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Mutagenic Dissection of Hemoglobin Cooperativity: Effects of Amino Acid Alteration on Subunit Assembly of Oxy and Deoxy Tetramers

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ABSTRACT **Free energies of oxygen-linked subunit assembly and cooperative interaction have been determined for 34 molecular species of human hemoglobin, which differ by amino acid alterations as a result of mutation or chemical modification at specific sites. These studies required the development of extensions to our earlier methodology. In combination with previous results they comprise a data base of 60 hemoglobin species, characterized under the same conditions. The data base was analyzed in terms of the five following issues. (1) Range and sensitivity to site modifications. Deoxy tetramers showed greater average energetic response to structural modifications than the oxy species, but the ranges are similar for the two ligation forms. (2) Structural localization of cooperative free energy. Difference free energies of dimer-tetramer assembly (oxy minus deoxy) yielded ΔG_c for each hemoglobin, i.e., the free energy used for modulation of oxygen affinity over all four binding steps. A structure-energy map constructed from these results shows that the $\alpha^1\beta^2$ interface is a unique structural location of the noncovalent bonding interactions that are energetically coupled to cooperativity. (3) Relationship of cooperativity to intrinsic binding. Oxygen binding energetics for dissociated dimers of mutants strongly indicates that cooperativity and intrinsic binding are completely decoupled by tetramer to dimer dissociation. (4) Additivity, site-site coupling and adventitious perturbations. All these are exhibited by individual-site modifications of this study. Large nonadditivity may be correlated with global (quaternary) structure change. (5) Residue position vs. chemical nature. Functional response is solely dictated by structural location for a subset of the sites, but varies with side-chain type at other sites. The current data base provides a unique framework for further analyses and modeling of fundamental issues in the structural chemistry of proteins and allosteric mechanisms.**

Key words: energetics of cooperativity, hemoglobin mutants, subunit assembly

INTRODUCTION

Human hemoglobin is an especially convenient system for studying the effects of amino acid alterations on protein interactions responsible for biological function.[1] Mutation frequencies of the human globin genes are sufficiently high that approximately one individual in 2,000 carries a variant subunit.[2] Over 450 such mutant hemoglobins have been isolated and their amino acid alterations identified.[3] The great majority of these have been found to occur in heterozygous individuals and as a minor hemolysate component. With certain exceptions (e.g., hemoglobins S and C) these mutated genes may be regarded essentially as "genetic noise" for use in structure-function studies. It is possible to obtain mutants at many regions of the molecule in substantial quantities, in highly purified, stable, and nondegraded form. This repertoire of mutant

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hemoglobins may be supplemented by a number of well-controlled chemical modifications whereby an amino acid side chain is altered in a desired way.[4–8] Increasingly effective expression systems are making it easier to generate altered human hemoglobins by site-directed mutagenic techniques following the pioneering work of Nagai and Thørgersen.[9–13] All of these mutant and chemically modified species can be studied by physical-chemical methods to determine the effects of the altered amino acid residues on protein stability and the mechanism of cooperativity in oxygen binding.

As illustrated by the present work, the interaction constants of interest to the molecule's biological functioning may exhibit very large changes (e.g., 100,000-fold) in response to a single-residue mutation carried by each α or β subunit of the hemoglobin tetramer. This sensitivity makes hemoglobin an ideal protein for studying effects of amino acid alterations on stability and function. The rigorous and systematic study of the energetics of mutant and chemically modified hemoglobins has been initiated during the past several years[14–17] and is still in its infancy. Considerable interest in this problem stems from the desirability of engineering hemoglobins with designated properties, e.g., in connection with blood substitutes.

Mapping by Structure-Function Perturbation

Most studies of mutational effects on proteins have employed a strategy that regards the effects of structural alterations as local, with the remainder of the molecular structure assumed to be unperturbed. Observed functional changes have often been attributed exclusively to the altered local chemistry resulting from the specific modification.[18–20] An alternative strategy for interpreting the effects of altered primary structures on functional responses is that of "Mapping by Structure Function Perturbation."[16] This approach is based upon evaluating the patterns of functional response from numerous site-specific residue alterations distributed throughout a molecular structure. This results in a map of functional response versus structural location. Evaluation of the specific chemistry of the amino acid side chain modifications is not required for interpreting certain properties of the structure-function map generated. This strategy has been used with hemoglobin for: (1) structural localization of cooperative free energy changes within the tetrameric structure;[15] (2) deciphering the coupling between multiple hemesite reactions and the cooperative free energy distributions;[21,22] (3) mapping the pathways of coupling between single residue sites within the tetrameric molecule;[17] and (4) defining the quaternary structures of partially ligated tetramers.[23,24]

The work presented here is an extension of the earlier study from this laboratory,[15] which analyzed the cooperative energetics of 23 mutant and chemically modified hemoglobins, i.e., 19 species characterized for the first time plus previous results on three species in addition to the normal molecule A_o. We now report studies on the energetics of cooperativity for an additional 34 mutant and chemically modified species. The data base contains properties of 48 mutants including three genetically engineered species,[12,13] plus 11 chemically modified species. Included in the latter category are three chemically modified mutant hemoglobins (each carrying four nonnative residues/tetramer). We thus compare the properties of all molecular species that have been similarly characterized to date comprising a total of 60 human hemoglobins. The structural modifications examined occur at 34 residue sites (see Table IV for comprehensive listings). Principal goals of this study were: (1) to explore further the ranges of mutational effects by extending the structural locations and types of modifications, (2) to develop a library for use in more detailed studies of the hemoglobin mechanisms, and (3) to begin the development of a data base for rational design by site-directed mutagenesis of hemoglobins with desired properties. The characterization of this wider range of sytems has required new extensions of experimental techniques that are described here. Several of the "subsequent" studies using results of the present work have already been completed,[17,23–25] and correlations have been established with several independent studies.[26,27]

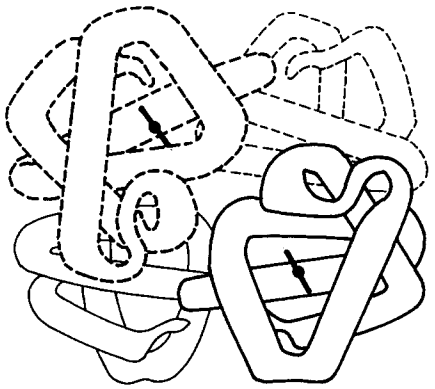


Fig. 1. Tetrameric hemoglobin (oxy quaternary structure) showing the dimeric half molecules (right and left) each consisting of an αβ dimer (α subunits in solid, β in dashed outlines). The true twofold axis lies in the vertical plane perpendicular to the page. The rightmost dimer ($\alpha^1\beta^1$) forms ligation-sensitive intersubunit contacts $\alpha^1\beta^2$, $\alpha^2\beta^1$, $\alpha^1\alpha^2$, and $\beta^1\beta^2$ with the structurally identical leftmost dimer, $\alpha^1\beta^2$. These intersubunit contacts are altered upon binding ligands at the four hemes (shown as thick solid lines for the α^1 and β^2 subunits in front).

Structural Features

Tetrameric hemoglobin ($\alpha_2\beta_2$) is comprised of two identical dimers $\alpha^1\beta^1$ and $\alpha^2\beta^2$ as shown in Figure 1 (see ref. 28 for review). The dimers assemble to form either quaternary structure T (also called "deoxy")

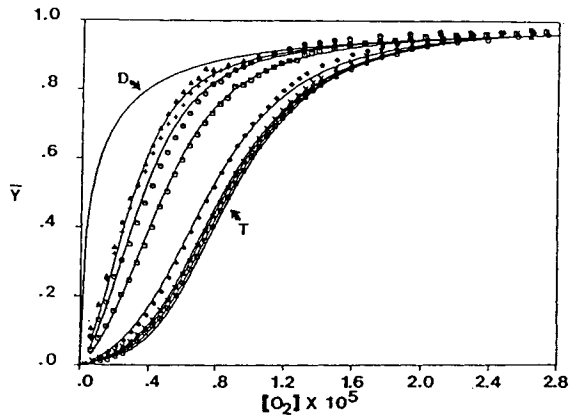


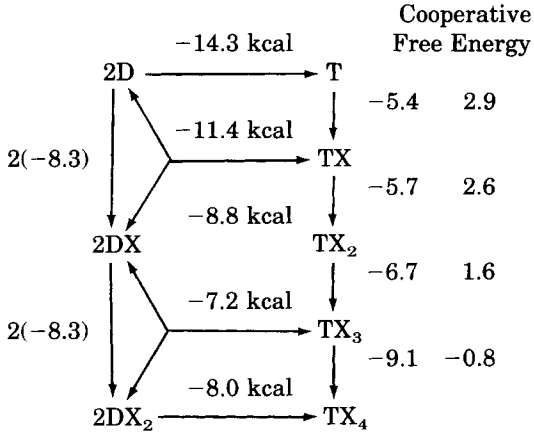
Fig. 2. Linkage between subunit assembly of noncooperative dimers (solid curve on left) and cooperative oxygen bindings.[35] The binding isotherm of tetrameric species is the solid sigmoidal curve on the right. Intermediate binding curves are for hemoglobin concentrations from 4×10^{-8} M to 1×10^{-4} M.

or quaternary R ("oxy"). The T→R switchover changes the relative disposition of the two dimeric halves of the molecule through a global shift of the $\alpha^1\beta^2$ intersubunit contacts. Within the dimer-dimer interface (designated the "$\alpha^1\beta^2$ interface") are specific intersubunit contacts $\alpha^1\beta^2$, $\alpha^2\beta^1$ and $\alpha^1\alpha^2$. An additional "loose" $\beta^1\beta^2$ contact is formed in the R quaternary structure.[29] The interface within each dimer ($\alpha^1\beta^1$) undergoes motion on a smaller scale and is usually classified as "tertiary." Through a combination of tertiary and quaternary structure changes that accompany ligand binding, a tetramer initially in unligated quaternary T will become more stable in quaternary R when all four subunits contain ligated hemes. The local hemesite binding energy has thus been used to pay for the structural rearrangement that forms quaternary R. Recent work[23,24,30] has shown that cooperativity arises only in part from the energetics of quaternary T→R switching; an additional component arises from sequential cooperativity within each of the quaternary forms.

