The Oxygen-Binding Intermediates of Human Hemoglobin: Evaluation of Their Contributions to Cooperativity Using Zinc-Containing Hybrids

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ABSTRACT Hemoglobin tetramers [Zn/FeO_2] containing oxygenated subunits (FeO_2), in combination with unligated subunits containing zinc-substituted hemes (Zn), were analyzed to determine their contributions to the cooperativity of oxygen binding at the Fe sites. Energetic consequences of possible perturbation by zinc substitution were evaluated in all combinations of unligated Zn/Fe hybrid tetramers. A general thermodynamic strategy that corrects for the energetic effects of substituting a second metal for Fe showed the perturbations of Zn substitution to be negligible. This permitted cooperativity parameters of the native Fe/FeO_2 intermediates to be calculated from data on the corresponding Zn/FeO_2 molecules. These parameters, determined explicitly for all eight oxygen-binding intermediates (Fe/FeO_2), were found to be identical to those predicted earlier from analyzing the O_2 binding data of normal hemoglobin according to the "molecular code" of hemoglobin allostery. The cooperativity parameters determined for this system showed the same distribution pattern found previously for five other oxygen analog systems (Fe/FeCN, Fe/Mn^{3+}, Co/FeCO, Co/FeCN, and Fe/FeCO).

INTRODUCTION

Metal-substituted hemoglobins have been used extensively as models for binding and cooperativity of the native human protein (Hoffman and Petering, 1970; Yonetani et al., 1974; Imai et al., 1980; Simolo et al., 1985; Hofrichter et al., 1985; Speros et al., 1991; Doyle et al., 1991; Shibayama et al., 1995; Unzai et al., 1996; Huang et al., 1996; Huang and Ackers, 1996). These model systems are made by replacing the heme iron (Fe^{2+}) with another metal (e.g., Co^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}) that has a different ability to bind O_2 or its analogs, while mimicking structural properties of normal hemes. By hybridizing the metal-substituted subunits with normal Fe subunits, a set of "mixed metal" hybrid tetramers can be constructed (see Fig. 1 for the site configurations of such a system). Stable site configurations can be formed by binding ligands to the iron hemes of the mixed-metal tetramer when the metal-substituted sites do not bind oxygen or its analogous ligands (CO, CN, NO). These tetramers serve as models for the corresponding oxygenation intermediates of normal hemoglobin (Hb). An additional type of metal-substituted model system uses unligated Fe^{2+} subunits in combination with subunits containing metals that mimic oxygenated heme sites, e.g., Mn^{3+} (Smith et al., 1987) or Cr^{3+} (Unzai et al., 1996).

The use of mixed-metal hybrids to evaluate the cooperativity properties of Hb oxygen binding raises the question of whether metal substitution itself alters or masks the basic mechanism of cooperative ligand binding. To address this possibility, a strategy was recently developed for evaluating and "correcting" the energetic contributions arising from metal substitution to the apparent cooperativity parameters of mixed-metal hybrids (Huang and Ackers, 1996). Application of this strategy to the Co/FeCO ligation system revealed that cobalt (Co^{2+}) substitution at all four heme sites generates altered constraints within the tetramer (as shown previously by a 600-fold reduction in the dimer-tetramer association equilibrium constant; cf. Speros et al., 1991; Doyle et al., 1991). These perturbations originate specifically from Co substitution in the α subunits; they are transduced within the same dimeric half-tetramer to the β subunit and alter the free energies for binding to the non-substituted (Fe) sites. Using a rigorous method of correcting for these free energy perturbations of Co substitution, the contributions to cooperativity by the species of normal FeHb in all partial states of carbon monoxide binding were determined (Huang and Ackers, 1996). These results were consistent with direct CO binding isotherm data (Perrella et al., 1990b) and with population distributions that were partially resolved by low-temperature electrophoresis (Perrella and Denisov, 1995).

The present study has applied this strategy to determine the apparent cooperativity parameters for intermediates of the Zn/FeO_2 ligation system, i.e., with unligated subunits containing Zn-substituted hemes in each combinatorial arrangement with subunits containing normal hemes to which O_2 molecules are bound (FeO_2). The five-coordinate Zn^{2+} porphyrins employed in these hybrid tetramers are essentially isostructural with the five-coordinate, high-spin Fe^{2+} porphyrins of native deoxy hemoglobin (Scheidt and Reed, 1981). Because Zn^{2+} does not react with O_2, ligation of the iron subunits does not affect the ligation state of the Zn-substituted heme sites (cf. Simolo et al., 1985). This strategy also entails an analysis for any energetic effects of "pure" zinc substitution in all site configurations of Zn/Fe hybrid tetramers in the absence of bound ligand (O_2). By employ-

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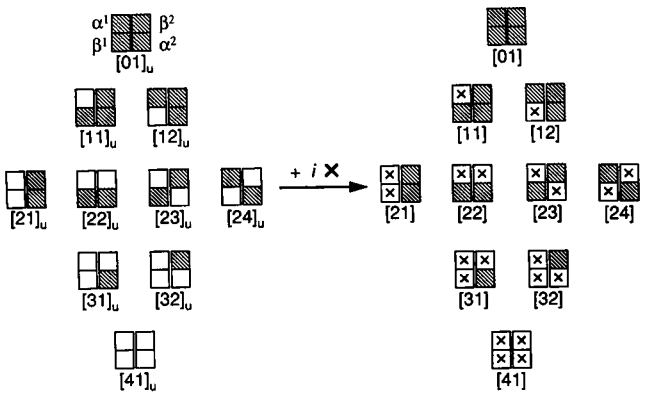

FIGURE 1 Topographic representation of unligated (M/Fe) and ligated (M/FeX) species for mixed-metal hemoglobin systems. Subunits within each tetramer are represented by squares with subunit arrangement as assigned in species $[01]_u$. Hemesite ligand is denoted by X. □, ⊠, Unligated and ligated Fe subunits, respectively. ▧, Subunits with ferrous hemes replaced by metal-substituted hemes that do not bind the ligand X. Each index $[ij]$ denotes the particular species j among those with i ligated iron sites (i = 0 to 4; j = 1 to 4) coexisting with 4-i metal-substituted subunits. Ordering of species with respect to j is arbitrary. Species $[ij]_u$ corresponds to species $[ij]$ with the Fe-bound ligand X removed.

ing the strategy for correcting any effects of metal substitution (Huang and Ackers, 1996), the measured apparent cooperativity parameters for the Zn/FeO_2 tetramers have yielded the true cooperativity parameters for oxygen-binding intermediates of the native Fe/FeO_2 system. These parameters are compared with data from direct oxygen binding measurements conducted under identical solution conditions.

Cooperative free energies

By using as a reference species the dissociated $\alpha\beta$ dimers that bind O_2 noncooperatively (Ackers and Johnson, 1990; Doyle and Ackers, 1992; Doyle et al., 1996), the energetic costs of tetrameric cooperativity are readily conceptualized as the difference between free energy of binding i oxygen molecules to the tetramer (in a specific configuration ij of reacted hemesites) and the free energy of binding the same sites within the ij-tetramer's dissociated half-molecules ($^{ij}\Delta G_x^D$):

$$^{ij}\Delta G_c = {}^{ij}\Delta G_x^T - {}^{ij}\Delta G_x^D. \quad (1)$$

Specifically, $^{ij}\Delta G_c = -RT\ln(^{ij}k_c)$, where $^{ij}k_c$ equals the binding constant (k_{ij}) for the formation of each species ij, from unligated tetrameric species [01], normalized to the intrinsic site values k_α and k_β:

$$^{ij}k_c = k_{ij}/(k_\alpha)^p(k_\beta)^q, \quad (2)$$

where p and q are the respective numbers of oxygenated α and β subunits. By path independence of free energy, the right side of Eq. 2 must also equal the equilibrium constant $^{ij}k_2$ for dimer-tetramer assembly of species ij, divided by the assembly constant $^{01}k_2$ of unligated Hb:

$$^{ij}k_c = {}^{ij}k_2/{}^{01}k_2. \quad (3)$$

Because of the thermodynamic cycle that couples the binding reactions and those of dimer-tetramer assembly, $^{ij}\Delta G_c$ may be measured as the difference between assembly free energies for the ligated and unligated tetrameric species (Ackers and Halvorson, 1974; Pettigrew et al., 1982; Smith and Ackers, 1985).

