shifts of the upper asymptotes are accompanied by gradual decrease of the apparent cooperativity (the slopes of the middle portion of Hill plots). When the upper asymptotes reach the minimal value of P_{50} = 100 torr, the apparent cooperativity is reduced to n_{max} ≈ 1 at the maximal allosteric constraint (Fig. 1F and 2F).

Thus, the O₂-affinity of Hb is modulated as much as >1,000-fold from P_{50} ≈ 0.1 torr (the upper asymptotes in Fig. 1A) to P_{50} ≈ 160 torr (the lower asymptotes in Figs. 1F) by the influence of heterotropic allosteric effectors.

Comparison of the Hill plots in Figs. 1 and 2 provides the following conclusions.

[i] The allosteric effect of H⁺ is additive or synergetic with those of all other heterotropic
effectors. [ii] The allosteric effect of Cl\(^-\) is competitive or antagonistic against those of other heterotropic effectors, as previously observed by others (13,28) (Fig. 1 versus Fig. 2). [iii] The effect of BZF is additive or synergetic with organic phosphates (DPG or IHP) (Fig. 1E-F and Fig. 2E-F), as previously noted (17,29-31) [iv] The O\(_2\)-affinity of unmodified Hb has an apparent lowest limit at \(P_{50} \approx 160\) torr under the present solution conditions. [v] The Bohr effect of Hb (or the pH-dependence of \(P_{50}\)) is increasingly enhanced by the heterotropic effectors in the order of their apparent allosteric potencies mentioned above.

**MWC allosteric and Adair oxygenation parameters -** The O\(_2\)-binding data shown in Figs. 1 and 2 have been analyzed by Equation 2 of the MWC model. Figure 3 lists the MWC parameters and related oxygenation parameters (\(\Delta G_0\), \(P_{50}\), and \(n_{max}\)) primarily from those in the presence of Cl\(^-\) (Fig. 1A and Fig. 2A-F). The MWC and Adair parameters obtained are comparable with those reported previously in the literature (13). The Hill plots of Figs. 1 and 2 are fully and quantitatively simulated by these MWC parameters \((K_T, K_R,\) and \(L_0\)) as well as by the Adair constants \((K_1, K_2, K_3,\) and \(K_4\)). For example, Fig. 4 illustrates simulation curves of Hill plots according to MWC and Adair models. Table I lists the selected sets of the MWC parameters and Adair constants in the presence of Cl\(^-\), BZF, and IHP, which were used for Fig. 4. The correlation of the MWC parameters are also expressed by the "global allostery" plots, namely, the log K versus log L plots (32,33) in Figs. 5 and 6. Such plots give explicitly the correlation among the MWC parameters under different effector conditions. The concentrations of allosteric effectors used are below saturation. However, in the MWC model, it is not the necessary condition that every state must be saturated with the effectors present in the solution. The important
condition is that the concentration of free effectors must be "virtually constant" during the oxygenation process. This condition is well met in this study since the [effector]/[Hb] ratio is sufficiently larger than unity. It should be pointed out that all the values of the MWC parameters obtained at higher concentrations of effectors or with stronger effectors lie on the closed distorted circular plot of Fig. 6, though the values are found at higher pHs than the corresponding values shown in Fig. 6. Thus, we consider the "global allostery" plot of Fig. 6 to be universally applicable to normal Hb under different concentrations of effectors and/or different effectors.

**Quaternary structures** - Exchangeable proton NMR spectra in the hydrogen-bonded region of deoxy and ligated Hb were measured at pH 7.0 with and without allosteric effectors at 15°C (Fig. 7) and 29°C. This was to determine whether the effector-induced shifts in the allosteric equilibrium toward T (R₉→T₀ and/or R₄→T₄, respectively) would alter the apparent T/R quaternary states of deoxy and oxy Hb. The pattern of the NMR spectra representing the quaternary structure-specific hydrogen-bonds (19-21) is not altered in the presence of various allosteric effectors. The quaternary structures were also probed by other methods, which are considered to indicate the quaternary structures of Hb in solution. Ultraviolet absorption fine structures showed larger troughs at 294 nm on the first derivative display (ΔA₂₉₄/Δλ) in R(ligated) states than in T (deoxy) states. They were not significantly affected by the addition of allosteric effectors (Table II). Circular dichroic spectra in the ultraviolet region (280 to 295 nm) showed deeper troughs at 293 nm (Θ) for T (deoxy) Hb than R (ligated) Hb. The trough of R (ligated) Hb was much less affected by the addition of allosteric effectors (Table II).

The SH groups of β93Cys of R (ligated) Hb are more solvent-exposed than those of T
(deoxy) Hb, so that the former react with SH reagents such as 4,4’-dithiopyridine approximately 10-times faster than the latter. The difference in their reactivity (log apparent k\text{on}) was hardly affected by the presence of allostERIC effectors (Table II). Therefore, it is qualitatively concluded that the T- and R-quaternary states of deoxy and ligated (oxy and carbonmonoxy) Hb, respectively, remain unaltered in the presence of various effectors, though K\text{T} and K\text{R} are reduced substantially in the presence of strong effectors (Table III). In other word, the conversion of deoxy Hb to oxy Hb is always accompanied, without exception, by the T→R quaternary transition under all the solution conditions we have tested.

_Tertiary Structure_ - Significant perturbations of the tertiary structure in the distal heme region of R (ligated) Hb was caused by the addition of hetrotropic effectors, as indicated changes in the proton NMR spectra in the ring-current region by hetrotropic effectors (Fig. 8). These changes represent the changes in geometry and mobility of the distal Val (E11) side chains (34).

**DISCUSSION**

_Oxygen-binding curves_ - Our comprehensive results of O\text{2}-binding curves of Hb are in full agreement with those reported previously (13), albeit they were limited in the scope and range, if differences in experimental conditions such as the type of buffers and measuring temperature are appropriately corrected. However, our O\text{2}-binding measurements are coupled with new structural data obtained under the same conditions. Thus, we have been able to deduce a new interpretation of the molecular mechanism of cooperativity and allostery of Hb. We show that “stripped” Hb is a surprisingly inert, moderately cooperative O\text{2}-carrier with a limited functional diversity, if hetrotropic
effectors are absent. Further, we show that Hb exhibits the amazing functional diversities in the O2-affinity, the cooperativity, and the Bohr effect only in the presence of heterotopic effectors. Such functional diversities are generated primarily by the tertiary structural constraints caused by the interactions of the effectors with Hb, especially by those with oxy Hb, rather than the T/R quaternary structural transition. We shall describe how we have arrived at this new interpretation, the “global allostery” model, that is starkly different from the current widely accepted view, particularly those of the T/R two-state concerted model of MWC/Perutz (1,3) and its extensions (35,36).

