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The Intermediate Compounds between Human Hemoglobin and Carbon Monoxide at Equilibrium and during Approach to Equilibrium*

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The procedure of Perrella *et al.* (Perrella, M., Benazzi, L., Cremonesi, L., Vesely, S., Viggiano, G., and Rossi-Bernardi, L. (1983) *J. Biol. Chem.* 258, 4511–4517) for trapping the intermediate compounds between human hemoglobin and carbon monoxide was validated by quantitatively determining during the approach to equilibrium all the species present in a solution containing large amounts of intermediates. An accurate estimate of the intermediate compounds at 50% carbon monoxide saturation in 0.1 M KCl, pH 7, at 22 °C, allowed the calculation, according to Adair's scheme, of the four equilibrium constants.

At 50% ligand saturation, the pool of intermediate species was about 12% of the total. A slightly greater concentration of tri-liganded than mono-liganded species was found. Carbon monoxide bound to β chains in slightly greater excess with respect to α chains in both the mono- and tri-liganded species. The symmetrical bi-liganded intermediates, $\alpha_2\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2$, were absent. The nature of the bi-liganded intermediate found to be present in detectable amounts by our technique has yet to be clarified: it could be either the asymmetrical species $(\alpha\beta)(\alpha^{CO}\beta^{CO})$ and $(\alpha\beta^{CO})(\alpha^{CO}\beta)$ or both of them. Such a finding on the functional heterogeneity among the four possible bi-liganded intermediates is consistent with hypotheses of the existence of more than two quaternary structures in the course of ligand binding to hemoglobin.

The molecular mechanism of the cooperative interaction between hemoglobin and its physiological ligands is still an elusive problem in spite of the vast body of information on the structure and function of this protein (1–4). Most of the hemoglobin functional studies have been carried out on either liganded and unliganded hemoglobin or on solutions containing these species and unknown amounts of intermediate ligated species. Little is known about the functional properties of such intermediates. Crystals of deoxyhemoglobin exposed to oxygen and showing only the α chains in liganded state have been studied by x-ray methods (5). However, most of the crystallographic information available on hemoglobin has been obtained from the liganded and unliganded forms.

The model of allosteric ligand binding to proteins by Monod *et al.* (6), which has been widely applied to the interpretation of the relationship between the functional and structural data on hemoglobin, assumes that the properties of the intermediate species can be deduced from those of the liganded and unliganded species. The model states that multimeric proteins at any stage of the ligation process exist in equilibrium between two conformations, T, with low affinity for the ligand, and R, with high affinity, and that the transition between these conformations occurs by some concerted mechanism. The conformations of unliganded and liganded hemoglobin have been assumed to be, respectively, T and R on the basis of crystallographic and functional studies. A detailed stereochemical mechanism has been formulated by Perutz to describe the concerted conformational transition in hemoglobin (7–9).

However, thermodynamic studies on the tetramer-dimer equilibrium properties of the cyanomet-/deoxyhemoglobin hybrids have suggested the existence of more than two energetic states in the course of the stepwise ligand binding to hemoglobin (10). High resolution NMR studies on normal hemoglobin (11), on cross-linked valence hemoglobin hybrids (12), and on cross-linked asymmetrically modified hemoglobins (13) have also suggested the existence of more than two structures.

Clearly, further advancement in the understanding of the mechanism of hemoglobin cooperativity is possible only if more information is gathered on the structure and function of the intermediate ligated species.

A fundamental question regards which species are present under definite equilibrium and kinetic conditions and in what proportions. In principle, the distribution of intermediate compounds can be calculated from experimental data on hemoglobin-ligand equilibrium isotherms and from the kinetics of ligand binding to hemoglobin (14–18). However, these indirect estimates are critically dependent on the nature and precision of the experimental techniques, use of sophisticated techniques for the numerical analysis of the data, and experimental conditions, such as the protein concentration (19). Moreover, indirect calculations give no information about the functional heterogeneity of the subunits in hemoglobin. The assembly of two different subunits, α and β, in a tetrameric unit allows for the existence of two functionally different mono- and tri-liganded species. Since two distinct intersubunit contacts, $\alpha^1\beta^1$ and $\alpha^1\beta^2$, occur in the hemoglobin tetramer, functional heterogeneity is also possible between the two symmetrical bi-liganded species, $\alpha_2\beta_2^L$ [1] and $\alpha_2^L\beta_2$, and between these and the asymmetrical bi-liganded species, $(\alpha\beta)(\alpha^L\beta^L)$ and $(\alpha\beta^L)(\alpha^L\beta)$.