Cooperative Free Energy

Isolated α or β subunits of normal hemoglobin bind ligands (e.g., O_2, CO, CN) each with a characteristic affinity ΔG_α or ΔG_β. These subunits assemble into αβ dimers whose free energy of ligation is merely the sum of the constituent values ($\Delta G_\alpha + \Delta G_\beta$).[31–33] Further assembly into tetramers ($\alpha_2\beta_2$) results in stepwise ligation energies that differ greatly from the stoichiometric sum of constituent values. The assembled tetramer binds ligands with successively increasing affinity over the four steps (yielding a sigmoidal binding curve), but the average affinity is greatly reduced. These effects are illustrated in Figure 2. It is clear from these features and the structural and mechanistic concepts outlined above that ligation-sensitive dimer-dimer interactions lie at the heart of the hemoglobin cooperativity problem. Advancement on this problem depends on strategies whereby hemoglobin cooperativity can be experimentally "dissected" in terms of the energetics of subunit interactions at the different stages of binding. We have carried out an extensive series of studies aimed toward this goal during the past several years.[34–37] In this study we describe the effects of structural modifications on the "total site-site interaction free energy" over all four binding steps, i.e., the energy in excess of that for binding to the constituent subunits. We shall refer to this quantity as *the cooperative free energy* (ΔG_c). This quantity has also been called the "regulatory free energy."[15] It represents the energetic cost of modulating the binding free energy from its value in the constituent α and β subunits (or noncooperative αβ dimers). ΔG_c arises from (1) concerted quaternary switching (T to R) and (2) sequential tertiary switching (t or r) within each quaternary form (T or R).[23,30]

Since dissociated αβ dimers bind hemesite ligands noncooperatively the thermodynamic linkage between binding and reversible dissociation of tetramers into dimers provides a way to measure the cooperative free energies.[15,34] This is shown in the linkage scheme below:



Totals −33.2	–	−26.9	6.3

The ligand (O_2, CO, etc.) is represented by "X". Equilibrium constants of this subunit assembly-oxygenation linkage are related by:

$$\frac{K_4}{(K_2)^2} = \frac{{}^4K_2}{{}^0K_2} \qquad (1)$$

where the binding constants are defined as follows in terms of species concentrations (in brackets):

$$K_4 = \frac{[(\alpha_2\beta_2)X_4]}{[\alpha_2\beta_2][X]^4} \quad ; \quad K_2 = \frac{[(\alpha\beta)X_2]}{[\alpha\beta][X]^2} \qquad (2)$$

and the dimer-tetramer association constants are:

$$^{0}K_2 = \frac{[\alpha_2\beta_2]}{[\alpha\beta]^2} \quad ; \quad ^{4}K_2 = \frac{[(\alpha_2\beta_2)X_4]}{[(\alpha\beta)X_2]^2}. \qquad (3)$$

The cooperative free energy, ΔG_c is given by:

$$\Delta G_c = \Delta G_4 - 2\Delta G_2 \qquad (4)$$

where $\Delta G_4 = -RT\ln K_4$ and $\Delta G_2 = -RT\ln K_2$. The cooperative free energy ΔG_c could, in principle, be determined from measured values[38] of the equilibrium constants for fully oxygenating tetramers and dimers as defined by equations 2 and 4 (e.g., from median values of their respective oxygenation curves). In practice, however, K_4 and K_2 cannot be determined separately because of their linkage to the assembly reactions of equation 3, which results in the presence of significant tetramer dissociation.[35,36] However, a practical means of determining ΔG_c for a given hemoglobin is through the independent measurement of $^{4}K_2$ and $^{0}K_2$.

$$\Delta G_c = {}^{4}\Delta G_2 - {}^{0}\Delta G_2 \qquad (5)$$

where $^{4}\Delta G_2 = -RT \ln (^{4}K_2)$ and $^{0}\Delta G_2 = -RT \ln (^{0}K_2)$. The crucial feature we exploit in this approach is the "differential experiment" built into the linkage scheme. Ligand affinity of the noncooperative dimers is automatically subtracted, leaving only the component of affinity that results from site-site interaction within the tetrameric molecule. Use of $^{0}\Delta G_2$ and $^{4}\Delta G_2$ in this differential experiment to study the effects of structural alterations on cooperative free energy takes advantage of the following principle: only if an amino acid alteration changes the ligation free energies of dimeric and tetrameric forms unequally will it affect the cooperative free energy. Any chemical or physical changes that alter the ratio of equilibrium constants on opposite sides of the linkage scheme (Fig. 1) must be energetically coupled to ΔG_c. It should be noted that the specific structural features of molecular interaction that generate individual terms of eq. (subunit assembly) are not necessarily identical to those that provide the individual terms of eq. 4 (oxygen binding). However, the difference free energy ΔG_c provides a rigorous thermodynamic measure of the molecular interactions responsible for cooperativity in oxygen binding whether evaluated by oxygenation or by subunit assembly.

In this study we have supplemented the determinations of assembly free energies with oxygen binding data for fifteen of the mutant hemoglobins, providing all four constants of the linkage system in those cases (eq. 1).

MATERIALS

Sources of Hemoglobins

Mutant and chemically modified hemoglobins were purified from samples of whole blood, hemolysates, or obtained in pure form, as gifts from the following: Pamela Como (Bunbury), Peter Dysert (Dallas), Virgil Fairbanks (Rush, British Columbia), Chien Ho (Malmo, Wood, O-Arab, Abruzzo, Sealy, Rampa, des His β146), Keiko Horano and T.H.J. Huisman (Kariya), Richard Jones (Cowtown), Claude Poyart (Chemilly, Barcelona), Helen Ranney (Rothschild), and David Weatherall (Radcliffe). Samples containing hemoglobins Tarrant, St. Claude, G-Georgia, Legnano, Pitie-Salpetriere and Ty Gard were obtained for these studies by F. Galacteros; those containing Austen, and Athens, GA, by W. Moo-Penn; and Ypsilanti by D. Rucknagel. Modified hemoglobins NES/Rush, NES/British Columbia, NES/des Arg, thiomethyl β93, and the 3 carboxymethylated species were prepared by B. Hedlund. All 60 hemoglobin species discussed in this study are listed in Table IV. Those characterized by other laboratories under comparable conditions to this study include Kansas,[33] Alberta,[40] and A_1c.[41] Values for the three recombinant hemoglobins (nos. 14–17 of Table IV) are from Doyle et al.[13] Original sources for identification of all the naturally occurring mutants can be found in Huisman et al.[3]

Hemoglobin Preparations

We employed a variety of ion exchange techniques in purifying the hemoglobins used in this study.[42] Except as noted, all procedures were performed at 4°C and all buffers included 1 mM Na_2 EDTA. Red cells were washed in isotonic saline solution and then lysed by dialysis against low ionic strength buffer. The lysates were centrifuged to remove stroma. Hemolysates were brought to buffer conditions appropriate for selective adsorption to the ion exchange matrix by dialysis or passage through an equilibrated Sephadex G-25 (Sigma) column.

Normal hemoglobin A_o and hemoglobin S were prepared by the method of Williams and Tsay (1973). Hemoglobins British Columbia, Barcelona, Bunbury, G-Georgia, and Legnano were purified on CM-Sephadex C50 columns (Sigma) with pH gradients formed using 0.05 M Tris-maleic acid buffers (adapted from Huisman and Dozy[43]). Hemoglobin Rothschild was purified by batchwise elution from CM Cellulose (CM52, Whatman) in 0.01 M phosphate buffers. With the exception of samples provided to us in purified form, the remainder of the mutant hemoglobins were isolated on CM cellulose (CM-52, Whatman) columns using pH gradients of 0.01 M phosphate.[44] Elution profiles were monitored spectrophotometrically. Fractions were identified and homogeneity determined by cellulose acetate electrophoresis and agarose gel isoelectric focusing (FMC).

Hemoglobin samples were concentrated using the column technique of Williams and Tsay[45] or by ultrafiltration (Amicon). When appropriate, hemoglobin was dialyzed or passed through Sephadex G-25

columns (Sigma) to match our "standard analytical buffer," and to remove organic phosphates. Samples were photolyzed to remove any bound carbon monoxide and then frozen as droplets, in liquid nitrogen, for long-term storage.[37]

Concentrations were determined spectrophotometrically, after conversion to the cyanomet form, using the standard extinction coefficient of 11.0 absorbance units per millimolar heme (per cm), at 540 nm.[46] Methemoglobin fraction was assayed by the method of Evelyn and Malloy.[47] Organic phosphate concentrations were checked by the methods of Ames and Dubin[48] and Gray and Gibson.[49]

Haptoglobin

Human haptoglobin types 1-1, 2-1, and 2-2 were identified in units of either fresh or outdated human plasma by 2–12% gradient polyacrylamide electrophoresis (Isolab). Samples were pooled by type and haptoglobin isolated according to the method of Connell and Shaw[50] with minor modifications. Binding capacity of each preparation for A_0 and mutant hemoglobins was determined by fluorescence quenching.[51] All abnormal hemoglobins of this study gave fluorescence titrations indistinguishable from normal hemoglobin A_0.

Buffers and Reagents

Standard buffer for analytical experiments consisted of: 0.1 M Tris-base (Sigma), 0.1 M NaCl (Baker), 1 mM Na_2EDTA (Sigma) titrated to pH 7.4, at 21.5°C, using reagent grade HCl (Baker). Sodium dithionite was generously provided by Virginia Chemical. Preparative buffers were made to include 1 mM Na_2EDTA.

EXPERIMENTAL METHODS

Deoxyhemoglobin Assembly Equilibrium Constants

Since the equilibrium constant, $^0K_2$, is usually too large to be measured by conventional techniques, a strategy was developed for its accurate determination from measurements of rate constants of the forward and reverse reactions.[51] This strategy combines kinetic results from trapping dissociated dimers by reaction with human haptoglobin with stopped flow results on the association of rapidly deoxygenated dimers in mixture with tetramers. Monitoring the time course of tetramer dissociation, or assembly, relies upon absorbance differences between deoxy dimers and tetramers. For normal hemoglobin A_0 the difference is approximately 17% at 430 nm. For the abnormal hemoglobins studied, the difference ranges between 5–20%, with most variations arising from extinction coefficients of the unligated tetramers. Application of the haptoglobin kinetics technique to the study of mutant and chemically modified hemoglobins has required the development of new controls for interpretation of biphasic time course data obtained. These are described briefly.

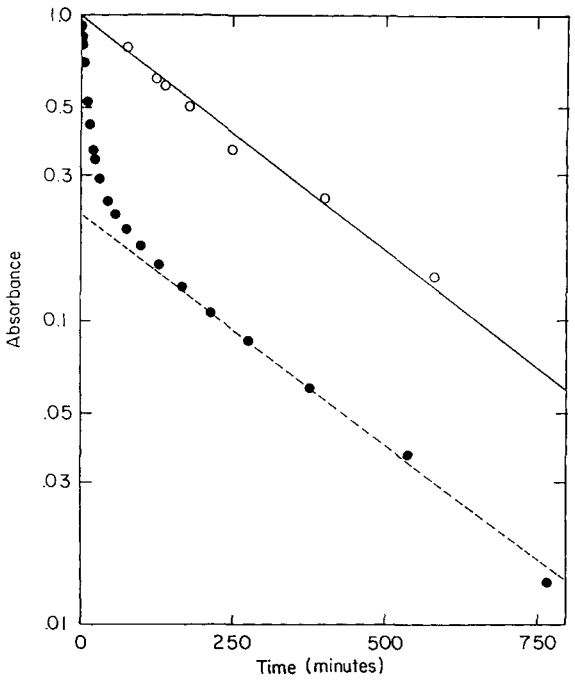


Fig. 3. First-order plot of absorbance at 430 nm for haptoglobin binding experiments with deoxyhemoglobin Chesapeake. Filled circles show results after mixing deoxyhemoglobin Chesapeake with haptoglobin. Open circles show results of an "oxy complex" experiment in which oxyhemoglobin Chesapeake is mixed with haptoglobin, then deoxygenated before kinetic data are collected. Conditions: 0.1 M Tris-base, 0.1 M NaCl, 1 mM Na_2EDTA, 21.5°C, pH 7.4, 0.1% sodium dithionite.

