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Experimental resolution of cooperative free energies for the ten ligation states of human hemoglobin

(hemoglobin mechanism/cooperativity/free energy coupling/protein interactions)

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ABSTRACT **Tetrameric human hemoglobin can assume ten molecular forms that differ in the number and configuration of ligands bound at the four heme sites. For each of these species we have determined the cooperative free energy—i.e., the deviation in free energy of ligation from that which would obtain for the same sites binding as independent α and β subunits. These cooperative free energies were resolved from measurements on the dissociation into dimers of tetramers in which each subunit is either unligated (Fe^{2+} deoxy) or "ligated" by conversion into the cyanomet form (Fe^{3+} CN). The results indicate that each hemoglobin tetramer acts as a three-level molecular switch. During the course of ligation, the total cooperative free energy (6 kcal/mol over all four binding steps) is expended in two transitions that are synchronized with particular ligation steps. Whether a cooperative energy transition occurs or not depends upon how the ligation step changes both the number and configuration of ligated subunits. The hemoglobin tetramer is thus a "combinatorial switch." The finding of three distinct free energy levels for the ten ligation states suggests the existence of three major structural forms of the hemoglobin tetramer.**

Tetrameric human hemoglobin has been studied extensively as a classic prototype for protein assemblies that undergo molecular "switching" during their functional behavior. In the course of binding a ligand species at its four heme sites the hemoglobin molecule switches from a state of low affinity for ligand to one of high affinity (e.g., an ≈250-fold change in the binding constant for oxygen). During the reverse process (unloading ligands) the molecule switches back to its original state of low affinity. Understanding the pathways whereby this alteration of thermodynamic state reflects energetic behavior of the individual tetrameric molecules is central to understanding the mechanism of hemoglobin function. How many distinct energetic states are assumed by each molecule? How are the transitions between these energetic states influenced by ligand binding? These fundamental questions have yet to be resolved for any cooperative multisubunit protein.

Analysis of binding isotherms (e.g., for oxygen or carbon monoxide) reveals that each of the four stoichiometric degrees of ligation occurs with a different Gibbs free energy (cf. ref. 1). This graduated change in the stepwise free energies does not mean that each molecule necessarily switches its affinity in synchrony with the binding of each ligand, since the binding isotherms measure only changes in average behavior over all species in the population. In a popular model of allosteric regulation (the Monod–Wyman–Changeux model), only two types of tetramers exist (a low-affinity "T state" and a high-affinity "R state"). The ratio of these species in the population is shifted during the course of ligand binding according to specified rules of the model, giving rise to the graduated overall thermodynamic property (2). While a large number of observations with hemoglobin are consistent with the Monod–Wyman–Changeux mechanism, other models are found to fit the same observations equally well (3–7). Thus in spite of an almost overwhelming body of experimental and theoretical studies, the molecular mechanism of cooperativity in human hemoglobin has remained unsolved. Progress on this problem has been greatly inhibited by the inability to study the thermodynamic properties that constitute cooperativity for hemoglobin tetramers at all the intermediate states of ligation. Here we report an experimental determination of the relative Gibbs free energies for the ten ligation states of tetrameric hemoglobin. In this paper the term "ligation" denotes that the heme iron has been oxidized and has reacted with cyanide. "Unligated" subunits have ferrous iron hemes with no ligands bound.

Conversion of the hemes into cyanomet derivatives permits the study of tetrameric hemoglobins with any desired combination of "ligated" subunits. To carry out such studies, however, a number of problems must be solved. While a cyanomet subunit is sufficiently stable to prevent ligand rearrangement through site dissociation *per se*, such high stability also precludes determination of ligand binding constants. Furthermore, site rearrangement can occur through subunit dissociation–association reactions. Thus the following two problems had to be solved: (*i*) to control the effects of subunit dissociation and disproportionation in such a way as to permit the study of cooperativity and (*ii*) to measure cooperativity of ligand binding without actually measuring the binding reactions. In this study we have utilized an approach wherein the solution of problem *i* also provides the tools for solving problem *ii*. We have developed a technique for studying the energetics of tetrameric "hybrid" hemoglobins in the presence of the "parent" tetramers and the constituent dissociated dimers (see Table 1). With this technique we have utilized the thermodynamic linkage between subunit dissociation and ligand binding to resolve the cooperative free energies of tetramer ligation. From these experimental results we have been able to evaluate the free energy of cooperativity for each of the ten molecular forms.

