Biochemistry **1984**, *23*, 2875–2882 2875

Woods, E. F. (1969) *Biochem. J. 113*, 39–45.
Wray, W., Boulikas, T., Wray, V., & Hancock, R. (1981) *Anal. Biochem. 118*, 197–203.
Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.
Yphantis, D. A., Correia, J. J., Johnson, M. L., & Wu, G.-M. (1978) in *Physical Aspects of Protein Interactions* (Catsimpoolas, N., Ed.) pp 275–303, Elsevier/North-Holland, New York.
Zackroff, R. V., Idler, W. W., Steinert, P. M., & Goldman, R. D. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 754–757.

Carbon Monoxide Binding to the Ferrous Chains of [Mn,Fe(II)] Hybrid Hemoglobins: pH Dependence of the Chain Affinity Constants Associated with Specific Hemoglobin Ligation Pathways[†]

Neil V. Blough[‡] and Brian M. Hoffman*

ABSTRACT: In mixed-metal [Mn,Fe] hybrid hemoglobins (Hb), the two chains of a single type, α or β, are substituted with manganese protoporphyrin IX, which does not bind CO in either the Mn(II) or Mn(III) valency states. Thus, CO binding by the two ferrous subunits of a hybrid with Mn of either valency represents a simplified two-step Hb ligation process in which ligands bind to a single-chain type. Considering the [Mn(II),Fe(II)] hybrids, which are deoxy T-state analogues, at pH 6.6 both types bind CO with low affinity (α-Fe, 0.38 mmHg; β-Fe, 0.71 mmHg) and noncooperatively (Hill coefficient $n = 1$). At elevated pH, both exhibit an increase in affinity (Bohr effect) and strong cooperativity, with the α-Fe hybrid having a higher degree of cooperativity ($n \simeq 1.6$) than β-Fe ($\simeq 1.3$) at pH 9.0. The CO association constants for the Hb ligation routes in which the first two ligands bind to the same chain type are obtained from these measurements, and their pH dependence provides estimates of the proton release at each step. Through studies of CO on- and off-rates, the [Mn(III),Fe(II)] hybrids are used to obtain the pH dependence of the association constants for binding the fourth CO to the individual Hb chains. These results are used to parameterize an extended form of the two-state, allosteric model for Hb cooperativity and provide the first direct determination of the pH dependence of the CO affinity constants for the individual chains in the T and R conformations, $K_{\mathrm{T}\alpha(\beta)}$ and $K_{\mathrm{R}\alpha(\beta)}$, the ratios of these constants, C_α and C_β, and the concentration ratio of the low- to high-affinity structural forms of unliganded Hb, L_0. The α and β chains show a similar T-state Bohr effect; at pH 7.1, ligation of either α or β chains releases ~0.4 proton. In contrast, ligation of the chains within the R state releases at most ~0.2 proton at this pH. From pH 6.6 to 9.0, C_α varies ~2-fold and C_β 4-fold. The analysis indicates that even the extended Monod–Wyman–Changeaux model, which accounts for chain differences, incompletely describes Hb cooperativity, since multiple values of L_0 are required to accommodate the data, and supports the suggestion of Weber regarding subunit interactions.

Hemoglobin A (Hb A) cooperatively binds four ligands via a complex sequence of intermediate liganded states. Valency and NO hybrid hemoglobins have been used to model the behavior of Hb intermediates in the latter stages of the ligation process (Shulman et al., 1975; Baldwin, 1975; Szabo & Karplus, 1975). In this and in the following paper (Blough et al., 1984), metal substitution in the form of mixed-metal [Mn,Fe][1] hybrids (Hoffman, 1979; Waterman & Yonetani, 1970; Gibson et al., 1974; Hoffman et al., 1975; Blough & Hoffman, 1982) is shown to offer a particularly advantageous and direct means with which to probe both initial and final stages of CO binding by Hb. In combination with our earlier work, the results presented here comprise the first self-consistent set of CO binding parameters for individual chains within the two major quaternary states of hemoglobin. The pH response of CO binding to the hybrids further permits us to estimate the proton release associated with individual stages in the ligation process, as well as the tertiary Bohr effect associated with ligation of an individual chain within the T or the R quaternary structure.

In [Mn,Fe] hybrids, two chains of a single type, α or β, are substituted with manganese protoporphyrin IX, and the two complementary chains are retained in the ferrous form. The Mn-containing subunits can exhibit either the Mn(II) or Mn(III) valency states, and in neither case do they bind CO. Thus, ligand binding by the two ferrous subunits of a hybrid with Mn of either valency represents a simplified Hb ligation process in which ligands bind to a single-chain type. The early steps of Hb ligation are investigated with the [Mn(II),Fe(II)] hybrids. Work to date has established the following: fully reconstituted Mn(II) Hb is functionally equivalent to native Hb A (Gibson et al., 1974; Hoffman et al., 1975; Gibson & Hoffman, 1979); [Mn(II),Fe(II)] hybrids are functional analogues of unliganded, T-state Hb A (Blough et al., 1980; Blough & Hoffman, 1982). This was expected because of the

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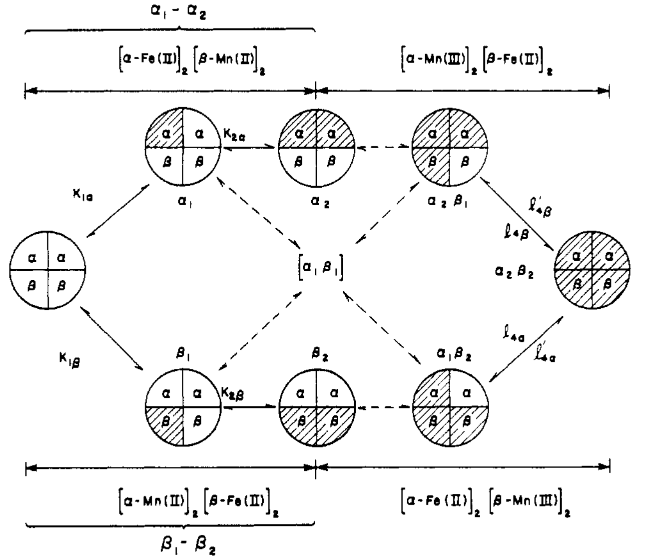
* Address correspondence to this author at the Department of Chemistry.

[‡] Present address: Laboratory of Chemical Biodynamics, University of California, Berkeley, CA 94720.

[1] Abbreviations: Hb, hemoglobin; met-Hb, ferric form of Hb; [Mn,Fe], hemoglobin derivative in which the two chains of a single type, α or β, are substituted with manganese protoporphyrin IX; Bis-Tris-HCl, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; MWC, Monod–Wyman–Changeaux model of cooperative ligand binding.