In the case of a mixed-metal hybrid, M/FeX, the apparent cooperative free energy ($^{ij}\Delta G_c^{M/FeX}$) for binding ligand X can be expressed as (Huang and Ackers, 1996)

$$^{ij}\Delta G_c^{M/FeX} = {}^{ij}\Delta G_2^{M/FeX} - {}^{ij}\Delta G_2^{M/Fe}. \quad (4)$$

Here $^{ij}\Delta G_2^{M/FeX}$ and $^{ij}\Delta G_2^{M/Fe}$ denote assembly free energies for the ligated M/FeX species $[ij]$ and the corresponding unligated M/Fe species $[ij]_u$, which has an identical configuration of Fe-containing subunits within the tetramer. For each of the eight ligated mixed-metal hybrid species $[ij]$, there is a configurationally identical unligated species $[ij]_u$ (see Fig. 1). The eight unligated mixed-metal tetramers thus differ by the number (1 to 3) and by the configuration of metal-substituted subunits. For normal FeHb, properties of the unligated species are always referenced to the deoxy species [01].

Thermodynamic linkages between ligand binding and perturbation by metal substitution

Because cooperativity arises from transduction of binding energy between heme sites, the structural and energetic perturbations of metal substitution at a given heme site may also propagate to the neighboring unmodified (Fe) hemes and thus alter ligand binding at those sites within the same tetramer. It is therefore necessary to quantitate the energetic contributions of metal substitution to accurately resolve the free energy effects that may be induced by ligand binding alone. This is especially important because the entire cooperativity effect of normal Hb entails a net free energy cost of only 4–7 kcal over the four oxygens bound (Chu and Ackers, 1981). For each mixed-metal hybrid M/Fe, it is useful to consider a "perturbation free energy" of metal substitution $^{ij}\Delta G_P^{M/Fe}$, defined as the energetic cost of replacing Fe by M at a particular set of heme sites within the tetramer, relative to that of similar replacement in the dissociated dimers (Huang and Ackers, 1996). Utilizing free energy linkages between the dimer-tetramer assembly reactions and the metal-substitution reactions, each value of $^{ij}\Delta G_P^{M/Fe}$ may be determined as

$$^{ij}\Delta G_P^{M/Fe} = {}^{ij}\Delta G_2^{M/Fe} - {}^{01}\Delta G_2^{Fe/Fe}, \quad (5)$$

where $^{ij}\Delta G_2^{M/Fe}$ is the measured assembly free energy for species $[ij]_u$. $^{ij}\Delta G_P^{M/Fe}$ measures the free energy of structural perturbation induced by metal substitution within the tetramer relative to that for dimers similarly undergoing metal

substitution. Likewise, for each mixed-metal species with bound ligand M/FeX, the perturbation free energy is measured by the difference between assembly free energies of the M/FeX and Fe/FeX species:

$$^{ij}\Delta G_P^{M/FeX} = {}^{ij}\Delta G_2^{M/FeX} - {}^{ij}\Delta G_2^{Fe/FeX}. \quad (6)$$

The perturbation free energies and cooperative free energies for a mixed-metal system M/FeX and for normal FeHb (Fe/FeX) satisfy a thermodynamic scheme such as that given in Fig. 2 for each species *ij* (illustrated for species [31]). Based solely on the principle of free energy conservation, the following relationship holds for each of the nine ligated species [*ij*] (for derivation, see Huang and Ackers, 1996):

$$^{ij}\Delta G_c^{Fe/FeX} = {}^{ij}\Delta G_c^{M/FeX} + ({}^{ij}\Delta G_P^{M/Fe} - {}^{ij}\Delta G_P^{M/FeX}). \quad (7)$$

This equation expresses the relationships between energetic contributions of metal substitution and ligand binding. It provides a rigorous and model-independent basis for translating the apparent cooperative free energies of a mixed-metal system into parameters of native FeHb.

METHODS AND RESULTS

Hemoglobin preparations

HbA_o and HbS preparations were based on the method of Williams and Tsay (1973). ZnHb was prepared by the method of Scholler et al. (1978). Zn^{2+} porphyrin IX used for reconstitution of ZnHb was obtained from Porphyrin Products (Logan, Utah). Species [23] and [24] of the Zn/FeO_2 system were kindly provided by B. Hoffman (Kuila et al., 1991). Buffer exchanges were carried out on all Hb samples to a standard buffer consisting of 0.1 M Tris-HCl, 0.1 M NaCl (0.18 M total chloride), and 1 mM EDTA, pH 7.4, at 21.5°C. All experiments with Zn-substituted hemoglobins were carried out in the absence of light.

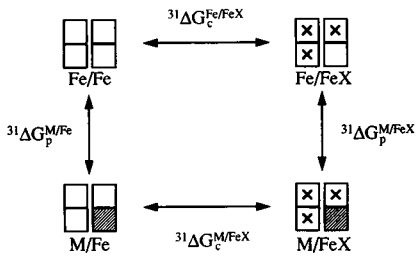

FIGURE 2 Thermodynamic relationships between metal substitution and ligation on FeHb and M/Fe hybrids, illustrated for species [31]. □, ▨, ☒, Unligated Fe, metal-substituted, and ligated Fe subunits, respectively. Cooperative free energies for ligand binding (left to right) equal the differences between assembly free energies of ligated and unligated species. Perturbation free energies for metal substitution (top to bottom) are measured by the differences between assembly free energies of metal-iron hybrids and normal iron hemoglobin with or without ligand X. A similar scheme may be constructed for each of the eight other ligation species.

Hybrid tetramers

Six of the 10 ligation species of the Zn/FeO_2 hemoglobin system ([11], [12], [21], [22], [31], and [32]) were studied in hybrid mixtures (see Fig. 3 for construction of hybrids). For example, species [11] $(\alpha_{FeO2}\beta_{Zn})(\alpha_{Zn}\beta_{Zn})$ is prepared by mixing parent species [01] $(\alpha_{Zn}\beta_{Zn})_2$ and [23] $(\alpha_{FeO2}\beta_{Zn})_2$. The two parents undergo a net dimer rearrangement via the dissociation and reassembly reactions, yielding species [11] as a hybrid tetramer in equilibrium with the two original parent species. To form a specific hybrid [*ij*], its two parent species were mixed and incubated at 21.5°C. Equilibrium incubation times ranged from a few minutes (for species [22], [31], and [32]) to 10–24 h (for species [11], [12], and [21]). To generate the six unligated Zn/Fe hybrid species ($[11]_u$, $[12]_u$, $[21]_u$, $[22]_u$, $[31]_u$, and $[32]_u$) the appropriate deoxygenated parent species (ZnHb, FeHb, Zn/Fe $[23]_u$, or Zn/Fe $[24]_u$) were mixed and incubated for 24–72 h at 21.5°C under anaerobic conditions. Anaerobicity was maintained by the addition of either 0.1% dithionite or an enzymatic system containing 1.8 mg/ml glucose oxidase (Sigma), 0.3 mg/ml *Aspergillus niger* catalase (Sigma), and 0.6% glucose (Sigma). Samples were enclosed in septum-sealed vials, which were individually immersed in 0.1% sodium dithionite solution within a crimp-sealed serum vial.

