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Probing the energetics of proteins through structural perturbation: Sites of regulatory energy in human hemoglobin

(structure–energy coupling/free energy transduction/hemoglobin cooperativity)

DONALD W. PETTIGREW*†, PAUL HENRI ROMEO‡, ANDREAS TSAPIS‡, JOELLE THILLET‡, MICHAEL L. SMITH*, BENJAMIN W. TURNER*, AND GARY K. ACKERS*§

*Department of Biology and McCollum–Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218; and ‡Unité de Recherches sur les Anemies, Institut National de la Sante et de la Recherche Medicale U. 91, Hôpital Henri Mondor, 94010, Creteil, France

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ABSTRACT **The sites of energy transduction within the human hemoglobin molecule for the regulation of oxygen affinity have been determined by an extensive study of the molecule's energetic response to structural alteration at individual amino acid residues. For 22 mutant and chemically modified hemoglobins we have determined the total free energy used by the tetrameric molecule for alteration of oxygen affinity at the four binding steps. The results imply that the regulation of oxygen binding affinity is due to energy changes which are mostly localized at the $\alpha^1\beta^2$ interface. They also indicate a high degree of "internal cooperativity" within this contact region—i.e., the structural perturbations at individual residue sites are energetically coupled. Cooperativity in ligand binding is thus a reflection of cooperativity at a deeper level—that of the protein–protein interactions within the $\alpha^1\beta^2$ interfacial domain.**

A fundamental problem of protein structure and function is the issue of how "local" properties of individual amino acid residues are related to "system" properties which reflect behavior of the molecule as a whole. Well-known manifestations of this problem include (*a*) the acid-base titration behavior of proteins, (*b*) the cooperative folding of tertiary structures, (*c*) the nucleated polymerization of self-assembling aggregates, and (*d*) the cooperative binding of ligands in allosteric systems. A large number of studies, including both experimental and theoretical work, have provided insights into the nature of these problems (cf. refs. 1–15).

One strategy for the exploration of structure–energy coupling and its role in biological function lies in perturbing a protein molecule through alteration of individual amino acid residues (i.e., deletion, substitution, or chemical modification) and determining the effects of these alterations upon appropriately selected system properties. By studying the effects on the system properties of a series of such changes, distributed throughout the molecular structure, one can determine which regions of the molecule are especially sensitive to structural perturbation. The power of this approach will be maximized when the system properties chosen reflect the energy states of the molecule as a whole and are at the same time directly related to its biological functions.

In this paper we present results of such a study with human hemoglobin. Using 23 different hemoglobins we have determined the effects of structural perturbation upon the energy invested by the molecule in altering the affinity at successive oxygenation steps. The results provide a structural "map" of energetic sensitivity related to the regulation of function.

The problem of structure–energy correlation in the human hemoglobin molecule is centered on the classic problem of resolving the cooperative mechanism of oxygen binding. A complete understanding of this problem must include (*a*) the structural and energetic changes at the heme site that accompany the binding of oxygen, (*b*) the changes of tertiary structure and energy for the individual subunit to which an oxygen molecule is bound, and (*c*) the coupling of tertiary changes to the changes in quaternary structure of the tetrameric molecule. Much of the necessary structural information regarding the tertiary and quaternary changes that accompany oxygenation has been provided by extensive x-ray crystallographic studies, beginning with the classic work of Perutz (11–13). Equally necessary to an understanding of the cooperative mechanism is a knowledge of the sources and manifestations of energy change that accompany the functional cycle of oxygenation/deoxygenation. A series of studies over the past 7 years, of which this work forms a part, has been aimed toward resolving the essential energetic aspects of the hemoglobin mechanism and of correlating the thermodynamic and structural information (cf. refs 15–20). Identification of the sites of regulatory energy change within the hemoglobin molecule, as provided by the results of this study, lies at the core of this problem.

First we present a summary of the thermodynamic relationships which form the conceptual basis for interpreting the experimental measurements.

