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An Allosteric Theory for Hemoglobin Incorporating Asymmetric States to Test the Putative Molecular Code for Cooperativity

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The two-state (MWC) model for cooperative oxygen binding by tetrameric ($\alpha_2\beta_2$) hemoglobin based on concerted transitions between symmetric states (T and R) is extended to include a third, asymmetric state with one $\alpha\beta$ dimer possessing high (R-like) oxygen affinity and the other $\alpha\beta$ dimer possessing low (T-like) oxygen affinity. The asymmetric state is assigned a stability that corresponds to the level reported by Ackers and colleagues in the studies on mixed valence hybrids that led to their proposed "molecular code for cooperativity in hemoglobin." However, this level of stability for the asymmetric intermediates significantly diminishes cooperativity in simulated oxygenation curves, to a degree (Hill $n = 2.1$) that is no longer compatible with the well-established oxygenation properties of normal ferrous hemoglobin (Hill $n \sim 3.0$). Therefore, the cyanomet derivatives do not appear to be reliable analogues of intermediate oxygenation states.

Keywords: hemoglobin cooperative oxygenation; two-state model; asymmetric hybrid state

The classical sigmoidal oxygen-binding curve for erythrocytes has long fascinated biologists, both in terms of the underlying physical basis related to the oxygen-transporter, hemoglobin, and the implications for vertebrate physiology. Several mechanistic models have been presented to serve as a basis for understanding cooperative oxygen binding and one of the most useful has been the two-state formulation due to Monod *et al*. (1965) the MWC model, according to which hemoglobin can occur in two symmetric conformational states, T and R, corresponding to the deoxy- and oxyhemoglobin conformations characterized in the X-ray crystallographic studies by Perutz (1989). The specific properties of oxygen equilibria can be related to the intrinsic affinities of the T and R states, as well as to the parameter L describing the equilibrium between the two states ($L = [\mathrm{T}]/[\mathrm{R}]$).

The concerted transitions between the T and R quaternary states account for the major equilibrium and kinetic features of the cooperative binding of oxygen by hemoglobin (Rubin & Changeux, 1966; Edelstein, 1971, 1975; Hopfield *et al*., 1971; Baldwin, 1975; Shulman *et al*., 1975; Sawicki & Gibson, 1976; Ackers & Johnson, 1981; Edelstein & Edsall, 1986; Perutz, 1989; Eaton *et al*., 1991; Rivetti *et al*., 1993). However, an inconsistency with the two-state model has been claimed from studies on analogues of asymmetric biliganded molecules produced with cyanomet derivatives representing oxygenated hemes (Smith & Ackers, 1985; Perrella *et al*., 1990a). The strength of the subunit interactions reported for these derivatives with the two cyanomet hemes on the same $\alpha\beta$ dimer is typical of the T state, whereas the values for the other biliganded analogues correspond to the R state. Based on this difference, a specific "molecular code for cooperativity" that abandons the symmetry principle of the two-state model has been invoked to describe the switching between states during oxygenation (Ackers *et al*., 1992; Doyle & Ackers, 1992; Ackers & Hazard, 1993; Holt & Ackers, 1995). Extensions of the two-state theory incorporating asymmetric molecules are presented here to test whether the proposed molecular code is compatible with the well-established oxygenation properties of normal ferrous hemoglobin.

The critical issue in evaluating the molecular code for hemoglobin cooperativity concerns the strength of subunit interactions at each stage of ligand binding. For the two-state model, the allosteric equilibrium constants, L_i, govern the distribution of