Analytical gel chromatography

The dimer-tetramer assembly equilibrium constants ($^{ij}k_2$) for Zn/FeO_2 species [23], [24], [22], and fully ligated normal Hb (species [41]) were determined by analytical gel chromatography (Ackers, 1970; Turner et al., 1982). Large-zone experiments were conducted with a G-100 Sephadex column at several plateau concentrations (C_t) of each protein species. Centroid boundary positions, determined from

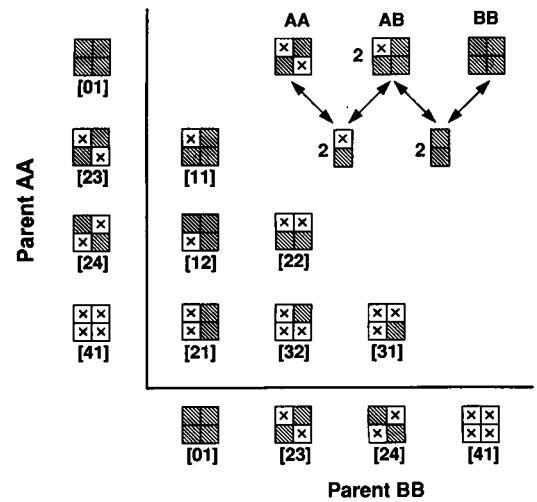

FIGURE 3 Hybridization scheme for construction of the six asymmetric tetramer species by combination of dimers from the two parent molecules AA and BB. Hybridization occurs through dissociation and reassociation of dimers (*upper right*). Species designations are as given in Fig. 1.

the large-zone elution profiles, were used along with void and internal volumes of the column to calculate weight-averaged partition coefficients, $\bar{\sigma}_w$ (Ackers, 1970). These coefficients are related to the dimer-tetramer equilibrium constant ${}^{ij}k_2$ by Eq. 8:

$$\bar{\sigma}_w = f_D\sigma_2 + (1 - f_D)\sigma_4 \tag{8a}$$

where

$$f_D = \frac{-1 + \sqrt{1 + 4C_t{}^{ij}k_2}}{2{}^{ij}k_2C_t}. \tag{8b}$$

Here f_D is fraction dimer, and σ_2 and σ_4 are partition coefficients for dimers and tetramers, respectively. Resolving the assembly equilibrium constant requires knowing the partition coefficients of pure dimeric and tetrameric molecules. Our approach involved measuring the elution profiles of normal oxy Hb (species [41]) as a function of protein concentration over the range 0.03–100 μM heme. Elution profiles of species [23] and [24] were then obtained at two or more Hb concentrations for each species. The data, after conversion to weight-averaged partition coefficients, were analyzed by global regression on Eqs. 8a and 8b with σ_2, σ_4, and ${}^{ij}k_2$ as fitting parameters, to resolve dimer-tetramer equilibrium constants for the Zn/FeO_2 species [23] and [24] as well as for oxy FeHb.

The determined ${}^{41}\Delta G_2$ for oxy Hb was -8.11 ± 0.14, in good agreement with -8.05 ± 0.1 kcal, measured under conditions identical to those of the present study (Chu et al., 1984). Species [22] was studied in a hybrid equilibrium mixture of species [23] and [24]. Elution profiles of this mixture were found to behave according to a dimer-tetramer stoichiometry as judged by individual large-zone profiles and by consistency with shapes of best-fit curves based on Eq. 8a. The best-fit equilibrium constant did not differ significantly from those of the parent species [23] and [24], indicating that the assembly reaction free energy for species [22] is closely similar to those of the parent species. Therefore, the determined equilibrium constant was also applicable for species [22]. A cryogenic isoelectric focusing experiment (discussed below) was also carried out with the [22] hybrid mixture. Separation of tetrameric species in the mixture was limited by the small pI difference (0.05 pH) between species [23] and [24]. Nevertheless, three species were clearly discernible in the gel tube, providing evidence for the presence of the species [22] hybrid (data not shown).

Dissociation rate constants

Tetramer-dimer dissociation rate constants were determined using the haptoglobin (Hp) kinetics technique (Ip et al., 1976). Mixing 50% molar excess of Hp with Hb results in a rapid, essentially irreversible binding of the Hb dimers to the Hp molecule and, consequently, a net dissociation of the Hb tetramer. Because the rate of forming haptoglobin-hemoglobin complex is much faster than that of tetramer-dimer dissociation, the latter is rate limiting, leading to a pseudo-first-order kinetic process, with the rate constant depending solely on tetramer dissociation. For unligated Zn/Fe species ($[11]_u$, $[12]_u$, $[22]_u$, $[23]_u$, and $[24]_u$) the standard buffer was deoxygenated for 24 h in a Coy anaerobic chamber with O_2 partial pressure maintained below the 1 ppm detection limit. Concentrated samples of Hb and haptoglobin (1 mM) were deoxygenated under a flow of humidified N_2 at 4°C for 1 h before dilution into the buffer. To ensure complete removal of O_2, 0.1% dithionite was added to each sample. The samples were then sealed in a split cell cuvette to maintain anaerobicity. Reaction between Hp and Hb was initiated by manual mixing. Net Hb dissociation was followed spectrophotometrically at 420 nm and $T = 21.5 \pm 0.1$°C, except as indicated. For species $[23]_u$ and $[24]_u$, the dissociation rate data were fit to single exponential functions to resolve the rate constants. For species $[11]_u$, $[12]_u$, and $[22]_u$ of the Zn/Fe system and species [11] and [12] of the Zn/FeO_2 system, the hybrid mixtures were incubated at 21.5°C for at least 24 h to populate the hybrid species before the Hp kinetics experiments (see Fig. 3 for hybrid construction). Upon mixing with haptoglobin, independent dissociation of the hybrid and the parent species leads to Eq. 9, by which the data were analyzed:

$$A = A_\infty + P_1e^{-k_{r1}t} + P_2e^{-k_{r2}t} + P_3e^{-k_{r3}t}. \tag{9}$$

Here, P_i is the "extent" of a dissociation phase, k_{ri} is the associated first-order rate constant, A is measured absorbance, and t is time. A_∞ is absorbance at the completion of dissociation reactions.

For the Zn/Fe species $[23]_u$ and $[24]_u$, dissociation kinetics were found, by using nonlinear regression, to be well characterized by a single exponential, resulting in dissociation rate constants of $1.8(\pm0.2) \times 10^{-5}$ s^{-1} and $2.0(\pm0.2) \times 10^{-5}$ s^{-1}, respectively. For hybrid species $[11]_u$, $[12]_u$, and $[22]_u$ of the Zn/Fe system, the Hp kinetics experiments were carried out by reacting each equilibrated hybrid mixture with haptoglobin at 21.5°C and monitoring the reaction for 70 h. Data for each mixture were analyzed by both single- and double-exponential functions (see Eq. 9). It was found that for each hybrid mixture a single-exponential function described the data well (cf. Fig. 4 *A* for data of the $[11]_u$ hybrid mixture). No significant improvement was found by using a double-exponential fit, as judged by variance of fit and randomness of residuals. A likely explanation of these results is that dissociation rates of all three tetramers in each hybrid mixture are indistinguishable, giving rise to a single apparent first-order kinetic phase. The existence of hybrids in these mixtures was confirmed, however, by cryogenic isoelectric focusing (LiCata et al., 1990), which partially separated the tetrameric species in each mixture. The resulting apparent first-order rate constants, i.e., $2.3(\pm0.5) \times 10^{-5}$, $1.9(\pm0.5) \times 10^{-5}$, and $2.2(\pm0.5) \times 10^{-5}$ s^{-1}, were assigned to species $[11]_u$, $[12]_u$, and $[22]_u$, respectively.

For hybrid species [11] and [12] of the Zn/FeO_2 system, the dissociation of tetrameric species in hybrid mixture was

