Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 3796–3800, June 1986
Biophysics

Linkage between ligand binding and the dimer-tetramer equilibrium in the Monod–Wyman–Changeux model of hemoglobin

(hemoglobin–O_2 equilibrium/subunit interactions/linked functions)

STUART J. EDELSTEIN* AND JOHN T. EDSALL†

*Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853; and †Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138

Contributed by John T. Edsall, January 21, 1986

ABSTRACT **G. Weber [(1984) *Proc. Natl. Acad. Sci. USA* 81, 7098–7102] has inferred that the Monod–Wyman–Changeux (MWC) model for ligand binding by hemoglobin would require (contrary to experimental evidence) that increased ligand binding must promote stabilization of $\alpha_2\beta_2$ tetramers with respect to dissociation into $\alpha\beta$ dimers. Reexamination of the MWC model, however, in the light of general linkage principles and the specific analysis by G. K. Ackers and M. L. Johnson [(1981) *J. Mol. Biol.* 147, 559–582] shows that the opposite relation must hold, in agreement with experiment. The T form of the tetramer, with low ligand affinity, must be destabilized and progressively dissociates into the high-affinity dimers, designated D, as ligand binding increases. Each ligand molecule bound shifts the standard Gibbs free energy ΔG_{2T} for the D–T equilibrium by approximately 3 kcal/mol in favor of the dimer. Thus, T must exist in (at least) five ΔG levels of cooperative free energy as it becomes progressively destabilized by successive binding of ligand molecules. Dissociation of the R tetramer to dimers, in contrast, is independent of the amount of ligand bound, so long as dimers and R-state tetramers possess the same (high) affinity for ligand. While the intrinsic ligand-binding constants of the T and R states (K_T and K_R) remain unchanged throughout by the postulates of the model, the model should not be regarded as a strictly two-state system in view of the multiple free-energy levels indicated above. The present analysis gives approximate, though not precise, agreement with experimental findings on the dimer–tetramer equilibrium considered by Weber and provides a rationale for interpreting other recent experiments concerning this equilibrium.**

Weber (1) has recently challenged the validity of the model for cooperative ligand binding in Hb proposed by Monod–Wyman–Changeux (2)—the MWC model—on the grounds that "the cooperative interaction of several liganded subunits within the tetramer should lead to its stabilization and not to facilitated dissociation" (ref. 1, p. 7102). There is abundant experimental evidence that binding of oxygen and other ligands does facilitate dissociation of Hb tetramers to dimers, whereas Weber concluded that a strictly two-state model could not reconcile such dissociation with cooperative ligand binding. This seeming paradox led us to reexamine the question, since numerous studies of the MWC model (e.g., refs. 3–12) have shown that it gives results in good general accord with a range of experimental findings for a reasonable choice of parameters. Although calculations based on the MWC model show some significant deviations from experimental data, there is no known case of such a gross qualitative discrepancy as Weber has inferred. Hence, the problem calls for further examination.

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Our analysis has led us to recognize an ambiguity in the definition of the MWC model for Hb as a two-state system. It is indeed such a system in the sense that each of the two interconverting forms of the tetramer—R with high and T with low ligand affinity—is characterized by a single intrinsic ligand-binding constant with no interaction between sites in the course of binding. It is not a two-state system in the sense that the dimer–tetramer association constant for both R and T can be independent of the amount of ligand bound. A ligand-independent constant must hold for one of the two forms (see below), but it is thermodynamically impossible that it should hold for both. Indeed, if the model is to be even approximately consistent with experimental data, this association constant for one form of the tetramer, which proves to be the T form, must vary systematically over a wide range with each additional ligand bound. In this sense the T tetramer must exist in five distinct free-energy levels, as ligand binding increases from 0 to 4 per tetramer. All of this is implicit in the rigorous thermodynamic analysis of the MWC model by Ackers and Johnson (13), whose equations show clearly the increasing dissociation of tetramers into dimers with increasing amounts of ligand bound. It appears that ligand binding to the T form must greatly increase its tendency to dissociate into dimers as well as to isomerize into R. When this is taken into account, the anomaly that Weber (1) has inferred does in fact disappear.