Hemoglobin in the absence of heterotopic allosteric effects - The Hill plots of Hb in the absence of heterotropic effectors at pH 7.8 ~ pH 9.0 are practically superimposable (Fig.1A). Thus, we consider that proton exerts no detectable allosteric effect on stripped Hb above pH 8.0. Thus, the property of stripped Hb above pH 8.0 is considered intrinsic to Hb itself with no heterotropic allosteric effect: $K_T = 0.34$ torr$^{-1}$, $K_R = 11$ torr$^{-1}$, $L_0 = 1.5 \times 10^3$, $n_{max} = 2.5$, $P_{50} = 0.55$ torr, $K_R/K_T \approx 31$, $\Delta H^+_{\text{average}} < -0.01$ H$/\text{heme}$, and $\Delta G_0 \approx 1.9$ kcal mol$^{-1}$. Stripped Hb is, thus, a high affinity, moderately cooperative O2-carrier with no alkaline Bohr effect. These features, particularly the intrinsic cooperativity, are generated by the homotropic O2-linked quaternary structural transition, involving the formation/dissolution of the critical salt bridges, as depicted by the stereochemical model of Perutz (3,35) and its extension by Szabo and Karplus (36). Our NMR examinations reveal that deoxy and oxy states of stripped Hb without allosteric effector at pH 9.0 exhibit exchangeable-proton NMR spectra of typical T and R quaternary structures, respectively (spectra not shown), which are indistinguishable from those at lower pH values in the presence of allosteric effectors (Fig. 7). It is, however, surprising to note
that its cooperative O₂-binding process is accompanied by no detectable proton release ($\Delta H^+_{\text{average}} < -0.01 \text{ H}^+/\text{heme}$) (16). Thus, one has to conclude that the T$\rightarrow$R quaternary-linked cooperativity and associated breakage of the critical salt bridges (3,35, 36) are not necessarily connected to the release of Bohr protons. Some of these features of stripped Hb were previously recognized. However, the present comprehensive comparison of Hill plots (Figs. 1 and 2) makes the relative functional inertness of stripped Hb in the absence of the effectors more explicit and quantitative.

**Heterotropic allosteric effects** - Below pH 7.8, the Hill plot of stripped Hb begins to shift to the right due to the allosteric effect of proton without noticeable changes in the cooperativity (as expressed by $K_R/K_T \approx 31$)(Fig. 1A). Approximately two-fold reduction in $K_T$ and $K_R$ and approximately two-fold increase in $L_0$ and $P_{50}$ are noted in the range of pH 7.8 to 6.6. A small alkaline Bohr effect is generated ($\Delta H^+_{\text{average}} = -0.23 \text{ H}^+/\text{heme}$) below pH 7.8, which may correspond to the so-called “Cl⁻-independent” alkaline Bohr effect (13).

All other heterotropic effectors examined shift the Hill plots of Hb to the right in the order of allosteric potencies. This tendency is increased as pH is decreased (Figs. 1 and 2). Increasingly downward shifts of the lower as well as the upper asymptotes accompany such right shifts. There is no difference in the apparent influence of allosteric effectors on the Hill plots between natural effectors like H⁺, Cl⁻, and DPG and unnatural effectors such as BZF, IHP, and combinations of BZF+DPG and BZF+IHP except for the difference in their allosteric potencies. The apparent allosteric effects on Hb of these effectors appear to be independent of their chemical structures, charges (positive or
negative), modes of interaction (electrostatic or hydrophobic), and the nature of their binding sites (α- or β-subunits) on Hb.

**MWC parameters under allosteric constraints** - Heterotropic effects have been traditionally interpreted primarily to shift the allosteric equilibrium toward T (R₀→T₀ and R₄→T₄) and, thus, to increase L₀ and L₄, respectively (1). However, the Hill plots of Hb in the presence and absence of DPG, where the DPG-induced right shift of the Hill plot is accompanied by a downward shift of the lower asymptote, could not be simultaneously simulated by Equation 2, unless both L₀ and K_T are varied (13,33,37). Thus, the role of a heterotropic effector was expanded from MWC’s original definition of the regulator of the allosteric equilibrium (1) to including a modulator of K_T (13,33,37) in the extended MWC/Perutz model (35,36,38).

When effectors shift the Hill plot of stripped Hb to the right, together with the concomitant downward shifts of both the upper and lower asymptotes (Figs 1 and 2), such Hill plots can not be simulated by varying L₀, or K_T, alone or both together. It was necessary to vary all the MWC parameters (L₀, K_T, and K_R) in order simultaneously to simulate all the Hill plots (Figs. 1, 2, and 4). The MWC parameters so obtained are listed in Figs. 3, 5, and 6 and Tables I and III.

Deoxy and oxy Hb always retain the T- and R-quaternary structures, respectively, without exception under all our solution conditions (Fig. 7 and Tables II and III). Thus, the T/R transition occurs always between deoxy and oxy Hb. Equation 3 is a mathematical expression of these structural data. It describes roughly that the T- and R-states are favored for deoxy and oxy Hb, respectively, and that the T/R quaternary transition takes place between deoxy and oxy Hb:
Although all the sets of the MWC parameters listed have been calculated with no restrictions attached, we observe that the restriction of Equation 3 is fully satisfied by all the $L_0$ and $L_4$ values we have obtained, as shown in Figs. 3, 5, and 6 and Tables I and III. In addition, the $K_T$ and $K_R$ values determined are in reasonable agreement, within the experimental error, with the $K_1$ and $K_4$ values of the Adair scheme, the model-independent O₂-affinities of Hb in the first and the last steps of the oxygenation process, respectively. (Table I). These facts give additional credence and reliability to our values of the MWC parameters.