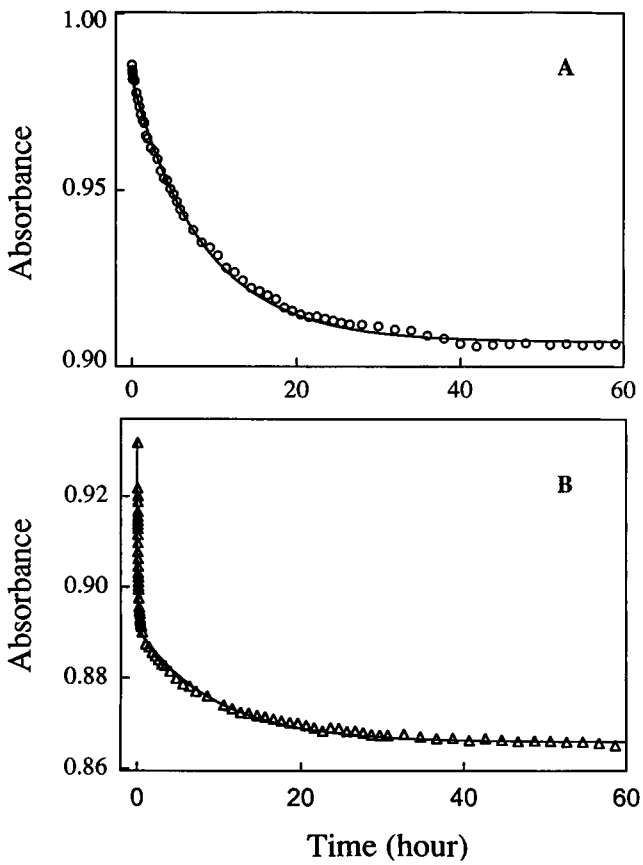

FIGURE 4 Tetramer-dimer dissociation kinetics of the Zn/Fe and Zn/FeO_2 hybrid species by the haptoglobin method. (*A*) ○, Time course of dissociation reactions for $[11]_u$ hybrid mixture containing Zn/Fe species $[11]_u$, $[23]_u$, and [01] (ZnHb). Solid curve depicts the best fit to a single-exponential. (*B*) △, Time course of dissociation for species [11] hybrid mixture containing Zn/FeO_2 species [11], [23], and [01]. Solid curve depicts best fit to a two-exponential process (see text). Hybridization reactions in both experiments proceeded 48 h at 21.5°C before quenching with haptoglobin. Solution conditions: 0.1 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 7.4, at 21.5°C. The unligated hybrid mixture contained 0.1% sodium dithionite.

monitored at 420 nm or 415 nm. These data were analyzed using single-, double-, and triple-exponential functions (Eq. 9). The best single-exponential fit failed to account for the data, resulting in systematic residuals and unreasonably large variance. Use of the two-exponential function substantially improved both the variance of fit and distribution of residuals. No further improvement in goodness of fit was attained from three exponentials. Fig. 4 *B* shows the data and best-fit curve from the two-exponential function for the species [11] hybrid mixture. Resolved apparent rate constants for the slow phase of the [11] and [12] mixtures were statistically identical to the dissociation rate constant of pure ZnHb [$k_r = 2.4(\pm 0.4) \times 10^{-5}\ s^{-1}$]. This value was therefore assigned to the ZnHb component in the hybrid mixture. For hybrid mixtures [11] and [12], the fast phase rates were found to be $2.7 \times 10^{-3}\ s^{-1}$ and $2.6 \times 10^{-3}\ s^{-1}$, respectively. To assign this phase unambiguously, we first estimated the dissociation rate constants for species [23] and [24] from their assembly free energies (discussed below) and the consensus assembly rate constant ($1.1 \times 10^6\ M^{-1}\ s^{-1}$; cf. Turner et al., 1992, and the following section of this work). Estimated rate constants for species [23] and [24] were 2.5 and 1.5 s^{-1}, respectively, differing significantly from the determined rate of the fast phase for the [11] or [12] mixtures. Therefore, the rate constant of the fast phase was assigned to species [11] or [12] for each mixture.

Equilibrium constants for dimer-tetramer assembly were evaluated as ratios of the respective association (k_f) and dissociation (k_r) rate constants. Association rates have previously been determined for normal hemoglobin over a wide range of conditions and for a large number of structurally altered hemoglobins, including mutant, chemically modified, and cobalt-substituted species (Pettigrew et al., 1982; Turner et al., 1992; Doyle et al., 1991). A value of $1.1(\pm 0.3) \times 10^6\ M^{-1}\ s^{-1}$ was found in all cases under solution conditions identical to the present study. This "consensus value" has therefore been employed for the other metal-substituted hemoglobins and partially ligated species to evaluate their equilibrium constants ($^{ij}k_2$) and corresponding assembly free energy ($^{ij}\Delta G_2 = -RT \ln {}^{ij}k_2$).

Cryogenic isoelectric focusing

In a hybrid mixture each of the three tetrameric species assembles according to its own dimer-tetramer free energy. The hybridization equilibrium can be analyzed in terms of the "deviation free energy" δ, which quantifies the difference between hybrid assembly free energy ($^{AB}\Delta G_2$) and the mean of assembly free energies for the two parent species AA and BB (LiCata et al., 1990):

$$\delta = {}^{AB}\Delta G_2 - \frac{1}{2}({}^{AA}\Delta G_2 + {}^{BB}\Delta G_2). \tag{10}$$

The deviation free energy δ is calculated directly from the experimentally measured equilibrium fractions of hybrid (f_{AB}) and parent species (f_{AA} and f_{BB}):

$$\delta = -RT \ln \frac{f_{AB}}{2\sqrt{f_{AA} f_{BB}}}. \tag{11}$$

For hybrid species [31] and [32] of the Zn/FeO_2 system and for the unligated species $[31]_u$ and $[32]_u$ of the Zn/Fe system, equilibrium fractions of the three tetrameric species were obtained by a cryogenic isoelectric focusing (cryo-IEF) protocol (LiCata et al., 1990), which was based on the pioneering work of Perrella (cf. Perrella and Rossi-Bernardi, 1981; Perrella and Denisov, 1995). An equilibrated hybridization reaction was first quenched rapidly by mixing 10 μl of the hybrid mixture with 150 μl of −30°C quench solution containing 50% ethylene glycol and 50% standard buffer. The mixture was then loaded onto a (prefocused) isoelectric focusing gel tube that was maintained at −25°C. The sample was focused for approximately 24 h, and the tetrameric species were separated according to their differences in isoelectric point. Finally, the gel tube was optically

scanned and the relative populations determined by integration of the species' absorbance peaks.

The approach to equilibrium of the hybrid mixture and its maintenance were verified by assaying the distribution of tetrameric species as a function of hybrid incubation time. To facilitate electrophoretic resolution, one of the parent species in each mixture was prepared using HbS (β6 Glu→Val), on which the mutation site is located at remote proximity from the dimer-dimer interface. Additional controls indicated no effect of this mutation on resolved energetics (LiCata et al., 1990). Total hemoglobin concentration during incubation of the hybrid mixture was approximately 1 mM in heme.

For hybrid species $[21]_u$ and [21], it was found earlier that our standard cryo-IEF procedure failed to detect the presence of these species in their respective mixtures (Huang et al., 1996). In the present study it was found that the absence of detectable species $[21]_u$ and [21] in such experiments resulted from an inability to quench the tetramer-dimer dissociation reaction of the species $[21]_u$ and [21] at the temperature (−25°C) where hybrids were normally separated from their respective parent species by isoelectric focusing. The origin of this "quenching instability" of the Zn/Fe hybrid species $[21]_u$ and of the Zn/FeO_2 species [21] at low temperature is not presently known. However, to further stabilize the hybrid species $[21]_u$ during cryo-IEF, the running temperature was lowered to the range −37°C to −40°C. In addition, sample quenching and loading were carried out anaerobically by the addition of 0.1% dithionite in the quench buffer, and the sample was focused for 3 days. Using this procedure, it was possible to separate species $[21]_u$ from its parent species before significant tetramer-dimer dissociation had taken place (see Fig. 5 *A*). For the Zn/FeO_2 species [21], cryo-IEF was likewise carried out at −37°C to −40°C and yielded 3–5% hybrid species (see Fig. 5 *B*). Because species [21] is expected to have a higher tendency to dissociate into dimers than the unliganded species $[21]_u$, it is not clear that the detected population of species [21] represents the true equilibrium abundance of the hybrid species. The IEF temperature was decreased even more in an attempt to further stabilize species [21]. However, at −45°C, the focusing time required for adequate separations increased to 6–7 days, resulting in gradual loss of hybrid during the isoelectric focusing process. Thus, although these experiments provided positive identification of the species [21] hybrid, it was necessary to employ a different strategy (described in the next section) for evaluating its cooperative free energy.