Partition Functions for Tetramers and Dimer

In our analysis we use the same set of data that Weber (1) used in his. They are taken from the paper of Ackers and Johnson (13), whose calculations made use of earlier experimental data from the same laboratory on human adult Hb (Hb A). We assume the simplest type of MWC model in which each of the two allosteric forms of the tetramer, R and T, is characterized by a single intrinsic binding constant for ligand, K_R or K_T. With these constants defined as association constants, their ratio $c = K_T/K_R$ is a fundamental parameter of the system, with the ratio defined to give $c < 1$. We use subscripts, R_i or T_i, to denote specific forms of R and T with i ligands bound (i runs from 0 to 4). In the absence of ligand, the T/R ratio, $L = [T_0]/[R_0]$, is a second fundamental parameter; it is a large number, about 10^4–10^5. The ligand-binding constant for the dimer D is taken as $K_D = K_R$, since it is known that both D and R have high and closely similar ligand affinity. Experimental evidence justifies the assumption that the two binding sites in D are equivalent and independent in ligand binding (7, 8, 13). The specific values of the parameters used (taken from ref. 13), are listed in Table 1.

The distribution of R, D, and T among the forms with different amounts of ligand bound, as a function of free ligand

Abbreviations: MWC, Monod–Wyman–Changeux; Hb A, adult hemoglobin.

Table 1. Assumed equilibrium constants for calculations of dimer–tetramer equilibrium in Hb A*

Ligand association	K, M^{-1}	ΔG, kcal/mol
Intrinsic		
T tetramer	$K_T = 1.0 \times 10^4$	$\Delta G_T = -5.4$
R tetramer	$K_R = 1.7 \times 10^6$	$\Delta G_R = -8.4$
D (dimer)	$K_D = K_R$	
Dimer–tetramer		
Unliganded tetramer (T)	${}^0K_{2T} = 4.6 \times 10^{10}$	$\Delta^0G_{2T} = -14.4$
Half-liganded tetramer		$\Delta^2G_2 = -8.4$
Liganded tetramer (R)	$K_{2R} = 8.5 \times 10^5$	$\Delta G_{2R} = -8.0$

MWC model values: $L = [T_0]/[R_0] = 5.2 \times 10^4$ and $c = [K_T]/[K_R] = 0.0061$.

*All of these assumed values are taken from Tables 1 and 2 of Ackers and Johnson (13) except for the value of the free energy of half-liganded tetramers, Δ^2G_2, which was taken from Table 6 of Mills *et al.* (14). The data are derived for the experimental conditions of 0.1 M Tris·HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, 21.5°C (14). The value of c derived from the values of K_R and K_T before rounding (13) has been retained, rather than the value of 0.0059 derived from the constants with two significant figures presented in the table.

at concentration $[x]$, is conveniently calculated in terms of the partition functions or binding polynomials of Wyman (15). Our equations are simpler than his because he assumed that the dimer, like the tetramer, might exist in two allosteric forms. This assumption (also considered but not used in ref. 13) now appears unnecessary, as indicated above. Since the binding sites are taken as equivalent and independent, the partition function for the R tetramer is given by the equation:

$$P_R = (1 + K_R[x])^4 = (1 + \alpha)^4. \quad [1a]$$

Here, for simplicity of notation following ref. 2, we write $\alpha = K_R[x]$, where α is a normalized concentration (a pure number). Expansion of the polynomial gives:

$$P_R = 1 + 4\alpha + 6\alpha^2 + 4\alpha^3 + \alpha^4 = \sum_{i=0}^{4} [R_i]. \quad [1b]$$

These terms give the successive Adair coefficients for the system, with statistical factors included. Taking the reference concentration of R_0 as unity, the term in α^i gives the relative amount of R_i in the system.