Deoxy tetramer to dimer dissociation. Haptoglobin forms essentially irreversible complexes with hemoglobin dimers but does not bind tetramers. Upon mixing deoxygenated solutions of hemoglobin A_0 and haptoglobin the absorbance at 430 nm decreases with time, as tetramers dissociate into dimers, which are trapped by the haptoglobin.[51] Formation of the hemoglobin-haptoglobin complex may also be observed by quenching of intrinsic haptoglobin fluorescence upon titration with oxyhemoglobin. All abnormal oxyhemoglobins of this study gave equilibrium fluorescence titrations indistinguishable from that of normal hemoglobin.[51]

Similar time-dependent absorbance changes were observed for all deoxy abnormal hemoglobins of this study, but many do not conform to first order kinetics. This is illustrated with data on hemoglobin Chesapeake (Fig. 3) where solid points of the lower curve show the time course of absorbance change after mixing deoxyhemoglobin Chesapeake with excess haptoglobin at zero time. Similar biphasic absorbance changes were observed for many of the abnormal hemoglobins of this study. The data conform well to a sum of two exponentials:

TABLE I. Analysis of Kinetic Data for Subunit Dissociation of Deoxyhemoglobin Chesapeake*

Experiment	No. rate terms	Variance $\times 10^{-3}$	P_1	k_1 (sec^{-1})	P_2	k_2 (sec^{-1})
"deoxy"	1	10.3	.181 ± .015	(1.0 ± .2) × 10^{-3}	—	—
"deoxy"	2	1.9	.149 ± .006	(1.8 ± .2) × 10^{-3}	.068 ± .005	(8.3 ± 2.2) × 10^{-5}
"oxy complex"	1	1.2			.050 ± .003	(6.5 ± 0.1) × 10^{-5}

*Rate constants and preexponential factors estimated from least-squares fits to eq. 6. Deoxy data of Fig. 3 (solid points) were analyzed in terms of single- and double-versions of eq. 6. The "oxy control" experiment (open circles, Fig. 3) was analyzed as a single exponential. Conditions: .1 M Tris-HCl, .1 M NaCl, 1 mM Na_2EDTA, pH 7.4, 21.5°C.

$$A_{(t)} = A_\infty + P_1 e^{-k_1 t} + P_2 e^{-k_2 t} \quad (6)$$

where $A_{(t)}$ is the absorbance at time t, A_∞ is the end point absorbance, P_1 and P_2 are amplitudes of the two exponentials, k_1 and k_2 are first-order rate constants. No indication of second-order processes was found when hemoglobin concentration was varied over the range of 2–15 μM.

Assignment of the first-order rate constant for dissociation of tetramers into dimers was accomplished by the following "oxy-control" experiment. Since all oxygenated hemoglobins dissociate rapidly and within a few seconds after mixing, all the hemoglobin is complexed with haptoglobin. The sample is then deoxygenated and any subsequent spectral changes monitored with time. The absorbance change observed for this "oxy-control" experiment conforms to a single first-order rate process, reflecting events following formation of the haptoglobin-hemoglobin dimer complex. Comparison of the oxy-control rate constant with those determined in the corresponding deoxyhemoglobin-haptoglobin experiment allows assignment of the tetramer dissociation rate of interest. Table I shows results of fitting the Chesapeake data (Fig. 3) to a single exponential (i.e., $P_2 = 0$ in eq. 6) or to the sum of two single exponentials. Clearly the data are not described well by a single exponential. As indicated by the improvement in variance of fit and narrower confidence limits of the fitted parameters, these data are well described by a sum of two exponentials.

Kinetic analysis of absorbance changes for the "oxy-control" experiment indicates a first-order process with rate constant identical to that for the slower process in the initial deoxy experiment (Fig. 3). The slower phase then reflects events that occur after formation of the haptoglobin-hemoglobin dimer complex. We conclude that for Chesapeake, the larger and faster absorbance change reflects dissociation of tetramers to dimers and provides the rate constant of interest in these studies.

Deoxy dimer to tetramer assembly. The technique for determining second-order rate constants of deoxy dimer assembly to tetramers is based upon perturbation of the dimer-tetramer equilibrium by rapid deoxygenation of a solution of oxyhemoglobin. When the equilibrium constant for deoxyhemoglobin ($^OK_2$) is larger than that for oxyhemoglobin ($^4K_2$), rapid deoxygenation will shift the oxyhemoglobin equilibrium toward formation of deoxy tetramers. The rate constant for association of deoxy dimers to tetramers can be determined from the increase in absorbance due to the different extinction coefficients of deoxy dimers and tetramers at 430 nm.[52–54] Experimentally, there are two distinct phases to the absorbance increase: a very rapid, initial increase due to deoxygenation, followed by a slower phase reflecting association of dimers to tetramers. When the rate of assembly is much greater than the rate of tetramer dissociation, the slower phase is described by a rate equation for an irreversible second order process with rate constant k_f:

$$A_{(t)} = A_\infty - \frac{(A_\infty - A_o)}{D_o k_f t + 1}. \quad (7)$$

A_∞ is the end point absorbance, A_o the initial absorbance, and D_o the initial dimer concentration calculated from $^4K_2$ and total protein concentration.

For normal hemoglobin A_o, where the deoxy equilibrium constant is on the order of 10^{10} the assumption is valid that, over the time course of the experiment and within the concentration range studied, the assembly reaction is irreversible. This assumption has also been found valid for many abnormal hemoglobins. However, for some species significant dissociation of deoxy tetramers may occur, and the rate of tetramer production is then:

$$\frac{d[\alpha_2\beta_2]}{dt} = k_f[\alpha\beta]^2 - k_1[\alpha_2\beta_2] \quad (8)$$

where $[\alpha\beta]$ and $[\alpha_2\beta_2]$ are initial concentrations of oxy dimers and tetramers, respectively, and k_1 is the previously determined deoxy tetramer dissociation rate constant. We carried out simulations to evaluate reliability of determining the deoxy dimer to tetramer association rate employing a wide range of oxy and deoxy equilibrium constants (see Results section).

Oxyhemoglobin Assembly

The analytical gel chromatography method for determining dimer-tetramer equilibrium constants[53,54] has been adapted for analysis of the mutant hemoglobins of this study. This technique accurately measures interactions between species

that lead to altered molecular size, reflected in the species partition coefficient, σ_i. For reversibly-associating systems, the composite partition coefficient (σ_w) determined from a "large zone experiment"[54] is a weighted average of the species partition coefficients. For a dimer-tetramer system:

$$\sigma_w = \sigma_4 + {}^4f_2\,(\sigma_2 - \sigma_4). \tag{9}$$

The weight fraction of dimers is:

$${}^4f_2 = \frac{-1 + \sqrt{(1 + 4\,P_t{}^4K_2)}}{2\,P_t{}^4K_2} \tag{10}$$

where P_t is total hemoglobin concentration (units of heme) and ${}^4K_2$ is the dimer-tetramer association constant (per mol dimer). Determination of ${}^4K_2$ for an individual hemoglobin involves obtaining weight average partition coefficients over a range of protein concentrations (see Fig. 5). Nonlinear regression techniques[36,55] are employed to obtain estimates of and confidence limits for the equilibrium constants and the two endpoint values σ_2 and σ_4.

The dissociation curves of weight average partition coefficient versus total hemoglobin concentration were found to conform to common values for the molecular partition coefficient of dimers and tetramers except for Rothschild (β37 trp→arg). This identity of σ values, in both dimers and tetramers, for hemoglobins with altered amino acid residues is the basis for a simultaneous analysis strategy using eq. 11.

$$\sigma_w = \sigma_4 + {}^4f_{2,i}\,(\sigma_2 - \sigma_4) \tag{11}$$

where i is an index relating an individual measurement to a particular hemoglobin and the corresponding equilibrium constants. In the simultaneous analysis the combined data from multiple hemoglobins contribute to the estimated endpoints and allow correct propagation of uncertainty into the confidence limits on the various equilibrium constants.

Oxygen Binding Studies

Oxygenation isotherms were measured with the continuous method of Imai[14] for samples at less than 600 μM heme and by the derivative method of Gill[56] at higher concentrations. Operation of the Imai cell for studies of this kind has been described previously[37,57] and is briefly summarized here. Initially the hemoglobin sample is equilibrated with humidified oxygen gas. Deoxygenation is achieved by flushing humidified nitrogen gas over the stirred sample. Fractional oxygen saturation is monitored at 415 or 577 nm with a Cary 118C spectrophotometer. Oxygen activity is measured with a Beckman 39065 electrode.

In the Gill cell the hemoglobin sample, contained within a gas-tight chamber, is initially equilibrated with humidified oxygen gas. Deoxygenation is achieved in a stepwise fashion by logarithmic dilutions of the oxygen with humidified nitrogen gas. These dilutions are performed by means of a precision gas dilution valve connected to the sample chamber. Upon reduction of the oxygen partial pressure the change in fractional saturation is monitored at 415 nm or 577 nm with a Cary 219 spectrophotometer. Oxygen partial pressure prior to and after each dilution step is known to high precision based on the independently determined dilution factor of the system.[56]

Each oxygenation isotherm obtained in this study was analyzed to determine its median ligand activity, $\bar{X}$. Each median is measured as the ligand activity where areas above and below the isotherm are equal.[38] Since the hemoglobins readily dissociate into dimers, the median of a particular isotherm reflects properties of both dimers and tetramers and thus varies with total concentration. However, it is straightforward to determine the equilibrium isotherm for oxygenation of pure dimers and pure tetramers (i.e., K_2 and K_4, eq. 2) from a binding isotherm at any total hemoglobin concentration, provided the dimer-tetramer association constants ${}^0K_2$ and ${}^4K_2$ (eq 3) are known independently.[36]

$$K_4 = \left[X^{-4}\,\frac{1 - {}^4f_2}{1 - {}^0f_2}\exp({}^0f_2 - {}^4f_2)\right] \tag{12}$$

Here the fraction of fully oxygenated dimers, ${}^4f_2$, is defined in eq. 10 and the deoxygenated fraction, ${}^0f_2$, is given by eq. 13.

$${}^0f_2 = \frac{-1 + \sqrt{1 + 4\,P_t\,{}^0K_2}}{2\,P_t\,{}^0K_2} \tag{13}$$

where P_t is total concentration of hemoglobin in heme units. The constant K_2, for dimer oxygenation is then calculated from eq. 1.