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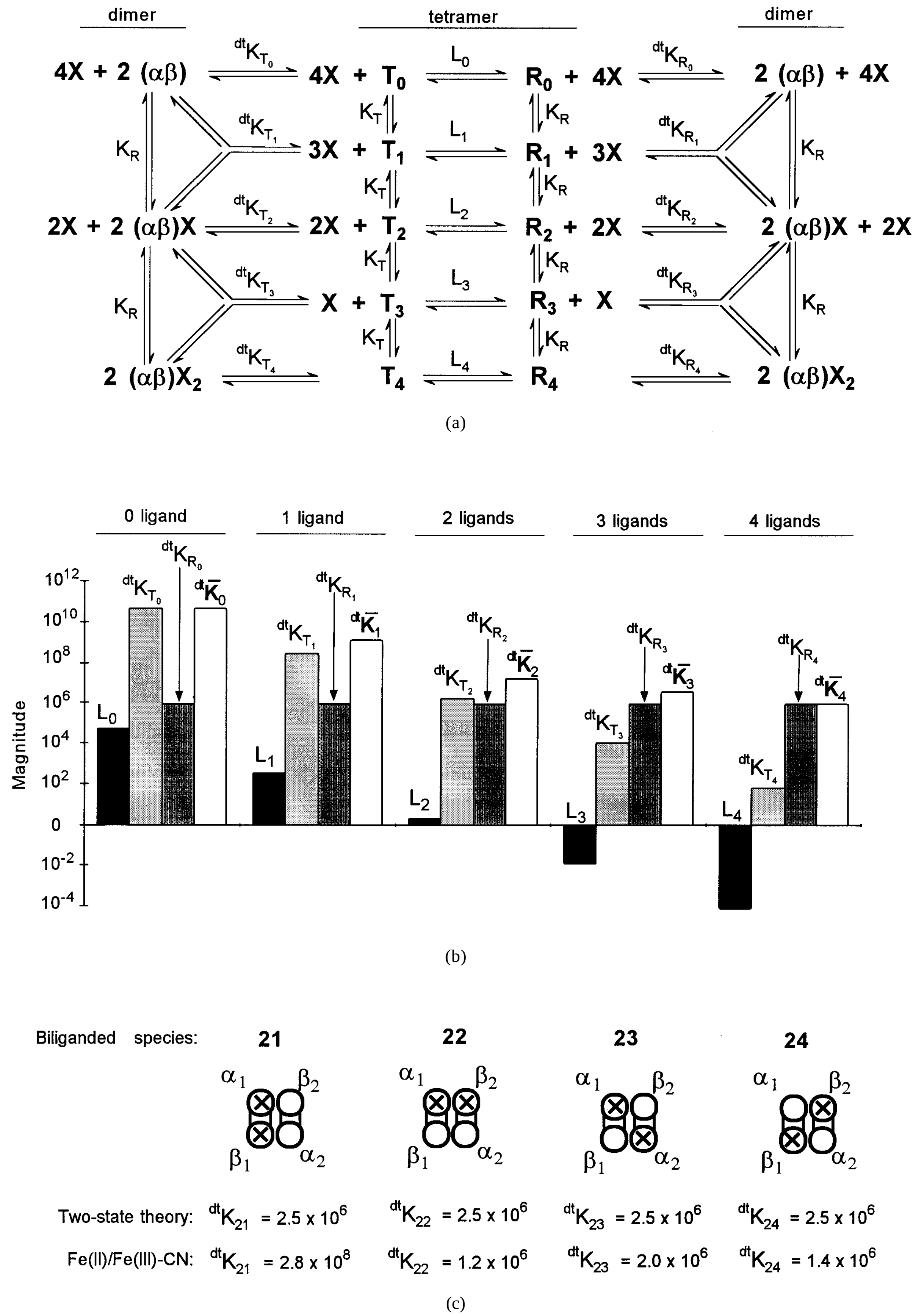

Figure 1(a–c) *legend opposite*

the T_i and R_i states as a function of ligand binding. These constants arise from quaternary constraint (Monod *et al*., 1965), as defined in Figure 1(a) by the ratio of the corresponding dimer-tetramer association constants. At each step (i) of ligand binding, the allosteric equilibria shift in favor of the R_i state ($L_i < L_{i-1}$), with a concomitant decrease in the subunit interaction strength of the T_i state, as described quantitatively in Figure 1(b) for the appropriate allosteric parameters. Cooperative binding of oxygen arises as a consequence of the transition from the intrinsically more stable T state (with low oxygen affinity) to an R state that is progressively stabilized by its stronger affinity for oxygen.

A deviation from the allosteric pattern was reported on the basis of the hyperstability of one of the asymmetric biliganded Fe^{3+}-CN/Fe^{2+} hybrids (species 21) defined in Figure 1(c). For this species a value of ${}^{dt}K_{21} = 2 \times 10^8\ M^{-1}$ was reported, in contrast to the three other biliganded species (22, 23, and 24), for which values of 1×10^6 to $2 \times 10^6\ M^{-1}$ were reported (Ackers *et al*., 1992). Since the value for species 21 is characteristic of the T state, whereas the values for the other biliganded species are characteristic of the R state, Ackers *et al*. (1992) proposed a molecular code for cooperativity, with T → R transitions occurring only when ligands are present on both αβ dimers.