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[1] The abbreviations used are: $\alpha_2\beta_2^L$, hemoglobin with α chain in deoxy form and β chain in liganded form, the ligand being CO, CN^-, etc., and numerical subscripts indicating stoichiometry; $\alpha_2^+\beta_2^L$, hemoglobin with α chain in ferric form and β chain in liganded form; $(\alpha\beta)(\alpha^L\beta^L)$, hemoglobin with $\alpha^1\beta^1$ dimer in deoxy form and $\alpha^2\beta^2$ dimer in liganded form, numerical superscripts indicating the separate subunits within the tetramer.

Models of hemoglobin function should account, under definite conditions, for both the proportion of each intermediate species and the functional heterogeneity of the species in the same state of ligation. Thus a direct method for the identification and quantitation of such species would provide an essential piece of evidence and serve the purpose of validating proposed theoretical models.

In a previous paper we described a technique by which intermediate compounds between hemoglobin and carbon monoxide are trapped by rapidly quenching an aqueous solution of hemoglobin into a cryosolvent at −25 °C containing ferricyanide. The valence hybrids formed by oxidation of the unliganded hemes of the intermediates are then separated from met- and carbonylhemoglobin by isoelectric focusing at −25 °C and identified (20). A significant, although very small, pool of intermediate species, approximately 10% of the total, was found under equilibrium conditions at 20 °C, pH 7, and 50% carbon monoxide saturation.

The purpose of this paper is to give a reliable quantitative estimate of the equilibrium distribution of intermediate compounds between hemoglobin and carbon monoxide and to answer the question of the functional heterogeneity under these conditions of the species in the same ligation state. Thus we report here new and definite evidence on the validity of our procedure for trapping the intermediates, based on the study of the approach to equilibrium in solutions containing large amounts of intermediates and in reacting mixtures of deoxy- and carbonylhemoglobin. We then use the data on the equilibrium concentration of the intermediates to predict the carbon monoxide equilibrium curve of hemoglobin.

EXPERIMENTAL PROCEDURES

Quench-flow Apparatus—Materials and methods used for the isolation of intermediate compounds between hemoglobin and carbon monoxide and the apparatus designed to trap the intermediates under kinetic conditions are described elsewhere (20, 21). Apparatus and procedure are shown in diagrammatic form in Fig. 1. Before filling the syringes of the quench-flow apparatus with reactants, the dead volume of the syringes and the tubes connecting the syringes to the mixer were filled with 20 mM phosphate buffer, pH 7.4, containing 20 mg/ml dithionite and then thoroughly rinsed with reactants. The reactants were prepared under anaerobic conditions in glass tonometers.

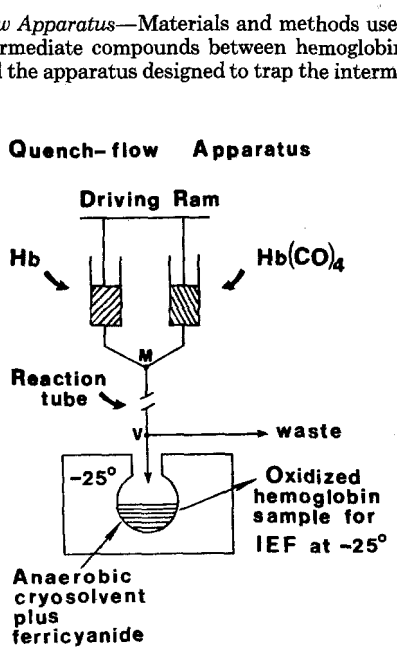

FIG. 1. **Diagrammatic representation of the quench-flow apparatus used for trapping intermediate compounds between hemoglobin and carbonmonoxide under kinetic conditions.** The syringes are filled with solutions of deoxy- and carbonylhemoglobin, respectively. Alternatively, one syringe is filled with a solution of partially oxidized carbonylhemoglobin and the other syringe with a solution of dithionite. *M* is a "ball-mixer" (22) and *V* is a sampling valve through which the reactants are expelled into a cryosolvent at −25 °C containing ferricyanide. The cryosolvent is contained in a glass tonometer thermostatted at −25 °C under anaerobic conditions and is mechanically shaken during sample quenching. A sample of quenched reactants is then transferred onto a gel tube for IEF at −25 °C.