Modulation of $K_R$ - Apparent binding of various heterotropic effectors such as Cl⁻ (39), DPG (40) and IHP (41) to ligated Hb as well as apparent reduction of the O₂ affinity of oxy Hb by some heterotropic effectors (13, 17, 32, 41-43) have been previously noted. In fact, Kister et al. (42, 43) proposed a reaction scheme of Hb, in which both $K_T$ and $K_R$ are modulated by heterotropic effectors. However, the degrees of observed reduction of $K_R$ (or $K_4$) by these heterotropic effectors were relatively small above pH 7.4. Further, no X-ray structure of a complex of ligated Hb with heterotropic effectors had been reported. Therefore, the reduction of $K_R$ by heterotropic effectors was not seriously integrated into the allosteric mechanism of Hb (3, 33, 35-38). This may have been in part inferred from the following consideration: [i] Since the inter-subunit space between the two β subunits narrows upon the T→R transition, effectors such as DPG and IHP cannot bind to R (oxy) Hb (3). [ii] In the absence of the critical salt bridges in the R structure, the O₂-affinity of R (oxy) Hb, or $K_R$, cannot be lowered (3, 35, 36, 38). [iii] As the critical salt bridges and hydrogen-bonds are broken in R (oxy) Hb (3, 35, 36, 38), each subunit in R (oxy) Hb
behaves like a high-affinity myoglobin (3). Thus, it was considered unlikely that heterotropic effectors can reduce the O₂-affinity of R (oxy) Hb, K_R. Instead, any apparent downward modulation of the upper asymptotes of Hill plots have been interpreted by assuming increasing L₀ values. Increasing L₀ values would gradually shift the T/R transition point toward higher values of pO₂. Thus, the transition point would eventually move from the normal position between deoxy and oxy Hb to those beyond oxy Hb. Then, oxy Hb would remain as T₄ due to the allosteric equilibrium shift (R₄ → T₄). Thus, the observed two-fold reduction in apparent K_R in oxy Hb with Cl⁻ between pH 7.0 and pH 6.5, for example, was interpreted due to a shift of the allosteric equilibrium toward T (R₄ → T₄) (44). The presence of >60% T₄ in oxy Hb at pH 6.5 was estimated under such a condition (44). Such an interpretation of the quaternary states of the ligated Hb was made without supporting structural proofs. In fact, it is incompatible with the above-mentioned restriction of Equation 3. Our data show that oxy Hb under the prescribed conditions is without doubt in the R quaternary state with L₄ = 0.003 (or >99.7% R₄) yet having substantially reduced K_R values (Figs 3, 5C, and 6 and Tables I and III).

Allosteric equilibrium and T/R quaternary structures - It should be noted that the allosteric effectors do shift the allosteric equilibrium toward T (R₀ → T₀ and R₄ → T₄). However, their effects on the actual quaternary states of Hb have been excessively overestimated. Allosteric equilibria of deoxy and oxy Hb are normally lopsidedly in favor of T₀ and R₄, namely, L₀ = 10³ ~ 10⁶ and L₄ ≈ 10⁻³, respectively. Therefore, even if the effectors shift the equilibria toward T as much as several orders of magnitudes, the actual quaternary states of deoxy and oxy Hb are hardly altered. For example, at the maximal allosteric constraints at pH 6.6 with Cl⁻+BZF+IHP, Hb has L₀ ≈ 15 and L₄ = 0.085 (Fig. 5C).
This means that deoxy and oxy Hb at our conditions of the maximal allosteric constraints are still >94% T₀ and >92% R₄ states, respectively, though their O₂-affinities are substantially reduced to Kₜ = 0.006 torr⁻¹ and Kᵣ = 0.02 torr⁻¹, respectively. These L-value-based assessments of the actual quaternary states of deoxy and oxy Hb are fully consistent with our structural data (Fig. 7 and Tables II and III). In other word, the shifts in the allosteric equilibria by the effectors have no practical consequence on the actual T and R quaternary states of two end products of the O₂-linked allosteric equilibrium, deoxy and oxy Hb, respectively. Of course, L values are important in defining the quaternary states of ligation intermediates (L₁, L₂, and L₃) and the T/R transition point (L = 1). We observed the T/R transition points (L = 1) to be between L₂ and L₃ under all the solution conditions tested. In other words, the T → R transition occurs between the second and third ligation steps under our conditions. Therefore, we must conclude that the observed varying degrees of reduction of Kₜ and Kᵣ values in the presence of allosteric effectors are due to the tertiary structural changes of Hb induced by its interactions with the effectors. It follows, therefore, that the observed downward shifts of the lower and upper asymptotes of Hill plots are the apparent indication of the binding of allosteric effectors to T (deoxy) and R (oxy) Hb, respectively, and reflect the associated reduction in the respective O₂-affinities, Kₜ and Kᵣ. Previously, Lee et al. (44) analyzed statistical-mechanically the O₂ equilibrium data of Hb in the presence of 0.1M Cl⁻ at pH 6.0 to 9.0 in order to decipher the contributions of the quaternary- and tertiary-linked structural changes to the cooperativity and the Bohr effect. Such a sophisticated method was necessary to obtain the answers under the conditions, where the “presumed” tertiary structural contributions toward the cooperativity would be relatively
small (approximately < 10%) in the absence of stronger effectors than Cl⁻ as well as in the absence of structural information. However, such a complex mathematical analysis has not been needed in the present study, since complete sets of MWC parameters and related structural information were available.

**MWC/Perutz model** - To recapitulate, the MWC/Perutz model (1,3) and its extension (35,36,38), assume that the cooperative feature of stripped Hb is regulated by the quaternary-linked formation/dissolution of critical salt bridges by Mode T/R in Fig. 9 (3,35,36,38). When the C-terminal His (β142) and Tyr (β141) residues which are involved in such salt bridges are removed by digestion with carboxypeptidase A (10), the resultant Hb (des-His-Tyr-Hb) becomes an essentially non-cooperative (Kₐ/K₉ ≈ 1), high affinity O₂-carrier, supporting such a hypothesis. Thus, the T (deoxy) and R (oxy) quaternary states have low and high O₂-affinities, respectively. Further, the T/R quaternary transition is primarily responsible for the cooperative behaviors of Hb. Heterotropic effectors are considered to act by shifting the allosteric equilibrium toward the T state (1) and providing tertiary perturbations on the T state to decrease K₉ by Mode T₁(3,33,35,38). The relative contributions of the quaternary-linked and the effector-linked structural changes to the overall cooperativity are > 80% and < 19%, respectively. Thus, such a hypothesis may be quite adequate in explaining the molecular mechanism of allostery of Hb under physiological conditions (pH ≥ 7.4), where natural allosteric effectors such as H⁺, Cl⁻, and DPG were considered to bind essentially to the T state, lowering K₉ by mode T₁ (Fig. 9). Under these conditions, as K₉ decreases at decreasing pH values, L₀ increases from L₀ ≈ 10³ up to L₀ = 1.7×10⁶, whereas L₄ and K₉ remain relatively constant. These behaviors of the MWC parameters result in a linear correlation
of \( \log L + 4 \log K = \text{constant of Imai (33)} \) in the "global allostery" plot (the log K versus log L plots), as shown by the dotted lines in Fig. 5A and B. Such results have been considered as a proof to validate the extended MWC/Perutz model (35,38).

