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Description of hemoglobin oxygenation under universal solution conditions by a global allostery model with a single adjustable parameter

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Abstract

The Monod–Wyman–Changeux allosteric model parameters evaluated from accurate oxygen equilibrium curves (OECs) of hemoglobin that were measured in an extremely wide range of structural constraints, imposed by allosteric effectors, yielded a closed circle when log K_T and log K_R were plotted against log L_0 and log L_4, respectively, showing novel phenomena that L_0 and L_4 have a maximal value and a minimal value, respectively, and K_T and K_R vary by more than three orders of magnitude. These phenomena were successfully described by a global allostery model, which mathematically keeps the frame work of the MWC model, but allows that K_T under a set of solution conditions becomes larger than K_R under another set of solution conditions and postulates that a representative allosteric effector binds to both the T and R states with a lower affinity but with a larger stoichiometry for the R state than for the T state. Thus, this global model can describe any given OEC measured under universal solution conditions with the single adjustable parameter, the concentration of the representative effector. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Monod–Wyman–Changeux allosteric model; Global allostery model; Hemoglobin oxygenation; Universal solution condition; Structural constraint; Allostery plot

1. Introduction

As an honorary enzyme, hemoglobin has been regarded as the model allosteric protein and its ligand binding properties have extensively been studied for many years [1–6]. The molecular mechanism of the allostery in ligand binding was given structural bases from X-ray crystallographic, nuclear magnetic resonance and other techniques [7–9]. Whereas many people thought that the physiological functions of both hemoglobin and myoglobin had already been established, new aspects and proposals of their functions were

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presented recently [10–22]. Thus, hemoglobin, as well as myoglobin, is the old and new subject of study, still attracting much interest by biochemists and biophysicists.

The Monod–Wyman–Changeux allosteric model that was proposed to explain comprehensive allosteric regulation by enzymes [23], as applied to the vertebrate tetrameric hemoglobin in its simplest form, postulates that (1) the four subunits occupy equivalent positions, so that the hemoglobin molecule can have a tetrad axis of symmetry, (2) the four oxygen binding sites (the heme groups) have the same symmetry as that of the molecule, (3) association of the subunits imposes constraints on the conformation of each subunit, (4) the molecule is in an equilibrium (allosteric equilibrium) between two alternative quaternary structures, which differ by distribution and energy of intersubunit bonds and consequently by oxygen affinity for the heme, (5) when the molecule goes from one state to another (allosteric transition), its molecular symmetry is conserved. Of the two quaternary structures, the one with stronger constraints is called the 'T state' (tense state) while the other with weaker (or non-) constraints is called the 'R state' (relaxed state). The oxygen association equilibrium constant for the T state (K_T) is lower than that for the R state (K_R). From the postulations stated above, it is deduced that the oxygen affinity solely depends on the quaternary state, but not on the number of the oxygen molecules bound, that is, the free energy of oxygen binding to each state is a linear function of the number of bound oxygen molecules (the 'linear energy binding' assumption). It is implicitly assumed that the oxygen binding cooperativity (the homotropic effect) arises from the T-R transition and oxygenation of neither the T nor the R state is cooperative. This model further postulates that the heterotropic effects, such as the Bohr effect and the effects of Cl^-, 2,3-diphosphoglycerate (DPG) and inositol hexaphosphate (IHP) are ascribed to shifts of the allosteric equilibrium that arise from preferential binding of these heterotropic ligands (effectors). This implies that neither K_T nor K_R is influenced by their binding. The oxygen equilibrium curve (OEC) of hemoglobin is described by the following equation:

$$Y = \frac{L_0 K_T p(1+K_T p)^3 + K_R p(1+K_R p)^3}{L_0(1+K_T p)^4 + (1+K_R p)^4} \quad (1)$$

Here, Y is the fractional oxygen saturation, p is the partial pressure of oxygen and L_0 is the allosteric constant, i.e. the ratio of the population of the T state to that of the R state in the total absence of oxygen ($L_0 = [T_0]/[R_0]$). Let us consider a simple case where an allosteric effector exclusively binds to the T state (allosteric inhibitor, I) with a stoichiometry of j per hemoglobin tetramer and another allosteric effector exclusively binds to the R state (allosteric activator, A) with a stoichiometry of k per hemoglobin tetramer. Then, the effective allosteric constant in the presence of these effectors is given by

$$L_0 = \frac{(1+K_I[I])^j}{(1+K_A[A])^k} L_{0,0} \quad (2)$$

where $L_{0,0}$ is L_0 in the total absence of I and A, and K_I and K_A are the binding constant of I and A, respectively. Here, it is assumed that the binding of I and A is statistic.

The MWC model was given a structural basis by the Perutz stereochemical mechanism [7], which was mathematically expressed by Szabo and Karplus [24]. Nowadays, the integrated treatment of these three is sometimes called the 'MWC-PSK model' [25,26].