Quenching Medium—Samples of reactants (0.1 ml) were quenched into a cryosolvent (0.8 ml) prepared by diluting in a 1 to 1 ratio 20 mM phosphate, pH 7.5, at 20 °C, and ethylene glycol. The concentration of hemoglobin in the samples was 4–5 mM (heme concentration). The pH of the cryosolvent was about 8.5 at −25 °C (23). Ferricyanide was added to the cryosolvent in a 10-fold molar excess with respect to the unliganded heme content of the samples. When the products of the reaction between partially oxidized carbonylhemoglobin and dithionite were quenched, the concentration of ferricyanide in the cryosolvent had to be enough to oxidize excess dithionite and also to reoxidize the unliganded hemes of hemoglobin. The optimal conditions for these experiments were checked in preliminary tests using solutions of methemoglobin.

Modified Quenching Procedure—Thirty s or 3 min after quenching a hemoglobin sample, the pH of the quenching medium at −25 °C was raised to 10–11 by the addition of 20–30 μl of a 0.5 M solution of Tris in 50% ethylene glycol (v/v). The purpose of such an addition was to slow down both the rate of oxidation of the deoxy hemes which could be formed by CO dissociation (24) and the rate of dimer exchange at subzero temperature (25).

We previously showed (26) that after removal of buffer salts and ferricyanide during the first electrophoretic phase of IEF at −25 °C (5–10 min), the partially oxidized compounds are stable for the next 20–25 h. Before salt removal, dimer exchange reactions can occur in the usual quenching medium at −25 °C, as indicated by the formation of about 20% hybrid in mixtures containing equal amounts of $\alpha_2^+\beta_2^{CO}$ and either met- or carbonylhemoglobin. Such hybrid formation accounted for less than 5% of the total when the modified quenching procedure was used.

Quantitation of the Quenched Hemoglobin Components—This was carried out after the IEF separation, by scanning at 465 nm color slides of the gels in a Cliniscan (Helena Laboratories, Beaumont, TX) (20). The accuracy of the method was tested by comparing the data from the scan of a well resolved mixture of carbonylhemoglobins A_0 and C and their hybrid with the concentrations of the same components eluted from the sliced gel, as measured by a micromethod for hemoglobin detection (21). The agreement was found to be within 2% of the total. Instead of using deconvolution for the integration of the areas under overlapping peaks, we assumed the border line to be at the point of inflexion. This simple, although not rigorous, procedure, reproducibly measured a value for the concentration of hemoglobin A_{1c} in the range of 3.5–4.5% in lysates where the peaks of hemoglobins A_0 and A_{1c} overlapped. This compares well with the average content of hemoglobin A_{1c} in samples of blood from nondiabetic donors (3.3–3.5%), as measured by chromatographic methods (27).

Curve Fitting—Data on the hemoglobin-carbon monoxide equilibrium were fitted according to Adair's equation (28) using the weighting procedure of Winslow *et al.* (29) and the nonlinear regression of Shrager (30).

RESULTS

Isolation of the Intermediates during Approach to Equilibrium

Quantitative Recovery of the Intermediates—In a previous paper (20) we described a test in support of the validity of our procedure for the determination of intermediate compounds. It was based on the spectrophotometric measurement of the deoxyhemoglobin content in different equilibrium mixtures of deoxy- and carbonylhemoglobin and the methemoglobin content in samples of these solutions quenched at subzero temperature in cryosolvent containing ferricyanide. Since the concentration of intermediate compounds at equilibrium was found to be very small, such a test has limited sensitivity. To test rigorously the accuracy of the determination of the intermediates, the isolation and quantitation procedures were car-

