Experimental Resolution of Cooperative Free Energies for the Ten Ligation Species of Cobalt(II)/Iron(II)-CO Hemoglobin†

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Received February 21, 1991; Revised Manuscript Received April 9, 1991

ABSTRACT: Cooperative free energies have been determined for the 10 ligation species of human hemoglobin in the Co(II)/Fe(II)-CO system. In this system, subunits containing unligated cobaltous hemes coexist in the same tetramer with naturally occurring ferrous hemes that are ligated with carbon monoxide. Tetramers comprising the 10 structurally unique combinations of ligated and unligated subunits were characterized in terms of their dimer-tetramer assembly free energies. By use of the thermodynamic linkage between assembly and ligation, the experimentally resolved values were used to obtain the corresponding cooperative free energies (i.e., the differences between actual free energies of ligation and the summed contributions of intrinsic values). The results obtained are in general accord with previous findings on this same system (Imai et al., 1980). The present study extends this earlier work by resolving the cooperative properties of each configurational isomer of the doubly ligated tetramers. The 10 ligation species were found to distribute into 5 discrete cooperative free energy levels according to a combinatorial code which includes, as a special case, the code found previously with cyanomethemoglobin and manganese-substituted hemoglobin (Smith et al., 1987; Daugherty et al., 1991). This distribution exhibits additional characteristics found in the oxygenation of normal ferrous hemoglobin including the quaternary enhancement effect (Mills & Ackers, 1979a,b). These results, and those of the following paper (Doyle et al., 1991), strongly support the premise that a common set of qualitative rules governs the cooperative interactions in hemoglobin irrespective of the metal carried by the heme and the ligands bound to it.

Human hemoglobin has long served as a prototype for attempts to understand regulatory interactions in complex multisubunit protein systems. Cooperative interactions among the four heme sites of this tetrameric molecule depend upon the energetic coupling among multiple combinations of tertiary and quaternary structural transitions. During the overall ligand binding process, the molecule assumes 10 structurally unique forms (Figure 1). These ligation species differ in the number and configuration of ligands bound at the four heme sites as indicated by the topographic diagrams. For each species the cooperative free energy is the deviation in free energy of ligation (relative to the fully unligated species, designated "01") from that which would be obtained for the same sites binding as independent α and β subunits. Since dissociation of tetramers into dimeric "half-molecules" eliminates the cooperative intersubunit interactions, ligation of the α and β subunits within the dissociated $\alpha^1\beta^1$ dimers provides a useful reference reaction for assessing these intrinsic values (Ackers & Halvorson, 1974; Mills & Ackers, 1979a).

Present experimental methods cannot distinguish oxygenation at each specific site within the tetramer so that individual-site binding isotherms (Ackers et al., 1983) are not obtainable. Furthermore, oxygen moves from site to site with such lability that the isolation and study of each partially oxygenated tetramer has not been possible. Only the overall oxygenation isotherms are usually attainable, and therefore only macroscopic binding constants reflecting averages of the microscopic properties have generally been resolvable.

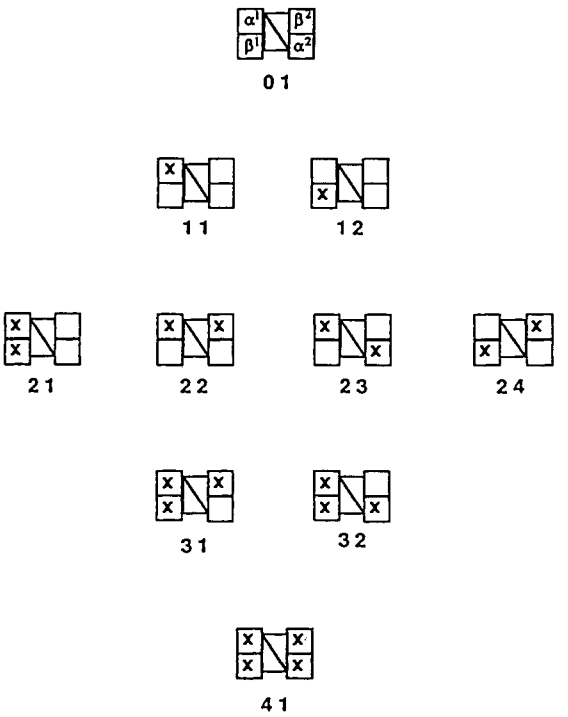

FIGURE 1: Topographic diagrams of the 10 microscopic ligation species of hemoglobin (microstates). In this study, unligated cobalt-substituted heme sites (open squares) coexist in the same tetramer with naturally occurring iron sites ligated with carbon monoxide (×), designated as the Co(II)/Fe(II)-CO system.

A strategy for partially circumventing this problem was developed by Yonetani and colleagues using cobalt-iron hybrid tetramers (Imai et al., 1980). Oxygen equilibrium curves were determined for $\alpha(Fe)_2\beta(Co)_2$ and the complementary α-

† This work was supported by NIH Grants R37-GM24486 (G.K.A.) and HL14508 (T.Y.) and NSF Grants DMB 86-15497 (G.K.A.) and DMB 87-6796 (T.Y.).

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0006-2960/91/0430-7254$02.50/0

$(Co)_2\beta(Fe)_2$ tetramers. Taking advantage of the different spectral changes for the normal and cobalt-substituted hemes, it was possible to measure a separate oxygen binding isotherm for each pair of hemes in each hybrid tetramer. By combining these data with oxygenation measurements on the "homogeneous" tetramers $\alpha(Co)_2\beta(Co)_2$ and $\alpha(Fe)_2\beta(Fe)_2$, it was possible to resolve a set of 12 microscopic equilibrium constants under the assumptions that (a) the subunit interactions responsible for cooperativity depend on their ligation state, but not the kind of metal carried by them, and (b) the two asymmetric configurations of doubly ligated tetramers (designated as species 21 and 22 in Figure 1) can be resolved as an average. Cobalt-substituted hemoglobin binds oxygen reversibly, cooperatively, and with full response to allosteric affectors (Hsu et al., 1972; Yonetani et al., 1974; Imai et al., 1977, 1980; Doyle et al., 1991; Ikeda-Sato & Yonetani, 1980; Ikeda-Sato & Verzili, 1981). Cobaltous hemoglobin has been characterized by X-ray crystallography (Fermi & Perutz, 1982), CD spectroscopy (Snyder & Chien, 1979), and EPR spectroscopy (Hoffman & Petering, 1970; Ikedo-Saito et al., 1974; Inubishi & Yonetani, 1983; Inubishi et al., 1983). It has been found to comprise a good structural analogue of normal hemoglobin in its respective deoxy and oxy forms. These studies have provided considerable evidence that the allosteric mechanism of cobalt hemoglobin and cobalt–iron hybrids closely resembles that of iron hemoglobin in qualitative behavior even though the ranges and magnitudes of functional parameters are quantitatively different.