A similar partition function holds for the T tetramer, with $K_T = cK_R = 0.0061\,K_R$. Hence, $P_T = (1 + c\alpha)^4$. However, in the absence of ligand, there are L molecules of T for each molecule of R. Hence, to obtain the total numbers of the different forms of T relative to R, we must multiply P_T by L:

$$\begin{aligned} LP_T &= L(1 + c\alpha)^4 \\ &= L(1 + 4c\alpha + 6c^2\alpha^2 + 4c^3\alpha^3 + c^4\alpha^4) \\ &= \sum_{i=0}^{4} [T_i]. \end{aligned} \quad [2]$$

Comparing the terms in powers of α in Eqs. 1 and 2, we obtain immediately a familiar relation:

$$[T_i]/[R_i] = Lc^i. \quad [3]$$

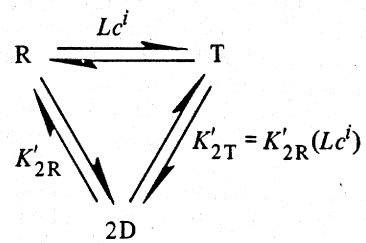
Thus, this ratio for unliganded tetramers ($i = 0$) is $L > 10^4$; for the completely liganded tetramers ($i = 4$), it is $Lc^4 < 10^{-4}$. This is the basis of the usual assumption that the properties of T_0 may be identified with those of deoxy-Hb, and those of R_4 may be identified with those of completely oxygenated (or, in general, of completely liganded) Hb.

The partition function of the dimer (D_0, D_1, D_2) is given by $[D_0]$, multiplied by the function P_D. Since $K_D = K_R$, we can write it as:

$$\sum_{i=0}^{2} [D_i] = [D_0]P_D = [D_0][1 + \alpha]^2. \quad [4]$$

Equilibrium Between Tetramers and Dimer

The linkage relations between R, T, and D are illustrated by Scheme **1**.



Scheme **1**

We follow the notation of ref. 13 by denoting the dimer–tetramer association constant for a tetramer with i ligands bound, by ${}^iK_2$, adding the subscript R or T if we refer to only one of the two tetramers. The symbol ${}^iK'_2$ denotes the corresponding intrinsic constant uncorrected for statistical factors.

In considering Scheme **1**, we note an important proposition stated by Ackers and Johnson (13): one of the two dimer–tetramer intrinsic association constants, ${}^iK'_{2T}$ or ${}^iK'_{2R}$, must be independent of i and, therefore, is a true constant, unchanged by ligand binding or removal.‡ Clearly this constant must be ${}^iK'_{2R}$, since both R and D have high and essentially equal ligand affinities (indeed, in the MWC model they in fact are required to be equal, as in Eq. **4** above). Thus, K'_{2R} can be written as a constant, independent of the superscript i.

Given this proposition, it is immediately apparent that the D–T equilibrium must be profoundly affected by ligand binding. The equilibrium ratio between T_i and R_i, from Eq. **3**, is Lc^i. We must obtain the same result by proceeding from R to T in Scheme **1** by way of the dimer intermediate D. This requires the relation:

$${}^iK'_{2T} = Lc^i \cdot K'_{2R}. \quad [5]$$

In the absence of ligand ($i = 0$), ${}^0K'_{2T}$ is thus L times as great as K'_{2R}. Given the identification of T_0 with deoxy-Hb and of R_4 with HbO_2, one of us (7) noted in 1975 that this relation provides a means of determining L but did not deduce the general equation, **5**. Since, from Table 1, $L = 5.2 \times 10^4$ and $Lc^4 = 7.2 \times 10^{-5}$, the D–T association constant must decrease by a factor of the order of 10^9 over the course of ligand binding, from $4.6 \times 10^{10}\ M^{-1}$ in T_0 to around $60\ M^{-1}$ in T_4. From Eq. **5** and Scheme **1**, it is clear that this change runs exactly parallel to the change in the [T]/[R] ratio from L to Lc^4. Thus, ligand binding profoundly destabilizes the T tetramer both with respect to isomerization to R and dissociation to D. The biliganded T_2 dissociates to dimer almost as readily as does R, and T_4, which can never be present except in very small amounts, should dissociate to dimer about 10^9 times as readily as T_0.