The predictions of the molecular code are explicitly evaluated below by introducing into the two-state model a value of L_2 that is increased due to the presence of a hyperstable biliganded T form (test 1), or by the more physically plausible assumption that the hyperstable biliganded molecules represent a distinct conformational state that could participate at all degrees of ligand binding (test 2). For the former, a modification of L_2 is imposed using a multiplying factor (Marden *et al*., 1990) that arbitrarily stabilizes this state. For the latter, a full-fledged third state, H, is formulated, with "hybrid" properties arising as a combination of one T-like αβ dimer and one R-like αβ dimer. The distinct occupancy pattern of the H state is presented in Figure 2. For each degree of ligand binding to the H state, stability increases by an amount dependent upon whether the liganded site is characterized by T-state or R-state affinity, leading to the energy diagram presented in Figure 3 for all T, R and H unliganded and liganded forms. In this formulation, the asymmetric hybrid (species 21, Figure 1(c)) would correspond predominantly to the species H_{2r}.

The consequences of the hyperstable asymmetric molecules on simulated oxygen binding properties are presented in Figure 4. The standard oxygen binding curve ($\bar{Y}_2$) is strongly cooperative, as indicated by the coefficient of the Hill equation, $n = 3.1$. For the original data sets upon which the values of the parameters are based, Hill values of 3.2 to 3.3 were reported (Mills *et al*., 1976). When the hyperstability of the asymmetric biliganded molecules is introduced through a stability factor, **s**, that multiplies the value of L_2 (test 1), a substantial anti-cooperative effect is produced in the oxygen binding curve ($\bar{Y}_{2,\mathbf{s}}$), dropping the Hill coefficient to $n = 1.6$. When the full three-state model is considered (test 2), the anti-cooperative effect of the hyperstable biliganded molecules on the oxygenation curve ($\bar{Y}_3$) is reduced, but still pronounced, with a Hill coefficient of $n = 2.1$. These anti-cooperative effects can be understood in terms of the fraction of biliganded intermediates, as presented in Figure 4, inset. For normal hemoglobin, with the two-state model the sum of all biliganded intermediates reaches a maximum (at $\bar{Y}_2 \sim 0.5$) of only 2.8%. When the hyperstable asymmetric biliganded molecules are introduced ($\bar{Y}_{2,\mathbf{s}}$) by increasing L_2, the anti-cooperative effect is reflected by an increase in the biliganded intermediates to a maximum value of 52%. For the full three-state model ($\bar{Y}_3$), the moderate anti-cooperative effect is reflected by a

Figure 1. Linkage between the allosteric transitions and the dimer-tetramer association constants. (a) Ligand binding reactions, allosteric transitions and dimer-tetramer equilibria for the T and R states. Equilibria are presented between T_i and R_i, where $L_i = [T_i]/[R_i]$ and the subscript (i = 0 to 4) indicates the number of ligand molecules (X) bound to each state with a characteristic intrinsic dissociation constant, K_T or K_R. For each ligand binding event, the $T_i \rightleftarrows R_i$ equilibrium shifts towards R as a consequence of the higher affinity for ligand, according to the relationship $L_i = L_0\, c^i$, where c is the ratio of the affinities of the R and T states ($c = K_R/K_T$). The magnitude of L_i reflects the differences in quaternary stability of the T and R states, as determined by their respective dimer-tetramer association constants, ${}^{dt}K_{T_i}$ and ${}^{dt}K_{R_i}$, where $L_i = {}^{dt}K_{T_i}/{}^{dt}K_{R_i}$. This relationship arises from the fact that the tetramers of both the T and R states are in equilibrium with an αβ dimer state possessing the same oxygen affinity (K_R) as the R state (Thomas & Edelstein, 1972; Ackers & Johnson, 1981; Edelstein & Edsall, 1986). (b) Quantitative relationships between L_i and the dimer-tetramer equilibria. With invariance of ${}^{dt}K_{R_i}$ as a function of ligand binding (Ackers & Johnson, 1981; Edelstein & Edsall, 1986), each successive change in L_i corresponds to a decrease in ${}^{dt}K_{T_i}$ by the factor c. At each degree of ligand binding a global dimer-tetramer association constant, ${}^{dt}K_i$ (corresponding to the experimentally observed value), is defined in the two-state model by the relationship ${}^{dt}\boldsymbol{K}_i = [R_i] + [T_i]/[D] = \binom{N}{i}{}^{dt}K_R(1 + L_i)$, where $\binom{N}{i}$ is the binomial coefficient, ($N!/(N-i)!i!$), with values of 1, 4, 6, 4 or 1 for $i = 0$ to 4, respectively (with $N = 4$). Hence for $i = 0$, ${}^{dt}\boldsymbol{K}_0 = [R_0] + [T]_0/[D] = {}^{dt}K_R\ (1 + L_0)$. The values for parameters of the two-state model (Ackers & Johnson, 1981; Edelstein & Edsall, 1986), $L_0 = 52{,}000$ and $c = 0.0061$, correspond to the pH 7.4 conditions summarized by Ackers *et al*. (1992) on the basis of the data reported by Mills *et al*. (1976). (c) Differences in stability of the four species of biliganded molecules according to the two-state model and the deductions from cyanomet hybrids (Ackers *et al*., 1992). For the former, X indicates a bound oxygen atom; for the latter X indicates a cyanomet heme.