A second approach to the energetics of hemoglobin cooperativity is based on the assembly reactions of $\alpha^1\beta^1$ dimers into tetramers at different stages of ligation. The assembly reactions for each ligation species (Figure 1) are thermodynamically linked to the ligation processes which both undergo. An extensive series of studies have accurately resolved the macroscopic binding constants for the tetrameric and dimeric species, as well as the equilibrium constants for assembly of partially oxygenated tetramers under a variety of conditions (Mills et al., 1976; Mills & Ackers, 1979a,b; Atha et al., 1979; Chu et al., 1984; Doyle et al., 1991). These studies have been particularly valuable in defining the roles of intersubunit contacts in the cooperative mechanism.

Recent extensions of this conceptual approach, based on the thermodynamics of ligand-linked subunit assembly, have made possible, for the first time, the study of cooperativity in all 10 microstates of human hemoglobin. In the first system to which this approach was applied (cyanomethemoglobin), ligated sites contain cyanide bound to ferric hemes, and ferrous heme sites comprise the unligated sites. [This is represented as Fe(II)/Fe(III)–CN, a nomenclature used throughout this paper to abbreviate the various ligation systems.] In this system the dimer–tetramer assembly free energies of all species have been resolved experimentally by using a combination of kinetic and equilibrium techniques (Smith & Ackers, 1985; Perrella et al., 1990; Daugherty et al., 1991). The assembly free energy $^{ij}\Delta G_2$ for species ij (Figure 1) is related to the cooperative free energy $^{ij}\Delta G_c$ by the definition:

$$^{ij}\Delta G_c = {}^{ij}\Delta G_2 - {}^{01}\Delta G_2 \quad (1)$$

where $^{01}\Delta G_2$ is the free energy of the reference reaction, i.e., for assembly of unligated dimers into tetramers. The experimentally resolved distribution of cooperative energies for cyanomethemoglobin is shown in Figure 2 at pH 7.4 and 21.5 °C. The species were found to distribute into three discrete levels. Doubly ligated species occupy two different cooperative free energy levels, with species 21 in the intermediate level and the remainder of the doubly ligated species in the third, fully

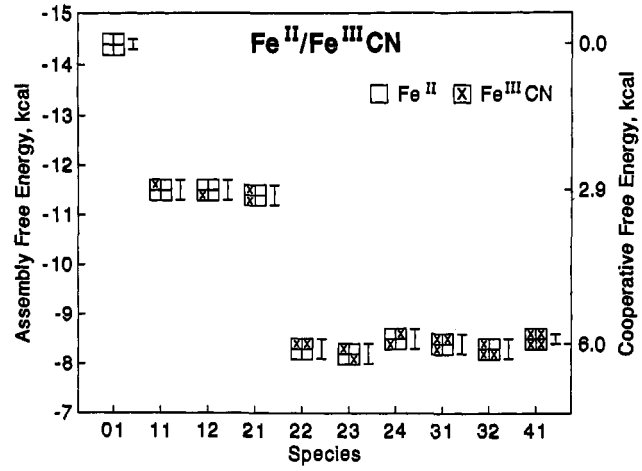

FIGURE 2: Cooperative free energy levels for the 10 microstates of the Fe(II)/Fe(III)–CN (cyanomet) system (Smith & Ackers, 1985; Perrella et al., 1990; Daugherty et al., 1991). The species are distributed among three evenly spaced levels according to a combinatorial code—i.e., the level for a given species depends not only on the number of ligands bound but also on the configuration of ligated sites. Two other ligand analogue systems, Fe(II)/Mn(III) and Mn(II)/Fe(II)–CO (not shown), exhibit similar spacing and distribution under the same conditions: pH 7.4, 21.5 °C, 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA (Smith et al., 1987). The spacing between cooperative free energy levels can vary dramatically with pH (Daugherty et al., 1991).

switched, level. This feature, and the actual spacing of the energy levels, rules out the two-state MWC mechanism (Monod et al., 1965) which requires that cooperative free energies depend solely on the number, and not the configuration, of bound ligands [for a rigorous analysis of this problem see Ackers (1990)]. These findings were in accord with subsequent experimental results on the Fe(II)/Mn(III) and Mn(II)/Fe(II)–CO ligand analogue systems (Smith et al., 1987), indicating that the combinatorial nature of the cooperative mechanism is not a special feature of cyanomethemoglobin.

An important step in understanding the molecular nature of the cooperative free energy transitions for partially ligated hemoglobins was the identification of the intermediate cooperativity state represented by species 21, Figure 2. Does this species represent a third quaternary structure, i.e., a dimer–dimer interface distinctly different from those of the two end states T and R? Or is the third structure an R or T quaternary tetramer with sufficient tertiary structure alteration to generate the 3 kcal of cooperative free energy? Recently, this problem has been solved by a combination of studies on the effects of pH, temperature, and single-site mutations on the energetics of quaternary assembly (Daugherty et al., 1991). The results of these studies indicate clearly that the intermediate allosteric tetramer has the deoxy (T) quaternary structure. This assignment, together with the observed distribution of the 10 microstate species (Figure 2), revealed a *symmetry rule* for quaternary switching, i.e., *switching from T to R occurs whenever a binding step creates a tetramer with one or more ligated subunits on each side of the $\alpha^1\beta^2$ intersubunit contact.* A further consequence of the cyanomet results has been the finding of substantial cooperativity within the $\alpha^1\beta^1$ dimer of the T-state tetramers. At pH 7.4, the ligand-induced tertiary free energy alters binding affinity within the T structure by 170-fold prior to quaternary switching (Daugherty et al., 1991).

