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The energetics of ligand-linked subunit assembly in hemoglobin require a third allosteric structure

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For partially ligated cyanomet hemoglobins, Smith and Ackers (Proc. Natl. Acad. Sci. U.S.A. 71 (1985) 4312) determined the free energies of dimer-tetramer assembly for all of the partially ligated species using a combination of kinetic and equilibrium methods. They found a third apparent cooperative free energy level in addition to those of deoxy- and cyanomethemoglobin. Using cryogenic methods, Perrella et al. (Biophys. Chem. 35 (1990) 97) confirmed the existence of the third cooperative free energy level, but found a different energy level assignment for one of the species. These combined studies have yielded a solid data base for considering mechanistic issues. The number of cooperative free energies ΔG_c can, in principle, be different from the number of molecular forms which have unique free energies of heme-heme interaction, since ΔG_c can be an average over conformational subspecies. Furthermore, since the ΔG_c values are determined from free energies of dimer-tetramer assembly, it is necessary to evaluate possible contributions from dimeric properties, and from quaternary constraint (or enhancement) effects associated with subunit assembly. In this paper we analyze the observed distributions of apparent ΔG_c values among the various ligation states in terms of mechanisms based on two interconvertible molecular forms (R and T) under the most general conditions in which (i) dimers may be cooperative, (ii) ligand affinities of α-subunits may be different within tetramers and dimers, and the same for β-subunit affinities, and (iii) dimers need not be halves of R-state tetramers. It is found that the experimental distributions are inconsistent with even the most general model of the two-state class; thus, at least *three molecular forms of tetramer are required*, each with an individually different value of cooperative free energy (heme-heme interaction). This result implies the existence of at least three corresponding molecular structures; while a degeneracy of multiple structures into only a few dominant free energy levels is frequently to be expected, the reverse situation is extremely unlikely.

1. Introduction

A major goal of biophysical chemistry is to understand the mechanisms of regulatory interactions in protein assemblies. Human hemoglobin has long served as a prototype for understanding how such assemblies can utilize subunit interactions to effect regulation of biological functions. Much of the work on allosteric proteins has been based upon, or utilized, the allosteric model of Monod et al. [1], in which the molecules exhibit only two allosteric states, i.e., two molecular conformations with different ligand affinities. Two-state models have traditionally been successful in decribing many aspects of highly cooperative processes (e.g., protein folding) where the properties of intermediates are inaccessible due to their low abundance. Recent work on partially ligated hemoglobin species, however, has indicated that the tetramer can assume more than two forms that have different affinities, i.e., more than two allosteric structures.

In 1985, Smith and Ackers [2] reported evidence for the existence of a third allosteric form.

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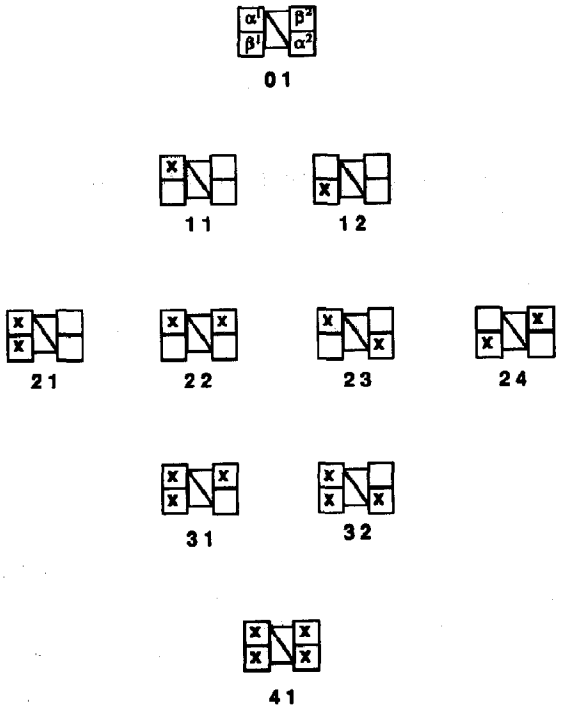


Fig. 1. Topographic representation of the 10 ligation species of tetrameric hemoglobin. The index *ij* denotes the particular species *j* among those with *i* ligands bound ($i = 0$–4; $j = 1$–4). Ordering of species with respect to *j* is arbitrary. Subunit positions are shown in species 01.

Using analytical gel chromatography in combination with kinetic methods they determined free energies of subunit assembly (dimers to tetramers) for each of the ten tetrameric species, with a structurally unique combination of ligated and unligated subunits using partially ligated cyanomethemoglobins (see fig. 1). These data yielded the striking result that only three distinct free energies of dimer-tetramer assembly exist among the ten species. The lowest free energy (−14.3 kcal/mol) was found uniquely for deoxyhemoglobin (species 01, fig. 1) and the highest value (−8.5 kcal/mol) was observed for five of the species: fully ligated (species 41), the two species with three subunits in cyanomet form (31 and 32) and the two symmetric doubly ligated species (23 and 24). A third free energy (−11.4 kcal/mol) was found for the two singly ligated species (11 and 12) and the two asymmetric doubly ligated species (21 and 22), i.e., with ligands on the α_1- and β_1-subunits or on the α_1- and the β_2-subunits. A subsequent study [3] using cryogenic quenching and low-temperature electrophoresis has confirmed the existence of these three cooperative free energy levels. These results, along with further kinetic analyses [3], have provided compelling evidence for reassignment of species 22 to the highest free energy level (i.e., −8.5 kcal). The current results are summarized in table 1 and fig. 2. Assuming the dissociated dimeric species to be noncooperative and the ligand affinities of the α- and β-subunits to be identical, it was shown [4,5] that this distribution of free energies was incompatible with the simplest two-state mechanism of allosteric control [6]. A quantitatively similar result was found with two other ligand-analog systems in which the hemes had been replaced with manganese [5].

In general, the existence of three distinct assembly free energies among the ten ligation species of hemoglobin tetramers does not by itself mean that there must be three corresponding allosteric structures for the following reasons:

(a) The tetrameric (MWC) system with only two molecular structures can have assembly free energies for partially ligated species that are intermediate between those of the end-state species 01 and 41. As shown by Ackers and Johnson [6], a value of −11.4 kcal for singly ligated molecules

Table 1

Free energies of dimer-tetramer assembly for the ten ligation species of tetrameric hemoglobin

Experimental conditions: 0.1 M Tris-HCl, 0.1 M NaCl (pH 7.4), 21.5° C. Values are in kcal.

