RESOLVING PATHWAYS OF FUNCTIONAL COUPLING WITHIN PROTEIN ASSEMBLIES BY SITE-SPECIFIC STRUCTURAL PERTURBATION

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ABSTRACT Site-specific structural modification is a powerful tool for studying functional mechanisms in proteins where the structures may be manipulated by direct chemical modification, by selection of naturally-occurring mutants, or by site-directed mutagenesis. Here, we present a general strategy for such studies, which we term "mapping by structure-function perturbation. " A series of functional perturbations (i.e., deviations of functional behavior from that of the native protein) are mapped against the structural locations of the modified sites, obtained over a range of locations. The modifications are treated as arbitrary perturbations of structure at specific locations, in contrast to the conventional approach of trying to interpret their local stereochemistry. The map yields information on structural locations of functional events and pathways of coupling within protein assemblies. We have applied this approach to the ligand-linked subunit assembly of human hemoglobin, using both chemically-modified heme sites (CN-met), and amino acid residues altered by mutation and chemical modification.

INTRODUCTION

A central issue in protein structure-function problems is understanding the roles played by the local parts of a molecular structure in the overall functional processes of interest. How do functional effects or their control arise from particular domains, local binding sites, or interfaces? Which amino acid side chains and specific residue locations are crucial to function? By what pathways within a molecular asssembly are the local changes in structure and noncovalent interaction coupled to each other? In this article we present a general rationale for studying these issues using local-site modification accomplished through chemical alteration of the native protein, selection of naturally-occurring genetic variants, or techniques of site directed mutagenesis. The new approach embodies a set of strategies based upon studying the effects of one type of perturbation (i.e., covalent structure modification) upon perturbations of a second type (i.e., interactions representing the functional process of interest). This method of combining structural and thermodynamic information is applicable to biological functions that reflect, directly or indirectly, relatively small changes in both structure and thermodynamic state of a protein molecule. We describe applications of this approach to studies on the ligand-linked subunit assembly and cooperative mechanism of human hemoglobin. The results are as follows: The 10 ligation states of normal hemoglobin assume only three cooperative free energy levels, transitions being synchronized with particular ligation steps according to a specific "code." The sites of cooperative free energy are found to be localized within the $\alpha^1 \beta^2$ intersubunit contact region within the tetramers. Functional perturbations arising from amino acid substitutions within this interface are not directly coupled to each other but are coupled indirectly through perturbations at the heme sites.

MAPPING BY STRUCTURE-FUNCTION PERTURBATION

In the normal function of a protein (e.g., ligand binding), local changes in structure and interaction energy occur within the molecule while a similar overall tertiary structure is maintained. Likewise, the alteration of a single site, e.g. by an amino acid substitution, generally preserves the protein's folded tertiary structure. It is reasonable to expect that the general modes of coupling between the local site and the rest of the molecule are frequently not altered by such modifications. (The interactions may often be altered quantitatively but not qualitatively). Therefore, structural modifications can be used as models for the changes that occur in the normal functional process. If, for example, interactions in the region of a given site are altered during function, it may be expected that structural modification at that site will lead to altered functional behavior. Functional processes that satisfy these criteria include subunit assembly, ligand binding, conformational changes, and steady-state enzymatic activities. Each of these functions is a "system property" reflecting observable behavior of the protein molecule or assembly as a whole. The goal of

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structure-function studies is to learn how the roles played by the local parts of the assembly contribute to its net functional behavior.

The functional interactions to be discussed may, in principle, be mediated by the following types of effects:

Local Effects—short-range steric and noncovalent bonding interactions, which are in the neighborhood of a local site. These include ionic bonds (salt bridges), H-bonds, hydrophobic and van der Waals interactions, as well as local conformational changes.

Global Effects—overall changes of conformation or solvation that may affect, or be propagated throughout, the molecule as a whole.

Specific Long-Range Effects—specific pathways whereby structural modifications at a site are transmitted to another site.

Strategies for using site modification to study functional mechanisms have usually followed an approach based on the interpretation of functional perturbation (the deviation from normal behavior due to the effects of amino acids modification) in terms of the local stereochemistry of the modified site. This approach requires knowledge on the atomic level of the structural changes at each site that accompany the molecule's functional transitions and the potentials of interaction (including those of solvent where relevant) for each atom involved in the structural alterations. Theories are then constructed to interpret the relevance of these properties to the functional process. This type of analysis is the classical approach to detailed structure-function problems in protein chemistry, and has yielded much useful insight into the possible roles of individual amino acid side chains in mechanistic processes.

By contrast the method of mapping by structure-function perturbation does not make use of detailed information on the local changes in structure and interactions. It is based, rather, on two entirely different kinds of information: experimental determinations of the functional perturbations for a series of structure modifications, and the locations within the molecular structure of the corresponding modified sites. In this approach, the modifications are treated as arbitrary perturbations of structure at specific locations. The observed functional perturbations vs. structural locations of the modified sites provide a map from which inferences may be drawn regarding the structural locations of functional events and structural pathways for coupling and transmission of functional events within the molecule. This approach is an especially powerful tool in systems where long-range coupling effects play important biological roles and where detailed stereochemical information throughout the entire molecular structure is not feasible. Indeed such information has yet to be obtained for any macromolecular assembly. Moreover it is not clear that a strictly atomic-level information base is required to understand the essential aspects of biologically important structure-energy correlations in many systems. It is certain that much can be learned from a knowledge of effects occurring at the level of subunits, domains, local binding regions and interfaces. In principle the two approaches, based on different types of information, should provide complementary and mutually reinforcing results.

Functional Filter

A major feature of the structure-function perturbation method is the incorporation of a "functional filter," based on the following rationale. Modification of a given site may generate local structure changes at other sites that may not be involved in the specific function of interest. Even if these local structure changes are monitored (e.g., spectroscopically) there remains the problem of determining which ones are relevant to function. This problem is bypassed when locations of the modified sites are mapped directly against a quantitative measure of the specific function. This procedure automatically selects contributions only from those sites where structural modification affects the functional behavior.