‡The proof of this proposition was given in the original manuscript (13), but it was omitted from the published paper by editorial request to reduce the length of the paper (personal communication from G. K. Ackers).

The total dimer–tetramer association constant, ${}^iK_2$, is the sum of ${}^iK_{2T}$ and the constant K'_{2R}, which is independent of i. Making use of Eq. **5**, we have:

$$ {}^iK'_2 = \frac{[T_i] + [R_i]}{[D]^2} = {}^iK'_{2T} + K'_{2R} = K'_{2R}(1 + Lc^i) . \quad [6] $$

This is identical with equation 24 of Ackers and Johnson (13) except for a statistical factor s_i for ligation state i. Table 2 lists the resulting values of ${}^iK_2$, including the corrections for s_i, and the corresponding values of $\Delta^iG_2 = -RT \ln {}^iK_2$. It also includes the values of ${}^iK_{2T}$, calculated from Eq. **5**, which are corrected by the same statistical factors. For comparison it also includes values from the experimental data of Mills *et al.* (18). The values calculated from the MWC model for $i = 1$, 2, and 3 are indeed in quite satisfactory agreement with the experimental evidence. (The values for $i = 0$ and 4 are assumed from experimental data by the postulates of the model.)

Another useful function is obtained by considering the total concentrations of the two tetramers and the dimer as a function of free ligand concentration, $[x]$. This is essentially the same function defined in equation 10 of ref. 13 and denoted there by the symbol ${}^xK'_2$, which we also employ. It can be written as the sum of two terms, ${}^xK'_{2T}$ and K'_{2R}. The latter, as we have seen, is a constant independent of $[x]$.

$$ {}^xK'_2 = \left(\sum_{i=0}^{4}[T_i] + \sum_{i=0}^{4}[R_i]\right) \Big/ \sum_{i=0}^{2}[D_i]^2 = \frac{[T_0]P_T + [R_0]P_R}{[D_0]^2 P_R} $$

$$ = {}^0K_{2T}\,(P_T/P_R) + K'_{2R} $$

$$ = K'_{2R}\,[1 + L(1 + c\alpha)^4/(1 + \alpha)^4] . \quad [7] $$

Hence, we have used the values of P_R and P_T from Eqs. **1** and **2**, the relation $P_D^2 = P_R$, and the relation ${}^0K_{2T} = L\,K'_{2R}$ from Scheme **1**. As $[x]$ becomes very large, the term in brackets in the last equality of Eq. **7** approaches a limiting value of $1 + Lc^4$ as when $i = 4$ in Eq. **6**. In Fig. 1 we have plotted $\Delta^xG'_2 = -RT \ln {}^xK_2$ as a function of $-\log[x]$ and also the corresponding values of $\Delta^xG'_{2T}$. At low ligand concentrations, $\Delta^xG'_2$ is virtually identical with $\Delta^xG'_{2T}$; at higher concentrations it becomes virtually identical with $\Delta G'_{2R}$, which is shown as a horizontal line. The transition zone for $\Delta^xG'_2$ lies in the range $-\log[x] = 5.0 \pm 0.5$. The midpoint ($\overline{Y} = 1/2$) of the ligand-binding curve for tetramers lies in the same range. The value of $\Delta^xG'_{2T}$ continues to drop, approaching a lower limiting value of approximately -2.4 kcal/mol at saturation with ligand, as also seen in Table 2.§ Thus, $-\Delta^xG'_{2T}$ decreases altogether by about 12 kcal/mol as i increases from 0 to 4; this occurs in four steps, each involving a decrease in $-\Delta^iG'_2$ of about 3 kcal/mol, corresponding to the term $-RT \ln c$.

