Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 7089–7093, October 1987
Biophysics

Three-state combinatorial switching in hemoglobin tetramers: Comparison between functional energetics and molecular structures

(cooperativity/ligand binding/protein interactions)

FRANCINE R. SMITH*, DAVID GINGRICH†, BRIAN M. HOFFMAN†, AND GARY K. ACKERS*

*Department of Biology and McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, MD 21218; and †Department of Chemistry and Department of Biochemistry, Molecular and Cell Biology, Northwestern University, Evanston, IL 60201

Communicated by Christian B. Anfinsen, May 5, 1987

ABSTRACT **In a previous study on cyanomethemoglobin the 10 tetrameric species (each with a unique combination of ligated and unligated subunits) were found to exhibit three distinct free energies of cooperative interaction. The distribution of these free energies among the partially ligated species is incompatible with a two-state mechanism of molecular switching and requires a minimum of three molecular structures with distinctly different free energies of heme–heme interaction. Ligand-linked transitions between the three cooperativity states were found to be "combinatorial"—i.e., dependent upon changes in both the number and specific configuration of bound ligands. Here we present results from two other chemical systems that mimic intermediate oxygenation states. In these systems the heme iron is replaced by manganese in certain of the subunits. We find the same distribution of cooperative free energies as reported for the cyanomethemoglobin system. These results demonstrate that the three-state combinatorial nature of cooperative switching is neither a special feature of the cyanomet reactions nor of the substitution of manganese for iron, but reflects a fundamental property of hemoglobin. These findings are compared with crystallographic structural results on partially ligated hemoglobins.**

During the course of ligand binding, tetrameric human hemoglobin can exist as 10 distinct molecular species (ligation states) reflecting the structurally unique combinations of ligated and unligated subunits (Fig. 1). While an understanding of the mechanism of cooperativity will ultimately require much information on the structures and functional energetics of tetrameric molecules in the intermediate states of ligation, such information has hitherto been largely inaccessible. Developments, however, have provided promising approaches to this problem. A study from this laboratory (1) using cyanomet species in all ligation states has demonstrated that the hemoglobin tetramer acts as a three-level combinatorial switch—i.e., each molecule assumes one of three cooperative free energies, depending on both the number of ligands bound and the specific configuration of ligated subunits (Fig. 2).

In view of the potential significance of these findings to the mechanism of hemoglobin cooperativity, it is essential to determine whether three-state combinatorial switching is a special property of the cyanomet reactions with hemoglobin and whether the three levels of cooperativity correspond to distinct molecular structures. In the present study we determined the cooperative free energies for partially ligated tetramers in two additional, chemically different, systems that mimic partially ligated tetramers. The species we have studied are depicted in Fig. 3: in system A the hemes are

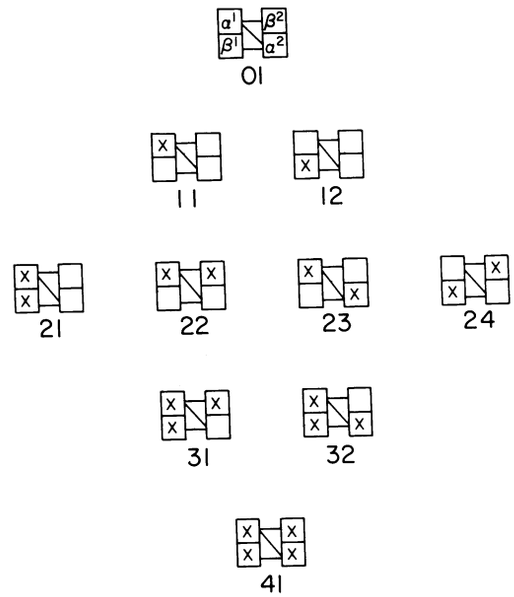


FIG. 1. Topographic representation of the 10 ligation states of tetrameric hemoglobin. The index *ij* denotes the particular species *j* among those with *i* ligands bound ($i = 0, 1, 2, 3, 4$; $j = 1, 2, 3, 4$). Ordering of species with respect to *j* is arbitrary. Subunit positions are shown in species 01.

replaced in some of the subunits by Mn^{2+} protoporphyrin IX, providing a functional analog of unligated subunits (2, 14) while the remaining subunits have normal hemes that are ligated with carbon monoxide (CO); in system B, the unligated subunits contain normal heme while the ligated subunits contain hemes replaced by Mn^{3+} protoporphyrin IX. Here we analyze the functional energetics of these systems in terms of the minimum number of molecular structures required. We will also compare the energetics with crystallographic results (3, 4).