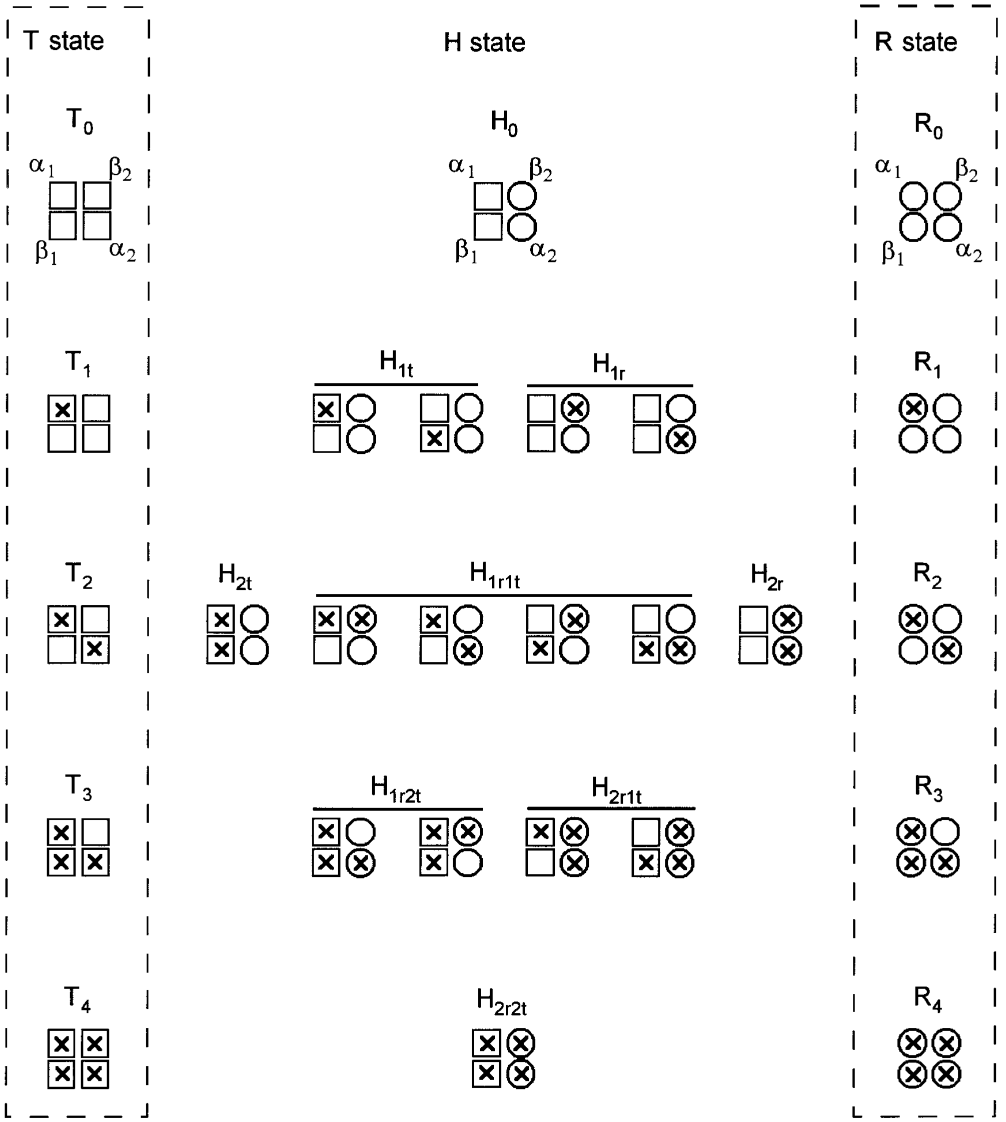

Figure 2. Individual ligation species with a model including a third hybrid state, H. The H state is formed with one αβ dimer in the T conformation and the other αβ dimer in the R conformation. Subscripts $i = 1$ to 4 for T and R indicate the number of ligands bound. For the H state subscripts $i = 1$ and 2 followed by t or r refer to ligands bound to the T-like or R-like subunit.

maximum fraction of biliganded intermediates of 26%.

The quantitative conclusions presented above depend, of course, on the values assigned to L_2 and $^{H}L_0$, as described in the legend to Figure 4. The sensitivity to these parameters can be described by noting that, for example, doubling the value of $^{H}L_0$ in the equation for $\bar{Y}_3$ reduces the Hill coefficient from $n = 2.1$ to $n = 1.7$, whereas halving the value of $^{H}L_0$ increases the Hill coefficient to $n = 2.5$. Reduction of $^{H}L_0$ by about tenfold restores cooperativity to normal levels. Cooperativity could also be restored by changing other parameters of the model, for example by decreasing c. However, a twofold decrease in c results in an increase of n only from 2.1 to 2.3 with the equation for $\bar{Y}_3$, indicating that large changes would be required to approach normal cooperativity. Such changes would be hard to reconcile with the values of K_R and K_T that have been well-established experimentally to within narrow limits (Edelstein, 1975; Gibson & Edelstein, 1987; Marden *et al*., 1989; Rivetti *et al*., 1993).

The results presented in Figure 4 would therefore appear to indicate that the reported hyperstability of the asymmetric biliganded cyanomet hybrids is incompatible with the oxygenation of normal ferrous hemoglobin, since it would produce a marked anti-cooperative effect. Cooperative ligand binding is a manifestation of the fact that intermediate forms are underpopulated; hence, augmenting the stability of intermediate forms must necessarily diminish cooperativity. In this respect, it is of interest to note that in the original presentation