"Global allostery" model - Diverse functional behaviors of Hb, as illustrated in Figs. 1 and 2, require a more global view than the MWC/Perutz model. The proposed mechanism of multifaceted modulation of the \( O_2 \)-affinity of Hb, the “global allostery” model, is illustrated in Fig. 9.

Below pH 7.4, both the lower and the upper asymptotes of the Hill plot of Hb in the presence of all the heterotropic effectors move downward at decreasing pH values (Figs. 1 and 2). This indicates increasing interactions of the effectors with both deoxy and oxy Hb, which are resulted in coordinated reduction of \( K_T \) and \( K_R \) by the Mode \( T_2 \) and Mode \( R \) mechanisms, respectively (Fig. 9). Through these effector-induced tertiary structural constraints (Modes \( T_1 + T_2 \) and Mode \( R \)), the intrinsic \( O_2 \)-affinities of stripped Hb are modulated down to \( K_T \approx K_R \approx 0.005 \text{ torr}^{-1} \). It has been traditionally assumed that the \( L_0 \) value would increase more and more in the presence of stronger effectors, as the effectors would shift the allosteric equilibrium more and more toward \( T \), as mentioned previously. However, this is not the case. The \( L_0 \) value reaches the maximum at \( 1.7 \times 10^6 \) in the presence of \( \text{Cl}^-+\text{DPG} \) at pH 7.4. Then, as soon as the effectors start to bind to \( R \) (oxy) Hb, the \( L_0 \) value begins to decrease as much as \( 10^5 \)-fold downward to \( L_0 =15 \) in the presence of a combination of \( \text{Cl}^-+\text{BZF}+\text{IHP} \) at pH 6.6 (Figs. 5C and 6, and Tables I and III). Consequently the \( L_4 \) value, which has been constant at \( \sim 10^{-3} \), rapidly increases as much as \( 10^2 \)-fold up to \( 10^{-1} \) in the presence of \( \text{Cl}^-+\text{BZF}+\text{IHP} \) at pH 6.6. These behaviors of the MWC parameters are reflected by the significant deviations from the linear
correlation of Imai (33) in the "global allostery" plot (the log K versus log L plots) (Figs. 5C, 5D, and 6). The plots show that the MWC parameters eventually converge at the point of $K_T \approx K_R \approx 0.005$ torr$^{-1}$ and $L_0 \approx L_4 \approx 1$ at an assumed “maximal” allosteric constraint (Figs. 5 and 6). We consider that the $O_2$-affinity of $K_T \approx K_R \approx 0.005$ torr$^{-1}$ or $P_{50} \approx 200$ torr to be the lowest possible limit of the $O_2$-affinity of normal Hb, the “low-affinity extreme” state (27). Deoxy Hb crystals exhibit such a low $O_2$-affinity of $P_{50} \approx 100 \sim 200$ torr even in the absence of heterotropic effectors (45). This was probably caused by structural constraints imposed by the crystal lattice forces.

The correlation of $K_R > K_T$, namely, R (oxy) Hb has a higher $O_2$-affinity than T (deoxy) Hb, is one of the fundamental tenets of the MWC/Perutz model (1,3). This correlation appears held up in view of the sigmoidal nature of the $O_2$-binding curves that we have obtained under all the solution conditions (Figs 1 and 2). However, strictly speaking, this correlation is applicable only to each one of the $O_2$-binding curves. Such a correlation of $K_R > K_T$ does not always hold from a global point of view. For example, $K_R$ values of Hb under strong allosteric constraints (say, Hb with $Cl^-+BZF+IHP$ at pH $< 7.4$) are smaller than $K_T$ values of stripped Hb (Fig. 3). Thus, the hypothesis that the quaternary structure determines and regulates the $O_2$-affinity (1) is no longer valid in absolute terms, though it may be applicable only to stripped Hb in the absence of heterotropic effectors or within a single $O_2$-binding curve at a fixed solution condition. Thus, we conclude that the effector-linked tertiary structural constraints are primarily responsible for all the observed modulations of intrinsic $K_T$ and $K_R$ of stripped Hb.

Therefore, the modulations of the $O_2$-affinity of Hb occur by several mechanisms. The large-amplitude (up to $\sim 65\sim 2,000$-fold, equivalent to $\Delta G_0 \approx 2.6$ and 4.5 kcal
mol\(^{-1}\), respectively) modulations of \(K_T\) and \(K_R\) via Modes \(T_1 + T_2\) and Mode \(R\), respectively, are brought about by the heterotropic effector-linked tertiary constraints. They are energetically more significant than the \(~31\)-fold (equivalent to \(\Delta G_0 \approx 1.9 \text{ kcal mol}^{-1}\)) modulation due to the homotropic effect of \(O_2\) through the T/R quaternary transition (via Mode \(T/R\)) of the MWC model (1). It has long been known that the binding of various heterotropic effectors modulates the \(O_2\)-affinity and cooperative function of Hb. The assumption has been that these all made perturbations on the essential cooperative behavior of the Hb molecule itself. It now appears that the reverse is actually true from a global point of view. Now Hb can be viewed much more like a classic enzyme, in which the fundamental and strongest interactions are heterotropic. The cooperative behavior of the Hb molecule exerts a small perturbation on that interaction.