Scheme I: Scheme for CO Binding to Hb in Which Equilibria That Are Characterizable through Use of [Mn,Fe(II)] Mixed-Metal Hybrid Hb Are Emphasized[a]



[a] The [α-Fe(II),β-Mn(II)] hybrid is used to measure the association constants, $K_{1\alpha}$ and $K_{2\alpha}$, for binding successive CO to the α chains of Hb; this binding route is labeled α_1–α_2. Similarly, [α-Mn(II),β-Fe(II)] gives the association constants, $K_{1\beta}$ and $K_{2\beta}$, for binding successive CO to the β chains of Hb; this route is labeled a β_1–β_2. The CO on- and off-rates (l_4' and l_4) for the α and β chains in the fourth CO binding step of Hb are obtained from the [α-Fe(II)(CO),β-Mn(III)] and [α-Mn(III),β-Fe(II)(CO)] hybrids, respectively. The ratio of these constants give the microscopic CO association constants $K_{4\alpha}$ and $K_{4\beta}$. The broken arrows represent microscopic ligation steps that are not directly probed.

similar stereochemistries of Mn(II) and Fe(II) porphyrins (Gonzales et al., 1975; Scheidt, 1977) and is confirmed by single-crystal X-ray diffraction studies of [Mn,Fe(CO)] hybrids.[2] Thus, as depicted in Scheme I, CO binding to the ferrous chains of the [Mn(II),Fe(II)] hybrids provides the pH dependence of the binding constants for the "symmetrical" Hb ligation routes in which the first two ligands bind to the same chain type, either the α_1–α_2 route or the β_1–β_2. In this way, we thereby characterize the CO affinity and proton release when a Hb tetramer binds its first ligand, either to an α or to a β chain, and two of the four ways in which the second CO can be bound. The processes cannot be directly characterized in Hb, nor in any of the previously studied hybrid systems.

The Mn(III) subunits within Mn(III) Hb are both structurally (Moffat et al., 1976) and functionally (Gibson et al., 1974; Hoffman et al., 1975) similar to liganded subunits of met-Hb. Thus, the [Mn(III),Fe(II)(CO)] hybrids are analogues of tetraliganded R-state Hb, and as with the valency hybrids, CO binding to the ferrous subunits of the [Mn(III),Fe(II)] hybrids is representative of the latter ligation stages of Hb (Scheme I). Through studies of CO on- and off-rates, these hybrids are used to obtain the pH dependence of the individual microscopic binding constants representing either an α or a β subunit binding the fourth ligand to Hb.

These results finally are used in combination to parameterize an extended form (Ogata & McConnell, 1971; Edelstein, 1974; Baldwin, 1975) of the two-state, allosteric model for Hb ligation of Monod, Wyman, and Changeaux (Monod et al., 1965) (MWC). Along with our previous study (Blough & Hoffman, 1982), this provides the first direct determination of the pH dependence of C_α and C_β, the ratios of the CO affinities for the individual chains in the T and R conformations, and of L_0, the concentration ratio of the low- to high-affinity structural forms of uniliganded Hb. Although the MWC model provides a good first-order description of the properties of Hb and the hybrids, these studies suggest that even the extended model, which accounts for chain differences, is not fully adequate to describe Hb cooperativity.

Experimental Procedures

Manganese(III) protoporphyrin IX was prepared from the sodium salt of protoporphyrin IX (Sigma Chemical Co.) by published procedures (Adler et al., 1970) and its purity confirmed by visible spectrophotometry and thin-layer chromatography, with the 1-butanol/acetic acid/water (50/1.5/1.4) system of Dinello & Dolphin (1975).

Hybrid samples for equilibrium and kinetic measurements of CO binding were prepared in 0.05 M buffer, either Bis-Tris-HCl at pH 6.6 or Tris-HCl at pH 7.6, 8.0, and 9.0. Stock dithionite solutions (0.1 M) were prepared daily with the appropriate deoxygenated buffer. A 1 mM stock solution of methylene blue was prepared with distilled, deionized water. β-Mercaptoethanol (Aldrich) was used without further purification. Gases employed were N_2 (Matheson, prepurified), CO (Matheson), and 4.988% CO in N_2 or 1.004% CO in N_2 (Matheson, primary standards). Then N_2 and CO were passed through Ridox (Fisher).

Preparation of [Mn(III),Fe(II)] Hybrids. Human hemoglobin was purified from pooled blood samples by methods previously described (Scholler et al., 1979). To isolate the individual α and β chains of hemoglobin, the chain preparation and two-column separation procedures of Geraci et al. (1969) were used. Both chain types were converted to the carbon monoxide form and were stored in liquid N_2 until needed. Unless otherwise noted, all preparative procedures were carried out at 4 °C.

Heme-free chain globin was prepared as described by Yip et al. (1977). Chain globin was combined anaerobically with an equimolar amount of the opposite heme-containing chain in a Schlenk tube under an atmosphere of CO. The Schlenk tube was sealed and placed on ice in a refrigerator for 72 h to allow combination of chains to form the half-substituted species or semiglobin. At the end of the incubation period, a 1.5 molar excess of Manganese(III) protoporphyrin IX over globin sites was added to the preparation; the Manganese(III) protoporphyrin IX solution was prepared and assayed by procedures previously published (Scholler et al., 1979). The preparation was stirred on ice for 15–30 min with the pH then adjusted to between 8 and 8.5 by the very slow addition of 0.1 M NaOH. At this point, the hybrid preparation was removed from the ice bath and allowed to warm to room temperature (15–20 min). Precipitate was then removed by centrifugation. The clarified solution was passed through a Sephadex G-25 column (2.5 × 58 cm; Sigma Chemical Co.) equilibrated with 0.01 M Tris, pH 8.6 buffer to adjust the pH and ionic strength and to remove excess porphyrin. To purify the hybrid from uncombined and incompletely reconstituted chains or semiglobin, the preparation was applied to a DE-52 (Whatman) ion-exchange column equilibrated with 0.01 M Tris, pH 8.6 buffer and eluted with a linear salt and pH gradient from 0.01 M Tris and pH 8.6 to 0.05 M Tris and pH 8.05. For a preparation containing ~20 μmol each of globin and the heme-containing chain in a volume of 80–100 mL, a column

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[2] [α-Mn(II),β-Fe(CO)] hybrids have been crystallized and found to be in the T quaternary structure by single-crystal X-ray diffraction techniques. Data collection has been extended to 3.5 Å (N. V. Blough, A. Arnone, J. McGourty, and B. M. Hoffman, unpublished results).

size of 5 × 10 cm and a total gradient volume of 2 L were used. All protein was initially bound in a thin band on top of the column. upon application of the gradient, the α chains were eluted first, followed by hybrid; β chains remained on the column. The hybrid was collected in fraction and concentrated by use of an Amicon ultrafiltration cell containing a PM 10 membrane. The individual fractions were analyzed by visible spectrophotometry and isoelectric focusing to determine their purity. Since the hybrids are prepared in the [Mn(III),Fe(II)(CO)] form, only those fractions that focused at the same position as the half-oxidized hemoglobin were used in subsequent measurements. The CO hybrids were stored under liquid N_2 until needed.