RESULTS

Kinetics of Subunit Dissociation and Association for Deoxyhemoglobins

Table II illustrates the range of rate constants for assembly of abnormal deoxyhemoglobins obtained in this study. In most cases, the time course of absorbance change at 430 nm after mixing deoxyhemoglobin with haptoglobin required analysis by a sum of two first-order processes. Those constants are listed as the second and third entries of Table II. The fourth entry shows rate constants from the respective "oxy-control" experiments. Agreement between these constants and those of the second phase in the deoxy experiment is very good, confirming the assignment of the slower and smaller phase to processes that occur after formation of the haptoglobin-hemoglobin dimer complex.

Second-order kinetics of the absorbance changes that accompany association of deoxyhemoglobin A_o dimers into tetramers is illustrated in Figure 4 for

TABLE II. Assembly Kinetics for Abnormal Deoxyhemoglobins*

Hemoglobin (modification)	k_1 $(sec^{-1})^{\dagger}$	$k_2 \times 10^5$ $(sec^{-1})^{\dagger}$	$k_2 \times 10^5$ $(sec^{-1})^{\ddagger}$	$k_f \times 10^{-6}$ $(M^{-1}sec^{-1})^{\S}$	$^{O}K_2$ $(M^{-1})^{\|}$
Normal A_o	$(1.90 \pm 0.3) \times 10^{-5}$	$0.5 \pm .1$	$1.0 \pm .5$	$0.90 \pm .07$	$(4.7 \pm .4) \times 10^{10}$
Winnipeg (α75 asp→tyr)	$(1.42 \pm 0.7) \times 10^{-5}$	$.8 \pm .5$	12 ± 2.8	$0.74 \pm .03$	$(5.2 \pm .3) \times 10^{10}$
Fort de France (α45 his→arg)	$(1.03 \pm .03) \times 10^{-4}$	$3.0 \pm .5$	5.5 ± 1.3	$1.02 \pm .05$	$(0.9 \pm .6) \times 10^{9}$
San Diego (β109 val→met)	$(1.5 \pm .1) \times 10^{-4}$	$3.3 \pm .7$	11.0 ± 1.0	$1.21 \pm .5$	$(8.0 \pm 3.3) \times 10^{9}$
Zurich (β63 his→arg)	$(3.3 \pm .1) \times 10^{-4}$	10.0 ± 3	18.0 ± 3.0	$0.99 \pm .09$	$(3.0 \pm .3) \times 10^{9}$
Chesapeake (α92 arg→leu)	$(1.9 \pm .3) \times 10^{-3}$	9.2 ± 2.5	$6.5 \pm .1$	$0.84 \pm .08$	$(4.5 \pm .9) \times 10^{8}$
Osler (β145 tyr→asp)	$(4.1 \pm .8) \times 10^{0}$	—	—	$1.18 \pm .32$	$(2.9 \pm .8) \times 10^{6}$
Kempsey (β99 asp→asn)	$(6.8 \pm .7) \times 10^{-1}$	—	—	—	$(1.7 \pm .7) \times 10^{6}$

*Conditions: 0.1 M Tris-base, 0.1 NaCl, 1 mM Na_2EDTA, pH 7.4, 21.5°C.
†Analysis to double exponential kinetics after mixing deoxyhemoglobin with haptoglobin.
‡Single exponential analysis to "oxy control" experiment.
§Association rate constants determined from stopped-flow technique.
‖Equilibrium constant evaluated as the ratio k_f/k_1.

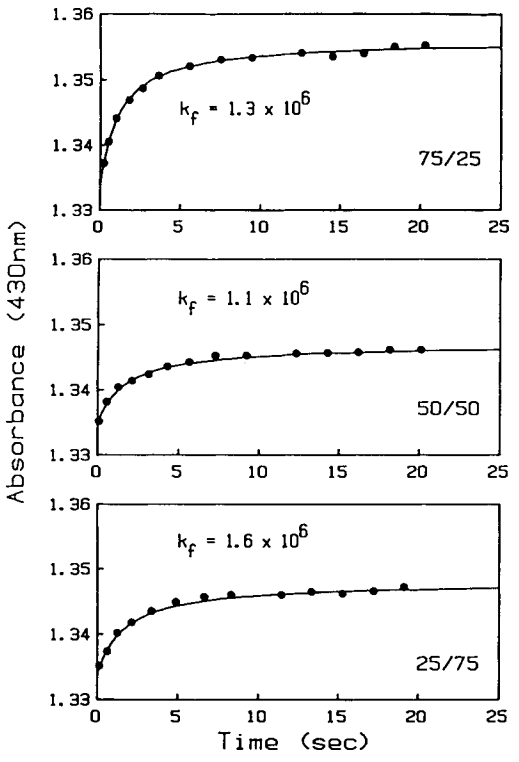


Fig. 4. Dimer-tetramer assembly rate measurements on mixtures of hemoglobins A_o and Chesapeake. All three sets of stopped flow data were analyzed according to a single second-order reaction. Solid lines are best fits.

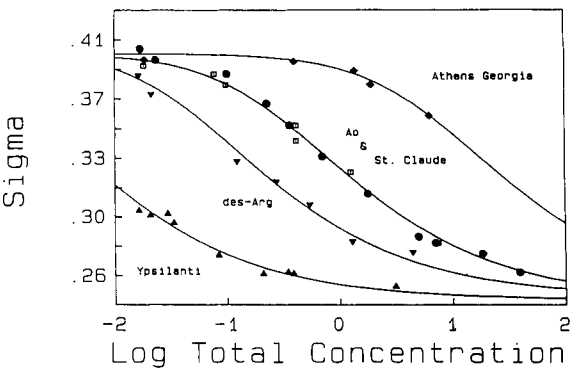


Fig. 5. Dependence of weight average partition coefficient (σ_w) on total hemoglobin concentration (μM dimer) for five hemoglobins with single amino acid differences. ●; A_o (native molecule), □; St. Claude (α127 lys→thr), ◆; Athens Georgia (β40 arg→ser), ▼; Des Arg (α141 arg deleted), ▲; Ypsilanti (β99 asp→tyr).

mixtures of A_o with Chesapeake. The identical behavior of A_o and Chesapeake (α92 arg-leu) (Fig. 4) is representative of the hemoglobins analyzed. The dimer-tetramer assembly rate determination for A_o and a number of abnormal hemoglobins are listed in Table II. In contrast to the wide range of values for the dissociation rate constant, k_1, the association rate, k_f, is essentially the same for all hemoglobins studied. The k_f for hemoglobin A_o was also found invariant over a range of pH values[58] and temperatures.[59] The k_f for a series of abnormal hemoglobins has been studied as a function of temperature and found identical with the corresponding rates determined for A_o.[42]

Numerical studies were carried out to evaluate the effect of including both the association and dissociation reactions in analyzing the rate of production of deoxy tetramers. Data were simulated using equation 8 with the value of k_f previously deter-

TABLE III. Association Equilibrium Constants for Unligated Hemoglobins ($^0K_2$ M^{-1} dimer) Determined by Various Techniques

Hb	Equilibrium gel permeation	Kinetic method	Analytical gel chromatography
A_o	$> 10^9$	$5.0 \pm 0.7 \times 10^{10}$	—
Kempsey (β99 asp→asn)	$2.7 \pm 0.4 \times 10^6$	$1.7 \pm 0.7 \times 10^6$	$2.3 \pm 0.4 \times 10^6$
Chesapeake (α92 arg→leu)	$5.3 \pm 3.5 \times 10^8$	$4.5 \pm 2.3 \times 10^8$	—
des-arg (α141)	$7.9 \pm 0.3 \times 10^6$	$2.6 \pm 0.9 \times 10^7$	—
Cyanomet	—	$1.7 \pm 0.7 \times 10^6$	$1.9 \pm 0.4 \times 10^6$

mined for hemoglobin A_o, and allowing significant rates of deoxy tetramer dissociation. Various pairs of $^4K_2$,$^0K_2$ were used. $^4K_2$ values determined the initial dimer and tetramer concentrations; $^0K_2$ values fixed both the equilibrium concentrations of deoxy tetramer, and the dissociation rate constant used in simulation ($^0K_2 = k_f/k_1$). The simulated data were then analyzed according to equation 7. Over the range of cases simulated, which represent the range of values encountered in this study, the rate constants resolved from fitting were never more than a factor of two different from the actual rate constants used in simulation. These results establish the validity of using equation 7 in the analysis of variant hemoglobins in this study. Moreover, k_f is invariant for a range of single amino acid substitutions, and solvent conditions. For hemoglobins where $^4K_2$ is greater than $^0K_2$ the association experiment is not feasible. The observed invariance of k_f (e.g., Table II) therefore validates use of the deoxy A_o rate for determining $^0K_2$.

Equilibrium Constants for Dimer-Tetramer Assembly

The results described above provide the basis for using the kinetic method to estimate equilibrium constants, $^0K_2$, calculated as the ratio k_f/k_t. Representative values for abnormal hemoglobins are illustrated in Table II. The corresponding Gibbs free energy $^0\Delta G_2$ for each hemoglobin of this study is given in Table IV. The values of $^0K_2$ found for all hemoglobins range from 10^{11} M^{-1} to 10^6 M^{-1}. Comparison of the relative values of k_f and k_1 shows that the structural alterations generated by amino acid replacement are reflected dramatically in the rates of tetramer dissociation, whereas essentially no change is observed in the rates of dimer-tetramer assembly.

Independent direct determinations of $^0K_2$ for hemoglobins Chesapeake, Kempsey, des-arg, Hirose, and Cyanomet, using equilibrium gel permeation methods[53,54] show agreement with values reported here (Table III). This agreement provides additional validation of the procedure for assignment of observed kinetic phases and the use of these particular kinetic methods for determining equilibrium constants.

For oxyhemoglobins the determination of $^4K_2$ by analytical gel chromatography is illustrated in Figure 5. The dependence on total hemoglobin concentration of the weight average partition coefficient σ_w is shown for a representative set of abnormal species. Approximately four orders of magnitude variation in protein concentration were employed. Shapes of the dissociation curves were tested against a range of stoichiometric models of subunit assembly and best estimates of the equilibrium constants were resolved. In all cases a dimer-tetramer stoichiometry was found to fit the data best, except for Pitié Salpetriérè (β34 val→phe), which exhibited a monomer-dimer-tetramer reaction scheme.[42] Implicit from Figure 5 are the dimer and tetramer endpoint partition coefficients determined by simultaneous analysis according to equation 11. Since all the hemoglobin experiments on a particular calibrated column yield information regarding the endpoints, it was not necessary to exhaustively cover the entire dissociation curve for each hemoglobin of interest. However, the gel permeation experiments for hemoglobin Rothschild (β37 trp→arg) had to be analyzed independently, as the apparent hydrated dimer partition radius was found to be significantly perturbed by this amino acid substitution.[60]

Results of the determinations on subunit assembly reactions for all hemoglobins of this study are given in Table IV. Listed are the Gibbs free energies, $^4\Delta G_2$, along with corresponding values of $^0\Delta G_2$ for each hemoglobin. Also tabulated for each molecule is the cooperative free energy ΔG_c, calculated as the difference ($^4\Delta G_2 - {}^0\Delta G_2$).