The cooperativity, as expressed by \(K_R/K_T\), changes from of \(~31\) in stripped Hb to a maximal value of \(~230\) in Hb with \(Cl^-+DPG\) at pH 7.4 (the physiological condition of the blood). Then, it moves downward to a minimal value of \(~1\) in Hb with \(BZF+IHP\pm Cl^-\) at or below pH 6.0. Thus, it is evident that the T/R quaternary transition does not require a fixed amount of free energy of cooperativity, which was previously equated to the sum of free energies of four \(O_2\)-binding reactions. The T/R quaternary transition can readily occur in a wide range of free energies of cooperativity from \(~3.1\) to less than 0.7 kcal mol\(^{-1}\) under our conditions, so that the current concept of the energetics associated with the allostery in Hb may need serious reexamination.

The minimal Bohr effect (\(\Delta H^+_{\text{average}} < -0.01/\text{heme at pH} > 8.0\)) of stripped Hb is enhanced \(~20\)-fold by proton (\(\Delta H^+_{\text{average}} = -0.23/\text{heme at pH} 7.6\)), \(~50\)-fold by \(Cl^-+DPG\) (\(\Delta H^+_{\text{average}} = -0.6/\text{heme at pH} 7.5\)), and further \(>100\)-fold by \(Cl^-+BZF+IHP\) (\(\Delta H^+_{\text{average}} = -\))
1.1/heme at pH 7.8) (26). Thus, the intrinsic alkaline Bohr effect of stripped Hb amounts to only < 1% of the maximal Bohr effect we have observed in Hb. Several previous studies (46-48), that have been generally ignored, had reached similar conclusions that the alkaline Bohr effect is created by and linked to the interaction of Hb with heterotropic effectors such as Cl⁻ and DPG. Therefore, it is evident that these effector-linked modulations of the O₂-affinity, the cooperativity, and the Bohr effect are responsible for bringing the diverse functionality to the “inert” high-affinity, stripped Hb.

The overall O₂-affinity of Hb (P₅₀) is approximately proportional to the average of the free energies of structural constraints in the T (deoxy) and the R (oxy) states of Hb. Therefore the P₅₀ value is affected more strongly by the downward modulation of Kᵣ than that of Kₜ, since Kᵣ is modulated much more than Kₜ. The highly sensitive pH dependence of the O₂-binding curves exhibited by Hb with BZF+IHP±Cl⁻ (Figs. 1F, 2F, and 4) reminds us of the Root effect of some fish Hbs in terms of the exaggerated pH sensitivity of P₅₀ accompanied by decreasing cooperativity at acidic pH values (49). This behavior of Hb must be created primarily by the Mode R mechanism, by which the O₂-affinity of oxy Hb (Kᵣ) is reduced as much as three orders of magnitude upon acidification. Such a mechanism may allow the fish oxy Hb in the presence of ATP, its effector, to release O₂ into the swim bladder, even under extremely high hydrostatic partial pressure of O₂ at deep sea, in order to adjust the buoyancy by local acidification. It is amazing to observe that such an extreme behavior can be mimicked in human Hb through the heterotropic allostery of Mode R.

In our “global allostery” model (Fig. 9), homotropic and heterotropic interactions of Hb are integrally linked with each other, as envisioned by Wyman (50). In the high-
affinity region \((P_{50} = 0.5 \sim 5.5 \text{ torr})\), the homotropic interaction of Mode \(T/R\) appears predominant and heterotropic interaction of Mode \(T_1\) plays only a minor role, as described in the extended MWC/Perutz model. However, in the low-affinity region \((P_{50} = 5.5 \sim 200 \text{ torr})\), the heterotropic interactions of Modes \(T_1 + T_2\) and Mode \(R\) play increasingly more dominant roles in modulating the \(O_2\)-affinity. They eventually wipe out the T/R quaternary-linked cooperativity (Mode \(T/R\)) completely at the maximal allosteric constraint of \(P_{50} \approx 200 \text{ torr}\) (Figs. 4, 5, 6, and 9), where the difference in the free energies of allosteric constraints between the T (deoxy) and R (oxy) states becomes zero.

The present “global allostery” model is based upon the thorough characterization of the structure and function of deoxy and oxy states of Hb, two end products of \(O_2\)-binding equilibria. Therefore, we have not discussed the molecular mechanism of allostery in terms of ligation intermediates such as the two-state concerted model (1,3,35,36,38), the sequential model (51), or the molecular code model (52) of the homotropic cooperativity of stripped Hb. We consider that two quaternary states of Hb, T and R states, possess a continuum of the \(O_2\) affinities, being regulated by the effector-linked tertiary structural constraints within the respective quaternary states. The T and R quaternary states may be closely associated with and may be merely an expression of the ligation states, deoxy and oxy states, respectively. They play a critical role in creating the intrinsic cooperativity in stripped Hb and regulating the differential binding of heterotropic effectors to Hb in appropriate quaternary states depending upon the solution conditions. However, the actual house-keeping functions of modulating the \(O_2\)-affinities and consequently the overall \(O_2\)-affinity, the cooperativity, and the Bohr effect are carried out principally by the effector-linked tertiary structural constraints, where the major
portion of the free energy of allostery is stored. We have presented a semi-quantitative
global view of the molecular mechanism of multifaceted allostery of Hb, the “global
allostery” model, which stresses the critical role of heterotropic effectors in modulating
allosteric functions of Hb in hitherto unimaginable extents. The present work provides
only a framework for more quantitative description of the multifaceted allostery in Hb.

Structural information - We have no information regarding the structural nature
of the effector-linked tertiary constraints, because the available crystallographic data have
not been thus far analyzed to probe such tertiary structural changes. However, our recent
study on the 1.47Å-resolution x-ray structure of carbonmonoxy Hb, which was
crystallized in the presence of BZF and IHP in low-salt polyethylene glycol media at pH
6 (unpublished results), gives a hint of possible perspectives regarding the nature of the
effector-induced tertiary structural changes. This study indicates that carbonmonoxy Hb
in the presence of BZF and IHP is without doubt in the “R” quaternary state. Two
bezafibrate molecules per Hb tetramer are hydrophobically bound to the E-helices of the
α-subunits in a piggyback fashion on the surface of the Hb molecule, shifting the E-
helices toward the α-hemes. The distal His (α58) side-chains are shifted by 0.3Å closer
to the bound CO. The distal Val (α62) side-chains are likewise shifted. This is probably
the first demonstration of the direct stereochemical consequence of the mode by which
how an effector can influence the distal heme environment and consequently the affinity
of Hb for the bound ligands. Two bezafibrates were reported to bind to T (deoxy) Hb at
the central cavity surrounded by two α- and two β-subunits (53). They are no longer
present at the interior sites in the R (ligated) state, indicating that the effectors have
moved out of Hb upon the T→R quaternary transition and attached themselves to the
above-mentioned new positions on the surface of the R (ligated) Hb molecule. This amazing acrobatic behavior of BZF is consistent with our hypothesis that the effectors can bind not only to the T (deoxy) state but also to the R (oxy) state, differentially lowering $K_T$ as well as $K_R$. We would stress here that these differential reductions of $K_T$ and $K_R$ by the heterotropic effectors involve neither the T/R quaternary transitions (1,3), consequently, the formation/dissolution of the critical salt bridges (3,35,36,38,44), nor the ligation changes (1,3,51,52). At this point we should recall that Shulman et al. (19) stated, in their review article supporting the MWC two-state model (1), “Certainly the model would be shown to be inadequate if controlled experiments showed a similar change in affinities of two orders of magnitude without accompanying quaternary change.” We find ourselves precisely in such a situation here; in fact, under a $\sim 10$-fold more exaggerated condition.