THERMODYNAMIC RELATIONSHIPS

Ligation States. The ten ligation states of tetrameric hemoglobin are represented schematically in Table 1. These molecular forms differ in the number and configuration of ligated subunits among the possibilities α^1, β^1, α^2, and β^2. Ligated subunits are represented each by a square containing an "X"; each unligated subunit is denoted by an open square and its position within the tetramer denotes the particular subunit among the four possibilities. The index, *ij*, designates the particular species *j* among those with *i* ligands bound.

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Table 1. Cooperative free energies for hemoglobin tetramers

ij	g_{ij}	Molecular forms: Tetramer	Molecular forms: Constituent dimers	Molecular forms: Parent species A	Molecular forms: Parent species B	Dissociation rate constants,* sec^{-1}: Parent A	Dissociation rate constants,* sec^{-1}: Parent B	Dissociation rate constants,* sec^{-1}: Tetramer	$^{i}\Delta G_{2j}$, kcal/mol†	Cooperative free energy ($^{i}\Delta G_{2j} - {}^{0}\Delta G_{21}$), kcal/mol
01	1	$\alpha^1\beta^2/\beta^1\alpha^2$	$\alpha^1/\beta^1 + \beta^2/\alpha^2$	None		—	—	2.1×10^{-5}	−14.4	0
11	2	X/	X/ + /	X/	+ /X	0.84	2.1×10^{-5}	3.0×10^{-3}	−11.5	2.9
12	2	/X	/X + /	/X	X/ + /	0.51	2.1×10^{-5}	5.7×10^{-3}	−11.2	3.2
21	2	X/X	X/X + /	X/X	+ /	0.59‡	2.1×10^{-5}	3.9×10^{-3}	−11.4	3.0
22	2	XX/	X/X + /	X/	+ /X	0.84	0.51	2.4×10^{-3}	−11.7	2.7
23	1	X/	X/ + /	None		—	—	0.84	−8.2	6.2
24	1	/X	/X + /	None		—	—	0.51	−8.5	5.9
31	2	XX/X	X/X + /	XX/X	+ /X	0.59‡	0.51	0.44	−8.6	5.8
32	2	X/XX	X/X + /	XX/XX	+ X/	0.59‡	0.84	0.68	−8.4	6.0
41	1	XX/XX	X/X + /	None		—	—	0.59‡	−8.5§	5.9

The index ij designates the particular species j among those with i ligands bound ($i = 0, 1, 2, 3, 4$). Ordering of species with respect to j values is arbitrary.

*Values accurate to 10%.

†Values accurate to ±0.2 kcal (1 kcal = 4.18 kJ).

‡Calculated from independently determined values of K_f and $^{4}K_{21}$.

§Determined by analytical gel chromatography (8).

Each tetrameric species is an assembly of constituent dimers of the $\alpha^1\beta^1$ type (identical with $\alpha^2\beta^2$). The placement of ligated subunits within these dimers is also shown in Table 1.