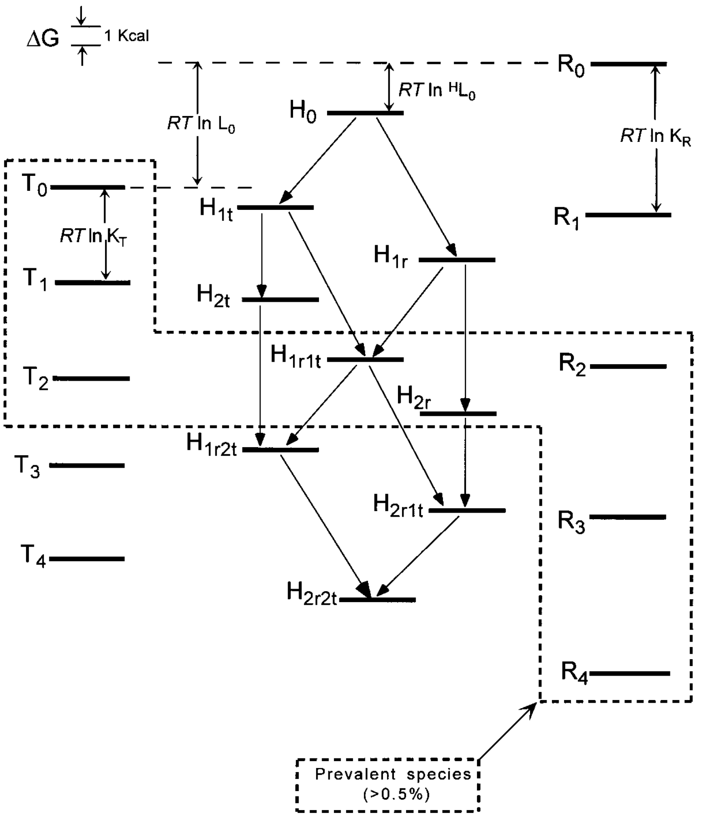

Figure 3. Energy diagram for the various T, H and R forms. Each state is shown at a vertical height corresponding to its relative free energy. With respect to each R_i, the corresponding T_i is characterized by a relative stability defined by L_0c^i and each H form by ${}^{H}L_0c^i$, where $L_0 = [T_0]/[R_0]$, ${}^{H}L_0 = [H_0]/[R_0]$, $c = K_R/K_T$. The subscripts ir and jt indicate the number of ligands bound to the R-like and T-like sites, respectively. For the unliganded states, the vertical spacing defines $RT \ln L_0$ and $RT \ln {}^{H}L_0$, as indicated on the diagram. Each liganded form is displaced downward from the preceding form to reflect the energy of ligand binding, $RT \ln K_T$ and $RT \ln K_R$ for the T and R states, respectively, or the T-like and R-like subunits of the H state. The prevalent species (representing at least 0.5% of the molecular population during oxygenation) are indicated within the boxed area.

of the two-state model (Monod *et al*., 1965), the principle of allosteric transitions with conservation of symmetry was not imposed as a condition *sine qua non*, but rather as a consequence of the assumption that asymmetric intermediate states would be significantly less stable than the symmetric T and R states. A hypothetical intermediate state is depicted in Figure 10 of the original article (Monod *et al*., 1965). The full evaluation of an asymmetric third state presented here provides a quantitative evaluation of this intuitive concept based on a virtual state and confirms the anti-cooperative effects of such a state.

Concerning the cyanomet derivatives as models for oxygenation, the hyperstable asymmetric biliganded molecules may not correspond to oxygenated intermediates. Conceivably, a novel state is created under the long incubation conditions (several days) required for formation of this intermediate (Perrella *et al*., 1990a; Doyle & Ackers, 1992; Huang & Ackers, 1995), but this state does not appear to be relevant to the mechanism of cooperative oxygenation of ferrous hemoglobin. Therefore, a specific