Available NMR data corroborate these structural findings. The overall pattern of exchangeable-proton NMR spectra of the hydrogen-bonded region, which represent the quaternary states in solution, does not change upon addition of various effectors (Fig. 7). However, we have noted that one hydrogen-bond resonance at 12.2 ppm in deoxy Hb is noticeably downfield-shifted in the presence of BZF (Fig. 7). On the other hand, the same signal exhibit no shift in ligated (oxy and carbonmonoxy) Hb. We interpret these NMR observations as follows: Bezafibrates bind in the central cavity [His ($\alpha_{103}$), Arg ($\alpha_{141}$), Lys ($\alpha_{99}$), and Asn ($\beta_{108}$)] (53), adjacent to the hydrogen bond [His ($\alpha_{103}$)....Asn ($\beta_{108}$)] responsible for the 12.2 ppm resonance (21), influencing the chemical shift of the resonance in deoxy Hb. However, BZF binds at a different location removed from this particular hydrogen-bond in ligated Hb, so that its chemical shift is no longer affected by
BZF in ligated Hb. Ring-current-shifted NMR spectra of ligated Hb (Fig. 8) indicate that the distal Val-resonance signals are significantly affected by the presence of all the allosteric effectors tested. Similar heterotropic effects on the ring-current-shifted proton NMR spectra of ligated Hb by buffers and heterotropic effectors were previously reported (34). These NMR results are consistent with the above-mentioned x-ray structural data on the mode of interaction of BZF in ligated Hb as well as our proposal of the critical roles of heterotropic effectors in modulating the O₂-affinities of deoxy and oxy Hb.

General comments - Although those critical salt bridges which are linked to the T/R quaternary transition are fully broken in R (oxy) Hb (3,35,36,38), the inter-subunit communications within R (oxy) Hb appear fully operational. All the Hill plots we have examined exhibit smooth sigmoidal curves (Figs. 1 and 2). This indicates that the O₂-affinities of unlike subunits are equally modulated up to ~2,000-fold by heterotropic effectors, though some effectors bind exclusively to one type of subunits in R (oxy) Hb. Our O₂-binding measurements can readily detect subunit-heterogeneity of >5-fold from the O₂-binding curves. Within this limit of sensitivity of our technique, we do not find significant differences in the O₂-affinity between the α- and β-subunits in Hb.

The R (ligated) Hb having a very low-affinity has been unthinkable in the MWC/Perutz model (1,3) and its extension (35,36,38,44), because it assigns T- and R-conformers of Hb to low- and high-affinity states, respectively (1,3). Therefore, the thermodynamic and kinetic characteristics of a very low O₂-affinity, which were observed in ligated Hb in the presence of potent heterotropic effectors, were previously attributed to ligated Hb being allosterically shifted to T (R₄→T₄) (29,30,44). In the light of our finding that heterotropic effectors can downward modulate the O₂-affinity (Kᵣ) of
R (ligated) Hb, such assignments of the quaternary state of Hb based upon the observed low-affinity thermodynamic and kinetic behaviors must be reexamined. Since the O₂-affinity is no longer directly related to the T/R quaternary states, there is no basis to assign any low-affinity behaviors solely to the T state. Available kinetic data of low ligand-affinity characteristics of ligated Hb in the presence of potent heterotropic effectors (30, 31) indicates that kinetic techniques alone cannot distinguish low-affinity behaviors of T-state Hb from those of R-state Hb. Coletta et al. (31) integrated their kinetic, thermodynamic, and structural data and came to a conclusion that the observed low-affinity kinetic behaviors of ligated (oxy and carbonmonoxy) Hb in the presence of BZF+IHP at pH 7.0 are derived from the effector-induced low-affinity state of R (ligated) Hb. This is in full agreement with our present interpretation and is a direct contrast to the solely kinetics-based interpretation of Marden et al. (30).

Since our present study has been based upon the MWC model (Equation 2), our results are still consistent with the basic tenet of the MWC model of the equal-energy separation of ligation intermediates (1), as a first approximation. However, the O₂-binding process of Hb is no longer considered as a simple equilibrium of the four-step reversible, successive oxygenation process, even under physiological conditions. We have to realize that it is tightly coupled to reversible dissociation of heterotropic effectors from the T (deoxy) state, followed by reversible differential binding of the effectors to the R (oxy) state. It is, therefore, difficult to imagine that such complex multi-faceted processes can take place in unison in accordance with the MWC concept of the equal energy ligation. The well-known fact that the amounts of protons released from Hb at successive steps of oxygenation are not equal (14, 16,28) is one of the cases to the point,
though it has not been thus far addressed to integrate it into the molecular mechanism of Hb.