Species	Fe(II)/ Fe(III) CN	Fe(II)/ Mn(III)	Mn(II)/ Fe(II) CO
01	−14.4± 0.1	−14.4±0.1	−15.6±0.5
11	−11.3± 0.2	−11.5±0.2	–
12	−11.2± 0.2	−10.7±0.2	–
21	−11.4± 0.2	−11.0±0.2	−13.1±0.5
22	−8.3± 0.2	−7.8±0.3	–
23	−8.2± 0.2	−7.6±0.2	−7.8±0.5
24	−8.5± 0.2	−8.2±0.2	−8.3±0.5
31	−8.6± 0.2	−7.9±0.2	–
32	−7.9± 0.2	−7.9±0.2	–
41	−8.5± 0.1	−7.5±0.1	−8.0±0.1

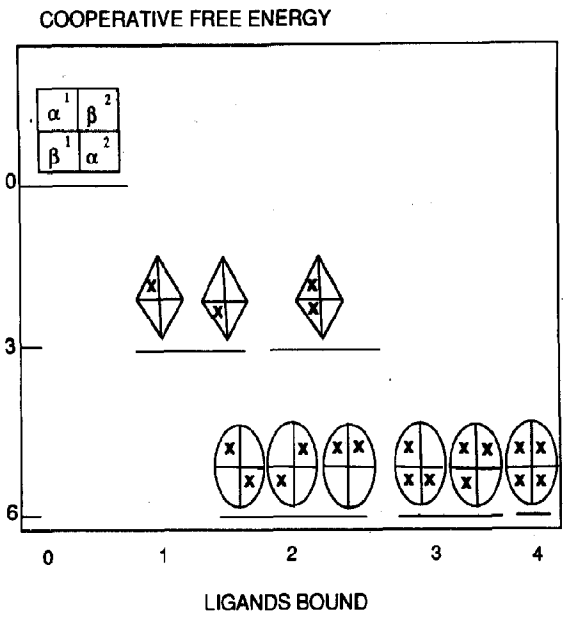


Fig. 2. The apparent cooperative free energy levels for partially ligated (cyanomet) tetramers in various ligation states. Configurations of ligated subunits (denoted by X) correspond to those of fig. 1. Differently shaped molecules indicate symbolically the possible distribution of different molecular.

can arise from the 'MWC average' of −14.4 and −8.0 kcal for unligated and fully ligated species, respectively. In that case, the intermediate free energy is a composite of values from the R- and T-state molecules and does not represent any single molecular species or structure. This follows from eq. 1 which is a principal result of the two-state MWC model as extended by Ackers and Johnson [6]. (For simplicity this model will be referred to as 'The AJ model'.)

$$ {}^{i}K_2' = K_{2R}'(1 + Lc^{i}) \qquad (1) $$

Here ${}^{i}K_2'$ is the intrinsic equilibrium constant (corrected for statistical factors) for assembly of a tetramer with i ligated subunits ($i = 0$–4) from noncooperative dimers, and K_{2R}' is that for forming an R-state tetramer from two dimers. The allosteric constant L characterizes the equilibrium between unligated conformers (R and T) while c is the ratio of their affinities for ligand (see refs 1 and 6). Each dimer is half of an R-state tetramer in the simplest version of the AJ model and the constant K_{2R}' is independent of the degree of ligation, i. With normal hemoglobin binding oxygen, the parameters L and c were evaluated as 5.2×10^4 and 6.1×10^{-3}, respectively, while K_{2R}' is 8.6×10^5. These values lead via eq. 1 to ${}^{1}K_2 = 2.7 \times 10^8$ or ${}^{1}\Delta G_2 = 11.4$ kcal.

(b) The ignoring of any cooperativity (or anti-cooperativity) in the two steps of dimer ligation could lead to an incorrect evaluation of tetrameric free energies for the ten ligation species. Although strong evidence indicates dimer cooperativity to be no more than a few tenths of a kcal with oxygen as ligand [7], virtually nothing is known about these effects in the cyanomet and manganese hemoglobin systems. As a formal possibility, significant dimer cooperativities must be allowed in the most general model.

(c) The intrinsic ligand affinities might be different for α-subunits within dimers compared with tetramers, and the same for the β-subunits. In principle, these 'quaternary constraint' or 'quaternary enhancement' effects need not even be the same for the two kinds of subunits.

Thus while the model of Monod et al. [1] as extended by Ackers and Johnson [6] to include the effects of dimer-tetramer assembly provides the theoretical background for treatment of this problem, it is necessary to extend the formal relationships even further in order to test rigorously the experimental findings against a most general two-state mechanism of cooperative switching. That is the primary goal of this paper. Because of the critical significance of a third allosteric structure to the mechanistic understanding of hemoglobin, the new 'polysteric' model developed here and its application to the recent experimental results (table 1) will be discussed. The derivation of the model itself is given in the appendix. Section 2 describes the fundamental result of that derivation and its application to the experimental findings. By way of introduction it will be desirable to consider some preliminary concepts regarding cooperative free energies and their relationship to subunit assembly.

1.1. Cooperative free energies

It is useful to transform an observed distribution of assembly free energies such as that in table

1 into a corresponding distribution of cooperative free energies. ${}^{ij}\Delta G_c$ is defined as the deviation in tetramer binding free energy from that for ligation of the same sites with their intrinsic free energies.

$$ {}^{ij}\Delta G_c = \Delta G_{ij} - i_\alpha \Delta G^\alpha - i_\beta \Delta G^\beta \quad (1) $$

where ΔG_{ij} is the standard Gibbs free energy for reacting i moles of ligand with a tetramer in configuration ij, and the last two terms are the intrinsic free energy per site for the same reaction in the absence of cooperativity (i_α and i_β denote the numbers of ligands bound to each type of subunit while ΔG^α and ΔG^β are the corresponding intrinsic free energies). In hemoglobin tetramers these last terms generally sum to a larger negative value (higher affinity) than ΔG_{ij} so that ${}^{ij}\Delta G_c$ is positive. A finding that ${}^{ij}\Delta G_c = 3.0$ kcal for species 21, for example, would mean that when 1 mol of species 01 is reacted with 2 mol of ligand to form species 21, the standard Gibbs free energy changes by $(\Delta G_\alpha + \Delta G_\beta + 3.0)$ kcal; in the absence of cooperative interactions the free energy change per mol tetramer would be just $\Delta G_\alpha + \Delta G_\beta$.