Functional Perturbation

Consider the following scheme depicting a (native, or wild-type) protein, P, which undergoes a functional process for which we can obtain a quantitative measure of its magnitude, F.

$$\begin{tikzcd} \mathrm{P} \arrow[r, "\substack{\text{functional process}\\ F}"] \arrow[d, "\substack{\text{structure}\\ \text{modification}}"'] \arrow[dr, phantom, "F_m"] & \mathrm{P}' \arrow[d] \\ \mathrm{P}_m \arrow[r] & \mathrm{P}'_m \end{tikzcd} \qquad (1)$$

The quantity F might represent a Gibbs energy ΔG of subunit assembly, an appropriate function of the rate constant for enzymatic reaction, etc. The lower part of the diagram depicts a modified form of the protein P_m which differs structurally from P at a single site. The protein P_m may be capable of the same functional process as P but with a quantitatively altered magnitude, F_m. There is thus, as a result of the structural modification, a perturbation δF_m in the functional property F:

$$\delta F_m = F_m - F. \qquad (2)$$

The possible ways in which a protein may respond to a single-site modification to yield the observed functional perturbation δF_m may be summarized as follows:

Function is Completely Eliminated by Structural Modification ($\delta F_m = -F$). This could result from any of the following:

(*a*) local structures directly required for function being eliminated (e.g., at an active site);

(*b*) the modification-precluding conformations that would permit function to occur, either through local steric effects or long-range "global" effects;

(*c*) alterations of noncovalent interactions being "coincidentally" of such magnitude that $\delta F_m = -F$.

Function is Unperturbed by Structural Modification ($\delta F_m=0$).

(*a*) The modification site is not involved in F;

(*b*) The modification site is involved in function but $\delta F_m = 0$ as a result of compensating effects; or because the magnitude of the perturbation resulting from the structure modification does not exceed a minimal threshold. This threshold might be large in cases where the functional response is mediated by "global" transitions of the molecule.

Function is Perturbed by Structural Modification ($\delta F_m \neq 0, \delta F_m \neq -F$).

(*a*) The site of modification is in a region of the molecule directly involved in function.

(*b*) The modification is at a site remote from the functional pathway, yet exerts an effect through long-range coupling either via a specific pathway, or via "global" conformational effects.

These possibilities define the nature of the problem encountered by the investigator in using single-site modification as a tool. What are the best strategies for design of experiments and interpretation of results?

Interpreting the Effects of Structure-Function Perturbation

It is obvious from the possibilities listed above that simplistic interpretations can be treacherous and that interpretation can be greatly aided if much is already known about the general nature of the functional process. Usually the functional perturbation δF_m resulting from only a single-site modification can only be interpreted in a speculative fashion. When a residue is altered and it is observed that the functional property of interest has also been changed it is hazardous to interpret the alteration solely in terms of the local interactions of the altered residue site. The observed functional property is a "system property," and may reflect contributions from any part of the molecule that is concomitantly altered during the overall functional process. A ligand-binding constant, for example, may reflect more than just the free energy of local interactions at the binding site if perturbations at that site are coupled to structural alterations in other parts of the molecule.

Whereas interpretation of the functional perturbation for a single modification is likely to be ambiguous, a pattern of behavior resulting from an extensive set of single-site modifications distributed throughout the molecular structure may provide a strong case for inferences regarding the localization and coupling of functional effects. The sites should be grouped according to structural regions of the molecule and the corresponding values of δF_m tabulated. If a striking pattern exists such that the only sites where modifications produce significant values of δF_m lie in a certain region of the molecular structure, then it may be reasonably inferred that these sites are altered during the functional process in the native molecule. We will use the concept of a "pathway of functional perturbation" within a protein assembly to denote that region of the molecule where local changes in structure and interaction occur during the normal functional process. In such a case it is desirable to explore the observed patterns by further modifications and by multiple modifications as described in the next section. If the patterns are not striking (if the magnitudes of differences are small) it may be necessary to obtain more data before conclusions may be drawn.

Structure-function perturbation mapping may be carried out using a randomized distribution of the structural locations of modified sites or by selection of specific combinations of sites already known to play functional roles (e.g., cooperative binding sites) to explore the coupling between those sites.

Functional Coupling Between Sites

Once a region or functional pathway has been identified, it is of great value to explore the coupling of effects within this region of structure through the interplay of multiple modifications. The simplest way to accomplish this is first to construct two separate proteins, P_1 and P_2 that are modified at different locations. The corresponding functional perturbations (Eq. 2) will be δF_1 and δF_2. Next construct a molecular species P_2 that has both modifications, and determine the resulting perturbation of function $\delta F_{1,2}$ for the doubly modified molecule. If the quantity F is proportional to a conserved property, say the Gibbs free energy, we can test for the independence of perturbations resulting from modifications at sites "1" and "2" by the relationship:

$$\delta F_{12} = \delta F_1 + \delta F_2. \qquad (3)$$

A finding that Eq. 3 is valid provides evidence that the two sites are not directly coupled in generating the functional response F. When Eq. 3 fails, it is useful to assess the magnitude of "functional coupling" by noting that P_{12} could be constructed along two routes depending on the order of introducing the modifications as shown in scheme 4.

$$\begin{tikzcd}
P \arrow[r, "\delta F_1"] \arrow[d, "\delta F_2"'] & P_1 \arrow[d, "\delta F_{2,1}"] \\
P_2 \arrow[r, "\delta F_{1,2}"'] & P_{12}
\end{tikzcd} \qquad (4)$$

In this scheme, $\delta F_{2,1}$ is the functional perturbation arising from modification at site "2," given that modification at site "1" has already been carried out. The analogous perturbation resulting from structural modification at site "1" for a molecule previously modified at site "2" is given by $\delta F_{1,2}$. We define a coupling constant δF_c by the relationships

$$\delta F_c = \delta F_{2,1} - \delta F_2 = \delta F_{1,2} - \delta F_1. \qquad (5)$$

If there is no site-site coupling so that $\delta F_c = 0$ then the relationship of Eq. 3 follows, because $\delta F_{12} = \delta F_1 + \delta F_{2,1} = \delta F_2 + \delta F_{1,2}$.

Indirect Coupling and Triangulation

Even if $\delta F_c = 0$ for two site-specific structure modifications (Eq. 5), it is entirely possible for both sites to lie within the functional pathway, if for example, they are both "driven" by changes at a third site. Directional or anisotropic effects in proteins are well-known (e.g., actin-myosin complexes) and can arise either through stereospecific pathways or through widespread conformational changes that may be "triggered" by perturbation of some sites but not others. We consider two sites, "1" and "2," each of which is coupled to a third site "3" as in Eq. 5. But sites "1" and "2," when modified in the absence of prior modification at site "3," generate perturbations that satisfy Eq. 3. Then it follows that modification of site 3 will simultaneously affect perturbations at sites "1" and "2," i.e., we have the simultaneous relationships

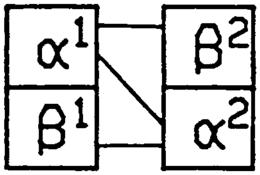
$$\begin{aligned} \delta F_{12} &= \delta F_1 + \delta F_2 \\ \delta F_{12,3} &= \delta F_{1,3} + \delta F_{2,3} \\ \delta F_{1,3} &\neq \delta F_1; \qquad \delta F_{2,3} \neq \delta F_2, \end{aligned} \tag{6}$$

where the subscripts denote the respective combinations of modified sites. By exploring all sites in the protein system it is possible, in principle, to map all such couplings by this triangulation procedure. Additional combinations of modifications can be usefully generated and studied in a hierarchical fashion. This type of coupling is illustrated by the mutant hybrid studies described below.