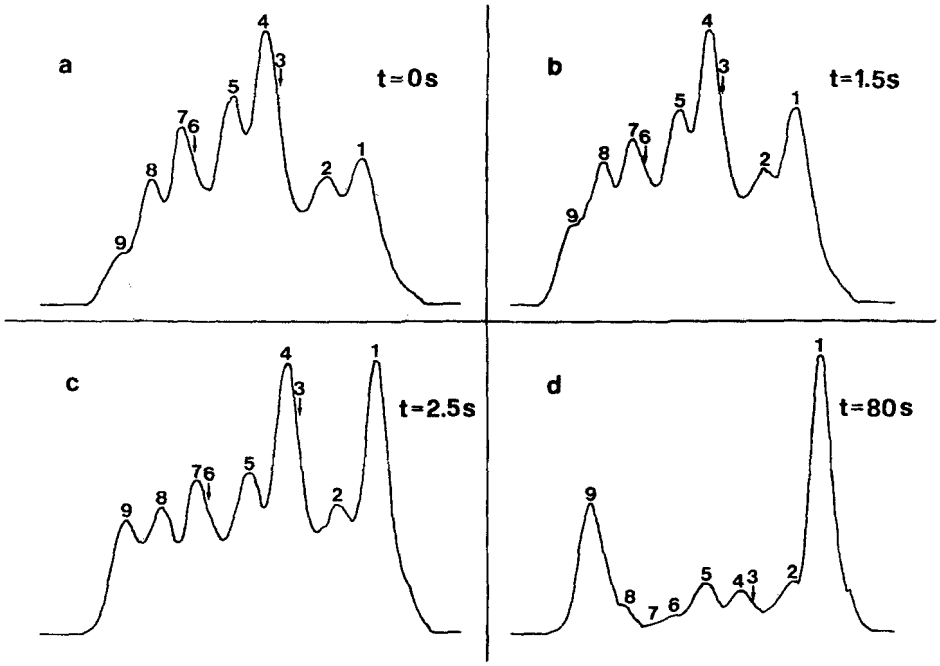

FIG. 2. **Densitometer scans of color slides of gel tubes used for the separation by IEF at −25 °C of the products of quenching a sample of partially oxidized carbonylhemoglobin (4 mM heme concentration in 10 mM phosphate, 0.05 M KCl, pH 7) before reaction at 22 °C with dithionite (*a*) and after reaction with dithionite for times varying from 1.5 to 80 s (*b–d*).** After a 15-min reaction a scan similar to *d* was obtained. Dithionite and ferricyanide were in 20- and 40-fold molar excess, respectively, with respect to the hemoglobin concentration. Areas under the peaks, in per cent, and identification of the components to which the peaks in scan *a* refer are as follows: 1, carbonylhemoglobin, 16%; 2, $\alpha_2^{CO}\beta^+\beta^{CO}$, 11%; 4, $\alpha^+\alpha^{CO}\beta_2^{CO}$, 27%; 5, $(\alpha^+\beta^+)(\alpha^{CO}\beta^{CO})$ and $(\alpha^+\beta^{CO})(\alpha^{CO}\beta^+)$, 18%; 7, $\alpha_2^+\beta_2^{CO}$, 15%; 8, $\alpha_2^+\beta^+\beta^{CO}$, 10%; 9, methemoglobin, 3%. The *arrows* indicate the focusing positions of components 3, $\alpha_2^{CO}\beta_2^+$, and 6, $\alpha^+\alpha^{CO}\beta_2^+$, which are present in trace amounts (<1%). The fractions of CO-bound hemes calculated from the scan data are *a*, 0.64; *b*, 0.63; *c*, 0.64; and *d*, 0.63.

ried out using a solution containing a large amount of intermediates, which was obtained by rapidly mixing at 22 °C a solution of carbonylhemoglobin in 0.1 M KCl, pH 7, containing 36% methemoglobin, and a solution of dithionite in 20 mM phosphate buffer, pH 7. A sample of reactants was then quenched after times varying from 1.5 s to 15 min.

Fig. 2*a* shows a densitometer scan of a color slide of a gel tube used for the IEF separation at −25 °C of the species present in the partially oxidized carbonylhemoglobin solution before reaction with dithionite. Five, possibly six, out of the eight theoretically predictable valence hybrids (20) are present, each in a concentration equal or greater than 10% of the total. The scans in Fig. 2, *b–d* refer to separations of the species formed by quenching samples of this solution after reaction with dithionite.

Under the conditions of this experiment, more than 95% of the ferric hemes were reduced to the ferrous state in less than 1 s. Thus the valence hybrids isolated after reacting the partially oxidized carbonylhemoglobin solution with dithionite for 1.5 and 2.5 s, Fig. 2, *b* and *c*, should correspond to the intermediate compounds present in a functional partially CO-saturated solution of deoxyhemoglobin approaching equilibrium with the ligand. Equilibrium was attained in about 2 min (Fig. 2*d*).

The total fraction of liganded hemes, which can be calculated for each scan in Fig. 2, *b–d* knowing the identity of the components and their relative proportion, remained equal to the fraction of CO-bound hemes in the partially oxidized hemoglobin solution (0.64 ± 0.01).

Accurate Determination of Small Amounts of Intermediates—Fig. 2*d* is representative of the small amount of intermediates at equilibrium, but cannot be used for their accurate determination under these conditions unless "blank" corrected for minor hemoglobin present in the sample and for by-products of the quenching and separation procedures, such as products of slight oxidation of liganded hemes and of hybridization reactions at subzero temperature.