In order to obtain a more complete understanding of the cooperativity states that are accessible to tetrameric hemoglobin when perturbed by multiple ligand induced tertiary

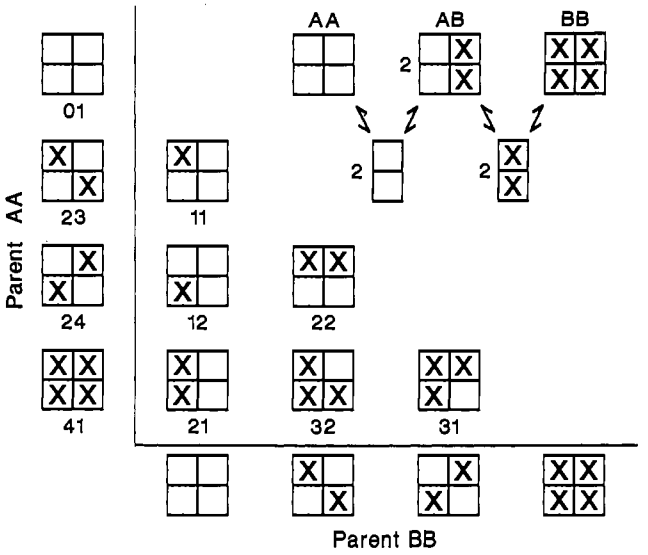

FIGURE 3: Hybridization of tetrameric hemoglobin molecules. Parent species are depicted topographically on left and bottom, while their respective hybrids are shown at the corresponding interior cross-coordinates.

structure changes, we have carried out studies, reported in this paper, using the Co(II)/Fe(II)–CO system. Since cobalt-substituted sites do not bind carbon monoxide (Hoffman & Petering, 1970), they have been studied in combination with carbon monoxide saturated (naturally occurring) iron sites. In this combination of sites, the Co(II)/Fe(II)–CO system provides a number of desirable characteristics. It is feasible to study the energetics of cooperative interactions for each species in isolation, or in a simple hybrid mixture as shown in Figure 3. The cobalt sites which serve as the unligated sites in this system are also known to be capable of participating in cooperative interactions in their binding of oxygen (Yonetani et al., 1974; Imai et al., 1977; Doyle et al., 1991). In the work described here cobalt-substituted sites were always unligated and cooperativity has been studied with the set of 10 tetrameric species in which cobaltous hemes are "replaced" in all possible combinations by ferrous hemes ligated with carbon monoxide.

Materials and Methods

All experiments were carried out in standard buffer consisting of 0.1 M Tris-HCl, 0.1 M NaCl (0.18 M total chloride), and 1 mM EDTA, pH 7.40, at 21.5 °C. Human haptoglobin was purified by the method of Connell and Shaw (1961; Ip et al., 1976). Phosphate-free hemoglobin A_0 was prepared by the method of Williams and Tsay (1973). Cobaltous hemoglobin and symmetric Co(II)/Fe(II) hybrids (i.e., species 23 and 24, Figure 1) were prepared in the laboratory of T. Yonetani as described previously (Yonetani et al., 1974). Sample purity was determined by polyacrylamide gel–isoelectric focusing. In all cases in which the cobalt hemoglobin was compared directly with samples prepared in a different laboratory, the results were identical. Hemoglobin samples were changed into the standard buffer by dialysis or by passage over a G-25 column equilibrated with standard buffer. The resulting solutions were shaken gently under constantly refreshed hydrated carbon monoxide, so that all cobalt sites were deoxygenated, and all iron sites were saturated with carbon monoxide. Progress of deoxygenation was complete in 45 min, as monitored spectrally between 390 and 430 nm. These solutions were placed in a glove bag (I^2R Inc., Cheltenham, PA) under nitrogen. Sodium dithionite (Virginia Chemicals) was added to the solutions to make them 0.1% (w/w). Haptoglobin solutions were treated in the same manner as those of hemoglobin to generate solutions of identical dissolved gas and sodium dithionite concentration. Data analysis was by nonlinear least-squares methods (Johnson et al., 1976) to determine best estimates of the parameters, goodness of fit, and 67% confidence limits.

Anaerobic Gel Permeation Chromatography. The equilibrium distribution of hemoglobin tetramers and dimers was determined as a function of concentration by anaerobic gel permeation chromatography, permitting determination of the equilibrium constant and assembly free energy (Turner et al., 1982). The apparatus employed utilized an LKB peristaltic pump, a Shimadzu spectrophotometer, operating at 401 nm with 5-nm band-pass, and an oxygen electrode (Beckman). The column bed was Sephadex G-100 resin (Pharmacia) (1-cm diameter) with porous polyethylene disks at the top and bottom. Data were collected by means of a Hewlett-Packard digital multimeter controlled by a Hewlett-Packard 150C microcomputer.

Large-zone experiments were carried out (Ackers & Thompson, 1965), and the centroid boundaries were constructed, defining the weight-average elution volume of each hemoglobin solution at its respective plateau concentration C_t. The weight average partition coefficients, σ_w, were determined from these values and the fixed volume constants of the column (Ackers, 1970). Elution behavior of the large-zone centroid depends on the equilibrium constant for dimer–tetramer assembly, K_2, the total concentration of hemoglobin, C_t, and the partition coefficients of dimers, σ_2, and tetramers, σ_4, according to (Valdes & Ackers, 1977)

$$\sigma_\omega = \frac{m_2\sigma_2 + 2K_2(m_2)\sigma_4}{C_t} \qquad (2)$$

where

$$m_2 = \frac{-1 + \sqrt{1 + 8C_tK_2}}{4K_2}$$

For each hemoglobin solution of different plateau concentration C_t, a different dimer concentration, m_2, applies. In practice, a number of large-zone samples are run on the column at different plateau concentrations, C_t, and the centroid elution volume (yielding σ_w) is determined for each. The best fit of eq 2 to the resulting values of σ_w vs C_t allows simultaneous estimation of K_2, σ_2, and σ_4.

In order to determine the assembly free energies of species 23 and 24, the cobalt sites must be free of oxygen, while maintaining saturation of the iron sites with carbon monoxide. This anaerobic and carbonmonoxy-saturated environment was attained by bubbling carbon monoxide through the column buffer, prior to entering the column. In addition, 0.1% (w/w) dithionite was added as an oxygen scavenger. Samples were prepared under carbon monoxide as described above. Sodium dithionite (Virginia Chemicals) was added to make the sample 0.1% (w/w). The samples were loaded into the column by means of an apparatus described elsewhere (M. C. Farmer and G. K. Ackers, in preparation). The anaerobic status of the column was verified in two ways: (1) an in-line oxygen electrode was used to monitor oxygen pressure in the column effluent; (2) hemoglobin spectra were measured during the plateau segment of the large-zone experiments.

Dissociation Kinetics Obtained by the Haptoglobin Technique. The haptoglobin technique (Ip et al., 1976) determines the dissociation rate of tetramers into dimers. Mixing of hemoglobin with haptoglobin results in a rapid and essentially irreversible binding of hemoglobin dimers. When the rate of

haptoglobin–dimer reaction is fast relative to the tetramer-to-dimer dissociation rate, the dissociation of tetramers is rate limiting, and pseudo-first-order kinetics arise. Subsequent progress of the reaction therefore depends only upon the dissociation rate of tetramers, since the binding of the dimers by haptoglobin is irreversible. These pseudo-first-order kinetic processes define the following relationship, by which the data were analyzed (Turner et al., 1982):

$$A = A_\infty + P_1 e^{-k_1 t} + P_2 e^{-k_2 t} + \ldots \quad (3)$$

where P_n is the preexponential or "extent" of a phase, k_n is the first-order rate constant, A is absorbance, and t is time. The first term, A_∞, corresponds to final equilibrium. The reactions were started by rapid mixing in a stopped-flow spectrophotometer (Gibson-Durrum or Biologic/Molecular Kinetics) which had been purged of oxygen by repeated washing with 0.1% sodium dithionite solutions. Dissociation of tetramers into dimers was monitored by absorbance changes at 401 nm. Data were analyzed by eq 3.