Assembly free energies of the Zn/FeO_2 tetramers

Assembly free energies ($^{ij}\Delta G_2$) for species [01] (ZnHb) and for the singly oxygenated species [11] and [12] were resolved by determining tetramer dissociation rates by the Hp kinetics technique and combining this value with the consensus "on" rate (Turner et al., 1992). For species [22], [23],

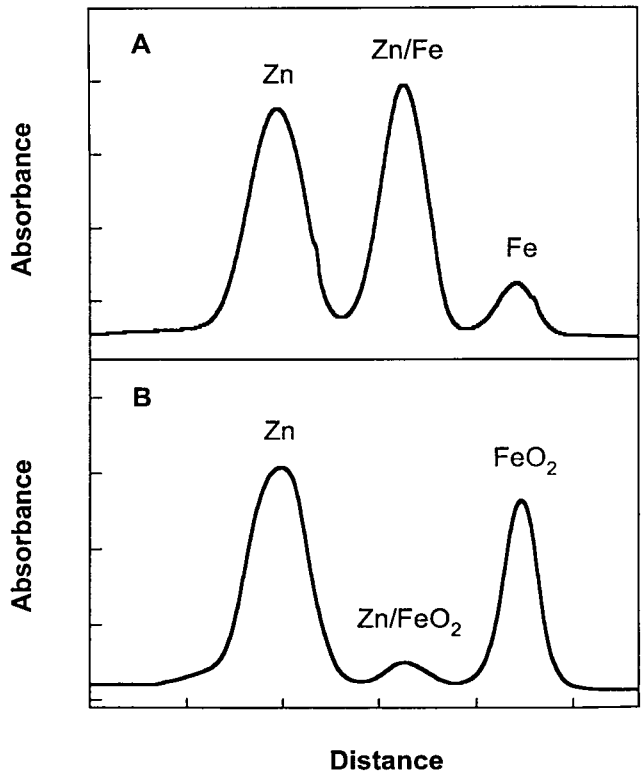

FIGURE 5 Population distribution of tetrameric species in hybrid mixtures resolved by a modified cryo-IEF method (see Materials and Methods). Solution conditions are as in Fig. 4. (*A*) Hybrid mixture of deoxy FeHbS and Zn-substituted HbA_0. (*B*) Hybrid mixture of Zn-substituted HbA_0 and oxy FeHbS.

[24], and [41], the $^{ij}\Delta G_2$ values were found by analytical gel chromatography, whereas species [21], [31], and [32] were analyzed by quantitative cryo-IEF. Results are given in Table 1. The determined $^{01}\Delta G_2$ for pure ZnHb was identical to that of normal FeHb (Huang et al., 1996), and the resolved $^{41}\Delta G_2$ (i.e., containing four O_2-bound Fe hemes) was identical to previous determinations (e.g., Ip and Ackers, 1977; Doyle et al., 1996).

Hybrid species [21] in this system was recently studied (Huang et al., 1996) by a standard cryo-IEF protocol (Li-

TABLE 1 Assembly free energies and cooperative free energies of the zinc/iron hybrid hemoglobin system

Species *ij*	$^{ij}\Delta G_2^{Zn/FeO_2}$	$^{ij}\Delta G_2^{Zn/Fe}$	$^{ij}\Delta G_c^{Zn/FeO_2}$
[01]	−14.4 ± 0.2	−14.4 ± 0.2	0
[11]	−11.6 ± 0.3	−14.4 ± 0.3	2.8 ± 0.3
[12]	−11.6 ± 0.3	−14.5 ± 0.3	2.8 ± 0.3
[21]	−9.4 ± 0.8	−14.4 ± 0.2	5.0 ± 0.8
[22]	−7.7 ± 0.4	−14.4 ± 0.3	6.7 ± 0.4
[23]	−7.6 ± 0.1	−14.5 ± 0.1	6.8 ± 0.2
[24]	−7.9 ± 0.1	−14.4 ± 0.2	6.5 ± 0.2
[31]	−7.5 ± 0.3	−14.1 ± 0.2	6.9 ± 0.3
[32]	−7.5 ± 0.3	−14.3 ± 0.2	6.9 ± 0.3
[41]	−8.1 ± 0.10	−14.4 ± 0.2	6.3 ± 0.2

Solution conditions: 0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4, 21.5°C.

Cata et al., 1990), which did not reveal a significant presence of the hybrid. In the present study it was found that the absence of species [21] in those experiments is a result of the inability to completely quench the tetramer-dimer dissociation of the species [21] at the −25°C conditions, even though this hybrid was separated from its parent species [01] and [41]. However, subsequent studies have revealed that by lowering the temperature to the range −37°C to −40°C, it was possible to clearly detect species [21] using the cryo-IEF method, verifying that species [21] had indeed been formed in the hybrid mixture at 21.5°C. Nevertheless, in spite of this positive verification of species [21] formation, uncertainties in the relative abundance of the species [21] tetramer could not be ruled out in this experiment, even at this lowest temperature range. The assembly free energy for this species, given in Table 1, was therefore determined by using a different approach (see Cooperative Free Energies of O_2 Binding Intermediates in Normal FeHb).

Assembly free energies for the Zn/Fe tetramers

Dimer to tetramer free energies for unligated species $[23]_u$, $[24]_u$, $[11]_u$, $[12]_u$, and $[22]_u$ of the Zn/Fe system were resolved by haptoglobin kinetics, in combination with the consensus association rate constant (Turner et al., 1992); these are given in Table 1. Assembly free energies of species $[21]_u$, $[32]_u$, and $[31]_u$ were determined by the cryo-IEF method. Species $[21]_u$ was formed by hybridizing ZnHbA with deoxy FeHbS, and $[32]_u$ and $[31]_u$ were formed upon mixing deoxy FeHbS with Zn/Fe species $[23]_u$ and $[24]_u$ of HbA, respectively. These hybridization reactions reached equilibrium in approximately 50 h at 21.5°C. Assembly free energies of species $[21]_u$, $[32]_u$, and $[31]_u$ were evaluated from Eqs. 10 and 11 in combination with assembly free energies for the corresponding parent species. Resulting values are also given in Table 1. As found with the species [21] hybrid, the $[21]_u$ hybrid showed uncertain stability in cryo-IEF experiments at the lowest temperature range and thus required a different procedure for quenching and separation (see Cryogenic Isoelectric Focusing).

Apparent cooperative free energies of Zn/FeO_2 intermediates

For each species $[ij]$ of the Zn/FeO_2 system, the apparent cooperative free energy (${}^{ij}\Delta G_c^{Zn/FeO_2}$) for binding O_2 to the unmodified Fe subunits was evaluated as the difference between the assembly free energy of this species and that of the corresponding unligated Zn/Fe species $[ij]_u$ (Eq. 4). The ${}^{ij}\Delta G_c^{Zn/FeO_2}$ value measures the "energetic penalty" for O_2 binding to Fe subunits of the Zn/Fe tetramer having site occupancy ij relative to binding to the constituent dimers (Eq. 1). It should be noted that the unligated constituent dimer for species [21] is identical to that of the dimers from the pure deoxy species [01], i.e., it contains only native Fe hemes.

As shown in Table 1, the assembly free energies of all Zn/Fe hybrid species fall within the narrow range −14.1 to −14.5 kcal (Table 1) and are statistically indistinguishable from that of normal FeHb (−14.35 ± 0.2 kcal), indicating no significant energetic perturbation by Zn substitution to the unligated tetramers. Using the mean value (−14.4 ± 0.2 kcal) obtained for species of the unligated Zn/Fe system, the apparent cooperative free energy for O_2 binding to form each Zn/FeO_2 species $[ij]$ was evaluated by Eq. 4 from its unligated counterpart Zn/Fe. These cooperative free energies are also listed in Table 1.