Functional Energetics. (*i*) *Cooperative free energy.* ΔG_c is the deviation of ligand binding free energy from that for ligation of the same sites with their intrinsic free energies.

$$\Delta G_c = \Delta G_i - i\Delta G_x, \qquad [1]$$

where ΔG_i is the standard Gibbs free energy for reacting *i* moles of ligand with a tetramer and ΔG_x is the intrinsic free energy per site for the same reaction in the absence of cooperativity (5). In hemoglobin tetramers $i\Delta G_x$ generally has a larger negative value (higher affinity) than ΔG_i so that ΔG_c is positive. A finding that $\Delta G_c = 3.0$ kcal (1 cal = 4.18 J) for

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: MWC, Monod–Wyman–Changeux.

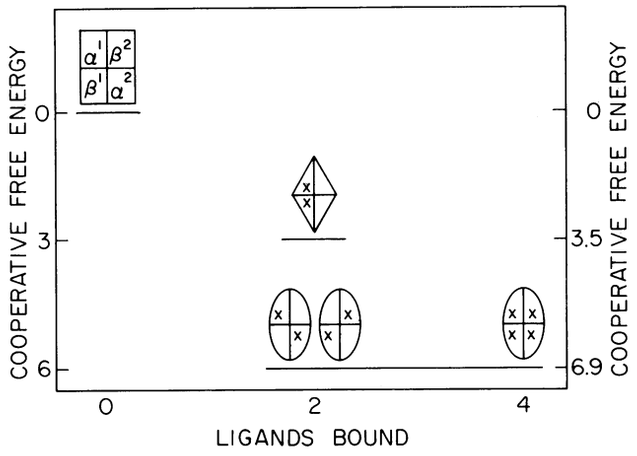


FIG. 2. Cooperative free energy levels for tetramers in various ligation states. Values on left ordinate pertain to system C, and those on right are for system B.

species 21, for example, means that when a mole of species 01 is reacted with 2 mol of ligand to form species 21, the standard Gibbs free energy changes by $(2\Delta G_x + 3.0)$ kcal; with no cooperativity the free energy change per mole of tetramer would be $2\Delta G_x$.

Subunit interactions that generate the cooperative free energies are decoupled by dissociation of the hemoglobin tetramer into $\alpha^1\beta^1$ dimers, which bind two ligands with intrinsic free energy ΔG_x (6–8). The thermodynamic coupling between ligand binding and reversible dissociation of tetramers into dimers has thus proved to be a powerful means of measuring cooperative free energies (5–8). The approach, illustrated in Fig. 4, shows how the determination of dimer–tetramer assembly free energies (upper and lower reactions) can be used to deduce the cooperative free energy ΔG_c for binding two ligands (each denoted by X) to the tetramer. Since the Gibbs free energy is a state function (independent of path), the free energies around the cycle of Fig. 4 must sum to zero. Thus, using the terms defined in Fig. 4, we have

$$^0\Delta G_{21} + (2\Delta G_x + \Delta G_c) - {}^2\Delta G_{21} - 2\Delta G_x = 0, \quad [2]$$

which reduces to

$$\Delta G_c = {}^2\Delta G_{21} - {}^0\Delta G_{21}. \quad [3]$$

Therefore, an experimental determination of the two free energies of dimer–tetramer assembly provides a determination of the cooperative free energy ΔG_c (i.e., free energy of heme–heme interaction) without actual measurement of the ligand binding equilibria.

(*ii*) *Cooperativity states.* Each distinct value of ΔG_c that occurs in the set of ligation state species (Fig. 1) defines a separate cooperativity state. ΔG_c is a thermodynamic parameter reflecting properties of all tetrameric species in a particular ligation state, *ij* (see Fig. 1). It must be emphasized that the number of cooperative free energies is not necessarily equal to the number of molecular forms with different free energies of heme–heme interaction (9). If a population of subspecies exists within a given ligation state (e.g., structural forms with different ligand affinities) the value of ΔG_c will be an average over those subspecies. The thermodynamically correct method for constructing this average was presented by Ackers and Johnson (9). Other methods of averaging have been proposed by Weber (10) and by Ferrone (11). Analysis of our experimental results in terms of an allosteric mechanism (9) based on two molecular structures, each with a separate ligand affinity, will be presented in *Results*.