Monod et al. (1) formulated the MWC two-state concerted model of Hb on the basis of limited O$_2$-equilibrium data available at that time (1965). It is, therefore, not surprising that the molecular mechanism of allostery of Hb is now being modified and expanded to the present “global allostery” model. This new model, which incorporates both ligation-linked quaternary and effector-linked tertiary structural changes, is formulated primarily on the basis of the detailed quantitative analyses of more extensive oxygenation data that were obtained by the same old-fashioned technique of O$_2$-equilibrium measurements. There are notions, which are rather unique in the field of Hb research, that the extended MWC/Perutz model (1,3,35,38) is sufficient enough to quantitatively explain the behaviors of Hb under physiological conditions. Further, any other findings that are obtained using synthetic effectors and/or beyond those conditions and that do not conform to the MWC/Perutz model, are un-physiological and thus can be ignored. These notions are quite contrary to the well-established convention in our strategy of the elucidation of enzyme mechanisms. We normally elucidate the molecular mechanism of an enzyme by examining the behaviors of the enzyme in the widest possible range of experimental conditions using not only natural but also synthetic substrates, activators and inhibitors, regardless of the physiological conditions where it functions. We consider that the MWC/Perutz model and its extension are merely an abbreviated version of the “global allostery” model to approximate behaviors of Hb under normal physiological conditions. It has been reported that during the high-altitude adaptation, the intra-erythrocyte concentration of DPG increases by ~50%, accompanied
by substantial increases in $P_{50}$ from 26.6 torr to 31 torr (54). Hemoglobin is nearly saturated with DPG inside of the erythrocytes at the sea level. Therefore, any increases in the intra-erythrocyte concentration of DPG beyond the sea level would decrease the $K_T$ value only slightly with minimally affecting the $P_{50}$ value, according to the MWC/Perutz model. The observed significant increases in $P_{50}$ can be explained only by the following assumption: At increased concentrations of DPG, this effector begins to bind to R (oxy) Hb to lower the $K_R$ value significantly even at the physiological pH, resulted in substantial increases in $P_{50}$. Thus, the binding of effectors to R (oxy) Hb, which has been ignored in the MWC/Perutz model, becomes significant in explaining a physiological phenomenon. Likewise, the proposed mechanism of the Root effect mentioned previously is another example of the utility of the “global allostery” model logically to explain an important physiological function of Hb.

In summary, we have demonstrated that the O$_2$-linked T/R quaternary transition provides merely the intrinsic cooperativity to stripped Hb in the absence of heterotropic effectors. On the other hand, the O$_2$-affinity and the cooperativity of Hb are modulated and the Bohr effect of Hb is generated primarily by the heterotropic effector-induced tertiary structural changes, as depicted by the “global allostery” model (Fig. 9).

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FOOTNOTES

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Abbreviations used are: Hb, human adult hemoglobin; des-His-Tyr-Hb, Hb from which terminal His and Tyr are removed by caboxypeptidase A-treatment; MWC, Monod-Wyman-Changeux; BZF, bezafibrate; DPG, 2,3-diphosphoglycerate (or 2,3-biphosphoglycerate); IHP, inositol hexaphosphate.
FIGURE LEGENDS

**Fig. 1.** Hill plots of O₂-binding equilibria of Hb in the absence of Cl⁻ at different pH values with and without heterotropic effectors (DPG, BZF, IHP, BZF+DPG, and BZF+IHP) at 15°C. The indicated experimental points represent only one thirds of the 90 experimental points in each of the O₂-binding experiments. The isotherms were measured at pH 6.6 (●), 7.0 (○), 7.4 (▲), 7.8 (△), 8.2 (●), 8.6 (◇), and 9.0 (■). Upper and lower broken lines represent approximate upper and lower limits of the O₂-affinity (P₅₀ = 0.1 torr and P₅₀ = 100 torr, respectively). Panel A: stripped Hb, Panel B: +DPG, Panel C: +BZF, Panel D: +IHP, Panel E: +BZF+DPG, and, and Panel F: +BZF+IHP.

**Fig. 2.** Hill plots of O₂-binding equilibria of Hb in the presence of 0.1M Cl⁻ at different pH values with and without heterotropic effectors. Explanations of symbols and Panels are the same as Fig. 1.

**Fig. 3.** MWC (K₇, K₉, L₀, and L₄), and related (ΔG₀, P₅₀, and n₉₉₉) parameters of the O₂-binding equilibria of Hb as functions of pH and heterotropic effectors. The distance between K₇ (■) and K₉ (○), which is indicated by a horizontal double-headed arrow between them, is proportional to the degree of cooperativity (K₉/K₇ and/or ΔG₀). The range of O₂-affinity is qualitatively color-coded: Red, blue, and yellow for high affinity, low affinity, and low affinity extreme, respectively.

**Fig. 4.** Simulation of Hill plots of O₂-binding equilibria of Hb using MWC and Adair parameters of Table I. Symbols indicate experimental points (only the 50% of the 90 data points in each experiment are shown), which were obtained in 0.1M HEPES buffer, pH 6.6-9.0 in the presence of Cl⁻, BZF, and IHP at 15°C (Fig. 2F). Under the conditions, large (~500-fold) pH-dependent modulations of K₉ were observed. Real and broken
curves represent simulated Hill plots based upon numerical values of the MWC and Adair models, respectively (cf. Table I).

**Fig. 5.** The global allosteric plots (the log K versus log L plots) of MWC parameters of O₂-binding equilibria of Hb. The numerical mark of each data point indicates the measuring pH (1, 2, 3, 4, 5, 6, and 7 refer to pH 6.6, 7.0, 7.4, 7.8, 8.2, 8.6, and 9.0, respectively). Panel A: with (■) and without Cl⁻ (○), Panel B: with Cl⁻+DPG (■) and Cl⁻+BZF (○); Panel C, with Cl⁻+IHP (○) and Cl⁻+IHP+BZF (■), and Panel D: with IHP+BZF.

**Fig. 6.** Summary of the global allosteric plots (the log K versus log L plots) of MWC parameters of Hb (circles) and des-His-Tyr-Hb (squares) at pH 6.6 to 9.0. The effectors used are color-coded. The data points shift downward continuously as pH is decreased from pH 9.0 to pH 6.6 (See examples in Fig. 5).

**Fig. 7.** Effects of allosteric effectors on exchangeable-proton NMR spectra in the hydrogen-bonded region of deoxy Hb, oxy Hb, and carbonmonoxy Hb at pH 7.0 and 15°C. (a) no effector, (b) +BZF, (c) +IHP, and (d) +BZF+IHP. Neither T (deoxy)- nor R (ligated)-quaternary states of Hb are affected by the presence of heterotropic effectors. It should be noted that oxy Hb with BZF+IHP is ~90% O₂-saturated even under the conditions of pO₂ = 760 torr. However, no significant T-state signals were observed, because the ~90% O₂-saturated Hb in the low-affinity, nearly non-cooperative conditions is statistically essentially a mixture of tetra- and tri-oxygenated states, both of which are in the R quaternary state. Alternatively, a small band observed at –14 ppm in oxy Hb with BZF+IHP [(d) in the middle panel] could be an indication of the presence of small amounts (<5%) of the T (deoxy) Hb.
Fig. 8. Effects of allosteric effectors on ring-current-shifted proton NMR spectra of oxy and carbonmonoxy Hb at pH 7.0 and at 15°C. (a) no effector, (b) +BZF, (c) +IHP, and (d) +BZF+IHP.