Subunit interactions that generate the cooperative free energies are decoupled by dissociation of the hemoglobin tetramer into $\alpha^1\beta^1$ dimers, which are generally believed to bind two ligands only with the intrinsic free energy $(\Delta G_\alpha + \Delta G_\beta)$. The thermodynamic coupling between ligand binding and reversible dissociation of tetramers into dimers has thus proved to be a powerful and conceptually simple means of measuring cooperative free energies [7]. When the dimers are noncooperative the determination of dimer-tetramer assembly free energies can be used to deduce the cooperative free energy directly. All that is required are two assembly free energies – one for the tetrameric species of interest and a second one for the unligated reference molecule. Since the Gibbs free energy is a state function (independent of path), the free energies around the appropriate cycle must sum to zero. Thus, for doubly ligated species 21, we have

$$ {}^{21}\Delta G_2 - {}^{01}\Delta G_2 = \left(\Delta G^\alpha + \Delta G^\beta + {}^{21}\Delta G_c\right) - \left(\Delta G^\alpha + \Delta G^\beta\right) \quad (2) $$

where terms in parentheses are ligation free energies of the tetramers and (noncooperative) dimers. Eq. 2 reduces to

$$ {}^{21}\Delta G_c = {}^{21}\Delta G_2 - {}^{01}\Delta G_2. \quad (2a) $$

Therefore, an experimental evaluation of the two free energies of dimer-tetramer assembly provides a determination of the cooperative free energy ${}^{21}\Delta G_c$ (i.e., free energy of heme-heme interaction) without actual measurement of the ligand binding equilibria. However, if the dimers are cooperative an additional term $\Delta G_{\alpha\beta}$ must be included in the rightmost sum of eq. 2, leading to the result:

$$ \left({}^{21}\Delta G_2 - {}^{01}\Delta G_2\right) = {}^{21}\Delta G_c - \Delta G_{\alpha\beta} \quad (2b) $$

Thus, the experimental quantity on the left, which we shall call the 'apparent cooperative free energy' ${}^{21}\Delta G_c'$ may differ from the true cooperative free energy ${}^{21}\Delta G_c$ by an amount $\Delta G_{\alpha\beta}$. In general, this situation may arise in four of the species (i.e., species 21, 31, 32, and 41) as illustrated in table 2. The general linkage expression for these species is

$$ \left({}^{ij}\Delta G_2 - {}^{01}\Delta G_2\right) = \left(i_\alpha \Delta G^\alpha + i_\beta \Delta G^\beta + {}^{ij}\Delta G_c\right) - \left(i_\alpha \Delta G^\alpha + i_\beta \Delta G^\beta + n_D \Delta G_{\alpha\beta}\right) \quad (3a) $$

where n_D is the number of $\alpha^1\beta^1$ dimers in the assembled tetrameric species of interest. This expression reduces to

$$ {}^{ij}\Delta G_c' = {}^{ij}\Delta G_c + n_D \Delta G_{\alpha\beta} \quad (3b) $$

Table 2

Tetrameric cooperative free energies as a function of dimer cooperativity $\Delta G_{\alpha\beta}$ and experimental assembly free energies

ij	${}^{ij}\Delta G_c$	n_D
11	$({}^{11}\Delta G_2 - {}^{01}\Delta G_2)$	0
12	$({}^{12}\Delta G_2 - {}^{01}\Delta G_2)$	0
21	$({}^{21}\Delta G_2 - {}^{01}\Delta G_2) + \Delta G_{\alpha\beta}$	1
22	$({}^{22}\Delta G_2 - {}^{01}\Delta G_2)$	0
23	$({}^{23}\Delta G_2 - {}^{01}\Delta G_2)$	0
24	$({}^{24}\Delta G_2 - {}^{01}\Delta G_2)$	0
31	$({}^{31}\Delta G_2 - {}^{01}\Delta G_2) + \Delta G_{\alpha\beta}$	1
32	$({}^{32}\Delta G_2 - {}^{01}\Delta G_2) + \Delta G_{\alpha\beta}$	1
41	$({}^{41}\Delta G_2 - {}^{01}\Delta G_2) + 2\Delta G_{\alpha\beta}$	2

2. General allosteric theory for ligand-linked assembly

It can be seen in table 2 that the apparent cooperative free energies of triligated tetramers might also be influenced by a contribution from dimer cooperativity ($n_D = 1$) while that of the fully ligated molecule could contain two such contributions. Thus, any significant value of $\Delta G_{\alpha\beta}$ could lead to a distorted picture of the cooperative free energy distribution and its interpretation in terms of the number of allosteric states for tetramers. It was therefore necessary to reformulate the AJ model [6] to take these effects into account. In the present generalization of the model the constraint of noncooperativity in dimers has been relaxed. An equally important feature is that the intrinsic ligand affinities are allowed to be different for α-subunits within dimers compared to tetramers (and the same for β-subunit affinities). In addition, these energetic effects, denoted by δ_α and δ_β need not be the same for the two kinds of subunits. These effects of quaternary constraint and quaternary enhancement have been shown to be necessary to explain the oxygenation-linked assembly of hemoglobin [6–8]. For ligands like cyanide and the metal-substituted hemoglobins there is presently no information regarding the magnitudes of these effects. It was thus necessary to allow for them in their most general form (see the appendix).

The generalized form of eq. 1 that results from these considerations (see the appendix for derivation) is:

$$^{ij}K_2 = S\Delta K_{2R}\left[1 + Lc_\alpha^p c_\beta^q\right] \tag{5}$$

where $^{ij}K_2$ is the equilibrium constant for assembly of species ij from its constituent dimers. K_{2R} is the equilibrium constant for formation of R-state tetramers of species 01, c_α represents the ratio of α-subunit affinities K_T^α/K_R^α while c_β is the corresponding ratio for β-subunits. The exponents p and q are integers given in table 3. The parameter Δ incorporates factors for dimer cooperativity ($\delta_{\alpha\beta}$) and for the ratio of intrinsic ligand affinities in dimers relative to R-state tetramers (δ_α) and the same for β-subunits (δ_β). The appropriate combination of these factors for each of the ten species is given in table 3. Note that eq. 5 reduces to eq. 1 under the appropriate simplifications discussed above.