By means of high precision measurement and analysis of the OECs for native and chemically modified hemoglobins, Imai [27] tested the applicability of the MWC model. He found that not only L_0 but also K_T was significantly influenced by DPG, and he insisted that the MWC model in its original form cannot describe the heterotropic effect by DPG and it needs modifications. Similar conclusions were reached by other investigators, who found that protons, DPG, ATP and IHP affected not only L_0 but also K_T [28–30]. Actually, an individual OEC measured under any given solution conditions can be fitted by the MWC equation [Eq. (1)], but any set of OECs measured under different solution conditions cannot be fitted simultaneously by adjusting L_0 only [3,31]. The multiplicity of the deoxy affinity state was also suggested by Colombo and Seixas [32].

This apparent discrepancy let Minton and Imai [33] propose an extension of the MWC model, a three-state model as the minimal description of the homotropic and heterotropic effects of hemoglobin. In this model, a third state (S state) was introduced to describe the effects of proton, chloride ion and DPG. It was stressed that their heterotropic effects were not regarded as second-order perturbations of the T state and any 'effective' two-state picture was meaningless. This extension implied that one more state was necessary to describe the effect of IHP, which lowered K_R significantly and, in a strict sense, the same number of affinity states as that of added allosteric effectors were needed.

Imai [31,34] showed that, in the framework of the original two-state MWC model, it describes a set of OECs measured under arbitrary solution conditions, with only one adjustable parameter, K_T or L_0 (these two parameters are not independent of each other). This approach was based on his observation that, as long as the conformational constraints were not extremely strong, (i.e. except for the conditions where IHP was present at acidic pHs), both K_R and L_4 were nearly constant [3]. Here, $L_4=[T_4]/[R_4]=(K_T/K_R)^4L_0$. When log K_T and log K_R were plotted against log L_0 and log L_4, respectively (we will call this log K vs. log L plot 'allostery plot' hereafter), all the data points except for the points for pH 6.5 with IHP lay on a straight line. On this plot, the paired two data points, [log K_T, log L_0] and [log K_R, log L_4], corresponding to each OEC were always related by the equation,

$$\log L_0+4\log K_T=\log L_4+\log K_R = \text{constant} \quad (3)$$

which was derived from the law of energy conservation.

Here, the constant value was -1.6, -1.2, -0.4 and -0.7 for human, rabbit, horse and bovine hemoglobins, respectively [34].

It was known that IHP, different from other effectors, actually reduced not only K_T but also K_R [3,35]. At low salt concentrations, even DPG reduced K_R as well as K_T [36]. This superior effect of IHP or the extra effect of DPG was not given much interest, and it was concluded that heterotropic ligands such as protons, CO_2, Cl^- and DPG made major contributions to cooperativity of hemoglobin by reducing K_T with K_R kept nearly constant [3,37]. This conclusion is valid at least under physiological conditions.

Recently, Yonetani et al. (Yonetani et al., manuscript in preparation) explored the oxygenation properties of hemoglobin under extremely wide varieties of solution conditions from pH 10.6 with no added allosteric effectors to pH 6.6 with 2 mM IHP plus 10 mM BZF (bazafibrate). The combination of IHP and BZF was advantageous to impose strong constraints on the hemoglobin molecule because they bind to different subunits of hemoglobin [38] and their effect is additive [39], as is true for the combination of DPG and BZF [40]. They further added the oxygenation data for isolated β chains and carboxypeptidase-treated hemoglobin (des-His-Tyr Hb) [41,42] as a model for completely constraint-free hemoglobin (Yonetani et al., manuscript in preparation). The most novel phenomenon was that the K_R value measured under extremely strong constraints became even lower than the K_T value measured under moderate physiological conditions, whereas separate experiments indicated that the T-R transition upon oxygenation actually occurred under both conditions. They concluded that (a) hemoglobin in total absence of allosteric effectors is physiologically inert, (b) the allosteric effectors that bind to both the T and R states are primary responsible for the regulation of oxygen affinity and cooperativity, which are relevant to physiological function of hemoglobin and (c) the heterotropic effector-linked tertiary structural changes, rather than the homotropic ligation-linked T-R quaternary structural transition, are primarily responsible for modulation of hemoglobin functions. The importance of effector binding to the R state was demonstrated by careful experiments using Cys93β sulfhydryl reactivity and UV circular dichoroism as structural probes (see Tsuneshige et al., in this issue).

In the present study we explored how far the MWC model can explain the novel behavior of hemoglobin under extremely wide variety of solution conditions (virtually under universal solution conditions). Here, this model was given more global scope to allow situations such that heterotropic effectors affect not only K_T but also K_R and

the K_T value under a set of solution conditions becomes larger than the K_R value under another set of solution conditions. We found that all the OECs measured under universal solution conditions can be described simultaneously by this 'global allostery model' with a single adjustable parameter.