molecular code for the cooperative oxygen binding to normal hemoglobin cannot be deduced from these data. Ackers *et al*. (1992) presented values for the dimer-tetramer interactions of the biliganded oxygenated intermediates at pH 7.4 in Table 1 of their article (Ackers *et al*., 1992), with the value (converted from free energy) for species 21 (${}^{dt}K_{21} = 6.5 \times 10^6$ M^{-1}) about 30-fold higher than the values for species 22, 23 and 24 (${}^{dt}K_{22} = {}^{dt}K_{23} = {}^{dt}K_{24} = 2.2 \times 10^5$ M^{-1}). However, this difference was based solely on the assumption that the pattern for cyanomet intermediates applies to oxygen, with values that satisfy ${}^{dt}\boldsymbol{K}_2$, the global dimer-tetramer association constant, ${}^{dt}\boldsymbol{K}_2 = 2{}^{dt}K_{21} + 2{}^{dt}K_{22} + {}^{dt}K_{23} + {}^{dt}K_{24} = 1.4 \times 10^7$ M^{-1} (Ackers *et al*., 1992). The global constant would also be satisfied with an identical value for each of the biliganded species (${}^{dt}K_{21} = {}^{dt}K_{22} = {}^{dt}K_{23} = {}^{dt}K_{24} = 2.5 \times 10^6$ M^{-1}; see Figure 1(c)), as predicted by the two-state model (Ackers & Johnson, 1981; Edelstein & Edsall, 1986). For data at pH 8.8 an anti-cooperative effect is also produced by the hyperstable asymmetric biliganded molecules, but the magnitude of the effect is smaller than at pH 7.4 because the differences in the strengths of the dimer-tetramer interactions among the various unliganded and liganded forms are smaller (Ackers *et al*., 1992).

In experiments measuring the various forms present in molecules partially liganded with CO, low concentrations of asymmetric hybrids were observed by an elegant low-temperature electrophoresis method, indicating that these forms are not preferentially stabilized to the extent implied by the mixed-valence hybrids (Perrella *et al*., 1990b; Perrella & Denisov, 1995). In contrast, resonance Raman spectra on kinetic intermediates in the reaction with CO were interpreted in a ''working model'' based on preferentially populating asymmetric hybrids (Jayaraman *et al*., 1995), but it is not