Equilibrium CO Binding to [Mn(II),Fe(II)] Hybrids. Reduction of the Mn(III) subunits of a hybrid greatly facilitates the release of CO from the ferrous chains, and thus, stock solutions of reduced, deoxy hybrid were prepared in the following manner. Approximately 10 mL of buffer, additionally containing β-mercaptoethanol and methylene blue at concentrations of 0.1% and 1 μM, respectively, was thoroughly deoxygenated within a tonometer by successive cycles of evacuation and back-filling with N_2. A sufficient quantity of concentrated hybrid was then diluted into the N_2-saturated buffer to give a final concentration of 3–4 μM in hybrid heme. The Mn-containing subunits were subsequently reduced with a minimal amount of dithionite. After complete reduction, only mild pumping and back-filling with N_2 were needed to remove the residual CO, as confirmed by spectrophotometric assay. A gas-tight syringe was used to anaerobically transfer 3-mL aliquots of the appropriate reduced hybrid to a tonometer that had been treated earlier with Prosil-28 (PCR Research Chemicals). Use of this organosilane provides a water-repellent glass surface and greatly improves sample stability, presumably by reducing surface denaturation effects. CO equilibria were measured on a Beckman Acta III spectrophotometer at 432 and 418 nm for the α-Fe hybrid and 432 and 420 nm for the β-Fe hybrid. After each gas addition, the sample was equilibrated with stirring in a darkened, 25 °C water bath until a constant optical spectrum was obtained. Sample integrity within an experiment was confirmed by the observation of isosbestic points. As a further test of sample integrity, flash photolysis was used to measure the CO recombination kinetics of the hybrid samples after completion of the equilibrium experiments. The results of these measurements were compared to those from samples prepared directly. In all cases, the CO recombination kinetics were identical.

Flash Photolysis Measurements. Samples of [Mn(III),Fe(II)(CO)] hybrid were prepared as above, but without dithionite, methylene blue, and β-mercaptoethanol. The CO concentration was 51 μM (5% CO) in all cases while the heme concentration varied between 3 and 5 μM. The CO recombination kinetics were measured on flash photolysis equipment previously described (Stanford et al., 1980), with a Sunpak 611 photographic flash screened by a Corning 3-71 filter as the photolysis source. Absorption changes were monitored at 436 nm in the Soret region. Attenuation of photolyzing light was accomplished with neutral density filters and changes in the power setting of the photographic flash. The decay traces were fit to either a single exponential or a sum of two exponentials by use of a nonlinear least-squares fitting routine (Bevington, 1969).

CO Dissociation Rate from the [Mn(III),Fe(II)(CO)] Hybrids Measured by NO Replacement. Small, air-tight vials of concentrated [Mn(III),Fe(II)(CO)] hybrid were thoroughly flushed with 1.004% CO in N_2. A total of 10–50 μL of the hybrid was then rapidly injected into a stoppered cuvette containing 2.5–3 mL of NO-saturated buffer equilibrated at 25 °C. The sample was then quickly positioned in a Beckman Acta III spectrophotometer, and the replacement of CO by NO was followed at 420 and 418 nm for the β- and α-Fe hybrid, respectively. At least three kinetic traces were obtained at each pH. In all runs, NO was in excess of 1 mM, whereas the heme concentration varied between 5 and 10 μM and the final concentration of CO was under 1 μM. Under these conditions, the observed rate-limiting step is that of CO dissociation from the liganded hybrid.

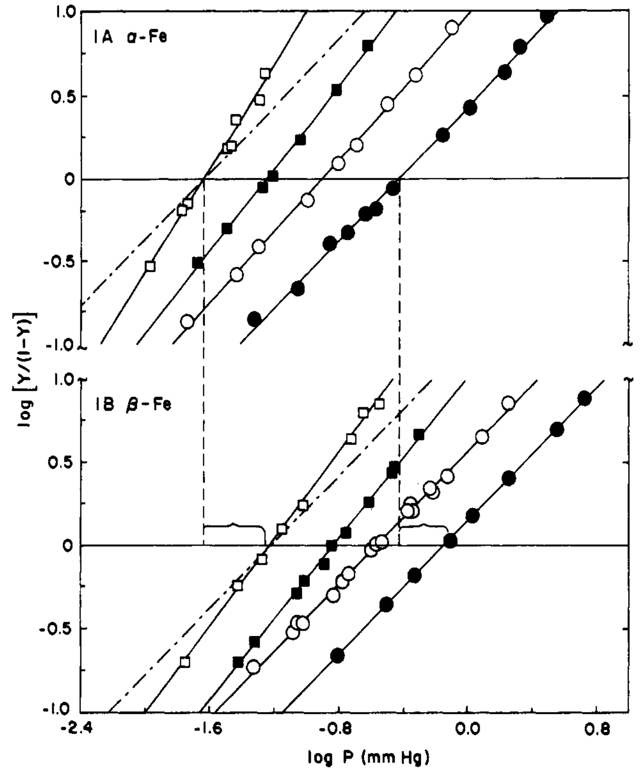

FIGURE 1: Hill plots of equilibrium CO binding to the ferrous chains within the mixed-metal [Mn(II),Fe(II)] hybrid hemoglobins. Y is fractional CO saturation of the ferrous subunits; P is partial pressure of CO in millimeters of Hg. Conditions are as follows: 25 °C; heme concentration 3–4 μM; pH 6.6 and 0.05 M Bis-Tris-HCl (●); pH 7.6 and 0.05 M Tris-HCl (○); pH 8.0 and 0.05 M Tris-HCl (■); pH 9.0 and 0.05 M Tris-HCl (□). (A) (Top) [α-Fe(II),β-Mn(II)] hybrid. (B) (Bottom) [α-Mn(II),β-Fe(II)] hybrid. Solid lines (—) represent least-squares fits to the data points. The slopes, n (Hill constant), and intercepts, which are the partial pressure of CO at half-saturation ($P_{1/2}$), are listed in Table I. Broken lines (---) represent Hill plots with slopes (n) equal to 1. The vertical tie lines are inserted to illustrate the differences in $P_{1/2}$ between the two hybrids.

Results

In this section, the results for CO binding to the Fe chains of [Mn(II),Fe(II)] hybrids are first presented. These data are then analyzed within a two-step Adair (thermodynamic) formulation. The pH dependence of the CO association constants thus obtained for the first and second binding steps in the α_1–α_2 and β_1–β_2 routes (Scheme I) is used to estimate the proton release associated with each step. The kinetics of CO binding and release by the Fe chains of [Mn(III),Fe(II)] hybrids are then shown to provide the individual chain binding constants of the fourth CO ligation step. Finally, these results are analyzed within an extended form of the MWC model (Ogata & McConnell, 1971; Edelstein, 1974).