2. Methods

Accurate OECs were measured by an improved version of the automatic polaro-spectrophotometric method [43–45] for hemoglobin samples dissolved in a Good buffer, 0.1 M HEPES (pH 6.6–9.0) or 0.1 M CAPSO (pH 10.6) at 15 °C. The temperature used was lower than 25 °C, which was used by many investigators, to attain high oxygen saturation at low pH values in the presence of strong allosteric effectors such as IHP and BZF (see below). The concentration of hemoglobin was 60 μM on a heme basis. Other experimental details will be fully described in a separate paper (Yonetani et al., manuscript in preparation).

It was pointed out that oxy hemoglobin in dilute solutions is dissociated in part into αβ dimers and this dissociation can exert influences on oxygen equilibrium parameters. One must work at high protein concentrations to avoid the effects of partial dissociation, or alternatively, one must incorporate the effects of the dimeric species into the analysis of OECs [46,47]. The Adair constants determined for hemoglobin solutions of 60 μM or higher concentrations were comparable to those predicted for tetrameric hemoglobin as far as DPG or IHP was present, whereas the K_1 value deviated somewhat from the true value in their absence [44]. The oxygen equilibrium parameters determined in the presence of DPG, IHP and/or BZF at neutral and acidic pHs are probably equal or close to the true values, since these effectors stabilize the hemoglobin tetramer by imposing strong structural constraints. In the absence of these effectors and at high pHs, where the constraints are weak, the dissociation into αβ dimers will become more marked and the oxygen equilibrium parameters can be significantly different from the true values. However, this apprehension is probably made moderate by the mechanism that, under these solution conditions, the allosteric equilibrium of hemoglobin is greatly biased to the R state and the partial dissociation into dimers during oxygen binding will cause no more significant changes in the oxygen equilibrium parameters. We obtained firm data for constraint-free conditions by using the isolated β chains (see below) to cover the inaccuracy in parameter values under weak constraints. Thus, the present oxygenation data are considered to express the essential features of tetrameric hemoglobin.

The OEC data used for analysis in the present study were: (a) those for normal human hemoglobin (Hb A) at eight different pHs (from 6.6 to 9.0 with 0.4 increment and 10.6) in the presence of six combinations of 0.1 M Cl^-, 2 mM DPG, 2 mM IHP and 10 mM BZF, i.e. in the presence of Cl^-, Cl^- + DPG, Cl^- + IHP, Cl^- + BZF, Cl^- + DPG + BZF or Cl^- + IHP + BZF, and in their total absence [56(=8×[6+1]) solution conditions as total]; (b) those for des-His-Tyr Hb under the same conditions as Hb A, except that the pH was 7.4 only; (c) those for isolated β chains at pH 7.4 with and without Cl^-.

Best-fit parameter values were obtained by fitting Eq. (1) or the equations derived in the extension of the MWC model (described later) to the experimental OECs by using a non-linear least-squares curve-fitting program of 'ORIGIN' (OriginLab Corp., ver. 6.1) which ran on a Windows computer. The curve-fitting was done on the Hill plot [log $(Y/[1-Y])$ vs. log p] so that the jth observed point was weighted by w_j according to the function,

$$w_j = N/[Y_j(1-Y_j)]$$

where Y_j was the observed fractional oxygen saturation for the jth point and N was a normalizing factor [3,48]. The present curve-fitting analysis fulfills the criterions described previously [49].

Graphic analysis was performed using 'Grapher' (Golden Software Inc., ver. 2).