Cooperative free energies of the O_2-binding intermediates of normal FeHb

The relative contributions to hemoglobin cooperativity by the eight O_2-binding intermediates had not been experimentally resolved before the present study. This has been accomplished by using the thermodynamic method summarized in Fig. 2. Transformation of cooperative free energies from the Zn/FeO_2 species to the Fe/FeO_2 species required evaluating the energetic perturbation (ΔG_P) induced by Zn substitution on dimer-tetramer assembly of the Zn/Fe species and of the Zn/FeO_2 species. Perturbation free energies (${}^{ij}\Delta G_P^{Zn/Fe}$) for the unligated Zn/Fe species $[ij]_u$ were each evaluated, using Eq. 5 as the difference between assembly free energies of each Zn/Fe species and that of normal deoxy FeHb. The finding that assembly free energies of all Zn/Fe hybrids are identical to those of normal deoxy FeHb indicated the absence of any significant perturbation by Zn substitution (${}^{ij}\Delta G_P^{Zn/Fe} = 0$ kcal). Although ${}^{ij}\Delta G_P^{Zn/FeO_2}$ for the Zn/FeO_2 species cannot be determined experimentally, the previous study of metal substitutional effects in the Co/FeCN system (Huang and Ackers, 1996) showed clearly that perturbations of the energetics of ligand binding can result from the propagation of energetic constraints induced by metal substitution to the neighboring Fe subunits. The complete absence of energetic perturbation by Zn substitution is not surprising, given that the Zn^{2+} porphyrins are essentially isostructural with the deoxy Fe^{2+} porphyrins of native deoxy Hb (Scheidt and Reed, 1981). These stereochemical features of the Zn substitution and our experimental finding of no Zn perturbation argue strongly that O_2 binding to the neighboring iron subunits of these hybrids is also unlikely to be altered by the Zn substitution. It is therefore reasonable to assume that ${}^{ij}\Delta G_P^{Zn/FeO_2}$ also has a value of essentially zero for each species $[ij]$. Then, according to Eq. 7, the cooperative free energy ${}^{ij}\Delta G_c^{Zn/FeO_2}$ for O_2 binding to native FeHb is equal to the determined ${}^{ij}\Delta G_P^{Zn/FeO_2}$ for each of the corresponding Zn/FeO_2 species $[ij]$.

Among the eight Zn/FeO_2 hybrids, only species [21] showed an apparent dynamic instability with respect to tetramer-dimer dissociation during the cryo-IEF experiments. This made it more difficult to accurately determine the assembly free energies (and cooperative free energy) for

this species and hence reduced the final accuracy of values for the Fe/FeO_2 species [21]. However, because the presence of Zn/FeO_2 species [21] was positively identified by the cryo-IEF experiment, a more accurate evaluation of the free energy $^{21}\Delta G_c^{Fe/FeO2}$ for Fe/FeO_2 species [21] was justified by using the following approach. Direct oxygen binding studies, in combination with independent measurements of dimer-tetramer assembly free energy for deoxy and fully oxygenated FeHb, had been conducted previously to resolve the stoichiometric cooperative free energies ($^{-i}\Delta G_c$) for the reaction of tetramers with each number i of oxygenated hemes (Mills et al., 1976; Mills and Ackers, 1979; Chu et al., 1984; Doyle et al., 1996). For the four doubly ligated species, the stoichiometrically averaged cooperative free energy $^{-2}\Delta G_c$ has been reliably found in several studies (cited above) to be 5.6 ± 0.7 kcal under conditions identical to those of the present study. The $^{-2}\Delta G_c$ value is a composite average of the site-specific equilibrium constants of oxygenation cooperativity ($^{ij}k_c$) for the four doubly oxygenated species (Ackers et al., 1992; Doyle and Ackers, 1992):

$$\overline{^{2}\Delta G_c} = -RT \ln\left[\frac{1}{3}(^{21}k_c) + \frac{1}{3}(^{22}k_c) + \frac{1}{6}(^{23}k_c) + \frac{1}{6}(^{24}k_c)\right]. \quad (12)$$

Using the microstate cooperative free energies $^{ij}\Delta G_c$ for the Fe/FeO_2 species [22], [23], and [24] (Table 3) that were obtained from data of the present study, we calculated the corresponding cooperativity constants $^{ij}k_c = \exp[-^{ij}\Delta G_c/RT]$. These microstate $^{ij}k_c$ values, along with $\overline{^{-2}\Delta G_c}$, determined from the stoichiometrically resolved oxygenation linkage system (cf. Doyle and Ackers, 1992; Doyle et al., 1996), yielded the value of $^{21}k_c$ by Eq. 12. The resulting cooperative free energy $^{21}\Delta G_c$ for species [21] of the Fe/FeO_2 was thus found to be 5.0 ± 0.8 kcal, and the species [21] assembly free energy was estimated to be −9.4 ± 0.8 by using the independently determined $^{01}\Delta G_2 = -14.4 \pm 0.2$ kcal (Ip and Ackers, 1977; Doyle et al., 1996).

The complete distribution of cooperative free energies for all O_2-binding species of normal FeHb is given in Table 3. For comparison, the cooperative free energies recently resolved for CO binding to normal FeHb (Huang and Ackers, 1996) are also listed.

DISCUSSION

Comparison with direct O_2 binding results

High-precision oxygen binding isotherms of normal FeHb have been studied extensively under solution conditions identical to the present work (e.g., Mills et al., 1976; Mills and Ackers, 1979; Chu et al., 1984; Doyle et al., 1996). The populations of tetramers with each stoichiometric number of heme sites occupied and the corresponding free energies of binding have been resolved from the analysis of concentration-dependent binding isotherms measured using the technique pioneered by Imai (cf. Imai et al., 1980), in combination with independently measured energetics of dimer-tetramer assembly (Ackers, 1970). However, in the absence of methods capable of distinguishing the configurational isomers at each degree of ligation, it has not been possible to determine explicitly the population distributions or the binding constants for the eight oxygenation intermediates. This limitation is inherent in all techniques that measure the fractional saturation of O_2 binding sites without resolving the configurational isomers, and in techniques that measure dimer-tetramer assembly parameters without differentiating the configurational isomers. However, an accurate set of cooperativity parameters, determined at the stoichiometric level of resolution, can provide important constraints when analyzed in conjunction with a corresponding set of microstate parameters. Such is the case here.

Each set of cooperative free energies (or assembly energies) resolved for the 10 oxygenation microstates may be translated into the stoichiometric (Adair) binding constants, K_{4i}, and related parameters that are obtainable from O_2 binding isotherms. For each stoichiometric degree i of binding, the Adair equilibrium binding constant K_{4i} may be written:

$$K_{4i} = \sum_{(ij)} g_{ij}(k_\alpha)^p(k_\beta)^q \exp(-^{ij}\Delta G_c/RT). \quad (13)$$

The right-hand sum of Eq. 13 is over all configurational isomers (ij) that have i sites bound, and the g_{ij} values are their statistical degeneracies.

The binding partition function for tetramers Z_4 may thus be written:

$$Z_4 = 1 + K_{41}(O_2) + K_{42}(O_2)^2 + K_{43}(O_2)^3 + K_{44}(O_2)^4, \quad (14)$$

and the binding isotherm $\bar{Y}_4$ is

$$\bar{Y}_4 = \frac{1}{4}\frac{d \ln Z_4}{d \ln(O_2)}. \quad (15)$$

The fractional population f_{4i} of bound sites among microstate species with i sites occupied is

$$f_{4i} = \frac{iK_{4i}(O_2)^i}{4\sum_{i=0}^{4}K_{4i}(O_2)^i}. \quad (16)$$

Finally, the Hill coefficient (n_H) reflecting the statistical variance in the population of ligated tetramers (cf. Edsall and Gutfreund, 1973) may be calculated from the binding isotherm $\bar{Y}_4$ as

$$n_H = \frac{4[\overline{\bar{Y}_4^2} - (\bar{Y}_4)^2]}{\bar{Y}_4(1 - \bar{Y}_4)}. \quad (17)$$

To examine the consistency of the present results with comparable solution studies of oxygen binding, we calculated the population (f_{4i}) of tetrameric species at each degree of oxygenation as a function of the overall fractional saturation ($\bar{Y}$), based on the cooperative free energies determined in this study and the experimentally measured dimer binding free energy of −8.35 kcal (Chu et al., 1984). The results are shown in Fig. 6. Maximum populations of singly, doubly, and triply ligated species were predicted to be 21%, 3.5%, and 4.5%,