Cooperative Free Energies. Cooperativity is the deviation in free energy of ligation from that which would obtain for the same sites if they bind independently—i.e., with their intrinsic free energies (9). Since dissociated dimers ($\alpha\beta$) bind ligand noncooperatively (i.e., with the same affinity as an isolated pair of α and β subunits) the linkage between ligand binding and reversible dissociation of tetramers into dimers provides a way to measure the cooperative free energies (10). Subunit interactions that generate the cooperative free energies are decoupled by dissociation into dimers (cf. refs. 1 and 9). For a tetrameric species representing a particular ligation state ij (Table 1), the free energy ΔG_{ij} of binding the i ligands may be written

$$\Delta G_{ij} = i\Delta G_x + {}^{i}\Delta G_{2j} - {}^{\circ}\Delta G_{21}, \qquad [1]$$

in which ΔG_x is the intrinsic free energy of binding ligand X to a site, such as one on the noncooperative dimers. The constituent dimers shown in Table 1 self-associate to form each tetramer ij with standard free energy $^{i}\Delta G_{2j}$. $^{\circ}\Delta G_{21}$ is the standard free energy of forming unligated tetramers from unligated dimers (Table 1). From Eq. 1 it is seen that the difference ($^{i}\Delta G_{2j} - {}^{\circ}\Delta G_{21}$) provides a measure of the deviation of ΔG_{ij} from that expected from the binding of i ligands in the absence of site–site interactions (i.e., $i\Delta G_x$). We may thus evaluate the *cooperative free energies* ($\Delta G_{ij} - i\Delta G_x$) by determining the free energies of dimer–tetramer association for species in all ligation states of Table 1 and forming the differences ($^{i}\Delta G_{2j} - {}^{\circ}\Delta G_{21}$).† The contributions of these cooperative free energies to the

†The quantities that we term "cooperative free energies" in this paper are identical to those called "regulatory energies" by Pettigrew *et al.* (9).

four standard Adair binding constants K_{4i} (11) are given by

$$K_{4i} = K_x^i \sum_j g_{ij} \exp[-({}^i\Delta G_{2j} - {}^\circ\Delta G_{21})/RT]$$
$$i = 0, 1, 2, 3, 4, \quad [2]$$

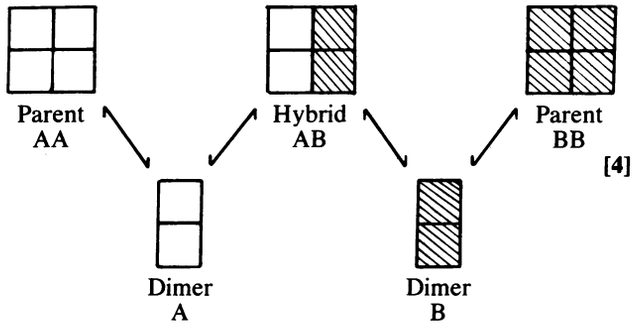
in which K_x is the intrinsic binding constant of ligand ($\Delta G_x = -RT \ln K_x$) and g_{ij} is the statistical degeneracy of species ij (see Table 1). The terms under the summation on the right comprise a factor that measures the overall cooperativity in binding i ligands. In the absence of cooperativity the right-hand side of Eq. 2 reduces to purely statistical terms Σg_{ij} with values of 1, 4, 6, 4, 1 for $i = 0, 1, 2, 3, 4$. The tetrameric binding isotherm is

$$\bar{Y} = \frac{\sum_{i=0}^{4} iK_{4i}[X]^i}{4\sum_{i=0}^{4} K_{4i}[X]^i}, \quad [3]$$

in which [X] is the concentration of ligand in equilibrium with hemoglobin tetramers. The stepwise binding constants k_{4i} are related to the Adair constants by the relationships $k_{4i} = K_{4i}/K_{4(i-1)}$.

METHODS

The approach we have used for determining the assembly free energies (${}^i\Delta G_{2j}$) is based on the energetics of forming hybrids between tetrameric species as shown below:



[4]

In a mixture of two types of hemoglobin the two "parent" tetramers AA and BB, the hybrid tetramer AB, and the constituent dimers A and B ($\alpha^1\beta^1$ type) are all in equilibrium (Scheme 4). If all tetramers had equal assembly free energies the fraction of hybrid (AB) would be 50%; with other energy distributions the fraction is higher or lower. If the hybrid were isolated from its parent species, it would disproportionate over time back to the original distribution of parent and hybrid molecules. The problem then is to ascertain the free energy of hybrid formation in the presence of these other molecular species. We have developed kinetic methods for achieving this goal.