THERMODYNAMIC RELATIONSHIPS

Cooperativity in Oxygen Binding. Isolated hemoglobin subunits have a single site where they bind oxygen with high affinity. Upon assembly into dimers ($\alpha^1\beta^1$)¶ the affinities of the subunits are unaltered and the binding is "noncooperative"—i.e., each of the two stepwise dimer binding constants is a simple statistical average of the individual chain constants (16). This is true even when the chains have widely different affinities (17). Upon assembly of subunits into tetramers ($\alpha_2\beta_2$), the equilibrium constants for the four stepwise binding reactions are altered from the statistical average values of the individual chains. The amount of energy by which they are altered at each step is given by

$$\delta_{4i} = \Delta G_{4i} - \langle \Delta G \rangle_{4i} \qquad [1]$$

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† Present address: Dept. of Biochemistry and Biophysics, Texas A & M Univ., College Station, TX 77843.

§ To whom reprint requests should be addressed.

¶ Superscripts denote the intersubunit contacts, whereas subscripts denote subunit composition. The tetramer $\alpha_2\beta_2$ has two types of contact planes, designated $\alpha^1\beta^1$ and $\alpha^1\beta^2$. The $\alpha^1\beta^2$ contact includes the contacts between $\alpha^1\beta^2$, $\alpha^2\beta^1$, and $\alpha^1\alpha^2$. Dissociated dimers are of the $\alpha^1\beta^1$ type.

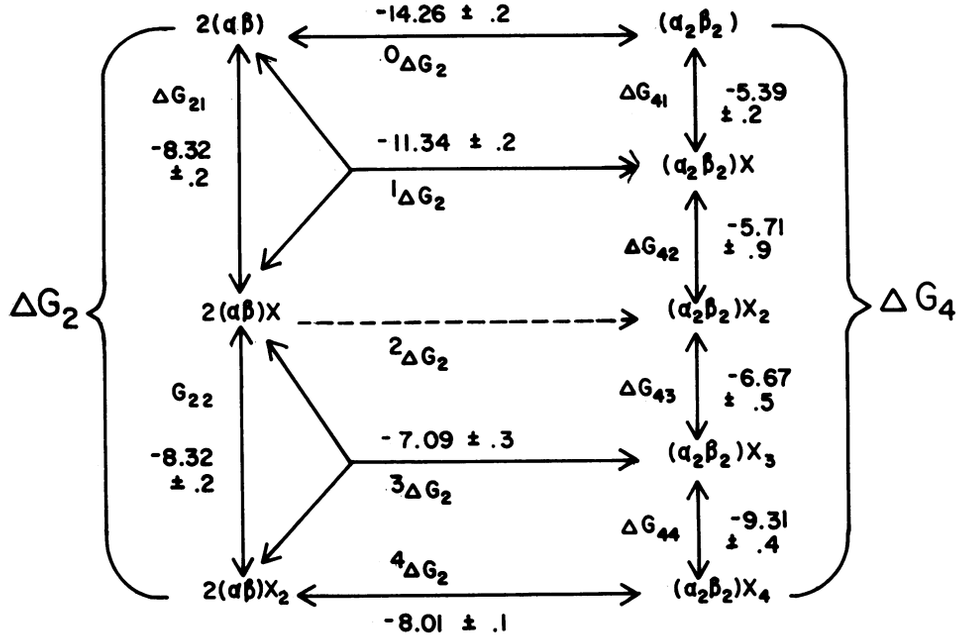

FIG. 1. Linkage between oxygen binding and dimer–tetramer assembly. The left side shows $\alpha_1\beta_1$ dimers at successive stages of ligation (X denotes an oxygen molecule), and the right side shows oxygenation of tetramers. ΔG_{ni} denote the stepwise free energies per mole of oxygen bound. The subunit assembly reactions (left to right) denote the appropriate combinations of dimers to form tetramers with i oxygens bound, and free energy of formation ${}^i\Delta G_2$. Numerical values are intrinsic free energies of reaction in kcal for normal hemoglobin A_0 under experimental conditions: pH 7.4, 0.1 M Tris, 0.1 M NaCl, 1 mM Na_2EDTA, and 21.5°C (21). This model-independent linkage scheme has been discussed theoretically (18) and its application to experimental studies has been described extensively (16, 17, 19, 20). Its relationship to mechanistic theories has also been presented (14, 15).