Table 3

Components of the subunit assembly equation [$^{ij}K_2 = S\Delta K_R(1 + Lc_\alpha^p c_\beta^q)$] for the ten ligation species of tetrameric hemoglobin

ij	S	Δ	p	q
01	1	1	0	0
11	2	δ_α	1	0
12	2	δ_β	0	1
21	2	$\delta_\alpha\delta_\beta\delta_{\alpha\beta}$	1	1
22	2	$\delta_\alpha\delta_\beta$	1	1
23	1	δ_α^2	2	0
24	1	δ_β^2	0	2
31	2	$\delta_\alpha\delta_\beta^2\delta_{\alpha\beta}$	1	2
32	2	$\delta_\alpha^2\delta_\beta\delta_{\alpha\beta}$	2	1
41	1	$\delta_\alpha^2\delta_\beta^2\delta_{\alpha\beta}^2$	2	2

By means of eq. 5 we can formulate expressions for the apparent cooperative free energy $^{ij}\Delta G_c'$ of each species:

$$^{ij}\Delta G_c' = -RT\ \ln\frac{^{ij}K_2}{^{01}K_2} = -RT\ \ln\frac{\Delta\left[1 + Lc_\alpha^p c_\beta^q\right]}{1 + L} \tag{6}$$

Specific formulas for each of the ten ligation species are given in table 4 along with experimental values for the three systems.

2.1. Application to the experimental results on CN-methemoglobin

Here we describe applications of the generalized AJ model to analysis of the distribution of $^{ij}\Delta G_c'$ values for the cyanomet system (table 4), because the most detailed information is presently available for this system and it is also representative of the observed behavior in the other two systems. The experimental values of $^{ij}K_2$ obtained in the cyanomet system lead, by the left-hand equality of eq. 6, to three distinct values of $^{ij}\Delta G_c'$ for the ten ligation species as shown in table 4 and fig. 2. Cooperative free energies for species 11, 12, and 21 are found to be equal, leading, after multi-

Table 4

Cooperative free energies for partially ligated hemoglobins in terms of the extended two-state MWC model

Apparent cooperative free energy expression [a]	Experimental values (kcal)		
	Fe(II)/Fe(III) CN	Fe(II)/Mn(III)	Mn(II)/Fe(II) CO
$^{01}\Delta G_c' = -RT \ln \frac{1+L}{1+L}$	0(ref.)	0(ref.)	0(ref.)
$^{11}\Delta G_c' = -RT \ln \frac{\delta_\alpha(1+Lc_\alpha)}{1+L}$	3.1±0.3	2.9±0.3	–
$^{12}\Delta G_c' = -RT \ln \frac{\delta_\beta(1+Lc_\beta)}{1+L}$	3.2±0.3	3.7±0.3	–
$^{21}\Delta G_c' = -RT \ln \frac{\delta_\alpha\delta_\beta\delta_{\alpha\beta}(1+Lc_\alpha c_\beta)}{1+L}$	3.0±0.3	3.4±0.3	2.5±0.7
$^{22}\Delta G_c' = -RT \ln \frac{\delta_\alpha\delta_\beta(1+Lc_\alpha c_\beta)}{1+L}$	6.0±0.3	6.6±0.4	–
$^{23}\Delta G_c' = -RT \ln \frac{\delta_\alpha^2(1+Lc_\alpha^2)}{1+L}$	6.2±0.3	6.8±0.3	7.8±0.7
$^{24}\Delta G_c' = -RT \ln \frac{\delta_\beta^2(1+Lc_\beta^2)}{1+L}$	5.9±0.3	6.2±0.3	7.3±0.7
$^{31}\Delta G_c' = -RT \ln \frac{\delta_\alpha\delta_\beta^2\delta_{\alpha\beta}(1+Lc_\alpha c_\beta^2)}{1+L}$	5.8±0.3	6.5±0.3	–
$^{32}\Delta G_c' = -RT \ln \frac{\delta_\alpha^2\delta_\beta\delta_{\alpha\beta}(1+Lc_\alpha^2 c_\beta)}{1+L}$	6.5±0.4	6.5±0.3	–
$^{41}\Delta G_c' = -RT \ln \frac{\delta_\alpha^2\delta_\beta^2\delta_{\alpha\beta}^2(1+Lc_\alpha^2 c_\beta^2)}{1+L}$	5.9±0.2	6.9±0.3	7.6±0.6

[a] The apparent cooperative free energy $^{ij}\Delta G_c'$ is the difference in dimer-tetramer assembly free energies ($^{ij}\Delta G_2 - {}^{01}\Delta G_2$).

plication by $(1+L)$, to the equalities given below as set A:

$$\delta_\alpha(1+Lc_\alpha) \underset{\text{(i)}}{=} \delta_\beta(1+Lc_\beta) \underset{\text{(ii)}}{=} \delta_\alpha\delta_\beta\delta_{\alpha\beta}(1+Lc_\alpha c_\beta) \tag{A}$$

We can also write five equalities from the observed identity in cooperative free energies for the six species 22, 23, 24, 31, 32, 41 (set B):

$$\delta_\alpha\delta_\beta(1+Lc_\alpha c_\beta) \underset{\text{(iii)}}{=} \delta_\alpha^2(1+Lc_\alpha^2) \underset{\text{(iv)}}{=} \delta_\beta^2(1+Lc_\beta^2) \underset{\text{(v)}}{=} \delta_\alpha\delta_\beta^2\delta_{\alpha\beta}(1+Lc_\alpha c_\beta^2) \underset{\text{(vi)}}{=} \delta_\alpha^2\delta_\beta\delta_{\alpha\beta}(1+Lc_\alpha^2 c_\beta) \underset{\text{(vii)}}{=} \delta_\alpha^2\delta_\beta^2\delta_{\alpha\beta}^2(1+Lc_\alpha^2 c_\beta^2) \tag{B}$$