Examination and Revision of Weber's Analysis of Ligand Binding and Subunit Association

Weber's analysis of the MWC model was based on consideration of the ΔG_2 values for macromolecules with 4 ligand-binding sites (i) in three states of ligand binding: unliganded, biliganded, and completely liganded. His criterion of what he termed the order of free-energy couplings is the quantity $I(4)$, defined in our notation by:

$$ I(4) = [\Delta^4G_2 - \Delta^2G_2]/[\Delta^2G_2 - \Delta^0G_2] . \quad [8] $$

From his analysis, he concluded that if $I(4) >> 1$, the order of free energy couplings is higher than the second, whereas if $I(4) << 1$, the coupling is second order or less. The values of Δ^4G_2 and Δ^0G_2 are well established (see Tables 1 and 2). The uncertainty in calculating $I(4)$ depends on the calculation of Δ^2G_2. Weber calculated this from the proportion of R and T tetramers present when $i = 2$, giving the T/R ratio $Lc^2 = 1.93/1$ or roughly 2/1. Weber assumed that $\Delta^iG'_{2T}$ was a constant, independent of i. Therefore, he multiplied the fraction of T (2/3) by Δ^0G_2 for deoxy-Hb (-14.3) and the fraction of R (1/3) by Δ^4G_2 for HbO_2 (-8.0), thereby obtaining $\Delta^2G_2 = 12.2$ kcal/mol. This gave a value of $I(4) = 1.8$. From this he concluded that the order of free-energy couplings was higher than the second and inferred that the

Table 2. Calculated and experimental dimer–tetramer association constants and standard free energies for Hb A at successive stages of ligand binding

Δ^iG_{2T}	i	s	${}^iK_2/M^{-1}$	Δ^iG_2 Calculated	Δ^iG_2 Experimental
−14.3	0	1	4.6×10^{10}	−14.3	−14.4
−11.7	1	2	5.4×10^{8}	−11.7	−11.9
−9.4	2	6	1.5×10^{7}	−9.7	−9.4
−5.8	3	2	1.7×10^{6}	−8.4	−8.2
−2.4	4	1	8.5×10^{5}	−8.0	−8.1

Values for the MWC model are calculated from parameters in Table 1 by the Ackers–Johnson equation (equation 24 in ref. 13; see our Eq. **6**):

$$ {}^iK_{24,tot} = s_i\,{}^iK'_{2R}\,(1 + Lc^i). $$

Here i denotes the number of ligands bound per tetramer; s is a statistical factor depending on the number of distinct microscopic forms in the total equilibrium for dimer and tetramer at the various stages of ligand binding. For $i = 2$, $s = 6$ because there are two ways to form a biliganded tetramer from a dimer—either from $DX_2 + D$, or from DX + DX. Also the biliganded tetramer can exist in six microscopic forms, either symmetric: $\alpha\beta X{\cdot}\alpha\beta X$ (four forms) or assymetric: $\alpha\beta X_2{\cdot}\alpha\beta$ (two forms). We do not attempt to present the complete statistical analysis here. The Δ^iG_2 values, from experimental data, are from table VI of ref. 14, which lists intrinsic Δ^iG_2 values as well as the statistically corrected values listed here. A later study (16) over a range of temperatures gives $\Delta^3G'_2$ at 21.5°C as -7.0, which gives -7.4 after correction for the statistical factor.

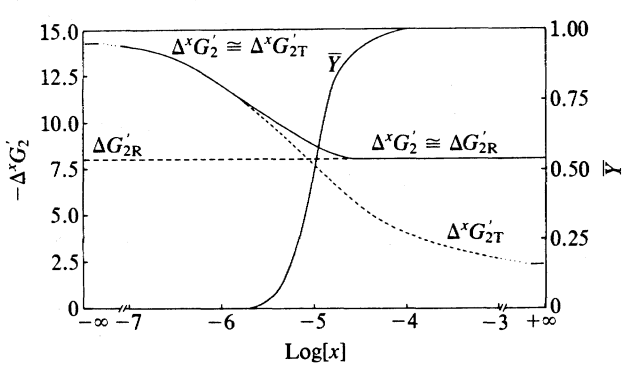


FIG. 1. Gibbs free-energy values for the tetramer–dimer equilibria as a function of ligand activity, log $[x]$, for the T and R tetramers and the system as a whole. Data were calculated using Eq. **7** with the numerical values for K_T and K_D and ${}^0K_{2T}$ given in Table 1. The $\overline{Y}$ curve shows the course of ligand binding by the tetramers as calculated from equation 2 of ref. 2.