where ΔG_{4i} (i = 1, 2, 3, 4) is the actual free energy of oxygenation, related to the stepwise binding constant K_{4i} ($\Delta G_{4i} = -RT\ln K_{4i}$) and $\langle\Delta G\rangle_{4i}$ is the energy corresponding to the statistical average $\langle K\rangle_{4i}$ of the individual binding constants: $\langle\Delta G\rangle_{4i} = -RT\ln\langle K\rangle_{4i}$. The statistical average binding constants $\langle K\rangle_{4i}$ are given (17) by:

$$\langle K\rangle_{41} = 2K_\alpha + 2K_\beta$$
$$\langle K\rangle_{42} = [(K_\alpha)^2 + (K_\beta)^2 + 4K_\alpha K_\beta]/\langle K\rangle_{41} \quad [2]$$
$$\langle K\rangle_{43} = [(2(K_\alpha)^2 K_\beta + 2(K_\beta)^2 K_\alpha]/[\langle K\rangle_{42}\langle K\rangle_{41}]$$
$$\langle K\rangle_{44} = [(K_\alpha)^2(K_\beta)^2]/[\langle K\rangle_{43}\langle K\rangle_{42}\langle K\rangle_{41}]$$

where $\langle K\rangle_{4i}$ are the stepwise binding constants that would obtain if each subunit chain within the tetramer were to react with oxygen according to its individual binding constant K_α or K_β. In this paper we shall use the term "regulatory energy" to denote the difference δ_{4i} between the actual energy of oxygenation and the statistical average value of the constituent chains. For oxygen binding we use the term "cooperativity" to denote the magnitudes of δ_{4i} with the successive binding steps. For example, the first oxygen will be bound cooperatively only if δ_{41} differs from 0. Only in this way are the changes in binding affinity corrected for the effects of different affinities of the individual chains. Cooperativity in oxygen binding is "paid for" by the regulatory energies δ_{4i}. The terms δ_{4i} might arise from (*a*) changes in chemical affinity of the heme iron for oxygen, (*b*) tertiary structure changes of the subunits, (*c*) interactions at the subunit interfaces, or (*d*) other effects (e.g., solvent interaction) that accompany the tertiary and quaternary structure changes.

The total energy used for regulation of oxygen affinity is the sum of the regulatory energies δ_{4i} over all the binding steps. We designate this sum $\delta\Delta G_{04} = \Sigma\delta_{4i}$ as the "total regulatory energy." From Eq. 1

$$\delta\Delta G_{04} = \sum_{i=1}^{4}\Delta G_{4i} - \sum_{i=1}^{4}\langle\Delta G\rangle_{4i}. \quad [3]$$

The first sum on the right of Eq. 3 is the total energy for oxygenation of all four sites in the tetramer and shall be designated ΔG_4. From Eqs. 1–3:

$$\delta\Delta G_{04} = \Delta G_4 - 2(\Delta G_\alpha + \Delta G_\beta) \quad [4]$$

where the individual chain energies are: $\Delta G_\alpha = -RT\ln K_\alpha$ and $\Delta G_\beta = -RT\ln K_\beta$. Thus, the second sum of Eq. 4 equals the total energy for oxygenation of two α chains and two β chains in the absence of cooperativity. This also equals the free energy for complete oxygenation of two dimers, ΔG_2, provided the individual chain binding constants K_α and K_β are the same in dimers and tetramers.‖ Thus, the total regulatory energy $\delta\Delta G_{04} = \Delta G_4 - \Delta G_2$. In this study we have determined $\delta\Delta G_{04}$ by making use of the linkage relationships for oxygenation and subunit assembly of dimers into tetramers.

The Linkage Scheme. The relationships between dimer–tetramer assembly and oxygen binding are depicted in Fig. 1. By conservation of energy it can be seen that

$$\Delta G_4 - \Delta G_2 = {}^4\Delta G_2 - {}^0\Delta G_2 = \delta\Delta G_{04}. \quad [5]$$

Thus $\delta\Delta G_{04}$ can be determined from the free energies of dimer–tetramer assembly in the unliganded (${}^0\Delta G_2$) and fully oxygenated (${}^4\Delta G_2$) states. These are the quantities that we have measured experimentally.

‖ The effect of possible nonidentity between K_α and K_β in dimers and tetramers on the results obtained in this study are discussed at the end of *Methods and Results*.