The two sets of equalities, A and B, provide a description of the experimental observations to within their experimental accuracy in terms of the generalized two-state allosteric mechanism. We note that each of the six terms in set B differs from each term of set A by approximately two orders of magnitude, corresponding to $e^{3/RT}$. The data also provide one additional piece of information in that the cooperative free energy for species 01 is also different from those of set A by 3 kcal. We shall make use of these facts shortly. Altogether there are six parameters in these equations:

L, c_α, c_β, δ_α, δ_β, $\delta_{\alpha\beta}$

Interpretation of the experimental results in terms of these parameters is most easily considered in two separate cases:

2.2. *The case of unequal δ_α and δ_β values*

In this case the quaternary constraint (enhancement) effects are different for the two kinds of subunit, so that $(\delta_\alpha - \delta_\beta)$ is nonzero. Then relationship (iv) of set B can be rewritten as:

$$(\delta_\alpha - \delta_\beta)(\delta_\alpha + \delta_\beta) = L(\delta_\beta c_\beta - \delta_\alpha c_\alpha)(\delta_\beta c_\beta + \delta_\alpha c_\alpha) \tag{7}$$

whereas from relationship (i) of set A

$$(\delta_\alpha - \delta_\beta) = L(\delta_\beta c_\beta - \delta_\alpha c_\alpha) \tag{8}$$

Substituting the right-hand side of eq. 8 into eq. 7, we obtain:

$$\delta_\alpha + \delta_\beta = \delta_\alpha c_\alpha + \delta_\beta c_\beta \tag{9}$$

Since $\delta_\alpha > 0$, $\delta_\beta > 0$, $c_\alpha \leq 1$, and $c_\beta \leq 1$, eq. 9 requires that

$$c_\alpha = c_\beta = 1 \tag{10}$$

A value of unity for c_α and c_β eliminates all allosteric effects of the heme site ligands and reduces the system to the trivial case where $K_T^\alpha = K_R^\alpha$ and $K_T^\beta = K_R^\beta$. The tetramers thus become a 'one-state system' in the allosteric sense which can still exhibit site heterogeneity, but no cooperativity. Regardless of whether the dimers are cooperative (or anticooperative) the experimental free energy distribution, when constrained by the requirement of only two allosteric states, does not permit the tetramers to differ in their stepwise free energies.

From the equalities of set A, along with eq. 10:

$$\delta_\alpha = \delta_\beta = \frac{1}{\delta_{\alpha\beta}} \tag{11}$$

This relationship is also consistent with the equalities of set B which, in lieu of eq. 10 become:

$$\delta_\alpha\delta_\beta = \delta_\alpha^2 = \delta_\beta^2 = \delta_\alpha\delta_\beta^2\delta_{\alpha\beta} = \delta_\alpha^2\delta_\beta\delta_{\alpha\beta} = \delta_\alpha^2\delta_\beta^2\delta_{\alpha\beta}^2 \tag{12}$$

The two-state mechanism applied to the experimental observations on the ten ligation species thus leads to two fundamental constraints, represented by eqs 10 and 11. The physical significance of eq. 11 is interesting, since δ_α is the binding constant of α-subunits in R-state tetramers divided by their binding constant in dimers (and δ_β represents the same for β subunits) while $\delta_{\alpha\beta}$ is the reciprocal of the equilibrium constant that measures dimer cooperativity (see eqs A10, A15 and A16). Consider the application of these relationships to species 21, for which the experimental value of ${}^{21}\Delta G_c'$ is 3 kcal (table 4). By eqs 6, 10 and 11,

$${}^{21}\Delta G_c' = -RT \ln \delta_\beta = 3 \text{ kcal} \tag{13}$$

and since $\delta_\beta \equiv K_R^\beta / K_D^\beta$, we have

$$\frac{K_R^\beta}{K_D^\beta} = e^{-3/RT} \tag{14}$$

Converting equilibrium constants to free energies ($\Delta G = -RT \ln K$), eq. 14 becomes:

$$\Delta G_R^\beta = \Delta G_D^\beta + 3 \text{ kcal} \tag{15}$$

i.e., the affinity for binding ligand to the β-subunit (negative value of ΔG) is reduced by 3 kcal when the subunit is within an R-state tetramer compared to the affinity for the same subunit when part of a dimer. This 'quaternary constraint' of 3 kcal is also operative in the α-subunit of species 21 by virtue of eqs 6, 10 and 11. By the same argument, it is easy to show that the quaternary effect of 3 kcal applies to all sites of the nine ligated species (table 1) relative to their constituent dimers. Furthermore, since $\delta_\alpha = \delta_\beta$ by eq. 11 and $K_T^\alpha = K_T^\beta = K_R^\alpha = K_R^\beta$ by eq. 10, there is no subunit heterogeneity for ligand binding to the tetramer.

The remaining point arising from application of eq. 11 to the experimental results is that the difference between free energy of binding to tetramers vs dimers must also equal the free energy of dimer cooperativity, $\Delta G^{\alpha\beta}$. Since $\delta_{\alpha\beta} = 1/K^{\alpha\beta}$ (see the appendix), eq. 11 may be written:

$$K^{\alpha\beta} = \delta_\alpha = \delta_\beta \tag{16}$$

So that,

$$-RT \ln K^{\alpha\beta} = -RT \ln \delta_\beta \tag{17}$$

Then, with eq. 13:

$$\Delta G^{\alpha\beta} = 3 \text{ kcal} \tag{18}$$

i.e., the dissociated dimers are required by the model to exhibit 3 kcal of negative cooperativity.

We thus find that when $\delta_\alpha = \delta_\beta$ the only version of the two-state MWC model consistent with the experimental results is the degenerate case in which: (a) the tetramers are not allosteric and are, in fact, noncooperative; (b) the tetramers bind ligands with an intrinsic free energy that differs by +3 kcal from that of the first binding step to

dimers; (c) the dimers are anticooperative by 3 kcal.

In simulation studies it was found that, whenever cooperativity terms for the dimers are altered from this value, some of the pathways through the ten tetrameric species become anticooperative, in violation of a two-state mechanism [4].