Consequences for oxygen binding studies. A consequence of the wide range of subunit association constants observed in this study is that oxygen binding experiments, performed at experimentally convenient protein concentrations on mutant and chemically modified hemoglobins, may not reflect tetrameric properties alone. Even at high protein concentrations, oxygen binding curves for many variant hemoglobins may be significantly perturbed by appreciable quantities of dimers. For example, hemoglobin Rothschild (β37 trp→arg) at a concentration of 1 mM heme contains 38% dimers. The ultrasensitive shapes of binding curves for hemoglobin A_o may be perturbed significantly even at 1 mM

TABLE IV. Free Energies of Dimer-Tetramer Assembly for Mutant and Chemically Modified Human Hemoglobins

Hemoglobin	Modification	Structural location	deoxy $^0\Delta G_2$	oxy $^4\Delta G_2$	ΔG_c
1. Normal A_o	none	—	−14.3 ± .2	−8.0 ± .1	6.3 ± .2
2. *Kariya†	(α40 lys→glu)	$\alpha^1\beta^2$ contact	−9.1 ± .1	−8.0 ± .1	1.1 ± .2
3. *Fort de France	(α45 his→arg)	external, heme contact	−13.6 ± .1	−8.0 ± .1	5.6 ± .2
4. *Sealy†	(α47 asp→his)	external	−15.1 ± .2	−7.8 ± .1	7.4 ± .3
5. *Winnipeg	(α75 asp→tyr)	external, near cavity	−14.5 ± .1	−7.9 ± .1	6.6 ± .2
6. *G. Norfolk	(α85 asp→asn)	external	−14.2 ± .1	−8.3 ± .1	5.9 ± .2
7. *Chesapeake	(α92 arg→leu)	external, $\alpha^1\beta^2$ contact	−11.7 ± .2	−9.6 ± .1	2.1 ± .3
8. *Rampa†	(α95 pro→ser)	internal, central cavity $\alpha^1\beta^2$	−15.0 ± .2	−7.6 ± .1	7.4 ± .3
9. G. Georgia†	(α95 pro→leu)	internal, central cavity $\alpha^1\beta^2$	−10.3 ± .2	−5.4 ± .6	4.9 ± .6
10. *Dallas†	(α97 asn→lys)	$\alpha^1\beta^2$ contact	−11.4 ± .2	−9.5 ± .1	1.9 ± .3
11. *Tarrant†	(α126 asp→asn)	$\alpha^1\beta^1$ contact $\alpha^1\alpha^2$ contact	−10.0 ± .2	−8.2 ± .1	1.8 ± .3
12. *St. Claude†	(α127 lys→thr)	$\alpha^1\alpha^2$ contact	−11.1 ± .1	−8.0 ± .1	3.1 ± .2
13. *Legnano†	(α141 arg→leu)	external, $\alpha^1\alpha^2$ & $\alpha^1\beta^2$ contacts	−9.5 ± .2	−7.9 ± .1	1.6 ± .3
14. *rβ1 + M‡	(add met to β1)	external	−14.5 ± .1	−7.4 ± .1	7.1 ± .2
15. rβV1A‡	(β1 val→ala)	external	−14.4 ± .1	−8.0 ± .1	6.5 ± .2
16. rβV1M‡	(β1 val→met)	external	−15.2 ± .1	−7.7 ± .1	7.5 ± .2
17. *S	(β6 glu→val)	external	−14.3 ± .2	−8.0 ± .1	6.3 ± .3
18. C	(β6 glu→lys)	external	−14.7 ± .1	−8.2 ± .2	6.5 ± .3
19. *Strasbourg	(β23 val→asp)	internal	−13.6 ± .1	−8.3 ± .1	5.3 ± .2
20. *Pitié Salpetrérè†	(β34 val→phe)	$\alpha^1\beta^1$ contact $\alpha^1\beta^2$ contact	−8.4 ± .1	−8.8 ± .5	−0.4 ± .5
21. *Linkoping†	(β36 pro→thr)	external, $\alpha^1\beta^2$ contact	−11.6 ± .2	−8.2 ± .2	3.4 ± .3
22. *Rothschild†	(β37 trp→arg)	external, $\alpha^1\beta^2$ contact	−13.3 ± .2	−3.6 ± .3	−9.7 ± .5
23. Hirose	(β37 trp→ser)	external, $\alpha^1\beta^2$ contact	−8.8 ± .1	−5.8 ± .3	3.0 ± .5
24. *Austin†	(β40 arg→ser)	external, $\alpha^1\beta^2$ contact	−12.0 ± .1	−4.5 ± .2	7.5 ± .3
25. Athens, GA†	(β40 arg→lys)	external, $\alpha^1\beta^2$ contact	−13.5 ± .1	−5.4 ± .5	8.1 ± .5
26. *Zurich	(β63 his→arg)	distal histidine	−12.9 ± .1	−6.9 ± .1	6.0 ± .2
27. *Creteil	(β89 ser→asn)	internal, near $\alpha^1\beta^2$ contact	−8.5 ± .2	−7.7 ± .1	0.8 ± .2
28. *Barcelona†	(β94 asp→his)	external, $\alpha^1\beta^2$ contact	−13.8 ± .1	−8.0 ± .2	5.8 ± .2
29. Bunbury†	(β94 asp→asn)	external, $\alpha^1\beta^2$ contact	−14.0 ± .1	−7.8 ± .1	6.2 ± .2
30. *Malmö†	(β97 his→gln)	external, $\alpha^1\beta^2$ contact	−12.1 ± .2	−8.2 ± .1	3.9 ± .3
31. Wood†	(β97 his→leu)	external, $\alpha^1\beta^2$ contact	−12.1 ± .2	−8.3 ± .1	3.8 ± .3
32. *Hôtel Dieu	(β99 his→gly)	internal, $\alpha^1\beta^2$ contact	−8.2 ± .1	−8.0 ± .1	0.2 ± .2
33. Radcliffe†	(β99 asp→ala)	internal, $\alpha^1\beta^2$ contact	−8.8 ± .1	−9.2 ± .1	−0.4 ± 2.
34. Chemilly†	(β99 asp→val)	internal, $\alpha^1\beta^2$ contact	−8.7 ± .1	−8.7 ± .1	0.0 ± .2
35. Kempsey	(β99 asp→asn)	internal, $\alpha^1\beta^2$ contact	−8.4 ± .2	−8.7 ± .1	−0.3 ± .2
36. Yakima	(β99 asp→his)	internal, $\alpha^1\beta^2$ contact	−9.8 ± .2	−9.5 ± .1	0.3 ± .2
37. Ypsilanti†	(β99 asp→tyr)	internal, $\alpha^1\beta^2$ contact	−8.7 ± .1	−11.3 ± .1	−2.6 ± .2
38. *Alberta§	(β101 glu→gly)	internal, $\alpha^1\alpha^2$ contact $\alpha^1\alpha^2$ contact	−11.5 (n.d.)	−7.4 (n.d.)	4.1 (n.d.)
39. British Columbia	(β101 glu→lys)	internal, $\alpha^1\beta^1$ contact $\alpha^1\beta^2$ contact	−11.5 ± .1	−5.9 ± .1	5.6 ± .2
40. Rush†	(β101 glu→gln)	internal, $\alpha^1\beta^1$ contact $\alpha^1\beta^2$ contact	−14.8 ± .2	−7.8 ± .2	7.0 ± .3
41. Kansas‖	(β102 asn→thr)	internal, heme & $\alpha^1\beta^2$ contact	−13.6 ± .1	−5.8 ± .1	7.8 ± .2
42. *Saint Mandé	(β102 asn→tyr)	internal, heme & $\alpha^1\beta^2$ contact	−14.9 ± .1	−6.4 ± .1	8.5 ± .2
43. *San Diego	(β109 val→met)	internal, $\alpha^1\beta^1$ contact	−13.4 ± .1	−7.6 ± .1	5.8 ± .2
44. *O-Arab†	(β121 gly→lys)	external, near $\alpha^1\beta^1$ contact	−15.4 ± .2	−8.2 ± .2	7.2 ± .3
45. *Ty Gard†	(β124 pro→gln)	external, $\alpha^1\beta^1$ contact	−14.3 ± .1	−8.0 ± .2	6.3 ± .3
46. *Hope	(β136 gly→asp)	central cavity	−15.1 ± .2	−7.6 ± .1	7.5 ± .3
47. *Abruzzo†	(α143 his→arg)	external, central cavity	−14.6 ± .2	−7.2 ± .2	7.4 ± .3
48. *Osler	(β145 try→asp)	$\alpha^1\beta^2$ contact	−8.8 ± .2	−7.4 ± .2	1.4 ± .3
49. *Cowtown†	(β146 his→leu)	$\alpha^1\beta^2$ contact C-terminal	−13.8 ± .1	−8.1 ± .1	5.7 ± .1

(continued)

TABLE IV. Free Energies of Dimer-Tetramer Assembly for Mutant and Chemically Modified Human Hemoglobins (Continued)

Hemoglobin	Modification	Structural location	deoxy $^0\Delta G_2$	oxy $^4\Delta G_2$	ΔG_c
Chemically modified species					
50. *Carboxy-methylated†	(α_1 val)	$\alpha^1\alpha^1$ contact	−13.8 ± .1	−7.2 ± .1	7.1 ± .2
51. Carboxy-methylated†	(β_1 val)	internal, DPG pocket	−14.3 ± .1	−7.8 ± .1	6.5 ± .2
52. Carboxy-methylated†	(α_1 val, β_1 val)	$\alpha^1\alpha^1$ contact, DPG pocket	−14.3 ± .1	−6.6 ± .1	7.7 ± .2
53. A_1c#	(β_1 val→gly)	internal, DPG pocket	−14.2 ± .1	−7.9 ± .1	6.3 ± .2
54. *NES	(β93 cys-NES)	$\alpha^1\beta^2$ contact	−11.5 ± .1	−8.5 ± .2	3.0 ± .2
55. *Thiomethylated*†	(β93 *cys-methyl*)	$\alpha^1\beta^2$ *contact*	−13.0 ± .1	−7.2 ± .1	5.8 ± .3
56. β93 NES/Rush†	(β101 glu→gln)	$\alpha^1\beta^2$ contact, $\alpha^1\alpha^1$ contact	−11.7 ± .1	−8.2 ± .1	3.5 ± .3
57. β93 NES/British Columbia†	(β101 glu→lys)	$\alpha^1\beta^2$ contact, $\alpha^1\alpha^1$ contact	−8.7 ± .1	−6.3 ± .1	2.4 ± .2
58. des arg	(α141 deleted)	$\alpha^1\beta^2$ contact, $\alpha^1\alpha^2$ contact	−10.1 ± .2	−9.0 ± .1	1.1 ± .2
59. NES/des arg†	(α141/β93)	$\alpha^1\beta^2$ contact, $\alpha^1\alpha^2$ contact	−8.8 ± .1	−9.3 ± .1	.-.5 ± .1
60. des his†	(β146 deleted)	$\alpha^1\beta^2$ contact	−11.8 ± .1	−8.0 ± .1	3.8 ± .2

*Species arbitrarily selected to assess cumulative energetic perturbation. Each asterisk references one of the 34 residue sites probed by structural modification (see text).
†Characterized in this study. Species not followed by superscripts were characterized in the previous study of this series, ref. 15. Conditions: 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM Na_2EDTA, pH 7.40, 21.5°C.
‡Recombinant hemoglobins described in refs. 12, 13.
§Ref. 40.
‖Ref. 33.
#Ref. 41 (oxy) and this study (deoxy).

heme where only 2% of species are dimers in the solvent conditions of these studies.[61] As a rule, one must always assess the effects of dimer populations in conjunction with performing oxygenation studies for each particular hemoglobin. It is not justified to assume that hemoglobin concentrations that may be adequate for minimizing the dimer population in normal hemoglobin will suffice for all abnormal hemoglobins.