Table 1. Free energies of dimer–tetramer assembly for human hemoglobins

Hb	Modification	Structural location (see ref. 29)	${}^0\Delta G_2$*	${}^4\Delta G_2$*	$\delta\Delta G_{04}$	Bohr effect	Oxygen affinity	Ref.
Normal A_o	—	—	−14.3 ± 0.2	−8.0 ± 0.1	6.3 ± 0.2	N	N	
Hôtel Dieu	(β99 Asp-Gly)	Internal, $\alpha^1\beta^2$ contact	−8.2 ± 0.1	−8.0 ± 0.1	0.2 ± 0.2	U	+	30
Kempsey	(β99 Asp-Asn)	Internal, $\alpha^1\beta^2$ contact	−8.4 ± 0.2	−8.7 ± 0.1	0.3 ± 0.2	−	+	31
Yakima	(β99 Asp-His)	Internal, $\alpha^1\beta^2$ contact	−9.8 ± 0.2	−9.5 ± 0.1	0.3 ± 0.2	N	+	32
Osler	(β145 Tyr-Asp)	Internal, $\alpha^1\beta^2$ contact	−8.8 ± 0.2	−7.4 ± 0.2	1.4 ± 0.3	−	+	33
Creteil	(β89 Ser-Asn)	Internal, near $\alpha^1\beta^2$ contact	−8.5 ± 0.2	−7.7 ± 0.1	0.8 ± 0.4	−	+	21
des-Arg A_o	(α141 deleted)	External, $\alpha^1\beta^2$ & $\alpha^1\alpha^2$ contacts	−10.1 ± 0.2	−9.0 ± 0.1	1.1 ± 0.2	−	+	34
Chesapeake	(α92 Arg-Leu)	External, $\alpha^1\beta^2$ contact	−11.7 ± 0.2	−9.6 ± 0.1	2.1 ± 0.3	N	+	35
NES A_o	(β93 modified)	Internal, $\alpha^1\beta^2$ contact	−11.5 ± 0.1	−8.5 ± 0.2	3.0 ± 0.2	−	+	17
Kansas	(β102 Asn-Thr)	Internal, heme & $\alpha^1\beta^2$ contact	−13.6 ± 0.1†	−5.8 ± 0.1†	7.8 ± 0.2	−	−	36
Saint Mandé	(β102 Asn-Tyr)	Internal, heme & $\alpha^1\beta^2$ contact	−14.9 ± 0.1	−6.4 ± 0.1	8.5 ± 0.2	−	−	37
Hirose	(β37 Trp-Ser)	External, $\alpha^1\beta^2$ contact	−8.7†	0†	8.7	−	+	38
$\alpha^c\beta$	(α1 carbamylated)	External, central cavity, $\alpha^1\alpha^2$ contact	−14.7 ± 0.2	−8.5‡	6.2	−	+	39
Fort de France	(α45 His-Arg)	External, heme contact	−13.6 ± 0.1	−8.0 ± 0.1	5.6 ± 0.2	N	+	25
Winnipeg	(α75 Asp-Tyr)	External, near cavity	−14.5 ± 0.1	−7.9 ± 0.1	6.6 ± 0.2	N	N	24
G Norfolk	(α85 Asp-Asn)	External	−14.2 ± 0.1	−8.3 ± 0.1	5.9 ± 0.4	N	+	40
$\alpha^c\beta^c$	(α1β1 carbamylated)	External, central cavity, $\alpha^1\alpha^2$ contact, DPG pocket	−14.4 ± 0.2	−9.4 ± 0.1‡	5.0 ± 0.2	−	+	38
S_o	(β6 Glu-Val)	External	−14.3 ± 0.2	−8.0 ± 0.1	6.3 ± 0.2	N	N	40
C	(β6 Glu-Lys)	External	−14.7 + 0.1	−8.2 + 0.2	6.5 + 0.2	N	N	41
Strasbourg	(β23 Val-Asp)	Internal	−13.6 + 0.1	−8.3 + 0.1	5.3 + 0.3	N	+	22
Zurich	(β63 His-Arg)	External, surface crevice, distal His	−12.9 ± 0.1	−6.9 ± 0.2	6.0 ± 0.2	N	+	42
San Diego	(β109 Val-Met)	Internal, central cavity, $\alpha^1\beta^1$	−13.4 ± 0.1	−7.6 ± 0.1	5.8 ± 0.2	N	+	43
Hope	(β136 Gly-Asp)	Central cavity	−15.1 ± 0.2	−7.6 ± 0.1	7.5 ± 0.3	N	−	44

Conditions: except as noted below, in 0.1 M Tris·HCl/0.1 M Na_2EDTA, pH 7.4, at 21.5°C.