COOPERATIVE INTERACTIONS IN HUMAN HEMOGLOBIN

We have applied structure-function perturbation mapping to the ligand-linked subunit assembly of human hemoglobin where numerous mutant and chemically-modified proteins are readily available for study. These studies have included site-specific modification of four general types: (*a*) normal hemoglobin with heme-site chemical modification (CN-met), representing all combinations of ligated sites within the tetramer; (*b*) mutant and chemically modified tetramers bearing two identical, symmetrically-disposed, modifications; (*c*) hybrids of these species with normal hemoglobin resulting in molecules that bear only one of the modified sites; and (*d*) hybrids of two hemoglobins bearing different modifications.

Human hemoglobin is a classic example of a macromolecule (or assembly) that regulates its own functions (e.g., oxygen-binding affinity) in response to interactions with ligands at specific sites, i.e., the heme sites (see reference 1 for a general review of hemoglobin structure and function). The hemoglobin molecule is a tetrameric structure comprised of four subunits (two α and two β chains) each of which contains a heme group where oxygen is bound. The topographic relationships of the subunits within the tetramer are shown in diagram 7.



(7)

The subunits are designated α^1, α^2, β^1, and β^2, denoting their relative locations within the tetramer. Each tetramer contains two types of intersubunit contact regions, the $\alpha^1\beta^1$ ($\alpha^2\beta^2$) interface, and the $\alpha^1\beta^2$ contact region, which contains three pairwise contacts $\alpha^1\beta^2$, $\alpha^2\beta^1$, and $\alpha^1\alpha^2$ (β chains are not in contact). The pairwise intersubunit contacts include hydrogen bonds, ion pairs (salt bridges), hydrophobic interactions, and van der Waals interactions. When oxygen or other ligands are bound at all four sites, major changes occur at the "$\alpha^1\beta^2$ intersubunit contact region" (2–4). The $\alpha^1\beta^1$ and $\alpha^2\beta^2$ contacts do not undergo appreciable alterations when oxygenated. The binding of oxygen at the heme sites within the tetramer leads to structure changes at the heme itself, tertiary changes within the oxygenated subunits, and quaternary changes affecting the subunit contacts mentioned above (4).

Ligation States

The 10 ligation states of teterameric hemoglobin are represented schematically in Table I. These molecular forms differ in the number and configuration of ligated subunits among the possibilities α^1, β^1, α^2 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, i_j, designates the particular species j among those with i ligands bound. Each tetrameric species is an assembly of constitutent 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 I.

Cooperative Free Energies

Cooperativity is the deviation in free energy of ligation from that which one would obtain for the same sites if they bind independently, i.e., with their intrinsic free energies (5). Because dissociated dimers (α,β) bind ligands 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 (6). Subunit interactions that generate the cooperative free energies are decoupled by dissociation into dimers (see reference 7). For a tetrameric species representing a particular ligation state i_j (Table I), the free energy ΔG_{ij} of binding of i ligands

TABLE I

COOPERATIVE FREE ENERGIES FOR HEMOGLOBIN TETRAMERS

		Molecular Forms				Dissociation Rate Constants*				
ij	g_{ij}	Tetramer	Constituent dimers	Parent species A	B	Parent A	Parent B	Tetramer	$i\Delta G_{2j}$	Cooperative Free Energy (${}^{i}\Delta G_{2j} - {}^{0}\Delta G^{2i}$)
						s^{-1}	s^{-1}	s^{-1}	*kcal/mol*‡	
01	1	$\begin{array}{cc}\boxed{\alpha^1}&\boxed{\beta^2}\\\boxed{\beta^1}&\boxed{\alpha^2}\end{array}$	$\begin{array}{c}\boxed{\alpha^1}\\\boxed{\beta^1}\end{array}+\begin{array}{c}\boxed{\beta^2}\\\boxed{\alpha^2}\end{array}$	none		—	—	2.1×10^{-5}	−14.4	0
11	2	$\begin{array}{cc}\boxed{X}&\boxed{}\\\boxed{}&\boxed{}\end{array}$	$\begin{array}{c}\boxed{X}\\\boxed{}\end{array}+\begin{array}{c}\boxed{}\\\boxed{}\end{array}$	$\begin{array}{cc}\boxed{X}&\boxed{}\\\boxed{}&\boxed{X}\end{array}$	$+\begin{array}{cc}\boxed{}&\boxed{}\\\boxed{}&\boxed{}\end{array}$	0.84	2.1×10^{-5}	3.0×10^{-3}	−11.5	2.9
12	2	$\begin{array}{cc}\boxed{}&\boxed{}\\\boxed{X}&\boxed{}\end{array}$	$\begin{array}{c}\boxed{}\\\boxed{X}\end{array}+\begin{array}{c}\boxed{}\\\boxed{}\end{array}$	$\begin{array}{cc}\boxed{}&\boxed{X}\\\boxed{X}&\boxed{}\end{array}$	$+\begin{array}{cc}\boxed{}&\boxed{}\\\boxed{}&\boxed{}\end{array}$	0.51	2.1×10^{-5}	5.7×10^{-3}	−11.2	3.2
21	2	$\begin{array}{cc}\boxed{X}&\boxed{}\\\boxed{X}&\boxed{}\end{array}$	$\begin{array}{c}\boxed{X}\\\boxed{X}\end{array}+\begin{array}{c}\boxed{}\\\boxed{}\end{array}$	$\begin{array}{cc}\boxed{X}&\boxed{X}\\\boxed{X}&\boxed{X}\end{array}$	$+\begin{array}{cc}\boxed{}&\boxed{}\\\boxed{}&\boxed{}\end{array}$	0.59§	2.1×10^{-5}	3.9×10^{-3}	−11.4	3.0
22	2	$\begin{array}{cc}\boxed{X}&\boxed{X}\\\boxed{}&\boxed{}\end{array}$	$\begin{array}{c}\boxed{X}\\\boxed{}\end{array}+\begin{array}{c}\boxed{X}\\\boxed{}\end{array}$	$\begin{array}{cc}\boxed{X}&\boxed{}\\\boxed{}&\boxed{X}\end{array}$	$+\begin{array}{cc}\boxed{}&\boxed{X}\\\boxed{X}&\boxed{}\end{array}$	0.84	0.51	2.4×10^{-3}	−11.7	2.7
23	1	$\begin{array}{cc}\boxed{X}&\boxed{}\\\boxed{}&\boxed{X}\end{array}$	$\begin{array}{c}\boxed{X}\\\boxed{}\end{array}+\begin{array}{c}\boxed{}\\\boxed{X}\end{array}$	none		—	—	0.84	−8.2	6.2
24	1	$\begin{array}{cc}\boxed{}&\boxed{X}\\\boxed{X}&\boxed{}\end{array}$	$\begin{array}{c}\boxed{}\\\boxed{X}\end{array}+\begin{array}{c}\boxed{X}\\\boxed{}\end{array}$	none		—	—	0.51	−8.5	5.9
31	2	$\begin{array}{cc}\boxed{X}&\boxed{X}\\\boxed{X}&\boxed{}\end{array}$	$\begin{array}{c}\boxed{X}\\\boxed{X}\end{array}+\begin{array}{c}\boxed{X}\\\boxed{}\end{array}$	$\begin{array}{cc}\boxed{X}&\boxed{X}\\\boxed{X}&\boxed{X}\end{array}$	$+\begin{array}{cc}\boxed{}&\boxed{X}\\\boxed{X}&\boxed{}\end{array}$	0.59§	0.51	0.44	−8.6	5.8
32	2	$\begin{array}{cc}\boxed{X}&\boxed{}\\\boxed{X}&\boxed{X}\end{array}$	$\begin{array}{c}\boxed{X}\\\boxed{X}\end{array}+\begin{array}{c}\boxed{}\\\boxed{X}\end{array}$	$\begin{array}{cc}\boxed{X}&\boxed{X}\\\boxed{X}&\boxed{X}\end{array}$	$+\begin{array}{cc}\boxed{X}&\boxed{}\\\boxed{}&\boxed{X}\end{array}$	0.59§	0.84	0.68	−8.4	6.0
41	1	$\begin{array}{cc}\boxed{X}&\boxed{X}\\\boxed{X}&\boxed{X}\end{array}$	$\begin{array}{c}\boxed{X}\\\boxed{X}\end{array}+\begin{array}{c}\boxed{X}\\\boxed{X}\end{array}$	none		—	—	0.59§	−8.5¶	5.9