§Equation **7** is an approximation, since it neglects the decrease in total tetramers that accompanies the increased dimer formation associated with ligand binding. It is a good approximation at relatively high Hb concentration, where the mole fraction of dimers is always small. The conservation equation, in terms of molar concentrations of $\alpha\beta$ subunits, is $2(\Sigma T_i + \Sigma R_i) + \Sigma D_i =$ a constant; but the refinements needed to take account of this relation in dilute Hb solutions need not concern us here. This statement also applies to Eq. **4**.

MWC model would incorrectly predict increased association of dimer to tetramer with increased ligand binding, if indeed the model represented a two-state system.

On inserting the corrected value of $\Delta^2G_2 = -9.7$ kcal/mol from Table 2, we see that $I(4)$ decreases to about 0.3—a value that Weber considers quite consistent with first-order coupling. However, as we have already indicated, the MWC system is not in fact a two-state system in the strict sense that Weber has assumed but must be considered as existing in at least six cooperative free energy levels: five for the T tetramer and one for R. Such a multistate system could well be compatible with Weber's interpretation of the observed findings on tetramer–dimer equilibrium.

It may be helpful to summarize the qualitative picture of the shift in the tetramer–dimer equilibrium as ligand binding proceeds. Starting in the absence of ligand, the model protein is nearly all in the form T_0, with a minute trace of R_0 and extremely little dimer. As ligand is added, the formation of dimer increases by two paths: (*i*) T is increasingly converted to R, which dissociates into dimer much more readily than does T_0, and (*ii*) as T_0 changes to T_1, T_2, T_3, and T_4, it becomes progressively destabilized. It isomerizes to R, and also increasingly dissociates directly to form D, as seen from Scheme 1. Fig. 2 presents a picture of the overall relationship of the energy levels of T, R, and D at various stages of ligand binding.

Discussion

The preceding analysis, we believe, demonstrates that the MWC model predicts correctly that dissociation of tetramer to dimer increases with increased ligand binding. Given this evidence, we comment on two major questions. (*i*) In what sense is the model to be regarded as a two-state system? (*ii*) What relation does the model bear to the actual Hb molecule?

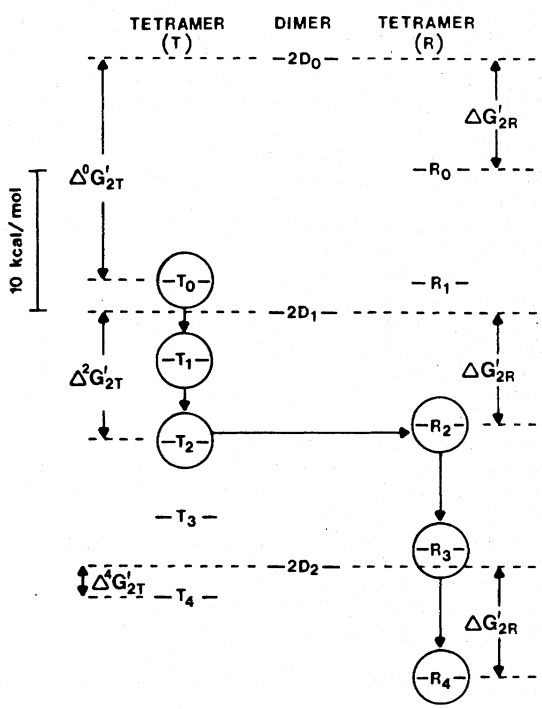


FIG. 2. The various states of Hb tetramers (R and T) dimers on a scale of Gibbs free energy. For dimers, D, and tetramers in the R and T states, the integer after the letter corresponds to the number of oxygen molecules bound. The species circled refer to the major states in the sequence of ligand-binding events. Energies of tetramer stabilization relative to the corresponding dimer species are indicated on the far left for the T state and on the far right for the R state. While the stabilizations for the R states indicated remain constant relative to the dimers, the energies of stabilization of T-state tetramers relative to the corresponding dimer states diminish with additional ligands bound.