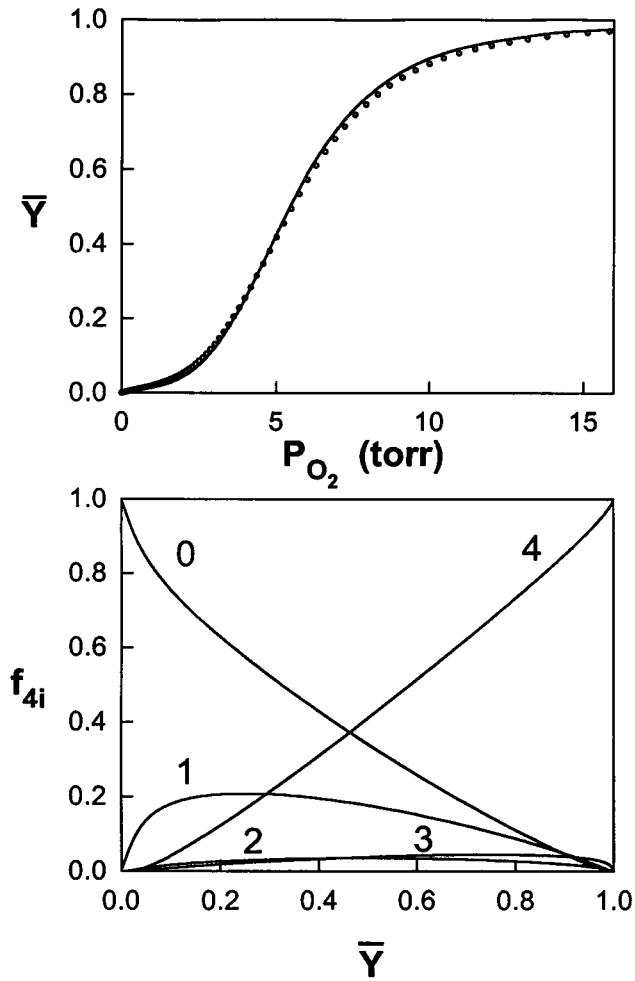

FIGURE 6 (*Top*) Oxygenation isotherm of normal hemoglobin at pH 7.4. O, Values calculated using stoichiometric binding free energies from Chu et al. (1984). Solid curve calculated from partition function (Eq. 18) with cooperative free energies of the 10 oxygenation species (Table 3) and intrinsic oxygen binding free energy (for dissociated dimer). (*Bottom*) Fractional population map of stoichiometric ligation states as a function of fractional saturation of O_2 to normal FeHb. Calculated values are based on the determined cooperative free energies of the 10 oxygenation species (Table 3).

respectively. These calculated populations and their variation with $\bar{Y}$ are in close agreement with the distributions resolved from direct oxygen binding isotherms measured under conditions identical to those of the present study (Chu et al., 1984; Doyle et al., 1996). A binding isotherm calculated from the microstate cooperative free energies of Table 3 and the intrinsic binding free energy of −8.35 (Fig. 6) is identical within experimental uncertainty to the tetrameric isotherm measured in oxygen binding studies (Chu et al., 1984; Doyle et al., 1996) under identical experimental conditions (Fig. 6). The maximum Hill coefficient (n_H) was calculated to be 3.3 and is statistically indistinguishable from that reported from direct oxygen binding data (e.g., Chu et al., 1984; Doyle et al., 1996).

Stoichiometric values of cooperative free energy ${}^{i}\Delta G_c$ were previously found to be 2.9 ± 0.2, 5.6 ± 0.7, 7.0 ± 0.6, and 6.2 ± 0.1 kcal for the averages of singly, doubly, triply, and fully ligated species, respectively, under the conditions of the present study (Mills et al., 1976; Chu et al., 1984; Doyle et al., 1996). Each value represents a statistical average over the relevant set of configurational isomers. The cooperative free energy of each oxygenation species was previously estimated from these average values (Ackers et al., 1992) based on the following assumptions: 1) both singly ligated species have the same ΔG_c; 2) both triply ligated species have the same ΔG_c; 3) species [22], [23], and [24] have a common ΔG_c value which also equals that of the triply ligated tetramers. With the last assumption, the cooperative free energy for species [21] may be calculated (Eq. 12) from the experimental parameters. These values and those estimated previously for the 10 oxygenation species (Ackers et al., 1992) are compared in Table 3. It is clear that, within experimental error, the ${}^{ij}\Delta G_c$ value for each oxygenation species estimated from fitting the "symmetry rule" partition function to oxygen binding data agrees well with that resolved independently in the present study. The assumptions used previously for estimating ${}^{ij}\Delta G_c$ from O_2 binding data are thus strongly supported by these results. The explicitly resolved experimental distribution of ${}^{ij}\Delta G_c$ for the O_2 binding microstates is found to conform to the symmetry rule mechanism that was proposed several years ago to account for hemoglobin allostery (Ackers et al., 1992).

Comparison between different oxygenation analog systems

Five ligation analog systems have previously been resolved under the same experimental conditions as the present study. Assembly free energies are given in Table 2 for these systems under identical solution conditions, along with the results of this study for the Zn/FeO_2 system. It is seen that the magnitudes of assembly free energies for the 10 ligation species are system dependent. Nevertheless, the six systems studied to date exhibit striking common features: 1) binding the first ligand generates unfavorable free energy, which destabilizes the tetramer relative to its constituent dimers; 2) species [21] occupies a different free energy level relative to the other doubly ligated species; 3) species [22], [23], and [24] are substantially destabilized compared to either of the singly ligated species or to species [21] (these three configurational isomers bearing two ligands occupy the same level as the two triply ligated species); 4) the last ligation step may generate an increased stabilization of the tetramer, compared with the triply ligated species. It has been found that this effect may become very large under low-salt conditions (Doyle et al., 1996).

The common distribution patterns observed for assembly free energies of the 10 ligation species have indicated a common mechanism for the pathways of ligand binding-induced changes in tertiary subunit conformation and quaternary interactions that are well known to drive hemoglobin cooperativity (Perutz, 1970). To relate the energetic distributions to a molecular mechanism for O_2 binding of

TABLE 2 Assembly free energy distributions of hemoglobin ligation analog systems

Species	Zn/FeO_2*	Co/FeCO#	Co/FeCN§	Fe/Mn^{3+}¶	Fe/FeCN‖	Fe/FeCO§
[01]	−14.4 ± 0.2	−10.6 ± 0.1	−10.6 ± 0.1	−14.4 ± 0.2	−14.4 ± 0.2	−14.4 ± 0.2
[11]	−11.6 ± 0.3	−9.0 ± 0.2	−8.9 ± 0.1	−11.5 ± 0.3	−11.3 ± 0.2	−11.1 ± 0.2
[12]	−11.6 ± 0.3	−8.4 ± 0.2	−8.5 ± 0.1	−10.7 ± 0.3	−11.1 ± 0.2	−11.0 ± 0.3
[21]	−9.4 ± 0.8	−8.3 ± 0.2	−9.0 ± 0.2	−10.8 ± 0.3	−11.3 ± 0.2	−10.2 ± 0.3
[22]	−7.7 ± 0.4	−7.4 ± 0.2	−7.5 ± 0.1	−7.8 ± 0.3	−8.0 ± 0.2	−7.9 ± 0.3
[23]	−7.6 ± 0.2	−7.5 ± 0.2	−7.7 ± 0.1	−7.6 ± 0.3	−8.3 ± 0.2	−7.8 ± 0.3
[24]	−7.8 ± 0.2	−7.4 ± 0.2	−7.5 ± 0.1	−8.2 ± 0.3	−8.0 ± 0.2	−7.9 ± 0.3
[31]	−7.5 ± 0.3	−7.7 ± 0.2	−7.7 ± 0.1	−7.9 ± 0.3	−8.1 ± 0.2	−7.9 ± 0.3
[32]	−7.5 ± 0.3	−7.6 ± 0.2	−7.9 ± 0.2	−7.9 ± 0.3	−8.2 ± 0.2	−7.8 ± 0.3
[41]	−8.1 ± 0.1	−8.0 ± 0.1	−8.3 ± 0.1	−7.5 ± 0.3	−8.3 ± 0.1	−8.0 ± 0.1

Solution conditions: 0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, pH 7.4, 21.5°C.
* This study.
# Speros et al. (1991).
§ Huang and Ackers (1996).
¶ Smith et al. (1987), Ackers (1990).
‖ Smith and Ackers (1985), Perrella et al. (1990a), Huang and Ackers (1995).