Equilibrium CO Binding to the [Mn(II),Fe(II)] Hybrids. The results of these measurements are presented in the form

Table I: Half-Saturation Pressures ($P_{1/2}$) and Hill Coefficients (n) for Equilibrium CO Binding to [Mn(II),Fe(II)] Hybrid Hemoglobins[a]

	[α-Mn(II),β-Fe(II)]		[α-Fe(II),β-Mn(II)]	
pH	$P_{1/2}$ (mmHg)[b]	n[c]	$P_{1/2}$ (mmHg)[b]	n[c]
6.6	0.71	1.00 (5)	0.38	1.01 (5)
7.6	0.27	1.00 (5)	0.13	1.11 (5)
8.0	0.15	1.22 (5)	0.057	1.24 (10)
9.0	0.061	1.29 (10)	0.023	1.56 (10)

[a] Conditions are given in detail in the legend to Figure 1 (T = 25 °C; 0.05 M Tris-HCl or Bis-Tris-HCl). [b] These values are reproducible to within 10%. [c] Uncertainties in the least significant figure(s) are given in parentheses.

of Hill plots in panels A (α-Fe) and B (β-Fe) of Figure 1, and the values of the Hill plot slopes (n values) and intercepts ($P_{1/2}$) are listed in Table I. At pH 6.6, both hybrids bind CO noncooperatively (n = 1). The affinities ($1/P_{1/2}$) are ~10–20-fold lower than that for Hb under similar conditions (Anderson & Antonini, 1968), with that of the α-Fe hybrid about twice that of the β-Fe hybrid. Raising the pH to 7.6 increases the CO affinity of both hybrids by about 2.5–3.0-fold (decrease in $P_{1/2}$) without the appearance of significant cooperativity. Between pH 7.6 and 9.0, the affinities of the α- and β-Fe hybrids increase another 5-fold; more striking, ligand binding becomes cooperative, as indicated by Hill constants of greater than unity (Table I, Figure 1). The Hill constants for the two hybrids differ significantly at pH 9.0, with the α-Fe hybrid exhibiting higher cooperativity ($n \simeq 1.6$) than β-Fe ($n \sim 1.3$). Recalling that the maximum Hill plot slope is n = 2 for a system that binds two ligands such as the hybrids emphasizes the high degree of cooperativity.

Adair Formulation. Ligand binding to the ferrous chains of the Mn(II) hybrids represents specific ligation routes that form part of the process by which Hb binds four ligands, but the Mn(II) hybrids themselves bind only two ligands and must therefore be treated formally as two-site cooperative systems that undergo ligation by the phenomenological or Adair, two-step scheme (Scheme II). As indicated in the introduction and represented in Schemes I and II, the association constants for binding two ligands to an α-Fe hybrid correspond to the Adair binding constants $K_{1\alpha}$ and $K_{2\alpha}$ for the α_1–α_2 binding route and equivalently for the β-Fe hybrids. For clarity, the subscript indicating the hybrid type usually is suppressed.

Scheme II

$$\mathrm{Hy} + \mathrm{CO} \overset{K_1}{\rightleftharpoons} \mathrm{Hy(CO)} + \mathrm{CO} \overset{K_2}{\rightleftharpoons} \mathrm{Hy(CO)_2}$$

The CO binding curves of Figure 1 were analyzed following Wyman (1948) and Szabo & Karplus (1975) to obtain the association constants for the two ligation steps of each Mn(II) hybrid. In the absence of dissociation into dimers, the intrinsic association constants of the two steps are given by the following expressions

$$K_1 = \frac{2-n}{n[\mathrm{CO}]_{1/2}} \qquad K_2 = \frac{n}{(2-n)[\mathrm{CO}]_{1/2}} \qquad (1)$$

where n is the slope of the Hill plot, and $[\mathrm{CO}]_{1/2}$ is the free-ligand concentration at half-saturation. At the concentrations of hybrid used in these studies, some dissociation into dimers is expected. Although this could lead to errors in the calculated K_1 and K_2 and to asymmetric Hill plots (Szabo & Karplus, 1975), in practice it does not; the binding curves are symmetric and calculations on the basis of estimates of dimerization (see paragraph at end of paper regarding supplemental material) reveal that eq 1 gives good approximations to the true binding constants.

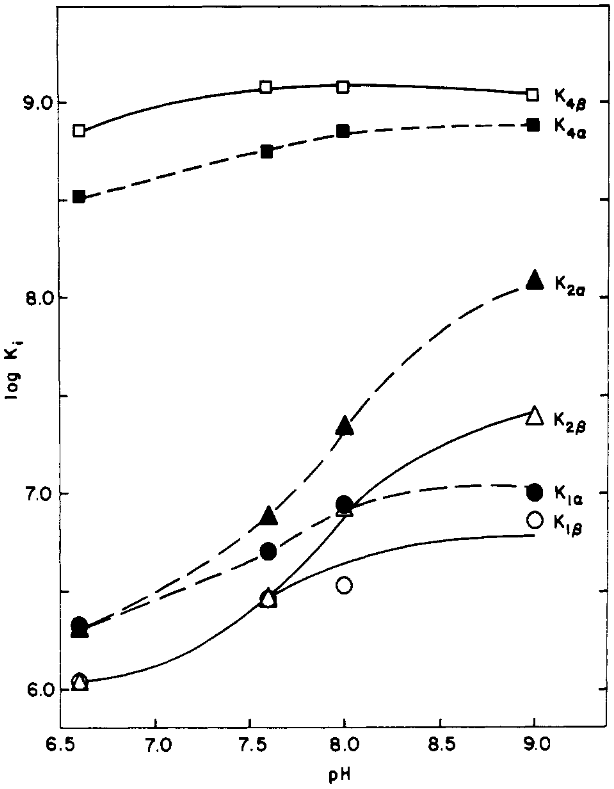

FIGURE 2: Effect of pH on the association constants [K (M^{-1})] for CO binding to Hb, obtained by use of [Mn,Fe(II)] hybrid hemoglobins. The constants are defined in Scheme I. Solid symbols indicate Hb α-chain CO association constants; open symbols denote Hb β-chain CO binding steps. K_{1i} and K_{2i} ($i = \alpha$ or β) were obtained from equilibrium CO binding measurements of the [Mn(II),Fe(II)] hybrids and are based on a CO solubility of 956 μM/atm at 25 °C. The K_{4i} values were obtained from the [Mn(III),Fe(II)(CO)] hybrids and were calculated from the measured on- and off-rate kinetic constants. Conditions are described in legend to Figure 1.

At pH 6.6, the α and β chains have unequal, low affinities, and for each hybrid the binding constant for the first and second ligand is the same ($K_1 = K_2$) (Figure 2). For either chain, an increase in pH from 6.6 to 9.0 increases the affinity for the first CO only by a factor of ~5. However, for α chains the affinity for the second CO ($K_{2\alpha}$) increases by a factor of ~60, whereas that for binding to the second β chain ($K_{2\beta}$) increases by ~20. Indeed, the observation of cooperative CO binding ($n > 1$) at high pH reflects the fact that the second ligand binds more strongly than the first: $K_2/K_1 > 1$ (Figure 2).

Proton Release (Bohr Effect) in the First and Second Hb Ligation Steps. The pH dependence of the CO affinity of the Fe chains within the [Mn(II),Fe(II)] hybrids reflects the heterotropic linkage between CO and proton binding and can be used to calculate an average value for proton release upon CO binding to the two ferrous chains of a hybrid. For two-site systems, such as these, which undergo an affinity change of $\Delta \log P_{1/2}$ over the pH interval ΔpH, the approximate total proton release upon binding two ligands, ΔH^+, and the average proton release per binding site, $\overline{\Delta H^+}$, are given by (Baldwin, 1975)

$$\overline{\Delta H^+} = \frac{\Delta H^+}{2} \doteq -\frac{\Delta \log P_{1/2}}{\Delta \mathrm{pH}} \qquad (2)$$

The proton release associated with a particular step in a given route, ΔH^+_j, j = 1 or 2, can be estimated from the pH dependence of the equilibrium constant

$$\Delta H^+_j \simeq \Delta \log K_j/\Delta \text{pH} \qquad (3)$$

with $\overline{\Delta H^+} = (\Delta H^+_1 + \Delta H^+_2)/2$.