The original authors of the model (2) defined it in terms of only two intrinsic ligand-binding constants, one for the R form and one for the T form, each being independent of the extent of ligand binding. In that paper they did not consider tetramer–dimer equilibria. In the absence of previous explicit discussion, some investigators have apparently assumed that both K'_{2R} and K'_{2T} must be unaffected by the state of ligand binding in a two-state system. Others including ourselves formerly shared this assumption; but examination of Scheme 1 and of Eqs. 5 and 6 shows that it is in fact thermodynamically impossible. When K'_{2R} is held constant, K'_{2T} must vary over a wide range as bound ligand increases from 0 to 4. Ackers and Johnson (13) obviously were aware of this fact, but did not emphasize it explicitly.

Thus, in the model, as Figs. 1 and 2 emphasize, the T isomer undergoes a progressive change in cooperative free energy as ligand is bound. Each added ligand changes $\Delta G'_{2T}$ by $\approx$3 kcal/mol. This change arises from the term $RT \ln c$ involved in each successive step in ligand binding and is close to 3. Thus T, unlike R, must exist in at least five different levels of cooperative free energy. Therefore, should we not consider the MWC model as at least a six-state system, with five levels for T and one for R? The answer must be to some extent a matter of definition; but in this broader sense, it would seem that we are justified in considering it a multistate system. Indeed, it cannot be otherwise, since the alternative is thermodynamically impossible. Since Weber's critique of the model was based largely on the assumption that it represented a two-state system in the strictest sense, these considerations would appear to remove the grounds for his concern.

This brings us to the question: what in fact are the relations between the MWC model and the actual Hb system? Contact with reality is established by the postulates that deoxy-Hb corresponds to the unliganded T tetramer, and HbO_2, to R_4. The T tetramer, as the present analysis shows, is progressively destabilized with respect both to the R tetramer and to the dimer at each step in ligand binding, with an increment of about 3 kcal/mol in $\Delta^iG'_{2T}$ for each unit increase in i. This suggests that even the first step in the binding of O_2 to deoxy-Hb must involve significant conformational alterations, whereas the second step brings the free energy of dissociation into dimers fairly close to the value for completely liganded Hb. Overall, the inescapable conclusion is that constant affinity for a ligand of the T state at all levels of saturation must be accompanied by an increased propensity for dissociation into dimers. Thus, to maintain a single ligand-binding constant for T at all stages of ligand binding would require a perfect compensation in increased dimer dissociation (Fig. 2). However, perfect consistency of one property (ligand binding) requiring the precise adjustment of another property (dimer–tetramer association–dissociation) would seem surprisingly arbitrary. Rather, it would seem more plausible for some compromise to be achieved, with ligand affinity rising slightly and therefore dimer–tetramer equilibrium constants falling less dramatically as ligation proceeds. New experimental findings bearing on this point will be awaited with interest.

In any case, the relation of the calculations on the liganded T tetramer in the model can be only suggestive in relation to the actual events associated with the binding of the first two ligands to deoxy-Hb. A recent important study by Smith and Ackers (17) identifies three distinct levels by cooperative free energy in ligand binding to Hb. In their system, each subunit is either in the unliganded state (Fe^{2+}, deoxy) or is "ligated" by conversion to the cyanomet form (Fe^{3+}, —CN). Besides the completely liganded and completely unliganded species,

there are eight distinguishable intermediates. The first upper level of cooperative free energy, relative to the deoxy state, differs from it by about 3 kcal/mol; this transition occurs on binding a single ligand to either an α or a β chain. The next level again differs from the first by about 3 kcal/mol; this is essentially the same level as that for completely liganded Hb. It is reached when either the second or the third ligand is bound, depending on the pattern of binding to the α and β subunits.