Oxygen Binding Parameters for Dimers and Tetramers

Table VI lists free energies of oxygenation for dimers and tetramers of 17 hemoglobins. These values were obtained from combined analysis of binding isotherms and independently determined constants K_2 and K_4 according to eqs. 10–13. The set of values ΔG_2, ΔG_4, $^0\Delta G_2$, and $^4\Delta G_2$ define the overall linkage scheme for each of these hemoglobins. The values found for dimer oxygenation free energy for the abnormal molecules were identical to that of normal hemoglobin A_o except for Athens Ga., Austin, Barcelona, and Kansas. Independent determinations of binding free energies for isolated α and β subunits of A_o under identical conditions yielded $\Delta G_\alpha = -8.1 \pm .1$ kcal; $\Delta G_\beta = -8.5 \pm .3$ kcal.[31,32] Their sum, −16.8 ± .3, is the same value found for dissociated dimers (Table VI). The only abnormal species for which values of the constituent isolated subunits have been determined is Kansas. Here a value of −7.2 ± .3 for the mutant β chain plus that of α subunits (−8.1 ± .2) sums to the experimental dimeric value of −15.7 ± .3 kcal.[33] All but three (above) of the other 14 abnormal hemoglobins of Table V are found to have ΔG_2 values identical within error to that of normal A_o. Although isolated subunits have not been characterized for these three species, all values to date strongly support the concept that primary function (i.e., oxygen binding) and its regulation (cooperativity) are "decoupled" by dissociation of tetramers into dimers. These findings validate further the dissociated dimers as the fundamental noncooperative unit and their use as a reference for assessing tetramer cooperativity. Recent work has shown that the dimers do become cooperative when assembled into tetramers.[30]

ANALYSES AND CORRELATIONS

Ranges of Energetic Response

The experimental findings summarized in Table IV show a dramatic range in the energetic responses to amino acid modification. Assembly free energies of the unligated molecules vary between −15.4 kcal (O-Arab) and −8.2 kcal (Hotel Dieu). However, the range of energetic perturbation is slightly greater for the oxygenated molecules. Although most of these have assembly energies similar to the normal molecule (−8.0 kcal), the $^4\Delta G_2$ values range between −3.6 kcal (Rothschild) and −11.3 kcal (Ypsilanti), reflecting over six orders of magnitude variation in $^4K_2$. The range of cooperative free energies ΔG_c spans 12.1 kcal (i.e., from −2.6 kcal for Ypsilanti to +9.7 for Rothschild). This corresponds to nearly 10 orders of magnitude change in the ratio of tetramer oxygen affinity relative to that of dimers.

Deoxy Molecules Are Most Sensitive

On average, the structural modifications produce a larger effect on stability of the unligated molecule than on the ligated species. For the 59 abnormal

TABLE V. Oxygen Binding to Dimers and Tetramers of Abnormal Hemoglobins*

Hemoglobin	ΔG_2	ΔG_4
A_o	−16.6 ± .2	−27.1 ± .1
Kariya (α40 lys→glu)	−17.1 ± .3	−33.2 ± .3
β1 val→ala	−16.6 ± .2	−26.6 ± .1
β7 val→met	−16.6 ± .4	−25.9 ± .2
β1 O + met	−17.0 ± .2	−26.8 ± .1
Rothschild (β37 trp→arg)	−16.7 ± .4	−23.9 ± .4
Athens, GA (β40 arg→lys)	−17.8 ± .6	−27.6 ± .3
Austin (β40 arg→ser)	−17.9 ± .3	−28.3 ± .3
Barcelona (β94 asp→his)	−17.4 ± .3	−29.1 ± .3
Hotel Dieu (β99 asp→gly)	−16.7 ± .3	−33.1 ± .3
Radcliffe (β99 asp→ala)	−17.0 ± .1	−33.9 ± .2
Chemilly (β99 asp→val)	−17.0 ± .1	−34.0 ± .1
Kempsey (β99 asp→asn)	−16.6 ± .2	−33.6 ± .1
Yakima (β99 asp→his)	−16.8 ± .1	−33.4 ± .1
Ypsilanti (β99 asp→tyr)	−16.5 ± .1	−35.6 ± .2
Kansas (β102 asn→thr)	−15.7 ± .3	−23.1 ± .1

*Conditions: 0.1 M Tris-HCl, 0.1 M NaCl (total chloride is 0.18 M), 1 mM N_2EDTA, pH 7.40, 21.5°C.

deoxyhemoglobins studied the average tetramer is destabilized by 2.1 kcal (a 40-fold change in equilibrium constant), whereas the oxyhemoglobins exhibit average destabilization of only 0.3 kcal (i.e., less than twofold change of equilibrium constant). The mean absolute deviations from normal (2.3 kcal for deoxy and 0.8 kcal for oxy) exceed the net values cited, reflecting the fact that a few mutations stabilize tetramers relative to dimers. The larger discrepancy between these averages is for the unligated molecules, consistent with the concept that the native tetramer is more "constrained" in its unligated form.

Normal and reverse linkages. Applying an experimentally based discrimination level of ± 0.6 kcal to the distribution of ΔG_c values reveals 14 species whose responses to oxygenation were in the "normal range" (i.e., 5.7–6.9 kcal). In four cases (Zurich, Barcelona, San Diego, and β93 thiomethyl cys) perturbations in both $^0\Delta G_2$ and $^4\Delta G_2$ compensate to bring ΔG_c into the normal range. At the other extreme, reverse linkages (i.e., negative values of ΔG_c) were found for five species (Radcliffe, Kempsey, Ypsilanti, Pitié Salpetriérè, and NES/des arg). Only Ypsilanti exhibits a highly significant reverse linkage. Ypsilanti is capable of forming a quaternary structure distinctly different from T or R under high salt conditions.[26] Detailed functional energetics of this, and five related mutants at the β99 position are described in the accompanying manuscript.[25]

Localization of Cooperative Free Energy

A major correlation from these studies is that the cooperative free energy ΔG_c is dramatically altered by amino acid modifications at or near the $\alpha^1\beta^2$ intersubunit contact region (including pairwise subunit contacts $\alpha^1\beta^2$, $\alpha^2\beta^1$, and $\alpha^1\alpha^2$), but is not affected similarly by alterations outside of this interface, including those at the external surface, heme pocket, β cleft, and $\alpha^1\beta^1$ contact. This correlation, found in the first 23 hemoglobins studied,[15] is strongly reinforced by the much larger set of experimental values from the present study. An apparent exception to this rule is Creteil (β89 ser-asn), which by crystallographic analysis eliminates a hydrogen bond between the β89 ser OH and carbonyl oxygen of β141 leu leading to destabilization of the carboxy terminal region (in the $\alpha^1\beta^2$ contact) in normal deoxy hemoglobin. Identical structural rearrangement occurs with the mutant hemoglobin Osler (β145 tyr→asp) although none of the side chains at β89, or β141 are close enough for contact with those at β145 (see ref. 15).

The composite results for all 60 hemoglobins are shown graphically in Figure 6. The ratio of oxy and deoxy assembly constants for each hemoglobin, normalized against the same ratio of equilibrium constants for A_o, are plotted in two structural regions of the tetrameric molecule: (1) the external surface, $\alpha^1\beta^1$ contact, heme pocket, and β cleft; and (2) the "$\alpha^1\beta^2$ interface," including $\alpha^1\beta^2$, $\alpha^2\beta^1$ and $\alpha^1\alpha^2$. A structure-function perturbation map has been constructed (Fig. 7) showing the structural location of responses in $\delta\Delta G_c$ values at three ranges of magnitude, denoted by separate colors.

Figures 6 and 7 show that the cooperative free energy ΔG_c is highly sensitive to structural alterations within the $\alpha^1\beta^2$ intersubunit contact but is generally much less sensitive to modifications at other regions of the molecule. Of greatest significance is the general absence of high sensitivity at the other structural locations. These findings suggest that a major source of ΔG_c is the free energy of ligand linked changes in interactions within the $\alpha^1\beta^2$ interface. The alteration of local amino acid side chain interactions within this subunit contact region, as the deoxyhemoglobin tetramer undergoes ligation, appears to provide a major component of the cooperative free energy. These findings are consistent with the crystallographic observations which have identified the $\alpha^1\beta^2$ interface as the region of the hemoglobin tetramer that exhibits the most extensive changes in pairwise amino acid contacts.[63–66]

Since the T to R quaternary transition provides a positive ΔG term, mutations that drive the unligated molecule toward quaternary R by lowering the energy barrier for the quaternary switch may produce large reductions in ΔG_c. Mutations at residue sites within or near the $\alpha^1\beta^2$ interface are, of course, prime candidates for this effect, e.g., hemoglobin Kempsey. Recent work on intermediate ligation species indicates that only part of the cooperative free energy arises from the quaternary T→R transition and that a significant portion (e.g., 50%)