The pH dependence of the $P_{1/2}$ values over the total pH interval examined, pH 6.6–9.0 (Table I), corresponds to an average proton release at the midpoint of the interval, pH 7.8, of $\Delta H^+ = 0.50$ and 0.45 per heme for the α-Fe and β-Fe hybrids, respectively. Since the total proton release in native Hb at this pH is ~1.9 (Imai & Yonetani, 1975), about one-half of the total Hb Bohr effect is provided in the first two binding steps along either of the ligation routes of Scheme I.

In the low pH portion of the range, pH 6.6–7.6, in each of the ligation pathways, the affinity constants, K_1 and K_2, show a similar variation with pH (Figure 2); thus, proton release is the same for the first and second CO binding steps and, consequently, is directly proportional to the extent of ligation. In this pH range, both hybrids remain largely in the T state while binding CO noncooperatively ($n = 1$) and with low affinity (Blough et al., 1980; Blough & Hoffman, 1982); thus, the associated proton release must arise from tertiary conformational changes within the T structure. The calculated value, ΔH^+ ~0.4 at pH 7.1, is in good agreement with the average proton release of the α and β subunits obtained from the pH dependence of the first Adair constant for O_2 binding and corresponds to ~80% of the average proton release per subunit for Hb, $\overline{\Delta H^+} = 0.5$, at this pH value (Imai & Yonetani, 1975).

The demonstration that ligation of either α or β chains within the T state gives a large proton release at low pH is unexpected. It is known that ligation of the β chain within the T state breaks a salt bridge involving His-146β and results in a proton release of the size measured here (Kilmartin et al., 1978). However, the only confirmed α-chain contribution comes from breakage of the Val-1α–Arg-141α salt bridge, and its magnitude is only half as large (Kilmartin et al., 1978, 1973). This suggests that additional groups within the α chains contribute to the T-state tertiary Bohr effect; they might correspond to the several α-chain histidines identified by Ohe & Kajita (1980) and Nishikura (1978).

In the high pH range, 7.6–9.0, $K_{1\alpha}$ and $K_{1\beta}$ increase similarly (Figure 2), indicating that the T-state Bohr effect remains the same for the α and β chains. However, the appearance of cooperativity ($n > 1$) at elevated pH reflects the fact that K_2 for each chain increases more with pH than the related K_1 (Figure 2). This implies unequal proton release at the successive CO binding steps ($\Delta H^+_2 > \Delta H^+_1$) and proton release that is not directly proportional to CO uptake. Since the pH dependence of $K_{2\alpha}$ appears greater than that of $K_{2\beta}$, the total proton release in the second binding step is greater for the α_1–α_2 route (eq 3).

Determination of K_4 for the Individual Chains within Hb. The affinity of the ferrous subunits within the [Mn(III),Fe(II)] hybrids is so high that direct equilibrium binding measurements were impossible with our experimental procedures; instead, CO binding was studied by kinetic methods. When the level of CO photolysis from either [Mn(III),Fe(II)(CO)] hybrid was 10% or below, CO recombination to the monoliganded photolysis product was fast and monophasic over the full pH range, 6.6–9. In addition, NO replacement studies showed CO dissociation from either hybrid to be monophasic and slow. The rate constants for both CO recombination and dissociation are similar to those previously measured for HbCO (DeYoung et al., 1976; McDonald & Noble, 1972), and thus we conclude that the monoliganded, [Mn(III)$_2$; Fe(II)CO,-Fe(II)] hybrids are fully in the high-affinity, or R structural form over the full pH range, 6.6–9. On the basis of the above results, the rate constant for the rapid CO recombination to a given chain at low photolysis is assigned to the on-rate of the fourth Hb binding step, l_4', and the ligand replacement rate is assigned to the off-rate for the fourth Hb CO binding step, l_4; the results are shown in Table II.

Table II: Fourth CO Association, l_4', and Dissociation, l_4, Rate Constants for the Individual Fe(II) Chains within Hb Obtained with the [Mn(III),Fe(II)] Hybrids[a]

	[α-Fe(II),β-Mn(III)]		[α-Mn(III),β-Fe(II)]	
pH	l_4 (×10^{-6} M^{-1} s^{-1})	l_4 (s^{-1})	l_4 (×10^{-6} M^{-1} s^{-1})	l_4 (s^{-1})
6.6	5.9 (5)	0.018 (2)	6.5 (5)	0.009 (1)
7.6	9.0 (5)	0.016 (2)	9.6 (5)	0.008 (1)
8.0	9.8 (5)	0.014 (2)	9.6 (5)	0.008 (1)
9.0	9.8 (5)	0.013 (2)	9.8 (5)	0.009 (1)

[a] Association rate constants obtained at 25 °C from flash photolysis measurements; dissociation rates were measured at 25 °C by NO replacement of CO.

The on-rates for the α- and β-Fe chains are large and indistinguishable at all values of pH, $l_4' \sim 6 \times 10^6$ M^{-1} s^{-1}. They increase minimally (~50%) between pH 6.6 and 7.6 and then remain constant until pH 9.0. These results are in good agreement with those obtained for HbCO by DeYoung et al. (1976). The off-rate for β-Fe remains constant, $l_4 = 0.009$ s^{-1}, throughout the pH interval 6.6–9.0 (Table II). The off-rate, l_4, for the α-Fe chain at low pH is twice that of β-Fe, and it decreases slightly with pH. For comparison, we find the overall CO off-rates for HbCo at 25 °C to be roughly pH independent with $l_4 \approx 0.01$ s^{-1}, which falls between and thus appears to be an average of the rates for the individual chains within a tetramer. In contrast, neither the on- nor off-rate constants of isolated chains appear to reproduce the tetramer rate constants for the fourth binding step (McDonald & Noble, 1972).

The microscopic association constants for the fourth CO ligation step of Hb were calculated from the rate ratios, $K_4 = l_4'/l_4$, and are plotted in Figure 2. At every pH measured, $K_{4\beta}$ is larger than $K_{4\alpha}$, because of the lower β-Fe chain CO off-rate. Both $K_{4\alpha}$ and $K_{4\beta}$ remain constant between pH 8 and 9 and thus show that at elevated pH neither chain releases protons at the fourth CO binding step (eq 3). However, both $K_{4\alpha}$ and $K_{4\beta}$ increase modestly (ca. 2-fold) in the range pH 6.6–7.6. This corresponds to the release of approximately 0.20 proton as a consequence of tertiary conformational changes when a CO binds to the high-affinity, R structure. The R-state Bohr effect is thus about half that of the T-state Bohr effect (see above) in this pH range. Its value is consistent with the data of DeYoung et al. (1976) but is about twice that calculated from the pH dependence of the fourth Adair constant for O_2 binding (Imai & Yonetani, 1975). The discrepancy could mean that the R-state tertiary Bohr effect is different for O_2 and CO, but it is more likely that the fitting procedures used to obtain the fourth Adair constant from Hb A O_2 binding curves are incapable of fully resolving these small differences.