METHODS

Sample Preparation. Normal hemoglobin A_0 was prepared as described (12). Mn^{3+} hemoglobin was prepared (13) and further purified by ion-exchange chromatography. The symmetric hybrid species $\alpha_2[Fe^{2+}]\beta_2[Mn^{3+}]$ and $\alpha_2[Mn^{3+}]\beta_2[Fe^{2+}]$ were prepared according to Blough and Hoffman (14). Sample purity was judged by polyacrylamide gel electrophoresis and isoelectric focusing.

Kinetic Methods. Values of the dimer–tetramer assembly free energies $^i\Delta G_{2j}$ were determined by analysis of rate constants for forward (k_f) and reverse (k_r) reactions (1, 15). Species 01, 23, and 24 were studied in pure form, while

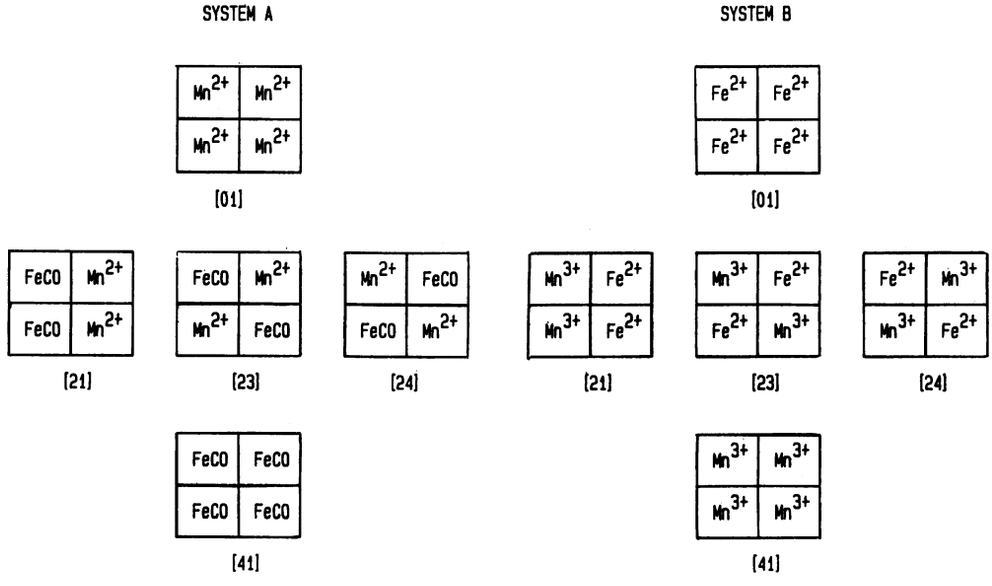
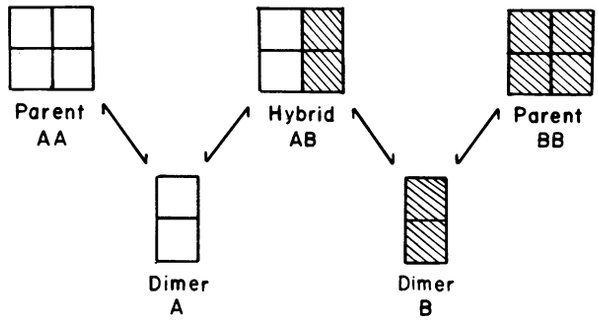


FIG. 3. Configurations of ligation state models utilizing hemes in which Fe is replaced by Mn. Position of small boxes within each tetramer indicates a specific subunit: α^1 (upper left box), β^1 (lower left box), α^2 (lower right box), and β^1 (upper right box). Symbols within each box denote the chemical state of the heme. The index below each tetramer denotes the ligation state, keyed to those of Fig. 1.

species 21 was studied by forming hybrid mixtures of the appropriate "parent" molecules, as shown below.



It is necessary to resolve the dissociation rate constant for the hybrid species AB (and, in combination with the assembly rate constant, the free energy of hybrid formation) in the presence of the other molecular species, as depicted above.