Fig. 9. Comparison of the “MWC”, “Extended MWC/Perutz” and “Global allostery” models of the allosteric mechanism of Hb. Mode T/R represents the ~31-fold reduction of the O₂ affinity of deoxy Hb (Kₜ). This is caused by the structural constraints in the T state, induced by the homotropic, ligation-linked quaternary structural changes. Modes T₁+T₂ and Mode R are the ~65- and ~2,000-fold reduction of Kₜ and Kₐ, due to the tertiary structural constraints in T and R states, respectively. These structural constraints are induced by the interactions of heterotropic effectors within the T and R states, respectively. The slopes of double-headed arrows are proportional to the degrees of cooperativity (Kₐ/Kₜ). The deoxy state of des-His-Tyr-Hb is considered in a R-quaternary state. The range of O₂-affinity is qualitatively color-coded, as in Fig. 3.
Table I. Comparison of MWC and Adair parameters of hemoglobin in O₂-binding equilibria.a.

<table>
<thead>
<tr>
<th>pH</th>
<th>K_T</th>
<th>K_R</th>
<th>L_0</th>
<th>K_1</th>
<th>K_2</th>
<th>K_3</th>
<th>K_4</th>
<th>ΔH°_average</th>
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<tr>
<td>9.0</td>
<td>5.5E-2</td>
<td>9.0E+0</td>
<td>5.2E+5</td>
<td>4.77E-2</td>
<td>4.97E-2</td>
<td>5.56E-1</td>
<td>9.44E+0</td>
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<tr>
<td>8.6</td>
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<td>5.3E+0</td>
<td>2.6E+5</td>
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<td>3.39E-2</td>
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<tr>
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<td>1.6E+3</td>
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</tr>
<tr>
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<td>3.0E-2</td>
<td>3.3E+1</td>
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<td>1.05E-2</td>
<td>1.15E-2</td>
<td>2.33E-2</td>
<td>-0.3</td>
</tr>
<tr>
<td>6.6</td>
<td>6.0E-3</td>
<td>2.0E-2</td>
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<td>1.15E-2</td>
<td>1.79E-2</td>
<td>-0.1</td>
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a: Oxygen-binding equilibria were measured in 0.1M HEPES buffer, pH 6.6-9.0 in the presence of 0.1M Cl⁻, 10mM BZF, and 2mM IHP at 15°C.
Table II. Parameters for the quaternary state indicators of hemoglobin in solution $^a$.

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<thead>
<tr>
<th>Ligation State</th>
<th>pH</th>
<th>deoxy</th>
<th>oxy</th>
<th>CO</th>
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<td>Technique</td>
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<td>stripped + effectors</td>
<td>stripped + effectors</td>
<td>stripped + effectors</td>
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<tr>
<td>UV-Fine Structure$^b$</td>
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<td>β93Cysteine-Sulfhydryl</td>
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<td>-3.09</td>
<td>-3.25</td>
<td>-2.13</td>
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<tr>
<td>Sulphhydryl</td>
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<td>-3.66</td>
<td>-2.31</td>
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<tr>
<td>Reactivity$^d$</td>
<td>6.6</td>
<td>-3.34</td>
<td>-3.72</td>
<td>-2.38</td>
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</tbody>
</table>

$^a$: Measurements were made in 0.1 M HEPES buffers at 15°C. The effectors added were 10 mM BZF and 2 mM IHP.

$^b$: The ultraviolet absorption fine structure at 294 nm: The value, $\Delta A_{294}/\Delta \lambda, \times 10^{-2}$ nm$^{-1}$), corresponds to the larger negative trough around 294 nm on the first derivative of the absorption spectra of Hb derivatives with respect to wavelength ($\lambda$) using [Hb] = 2 mM.

$^c$: The ultraviolet circular dichroic molar ellipticity ($\Theta, \times 10^6$ deg.cm$^2$/dmol) at 293 nm using [Hb] = 500 µM.

$^d$: The reactivity of β93Cys-SH groups toward 4,4'-dithiopyridine (log k$_{on}$ [sec$^{-1}$]) at [Hb]=40 µM or [β93Cys-SH]=20µM and [4,4'-dithiopyridine] = 160 µM.
Table III. Correlation between the MWC parameters and the quaternary states of hemoglobin in solution.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Effector</th>
<th>MWC Parameters</th>
<th>Analytical Methods</th>
<th>To Probe UV Fine</th>
<th>Quatern. States β93 Cys SH reactivity</th>
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<td>$L_0=1.4E+5$</td>
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<td>$L_0=2.4E+5$</td>
<td>T</td>
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<tr>
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<td>Cl$^+$IHP</td>
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<td>$L_0=2.0E+4$</td>
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<tr>
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<td>Cl$^+$BZF+IHP</td>
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<tr>
<td></td>
<td>Cl$^+$BZF+IHP</td>
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<td>Cl$^+$BZF+IHP</td>
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<td>$L_4=1.2E-1$</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

$^a$: Measured at both 15°C and 29°C.
Fig. 1
Fig. 2
Fig. 3

( and ): T and R-quaternary states, respectively, as determined by hydrogen-bonded proton NMR.
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Extended model

Global model

51 MWC

"model

Removal of C-terminal constraint by carboxypeptidase A digestion

Global allostery model

Extended MWC/Perutz model

Log K (torr⁻¹)

pH 9.0:

pH 7.4:

pH 6.6:

pH < 6.0:

pH 7.4: +Cl⁻, IHP

pH 6.6: +Cl⁻, DPG

pH 7.4: +Cl⁻, BZF, IHP

pH 6.6: +Cl⁻, BZF, IHP

Fig. 9
Global allostery model of hemoglobin: Modulation of O2-affinity, cooperativity, and Bohr effect by heterotropic allosteric effectors
Takashi Yonetani, SungIck Park, Antonio Tsuneshige, Kiyohiro Imai and Kenji Kanaori

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