* ${}^0\Delta G_2$ and ${}^4\Delta G_2$ are free energies for assembly of 1 mol of tetramer from 2 mol of dimer in the unliganded and fully liganded states, respectively. Values are based on a reference state of 1 mol of dimer per liter at 21.5°C.

† Values are for the conditions 0.05 M Tris·HCl/0.1 M NaCl/1 mM Na_2EDTA, pH 7.5 at 20°C.

‡ Values were measured on the carbonmonoxy form and are reported on a molar heme concentration scale (9). These values have been adjusted to the same standard state as the other values of the table (molar dimer concentration scale).

METHODS AND RESULTS

Samples of whole blood for purification of Hb S, C, Chesapeake, and Osler were provided by Samuel Charache (The Johns Hopkins University School of Medicine, Baltimore, MD). Hb Kempsey and Yakima were purified from whole blood provided by H. F. Bunn (Harvard Medical School, Boston, MA), and R. T. Jones (University of Oregon Medical School, Portland, OR), respectively. Detailed methods for purification and assessment of homogeneity of Hb Yakima, Saint Mandé, Hôtel Dieu, S, C, Chesapeake, Osler, Kempsey, San Diego, and Fort de France will be described elsewhere. Hb Strasbourg (22), Hope (23), G., Norfolk (24), Winnipeg (25), and Creteil (21) were prepared as described. Purified Hb Zurich was a gift from Ernesto di Iorio (ETH Zentrum, Zurich, Switzerland). Carbamylated hemoglobins were a gift of Todd Schuster (University of Connecticut, Storrs, CT). Normal Hb A_o, used for preparation of chemically modified hemoglobins and for the determinations on Hb A_o, was purified as described (26). Determinations of the free energies of dimer–tetramer assembly were carried out by methods described in detail elsewhere (27, 28).

The experimentally determined free energies of dimer–tetramer assembly are given in Table 1. This table is comprehensive in the sense that it contains all of the known thermodynamic constants for dimer–tetramer assembly of deoxy- and oxyhemoglobins obtained under comparable conditions. Values for 20 of the hemoglobins were determined in this study. Table

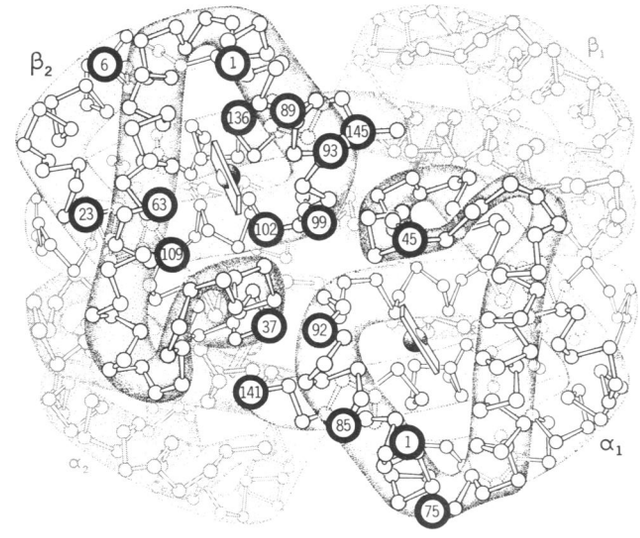

FIG. 2. Locations of amino acid alterations for hemoglobins listed in Table 1. Each numbered heavy circle shows the location of one of the two symmetry-related alterations that occur in each tetramer. (Adapted by permission from ref. 45; illustration copyright, Irving Geis.)