Analysis of Binding Data within the Extended MWC Model. The experimental results presented here have been discussed within the fundamental framework of the MWC model for Hb cooperativity, namely, that Hb can adopt either a low- (T) or high- (R) affinity form. The model was originally parameterized in terms of the concentration ratio of the unliganded structures, $[T_0]/[R_0] = L_0 \gg 1$, and the ratio of ligand affinities in the T and R states, $C = K_T/K_R < 1$. The observed differences between α- and β-chain association re-

Table III: CO Binding Parameters according to the Extended MWC Allosteric Model for Hb Cooperativity[a]

pH	$K_{T\beta}$ ($M^{-1} \times 10^{-6}$)	$K_{R\beta}$ ($M^{-1} \times 10^{-8}$)	C_β	$K_{T\alpha}$ ($M^{-1} \times 10^{-6}$)	$K_{R\alpha}$ ($M^{-1} \times 10^{-8}$)	C_α
6.6	1.1 (2)	7.2 (10)	0.0015 (4)	2.1 (3)	3.3 (5)	0.0064 (7)
7.6	2.9 (4)	12 (2)	0.0024 (7)	4.9 (7)	5.6 (8)	0.0089 (27)
8.0	3.4 (7)	12 (2)	0.0028 (10)	8.6 (17)	7.0 (10)	0.012 (4)
9.0	7.2 (14)	11 (2)	0.0065 (22)	9.8 (20)	7.5 (11)	0.013 (4)

[a] Parameters are defined and obtained from the data in Table I and Figure 2 as described in the text and eq 4–7. The equilibrium constants are based on a CO solubility of 956 μM/atm at 25 °C. Parentheses contain uncertainties in least significant figure(s).

quire the use of an extended MWC model, such as that of Ogata & McConnell (1971), in which the α and β chains within the T and R structures are allowed to have different ligand affinities represented by the *association* constants $K_{T\alpha}$ and $K_{T\beta}$ and $K_{R\alpha}$ and $K_{R\beta}$, respectively. The single affinity ratio, C, is replaced by the ratios of the affinities for the individual chains in the T and R state:

$$C_i = K_{Ti}/K_{Ri} \qquad i = \alpha \text{ or } \beta \tag{4}$$

If $K_{T\alpha} = K_{T\beta}$ and $K_{R\alpha} = K_{R\beta}$, then this model reduces to the original MWC model.

The Adair constants for binding the first, second, and last (fourth) ligand to the α-Fe hybrid, as defined in Scheme I and measured in this study, can be analyzed within the model (Baldwin, 1975):

$$K_{1\alpha} = K_{R\alpha}(L_0C_\alpha + 1)/(L_0 + 1) \tag{5}$$

$$K_{2\alpha} = K_{R\alpha}(L_0C_\alpha^2 + 1)/(L_0C_\alpha + 1) \tag{6}$$

$$K_{4\alpha} = K_{R\alpha}(L_mC_\alpha^2 + 1)/(L_mC_\alpha + 1) \tag{7}$$

where $L_m \equiv L_0C_{\beta^+}^2$, with C_{β^+} being the "affinity ratio" associated with oxidation of the Mn-substituted β chains (Szabo & Karplus, 1975). Switching subscripts gives the expressions for the β-Fe hybrid.

Through use of these equations, the Adair constants for the [Mn(II),Fe(II)] hybrids and the association constants for the [Mn(III),Fe(II)] hybrids can be used to obtain the MWC parameters. From the discussion in the previous section, one sees that the K_{Ri} values are obtainable from the data on the Mn(III) hybrids. For these species, eq 7 and its partner for the β-Fe hybrid reduce to the result $K_{Ri} = K_{4i}$ at all values of pH. With the K_{Ri} values thus established, the binding data for the Mn(II) hybrids are used to evaluate K_T and L_0, the evaluation being performed differently at low and at high pH. At pH 6.6, CO binding to the ferrous chains via either the α_1–α_2 or the β_1–β_2 ligation routes (Scheme I) occurs with low affinity and noncooperatively ($n = 1$, $K_1 = K_2$). The MWC, T-state binding constants are simply equal to the Adair constants, $K_{1i} = K_{2i} = K_{Ti}$, and the tetramer remains predominantly in the T, or low-affinity, structure even after binding two ligands, $L_0 \gg 1$, $L_0C_i >> 1$, and $L_0C_i^2 > 1$ (eq 5 and 6). Lower limits for L_0 were determined by systematically varying L_0 in eq 5 and 6, seeking the smallest value that can maintain $K_{1i} = K_{2i}$, within experimental error. At higher pH, CO binding by the [Mn(II),Fe(II)] hybrids is cooperative, which requires that there be T → R conformational conversion during the ligation process. From eq 6, the inequality $K_2/K_1 > 1$ implies that $L_0C^2 \leq 1$, which means that conversion to R is 50% or greater after the second CO binding step. However, analysis of the Adair constants according to eq 5 and 6 shows that in all cases the T → R conversion is negligible after a single ligand is bound, and thus, the MWC parameters, K_{Ti}, are equal to the phenomenological constants, K_{1i}. With K_{Ti} and K_{Ri} established, their ratio gives C_i; L_0 is then directly calculable from the phenomenological constants, K_{2i}, with eq 6. *Significantly*, the higher degree of cooperativity exhibited by the α-Fe hybrid reveals that T → R conversion is greater with CO binding to the α-chains than to the β-chains.

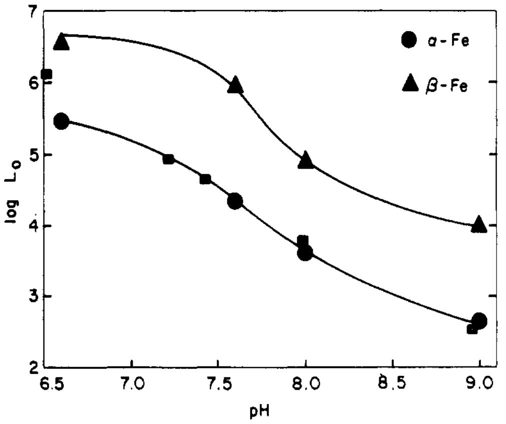

FIGURE 3: Effect of pH on the MWC allosteric constant, L_0, calculated from the binding data for the α_1–α_2 (●) and for the β_1–β_2 (▲) Hb ligation routes (Scheme I) with the measured values of K_{Ri} and C_i (Table III) and K_{2i} (Figure 2). The values of L_0 at pH 6.6 and 7.6 for the β_1–β_2 route and at pH 6.6 for the α_1–α_2 route represent lower limits for this parameter. Values for Hb from the data of Chu & Ackers (1981), representing the ratio of dimer–tetramer association constants for deoxy and liganded Hb, $L_0 = {}^0K_2/{}^4K_2$ (Edelstein, 1975) (see supplementary material), are included for comparison (■).