Kinetic Methods. The dimer–tetramer association equilibrium constants (${}^iK_{2j}$) for partially ligated (cyanomet) tetramers were determined from values of the rate constants for assembly (k_f) and dissociation (k_r). The equilibrium constant ${}^iK_{2j} = k_f/k_r$.

The rate constant k_r was determined using the haptoglobin binding technique (11, 12). In this method the time course for dissociation of tetramers into dimers is determined using haptoglobin as a dimer trap. At 430 nm, unligated dimers and tetramers have different extinction coefficients. After mixing samples of deoxygenated hemoglobin with haptoglobin under anaerobic conditions, the specific absorbance at 430 nm decreases with time because of the irreversible formation of a complex between hemoglobin dimers and haptoglobin. A similar technique is used for partially ligated (cyanomet) molecules.

For a mixture of parent and hybrid molecules the absorbance at 430 nm is given by:

$$A(t) = A_\infty + P_1e^{-k_{AA}t} + P_2e^{-k_{AB}t} + P_3e^{-k_{BB}t}, \quad [5]$$

in which k_{AA}, k_{BB}, and k_{AB} refer to the dissociation rate constants for the two parent molecules and the hybrid tetramers, respectively. The preexponential factors P_1, P_2, and P_3 are complex functions of the rate constants, extinction coefficients, and initial species distributions, but they have constant values for a given experiment.

The parent species used to generate each partially ligated tetramer ij are shown in Table 1. With the exception of species [01], [23], [24], and [41] (Table 1), these tetramers were studied in complex mixtures containing parent as well as hybrid molecules. For each kinetics experiment deoxygenated solutions of hemoglobin and haptoglobin were mixed and the decrease in absorbance at 430 nm was recorded with time. As shown in Table 1, the parent and hybrid tetramers display a wide range of dissociation rates. For slowly dissociating species ($t_{1/2} \approx$ minutes to hours) the reaction was carried out in a Varian 219 spectrophotometer. For rapidly dissociating species ($t_{1/2} \approx$ seconds) the dissociation rate was determined with a Dionex stopped-flow spectrophotometer.

All experiments were carried out in "standard buffer," consisting of 0.1 M Tris·HCl, 0.1 M NaCl, 1 mM EDTA, 10 μM KCN, 0.3% D-glucose, pH 7.4 at 21.5°C. Hemoglobin solutions were equilibrated with standard buffer by dialysis or gel chromatography, to maintain the free cyanide concentration at 10 μM. This concentration of free cyanide is 10-fold greater than that required for complete saturation, as calculated by using the binding constants for cyanide to a mixture of hydroxy- and aquomethemoglobin (14). Hemoglobin and haptoglobin solutions were deoxygenated with humidified nitrogen. To maintain deoxy conditions throughout all experiments, glucose oxidase (1.8 mg/ml) and catalase (0.3 mg/ml) were used in the presence of the substrate 0.3% D-glucose. Spectra obtained after deoxygenation (400–450 nm) were as expected for mixtures of deoxy and cyanomet hemoglobins.

A typical experiment is shown in Fig. 1 for a mixture of species [01] and [41]. Analysis of these data by a nonlinear least-squares fitting method (8) yielded values for the three rate constants of Eq. 5. Two of these constants were in close agreement with independent determinations carried out for pure samples of each parent species, permitting assignment of the third constant to the hybrid species.

The equilibrium constants ${}^iK_{2j}$ were calculated by using a rate constant for dimer–tetramer association (k_f) of 1.1×10^6 M^{-1} sec^{-1} (15). In an extensive series of measurements of the rate constants for association of normal, mutant, and chemically modified hemoglobins over a wide range of pH, this value was found to be invariant (9, 15). The value of k_f used in this study was also found to be in good agreement with that calculated from the values of ${}^4K_{21}$ and k_r determined independently for cyanomet hemoglobin with all four subunits ligated.