1 also lists the locations of residues at the sites of modification and the Bohr effect and oxygen affinities relative to Hb A. The location, within the molecule, of each altered amino acid residue is shown in Fig. 2. The majority of the alterations are either in the $\alpha^1\beta^2$ contact or on the external surface. One of the mutants (Hb San Diego) specifically involves the $\alpha^1\beta^1$ contact, and one of them (Hb Zurich) is distal to the heme iron. Other regions represented in the list include the $\alpha^1\alpha^2$ contact and the β cleft. Several sets of mutants involve different amino acid substitutions at the same position (i.e., Hb Kempsey, Hôtel Dieu, and Yakima; Hb S and C; Hb Kansas and Saint Mandé).

Structural Location of the Regulatory Energy. The only alterations that were found to have significant effects on the regulatory energy, $\delta\Delta G_{04}$, were those in or near the $\alpha^1\beta^2$ interface including the subunit contacts $\alpha^1\beta^2$ and $\alpha^2\beta^1$. Structural perturbations in this region give rise to major changes in regulatory energy; most modifications lead to alterations in $\delta\Delta G_{04}$ greater than half its normal value. In several cases the value of $\delta\Delta G_{04}$ is decreased nearly to zero. By contrast, the alterations on the external surface of the molecule, at the $\alpha^1\beta^1$ interface, and at the heme site all lead to values of $\delta\Delta G_{04}$ that are essentially normal. All but one of the deviations in $\delta\Delta G_{04}$ arising from modifications in the $\alpha^1\beta^2$ contact are larger than any of the deviations arising from alteration at any other regions of the structure. Most of the deviations in the former category are more than 3 times as great as those of the latter—i.e., a factor of approximately 200 in altered stability.

As a general rule we assume that the regions of the molecule that give the largest energetic response to structural perturbations are also the major sites of expression for the regulatory energy in normal hemoglobin. There are circumstances in which a given amino acid alteration might not follow this rule (see *Discussion*). However, for a number of perturbations, taken at random throughout the molecule, we may expect the rule to hold.

We cannot be certain that our sampling of the possible structural variations is random or statistically representative. With few exceptions (Hb S and C) the variants of Table 1 are found in minute populations and in heterozygous form, and they do not appear to be subject to any strong selection pressures. Among available hemoglobins, only those that are highly unstable were excluded from study. Clearly, an individual value of $\delta\Delta G_{04}$ might be misleading. In addition to reflecting the cooperative energies, values of $\delta\Delta G_{04}$ may also include energies arising from subunit assembly that do not vary with oxygenation state. For example, K_α and K_β might have different values in dimers and tetramers (cf. refs. 16 and 17). It is thus possible for $\delta\Delta G_{04}$ to have a finite value without variations in the δ_{4i} terms. It is also possible for $\delta\Delta G_{04}$ to be small (or zero) in spite of large variations in ΔG_{4i} (through cancellation of positive and negative values of δ_{4i}). However, for 22 structural alterations distributed throughout the molecule, we consider the likelihood vanishingly small that these effects would be of significant magnitude and yet cancel in a precise fashion to produce the striking correlation observed. We therefore conclude from the results shown in Table 1 that the regulation of oxygen affinity is the result of energy changes that are mostly localized at the $\alpha^1\beta^2$ contact region.

DISCUSSION

Up to the present there has been no compelling evidence as to the sites of cooperative energy change within the hemoglobin molecule, as indicated by the extreme diversity of current theories (5, 10–12, 35, 46). We believe that the results of this study demonstrate the $\alpha^1\beta^2$ intersubunit contact region to be the principal location of the energy changes in human hemoglobin that constitute cooperativity in ligand binding. Our finding that the $\alpha^1\beta^2$ interface is the only region where structural perturbation leads to altered regulatory energies argues strongly that this region is the actual site where the regulatory energy is "spent." We note that this experimental finding does not constitute absolute proof of our conclusion since it is theoretically possible that the $\alpha^1\beta^2$ interface acts merely as a "switching device" to control energy changes at other locations within the molecule. We believe this interpretation to be highly unlikely, however, based on the following considerations. None of the other regions of the molecule (including the heme pocket) was found to exhibit significantly altered regulatory energies upon structural perturbation at individual amino acid sites. Thus, the regulatory energy would have to be extensively delocalized (10) over many residues such that none contributes appreciably more to $\delta\Delta G_{04}$ than its thermal energy. For such a specific molecular process of precise biological regulation to have evolved in this form has no known precedent and would appear unlikely. The hydrogen exchange studies of Englander and associates (5) indicate highly localized energies, each several kilocalories in magnitude, for the oxygenation-linked structure changes of tetrameric hemoglobin. Theoretical calculations by Gelin and Karplus (9) also indicate local pathways for the structure–energy coupling within an individual subunit.