*Values accurate to ±10%.

‡Values accurate to ±0.2 kcal.

§Calculated from independently determined values of k_f and ${}^{4}K_{21}$.

¶Determined by analytical gel chromatography (11).

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.

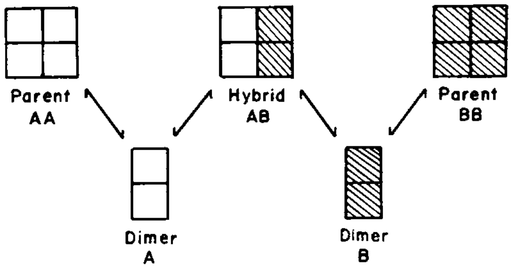
may be written

$$\Delta G_{ij} = i\Delta G_x + {}^{i}\Delta G_{2j} - {}^{0}\Delta G_{21}, \tag{8}$$

where ΔG_x is the intrinsic free energy of binding X to a site, such as one on the noncooperative dimers. The constituent dimers shown in Table I self-associate to form each tetramer i_j with standard free energy ${}^{i}\Delta G_{2j}$. ${}^{0}\Delta G_{21}$ is the standard free energy of forming unligated tetramers from unligated dimers (Table I). From Eq. 8 it is seen that the difference (${}^{i}\Delta G_{2j} - {}^{0}\Delta G_{21}$) 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 I, and forming the differences ${}^{i}\Delta G_{2j} - {}^{0}\Delta G_{21}$). (The term "cooperative free energy" used here is identical to the "regulatory free energy" discussed in reference 5.)

METHODS

The approach we used for determining the assembly free energies is based on the energetics of forming hybrids between tetrameric species as shown below:



(9)

In a mixture of two types of hemoglobins the "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 9). If all tetramers had equal assembly free energies, the fraction of hybrid (AB) would be 50% for an equal mixture of parent molecules; 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 (Smith and Ackers, unpublished results).

In studies discussed here the heme sites were oxidized and reacted with cyanide (CN-met) in various combinations of "ligated subunits. " The "parent" species used to generate each partially-ligated tetramer i_j are shown in Table I. Here X denotes that the heme iron has been oxidized and reacted with cyanide. With the exception of species [01], [23], [24], and [41] (Table I), these tetramers were studied in complex mixtures containing "parent" as well as hybrid molecules. Pairwise mixtures of normal hemoglobin A_0, mutant and chemically-modified hemoglobins were used to generate the mutant hybrid tetramers discussed below. For example, the hybrid A_0/Yakima was studied in a mixture containing this hybrid along with hemoglobin A_0 and Yakima tetramers and the constituent dimer species.

RESULTS AND DISCUSSION

How Cooperative Free Energy Transitions Are Coupled to Ligation of Hemes

The standard Gibbs energies ${}^i\Delta G_{2j}$ of dimer-tetramer assembly are given in Table I (8) for all combinations of modified sites. Also shown are values of the cooperative free energy calculated for each species according to Eq. 8. The information of Table I thus provides a map of structure-function perturbation in which the same modification is made at multiple combinations of sites. A striking feature of these results is that the 10 ligation states of tetrameric hemoglobin exhibit only three values of the cooperative free energy, i.e., 0 kcal, 3 kcal, and 6 kcal. 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. 8).

Transitions among the three levels of cooperative free energy are seen to occur in synchrony with single steps of ligation or deligation, but also depend on the configuration of ligated subunits within the rectant and product species. This leads us to the concept that the hemoglobin tetramer acts as a "combinatorial switch." Altering 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 asmong 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 I.

While we do not assume the effects of CN-met ligation to be quantitatively the same as those of oxygenation, we expect that the basic modes of free energy coupling within the hemoglobin tetramer will be the same when heme sites are perturbed with different ligands. Based on the structure-function perturbation map of Table I, 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.