When the approach to equilibrium of rapidly mixed deoxy- and carbonylhemoglobin was studied, as in Fig. 3, the concentration of components found after reaction times so short that no significant ligand exchange had occurred (31) (Fig. 3*a*) was used to correct the concentration of intermediates found at equilibrium (Fig. 3*d*). Thus the small amount of intermediates at equilibrium was accurately determined, since the same solutions of deoxy- and carbonylhemoglobin were used for the equilibrium and blank run; the blank sample was quenched in the same way as the equilibrium sample, and equilibration of deoxy- and carbonylhemoglobin occurred in the reaction tube of the quench-flow apparatus under rigorously anaerobic conditions before being directly injected into the quenching medium.

Concentration of the Intermediate Compounds at Equilibrium

Table I summarizes some of the data obtained at equilibrium using different hemoglobin preparations, various times of equilibration, and different conditions for the quenching reaction. The new data are compared with those from the work previously reported (20) (Column a). The agreement between the theoretical value of $Y = 0.5$ and the value calculated from the scan percentages was within 1% saturation (Columns a, b, d, and e).

Table I also shows the slight changes in the concentration of the intermediates that occurred when the quenched sample was exposed to the oxidant at −25 °C for 1 h before its removal by IEF using the normal and the modified quenching procedure, which consisted in raising the pH of the medium after

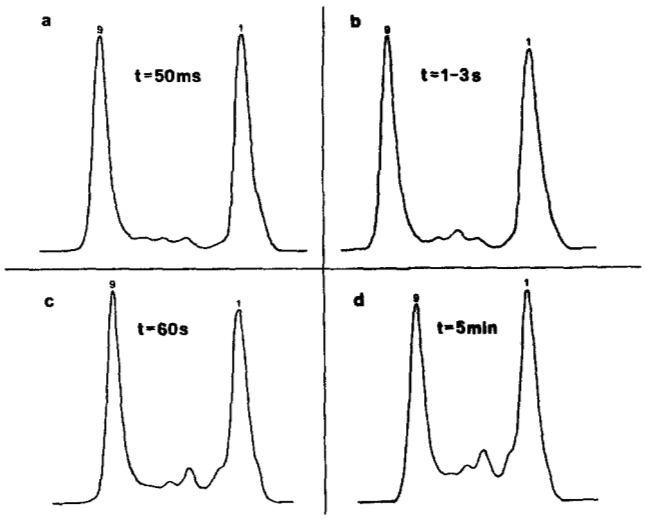

FIG. 3. **Densitometer scans of color slides of the gel tubes used for the separation by IEF at −25 °C of samples of 1 to 1 mixtures of deoxy- and carbonylhemoglobin in 0.1 M KCl, pH 7, reacted at 22 °C for times varying from 50 ms to 5 min before quenching.** The major peaks, labeled 1 and 9, correspond to the focused bands of carbonyl- and methemoglobin, respectively. The minor peaks are identified in Table I.

TABLE I

Equilibrium per cent concentrations of intermediate compounds between carbonmonoxide and hemoglobin (~5 mM) in 0.1 M KCl, pH 7, 22 °C at Y = 0.5

Valence hybrid	Intermediate	Equilibrium % concentration					
		a[a]	b[b]	c[c]	d[d]	e[e]	f[f]
1 $\alpha_2^{CO}\beta_2^{CO}$	$\alpha_2^{CO}\beta_2^{CO}$	44.6	43.6	35.0	42.0	43.5	42.3
2 $\alpha_2^{CO}\beta^+\beta^{CO}$	$\alpha_2^{CO}\beta\beta^{CO}$	2.5	2.5	2.0	3.8	1.1	1.8
3 $\alpha_2^{CO}\beta_2^+$	$\alpha_2^{CO}\beta_2$						
4 $\alpha^+\alpha^{CO}\beta_2^{CO}$	$\alpha\alpha^{CO}\beta_2^{CO}$	3.8	3.6	6.2	4.5	4.2	4.3
5 $(\alpha^+\beta^+)(\alpha^{CO}\beta^{CO})$ $(\alpha^+\beta^{CO})(\alpha^{CO}\beta^+)$	$(\alpha\beta)(\alpha^{CO}\beta^{CO})$ $(\alpha\beta^{CO})(\alpha^{CO}\beta)$	0.8	1.0	4.8	0.3	0.6	0.2
6 $\alpha^+\alpha^{CO}\beta_2^+$	$\alpha\alpha^{CO}\beta_2$	1.6	0.8	2.2	2.0	2.2	1.6
7 $\alpha_2^+\beta_2^{CO}$	$\alpha_2\beta_2^{CO}$						
8 $\alpha_2^+\beta^2\beta^{CO}$	$\alpha_2\beta\beta^{CO}$	3.7	2.7	6.7	3.3	3.2	5.7
9 $\alpha_2^+\beta_2^+$	$\alpha_2\beta_2$	43.2	45.6	43.1	44.1	45.2	44.2
Y %[g]		50.8	49.6	45.8	49.7	49.1	48.8