Dissociation Kinetis of Hybrid Mixtures. The kinetic experiments involving species 01 in isolation are described elsewhere (Speros et al., 1990; Doyle et al., 1991).

(*A*) *Species 11.* Oxygenated cobaltous hemoglobin was mixed with species 23, resulting in three tetrameric species: the two pure parent species, and hybrid species 11 composed of a dimer from each parent molecule. The mixture was gently shaken under hydrated carbon monoxide, which was constantly refreshed. This accomplished two things: all cobalt sites were deoxygenated, and all iron sites were saturated with carbon monoxide, which was monitored spectrally. The samples were rapidly mixed and absorbance was monitored at 401 nm. The resulting data set was analyzed by nonlinear least-squares parameter estimation to the rate eq 3. As a control for this experiment, species 23 was reacted with haptoglobin and absorbance was monitored at 401 nm.

(*B*) *Species 12* was studied in analogous fashion to that for species 11, with the hybrid mixture composed of species 01, 12, and 24 (see Figure 3). As a control, species 24 was reacted with haptoglobin and the absorbance monitored at 401 nm.

(*C*) *Species 21* was also studied in analogous fashion to that for species 11, with the hybrid mixture composed of species 01, 21, and 41. Species 41 exhibited no absorbance change at 401 nm upon mixing with haptoglobin solutions. Therefore, it makes no contribution to the absorbance signal in these hybrid kinetics experiments.

(*D*) *Species 22.* The experiment was analogous to that for species 11, except that the hybrid mixture was composed of species 23, 22, and 24. The isolated reactions of species 23 with haptoglobin and species 24 with haptoglobin (described above) were used as controls for this experiment.

Cryogenic Isoelectric Focusing. In hybrid mixtures each of the three tetrameric species associates according to its own assembly free energy. For given total concentrations of components at equilibrium, the relative values of the assembly free energies define the relative populations of the three tetrameric forms, according to

$$\Delta G_{AB} = -RT \ln \left[\frac{f_{AB} \sqrt{\exp\left(\frac{\Delta G_{BB}}{-RT}\right) \exp\left(\frac{\Delta G_{AA}}{-RT}\right)}}{2\sqrt{f_{AA} f_{BB}}} \right] \quad (4)$$

where f_{AA} is the fraction of tetramer composed of two "A" dimers, ΔG_{AA} is its assembly free energy, and the analogous symbols represent corresponding properties of the other tetramers BB and AB. It is thus possible to determine ΔG_{AB} from the experimentally measured population fractions f_{AA}, f_{BB}, and f_{AB}, provided assembly free energies of the parent species, ΔG_{AA} and ΔG_{BB}, are known. In practice, these quantities are determined either by kinetic methods or by equilibrium techniques such as analytical gel permeation chromatography.

When the parents are mixed in a 1:1 ratio, and the hybrid assembly free energy is additive (i.e., the arithmetic mean of the parents' assembly free energies), the population ratio of the three tetrameric species at equilibrium will be 1:2:1 [(parent 1):hybrid:(parent 2)]. A useful experimental parameter then is the "deviation free energy", δ, given by

$$\delta = -RT \ln \frac{f_{AB}}{2\sqrt{f_{AA} f_{BB}}} \quad (5)$$

This quantity is directly measured by the experiment and provides a measure of the deviation from additivity of the hybrid species assembly free energy:

$$\delta = \Delta G_{AB} - \frac{1}{2}(\Delta G_{AA} + \Delta G_{BB}) \quad (6)$$

The relative populations of the three tetrameric species in each hybrid mixture were analyzed by cryogenic isoelectric focusing (Perrella et al., 1983; LiCata et al., 1990). The two parent molecules are mixed and allowed to incubate at 21.5 °C for various periods of time so that net assembly of hybrid tetramers occurs to an increasing extent. The reactions are then quenched by rapid mixing of 20 μL of the hybrid mixture into 200 μL of a quenching solution (50% ethylene glycol and 50% standard buffer, saturated with carbon monoxide) at −25 °C. The cold quenched hybrid mixture is loaded onto a (prefocused) isoelectric focusing tube gel which is also maintained at −25 °C. The sample is focused for 18–30 h, and the tetrameric species separate according to their differences in net charge. One of the parent species carries a single-site mutation (Hb S or Hb C) which is not of functional significance in that the altered molecules exhibit assembly free energies identical with that of the normal molecule (Pettigrew et al., 1982). After focusing, the gel tube is directly scanned (LiCata et al., 1990) and the relative populations are obtained by integration of the absorbance peaks. A series of experiments, representing increasing times of incubation, allows assessment of approach to equilibrium values of the populations.

Hybrid mixtures of hemoglobin were prepared by using the appropriate parent molecules, described below (see Figure 3). Hemoglobin solutions were 1 mM (heme) or greater so that the presence of dimers could be neglected. Neslab KT-50 baths were used to maintain the required −25 °C temperatures. Scans were performed at 401 nm, with a 5.5-nm half-band-pass. With this band-pass, 401 nm was effectively isobestic for deoxy cobalt sites and carbonmonoxy iron sites. Linear resolution was 0.2 mm.

(*A*) *Species 12.* The parent species 01 was cobalt Hb C (β6 Glu → Lys) and was mixed with species 24.

(*B*) *Species 21* was made by hybridizing carbonmonoxy-Hb S (β6 Glu → Val) as parent species 41 with deoxy cobalt hemoglobin, which served as parent species 01. Alternatively, parent species 01 was cobalt Hb C (β6 Glu → Lys) and was used in a mixture with normal (carbonmonoxy)hemoglobin A_0 as parent species 41.

(*C*) *Species 31.* Carbonmonoxy-Hb S (β6 Glu → Val) was parent species 41 and was mixed with species 24.

(*D*) *Species 32.* Carbonmonoxy-Hb S (β6 Glu → Val) was parent species 41 and was mixed with species 23.