Local Pathway Mechanism. The transition in cooperative free energy after the first ligand is bound 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.

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 that only three cooperative free energy levels are found for the 10 molecular forms of tetramer, and that within a single energy level the molecular forms differ with respect to the symmetry of sites altered due to ligation. That is, tetramers with singly-ligated subunits show identical energy levels regardless of the placement (α or β) of ligand (species [11] and [12]). The same is true of the doubly-ligated tetramer pairs [21], [22], and [23], [24]. Furthermore, tetramers with different numbers of ligated subunits bound show identical cooperative energy levels.

Locations of Cooperative Free Energy

The total cooperative free energy in human hemoglobin measures the free energy used by the molecule to modulate binding affinity over all four binding steps (5). Experimentally, this quantity equals the difference in free energy of dimer-tetramer assembly between fully-oxygenated (${}^4\Delta G_2$) and unligated (${}^0\Delta G_2$) hemoglobins. For normal hemoglobin A_0 under a standard set of conditions (7), these assembly free energies are: ${}^0\Delta G_2 = -14.3$ kcal (deoxy) and ${}^4\Delta G_2 = -8.0$ kcal (oxy).

Recent studies have determined the values of ${}^0\Delta G_2$ and ${}^4\Delta G_2$ for a series of mutant and chemically-modified hemoglobins in which each tetrameric molecule bears two structural modifications, either in both α chains or both β chains (5). Table II lists values of functional perturbations δF_i and δF_i^{xy} for 27 human hemoglobins that have these site-specific structural modifications. δF_i denotes the change in assembly free energy in the deoxy state (${}^0\Delta G_2$) resulting from the structural modifications at site i of the appropriate subunits, and δF_i^{oxy} is the corresponding difference for the fully-oxygenated state. Among these hemoglobins there are 21 sites of modification. With few exceptions (HbS, and C) the variants of Table II are found in minute populations and in heterozygous form, and they do not appear to be subject to any strong selection pressures. Of the more than 450 known mutant human hemoglobins the great majority may be viewed as "genetic noise."

The hemoglobins are listed in Table II in two series of entries. The first (*A*) includes modification of residues within 4 Å of the $\alpha^1\beta^2$ intersubunit contact region (9). The second (*B*) lists modifications at other regions including the heme pocket; the $\alpha^1\beta^1$ intersubunit contact; the external surface; the interior of the molecule; and the "central cavity" (see references 1 and 9). The combination of Table II and structural models of the hemoglobin tetramer in its deoxy and oxy forms constitutes a "map of structure-function perturbation" from which inferences have been drawn regarding the location of the residue sites responsible for cooperativity in oxygen binding.

TABLE II

EFFECTS OF SITE-SPECIFIC STRUCTURE MODIFICATION ON SUBUNIT INTERACTIONS IN HUMAN HEMOGLOBINS

Hb	Modification	Perturbations of interaction free energies		
		δF_i	δF_i^{oxy}	$(\delta F_i^{oxy} - \delta F_i)$
A. Modified sites in the $\alpha^1\beta^2$ intersubunit contact region.				
*Hotel Dieu	(β99 Asp-Gly)	6.1	0.0	−6.1
Kempsey	(β99 Asp-Asn)	5.9	−0.7	−6.6
Yakima	(β99 Asp-His)	4.5	−1.5	−6.0
*Osler	(β145 Tyr-Asp)	5.5	0.6	−4.9
*Creteil	(β89 Ser-Asn)	5.8	0.3	−5.5
des Arg	(α141 deleted)	4.2	−1.0	−5.2
*Legnano	(α141 Arg-Leu)	4.8	0.1	−4.9
*Chesapeake	(α92 Arg-Leu)	2.6	−1.6	−4.2
*G-Georgia	(α95 Pro-Leu)	4.0	2.6	−1.4
*NES	(β93 modified)	2.8	−0.5	−3.3
Kansas	(β102 Asn-Thr)	0.7	2.2	1.5
*Saint Mande	(β102 Asn-Tyr)	−0.6	1.6	2.2
Rush	(β101 Glu-Gln)	−0.5	0.2	0.7
*British Columbia	(β101 Glu-Lys)	2.8	2.1	−0.7
*Hirose	(β37 Trp-Ser)	5.6	n.d.	n.d.
*$\alpha^c\beta$	(α1 carbamylated)	−0.4	−0.5	−0.1
$\alpha^c\beta^c$	($\alpha 1\beta 1$ carbamylated)	−0.1	−1.4	−1.3
B. Modification sites not in the $\alpha^1\beta^2$ contact region.				
*Fort de France	(α45 His-Arg)	0.7	0.0	−0.7
*Winnipeg	(α75 Asp-Tyr)	−0.2	0.1	0.3
*G Norfolk	(α85 Asp-Asn)	0.1	−0.3	−0.4
S_0	(β6 Glu-Val)	0.0	0.0	0.0
*C	(β6 Glu-Lys)	−0.4	−0.2	0.2
*Strasbourg	(β23 Val-Asp)	0.7	−0.3	−1.0
*Zurich	(β63 His-Arg)	1.4	1.1	−0.3
*San Diego	(β109 Val-Met)	0.9	0.4	−0.5
*Hope	(β136 Gly-Asp)	−0.8	0.4	1.2
*Barcelona	(β94 Asp-His)	0.5	0.0	−0.5

*Values used in statistical calculations (see text).

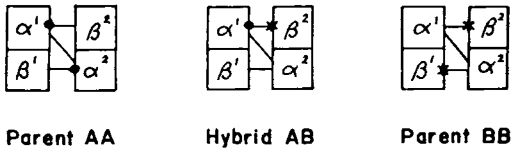
A striking pattern is seen in Table II for the effects of structure modifications on stabilizing interactions of the deoxy tetramer. For nearly all of the sites in the $\alpha^1\beta^2$ contact region the values of δF_i are large and correspond to changes in the equilibrium constant ${}^0K_2$ by factors of $10^2 - 10^5$. By contrast, the values of δF_i for sites outside of this region are very small. Modifications denoted by asterisks at 11 sites in the $\alpha^1\beta^2$ contact region destabilize the molecule by 3.6 ± 2.3 kcal (mean and standard deviation). This corresponds to a 470-fold less stable molecule on the average. Modifications at the nine sites not in the interface region destabilize by 0.3 ± 0.7 kcal corresponding to a 1.7-fold less-stable molecule. On the average then, a modification in the $\alpha^1\beta^2$ intersubunit contact region provides a molecule that is less stable than one modified elsewhere by a factor of 276-fold. Stability of the deoxy molecule is thus seen to be hypersensitive to structural modifications within the $\alpha^1\beta^2$ region but very insensitve to modifications elsewhere in the molecule. These results strongly suggest that the interactions responsible for stability of the deoxy tetramer are localized at the interface itself and are not the result of an assembly mechanism that depends on a change in conformation of the $\alpha^1\beta^1$ dimers between their dissociated and assembled deoxy states.