The ratios of the T- and R-state CO affinity constants, C_α and C_β, are presented in Table III. They are significantly smaller than previous estimates (Sharma et al., 1976) and range from $C_\beta = 0.0015$ (pH 6.6) to 0.0065 (pH 9) for the β-Fe chain and, correspondingly, $C_\alpha = 0.0064$–0.013. At every pH employed, C_α is larger than C_β, although these quantities become closer at high pH. At pH 6.6, where $C_\alpha \sim 4C_\beta$, examination of the individual constants shows that about half the difference arises from the differences in K_R and half from differences in K_T; the α-chains have a higher affinity than the β-chains within the T state but lower affinity in R state. With increasing pH, the difference between R- and T-state affinities decreases (both C_α and C_β increase), a result due primarily to the increase in K_T. In the interval, pH 6.6–9.0, C_β increases ca. 4-fold, while C_α increases by a factor of ~2. This translates to a pH-dependent decrease in the binding free energy difference between the T and R states ($\Delta G° = -RT \ln C$) of about 0.9 kcal/mol for the β chains and ~0.4 kcal/mol for the α chains.

The pH dependence of L_0 is plotted in Figure 3. The allosteric constant obtained from either route is quite large ($L_0 \sim 10^6$) at pH 6.6. A reduction of over 2 orders of magnitude by pH 9 corresponds to a major coupling between proton binding and quaternary structure stability and is the chief reason CO binding to the [Mn(II),Fe(II)] hybrids at high pH is cooperative ($n > 1$) and accompanied by a T → R conversion. The proton release upon T → R transition of the unliganded tetramer (ΔH^+_Q) is approximated by $\Delta H^+_{Q_0} \simeq -\Delta \log L_0/\Delta \text{pH}$, in analogy to eq 2 and 3. The large quaternary proton release indicated by the data in Figure 3 is not inconsistent with the rather large tertiary T-state Bohr effect observed in both hybrids. Rather, it merely requires that these protons can be released either through ligation *or* quaternary structure change (Shulman et al., 1975; Baldwin, 1975).

The use of this model to interpret the present results, however, does suffer from an internal inconsistency. Equations 5 and 6 indicate that the [Mn(II),Fe(II)] hybrid containing the ferrous chain with the smaller value of C (greatest T − R affinity difference) will exhibit the largest cooperativity. However, $C_\beta < C_\alpha$, yet the Hill constants for the α_1–α_2 ligation route are equal to or greater than those for the β_1–β_2 route (Tables I and III). This problem becomes apparent when eq 6 is used to calculate L_0 from K_{2i}, C_i, and K_{Ri}. Although the MWC model admits but a single value for the allosteric constant, the L_0 values obtained from constants for the α_1–α_2 pathway are more than 10-fold lower than those for β_1–β_2 (Figure 3). This difference cannot be explained by dimer formation (see paragraph at end of paper regarding supplementary material).

Discussion

In this study, [Mn,Fe] hybrid hemoglobins have been used to examine microscopic CO ligation routes not directly observable in hemoglobin A. The measurements provide model-independent, association constants for binding the first and fourth CO to the individual Hb chains, as well as for binding the second ligand to the same chain type. These results also have been used to calculate the affinity ratios C_α and C_β for an extended allosteric (MWC) model for cooperativity. By further examination of the pH dependence of these constants, estimates of the chain contributions to both the T and the R-state tertiary CO Bohr effect have been obtained.

The T- and R-state CO affinity constants (Table III) show only modest, 2-fold, $\alpha - \beta$ affinity differences within a particular quaternary structure; inequivalence within the T state is accentuated in the presence of inositol hexaphosphate, with the α-Fe subunit binding with an ~3-fold greater affinity than β-Fe (Blough & Hoffman et al., 1982). Although the chain affinities differ by only 2-fold within the individual quaternary structures, these differences compound so as to magnify the inequivalence in the ratios of T- and R-state affinities (C_α vs. C_β). For example, at pH 6.6, the affinity of the T-state β chains is reduced by a factor of $C_\beta^{-1} \sim 670$ relative to the R state, but the α chains show only a reduction of $C_\alpha^{-1} \sim 160$. At pH 9.0, affinity reduction in T state is less, as is the chain difference: $C_\beta^{-1} \sim 150$; $C_\alpha^{-1} \sim 80$. The pH dependence of the T/R affinity ratio primarily, but not exclusively, reflects changes in the T-state CO binding constants (Figure 2 and Table III).

The present results are consistent with the NMR measurements of Johnson & Ho (1974) on Hb and with kinetic measurements of CO binding (Parkhurst, 1979) and release (Sharma et al., 1976). However, the Hb spin-label study of Lau & Asakura (1980) is not in accord, presumably because of problems associated with the bulky reporter group. More important is the discrepancy presented by equilibrium CO binding measurements on the [Co(II),Fe(II)] hybrids (Ikeda-Saito & Yonetani, 1980). Ikedo-Saito & Yonetani (1980) have proposed that CO binding to the ferrous chains in the [Co(II),Fe(II)] hybrids represents the ligand affinities for the α- and β-Fe subunits in the first ligation step of Hb. However, although their affinities for the α-Fe hybrid are within 20% of ours, they find that the [α-Co(II),β-Fe(II)] hybrid binds CO with an affinity that is at least 10-fold greater than the [α-Fe(II),Co(II)] hybrid; this is close to, if not greater than, that for CO binding to Hb. This high affinity must reflect the fact, noted previously (Blough et al., 1980), that the replacement of Fe(II) by Co(II) in the two α subunits destabilizes the T state (reduces L_0) so effectively that the unliganded [Co(II),Fe(II)] hybrids are not fully in this state.

The [Fe(II),Fe(III)(CN)], deoxycyanomet valency hybrids cannot be used to probe the first two Hb ligation steps, but they do provide models for diliganded Hb intermediates. In contrast to the diliganded [Mn(II),Fe(II)(CO)] hybrids, the deoxycyanomet derivatives are largely in the R state at pH 6.6 (Cassoly & Gibson, 1972). Because of this, the MWC model predicts that their dimer–tetramer association constants will closely approach that for oxyhemoglobin (see paragraph at end of paper regarding supplementary material). However, Cassoly & Gibson (1972) found that the extent of dimerization actually is very small. The discrepancy suggests that oxidation and CN$^-$ binding to the Fe chains in the valency hybrids produce an intermediate liganded state that is not describable by the MWC model and that is different from that obtained by CO binding to the ferrous chains of the Mn(II) hybrids.

In contrast to the valency hybrids, the [α-Fe(II),β-Mn(II)] hybrid appears well behaved. When hemoglobin binds the first two ligands to α chains, as represented by CO binding to the α chains of this hybrid, the allosteric constants (L_0) calculated from the equations of the modified MWC model fall very close to those for Hb (Chu & Ackers, 1981; Figure 3) and also to those from curve fitting of O_2 equilibrium binding data (Imai & Yonetani, 1975; Ilmartin et al., 1978; Imai, 1979, 1973); in particular, note the expected decrease with increasing pH (Baldwin, 1975). Moreover, our crude estimates of the tetramer–dimer association constants give reasonable values (see paragraph at end of paper regarding (supplementary material).