Table I: Kinetic Determinations of Assembly Free Energy

species *ij*	in mixture with	k_r (s^{-1})	half-time (s)	k_f (M^{-1} s^{-1})	${}^{ij}K_2$ (M^{-1})	${}^{ij}\Delta G_2$ (kcal/mol)
01	21, 41	1.50×10^{-2}	50	1.1×10^6	7.3×10^7	-10.6 ± 0.1
11	23, 01	0.18	3.9	1.1×10^{6} [a]	6.1×10^6	-9.1 ± 0.2
21	01, 41	0.45	1.5	1.1×10^{6} [a]	2.4×10^6	-8.6 ± 0.2

[a] For a large set of mutant, chemically modified, and metal-substituted hemoglobins, this rate has been found constant within 25% of the value listed. The reassociation constant used here was thus assumed.

Results

Species 01. Association and dissociation kinetics for unligated cobaltous hemoglobin are described in the following paper (Speros, 1990; Doyle et al., 1991) and are listed in Table I. The assembly free energy for this species was found to be -10.6 ± 0.1 kcal/mol.

Species 11. The assembly free energy for this singly ligated tetramer was successfully resolved by the haptoglobin kinetics method. The mixture (containing species 01, 23, and 11) in reaction with haptoglobin produced a 4.3% absorbance increase at 401 nm. The data were analyzed to sum-of-exponential equations (eq 3) containing one, two, and three independent components. The single-exponential equation failed to account for the data, resulting in systematic residuals and unreasonably large variance of fit. The two-term version of eq 3 yielded significant improvement in the variance, and in randomness of residuals, while no further improvement resulted from the three-component exponential fit. The first, slower, kinetic process was assigned to species 01 on the basis that its dissociation rate was identical with that found for this species studied in isolation (Speros 1990; Doyle et al., 1991), and the extent of reaction was consistent with the expected value. The second (faster) process was thus attributed to species 11. In other cases, in which very early data were included, an additional very fast phase, corresponding to the expected rate for species 23, was also resolved.

The reassociation rate for species 11 was not measured. However, for a large number of hemoglobins that vary with ligation state, mutation, chemical modification, and metal substitution, the reassociation rate has been found to be within 25% of 1.1×10^6 M^{-1} s^{-1}. This same value was found to hold for the measured species 01 rate (Speros, 1990; Doyle et al., 1991). The reassociation rate for the singly ligated species 11 was thus assumed to have this same value, yielding an assembly free energy of -9.1 ± 0.1 kcal/mol (Table I). The finding of the same rate constant for both species 01 and 21 supports the validity of its application to species 11.

Species 12. A haptoglobin kinetics experiment was carried out in analogous fashion to the experiment for species 11 using a hybrid mixture of species 01 and 24, both of which exhibit only small absorbance changes upon dissociation. However, it was not possible to resolve any absorbance changes that could be assigned to the dissociation of species 12.

Reaction of the two parent species 01 (Hb C) and 24 was followed by cryogenic isoelectric focusing analysis for 70 h after mixing. The deviation free energy was 0.4 ± 0.2 kcal/mol, and the resulting assembly free energy of species 12 is thus -8.6 ± 0.4 kcal/mol (Table II).

Species 21. Reaction with haptoglobin of the hybrid mixture containing the three species 01, 41, and 21 produced a significant absorbance change at 401 nm. Data analysis was analogous to that for species 11. Two kinetic processes were resolved (see Figure 4), which were attributed to dissociation of species 01 and 21. The slowest process matched the kinetic behavior of species 01 in isolation. The second, faster process could be assigned to species 21, since species 41, the only other component of the mixture, had no signal when studied in isolation. The extent of each phase was consistent with the expected magnitude, on the basis of the number of deoxy cobalt subunits dissociating. Assuming the same association rate constant as for species 01, the calculated assembly free energy of species 21 is -8.6 ± 0.2 kcal/mol (Table I).

Table II: Determination of Hybrid Tetramer Assembly Free Energies by Cryogenic Isoelectric Focusing

hybrid species *ij*	in mixture with	deviation free energy (kcal/mol)	additive assembly free energy (kcal/mol)	assembly free energy (kcal/mol)
12	01, 24	0.4	−9.0	-8.6 ± 0.4
21	01, 41	0.88	−9.3	-8.5 ± 0.2
31	24, 41	0.16	−7.7	-7.6 ± 0.2
32	23, 41	0.27	−7.8	-7.5 ± 0.2

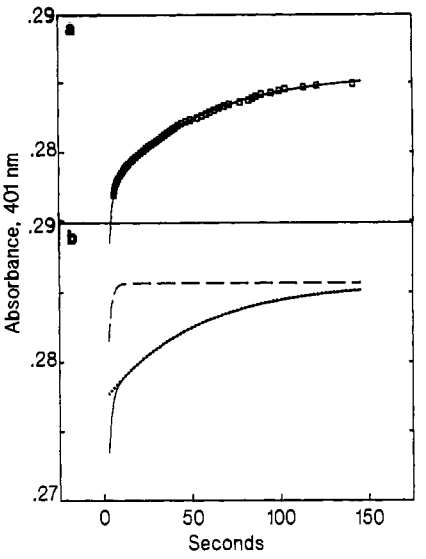

FIGURE 4: Kinetics of tetramer–dimer dissociation for hybrid species 21 by haptoglobin kinetics. The reaction mixture includes species 21, 01, and 41 (which exhibits no absorbance change upon dissociation). Analysis of the data (squares, part a) to the double-exponential form of eq 3 (solid line, parts a and b) yielded two dissociation rate constants. The slower process has a half-time of 50 s (dotted line, part b), which is identical with that resolved independently for species 01 in isolation (Doyle et al., 1991). The faster process has a half-time of 1.5 s (dashed line, part b), assigned to the dissociation rate of species 21 (see Table I).

Because of the central importance of species 21 found in the cyanomet system, the assembly free energy of this species in the Co(II)/Fe(II)–CO system was also studied by the cryogenic isoelectric focusing method. Species 01 and 41 were mixed, and the time course of the ensuing reaction was followed by observation of the relative population of each species in the mixture. The fraction of hybrid tetramer, f_{AA}, rose sharply over the first 80 min after mixing, reaching an equilibrium value of 17%. Figure 5 shows the time course for experiments in which the initial parent ratio was 1:1. Figure 5A exhibits the approach to equilibrium as seen in the actual scans. The absorbance versus distance plane shows the gel scans at given times of reaction. As time increases, the hybrid peak between the parent peaks grows in area and then reaches its equilibrium value. These hybrid peak areas, normalized