normal hemoglobin, it must be recognized that structural modification by metal substitution at the heme could in principle generate energetic perturbations on ligand binding. This phenomenon has recently been documented in detail (Huang and Ackers, 1996). Such perturbations are evident in Table 2 from variations in the apparent overall cooperative free energies ($^{41}\Delta G_c = {}^{41}\Delta G_2 - {}^{01}\Delta G_2$) among the various ligation systems, ranging from 6.4 kcal for Zn/FeO_2 to 2.3 kcal for Co/FeCN under conditions of the present study. A general strategy for thermodynamic dissection of the metal substitutional effects in each metal/iron hybrid species was previously applied to the Co/FeCO system and led to the resolution of cooperative free energies for all species of CO binding to the normal FeHb molecule (Huang and Ackers, 1996). In the present study this same method of "correcting" the cooperative free energies from metal/iron hybrid species to those of normal FeHb has been applied to the Zn/FeO_2 system. To resolve the cooperative free energies of O_2 binding to normal FeHb, the Zn/FeO_2 system was chosen because it appeared to show the smallest energetic effects of metal substitution (Huang et al., 1996) and was expected to resemble most closely the Fe/FeO_2 system. In principle, any structural modification of the Hb tetramer, such as covalent cross-linking, may be expected to cause perturbations to mechanistic behavior. In minor cases, such as the systems of Table 2, the perturbations will alter distributions of functional parameters in a quantitative (but not qualitative) fashion. The comparisons presented in Table 2 document the importance of addressing this issue in any proposed model system.

Cooperative mechanism of hemoglobin oxygenation

The mechanism of hemoglobin cooperativity is manifested as the thermodynamic coupling between ligation and structural transitions (tertiary and quaternary). Physiologically the loading and unloading of oxygen closely follow the equilibrium binding isotherm. This isotherm is a direct reflection of the cooperative free energy distribution determined in the present study. Its resolution was thus essential for understanding the detailed nature of couplings throughout the 16 reactions of the ligand binding cascade (Huang and Ackers, 1995). The determined distributions for O_2 binding as well as CO binding (Huang and Ackers, 1996) to unmodified FeHb have revealed the following features:

1) The two singly ligated species exhibit the same cooperative free energy, which accounts for approximately half of the total.

2) The two triply ligated species show identical cooperative free energies.

3) Dramatic splitting is seen in the distribution of doubly ligated tetramers: species [21] exhibits significantly lower cooperative free energy than the other three doubly ligated species, [22], [23], and [24]. This finding indicates that binding the second O_2 to the same dimeric half-tetramer with which the first O_2 reacted is enhanced by 2.2 kcal, which corresponds to a 40-fold higher affinity for the second binding step compared with the first. This cooperativity within the $\alpha^1\beta^1$ dimeric half-tetramer is found in all ligation systems of Table 2. Its value ranges from 3.1 kcal (170-fold) for Fe/FeCN to 0.5 kcal (2.5-fold) for Co/FeCO.

4) Species [22], [23], and [24] showed free energies identical to those of the two triply ligated tetramers.

5) The final ligation may generate a significant quaternary enhancement effect (e.g., binding the last ligand to the tetramer is more favorable than similar binding to the dissociated $\alpha\beta$ dimer), as reflected in the reduction in cooperative free energy at the final step.

It is notable that the intrinsic affinity for CO binding is more than 300-fold higher than for O_2, corresponding to a 3.4-kcal increase in ligation free energy, whereas the two systems show remarkably similar distributions of cooperative free energies (Table 3). The entire range for free energy regulation of cooperativity (6.3 kcal) is essentially identical for O_2 binding, CO binding, and CN-met ligation. We believe that this arises as the

TABLE 3 Cooperative free energy distributions of carbon monoxide and oxygen ligation of iron hemoglobin

Species	Fe/FeCO*	Fe/FeO_2#	Fe/FeO_2§
[01]	0	0	0
[11]	3.3 ± 0.2	2.8 ± 0.3	2.9 ± 0.2
[12]	3.4 ± 0.3	2.8 ± 0.3	2.9 ± 0.2
[21]	4.2 ± 0.3	5.0 ± 0.8	5.0 ± 0.8
[22]	6.5 ± 0.3	6.7 ± 0.4	7.0 ± 0.6
[23]	6.6 ± 0.3	6.8 ± 0.2	7.0 ± 0.6
[24]	6.5 ± 0.3	6.6 ± 0.2	7.0 ± 0.6
[31]	6.5 ± 0.3	6.9 ± 0.3	7.0 ± 0.6
[32]	6.6 ± 0.2	6.9 ± 0.3	7.0 ± 0.6
[41]	6.3 ± 0.1	6.3 ± 0.1	6.2 ± 0.1

Solution conditions as in Table 1.

* Huang and Ackers (1996).

This study. The $^{ij}\Delta G_c$ values are evaluated based on Eq. 5 and data from the Zn/FeO_2 system.

§ Predictions based on O_2 binding data. See text for detail.

difference between net energies of the T and R interfacial bonds and is thus largely independent of which heme site ligand has bound. When crystal lattices of deoxy Hb prevent this transition (and the tertiary transitions), as in the oxygenation studies of Mozzarelli and co-workers (1991), it is not surprising that the crystals bind noncooperatively with extremely low affinity and show no Bohr effect. These fascinating observations cannot be used to prove an absence of cooperativity within quaternary T molecules under solution conditions (i.e., where the tetramers do exhibit cooperativity, Bohr proton release, and normal O_2 affinity), contrary to recent assertions by Eaton and co-workers (Rivetti et al., 1993; Bettati et al., 1996).

A molecular partition function describing the equilibrium processes of ligand binding and the cooperative structural transitions in solution was proposed (Ackers et al., 1992) on the basis of the first three systems (Table 2) that were resolved energetically (Fe/FeCN, Fe/Mn^{3+}, and Co/FeCO), in combination with quaternary structural assignments from a variety of probes:

$$\begin{aligned} Z_4(X) = 1 &+ 2K_{tc}(k_\alpha + k_\beta)X + (2k_\alpha k_\beta K_{tc}^{21} \\ &+ 2k_\alpha k_\beta K_c K'_{tc} + k_\alpha{}^2 K_c K'_{tc} + k_\beta{}^2 K_c K'_{tc})X^2 \\ &+ 2K_c K'_{tc}(k_\alpha k_\beta{}^2 + k_\alpha{}^2 k_\beta)X^3 \\ &+ k_\alpha{}^2 k_\beta{}^2 K_c X^4. \end{aligned} \quad (18)$$

Here K_α and K_β are intrinsic binding constants for the α and β subunits within the tetramer. K_{tc} is a "tertiary constraint" equilibrium constant for the first ligation (i.e., which produces a ligated tertiary structure within the quaternary T tetramer). Similarly, K_{tc}^{21} characterizes a tertiary conformational change that accompanies the formation of species [21] from species [01] within the T quaternary structure; K'_{tc} reflects the energetic constraint arising from an unligated subunit's tertiary structure within an "oxy" quaternary structure R or R2 (e.g., upon dissociation of an O_2 from fully ligated Hb). This accounts for the quaternary enhancement effect when K'_{tc} is less than unity. K_c reflects the overall cost of cooperative interactions for complete oxygenation of the tetramer.

The molecular partition function, Eq. 18, was initially formulated on the basis of 1) the discovery that the detailed energetic distributions resolved for the first three ligation systems (Fe/FeCN, Fe/Mn^{3+}, Co/FeCO; Table 2) showed a common and highly specific pattern; 2) assignments of ligation intermediates of the Fe/FeCN system to the T or R quaternary structures using a series of structure-sensitive probes (LiCata et al., 1993; Daugherty et al., 1994; Doyle and Ackers, 1992; Huang and Ackers, 1995). In fact, all of the intermediate state ligation systems resolved to date (Table 2) conform to the symmetry rule partition function, Eq. 18. Most significantly, the distribution of $^{ij}\Delta G_c$ values for O_2 binding by normal Hb conform to this partition function. The ability to predict common thermodynamic relationships between the various states of diverse ligation systems (including O_2 binding to normal Hb) verifies that basic rules of the hemoglobin mechanism are encoded by this partition function, eq. 18 and that the perturbations generated by the oxygen analogs of Table 2 produce changes only in the relative magnitudes of the effects, without altering fundamental mechanistic rules.

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