[a] Data from published work (20).
[b] Deoxy- and carbonylhemoglobin equilibrated for 5 min.
[c] Same as [b], quenched hemoglobin sample left at −25°C in presence of ferricyanide for 1 h before IEF.
[d] Same as [b], 3 min after quenching the hemoglobin sample 0.5 M Tris buffer in 50% ethylene glycol (v/v) (30 μl) was added to the cryosolvent.
[e] Deoxy- and carbonylhemoglobin equilibrated for 10 min, same quenching procedure as [d].
[f] Same as [e], quenched sample left at −25°C in presence of ferricyanide for 1 h before IEF.
[g] Ligand per cent saturation calculated from scan data.

quenching (see "Experimental Procedures"). Clearly, the decrease in the value of *Y*% (where *Y* is hemoglobin fractional saturation with ligand) (Column c) was indicative of some oxidation of CO-bound hemes, and the increase in the amount of component 5 (Column c) was likely due to some hybridization between met- and carbonylhemoglobin. These side reactions were inhibited when the modified procedure was used (Column f). However, since ferricyanide removal by IEF required only 5–10 min (20), both procedures were equivalent when overexposure of the sample to the oxidant was avoided.

The Equilibrium Curve of Hemoglobin and Carbon Monoxide

According to Adair's classical scheme (28) the equilibrium between hemoglobin and its gaseous ligands is described by four constants K_{4i} (where K_{4i} (i = 1–4) are equilibrium constants for binding the ith ligand molecule). These are usually calculated from the data on the ligand saturation of hemoglobin at known free ligand concentrations, since the constants K_{4i} are related to the coefficients a_i of the following general form of Adair's equation (28), which is fitted to such data by numerical methods.

TABLE II

Equilibrium constants (K'_{4i}) and Hill coefficient (n) for the reaction of human hemoglobin with carbon monoxide and oxygen at pH 7 and 22 °C

	$(K'_{4i})_{CO}$[b]		$(K'_{4i})_{O_2}$[c]
K'_{41}[a]	0.027	(0.035)	0.11
K'_{42}[a]	0.09	(0.16)	0.07
K'_{43}[a]	14	(8.0)	1.5
K'_{44}[a]	26	(21)	85
n	3.5	(3.4)	2.8

[a] Constants referred to unitary ligand concentration at $Y = 0.5$. Assuming [CO] = 5.6 10^{-8} M at $Y = 0.5$, $K'_{4i} = K_{4i}\ M^{-1} \times 5.6\ 10^{-8}$ M.
[b] Values calculated from averaged data on the concentration of CO intermediates at $Y = 0.5$, as obtained in this work. Values in parentheses were recalculated assuming a 100% increment in the concentration of bi-liganded species and 50% increments in the concentrations of the mono- and tri-liganded species at $Y = 0.5$ with respect to the averaged data.
[c] Values calculated from the oxygen-binding isotherm at 21.5 °C and the median ligand concentration, as measured by Chu *et al.* (16).

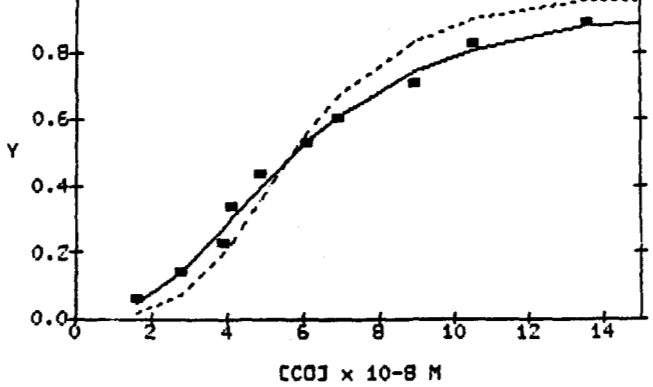

FIG. 4. **Carbon monoxide equilibrium curve of human hemoglobin.** *Full line*, curve fitted according to Equation 1 to equilibrium data of 2.34 10^{-7} M hemoglobin in 0.1 M phosphate, pH 7, 25 °C, adapted from Anderson and Antonini (33), who measured a value of $n = 2.5$. The concentration of CO corresponding to $Y = 0.5$ was 5.6 10^{-8} M. *Dashed line*, curve calculated using the averaged data on the intermediate concentration in 5 mM hemoglobin, 0.1 M KCl, pH 7, 22 °C, at $Y = 0.5$. The value calculated for n was 3.5. The equilibrium constants for the calculation of the coefficients a_i in Equation 1 are shown in Table II. The curve corresponding to the constants shown in parentheses in Table II was not plotted because it differs from the dashed line by less than 1% CO saturation.