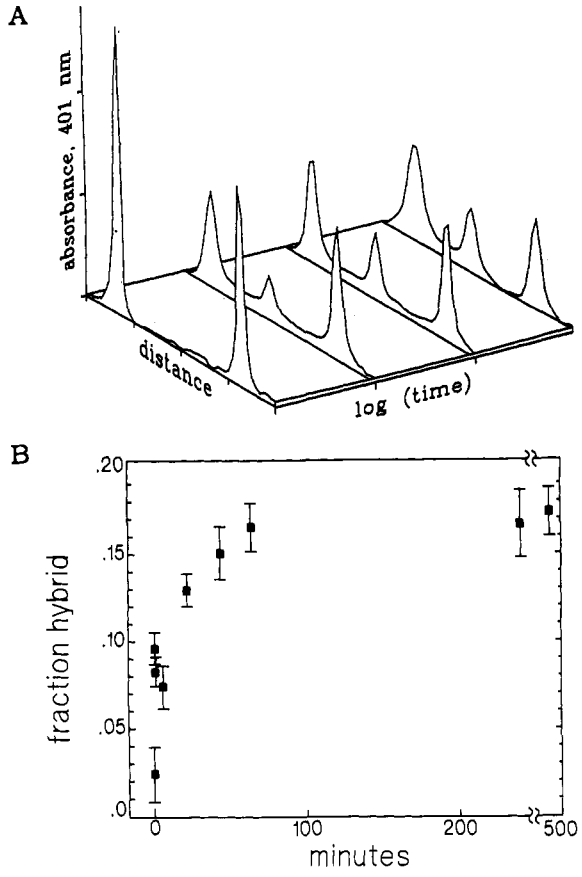

FIGURE 5: Time course of population distributions determined by low-temperature quenching and cryogenic isoelectric focusing. Parent species 01 and 41 were mixed and incubated, allowing dimers to dissociate from each parent and to reassemble into hybrid tetramer 21. After an initial incubation period, the reaction was quenched and the populations were determined by separating and quantitating each species from the mixture. Part A shows isoelectric focusing position (distance) versus absorbance, as a function of incubation time (in logarithmic increments). At very early reaction times, equilibrium is achieved. Part B shows the fractional integrated areas of the hybrid peak as a function of time. The data are averages of 2–6 tubes, for the initial 1:1 mixing ratio for the parent species. From the population distribution at equilibrium, relative assembly free energies are obtained according to eqs 5 and 6.

to the total area of all of the peaks in the scan, are plotted versus time in Figure 5B.

The equilibrium fractions for the three species yield (by eq 5) a deviation free energy of 0.8 ± 0.1 kcal/mol. This is the deviation of the hybrid assembly free energy from the additive value of the two parent assembly free energies. The assembly free energy of species 21 was thus found to be −8.5 ± 0.2 kcal/mol, in close agreement with the value determined by the dissociation kinetics method, described above. This agreement also establishes the correctness of the value used for the association rate constant of this species (Table I).

Species 22. This species is formed in the hybrid mixture of parent species 23 and 24, each of which exhibits a characteristic exponential change of absorbance upon mixing with haptoglobin. These characteristic changes are very fast and identical. The hybrid mixture, containing species 23, 24, and the hybrid molecule 22, showed no additional absorbance changes that could be resolved. Species 22 is therefore inferred to lie, within experimental error, at the same assembly free energy as the parent species 23 and 24 (which are energetically identical with each other).

Species 23 and 24. In haptoglobin kinetics experiments these species show very fast characteristic absorbance changes

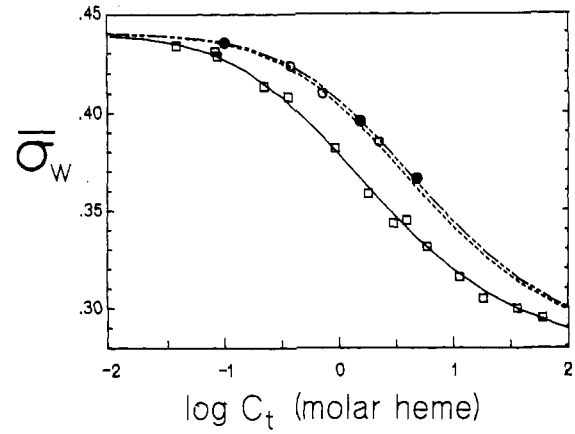

FIGURE 6: Anaerobic gel permeation chromatography was used to determine assembly free energies of species 23 and 24. The column was calibrated for partition coefficients of dimeric and tetrameric species with oxygenated normal hemoglobin A_0. Those data (open squares) fully determine the end points σ_2 and σ_4 and assembly equilibrium constant for oxyhemoglobin. The solid curve and open squares represent the behavior of (carbonmoxy)hemoglobin on this column. Since the dimer and tetramer end points were known, determination of only the position of the transition for species 23 (open circles) and species 24 (filled circles) was necessary. The data were analyzed simultaneously by eq 2. Conditions were pH 7.4, 21.5 °C, 0.1 M Tris, 0.1 M NaCl, 1 mM EDTA, and 0.1% (w/w) sodium dithionite.

which conform to a single exponential. Since the dissociation was so fast, a nonkinetic method was used to determine the assembly free energies of these species.

Determination of the assembly free energies of species 23 and 24 was achieved by anaerobic gel chromatography. The column was "calibrated" by large-zone experiments at varying concentrations of oxyhemoglobin A_0 (see Figure 6). These data provided partition coefficients of dimers and tetramers, as well as the position on the concentration scale of the characteristic dissociation curve between these "end points". This curve also defines the association equilibrium constant and corresponding assembly free energy. Since the dimeric and tetrameric partition coefficients were established by this calibration, accurate determination only of the position of the transition curve for the hybrid species was necessary. Simultaneous analysis of all of the data to eq 2 determined the end points, the assembly equilibrium constants, and their respective assembly free energies. The assembly free energies of species 23 and species 24 were found to be −7.5 ± 0.2 and −7.4 ± 0.2 kcal/mol, respectively.

Species 31. Reaction of parent species 24 and 41 (Hb S) was followed by cryogenic isoelectric focusing analysis for 50 h after mixing. Initial parent ratios of either 30:70 or 40:60 were used. For each ratio, the approach to equilibrium was observed. The deviation free energy for both ratios was 0.16 ± 0.1 kcal/mol, and the resulting assembly free energy of species 31 is −7.6 ± 0.2 kcal/mol (Table II).

Species 32. Species 23 and 41 (Hb S) were mixed in an initial ratio of 60:40, and the increase of hybrid fraction was followed to equilibrium by cryogenic isoelectric focusing. The deviation free energy was 0.27 ± 0.1 kcal/mol, resulting in an assembly free energy of −7.5 ± 0.2 kcal/mol (Table II).

Species 41. The assembly free energy of species 41, as determined by analytical gel permeation chromatography, has been found previously (Smith & Ackers, 1985), to be −8.0 ± 0.1 kcal/mol under conditions of the present study.