The rate constant for tetramer–dimer dissociation (k_r) was determined for each species using the haptoglobin binding technique (16). In normal hemoglobin A_0, deoxy dimers, and deoxy tetramers have different extinction coefficients at 430 nm. Upon mixing deoxygenated solutions of hemoglobin and haptoglobin, the absorbance at 430 nm decreases with time, as tetramers dissociate into dimers that complex with haptoglobin. A similar technique is used for Mn-substituted hemoglobins, including those partially ligated where some subunits contain Mn^{2+} or Mn^{3+}.

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

$$A(t) = A_\infty + P_1 e^{-k_{AA}t} + P_2 e^{-k_{AB}t} + P_3 e^{-k_{BB}t}, \quad [4]$$

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

All experiments were carried out in 0.1 M Tris·HCl/0.1 M NaCl/1 mM Na_2EDTA, pH 7.4, at 21.5°C. Experiments on hemoglobins containing Mn^{2+} or Mn^{2+}/Fe-CO were carried out as follows. Prior to each kinetics experiment, samples of hemoglobin containing Mn^{3+} or Mn^{3+}/Fe^{2+} were reduced to the Mn^{2+} form with 0.1% sodium dithionite under anaerobic conditions. In experiments on Mn^{2+}/Fe-CO samples (species 21, 23, and 24) the solutions were equilibrated with CO after reduction, to ensure saturation of Fe^{2+} hemes with CO. Spectra obtained after reduction and saturation with CO were as expected for mixtures of Mn^{2+} and Fe-CO hemoglobins.

For kinetics experiments on Mn^{3+} or Mn^{3+}/Fe^{2+}, the hybrid samples were deoxygenated using humidified nitrogen in combination with glucose oxidase (1.8 mg/ml) and with catalase (0.3 mg/ml) in the presence of the substrate 0.3% *d*-glucose (1). Spectra obtained after deoxygenation (400–500 nm) were as expected for mixtures of Mn^{3+} and Fe^{2+} (deoxy) hemoglobins.

For each experiment, Mn-substituted hemoglobin or Mn/Fe hybrids were mixed with solutions of haptoglobin under anaerobic conditions. The absorbance change with time was monitored at 434 nm for system A and at 430 nm for system B. For slowly dissociating species ($t_{1/2}$, from minutes to hours) the reaction was carried out on a Varian 219 spectrophotometer. For rapidly dissociating species ($t_{1/2}$, approximately seconds), the reaction was monitored on a Dionex stopped-flow spectrophotometer.

The equilibrium constants ${}^iK_{2j}$ were calculated using the resolved values of the dissociation rate constant k_r and the known value of the dimer–tetramer assembly rate constant (k_f, $1.1 \times 10^6\ M^{-1}\cdot sec^{-1}$). In an extensive series of studies of normal hemoglobin over a wide range of pH values (8) and with mutant or chemically modified hemoglobins, including hybrids (5, 15), the value of the assembly rate constant remained unchanged.

Analytical Gel Chromatography. Dimer–tetramer equilibrium constants for normal hemoglobin A_0 ligated with CO and for Mn^{3+} hemoglobin (species 41) were determined directly by analytical gel chromatography (15). The resulting value of ${}^4K_{21}$, in combination with the dissociation rate constant from the haptoglobin experiment, provides a determination of the association rate constant for species 41 in agreement with that obtained kinetically.

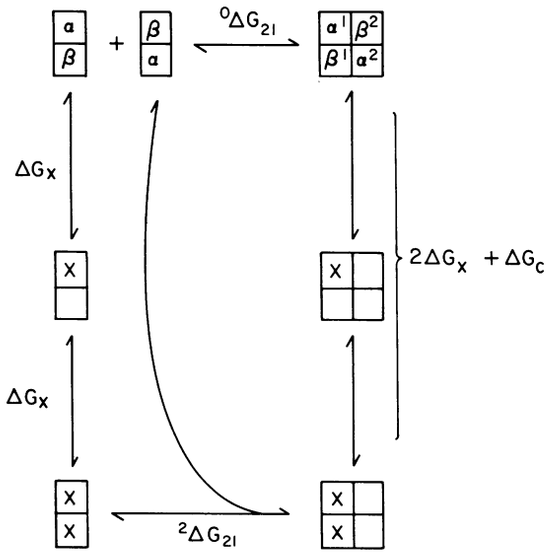


FIG. 4. Thermodynamic linkage between subunit assembly (*Left* to *Right*) and ligand binding (*Top* to *Bottom*). ΔG_x is the intrinsic binding free energy as determined by singly ligating the dimer. The expression $2\Delta G_x + \Delta G_c$ is the free energy difference between a mole of doubly ligated tetramers (*Bottom*) and a mole of unligated tetramers (*Top*). $^\circ\Delta G_{2j}$ is the free energy of forming the unligated tetramer from two unligated dimers. ${}^2\Delta G_{2j}$ is the free energy of forming doubly ligated tetramer by combination of a doubly ligated dimer and an unligated dimer.