3. Results

3.1. Allostery plot (Log K vs. log L plot)

The allostery plot using the best-fit values of

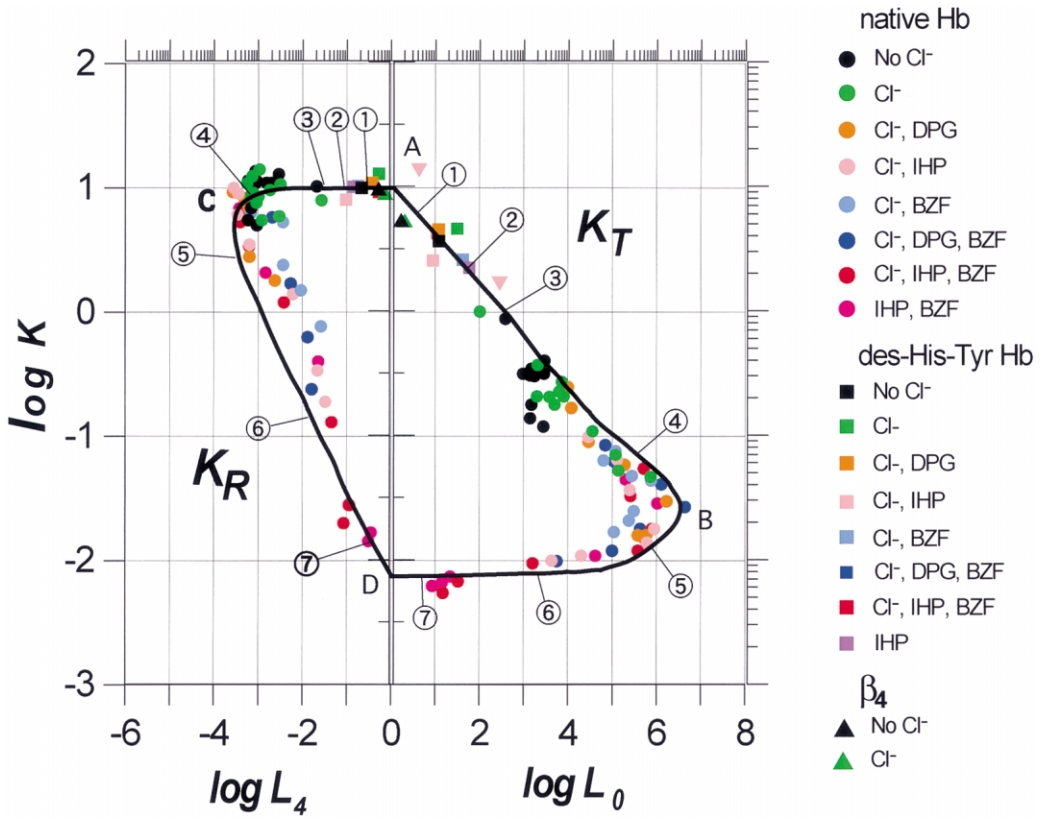

Fig. 1. The allostery plot (log K vs. log L plot) for the MWC parameters evaluated in extremely wide range of structural constraints imposed by allosteric effectors. Log K_T and log K_R are plotted against log L_0 and log L_4, respectively. Each OEC gives paired two points, [log K_T, log L_0] and [log K_R, log L_4], which lie on a straight line with a slope of $-1/4$ (see text). For native Hb (Hb A), each symbol stands for different pH values from 6.6 to 10.6 with 0.4 increment in the presence of any combination of Cl^-, 2 mM DPG, 2 mM IHP and 10 mM BZF or in total absence of them. For des-His-Tyr Hb and β chain tetramer, pH was 7.4. Temperature was 15 °C in all cases. The experimentally obtained closed circle was simulated by the present global allostery model, as shown by the continuous closed circle. The numbers attached to the continuous circle correspond to the data set numbers in Table 1.

the MWC parameters yielded a closed circle (Fig. 1). Note that each set of solution conditions gives the paired two data points that are related by Eq. (3) and lie on a straight line with a slope of $-1/4$ [31]. From this plot the following picture emerges.

3.1.1. Phase 1 (*point A*)

When conformational constraints are absent or extremely weak (in the cases of an isolated β chain and of des-His-Tyr Hb without added effectors), both K_T and K_R are nearly equal to 10 $torr^{-1}$ and both L_0 and L_4 are ≈ 1.

3.1.2. Phase 2 (*segments A–B and A–C*)

As the constraints are strengthened by lowering the pH and/or adding allosteric effector(s), K_T becomes smaller, L_0 larger, and L_4 smaller, while K_R remains constant at approximately 10 $torr^{-1}$. The paired two points, [log K_T, log L_0] and [log K_R, log L_4] for each OEC move off the point [log $K=1$, log $L=0$] and get apart more and more along the right upper half of the closed circle (A to B and A to C). In this phase, K_T is lowered by approximately 500-fold. The maximal value of L_0 and minimal value of L_4, are $\approx 10^6$ and $\approx 10^{-4}$, respectively.

3.1.3. Phase 3 (*segments B–D and C–D*)

As the constraints become much stronger, especially by adding DPG, IHP and/or BZF in combination at acidic pH values, K_T still becomes a little smaller, L_0 smaller, L_4 larger and K_R decreases by approximately 3 orders of magnitude.

3.1.4. Phase 4 (*point D*)

Under the extremely constrained conditions (in the presence of Cl^-, IHP and BZF at the lowest pH), the data points tend to approach the point [log $K=-2.2$, log $L=0$].

From these behaviors of the MWC parameters, we can summarize the following features of the allosteric properties of hemoglobin.

1. Not only K_T but also K_R is lowered by allosteric effectors.
2. As the constraints become stronger, L_0 does not increase infinitely but becomes smaller beyond some degree of constraints, yielding a maximum.
3. As the constraints become stronger, L_4 does not decrease infinitely but becomes larger beyond some degree of constraints, yielding a minimum.
4. Under extreme constraint-free conditions, the oxygen affinity for the T and R states are equal and the highest, and virtually these two states are indistinguishable ($L_0 \approx 1$, $L_4 \approx 1$).
5. Under the extremely constrained conditions, the oxygen affinity for the T and R states are again equal and the lowest, and virtually these two states are indistinguishable ($L_0 \approx 1$, $L_4 \approx 1$).

It is well established that proton, Cl^-, DPG, IHP and BZF bind preferentially to the T state and act as allosteric inhibitors. Then, Eq. (2) indicates that the addition of or the increase in the concentration of these effectors solely cause monotonic increases in L_0 value, contrary to the observation stated above. The concomitant reduction of K_R and increase in L_4 with strengthening of constraints cannot be explained by the preferential binding of the effectors to the T state.