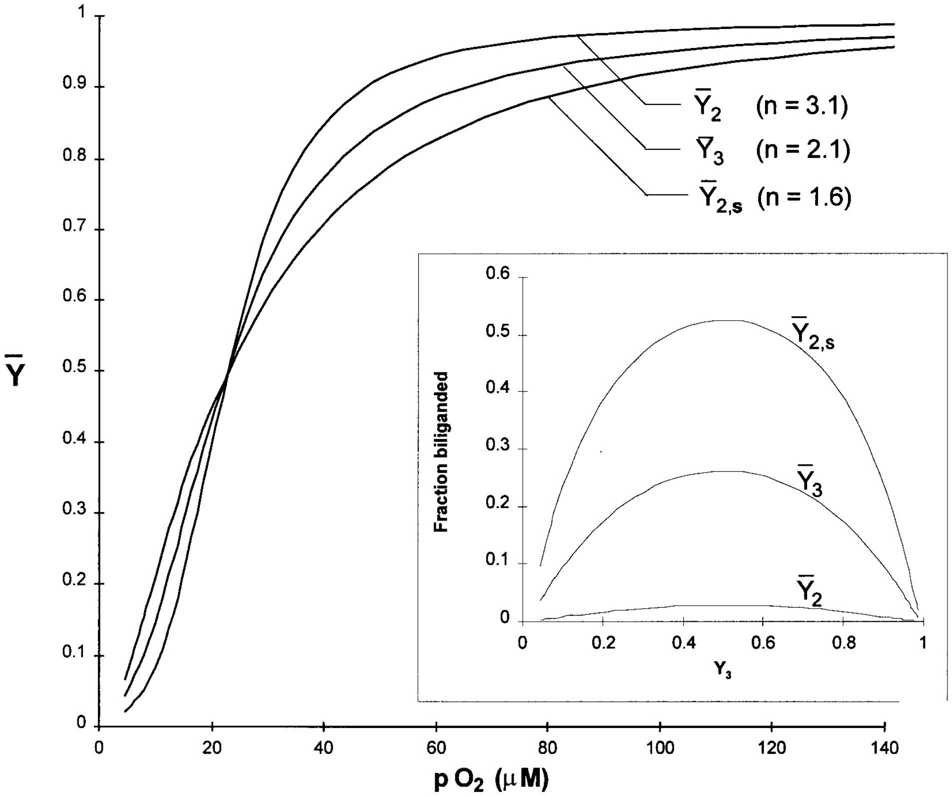

Figure 4. Simulated oxygen binding equilibria. Curves of ligand saturation as a function of pO_2 are presented with the conventional two-state model ($\bar{Y}_2$), an extended two-state model incorporating a high value of L_2 to account for the hyperstability of the asymmetric biliganded form ($\bar{Y}_{2,s}$), and a three-state model incorporating the H state at all levels of ligation ($\bar{Y}_3$). For each curve the n value presented corresponds to the coefficient for the Hill equation. For the two-state model, the binding equation, is:

$$\bar{Y}_2 = \frac{\alpha(1 + \alpha)^3 + L_0 c\alpha(1 + c\alpha)^3}{(1 + \alpha)^4 + L_0(1 + c\alpha)^4}$$

where $\alpha = [O_2]/K_R$ and the other parameters are as defined for Figure 3. In order to test the hypothesis that the hyperstability of the asymmetric biliganded molecules increases the value of L_2 above the level predicted by the two-state model (test 1), the terms for each L_i are explicitly introduced into an expanded binding equation, $\bar{Y}_{2,s}$, with L_2 multiplied by a hyperstability factor $\mathbf{s}$:

$$\bar{Y}_{2,s} = \frac{\alpha(1 + \alpha)^3 + L_1\alpha + 3\mathbf{s}L_2\alpha^2 + 3L_3\alpha^3 + L_4\alpha^4}{(1 + \alpha)^4 + L_0 + 4L_1\alpha + 6\mathbf{s}L_2\alpha^2 + 4L_3\alpha^3 + L_4\alpha^4}$$