RESULTS

Table 1 gives the free energies of assembly and the cooperative free energies for five ligation states of systems A and B, as obtained in this study. Also listed for comparison are the corresponding free energies found (1) for the cyanomet system (system C).

In considering these three systems as functional analogs of normal hemoglobin oxygenation, we note that assembly free energies for the "deoxy" species 01 have similar values, while stability of the fully ligated tetramer species 41 is decreased by approximately five orders of magnitude. While we do not expect exact quantitative agreement between different ligation state models, the energetic ranges found here support the premise that systems A, B, and C are valid functional analogs for hemoglobin oxygenation.

Table 1 shows values of the cooperative free energy ΔG_c as determined by Eq. 3. The distribution of ligation state species among the three cooperativity states is shown in Fig. 2. We find three distinct values of ΔG_c for each system. While the unligated and fully ligated tetramers (species 01 and 41, respectively) are found in cooperativity states with the smallest and largest ΔG_c, we also find a third distinct value of ΔG_c that is assumed by species 21. Furthermore, the two

Table 1. Gibbs energies of assembly and cooperative free energies for hemoglobin tetramers

Ligation state (*ij*)	System A (Mn^{2+}/Fe-CO)			System B (Fe^{2+}/Mn^{3+})			System C (Fe^{2+}/Fe-CN)		
	${}^{i}\Delta G_{2j}$	Cooperative free energy	MWC* c	${}^{i}\Delta G_{2j}$	Cooperative free energy	MWC* c	${}^{i}\Delta G_{2j}$	Cooperative free energy	MWC* c
01	−15.6 ± 0.5	0 (ref.)	—	−14.4 ± 0.1	0 (ref.)	—	−14.4 ± 0.1	0 (ref.)	—
21	−13.1 ± 0.5	2.5 ± 0.7	0.118	−11.0 ± 0.2	3.4 ± 0.3	0.055	−11.4 ± 0.2	3.0 ± 0.3	0.077
23	−7.8 ± 0.5	7.8 ± 0.7	0.0013	−7.6 ± 0.2	6.8 ± 0.3	0.003	−8.2 ± 0.2	6.2 ± 0.3	0.005
24	−8.3 ± 0.5	7.3 ± 0.7	0.0016	−8.2 ± 0.2	6.2 ± 0.3	0.005	−8.5 ± 0.2	5.9 ± 0.3	0.006
41	−8.0 ± 0.1	7.6 ± 0.6	—	−7.5 ± 0.1	6.9 ± 0.2	—	−8.5 ± 0.1	5.9 ± 0.2	—

${}^{i}\Delta G_{2j}$ values are standard Gibbs energies of assembling the tetrameric species *ij* from constituent dimers. Cooperative free energies were calculated by Eq. **3**.

*Allosteric constants, L, of two-state MWC model calculated by Eq. **7** are as follows: 4.4×10^5 (system A), 1.3×10^5 (system B), and 2.4×10^4 (system C). Values of the allosteric parameter, c, were calculated by Eq. **6** for each experimental value of ΔG_c.

Mn-substituted systems (systems A and B), like the cyanomet system (system C), are seen to behave according to an apparent combinatorial code—i.e., the observed cooperativity state depends not only on the number of ligands bound but also on the specific configuration of ligated subunits. We emphasize that this observation does not, by itself, imply any specific molecular mechanism (see below). A striking feature of these results (Table 1) is that the five species we have studied for each of the Mn systems (systems A and B) show the same distribution of energetic effects found using cyanomet hemes as the ligated subunits (system C). These findings demonstrate that the three-state combinatorial nature of switching between cooperativity states is not a special feature arising from the reactions of hemoglobin with cyanide or from the substitution of Mn for Fe. Our results lend strong support to the premise that the observed combinatorial switching is a fundamental property of the intra- and intersubunit interactions that accompany ligation-like perturbations at the hemes.