3.2. Extension of the MWC model: formulation of the global allostery model

The discrepancies stated above let us consider the following extensions of the original model. We will preserve the following assumptions.

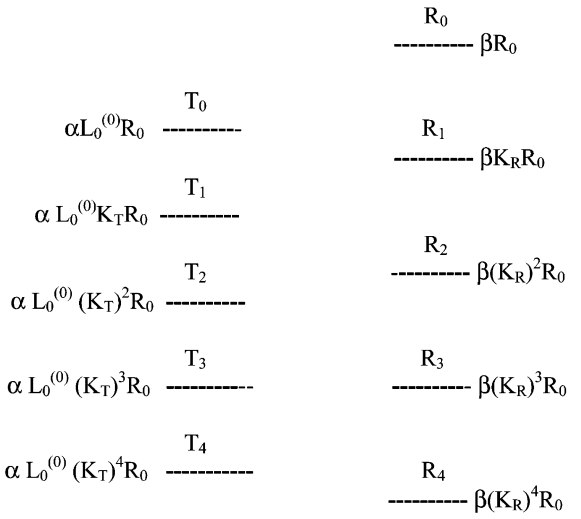

Fig. 2. Energetic expression of the global MWC model. The quantity attached to each state is the population of that state relative to the population of R_0.

1. The existence of two states, T and R, that correspond to two distinct protein conformations.
2. The oxygen affinity does not depend on the number of bound oxygen (the linear energy assumption).
3. Allosteric effectors shift the equilibrium between the T and R states by preferential binding to either the T or R state.

We will introduce one assumption.

1. Allosteric effectors change the K_T and K_R values by binding to the T and R states, respectively.

In Fig. 2, α and β represent the binding polynomial [50] for the binding of allosteric effectors, A, B, … to the T state and R state, respectively. Then,

$$\alpha = (1 + {}^{T}k_{a1}[A] + {}^{T}k_{a2}[A]^2 + \ldots)(1 + {}^{T}k_{b1}[B] + {}^{T}k_{b2}[B]^2 + \ldots)(1 + \quad)\ldots$$

$$\beta = (1 + {}^{R}k_{a1}[A] + {}^{R}k_{a2}[A]^2 + \ldots)(1 + {}^{R}k_{b1}[B] + {}^{R}k_{b2}[B]^2 + \ldots)(1 + \quad)\ldots$$

$$\text{Observed } L_0 = \alpha L_0^{(0)} \quad R_0/\beta R_0 = (\alpha/\beta)L_0^{(0)}$$

$$\text{Observed } K_T = f([A];\ K_T^{(0)},\ K_T^{(\infty)})$$

$$\text{Observed } K_R = f([A];\ K_R^{(0)},\ K_R^{(\infty)})$$

and

Observed $L_4 = (\alpha L_0^{(0)}(K_T)^4 R_0)/(\beta(K_R)^4 R_0)$
$= (\alpha/\beta)(K_T/K_R)^4 L_0^{(0)} = (K_T/K_R)^4 L_0$

Here, $K_T^{(0)}$, $K_R^{(0)}$, $L_0^{(0)}$ and $L_4^{(0)}$ are the parameters defined in the total absence of conformational constraints imposed by the effectors, (i.e. [A] = 0, [B] = 0, …), where $\alpha = \beta = 1$. $K_T^{(\infty)}$ and $K_R^{(\infty)}$ are K_T and K_R, respectively, under the extremely constrained conditions.

Let us consider such a situation that an allosteric effector binds to the T state with a 1:1 stoichiometry and a higher affinity while it binds to the R state with a 1:2 (or larger) stoichiometry and a lower affinity. As the constraints are strengthen on the increase in the concentration of that effector, L_0 is increased once by the preferential binding of the effector to the T state and then decreased by more pronounced binding to the R state (note that β becomes larger than α at sufficiently high effector concentrations).

For simplicity let us assume that an allosteric effector, A, represents all the effectors present in the solution. It binds to the T state with a higher affinity than to the R state, and it has two binding sites on the R state molecule while it has one binding site on the T state molecule. The binding of *A* to the R state is statistical. Then, the binding polynomials are:

$$\alpha = 1 + {}^{T}k_{a1}[A] \quad (4)$$

$$\beta = 1 + {}^{R}k_{a1}[A] + {}^{R}k_{a2}[A]^2 \quad (5)$$

The observed L_0 is:

$$L_0 = (\alpha/\beta)L_0^{(0)} \quad (6)$$

Let assume that effector A changes K_T and K_R in such the fashion that

$$K_T = \gamma(K_T^{(0)} - K_T^{(\infty)}) + K_T^{(\infty)} \quad (7)$$

$$K_R = \delta(K_R^{(0)} - K_R^{(\infty)}) + K_R^{(\infty)} \quad (8)$$

where

$$\gamma = \alpha^{-g} \quad (9)$$

$$\delta = \beta^{-h} \quad (10)$$

and

g = constant (>0)

h = constant (>0)

Note that Eqs. (7) and (8) are defined so that, as [A] changes, γ and δ vary within the range of 0 to 1 and consequently K_T and K_R vary in the range of $K_T^{(0)}$ to $K_T^{(\infty)}$ and $K_R^{(0)}$ to $K_R^{(\infty)}$, respectively. The parameters, g and h, are introduced to give γ and δ some freedom in defining the curvature of the variation of K_T or K_R between their two extreme values. The observed L_4 is related to these K_T and K_R, i.e.

$$L_4 = (K_T/K_R)^4 L_0 \quad (11)$$

3.3. Simulation of experimental data

From the experimental data, some of the parameter values are reasonably assumed as follows.