$$Y = \frac{a_1p + 2a_2p^2 + 3a_3p^3 + 4a_4p^4}{4(1 + a_1p + a_2p^2 + a_3p^3 + a_4p^4)} \quad (1)$$

The constants K_{4i} can also be calculated from the concentration of the intermediates at any value of the ligand saturation (32). We assumed here that heterogeneity between the mono- and tri-liganded species (Table I) was negligible, and that the free ligand concentration at $Y = 0.5$ was the same as that determined by Anderson and Antonini (33) in their study of the carbon monoxide equilibrium curve of hemoglobin under comparable conditions of pH and temperature. Calculations were carried out using the averaged data on the concentration of the intermediates as obtained in this work, and their values corrected for some estimated errors. The constants K'_{4i}, shown in Table II, refer to unitary ligand concentration at $Y = 0.5$ for the purpose of comparison with the data obtained by Chu *et al.* (16) in their study of the hemoglobin equilibrium with oxygen under comparable conditions.

Fig. 4 shows the data adapted from Anderson and Antonini (33) and fitted according to Equation 1, together with the curve predicted using the experimental data on the intermediate concentration. This curve agreed within 1% CO saturation with the curve corresponding to the constants shown in parentheses in Table II, which were calculated assuming a 100% increment in the concentration of the bi-liganded species and 50% increments in the concentrations of the mono- and tri-liganded species at $Y = 0.5$ with respect to the averaged data.

DISCUSSION

Our procedure for trapping the intermediate compounds between hemoglobin and carbon monoxide is not entirely a direct method, since it is not the intermediates but the products of their chemical quenching that are separated by low temperature focusing. However, the study reported here on the determination of the intermediates during the approach to equilibrium proves the quantitative correlation between the original intermediates and the valence hybrids isolated by focusing. In fact, changes in the patterns shown in Fig. 2, *b* and *c* with respect to the pattern in Fig. 2*a* were due to the ligand binding reactions in the course of approach to equilibrium. All five, possibly six, intermediate compounds formed by dithionite reduction of the corresponding valence hybrids (*i.e.* $\alpha_2\beta\beta^{CO}$, $\alpha_2\beta_2^{CO}$, $\alpha\alpha^{CO}\beta_2^{CO}$, $\alpha_2^{CO}\beta\beta^{CO}$, $(\alpha\beta)(\alpha^{CO}\beta^{CO})$, and $(\alpha\beta^{CO})(\alpha^{CO}\beta)$) were trapped and quantitatively determined within the experimental error of the technique (±1% of the total), since the total fraction of liganded hemes remained constant throughout the equilibrium process. The scan in Fig. 2*d* is indeed representative of the low amount of intermediate species at equilibrium. Thus the similar finding at $Y = 0.5$, when the equilibrium is approached by reacting solutions of deoxy- and carbonylhemoglobin (Fig. 3), becomes validated.

We can now turn to comment on the nature and significance of the intermediate compounds observed under equilibrium conditions at pH 7, 22 °C, and 50% CO saturation.

More carbon monoxide bound to the β than to the α chains in both the mono- and tri-liganded species. Since such a heterogeneity was slight and the amount of intermediates was also small, it is not surprising that Davis *et al.* (34) could not find evidence of heterogeneity using high resolution NMR techniques.

A greater amount of tri-liganded than mono-liganded compounds was found. Thus, at $Y = 0.5$, the concentration of deoxyhemoglobin slightly exceeded that of carbonylhemoglobin. The exact nature of the bi-liganded intermediate found to be present in detectable amounts is unknown. Our work indicates that only two out of the four possible bi-liganded intermediates have to be taken in consideration, namely $(\alpha\beta)(\alpha^{CO}\beta^{CO})$ and/or $(\alpha\beta^{CO})(\alpha^{CO}\beta)$.