Why a Minimum of Three Molecular Structures Is Required. A finding of three cooperativity states for hemoglobin tetramers does not by itself mean that there must be three corresponding molecular structures. It has been shown (9) that a tetrameric 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. This possibility appears to have been rediscovered by Ferrone (11). The minimum number of significant molecular structures can be determined by analysis of the distribution of ΔG_c values among the ligation species. The allosteric Monod–Wyman–Changeux (MWC) model provides a rigorous theoretical treatment for the simplest mechanism of cooperative switching in a tetrameric system that has only two molecular structures (17). Here we show briefly why the cooperative free energy distributions determined experimentally for the intermediate state tetramers are incompatible with this MWC model (see refs. 9 and 18 for more detailed treatment).

Theoretical treatment of this problem (9) provides the fundamental relationship

$$ {}^{i}K'_2 = K'_{2R}\,(1 + Lc^i). \qquad [5] $$

Here ${}^{i}K'_2$ is the intrinsic equilibrium constant for dimer–tetramer assembly for a tetramer with i ligated subunits (i = 0, 1, 2, 3, 4), and K'_{2R} is the intrinsic assembly constant for forming an R-state tetramer from two dimers. The allosteric constant, L, represents the equilibrium between unligated conformers (R and T) while c is the ratio of their affinities for ligand (see ref. 18). Each dimer is half of an R-state tetramer in the simple two-state MWC model, and the constant K'_{2R} is independent of the degree of ligation, i. Eq. **5** may be compared with the corresponding relationships postulated by Ferrone (11) and by Weber (10). From Eqs. **3** and **5** we see that values of cooperative free energy ΔG_c allowed by the MWC model must satisfy

$$ \Delta G_c = -RT \ln\left(\frac{1 + Lc^i}{1 + L}\right). \qquad [6] $$

For each of the three systems of Table 1, the set of relationships prescribed by Eq. **6** may be used to determine values of L and c from the four nonzero values of ΔG_c. For each system, L may be fixed independently using Eq. **5** by

$$ \frac{{}^{0}K_{21}}{{}^{4}K_{21}} \approx L \qquad (L>>1,\ Lc^4<<1). \qquad [7] $$

Results of these calculations are given in Table 1, where a wide discrepancy is shown in values of the allosteric parameter c within each system. Validity of the two-state mechanism requires that all the data on a given system (e.g., system B) conform to a single value of c, as given by Eq. **5**. However, this condition is not met, and we see that Eq. **5** is incompatible with behavior of each of the three hemoglobin systems. Hence the model is ruled out for each of them separately.

As noted earlier, this test of the two-state model assumes that each dimer is half of an R-state tetramer and that affinities of the α and β subunits are identical. Relaxation of these restraints requires a more elaborate theoretical treatment but leads to the same conclusion. This conclusion is also reached if we attempt to fit the experimental data of Table 1 to Eq. **6**, evaluating L and c simultaneously (calculation not shown). Failure of the two-state allosteric model to describe the distribution of all ΔG_c values found in any one of the hemoglobin systems (Table 1) implies that each tetrameric molecule is capable of at least three molecular structures with separate free energies of cooperative interaction. This conclusion is a consequence of the following consideration. The values of ΔG_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 contacts; and 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.

DISCUSSION

The relationship of these findings to the molecular mechanism of oxygenation is of paramount interest. From the

results of this study we see that either (*i*) the hemoglobin tetramer has a minimum of three molecular cooperativity states that may control oxygen affinity or (*ii*) ligation state "models" studied extensively during the last two decades (i.e., cyanomet and metal-substituted hemoglobins) are not valid functional analogs for hemoglobin oxygenation. Even if this were the case, there remains the question of the structural and mechanistic origins of the three-level cooperative switch found in these nonoxygen hemoglobin systems. Evidence for three cooperativity states with ligands CO and NO has been discussed (18).

Comparison with Crystallographic Results. A central concept of the two-state paradigm has been that each of the two affinity states is uniquely associated with a major quaternary structural form of the tetrameric molecule. The finding that each tetrameric molecule assumes at least three major cooperative free energies requires that either (*i*) a third major structural form of the tetrameric molecule exists or (*ii*) the concept of a one-to-one correspondence between cooperativity states and major structural forms of the tetramer must be abandoned. This would be the case if the intermediate energetic state should turn out to represent a structure only slightly different from either the deoxy or the oxy quaternary structures.