$L_0^{(0)} = 1$
$K_T^{(0)} = K_R^{(0)} = 10$ torr^{-1}
$K_T^{(\infty)} = K_R^{(\infty)} = 0.007$ torr^{-1}
(log K_T^{∞} = log $K_R^{\infty} = -2.15$)
$L_4^{(0)} = (K_T^0/K_R^0)^4$ $L_0^{(0)} = 1$

Furthermore, we will assume that

${}^{T}k_{a1} = 10^8$ M^{-1}
${}^{R}k_{a1} = 10$ M^{-1}
${}^{R}k_{a2} = 10^2$ M^{-2}
$g = 1/2.5$
$h = 1/4$

The ${}^{T}k_{a1}$ and ${}^{R}k_{a1}$ values were guessed from the binding constant values for DPG, IHP and BZF [3,51–54].

Then, Eqs. (4), (5), (7) and (8) become

$$\alpha = 1 + 10^8[A] \quad (12)$$

$$\beta = 1 + 10[A] + 10^2[A]^2 \quad (13)$$

$$K_T = 9.993\alpha^{-1/2.5} + 0.007 \quad (14)$$

$$K_R = 9.993\beta^{-1/4} + 0.007 \quad (15)$$

Eq. (6) becomes

$$L_0 = \alpha/\beta \quad (16)$$

Finally, this global allostery model is described by the four simultaneous equations: Eqs. (1), (14)–(16).

3.4. Simulation of the experimental allostery plot

By substituting arbitrary values of [A] into Eq. (12) and Eq. (13), K_T, K_R, L_0 and L_4 were calculated from Eqs. (11), (14)–(16). These values yielded a continuous closed circle on the allostery plot (Fig. 1). This simulated curve expresses the essential feature of the experimentally obtained closed circle very well. It is noteworthy that ${}^{T}k_{a1}$ ($=10^8$ M^{-1}) and ${}^{R}k_{a2}$ ($=10^2$ M^{-1}) were adjusted to realize the maximal value of L_0 ($\approx 10^6$) and the minimal value of L_4 ($\approx 10^{-4}$), respectively, and g ($=1/2.5$) and h ($=1/4$) were adjusted to simulate the shape of the right and left half, respectively, of the plot. The term, ${}^{R}k_{a1}$ ($=10$ M^{-1}, in Eq. (5)) contributed little and could virtually be omitted.

Eq. (1) was fitted to the individual OECs used in the present study and best-fit A values for them were obtained. Table 1 summarizes the best-fit values of A and the MWC parameters of Eq. (1) obtained for seven representative sets of solution conditions that span the lowest and highest extremes of conformational constraints (see the data set numbers attached to the simulated closed circle in Fig. 1). In Fig. 3, the Hill plots calculated from the best-fit A values that were obtained for the seven representative solution conditions are compared with their corresponding experimental plots. The agreement is excellent.

4. Discussion

In the previous study using the allostery plot [31], mainly Point B, Point C and the late stage of the Phase 2 were observed. Paired Points 10 (pH 6.5, +2 mM IHP) in figure 2 of [31] were indicative of Phase 3, but no attention was paid to its meaning. The present study, using universal solution conditions, revealed the existence of the closed circular allostery plot.

The present analysis indicates that the whole allostery plot can be simulated by the global allostery model with a few parameters, ${}^{T}k_{a1}$, ${}^{R}k_{a1}$, ${}^{R}k_{a2}$, g, h, $K_T^{(0)}$ ($=K_R^{(0)}$), and $K_T^{(\infty)}$ ($=K_R^{(\infty)}$), some of which have definite physical meanings and experimentally determined. This further means that, once these parameters have been determined, a given OEC of Hb A measured under any solution conditions that have been explored so far can be described by the global allostery model with only one adjustable parameter, [A]. The term, [A], which was defined as the concentration of the representative allosteric effector, can be regarded as the degree of constraints imposed on the hemoglobin molecule.

The determination of the MWC parameter values under extreme solution conditions revealed that, as the constraints are extremely strengthened, L_0 is decreased and L_4 is increased, both approaching unity (Yonetani et al., manuscript in preparation). Such the case has never been explored in previous studies. The constraint-dependent variation of K_R by more than 3 orders of magnitude is worthy of special note. The decrease in K_R value could be expected by postulating binding of allosteric effectors to the R state. However, the presence of a maximal value of L_0 was the feature that was hardly understood in the framework of the original MWC model (Yonetani et al., manuscript in preparation). We explained this maximization followed by decreases of L_0 with extreme strengthening of constraints in terms of a larger stoichiometry of effector binding for the R state than for the T state [Eqs. (4) and (5)].