2.3. The case where $\delta_\alpha = \delta_\beta \equiv \delta$

In this case the quaternary enhancement (or constraint) is the same for both α- and β-subunits. The expressions of table 4 reduce to simpler form so that eqs (i) and (iv) each require that $c_\alpha = c_\beta \equiv c$. Then we may write from the expressions for ${}^{01}\Delta G_c$ and ${}^{11}\Delta G_c$:

$$(1 + L) = \delta e^{3/RT}(1 + Lc) \tag{19}$$

For species 12, 21, and 22:

$$\delta(1 + Lc) \underset{\text{(ii)}}{=} \delta^2\delta_{\alpha\beta}(1 + Lc^2) = e^{3/RT}\delta^2(1 + Lc^2) \tag{20}$$

From the right-hand equality of eq. 20 we can evaluate the dimer cooperativity parameter:

$$\delta_{\alpha\beta} = e^{3/RT} \tag{21}$$

Then from eq. (v) of set B, and eq. 21:

$$(1 + Lc^2) \underset{\text{(v)}}{=} \delta e^{3/RT}(1 + Lc^3) \tag{22}$$

Similarly eq. (vii) leads to

$$(1 + Lc^3) \underset{\text{(vii)}}{=} \delta e^{3/RT}(1 + Lc^4) \tag{23}$$

From eqs 19–23 we construct the following terms, all of which are equal to $K_R e^{-3/RT}/\delta$ (note that $K_R^\alpha = K_R^\beta \equiv K_R$):

$$\frac{1 + Lc}{1 + L}K_R = \frac{1 + Lc^2}{1 + Lc}K_R = \frac{1 + Lc^3}{1 + Lc^2}K_R = \frac{1 + Lc^4}{1 + Lc^3}K_R \tag{24}$$

We note that these ratios are simply the MWC expressions for the sequential tetrameric binding constants [1,4,6]. Hence, with all of these equal, the system has no cooperativity and reduces to the same degenerate case as in section 2.2.

3. Evidence for cooperativity in partially ligated cyanomet tetramers

The analysis just given demonstrates incompatibility of the experimental results with even the most general MWC-type of allosteric model, but does allow for the degenerate case where the tetramers have no stepwise cooperativity while dimers have 3 kcal of negative stepwise cooperativity, exactly compensated by 3 kcal of quaternary constraint in the assembly process. Although this set of circumstances would seem highly contrived and unlikely it cannot be ruled out as a formal possibility from the distribution of assembly free energies alone. Additional information on the binding properties of partially ligated cyanomet hemoglobin is needed. While the free energy of heme oxidation plus cyanide binding (the 'ligation process' here) has proved too large for accurate measurement, it has been possible to probe the cooperativity of partially ligated cyanomet tetramers by studying the reaction of oxygen with the remaining sites. Miura et al. [8] found significant cooperativity in the binding of oxygen to the remaining three sites of cross-linked hemoglobin tetramers containing single cyanomet hemes, i.e., species 11 and 12. A study by Imai [9] on oxygen binding to the two vacant sites in species 21 cyanomethemoglobin (uncross-linked) yielded a Hill coefficient n_H of 1.7. This, and the studies by Miura et al. [8] indicate a high degree of positive cooperativity in the partially ligated tetrameric molecules. From the species 21 Hill coefficient of 1.7 we may calculate the free energy increment between the third and fourth binding steps [10].

$$\Delta G_{3,4} = -RT \ln \frac{n_H^2}{(2 - n_H)^2} \tag{25}$$

which yields a value of -2.03 kcal. This may be compared with the corresponding stepwise dif-

ference of -3.0 kcal for filling the same sites with cyanomet 'ligands' calculated from the assembly free energies assuming no cooperativity in ligation of the dissociated dimers (table 1). Given the uncertainty in estimation of the Hill coefficient (about ± 0.2) the two free energies could be identical. Taken together these results strongly suggest that oxygen and cyanide may modulate the binding affinities in a similar way during the last two steps and that in any case the molecule does not appear to be locked into a noncooperative state by the binding of the first cyanide as would be required to satisfy the degenerate MWC case discussed above.

4. Conclusions and discussion

Failure of the two-state allosteric model to describe the distribution of ${}^{ij}\Delta G_c$ values in the cyanomet system along with the observation that the cyanomet system is still cooperative implies that each tetrameric molecule is capable of at least three molecular structures with separate free energies of cooperative interaction. This conclusion follows from the 'directionality' of degeneracies involving molecular structures and Gibbs free energies. The values of ${}^{ij}\Delta G_c$ include all ligand-linked alterations in energy and entropy arising from vibrational, rotational and translational degrees of freedom; electronic states; covalent and hydrogen bonds; ion pairs; van der Waals contracts; ion-dipole, dipole-dipole, and solvent interactions. While a degeneracy of multiple structures into only a few dominant free energy levels is frequently to be expected, the reverse situation is extremely unlikely. It is thus highly probable that each of the three (or more) distinct cooperative free energies found in this study represents one (or more) molecular structure.

The central strategy of this work has been to use the tetrameric MWC model and its linkages to processes involving dimers as a tool for determining the minimum number of allosterically distinct structural forms that are accessible to the hemoglobin tetramer's ten ligation species. While previous work [4,5] has provided significant tests indicating that the minimum number must be three, there now exists a much more solid data base for the cyanomet system thanks to the use of cryogenic methods developed by Perrella and associates, in combination with the kinetic and gel permeation methods developed in our laboratory [2]. It is, therefore, desirable to eliminate certain assumptions and approximations of the previous analyses of these data in order to provide a correspondingly more rigorous test of the two-state hypothesis. The model discussed in this paper incorporates virtually every plausible process associated with the linkage system involving dimers and tetramers at all states of ligation and assembly. Analysis of the cyanomet data indicates clearcut and gross incompatibility; even this most general two-state model does not have enough 'adjustable degrees of freedom' to accommodate the data. The cyanomet hemoglobin system unequivocally exhibits a minimum of three molecular forms, each with a distinctly different cooperative free energy that cannot be described as an average of R- and T-state values.