Arnone *et al.* (3) have determined a crystallographic structure for the intermediate species 23 of system A (Fig. 3). The tetramers in these crystals exhibited a deoxy quaternary structure. The Mn^{2+}-substituted β subunits were isomorphous with deoxy Fe^{2+} β subunits, whereas CO binding to the α subunits was accompanied by small, localized changes in tertiary structure. Similar local structure perturbations had been observed in a crystallographic study of hemoglobin tetramers with α subunits ligated by oxygen and β subunits in the normal deoxy Fe^{2+} form (4). In contrast, the solution studies presented here and earlier (1) clearly demonstrate that species 23 has cooperativity properties like the fully ligated (oxy) species 41, under our experimental conditions.

The apparent difference between behavior in the crystal and in solution may result from differences in experimental conditions. Spectral and kinetic studies of species 23 and 24 have shown that conformation in solution is sensitive to phosphate concentration and pH (19, 20). In the studies of Arnone *et al.* (3) crystallization was carried out in solutions of 2.3 M ammonium sulfate plus 0.3 M ammonium phosphate buffer (pH 6.5). It was not possible to crystallize species 23 in the presence of CO; the preformed crystal was exposed to CO in the capillary. It seems plausible that, under these circumstances, the conformation in the crystal may be influenced by packing interactions and crystal lattice forces that stabilize the deoxy quaternary form, essentially forcing the molecule to maintain the deoxy structure (3, 4). By contrast, our solution studies were carried out in 0.18 M Cl^-, pH 7.4. Under these conditions the difference in relative stability for the transition between the oxy species 41 and the deoxy species 01 (system A) is 4.4×10^5, a value almost identical with that obtained with oxygen as ligand (8).

This work was supported by Grant GM 24486 from the National Institutes of Health and Grant DMB 84-03533 from the National Science Foundation. F.R.S. acknowledges a postdoctoral fellowship from the American Heart Association.

1. Smith, F. R. & Ackers, G. K. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5347–5351.
2. Scheidt, W. R. (1977) *Acc. Chem. Res.* **10,** 339–345.
3. Arnone, A., Rogers, P., Blough, N. V., McGourty, J. L. & Hoffman, B. M. (1986) *J. Mol. Biol.* **188,** 693–706.
4. Brzozowski, A., Derewenda, Z., Dodson, E., Dodson, G., Grabowski, M., Liddington, R., Skaryznski, T. & Vallely, D. (1984) *Nature (London)* **307,** 74–76.
5. Pettigrew, D. W., Romeo, P. H., Tsapis, A., Thillet, J., Smith, M. L., Turner, B. W. & Ackers, G. K. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1849–1853.
6. Ackers, G. K. & Halvorson, H. R. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4312–4315.
7. Mills, F. C., Johnson, M. L. & Ackers, G. K. (1976) *Biochemistry* **15,** 5350–5362.
8. Chu, A. H., Turner, B. W. & Ackers, G. K. (1984) *Biochemistry* **23,** 604–617.
9. Ackers, G. K. & Johnson, M. L. (1981) *J. Mol. Biol.* **147,** 559–582.
10. Weber, G. (1985) *Proc. Natl. Acad. Sci. USA* **81,** 7098–7102.
11. Ferrone, F. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6412–6414.
12. Williams, R. C. & Tsay, K. Y. (1975) *Anal. Biochem.* **54,** 137–145.
13. Scholler, D. M., Wang, M.-Y. R. & Hoffman, B. M. (1979) *Methods Enzymol.* **52C,** 487–493.
14. Blough, N. V. & Hoffman, B. M. (1984) *Biochemistry* **23,** 2875–2882.
15. Turner, B. W., Pettigrew, D. W. & Ackers, G. K. (1982) *Methods Enzymol.* **76,** 596–628.
16. Ip, S. H. C., Johnson, M. L. & Ackers, G. K. (1976) *Biochemistry* **15,** 654–660.
17. Monod, J., Wyman, J. & Changeux, J. P. (1965) *J. Mol. Biol.* **12,** 88–118.
18. Ackers, G. K. & Smith, F. R. (1987) *Annu. Rev. Biophys. Biophys. Chem.* **16,** 583–609.
19. Ogawa, S. & Shulman, R. G. (1972) *J. Mol. Biol.* **20,** 315–366.
20. Cassoly, R. (1978) *J. Biol. Chem.* **253,** 3602–3606.