In the global model, the quaternary effect, (i.e. the shift of the T-R equilibrium) by allosteric effectors [Eq. (16)] is linked to the tertiary effect, (i.e. the change in K_T and K_R) by effectors [Eqs. (14) and (15)] through the α and β terms [Eqs. (12) and (13)]. This is the mechanism by which allosteric effectors exert influences on not only L_0 but also K_T and K_R.

Apparently, the whole allostery plot (Fig. 1) is asymmetric with respect to the ordinate at $\log L = 0$. This asymmetric shape, which is exactly reproduced by our global model, arises from the differ-

Table 1
Parameter values of the MWC and global allostery models for representative OECs

Data set no.	Hemoglobin	Solution conditions		K_T (torr^{-1})	K_R (torr^{-1})	L_0	L_4	P_{50} (torr)	n_{max}	Best-fit [A] (M)
1	β chain	PH 7.4, no additives	Observed	5.5	9.9	1.7E+00	5.2E−01	0.11	1.00	
			Calculated	5.3	10	4.9E+00	3.9E−01			3.9E−08
2	Des-His-Tyr Hb	PH 7.4, IHP	Observed	2.3	10	5.9E+01	1.4E−01	0.27	1.36	
			Calculated	2.1	10	5.2E+01	9.4E−02			5.1E−07
3	Hb A	PH 10.6, no additives	Observed	0.88	10	3.9E+02	2.1E−02	0.46	1.84	
			Calculated	0.96	10	3.5E+02	3.0E−02			3.5E−06
4	Hb A	PH 7.4, Cl^-	Observed	0.071	7.6	1.2E+05	9.1E−04	2.6	2.90	
			Calculated	0.066	9.9	3.6E+05	7.1E−04			3.7E−03
5	Hb A	PH 7.0, Cl^-, DPG	Observed	0.016	2.8	5.9E+05	6.3E−04	14	2.80	
			Calculated	0.013	2.9	8.1E+05	3.2E−04			1.1E+00
6	Hb A	pH 6.6, Cl^-, IHP	Observed	0.01	0.19	4.3E+03	3.3E−02	47	1.88	
			Calculated	0.0075	0.15	1.9E+03	1.4E−02			5.3E+02
7	Hb A	pH 6.6, Cl^-, IHP, BZF	Observed	0.006	0.02	1.5E+01	8.6E−02	97	1.29	
			Calculated	0.007	0.014	5.1E+00	3.0E−01			2.0E+05

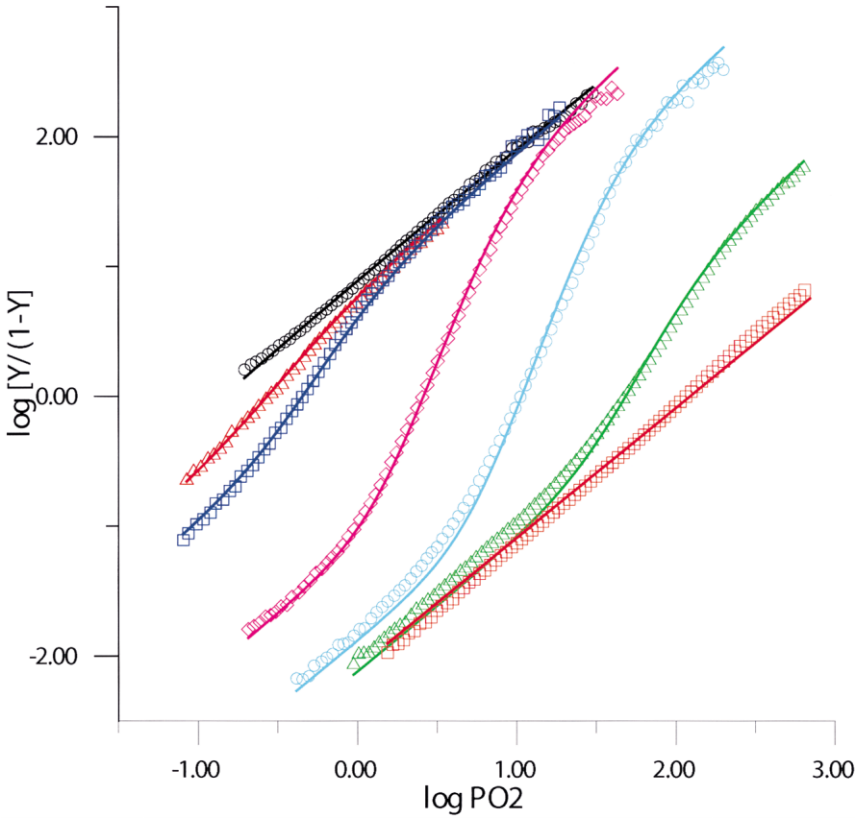

Fig. 3. Hill plots of oxygen binding observed under the seven representative solution conditions (Table 1). *Y*, fractional oxygen saturation; P_{O_2}, partial pressure of oxygen in torr. Symbols indicate observed points and lines indicate the OECs simulated by the present global allostery model. The plots from the left to the right correspond to the data set numbers from 1 to 7, respectively, in Table 1.