Preparation of Parent Hemoglobins. Normal hemoglobin A_0 was prepared and characterized as described (16). Cyanomet hemoglobin was prepared from oxyhemoglobin A_0 by oxidation with potassium ferricyanide in the presence of potassium cyanide. The ferrocyanide resulting from the oxidation was

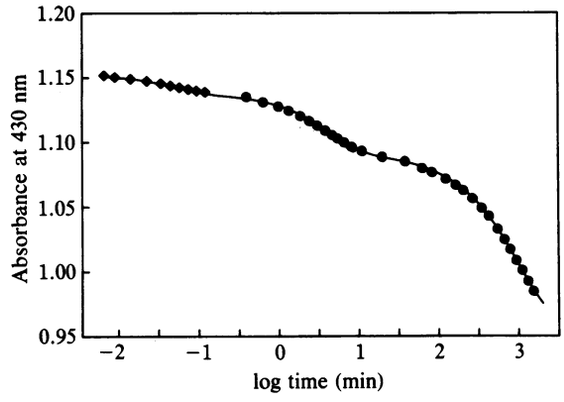


FIG. 1. Kinetics of tetramer to dimer dissociation for a mixture of species [01] and [41] (see Table 1). ◆, Stopped-flow experiment; ●, Varian 219 experiment. Solid lines represent best fit of data to Eq. 5, yielding rate constants given in Table 1.

removed by gel chromatography using Sephadex G-25. Hemoglobin species [23] and [24] were prepared by using a modified version of the Blough and Hoffman method (17).

RESULTS

The rate constants obtained for all ten molecular forms of tetrameric hemoglobin are given in Table 1 along with the corresponding standard Gibbs energies ${}^{i}\Delta G_{2j}$ of dimer–tetramer assembly. Each rate constant reflects a discrete molecular species, although we cannot rule out very small fractions (e.g., 5%) of additional kinetic species. From these experimental values the cooperative free energy was calculated for each species, and these values are listed in the right-hand column of Table 1. These values are accurate to ±0.2 kcal.

A striking feature of these results is that the ten ligation states of tetrameric hemoglobin exhibit essentially only three values of the cooperative free energy—i.e., 0, 3, and 6 kcal/mol. There are thus found to be three principal energetic levels of the tetrameric molecule after subtraction of the intrinsic $i\Delta G_x$ terms. For any molecular species the free energy relative to the unligated (or fully ligated) molecule is a simple combination of $i\Delta G_x$ and one of these three cooperative free energies (Eq. 1). The cooperative free energies reflect molecular alterations that oppose ligand binding. For example, in binding two ligands to form species [23] from species [01] (Table 1) the free energy is $(2\Delta G_x + 6)$ kcal/mol. The negative value of ΔG_x is opposed by the positive 6 kcal/mol of cooperative free energy. For the reaction carried out in reverse (deligation) the free energy is $(-2\Delta G_x - 6)$ kcal/mol.

Each Tetramer Acts as a Combinatorial Switch. The results summarized in Table 1 provide considerable insight into the "rules" or "code" whereby cooperative energy levels are controlled by ligand binding states. Transitions between the three levels of cooperative free energy are seen to occur in synchrony with single steps of ligation or deligation, but they also depend on the configurations of ligated subunits within the reactant and product species. This leads us to the concept that the hemoglobin tetramer acts as a "combinatorial switch." Changing the combination of bound ligands (i.e., the number bound and/or their configuration with respect to the four binding sites) generates perturbations within the tetrameric molecule that cause it to shift between the three cooperative energy levels according to a specific code. We believe the essence of this code is displayed in the correspondence between molecular states and cooperative free energies given in Table 1.