Discussion

In this study we have employed four experimental techniques (i.e., two equilibrium methods and two kinetic methods) in

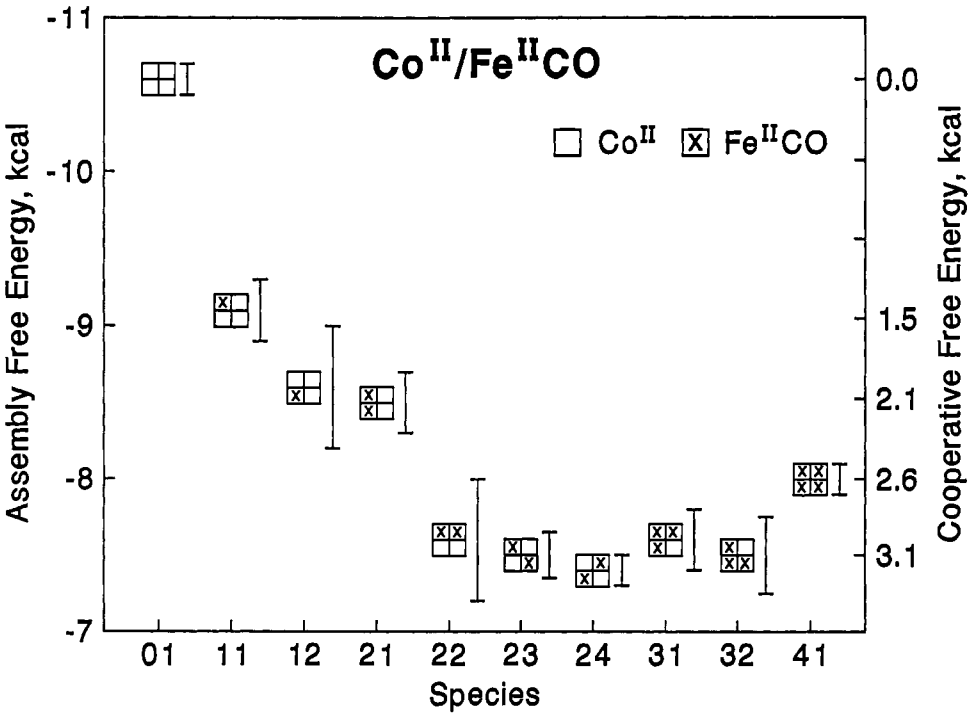

FIGURE 7: Tetrameric free energy levels versus ligation species, for the Co(II)/Fe(II)–CO system, at pH 7.4, 21.5 °C, 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA. The 10 species of this system define 5 discrete cooperative free energy levels. The combinatorial nature of cooperative switching and quaternary enhancement is exhibited in this distribution.

various combinations to resolve assembly free energies of partially ligated species in the Co(II)/Fe(II)–CO system (see Figure 7). The results obtained for this system are in general accord with the previous findings of Yonetani and colleagues, on this same system (Imai et al., 1980) and provide strong support for their conclusion that the cooperative mechanism in cobalt–iron hemoglobin is qualitatively the same as that of normal hemoglobin binding oxygen. A unique feature of the present work, however, lies in the resolution of cooperative energetics for species 21, in comparison with the other doubly ligated species. The microstate energetics of all 10 species are summarized in Table III and Figure 7. These results, in combination with previous studies of the 10 microstate energetics, provide a number of insights and generalizations.

Patterns in the Distribution of Ligation Species into Cooperative Free Energy Levels. The 10 ligation species are found to distribute into 5 discrete assembly free energy levels. These levels are defined, for example, by the results for species 01, 11, 21, 23, and 41. The only component of the assembly free energies obtained for these species (Table III) that has not been determined explicitly is the forward rate constant for species 11. The basis for assigning the value of 1.1×10^6 M^{-1} s^{-1} (Table I) for this rate has been presented in the previous section. It is possible, though not likely, that an unprecedented anomaly in the value for this constant could bring the assembly free energy of species 11 to the same level as that of species 21 so that the total number of levels would be four rather than five. In either case, this distribution differs at least in detail from that found previously for the Fe(II)/Fe(III)–CN, Mn(II)/Fe(II)–CO, and Fe(II)/Mn(III) systems, which exhibited three equally spaced levels of assembly free energy under these same conditions. As in the previously studied systems, species 21 and the other doubly ligated species in the Co(II)/Fe(II)–CO system are found to lie at different assembly free energy levels. However, species 11 and 21, in this system, clearly occupy different free energy levels, unlike the case of cyanomethemoglobin (Figure 2) and met-manganese hemoglobin systems. Because of the large experimental uncertainty in the estimated assembly free energy of species 12 it is not possible to ascertain whether it occupies the same level as species 11 or 21.

Table III: Assembly Free Energies of the Co(II)/Fe(II)–CO System

species	assembly free energy (kcal/mol)	methods
01	−10.6 ± 0.1[a]	dissociation and association kinetics
11	−9.1 ± 0.2	dissociation kinetics[c]
12	−8.6 ± 0.4	cryo-isoelectric focusing
21	−8.5 ± 0.2	cryo-isoelectric focusing
	−8.6 ± 0.2	dissociation kinetics[c]
22	−7.6 ± 0.4	dissociation kinetics[c]
23	−7.5 ± 0.2	gel permeation chromatography
24	−7.4 ± 0.2	gel permeation chromatography
31	−7.6 ± 0.2	cryo-isoelectric focusing
32	−7.5 ± 0.2	cryo-isoelectric focusing
41	−8.0 ± 0.1[b]	gel permeation chromatography

[a] Speros (1990); Doyle et al. (1991). [b] Smith et al. (1987). [c] In combination with the association rate constant found to be identical for 20 mutant hemoglobins under conditions of this study (Pettigrew et al., 1982; Chu & Ackers, 1981; Turner, 1989).

Quaternary Enhancement in Species 23, 24, 31, and 32. In normal hemoglobin the ligation free energies have been found to increase with each successive binding step until, at the last step, the tetrameric value exceeds the intrinsic ligation free energy exhibited by the noncooperative dimers. This effect, i.e., that the assembly of dimers into tetramers causes the binding free energy at the last step to surpass the intrinsic binding free energy, was termed "quaternary enhancement" (Mills & Ackers, 1979a,b). A thermodynamic correlate of this phenomenon is that triply ligated tetramers are more weakly assembled than the fully ligated tetramers. This same effect was observed with the ligand-linked assembly of tetrameric β_4 subunits (Valdes & Ackers, 1978a,b). Using a combination of four different techniques, quaternary enhancement has been found in the oxygenation-linked dimer–tetramer assembly of normal hemoglobin at temperatures between 15 and 35 °C (Mills & Ackers, 1979a; Di Cera et