ence in the two binding polynomials, α and β [Eqs. (4) and (5)]. Interestingly, the MWC parameters for Hb A under physiological conditions, pH 7.4 with 0.1 M Cl^- and 2 mM DPG (K_T= 0.030 $torr^{-1}$, K_R=6.9 $torr^{-1}$, $L_0=1.7\times10^6$ and $L_4=6.1\times10^{-4}$) span the largest diameter of the allostery plot, that is, the paired two data points, [log K_T, log L_0] and [log K_R, log L_4], are located at the most rightward (Point B) and most leftward (Point C) positions, respectively, giving a large ratio of K_R:K_T. Thus, the physiological conditions are optimized to produce large cooperativity. Hb A seems to utilize this mechanism.

Even at extremely high pH, some pH-independent C-terminal salt-bridges involving the α-carboxyl groups of the α and β chains and the guanidinium group of Arg-141α can remain intact as 'inherent' conformational constraints. The imaginary Hb A, which is free of any allosteric effectors added and lacks the pH-independent C-terminal salt bridges would be totally non-cooperative, as demonstrated by des-His-Tyr Hb. Hence, the total part of the cooperativity of Hb A seems to arise from the constraints imposed by allosteric effectors, including the inherent salt bridges.

It is rather surprising that the present global allostery model with only one adjustable parameter can describe a given OEC measured under any set of universal solution conditions. It contains several simplifications as well as several assumptions. The representation of allosteric effectors by a single effector (A) may be an audacious simplification,

which in turn made the binding polynomials very simple. The present global model could be made more elaborate by taking individual effectors in the formulation. However, such a treatment would cause no essential difference, as suggested by the fact that the second term of Eq. (13) could virtually be omitted. To know to what extent the present model is supported by experiments, it is important to examine the binding stoichiometry and affinity of allosteric effectors for the T and R states. It is reported that DPG is bound to the same site on deoxy and oxyHbs, with a stoichiometry of one mole for one mole Hb tetramer [54,55]. IHP has two classes of binding sites on both oxyHb and HbCO [38,53]. It was suggested that more than two molecules of BZF per Hb tetramer were bound to deoxyHb but not to oxyHb [38]. These results must be re-examined at a much wider range of pH and/or in their coexistence. According to our recent X-ray crystallographic observation, carbonmonoxy Hb A in the presence of BZF and IHP at pH 6.0 is without doubt in the R quaternary state and two BZF molecules per Hb tetramer are bound to the α subunits (Yonetani et al., manuscript in preparation). In the present global allostery model, it is also important to clarify the physical meaning and mechanism of the linkage between the quaternary and tertiary effects through the α and β terms. Further structural studies are needed for better understanding of the identity of the T and R structures at both extremes of the degree of structural constraints (Points A and D in Fig. 1).

We point out here that the present analysis is highly model-dependent. It is known that the actual hemoglobin system shows some properties that do not accord with some of the assumptions made in the original MWC model. Through their extensive studies, Ackers et al. showed that cooperativity of hemoglobin depends on site-configuration and the cooperative energies have a combinatorial character through the oxygen binding cascade, and hence, cooperativity does not depend solely on the number of oxygen bound, nor the occupancy of the α vs. β chains [6,56]. These properties are apparently not consistent with the assumptions of the conservation of tetrad-axis symmetry and linear energy binding in the two-state MWC model described above [57]. Also, the quaternary enhancement observed by them [58] is not consistent with the MWC model. Here, we insist that the approach and aim of our study are different from those of Ackers et al. We intended to see what happened in hemoglobin when solution conditions were changed in the extremely wide range that had never been explored yet, whereas Ackers et al. studied the energetics of hemoglobin cooperativity mostly under a single set of solutions conditions, i.e. 0.1 M Tris–HCl (pH 7.4), 0.1 M NaCl, 1 mM Na_2EDTA, 21.5 °C. We intended to examine what extensions or modifications of the original MWC model were required to describe the oxygenation properties observed in universal solution conditions. The only extensions made in the present study were that allosteric effectors change the K_T and K_R values by binding to the T and R states, respectively. A model-independent interpretation of our oxygenation data will be presented in a separate paper (Yonetani et al., manuscript in preparation). The present 'global allostery model' with a single adjustable parameter could further be extended to accept chain heterogeneity [59], to introduce more than two affinity states [33] and/or to take into account the ligand-linked tetramer–dimer equilibrium [60]. These extensions need additional experimental data of functional difference between the α and β chains and tetramer–dimer equilibrium under universal solution conditions, and will be future subjects to be studied.

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