4.1. Plasticity of the system

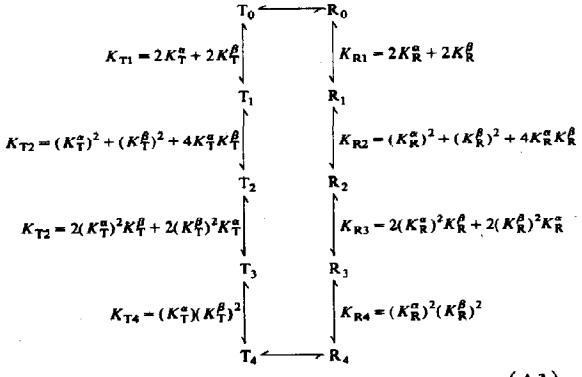
When this fundamental discovery was first made in 1985 [2], we immediately wondered how atypical these effects seen in the cyanomet system might be in light of the earlier work showing no inconsistency of oxygen-linked assembly data with a much more restricted two-state model [6]. (Of course, consistency with a model does not provide positive verification of the model's validity.) It was clear from this comparison and the comparison of species population maps for oxygen and cyanomet ligands that the quantitative distribution of species is highly dependent on the heme site ligand employed. Our fundamental premise, reinforced by the studies with manganese-substituted hemes [4], is that the various heme-site ligands and ligand analogs trigger the same basic modes of intersubunit coupling that generate cooperativity, even though these may be manifested to varying degrees. It has long been known that the isotherm shapes for oxygen binding, carbon monoxide binding and heme oxidation are different and that the cooperativity generated by these ligands is not quantitatively identical. At the cur-

rent stage of our understanding of intermediate state species the central problem is to define the molecular states that can be accessed by the tetrameric molecule. It may be that in our choice of the cyanomet system for the first complete resolution of the intermediate species, we may have serendipitously forced the molecule to reveal, in bold caricature, a state that is usually manifested with more subtlety in other ligands.

Appendix: General two-state allosteric model for ligand-linked assembly of human hemoglobin in intermediate ligation states

A1. Basic relationships for tetramers in the extended MWC model

Consider the MWC scheme for Hb tetramers with nonequivalent binding of α- and β-subunits, as analyzed by Ackers and Johnson [6].



(A1)

The binding constants K_{Ri} and K_{Ti} describe the binding of i ligands onto an R-state or T-state tetramer, respectively; e.g.,

$$K_{R3} = \frac{\left[(\alpha_2\beta_2)^R X_3\right]}{\left[(\alpha_2\beta_2)^R\right][X]^3} \tag{A2}$$

The Adair binding constants each representing the binding of i ligands onto tetramers of all forms are, therefore:

$$K_{4i} = \frac{\left[(\alpha_2\beta_2)X_i\right]}{\left[(\alpha_2\beta_2)[X]^i\right]}$$

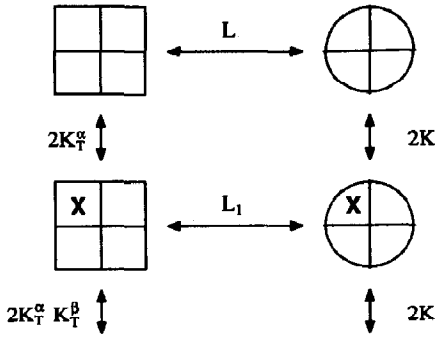
$$= \frac{\left[(\alpha_2\beta_2)^T X_i\right] + \left[(\alpha_2\beta_2)^R X_i\right]}{\left[(\alpha_2\beta_2)^T + (\alpha_2\beta_2)^R\right][X]^i}$$

$$= \frac{1 + c_i L}{1 + L} K_{Ri};\ i = 1\text{–}4 \tag{A3}$$

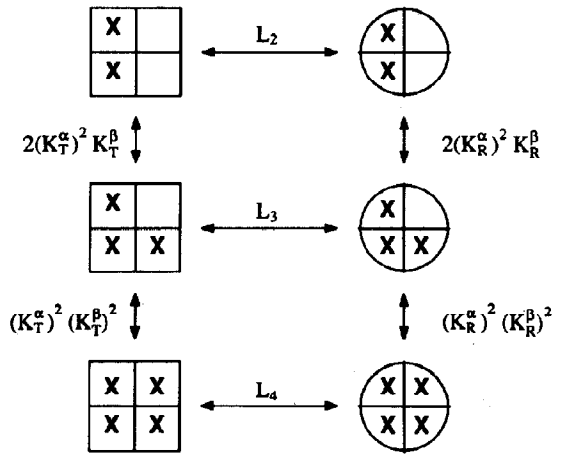
where

$$c_i = \frac{K_{Ti}}{K_{Ri}};\ L = \frac{[T_0]}{[R_0]} \tag{A4}$$

Note that the component terms of K_{Ti} or K_{Ri} are just the statistical weights (i.e., microscopic constants) for intermediate ligation state species at degree of ligation i in their R and T conformations, respectively. These are also 'product binding constants,' i.e., $(K_R^\alpha)^2$ is the product constant for formation of 'symmetric' doubly ligated R-state tetramers (species 23), whereas $2K_R^\alpha K_R^\beta$ is the product constant for either species 21 or 22 and includes the statistical factor. Both the right and left sides of scheme A1 contain all ten ligation species in this fashion. For any pathway through the species of fig. 1 we could write an MWC scheme like eq. A1 but only containing the terms of K_{Ti} and K_{Ri} that correspond to members of the pathway. For example, if our pathway consists of 01 – 11 – 21 – 32 – 41 the MWC scheme would look like:



(A5)



Now we define:

$$c_\alpha = \frac{K_T^\alpha}{K_R^\alpha};\ c_\beta = \frac{K_T^\beta}{K_R^\beta} \tag{A6}$$

Then it follows from eq. A5 that

$$L_1 = \frac{[T_1]}{[R_1]} = Lc_\alpha;\ L_2 = \frac{[T_2]}{[R_2]} = Lc_\alpha c_\beta$$
$$L_3 = \frac{[T_3]}{[R_3]} = Lc_\alpha^2 c_\beta;\ L_4 = \frac{[T_4]}{[R_4]} = Lc_\alpha^2 c_\beta^2 \tag{A7}$$

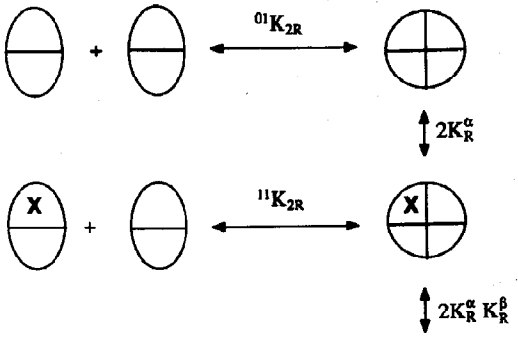
Generalizing, we see that for this or any similar scheme (or sub-scheme) we can always write