al., 1987) and pH values between 7.4 and 9.5 (Chu et al., 1984). This effect has also been found in the oxygen binding to cobaltous hemoglobin (Doyle et al., 1991). Results of the studies described in this paper clearly demonstrate quaternary enhancement in the cobalt–iron hybrid hemoglobins. Quaternary enhancement in hemoglobin has been considered controversial due to kinetic data which suggested that the quaternary enhancement effect must be negligibly small or was an artifact of incorrect oxygenation curve analysis [Gibson & Edelstein, 1987; Philo & Lary, 1990; see, however, Ackers and Johnson (1990)]. The present study establishes that four species of the Co(II)/Fe(II)–CO system exhibit significant quaternary enhancement. These determinations, carried out by direct equilibrium methods, provide values that are insensitive to minute variations in uncertainty of the data. In particular, the anaerobic analytical gel permeation chromatography studies performed on pure species 23 and 24 present an especially uncomplicated observation of the quaternary enhancement effect. The magnitude of this effect in iron–cobalt hybrids with bound carbon monoxide is nearly identical with that found for oxygen binding to pure cobaltous hemoglobin (Doyle et al., 1991).

The Combinatorial Switching Code. There are now four hemoglobin ligation systems for which cooperative free energies of species 21 have been measured in comparison with other doubly ligated species [i.e., Fe(II)/Fe(III)–CN, Fe(II)/Mn(III), Mn(II)/Fe(II)–CO, and Co(II)/Fe(II)–CO]. Every one of these systems exhibits a combinatorial switching pattern in which species 21 occupies a separate free energy level from the other doubly ligated species. This same pattern is found even though spacings of the energetic levels vary widely. This and other similarities in the observed cooperative free energy distributions reinforce the premise that the basic modes of energetic coupling within the tetrameric structure are identical, even though their quantitative manifestations may vary with heme site ligand and with conditions. It is therefore reasonable to assume that the symmetry rule for quaternary switching that has been inferred from studies on cyanomethemoglobin (Daugherty et al., 1991) may also be applicable to these other ligation systems. On that basis the energetics determined in the present study for species 11 and 21 of the Co(II)/Fe(II)–CO system would both represent quaternary T structures even though they occupy two different cooperative free energy levels. These levels represent two successive increments of positive free energy that are "spent" in the sequential binding steps prior to quaternary structural transition. The switchover from T to R occurs, according to the symmetry rule, when ligands are first bound on both sides of the $\alpha^1\beta^2$ interface. The cyanomet system appears to represent a special case of the more general mechanism since ligand-induced "tertiary" energy is spent only at the first binding step leading to identical cooperative free energies for species 11 and 21 (Figure 2).

Applying this general mechanism to the results of the present study, we may assign energetic properties of the remaining doubly ligated species (i.e., 22–24), both triply ligated species (31 and 32), and the fully ligated species 41 to the quaternary R structure. The quaternary enhancement exhibited by species 23, 24, 31, and 32 (and presumably applicable to species 22) then must represent ligand-induced effects within the quaternary R structure. Here, however, the sequential ligand-induced increment of free energy within the R structure has the opposite sign from that of the two analogous increments discussed above for the T structure. A simple concept for rationalizing these oppositely directed effects of ligand binding is that the tertiary energies are unfavorable (i.e., have positive sign) when ligand binding (or unbinding) generates a "mismatch" between tertiary and quaternary structures. The mismatch can occur either by (a) the creation, by binding a ligand, of an "oxy" tertiary subunit within the deoxy (T) quaternary structure or (b) the creation of a "deoxy" tertiary subunit within the oxy quaternary R structure by releasing a ligand from species 41. Thus the observed quaternary enhancement effect in the Co(II)/Fe(II)–CO system may be viewed as the release of such unfavorable free energy when the tertiary–quaternary mismatch is eliminated by fully ligating the R-state tetramer. This concept is supported by work on quaternary enhancement in isolated β subunits and their ligand-linked assembly into R-state β_4 tetramers (Valdes & Ackers, 1978a,b).

The previously studied cyanomet and met-manganese systems thus appear to present a limiting special case of the more general combinatorial code which gives rise to the five cooperative free energy levels found for the cobalt–iron hybrid system. The finding that doubly ligated tetrameric species (e.g., 21 and 23) may occupy different cooperative free energy levels (as determined from the subunit assembly results) would appear to rule out a simple two-state MWC mechanism. However, a detailed analysis via the comprehensive MWC model as extended to incorporate properties of the dissociated dimers (Ackers, 1990) indicates that the five levels found in the Co(II)/Fe(II)–CO system could, in theory, collapse into three levels of tetramer cooperativity, as found in cyanomethemoglobin. If substantial dimer anticooperativity and quaternary enhancement of the tetramer were present along with "MWC averaging", then the five assembly free energy levels observed in the cobalt–iron system could even reduce to a two-state MWC averaged mechanism (Ackers, 1990; detailed analysis to be presented elsewhere). This possibility of collapse into two states appears less likely than collapse into three states since the distributions found with cyanomethemoglobin and met-magnanese hemoglobin cannot be interpreted as two-state behavior (Ackers, 1990).

Validity of cobaltous hemoglobin as an analogue of native deoxyhemoglobin is supported by X-ray crystallographic studies which indicate full capability to assume the deoxy T quaternary structure (Fermi & Perutz, 1982), as well as CD spectroscopic measurements (Snyder & Chien, 1979), and by numerous other studies including those cited in the introduction. Under conditions of this study, the assembly free energy for cobaltous species 01 is approximately 1 kcal lower than that of normal deoxyhemoglobin at pH 9.5 (Chu & Ackers, 1981) where the latter molecule is virtually all T structure. Recent studies on the cyanomet system as a function of pH (Daugherty et al., 1991) demonstrate a wide range of thermodynamic stabilities for molecules that assume the quaternary T packing of dimers. However, since the quaternary T structure can also exist for molecules with intermediate allosteric properties (e.g., species 21 of the cyanomet system), it is possible that unligated cobaltous hemoglobin (species 01) is partially switched to one of these levels, even though its quaternary structure is still T. Further studies will be required to assess this possibility, and to define the exact correspondences between tetrameric and dimeric properties in the Co(II)/Fe(II)–CO system. The work presented in the following paper (Doyle et al., 1991) defines the relationships between dimer–tetramer assembly and oxygen binding in cobaltous hemoglobin. Results of that study, and the one described in this paper, provide complementary information in strong support of the premise that a common set of rules governs the cooperative interactions in hemoglobin regardless

of the metal carried by the heme and the ligands bound to it [see Imai et al. (1980) and Ackers and Smith (1987)]. Only by studying a range of systems and conditions at the level of detail of the present work can these rules be fully elucidated.

Registry No. HbC, 9008-00-8; HbS, 9035-22-7; CO, 630-08-0.

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