For the conventional two-state model, $L_2 = L_0 c^2$ and for the standard parameters of $L_0 = 52{,}000$ and $c = 0.0061$, a value of $L_2 = 1.9$ is obtained. In order to correct this value by the factor $\mathbf{s}$ corresponding to the contribution of the hyperstable asymmetric biliganded molecules, the individual dimer-tetramer association constants derived from the cyanomet hybrids 21, 22, 23 and 24 with the values listed in Figure 1(c) are converted to a global dimer-tetramer constant, ${}^{dt}\boldsymbol{K}_2$, where ${}^{dt}\boldsymbol{K}_2 = 2{}^{dt}K_{21} + 2\ {}^{dt}K_{22} + {}^{dt}K_{23} + {}^{dt}K_{24} = 5.6 \times 10^8\ M^{-1}$. The value of ${}^{dt}\boldsymbol{K}_2 = 5.6 \times 10^8\ M^{-1}$, reflecting the presence of the hyperstable species 21, may be compared with the value predicted by the two-state model (Ackers & Johnson, 1981; Edelstein & Edsall, 1986), according to the equation: ${}^{dt}\boldsymbol{K}_2 = 6{}^{dt}K_R(1 + L_2) = 1.5 \times 10^7\ M^{-1}$ (for $L_2 = 1.9$ and ${}^{dt}K_R = 8.4 \times 10^5\ M^{-1}$). The value of ${}^{dt}\boldsymbol{K}_2 = 5.6 \times 10^8$ corresponds to a value of $L_2 = 110$, or a stability factor for use in the equation for $\bar{Y}_{2,s}$ of $\mathbf{s} = 110/1.9 = 56.9$. In order to examine the hypothesis that the hyperstable asymmetric molecules represent one of the liganded forms of a complete third state, H, as described in Figures 2 and 3 (test 2), a three-state ligand binding function, $\bar{Y}_3$, is defined:

$$\bar{Y}_3 = \frac{\alpha(1 + \alpha)^3 + {}^{H}L[\alpha(1 + \alpha)(1 + c\alpha)^2 + c\alpha(1 + c\alpha)(1 + \alpha)^2] + L_0 c\alpha(1 + c\alpha)^3}{(1 + \alpha)^4 + 2{}^{H}L[(1 + \alpha)^2(1 + c\alpha)^2] + L_0(1 + c\alpha)^4}$$

In this case, the standard values for the allosteric parameters are used, with the addition of the value for the allosteric constant of the H state, ${}^{H}L_0$. This value may be calculated by assuming that the global dimer-tetramer association constant for all biliganded molecules, ${}^{dt}\boldsymbol{K}_2 = 1.5 \times 10^7\ M^{-1}$, is derived from contributions of the three states according to the equation ${}^{dt}\boldsymbol{K}_2 = 6{}^{dt}K_R(1 + {}^{H}L_2 + L_2)$, leading to a value of ${}^{H}L_2 = 108.2$. Since the biliganded H state is dominated by H_{2r} and this state is stabilized by the same energy as R_2 (i.e. $2\ RT \ln K_R$), the value ${}^{H}L_0 = 108.2$ is assigned for use in the equation for $\bar{Y}_3$. In order to obtain a characteristic Hill coefficient (n) for each oxygenation curve simulated, in spite of the variation of n between 1 at the extremes of Y and n_{max} (Rubin & Changeux, 1966), the apparent n value was determined by a least-squares fit to the Hill equation in the range $Y = 0.05$ to 0.95, yielding the values $n = 3.1$ for $\bar{Y}_2$, 1.6 for $\bar{Y}_{2,s}$ and 2.1 for $\bar{Y}_3$. In this range the data are adequately described by a constant n value, since the average deviations of Y between the simulated curves and the Hill equation were less than 10^{-4} in each of the three cases. Inset: for each oxygenation curve the fractional population represented by the sum of all biliganded species is presented as a function of the fractional saturation values scaled to the curve for $\bar{Y}_3$.

clear whether a more complete quantitative analysis would exclude alternative interpretations.

Other implications of the molecular code, such as cooperativity within the T state (Ackers *et al*., 1992) are ruled out by the observation of non-cooperative oxygen binding by crystals of hemoglobin in the T state (Rivetti *et al*., 1993) and in silica gels (Shibayama & Saigo, 1995). Small α-β differences in affinity should also be taken into account for a more precise modeling of oxygenation (Edelstein, 1975), but they represent a relatively minor perturbation to the two-state model (Rivetti *et al*., 1993). Adjustments of the parameters for a slightly higher affinity of R state tetramers compared with αβ dimers due to an effect designated quaternary enhancement (Edelstein, 1975; Ackers *et al*., 1992) have also been invoked, but this effect is close to the limits of experimental accuracy (Gibson & Edelstein, 1987; Doyle & Ackers, 1992). Overall, structural perturbations may produce modified conformational states, but only those correlated with functional changes would be relevant to the mechanism of cooperative oxygen binding.

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