It appears not merely a coincidence that the energy levels found by Smith and Ackers correspond roughly to those of T_0, T_1, and T_2 in the present paper. In the MWC model, of course, the factor of 3 kcal/mol arises simply from the factor $RT \ln c$ that relates the different levels of ligand binding. However, the chosen value of c does arise from the attempt to fit the model to experimental data; so it is not surprising that the value of 3 kcal/mol emerges. Thus, the T-state tetramer with even a single molecule of ligand bound can no longer be considered as virtually identical in atomic structure to unliganded T. Conclusions along these lines have already been implicit in the studies of partially oxidized or liganded T-state Hb crystals (18, 19). In general, the MWC model does behave like the actual Hb system in predicting increased dissociation of tetramers to dimers as ligand binding increases. It also emerges as a multistate, not a two-state, model in the particular sense that we have explained above. In this respect we are definitely in accord with Weber's views that the two-state model as narrowly formulated must be rejected and is indeed thermodynamically impossible.

We make no claim that the MWC model provides a fully adequate explanation for the properties of Hb A or other Hbs. Models are of use as heuristic devices, and the MWC model has provided a convenient first-order description of many of the properties of Hb. Ackers and Johnson (13) found that they could not obtain a really satisfactory fit to their experimental data using the best achievable MWC parameters (for their values see our Table 1), whereas they could obtain a fully satisfactory fit by a model-independent analysis. A significant divergence between the model and experimental data appears in the phenomenon of quaternary enhancement: the binding of the fourth O_2 molecule to triliganded Hb occurs with a value of $-\Delta G$ significantly greater than that characteristic of O_2 binding to the dimer. The difference in $-\Delta G$ is substantial, $\approx$0.8 kcal/mol on the average, and the phenomenon persists over a range of temperatures from 10°C to 37°C (16) and of pH from 7.4 to 9.5 (20). In contrast the MWC model predicts that the difference should be zero—i.e., that $K_R = K_D$—as we have seen. Corresponding to the quaternary enhancement of ligand binding is the fact that $Hb(O_2)_4$ has less tendency to dissociate to dimer than does $Hb(O_2)_3$. The binding of the fourth ligand stabilizes the tetramer (20), a finding that could not have been predicted from the model.

Our discussion has concentrated on the simplest type of MWC model, with all four ligand-binding sites equivalent. We have not mentioned the changes in tertiary structure that accompany ligand binding and the interactions of such changes in individual subunits with the constraints imposed by the quaternary oxy and deoxy structures (8, 18, 21). Extensive studies (22, 23) have probed such interactions in penetrating detail. Statistical mechanical models depending on evidence of this sort have gone far to describe cooperative binding and quaternary enhancement (24). These studies have involved the tetramer only, without need to consider the dimer. This work lies outside the central focus of our discussion, which deals with the internal logic of the MWC model and its relation to the dimer–tetramer equilibrium.

This article was initiated while one of us (S.J.E.) was on sabbatical leave in Paris for the 1984–1985 academic year; he thanks colleagues J. Rosa, Y. Beuzard, C. Poyart, M. Goossens, and D. Bonne for their hospitality and discussions and A. M. Dulac for assistance in preparation of the early drafts of this article. We give special thanks to G. Ackers for his extensive discussions and comments on earlier versions of this article and to J. Wyman, G. Guidotti, J. M. Baldwin, M. Karplus, S. Gill, Q. Gibson, and G. Weber for reading earlier versions. We thank J. Broadhead and J. Guidotti for help in preparing successive drafts of the manuscript. Support of a Senior International Fellowship from the Fogarty Center (to S.J.E.) is also gratefully acknowledged. J.T.E. acknowledges support from NEH Grant RH-20593-85.

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