There is one dramatic exception to this insensitivity of the intersubunit interactions to perturbations at sites remote from the intersubunit contact region. Perturbations brought about by complete oxygenation at all four sites destabilize the dimer-dimer interactions by 6.3 kcal, corresponding to a factor of over 10^5 in the equilibrium constant (7). The effect of CN-met modification is similar in magnitude, as discussed above. Furthermore, these heme site alterations bring about large and simultaneous alterations in the perturbations δF_i resulting from the site modifications within the $\alpha^1\beta^2$ interface. The perturbation values for oxyhemoglobin are listed in Table II (δF_i^{oxy} values) as well as the magnitudes of the shifts ($\delta F_i^{oxy} - \delta F_i$) brought about by oxygenation. It should be noted that the difference ($\delta F_i^{oxy} - \delta F_i$) is just the perturbation in cooperative free energy resulting from the structure modification. In sharp contrast to the sites within the $\alpha^1\beta^2$ interface, oxygen binding has very little effect on values of δF_i for mutations outside of the $\alpha^1\beta^2$ intersubunit contact region, i.e., the perturbations in cooperative energies for these sites are very small. The average shift in δF_i for $\alpha^1\beta^2$ modifications is -2.9 ± 2.6 kcal, whereas for sites elsewhere it is only -0.2 ± 0.6 kcal. These results indicate that the sites of cooperative free energy are localized within the $\alpha^1\beta^2$ intersubunit contact.

Cooperative Free Energy Coupling Within the Regulatory Interface

From the studies just discussed it was inferred that the $\alpha^1\beta^2$ interface region is the location of most cooperative free energy change within the tetrameric molecule and that this energy change is controlled by ligand binding perturbations at the heme sites. These results raise the important question of how much coupling exists between the residue sites that lie within the $\alpha^1\ \beta^2$ contact region.

The possible pairwise coupling between modified residue sites within the $\alpha^1\beta^2$ interface has been explored by constructing a series of hybrid tetramers (Smith and Ackers, unpublished results). These hybrids consist of one $\alpha^1\ \beta^1$ dimer from each of the parent molecules, as shown topographically in the following diagrams.



(10)

Here the three pairwise contacts $\alpha^1\beta^2$, $\alpha^2\beta^1$, and $\alpha^1\alpha^2$ are indicated by lines connecting the respective subunits. The filled circles and asterisks indicate mutation sites.

Free energies of formation for the hybrid species are given in Table III. The first set of values (Table III*A*) are for hybrids between normal hemoglobin A_0, and a mutant or chemically-modified hemoglobin, so that each tetramer carries only a single modified site. This allows one to test for energetic coupling between identical sites within the molecule, by examining whether δF for the hybrid tetramer with one altered subunit is simply half that obtained for the "parent" tetramer with two identically altered subunits, as would be predicted if the two modifications were independent. Values of δF listed are the differences between ${}^0\Delta G_2$ values for the hybrid molecules and the "parent" hemoglobin A_0 which bears no modified sites. For comparison, values listed as $\delta F_{i/2}$ in Table III*A* are half the δF_i values given in Table II for the corresponding abnormal "parent" molecules carrying the two identical modifications.

The second group of hybrids (Table III*B*) provides data on cases of two different modifications within the same tetramer. The values listed as $(\delta F_{AA} + \delta F_{BB})/2$ are the sum of half the δF_i values given in Table II, for the two mutant or chemically modified "parent" tetramers. These terms correspond to the free energy perturbations expected if the effects of the two modifications were independent (i.e., $\delta F_{12} = \delta F_1 + \delta F_2$). Also listed for both types of hybrids are the distances between the residues in the deoxy hemoglobin A tetramer (10) corresponding to the two different alteration sites in Table III*B*, and the two identical sites in the abnormal "parent" tetramer for hybrids in Table III*A*.

It was found in these studies that, to within limits of experimental accuracy, the individual-site perturbations appear to be "additive" and hence to exhibit pairwise independence over all the combinations tested. There thus appears to be no direct long-range coupling within the interface itself. Additional studies will be required with multiple modifications in closer proximity to locate the ranges of coupling within the regulatory interface.

Of major significance regarding the nature and pathway of functional perturbation in the hemoglobin system is the observation that long-range coupling does exist between the heme site and each of the sites represented in Table III. Even though these sites do not appear to affect each other they are all simultaneously affected by oxygen binding at the heme sites, as shown in Table II. We thus

TABLE III
FREE ENERGY PERTURBATIONS OF DIMER-TETRAMER ASSEMBLY FOR HYBRIDS OF MUTANT HEMOGLOBINS

Hybrid (site)	δF_{hybrid}	$\delta(F_{i/2})$	Distance between sites
A. Hybrids With Normal Hemoglobin A_0			
A_0/Yakima (β99)	2.3	2.3	23Å
A_0/Kempsey (β99)	3.1	3.0	23Å
A_0/NES (β93)	1.1	1.4	35Å
A_0/Hirose (β37)	2.4	2.8	22Å
A_0/Chesapeake (α92)	0.8	1.3	33Å
A_0/G. Georgia (α95)	1.9	2.0	16Å
A_0/des Arg (α141)	2.2	2.1	18Å

Hybrid	δF_{hybrid}	$\frac{\delta F_{AA} + \delta FF_{BB}}{2}$	Distance between sites
B. Hybrids With Double Modifications at The Regulatory Interface			
St. Mande/Kempsey (β102-B99)	2.6	2.7	24Å
Chesapeake/Kempsey (α92-β99)	3.6	4.3	8Å
des-Arg/Kempsey (α141-β99)	5.0	5.1	16Å
Chesapeake/Hirose (α92-β37)	3.7	4.1	5Å
des-Arg/NES (α141-β93)	3.3	3.5	24Å
Yakima/Fort de France† (β99-α45)	2.9	2.6	14Å

†The altered residue in Hb Fort de France (α_{45} His-Arg) lies in the heme contact and not at the $\alpha^1\beta^2$ contact region. This hybrid is listed here for comparison.

have indirect coupling among these sites through perturbations at the heme, as described by the "triangulation relations" Eq. 6. These triangulation pathways will be explored further by additional combinations of modifications, particularly those involving modifications at only some of the heme sites.