Several features of the apparent code for this combinatorial switch require comment. (*i*) Binding the first ligand generates a transition between the first two levels of cooperative free energy. The molecule switches between these levels through the "expenditure" of 3 kcal of free energy whether the ligated subunit is α or β. (*ii*) The resulting altered tetramer (species [11] or [12] of Table 1) undergoes a second transition in cooperative free energy at either the second or the third binding step, depending on where the second ligand is bound. When the first two sites ligated are on different types of subunits (species [21] and [22], Table 1) the molecule does not switch until the third ligand is bound. There appears to be a "selection rule" for the expenditure of cooperative energy: if an α subunit is ligated first there is no further expenditure of cooperative energy on subsequent ligation of a β subunit and *vice versa*. The selection rule is operative whether the ligated $\alpha\beta$ subunit pair is within the same dimer ($\alpha^1\beta^1$) or across the dimer–dimer interface ($\alpha^1\beta^2$). The net result is a pattern of alternating affinity values (Eq. 2) for the ligation steps that include species [21] and [22]. This pattern is supported by experiments (18) on mixtures of species [01] and [41] in varying ratios that showed cooperativity in oxygenation of the two vacant sites of species [21]. (*iii*) If the second ligand binds to the same kind of subunit (α or β) the tetramer undergoes the second energy transition, reflecting 6 kcal of cooperative free energy (species [23] and [24]). We note that occupancy of sites $\alpha^1\alpha^2$ or $\beta^1\beta^2$ represents structurally symmetric perturbations of the tetramer whereas species [21] and [22] are asymmetric. In an equilibrium mixture the asymmetric species would dominate the distribution of doubly ligated tetramers (Eq. 2). (*iv*) Whenever three ligands are bound the tetramers are switched to the third level of cooperative free energy. (*v*) Because the increments of cooperative free energy are approximately identical (3 kcal) for the two stages of tetrameric transition, only two stepwise binding free energies would be experimentally observed: when a binding step is not accompanied by expenditure of cooperative energy, the observed binding energy will be ΔG_x; when accompanied by an expenditure of cooperative energy (either at the first or the second transition of the tetramer) the ligand binds with an affinity equal to $(\Delta G_x + 3)$ kcal. Thus the system exhibits only two stepwise binding affinities even though each molecule undergoes transitions between three levels of cooperative energy. This provides a rationale for the numerous studies of hemoglobin in which essentially only two binding affinities have been observed.

We note that the above rules for combinatorial switching entail synchronization between cooperative energy transitions and particular ligation steps. This concept contrasts sharply with the premises of the simple two-state Monod–Wyman–Changeux model in which no such synchronization is present.

DISCUSSION

In a previous study from our laboratory (9) using mutant and chemically modified hemoglobins, it was inferred that the total cooperative energy (i.e., over all four binding steps) consists of the free energies of altered interaction at the $\alpha^1\beta^2$ intersubunit contact region. The present study is concerned with the question of how (by what rules) the expenditure of cooperative free energy is controlled by ligation of the heme sites in various combinations. We have used cyanomet ligation as a probe of this control system. While we do not assume the effects of cyanomet ligation to be quantitatively the same as those of oxygenation, there are strong reasons to believe that the basic modes of free energy coupling within the hemoglobin tetramer will be the same when heme sites are perturbed with different ligands: (*i*) The crystallographic structures of cyanomet (horse) hemoglobin (19), human

oxyhemoglobin (20), and human carboxyhemoglobin (21) are closely similar in regard to quaternary structure, although minor tertiary structure differences exist. (*ii*) The total cooperative free energy (over all four steps) differs by only a few tenths of a kcal/mol (out of ≈6) for these three ligands (9). (*iii*) The pattern of energetic effects we find for cyanomethemoglobin is supported by results of kinetic and spectral studies with the ligand NO (22, 23) and by cryogenic isoelectric focusing studies with CO (24), all of which indicate species [21] and [22] to dominate the distribution of doubly ligated tetramers and to have different properties from the other members of this set. (*iv*) Our finding that the ten structural states exhibit such high degeneracy with respect to energetic levels suggests a corresponding degeneracy in the mechanisms of molecular alteration responsible for cooperativity. We thus expect that the general trends and the concept of hemoglobin as a combinatorial switch derived from this work will be applicable to other ligands and other conditions, even though the actual free energy values and distributions of cooperativity levels with respect to the ligation states may vary.