The [α-Mn(II),β-Fe(II)]hybrids also behave as a faithful hemoglobin analogue. However, the values of L_0 calculated when hemoglobin binds the first two ligands to β chains, as represented by CO binding to the β chains of [α-Mn(II),β-Fe(II)], are more than 10-fold larger than those for the α-Fe hybrid (Figure 3). Calculation of different values of L_0 from the binding data for the [α-Fe(II),β-Mn] and [α-Mn,β-Fe(II)] hybrids indicates that the ligation process is not fully described by the MWC model, eq 5–7, and implies the presence of direct subunit interaction. In fact, Weber (1981) recently has argued that the positive asymmetry observed for ligand binding to Hb is possible only if α–α and β–β subunit interactions change by different amounts upon ligation and that the β–β subunit interaction (constraint) is larger than that for α–α. Our calculation that L_0 is largest for the β-Fe hybrid, reflecting the observation that the CO affinity and cooperativity are less for the β_1–β_2 ligation route than those for α_1–α_2, supports this idea. These "diagonal", β–β constraints, which must be small with respect to the symmetric constraints in stripped Hb (Weber, 1981), would have been masked in earlier studies of hemoglobin, because it was not possible to separate the binding behavior of the α and β chains and to study individual ligation intermediates directly.

Supplementary Material Available

pH dependence of estimated dimer–tetramer binding constants for the diliganded [Mn(II),Fe(II)CO] hybrids and an analysis of possible effects of this dimerization on the measurement of $K_{1\alpha(\beta)}$ and $K_{2\alpha(\beta)}$, shown to be of minimal importance (4 pages). Ordering information is given on any current masthead page.

Registry No. CO, 630-08-0.

References

Adler, A. D., Longo, F. R., Kampas, F., & Kim, J. (1970) *J. Inorg. Nucl. Chem. 32*, 2443–2445.

Anderson, S. R., & Antonini, E. (1968) *J. Biol. Chem. 243*, 2918–2920.

Baldwin, J. M. (1975) *Prog. Biophys. Mol. Biol. 29*, 225–320.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Blough, N. V., & Hoffman, B. M. (1982) *J. Am. Chem. Soc. 104*, 4247–4250.

Blough, N. V., Zemel, H., Hoffman, B. M., Lee, T. C. K., & Gibson, Q. H. (1980) *J. Am. Chem. Soc. 102*, 5683–5685.

Blough, N. V., Zemel, H., & Hoffman, B. M. (1984) *Biochemistry* (following paper in this issue).

Cassoly, R., & Gibson, Q. H. (1972) *J. Biol. Chem. 247*, 7332–7341.

Chu, A. H., & Ackers, G. K. (1981) *J. Biol. Chem. 256*, 1199–1205.

DeYoung, A., Pennelly, R. R., Tan-Wilson, A. L., & Noble, R. W. (1976) *J. Biol. Chem. 251*, 6692–6698.

Dinello, R. K., & Dolphin, D. (1975) *Anal. Biochem. 64*, 444–449.

Edelstein, S. J. (1974) *Biochemistry 13*, 4998–5002.

Edelstein, S. J. (1975) *Annu. Rev. Biochem. 47*, 209–232.

Geraci, G., Parkhurst, L. J., & Gibson, Q. H. (1969) *J. Biol. Chem. 244*, 4664–4667.

Gibson, Q. H., & Hoffman, B. M. (1979) *J. Biol. Chem. 254*, 4691–4697.

Gibson, Q. H., Hoffman, B. M., Crepeau, R. H., Edelstein, S. J., & Bull, C. (1974) *Biochem. Biophys. Res. Commun. 59*, 146–152.

Gonzales, B., Kouba, J., Yee, S., Reed, J. F., Keiner, J. F., & Sheidt, W. R. (1975) *J. Am. Chem. Soc. 97*, 3247–3249.

Hoffman, B. M. (1979) *The Porphyrins* (Dolphin, D., Ed.) Vol. VIIB, pp 403–444, Academic Press, New York.

Hoffman, B. M., Gibson, Q. H., Bull, C., Crepeau, R. H., Edelstein, S. J., Fisher, R. G., & McDonald, M. J. (1975) *Ann. N.Y. Acad. Sci. 244*, 174–186.

Ikeda-Saito, M., & Yonetani, T. (1980) *J. Mol. Biol. 138*, 845–858.

Imai, K. (1973) *Biochemistry 12*, 798–807.

Imai, K. (1979) *J. Mol. Biol. 133*, 233–247.

Imai, K., & Yonetani, T. (1975) *J. Biol. Chem. 250*, 2227–2231.

Johnson, M. E., & Ho, C. (1974) *Biochemistry 13*, 3653–3661.

Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., & Ho, C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1246–1249.

Kilmartin, J. V., Imai, K., Jones, R. T., Faruqui, A. R., Fogg, J., & Baldwin, J. M. (1978) *Biochim. Biophys. Acta 534*, 15–25.

Lau, P., & Asakura, T. (1980) *J. Biol. Chem. 255*, 1617–1622.

McDonald, M. J., & Noble, R. W. (1972) *J. Biol. Chem. 247*, 4282–4287.

Moffat, K., Loe, R. S., & Hoffman, B. M. (1976) *J. Mol. Biol. 104*, 669–685.

Monod, J., Wyman, J., & Changeaux, J. P. (1965) *J. Mol. Biol. 12*, 88–102.

Nishikura, K. (1978) *Biochem. J. 173*, 651–657.

Ogata, R. T., & McConnell, H. M. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 325–336.

Ohe, M., & Kajita, A. (1980) *Biochemistry 19*, 4443–4450.

Parkhurst, L. J. (1979) *Annu. Rev. Phys. Chem. 30*, 503–546.

Scheidt, W. R. (1977) *Acc. Chem. Res. 10*, 339–345.

Scholler, D. M., Wang, M.-Y. R., & Hoffman, B. M. (1979) *Methods Enzymol. 52C*, 487–493.

Sharma, V. S., Schmidt, M. R., & Ranney, H. M. (1976) *J. Biol. Chem. 251*, 4267–4272.

Shulman, R. G., Hopfield, J. J., & Ogawa, S. (1975) *Q. Rev. Biophys. 8*, 325–410.

Stanford, M. A., Swartz, J. C., Phillips, T. E., & Hoffman, B. M. (1980) *J. Am. Chem. Soc. 102*, 4492–4499.

Szabo, A., & Karplus, M. (1975) *Biochemistry 14*, 931–940.

Waterman, M. R., & Yonetani, T. (1970) *J. Biol. Chem. 245*, 5847–5852.

Weber, G. (1981) *Nature (London) 300*, 603–607.

Wyman, J. (1948) *Advances in Protein Chemistry*, pp 407–531, Academic Press, New York.

Yip, Y. K., Waks, M., & Beychok, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 64–68.