Huestis and Raftery (35) used ^{19}F NMR to study the equilibrium between carbon monoxide and a trifluoroacetonyl derivative of hemoglobin. At near 50% ligand saturation, they observed the presence of two main intermediate compounds accounting for about 12% of the hemoglobin in solution, which were identifed as $\alpha_2^{CO}\beta_2$ (4%) and $\alpha_2^{CO}\beta\beta^{CO}$ (8%). Our work is in agreement with their finding that the tri-liganded species are more abundant than the mono-liganded species at $Y = 0.5$. The identification of the tri-liganded species as $\alpha_2^{CO}\beta\beta^{CO}$ claimed by these authors was the result of their identification of $\alpha_2^{CO}\beta_2$ as the bi-liganded species. The basis for such an identification was the observation that the addition of 2,3-diphosphoglycerate induced chemical shifts in the same direction on the NMR resonances of the bi-liganded intermediate and of the cyanomet hybrid $\alpha_2^{+CN-}\beta_2$. However, it cannot be excluded that a similar effect could be induced by 2,3-diphosphoglycerate on the resonance of $(\alpha\beta)(\alpha^{CO}\beta^{CO})$ or $(\alpha\beta^{CO})(\alpha^{CO}\beta)$, which may have been the true intermediates observed by Huestis and Raftery (35). The methodology of these authors, which has been questioned by Reisberg *et al.* (36), is more likely to be suitable to identify the conformations of the intermediates, whereas the data reported here, once the quantitative correlation between the intermediates and the valence hybrids separated at subzero temperature are established, are based on the physical isolation and identification of the intermediates.

The carbon monoxide equilibrium curve of hemoglobin calculated from the concentration of intermediate compounds at $Y = 0.5$ predicted a highly cooperative system since $n = 3.5$. Such a prediction was not significantly affected by an estimated 50–100% error in the measurement of the concentration of some or all the intermediates. The uncertainty in the predicted curve introduced by such an error was less than the uncertainty in curve fitting the equilibrium saturation data due to errors in the measurements by current techniques. This strongly supports our method for determining both the intermediate concentration and the true equilibrium constants according to Adair's scheme (28). However, the predicted curve requires confirmation by measurements of the CO equilibrium of hemoglobin other than those reported by Anderson and Antonini (33), who used dilute hemoglobin, since under these conditions a large fraction of the protein is present as uncooperative dimers (19).

Quantitative testing of models of hemoglobin function have made use of the available data on the oxygen-hemoglobin equilibrium under various conditions of pH and temperature. The determination of the concentration of the intermediates under these conditions by our technique, if at all possible, would require extensive technological modifications. Our finding that the bi-liganded species were the least abundant at $Y = 0.5$ agrees with the prediction made by Chu *et al.* (16) for the hemoglobin-oxygen equilibrium. It is also consistent with the predictions of several models, such as that of Monod *et al.* (6) and the Szabo and Karplus model (37).

The functional difference between α and β chains in hemoglobin is usually discussed in studies of hemoglobin interaction with ligands. However, the possibility that the properties of the symmetrical and asymmetrical bi-liganded species may differ has been specifically considered only recently (38). Functional and NMR studies have been carried out on the symmetrical cyanomet-/deoxyhemoglobin hybrids $\alpha_2\beta_2^{+CN^-}$ and $\alpha_2^{+CN^-}\beta_2$ (2). It has been suggested that these molecules exist in a conformational equilibrium, which, in the absence of organic phosphates, is essentially shifted toward the conformation of fully liganded hemoglobin. Miura and Ho (12)

have found that the asymmetrical cyanomet-/deoxyhemoglobin hybrids stabilized by cross-linking have NMR properties different from those of the symmetrical species. Differences in the energetic properties between the bi-liganded symmetrical and asymmetrical cyanomet-/deoxyhemoglobin hybrids have been reported by Smith and Ackers (10). Our finding that, under equilibrium conditions with CO, only the asymmetrical molecules are detectable among the bi-liganded species shows that the functional differences between such symmetrical and asymmetrical species exist also in chemically unmodified hemoglobin. Such a finding does not confirm that the hypothesis made by Smith and Ackers (10) and by Miura and Ho (12) on the existence of more than two energetic states or structures in the hemoglobin oxygen binding process is correct, but is consistent with it.

Our study of the equilibrium of hemoglobin with carbon monoxide at pH 7 and 22 °C was focused on a single value of the ligand saturation because at $Y = 0.5$ the accuracy and reproducibility of the measurements were found to be maximal. It would be worth extending the analysis to other conditions if such measurements could be supplemented with experimental data on the equilibrium between CO and hemoglobin similar to those now available on the oxygen-hemoglobin equilibria. This would significantly improve the efficacy of models in the elucidation of the mechanisms of cooperative ligand binding to hemoglobin, since we have shown here that two basic features of such an interaction, the quantitative distribution of the intermediates and the functional heterogeneity of the species, can be clarified by our approach.

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