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DISCUSSION

Session Chairman: Adrian Parsegian *Scribe*: Jacquelyn Leszczynski

FERRONE: How is the synchrony of the Monod, Wyman, and Changeux model (MWC) being violated in what you describe in the case of CN-met hemoglobin?

ACKERS: In sequential models, such as the KNF model (Koshland, Némethy, and Filmer, 1966. *Biochemistry* 5:364–385) one has a sequence of molecular states that change in synchrony with the ligand binding steps. There is no pre-existing equilibrium between conformational forms at a given ligation state; cooperativity states are determined by changes in local regions of the molecule that are synchronized with the ligand-binding steps. By contrast, the classical concerted mechanism (Monod, Wyman, and Changeux. 1965. *J. Mol. Biol.* 12:88–118) has two or more global conformational states that are in pre-existing equilibrium at every stage of ligand binding. The coupling between ligand binding steps and changes in cooperativity states is not synchronous. In CN-met hemoglobin, we apparently have global cooperativity states (corresponding to the three free energy levels) with synchronization between the changes in cooperativity states and ligand binding steps. These comparisons are summarized in Table A.

TABLE A
CHANGES IN COOPERATIVITY STATES
AND LIGAND BINDING STEPS

Cooperativity States		Coupling to Ligand Binding
LOCAL	GLOBAL	
KNF model	hemoglobin	SYNCHRONOUS
	MWC model	ASYNCHRONOUS

FERRONE: If one does a standard MWC analysis and the levels are set up correctly, there is a kind of synchrony in that at equilibrium, a given subunit can be either in an unligated or ligated state.

ACKERS: In MWC, there can be an apparent synchrony, but if you try to analyze the CN-met hemoglobin data in terms of MWC theory, it does not fit; not all ligation pathways through the population of species give positive cooperativity, which is a requirement of MWC theory. This is true for any ligation pathway through the two asymmetric doubly-ligated tetramers (Species [21] and [22]), shown diagrammatically in Fig. *A*.

FERRONE: You can, however, account for eight of the ten states by allosteric theory. In the absence of another global competing model, the allosteric (MWC) model seems to be doing pretty well.

ACKERS: Any minimal model has to explain all the reliable data. We don't feel free to throw out any of the experimental information, because it is all equally reliable.

SALEMME: Could you explain Fig. *A* in more explicit terms?

ACKERS: Fig. *A* shows diagrammatically the three presumed structural forms of the molecule, each denoted by a different shape, that correspond to the three cooperative free energies shown in Fig. *B*. Within each tetramer we have drawn a topographic map of the four subunits to illustrate the relationship between configurations of ligated subunits and the three global structures.

PARSEGIAN: Do you think that the specific identification of groups (the study of local stereochemistry) is a futile objective?

ACKERS: Not at all. Our method is different from the classical approach of interpreting the local stereochemistry, which is difficult to do

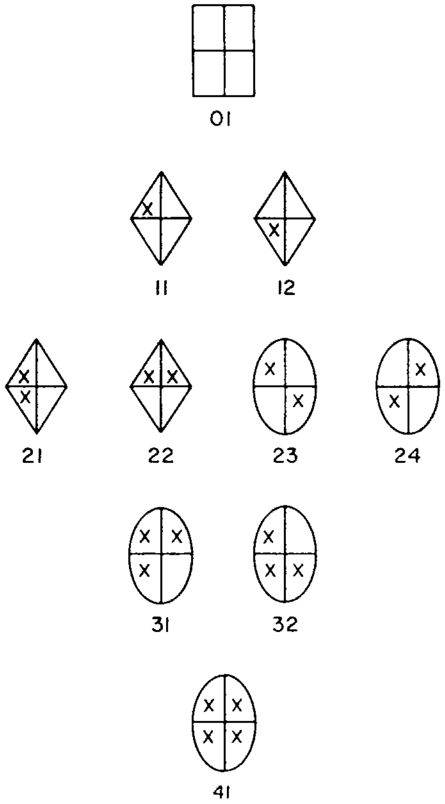

Figure A Structure of ligation pathways through species [21] and [22]

in large molecular assemblies with long-range coupling effects. Furthermore, the stereochemical approach requires construction of theories to connect the local stereochemistry with biological function. Our method relies instead on directly mapping the biological function against the structural locations of perturbing effects. It provides a complementary approach to the classical method.

HENDRICKSON: There are only three cooperative free energy levels, but that does not necessarily mean that there are only three structures. Do you have any evidence showing that there are only three structures?

ACKERS: There could be ten structural forms, if they partition in a 1, 4, 5 distribution with respect to those energy levels. In principle there could be even more, with appropriate degeneracies. The data imply a minimum of three. We have no additional structural information at this time.

HO: We have support for your model from our proton NMR studies on the oxygenation of hemoglobin. First, our results clearly show that the structural changes of the hemoglobin molecule associated with the oxygenation process are not concerted (Viggiano and Ho. 1979. *Proc. Natl. Acad. Sci. USA*. 76:3673–3677; Viggiano et al. 1979. *Biochemistry*. 18:5238–5247). Second, our more recent proton NMR studies on the ligation of cross-linked mixed valency hybrid hemoglobins also show that there are more than two structures per hemoglobin in going from the deoxy- to the CO state (Miura and Ho. 1982. *Biochemistry*. 21:6280–6287).

ACKERS: Your elegant NMR studies provide a convincing case for the existence of more than two structural forms during the course of ligand binding. I think it is always difficult to determine whether a given