Range of Coupling Between Individual Residue Effects. Our results strongly suggest that individual residue effects are propagated extensively throughout the $\alpha^1\beta^2$ interface.

a. Structural perturbations at widely separated regions of this interface lead to similar alterations of regulatory energy (e.g. Hb Hôtel Dieu and des-Arg.) The magnitudes of the largest energy changes resulting from perturbations at different locations are strikingly similar (e.g., Hb Kempsey, Creteil, des-Arg, and Osler). Furthermore, the alterations in regulatory energy never exceed the magnitude of $\delta\Delta G_{04}$ for normal hemoglobin. These observations suggest a common set of delicately balanced interactions which act in an interdependent way.

b. Substitutions of different amino acid residues at the same position lead to identical changes in regulatory energy. At the β99 position, Hb Kempsey, Yakima, and Hôtel Dieu have amino acid side chains of widely different steric and chemical natures. Yet they show exactly the same complete loss of regulatory energy (Table 1). Analogous results are obtained at the β102 position.

c. Recent structural studies (47) have shown Hb Osler and Creteil to have similar deoxy structures with identically altered COOH-terminal tetrapeptides. Our results show identical energetics for Hb Osler and Creteil (Table 1). Thus the structural and energetic changes that result from altering local interactions at the β89 and β145 positions are found to be highly interdependent.

These results argue strongly against strictly local effects and suggest long-range cooperativity within the interface between the dimers. Cooperativity in the energetics of ligand binding for normal hemoglobin thus appears to reflect cooperativity at a deeper level—that due to interactions within the $\alpha^1\beta^2$ interface.

Salt Bridges as the Source of Cooperative Energy. A set of oxygenation-sensitive salt bridges that lie mostly within the $\alpha^1\beta^2$ interface have been proposed as the source of cooperative energy (11–13, 48, 49). The main evidence in support of this proposal (48, 49) derives from the finding that alterations (e.g., mutation, chemical modification) that disrupt the salt bridges lead to a loss of cooperativity. In light of the present work, such effects are understandable as arbitrary perturbations of structure at the $\alpha^1\beta^2$ interface. Because other types of structural

perturbations at this interface also lead to loss of cooperativity, the argument specifically favoring salt bridges does not appear to be compelling. Recent thermodynamic and other evidence (20, 50, 51) indicates the salt bridges to be unlikely as the dominant source of the regulatory energy and suggest hydrogen bonding and van der Waals interactions for this role.

Pathways for Transduction of Cooperative Energy. Ever since the structural work of Perutz (11, 12) a classic problem of the hemoglobin mechanism has been to understand the roles of tertiary and quaternary structural changes in cooperativity. The results of this study provide a delineation of these roles and suggest a general mechanism for the generation of cooperative energy in the hemoglobin molecule.

First, we note that processes that do not involve appreciably altered energies upon successive oxygenation steps can make no contribution to cooperativity, even if the energies are large. Our results indicate that the energies of tertiary change for binding oxygen to each of the four subunits of a tetramer fall into this category—they are essentially the same except for effects at the $\alpha^1\beta^2$ interface. When each successive oxygen is bound, the tertiary change of the oxygenated subunit is propagated into the $\alpha^1\beta^2$ interface, leading to a cooperative transition in the interfacial domain itself. The resulting change in energy of the interfacial domain is δ_{4i} which contributes to cooperativity in ligand binding as prescribed by Eq. 1. The high energetic sensitivity observed in this study for the $\alpha^1\beta^2$ interface indicates that the small perturbations of tertiary structure that accompany oxygenation of individual chains (9, 52) may be sufficient to generate the energy changes δ_{4i} that pay for cooperativity in ligand binding.

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