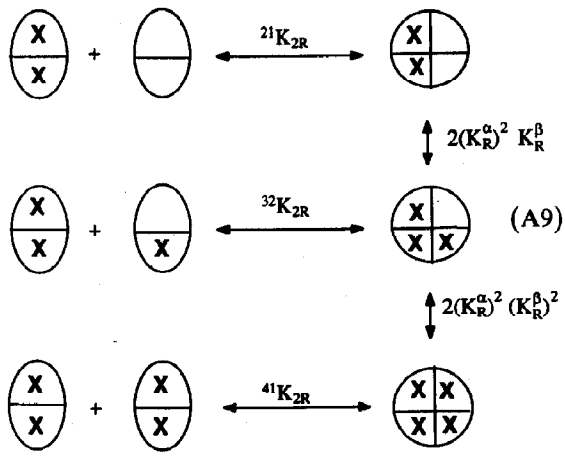
$$[T_i] = [R_i] L [C_\alpha]^p [C_\beta]^q \tag{A8}$$

where p and q are integers given in table 2:

A2. Subunit assembly relationships

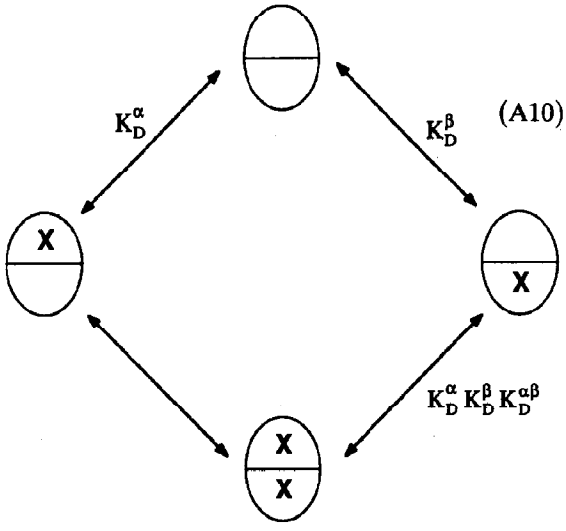
For the pathway of scheme A5 consider dissociation of the R-state tetramers into dimers





(A9)

Note that the dimers are not necessarily halves of the corresponding R-state tetramers. Instead they have the following energetic relationships:



(A10)

where K_D^α and K_D^β are intrinsic binding constants to α- and β-subunits within the dimer, and $K_D^{\alpha\beta}$ is the product constant for forming the doubly ligated dimer assuming that the intrinsic constants (K_D^α and K_D^β) may be different from the corresponding constants of the R-state tetramers and that the dimer may also be cooperative.

Using schemes A10 and A8 we can connect all species of scheme A9, or any similar scheme for a different pathway, with closed thermodynamic boxes that define the cooperative free energies.

For example, we wish to calculate the cooperative free energy for the first ligation step of scheme A9. We know that this will be given by

$$^{11}\Delta G_2 - {}^{01}\Delta G_2 = -RT \ln \frac{^{11}K_2}{^{01}K_2} \tag{A11}$$

so the problem reduces to that of calculating the $^{ij}K_2$ in terms of MWC parameters, L, c_α, c_β, and the other constants of the system, K_D^α, K_D^β, and $K_D^{\alpha\beta}$.

We assume that the dimeric species formed by dissociating R-state tetramers R_i and T-state tetramers T_i are the same. Note that since our dimers are allowed to be cooperative and also to differ in affinity from both tetrameric species this assumption is simply equivalent to stating that all dimeric species may equilibrate with each other and that we take their appropriate average energetic properties.

A3. Formation of species 01

$$^{01}K_2 = \frac{[R_0] + [T_0]}{[D]^2} = \frac{[R_0](1+L)}{[D]^2} \tag{A12}$$

$$= {}^{01}K_{2R}(1+L) \tag{A13}$$

where

$$^{01}K_{2R} = \frac{[R_0]}{[D]^2} \tag{A14}$$

and [D] is unligated dimer concentration.

Similarly for the other species of the pathway, eq. A5:

$$^{11}K_2 = \frac{[R_1] + [T_1]}{[D][D^\alpha X]} = \frac{[R_1](1+Lc_\alpha)}{[D]2K_D^\alpha[D][X]}$$

but since $[R_1] = [R_0]2K_R^\alpha[X]$

$$^{11}K_2 = \frac{2K_R^\alpha[X](1+Lc_\alpha)[R_0]}{2K_D^\alpha[X][D]^2} \tag{A15}$$

$$^{11}K_2 = \delta_\alpha {}^{01}K_{2R}(1+Lc_\alpha)$$

where $\delta_\alpha = K_R^\alpha / K_D^\alpha$.

Similarly

$$^{21}K_2 = \frac{[R_2] + [T_2]}{[D][DX_2]} = \delta_\alpha\delta_\beta\delta_{\alpha\beta} {}^{01}K_{2R}(1+Lc_\alpha c_\beta) \tag{A16}$$

where $\delta_\beta = K_R^\beta / K_D^\beta$ and $\delta_{\alpha\beta} = 1/K_D^{\alpha\beta}$

$$^{32}K_2 = \frac{[R_3] + [T_3]}{[DX][DX_2]} = \delta_\alpha^2\delta_\beta\delta_{\alpha\beta} {}^{01}K_{2R}(1+Lc_\alpha^2c_\beta) \tag{A17}$$

$$^{41}K_2 = \frac{[R_4] + [T_4]}{[DX_2]^2} = \delta_\alpha^2\delta_\beta^2\delta_{\alpha\beta}^2 {}^{01}K_{2R}(1+Lc_\alpha^2c_\beta^2) \tag{A18}$$

Following this approach and generalizing for all ten ligation state species we may write the following fundamental relationship for dimer-tetramer assembly:

$$^{ij}K_2 = S\Delta K_R(1 + Lc_\alpha^p c_\beta^q) \tag{A19}$$

where S is a statistical factor, and Δ is the product of appropriate factors δ_α, δ_β, $\delta_{\alpha\beta}$ as given in table 2; K_R is an intrinsic K. Eq. A19 is a generalized form of the fundamental relationship of Ackers and Johnson given as eq. 4 of this paper. Eq. A19 is given in the body of this paper as eq. 5.

Acknowledgements

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