TABLE VI. Energetic Effects of Multiple Modifications

	deoxy Hb		oxy Hb		Cooperative energy	
	$\delta^0\Delta G_2$	$(\delta_1 + \delta_2)$	$\delta^4\Delta G_2$	$(\delta_1 + \delta_2)$	$\delta\Delta G_c$	$(\delta_1 + \delta_2)$
(A) Two-site modifications						
British Columbia (β101 glu→lys)	2.8 ± 0.1	—	2.1 ± 0.1	—	−0.7 ± 0.2	—
Rush (β101 glu→gln)	−0.5 ± 0.2	—	0.2 ± 0.2	—	0.7 ± 0.3	—
NES (β93 NES)	2.8 ± 0.1	—	−0.5 ± 0.2	—	−3.3 ± 0.2	—
des-arg (α141 deleted)	4.2 ± 0.2	—	−1.0 ± 0.1	—	−5.2 ± 0.2	—
Carboxymethyl (α1 val)	0.5 ± 0.2	—	0.8 ± 0.2	—	0.3 ± 0.3	—
Carboxymethyl (β1 val)	0.0 ± 0.2	—	0.2 ± 0.2	—	0.2 ± 0.3	—
(B) Four-site modifications						
NES/British Columbia (β101 glu→lys/β93 NES)	5.6 ± 0.2	5.6 ± 0.2	1.7 ± 0.2	1.6 ± 0.3	−3.9 ± 0.2	−4.0 ± 0.3
NES/Rush (β101 glu→gln/β93 NES)	2.6 ± 0.2	2.3 ± 0.3	−0.2 ± 0.2	−0.3 ± 0.3	−2.8 ± 0.2	−2.6 ± 0.3
NES/des-arg (α141/β93 NES)	5.5 ± 0.2	7.0 ± 0.3	−1.3 ± 0.2	−1.5 ± 0.3	−6.8 ± 0.2	−8.5 ± 0.3
Carboxymethyl (α1 val, β1 val)	0.0 ± 0.1	0.5 ± 0.3	1.4 ± 0.2	1.0 ± 0.3	1.4 ± 0.3	0.5 ± 0.4

may also arise from "sequential" cooperativity within T and within R. Since these latter terms may contain partially cancelling terms, it is quite possible for a mutant whose ΔG_c is nearly normal to have an altered distribution of stepwise contributions to ΔG_c. These findings place a more complex interpretation on Figure 6 and 7 than would exist if, for example, a two-state concerted mechanism[67] were valid. In that model there is no sequential cooperativity in either T or R and ΔG_c bears a simple relationship to the T→R equilibrium constant.[68] The ΔG_c values in Table IV provide the net free energy used by each variant hemoglobin to modulate oxygen affinity over its four binding steps. The present results provide a unique data base that may be dissected further into energetic components for the various processes and correlated with structural information.

Correlation With Crystallographic Temperature Factors

Figure 8 shows the perturbations in subunit assembly energetics for hemoglobins of this study, versus the temperature factors (B) for the oxy[66] and deoxy[64] crystallographic structures of hemoglobin A_o. The value plotted for each residue is the average crystallographic thermal factor, B, of the four backbone atoms, Cα, C, O, and N.[69] The deoxy molecules have smaller B factors suggestive of less freedom of motion of the backbone atoms, presumably as a result of highly coupled sets of side chain interactions. Although a part of this effect may result from differences in resolution of the two data sets, the deoxy

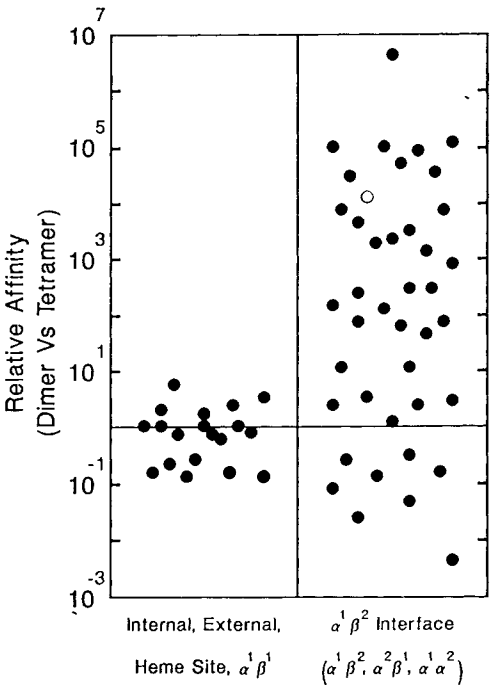
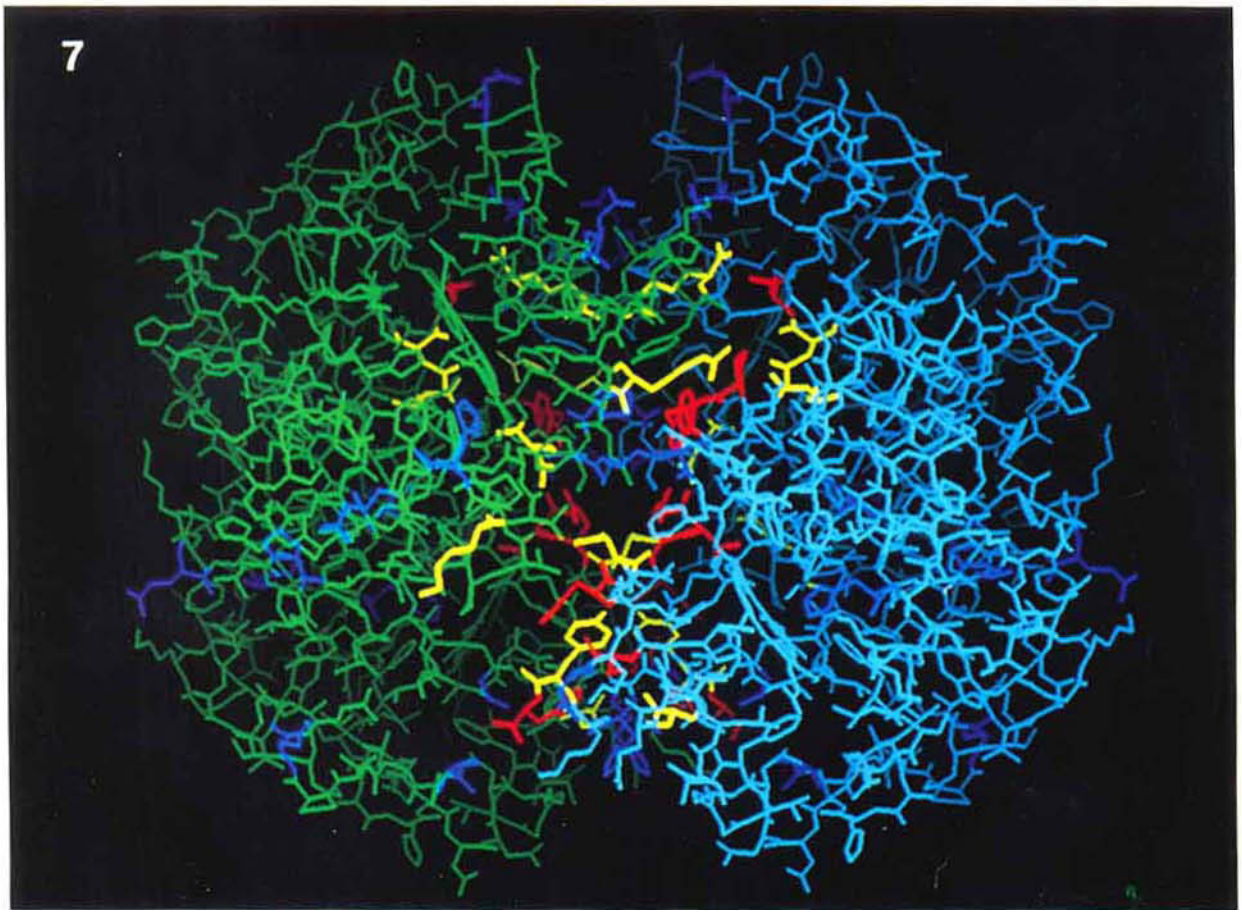
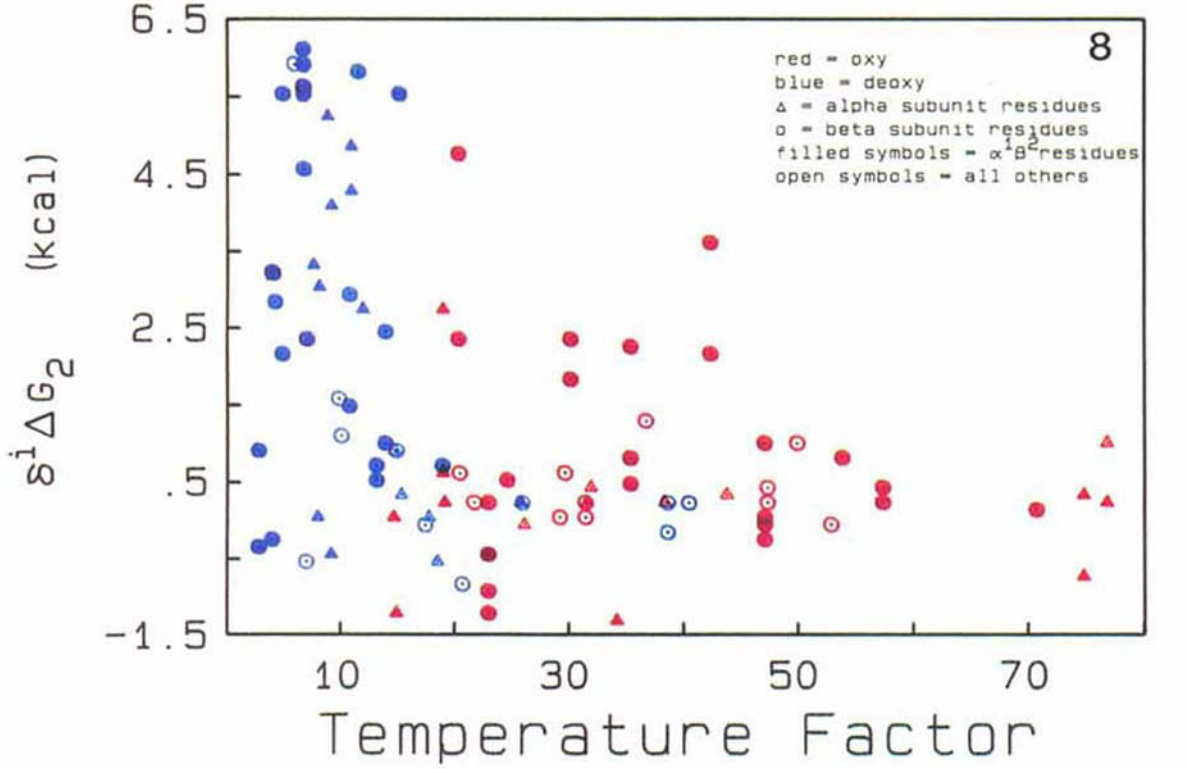


Fig. 6. Correlation between perturbation in cooperative free energy and molecular location of the altered side chains. The ordinate is $K_4/(K_2)^2$ for each variant hemoglobin, normalized to the same value for hemoglobin A_o. The absissa is composed by an arbitrary numbering of the hemoglobins characterized in the present study. These were subsequently grouped by amino acid residue location in the native tetramer. The groupings include: variants located at the $\alpha^1\beta^2$ interface, including the intersubunit contacts $\alpha^1\beta^2$, $\alpha^2\beta^1$, and $\alpha^1\alpha^2$ (right) and variants located at the external surface, β cleft, the heme site, and the $\alpha^1\beta^1$ intersubunit contact (left). The open circle represents Hb Creteil (see text).