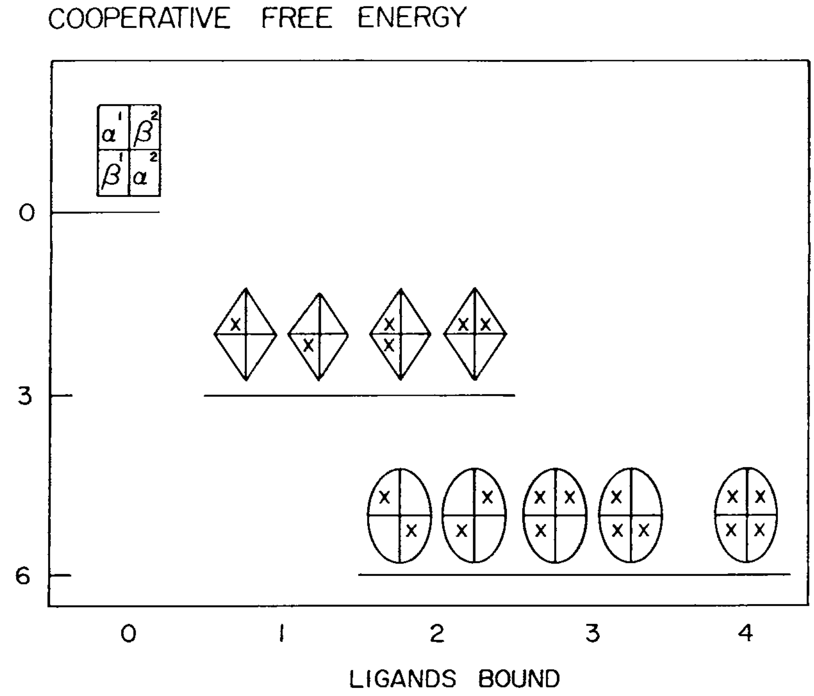

Figure B Cooperative free energies of the ligation pathways shown in Fig. *A*.

structural difference observed in a local part of the molecule is really of functional significance. If the structural difference has only small energetic consequences in relation to that required for function (i.e., cooperative ligand binding), or if the local energetic effect is compensated within the molecule (e.g., by entropic effects), the NMR-detected structural differences could be ancillary. Because the function of interest in hemoglobin is an energetic property (i.e., the Gibbs energy of cooperative ligation), this problem is bypassed by direct determinations of the cooperative free energies. To establish the structural origins of these cooperative energies we need a better correlation between our studies and yours.

To respond to your other point, our principal finding here is not a model, but an experimental fact. There are ten ligation states that partition into three energy levels. There is no simple pairwise algorithm that generates these effects; therefore, we postulate a global model.

HO: We have carried out some preliminary measurements of the oxygen-binding curves of our cross-linked mixed-valency hybrid hemoglobins (Miura, Ikeda-Saito, Yonetani, and Ho, unpublished results). We have found that the Hill coefficient for the oxygenation of the singly CN-met α chain cross-linked mixed-valency hybrid hemoglobin $[(\alpha^{tCN}\beta)_A (\alpha\beta)_c \mathrm{XL}]$, corresponding to species [11] of your Fig. *A*, is ~ 1.8, and that of $[(\alpha\beta)^{tCN}{}_A (\alpha, \beta)_{CXL}$, corresponding to species , is ~ 1.3. It should be mentioned that the Hill coefficient for the oxygenation of cross-linked hemoglobin is ~ 2.3. Thus, the singly CN-met α chain cross-linked hemoglobin is quite cooperative in its oxygen binding. The Hill coefficients for the oxygenation of doubly CN-met hemoglobins, $[(\alpha\beta^{tCN})_A (\alpha^{tCN}\beta)_C \mathrm{XL})]$, and $[(\alpha^{tCN}\beta^{tCN})_A (\alpha\beta)_C \mathrm{XL}]$, species [21], are ~ 1.2 and 1.1, respectively.

ACKERS: These Hill coefficients are in good qualitative agreement with our free energy determinations on the cross-linked species, although it must be remembered that our results pertain to a single set of conditions which are slightly different from yours. When we translate via Eq. 8 from energetics of subunit association into ligand binding, the ligation of species [21] and [22] are predicted to exhibit stepwise cooperativity. However, ligation of species [23] and [24] should exhibit no such cooperativity. What Hill coefficients do you find for these species? This is a critical comparison.

HO: We do not have those data at the moment.

NOVOTNY: What is the enthalpic and entropic contribution to the cooperative free energies? Are these comparable for the ligation states of identical cooperative free energy?

ACKERS: We have not studied the enthalpic and entropic distributions for the ten ligation states yet.

DURKIN: The first three kcals of cooperative free energy enter when the first ligand binds. The second three kcals of energy enter when another ligand is bound to a subunit whose corresponding subunit is already bound (α^1 or β^2), whether the resulting state is doubly or triply liganded. Do you have any idea what kind of intersubunit interactions account for that particular path? In particular, there is no direct β^1—β^2 interaction.

ACKERS: We don't know the answer to that. The disposition of these contacts in the unligated and fully ligated molecules are known, but the structures corresponding to the intermediate cooperative free energy are unknown.

KAPLAN: Lactate dehydrogenase has parallels to this system. LDH 1 dimers, however, are held together predominantly by hydrogen bonds. LDH 1 isolated from different tissues have different kinetic behaviors (Kaplan et al., unpublished results).

ACKERS: Hemoglobin dimers are held together by ionic and hydrogen bonds, and by van der Waals contacts. If you could generate all of the intermediate forms of LDH and measure their properties, perhaps you could sort out a distribution of effects that would correspond to these found with hemoglobin. This would be a valuable thing to do in many systems.

KAPLAN: All the monomers in LDH1 are considered to be identical. The same is true on LDH5; however, in hemoglobin the monomers are not identical.

ACKERS: Even if you have identical monomers, you may generate asymmetries within the tetrameric molecule by partial ligation, and you might have propagated effects that alter the subunit interactions asymmetrically.

POLLARD: Would your approach work for a very large system, such as binding an antibody to a specific site on a protein?

ACKERS: It certainly seems to work for putting two 34,000 M_w subunits together, but nothing can be said about the limits at this point. The only criterion we apply is that the relative changes in the network of local structural interactions are small enough for the subunits to retain approximately the same tertiary structure and that the corresponding energetic changes be small in comparison to the total energetics of the system in absolute terms. Antibody binding would probably meet that criterion.

BINA: To what extent would your analysis be applicable to systems where you make site specific temperature-sensitive mutants, then induce the perturbation by a temperature shift?

ACKERS: That's a good approach. John Schellman and co-workers (Schellman et al. 1984. *J. Mol. Biol.* 175:195–212) have done thermodynamic studies of many temperature sensitive mutants of T4 lysozyme using this approach.

WEISS: Jointly with Robert Sauer and co-workers, we have looked at site specific mutants which affect the thermostability of the phage λ C1 repressor. We have found that some mutations have no noticeable structural effects in a global sense; nevertheless, there are global effects on the thermostability of the molecule. Thermostability and global structural perturbation are, therefore, different. Also, global effects and local perturbation can be the same, for example in mutations at the dimer interface.

ACKERS: Such observations are the reason for the caution we give in this paper regarding the possible effects arising from a given mutation. One cannot take a single modification and interpret it in anything but a speculative fashion. To get a general picture, one has to do an extensive series of modifications and a correspondingly extensive series of physical characterizations.