To relate our energetic findings to structural features of hemoglobin, several experimental results are of interest. Comparison between crystallographic structures for unligated human hemoglobin (25) and cyanomet horse hemoglobin (19) indicates major differences in (*i*) tertiary structures of the individual subunits, (*ii*) quaternary structure (the relative disposition of $\alpha^1\beta^1$ dimer pairs within the tetramer), and (*iii*) noncovalent interactions (hydrogen bonds, ion pairs, van der Waals contacts) within the $\alpha^1\beta^2$ contact region (including subunit contacts $\alpha^1\beta^2$, $\alpha^2\beta^1$, and $\alpha^1\alpha^2$). Contacts $\alpha^1\beta^1$ and $\alpha^2\beta^2$ are essentially unaffected by complete ligation of the tetramer. The crystallographic structure of methemoglobin tetramers constrained in a "deoxy quaternary structure" (26) shows significant alterations both in tertiary structure and in interactions at the $\alpha^1\beta^2$ contact. These include alteration of hydrogen bonds involving the Tyr-42α and Asp-99β residues. Proton NMR studies of cross-linked singly ligated cyanomet hemoglobin (27) indicate a partial alteration of this same set of hydrogen bonds. Upon further ligation the resonances associated with the exchangeable hydrogens disappear, indicating complete elimination of these hydrogen bonds.

Taking these structural findings into account along with our energetic results, it is of interest to consider two possible mechanisms for switching between the cooperative energy levels. Both mechanisms require three major structural forms of the hemoglobin tetramer.

(i) *Local Pathway Mechanism.* The transition in cooperative free energy on binding the first ligand may represent essentially the effects of tertiary structure change of the ligated subunit and altered pairwise interactions at the nearby $\alpha^1\beta^2$ subunit contact while the molecule remains in an essentially deoxy quaternary form. The second cooperative transition of the tetramer may then reflect essentially a quaternary structural transition whereby the relative disposition of dimer pairs is altered and along with it the other set of contacts ($\alpha^2\beta^1$). This mechanism requires a specific pathway of local "communication" within the hemoglobin tetramer.

(ii) *Global Transition Mechanism.* It appears more likely that each of the three cooperative energy levels may represent a "global" structural state of the tetramer; i.e., all of the local interactions contributing to the cooperative free energy are altered when the molecule switches between any two cooperativity levels. A global mechanism is suggested strongly by the experimental findings: (*i*) only three cooperative free energy levels are found for the ten molecular forms of tetramer; (*ii*) within a single energy level the molecular forms differ with respect to the symmetry of sites altered by ligation; i.e., tetramers with singly ligated subunits show identical free energy levels regardless of the placement (α or β) of ligand (species [11] and [12]), and the same is true of the doubly ligated tetramer pairs [21] [22] and [23] [24]; and (*iii*) tetramers with different numbers of ligated subunits show identical cooperative energy levels, as described by the "selection rule".

The results of Table 1 suggest a distribution between structural forms for the doubly ligated molecules that is dominated by species [21] and [22] (see Eq. **2**). This distribution may differ with conditions of pH, temperature, heme-site ligand, and allosteric effectors (17, 28). The methods developed in this study provide a means to further investigate these effects in all ten ligation states.

We thank Benjamin W. Turner for helpful suggestions. The equilibrium constant for dimer–tetramer assembly of cyanomethemoglobin, species [41], was determined by George J. Turner in our laboratory. This work has been supported by Grant GM24486 from the National Institutes of Health and by Grant PCM 84-03533 from the National Science Foundation.

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