7


8
red = oxy
blue = deoxy
Δ = alpha subunit residues
o = beta subunit residues
filled symbols = α1β2 residues
open symbols = all others
6.5
4.5
2.5
.5
-1.5
10
30
50
70
Temperature Factor
δiΔG2 (kcal)

Figs. 7 and 8.

$\alpha^1\beta^2$ interface residues are clearly the most structurally constrained positions and correlate with the largest average energetic perturbations observed in this study.

The oxy molecules, in contrast, which seem more flexible in their responses to residue modifications exhibit B factors for the residues investigated that randomly span the entire range of observed values. A greater range of subunit assembly perturbation is found for residues involving the $\alpha^1\beta^2$ interface, but the correlation is not as dramatic as for the deoxy species. The noncovalent bonding interactions of sidechains in the oxy tetramers may reflect a virtual continuum of energetically degenerate structural forms, with nearly equivalent stabilities. Transitions between these iso-energetic states may accommodate alternative sets of local side chain interactions. A general picture arising from the composite results of this study is that the doexy tetramer is an energetically more constrained structure than the oxy tetramer. The degree of coupling between side chain interactions for the oxy tetramer is such that single amino acid alterations are generally insufficient to globally perturb tetrameric stability.

Nonadditivity, Site-Site Coupling, and Adventitious Perturbation

When free energy perturbations are summed over an arbitrarily chosen set of hemoglobins in which only one modification at each site is included (indicated in Table IV by asterisks), the sum of free energy perturbations, i.e., of $[^0\Delta G_2{}^* - {}^0\Delta G_2]$ or $[^4\Delta G_2{}^* - {}^4\Delta G_2]$ greatly exceeds the assembly free energy of the corresponding native molecule (i.e., fivefold for deoxy and threefold for oxy). This is not surprising in view of possible overlap between short-range effects arising from alteration at each residue site (steric interaction, charge, solvation, and hyrogen bonding) as well as interactions propagated over larger distances.

Even if the sites are not coupled an apparent "non-additivity" in the observed free energy effects may arise from "adventitious perturbations," i.e., the free energy alterations need not "add up" to the stabilization energy of the native molecule unless the effects of structural modification were solely to eliminate the stabilizing interactions present in the native structure. If the modifications replace local, "native" interactions with "arbitrary" ones that are, *on average, more destabilizing,* an apparent "nonadditivity" of the observed free energies will result. It is, of course, possible for "adventitious perturbation" and site-site coupling to occur simultaneously.

Molecular dynamics calculations have been carried out on the energetics of substituting the normal β99 aspartic acid by alanine[27] to assess the origins and extent of the changes in atomic interactions that produce the $\delta\Delta G_c$ observed for hemoglobin Radcliffe (β99 asp→ala). The magnitude reported for the calculated $\delta\Delta G_c$ was remarkably similar to that which is observed experimentally (Table IV). Particularly interesting was the finding that the net free energy change accompanying the single residue modification originates from numerous energetic perturbations in both the deoxy and oxy tetrameric species. The altered energetics included interactions from multiple inter-, and intra-amino acid side chain contributions and large solvent effects.

Quadruply Modified Tetramers

To assess the independence of altered amino acid residues, we constructed and studied several hemoglobins that have multiple types of structural modifications within the same tetramer. Hemoglobins British Columbia, Rush, and des-arg α141 were chemically modified at β93 with N-ethylmaleimide to obtain NES/British Columbia, NES/Rush, and NES/des arg. Shown in Table VI are the experimentally determined perturbations in $^0\Delta G_2$, $^4\Delta G_2$ and ΔG_c relative to hemoglobin A_o, that result from: (1) each single type of amino acid change (on both α chains or both β chains); and (2) the simultaneous presence of both types of alterations (i.e., four total per tetramer). The quantity $(\delta_1 + \delta_2)$ represents the sum of perturbations expected if the two types of modifications affected the hemoglobin tetramer independently. The values thus predicted of $(\delta_1 + \delta_2)$ are compared in Table VI with the respective actual values $\delta(^0\Delta G_2)$ or $\delta(^4\Delta G_2)$. A fourth system was comprised of normal hemoglobin carboxymethylated on amino terminal residues of the α subunits, β subunits, or both kinds of subunits.

In NES/British Columbia and NES/Rush we see exact additivity of the two types of perturbations. The β93 and β101 residues are located near the switch region of the $\alpha^1\beta^2$ interface, and their respective Cα carbons are separated by ~12 Å in both fully ligated and unligated tetramers.[64,66] Additivity of the two perturbations is also manifested by ligated tetramers of NES/des-arg. Although the effects of

Fig. 7. Structure-energy map showing perturbations in the cooperative free energy of human hemoglobin for site specific structure alterations. Orientation of the tetramer is the same as Figure 1. The symmetric view of the $(\alpha\beta)_2$ tetramer shows one αβ dimer in green (left) and the other αβ dimer in grey (right). β subunits are in the lower front. The magnitude of perturbations by mutations at a given location on the cooperative free energy is color coded: Yellow, > 4 kilocalories; Orange, 2–4 kilocalories; Blue, 0–2 kilocalories. This pattern of energy perturbations demonstrates the critical functional importance of the amino acid residues along the interface between the two αβ dimers.

Fig. 8. Changes in subunit association energetics for the abnormal hemoglobins of the present study versus the crystallographically determined temperature (B factors) for deoxy[64] and oxy[66] hemoglobin A_o. The temperature factors are tabulated as averages of the backbone peptide atoms for hemoglobin A_o at positions of the altered amino acids in the abnormal hemoglobins.

TABLE VII. Energetic Perturbations by Mutants at Distant Sites

Hemoglobin	Structural modification	$({}^0\Delta G_2{}^* - {}^0\Delta G_2)$	$({}^4\Delta G_2{}^* - {}^4\Delta G_2)$
Kariya	α40 lys→glu	5.2	0.0
Tarrant	α126 asp→asn	4.3	−0.2
Hirose	β37 trp→ser	5.6	2.2
Creteil	β89 ser→asn	5.8	0.3
British Columbia	β101 glu→lys	2.8	2.1
Net destabilization		23.7	4.4
Native stabilization		−14.3	−8.0

*Denotes assembly free energies for abnormal hemoglobins. Values in kcal.

both perturbations are individually large, they are strikingly not coupled to each other.

Long-range cooperativity without hemesite ligation. A very different result is observed for unligated NES/des-arg hemoglobin. The measured energetic perturbation resulting from simultaneous modifications in the deoxy form of NES/des-arg differs by ~1.5 kcal from the sum of perturbations (7.0 kcal) produced by the same modifications when introduced separately (Table VI). Introduction of the second pair of modifications to either the pure NES or pure des arg molecules is energetically favorable compared to the additive case. The system thus exhibits long-range cooperativity among pairs of sites. The closest approach is 26 Å between the α carbons for β93 and either of the α141 residues in the oxy or deoxy tetramers. Why does the oxygenated species exhibit additivity, whereas the unligated species does not? A plausible explanation is that deoxy NES/des arg is predominantly in quaternary R, whereas the constituent singly modified NES and des-arg species are in quaternary T under these conditions.

A similar but reciprocal order of energetic effects is found with the carboxymethylated molecules. Here the deoxy species exhibit strict additivity between identical sets of modifications to both kinds of subunits while nonadditivity is shown by the corresponding oxygenated species. The distance between C_α of α and β subunits is 38 Å. In this system the 1.4 kcal of nonadditivity appears to be solely a manifestation of long-range coupling within quaternary R.

In a recent extension of the present work[17] hybridization and cryogenic separation techniques were used to measure nonadditivity between identical single amino acid modifications within the same tetramer. For example, with oxyhemoglobin St. Mandé the substitution asp→tyr at the second β102 site is energetically more favorable by 1.8 kcal than the chemically identical modification of the first β102 site 24 Å away (C_α to C_α). The observed energetic nonadditivity between these very distant sites is a dramatic example of the global character of molecular interactions linked to hemoglobin function. Other manifestations of this same global character appear to involve sites less remote. In hemoglobin St. Claude, the closest approach of side chains at the two α127 sites are within 12 Å. Here the second residue altered is 3.0 kcal more favorable than the first.[17]

Table VII gives free energy perturbations for an arbitrary set of modifications at sites that are sufficiently distant to avoid contact between side chains. Therefore the effects of local steric and other short range interactions should not exist. It is seen that the sum of destabilization free energies for the oxy species is approximately half that required to dissociate normal oxyhemoglobin into constituent dimers. For the unligated molecules the sum of destabilization energies is considerably larger than the 14.3 kcal for complete dissociation of the tetramers. The magnitude of the cumulative destabilization of the deoxy molecules thus suggests long range, or global coupling between the residue sites. Except for British Columbia, these values may be presumed to reflect a T→R transition term. If so, this term is threefold overcounted in the net summation of Table VII. The discrepancy between net destabilization and wild-type A_o assembly would then be accounted for by an average T→R energy of 3.1 kcal.

Residue Position vs. Chemical Nature

To explore whether the location or local chemistry of the perturbation determines functional response we consider sites where the effects of multiple alterations have been measured. These include positions: α95, α141, β1, β6, β37, β40, β93, β94, β97, β99, β101, β102, and β146. For sets of modifications at a given residue position within the $\alpha^1\beta^2$ interface two types of results are observed. For one, at certain sites the location of the structural modification solely dictates the functional response. These include positions β1, β40, β99, β97, and α141 where the responses for each modification are essentially identical. For example, the β99 mutants include charged (histidine), uncharged (glycine), nonpolar (valine), and polar (asparagine) side chains of wide-ranging size. Five of these hemoglobins respond identically to the replacement of aspartic acid at β99, i.e., ΔG_c is essentially zero (the sixth member of the set, Ypsilanti (β99 asp→tyr), exhibits radically different behavior). In the other type of result, for certain residue positions within $\alpha^1\beta^2$, the specific

side chain chemistry of the amino acid modification mediates the functional response. Hemoglobins modified at positions α95, β37, β93, and β101 show dramatic differences in ΔG_c depending on the side chain. For example, Rothschild (β37 trp→arg) and Hirose (β37 trp→ser) exhibit cooperative free energies that differ by nearly 5 kcal. No simple correlation exists between the predicted local chemistry changes and the exclusive involvement of either the deoxy or oxy tetrameric stabilities.

For those residues not in the $\alpha^1\beta^2$ interface, the local chemistry of the specific amino acid modification may have a less dramatic effect as discussed earlier.

More detailed dissection of the noncovalent bonding interactions responsible for the observed sensitivity of the $\alpha^1\beta^2$ interface may become possible with increased availability of high resolution structures. In order to make the most valid comparisons with solution properties, a series of low-salt crystallographic structures will be required. The current data base of functional energetics related to structural modification provides a unique framework for further analyses and modeling of fundamental issues in the structural chemistry of proteins and of their allosteric behavior.

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