THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 262, No. 10, Issue of April 5, pp. 4528–4533, 1987
Printed in U.S.A.

The Dissociation of Carbon Monoxide from Hemoglobin Intermediates*

(Received for publication, April 21, 1986)

Michele Samaja, Ermanna Rovida‡, Michela Niggeler, Michele Perrella, and Luigi Rossi-Bernardi

From the Dipartimento di Scienze e Tecnologie Biomediche, Universitá degli Studi di Milano and the ‡Centro di Fisiologia del Lavoro Muscolare del Consiglio Nazionale delle Ricerche, Ospedale San Raffaele, via Olgettina 60, 20132 Milano, Italy

To investigate the mechanism of allosteric switching in human hemoglobin, we have studied the dissociation of the ligand (CO) from several intermediate ligation states by a stopped-flow kinetic technique that utilizes competitive binding of CO by microperoxidase. The hemoglobin species investigated include $Hb(CO)_4$, the diliganded symmetrical species (α β-CO)$_2$ and (α-CO β)$_2$, and the di- and monoliganded asymmetrical species (α-CO β-CO)(α β), (α-CO β)(α β-CO), (α β-CO)(α β), and (α-CO β)(α β). They were obtained by rapid reduction with dithionite of the corresponding valence intermediates that in turn were obtained by chromatography or by hybridization. The nature and concentration of the intermediates were determined by isoelectric focusing at −25 °C. The study was performed at varying hemoglobin concentrations (0.1, 0.02, and 0.001 mM [heme]), pH (6.0, 7.0, 8.0), with and without inositol hexaphosphate. The results indicate that: (*a*) hemoglobin concentration in the 0.1–0.02 mM range does not significantly affect the kinetic rates; (*b*) the α chains dissociate CO faster than the β chains; (*c*) the symmetrical diliganded intermediates show cooperativity with respect to ligand dissociation that disappears in the presence of inositol hexaphosphate; (*d*) the monoliganded intermediates dissociate CO faster than the diliganded intermediates; (*e*) the asymmetrical diliganded intermediates are functionally different from the symmetrical species.

Tetrameric human hemoglobin may form two monoliganded and two triliganded species during reaction with heme ligands. Moreover, two distinct interfaces between the α and β chains allow for the presence of four diliganded species. Some of these intermediates have been shown directly by experiments with cryogenic methods for hemoglobin at equilibrium with CO (1). Investigations into the functional properties of these species are central in understanding the molecular mechanism of the hemoglobin reaction with heme ligands.

There are two main difficulties when investigating the properties of the native intermediates of the reaction of hemoglobin with heme ligands. (*a*) Under equilibrium conditions, the amount of intermediates is low, due to the positive cooperativity of hemoglobin; and (*b*) under nonequilibrium conditions, ligand and dimer exchange is competitive with most experimental techniques.

Experimental approaches proposed to overcome these difficulties have included the use of variant hemoglobins (2, 3), hemoglobins with iron substituted by other metals (4–7), and cross-linked hemoglobins (8), thus not dealing with native normal human hemoglobin. Other authors used unmodified hemoglobin and CO or NO as ligands (9–12), but the unknown distribution of the intermediates in the mixtures under study required arbitrary assumptions regarding hemoglobin reactivity. Still other studies employed the CN^- ion as ligand; the species (α β-CN)$_2$ and (α-CN β)$_2$[1] have been studied by several techniques, including NMR and EPR (see Ref. 13 for a review), and the equilibrium constants for the tetramer to dimer association of all the CN-methemoglobin intermediates were recently obtained (14). Comparing these results with the properties of the corresponding intermediates obtained with gaseous ligands is of great importance to evaluate if the features observed on CN-met species may be extended to gaseous ligands.

Kinetic studies of the ligand dissociation from hemoglobin and its intermediates yield useful information into the electronic and steric factors affecting the reactivity of hemoglobin, because faster or slower rates correspond to a more "tense" or "relaxed" quaternary structure of the protein, respectively. Carbon monoxide has the highest cooperativity, compared with O_2 and NO, in the "on" reaction ($l_4'/l_1' = 17$ (15), $l_4'/l_1' = 2$ (16), and $l_4'/l_1' < 1$ (17) for CO, O_2, and NO, respectively), and the rate of the dissociation of the ligand is considered a valid probe to study the mechanism of quaternary structure changes.

Earlier studies of the dissociation of the first CO from $Hb(CO)_4$,[2] obtained with NO replacement techniques (18, 19), gave little insight into the quaternary changes of the protein. In contrast, studies using microperoxidase (MP), an endecapeptide that binds CO stronger and faster than hemoglobin, allowed the investigation of the successive steps of ligand dissociation (9). However, this study, which used diluted hemoglobin solutions, could not distinguish the functional differences between α and β chains nor the effects of dimers on the kinetics.

In this work, we have investigated the dissociation of CO

* This work has been supported in part by Consiglio Nazionale delle Ricerche Grant 83.02815.56. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The rate constants of the reaction of Hb with CO (l'_{nx}) are so keyed: n (1–4) is the number of ligands bound to the molecule; x (α or β) is the hemoglobin chain involved in the reaction; the prime mark (′) indicates that it is an "on" reaction (otherwise, the reaction is "off"). The species that are partially oxidized and partially liganded to CO, for example (α^+ β-CO)$_2$, are referred to as the partially oxidized compounds. The reduced forms of the partially oxidized compounds, as (α β-CO)$_2$, are referred to as the intermediates of the reaction of hemoglobin with CO.

[2] The abbreviations used are: Hb, deoxy (unliganded) hemoglobin; $Hb(CO)_4$, carboxyhemoglobin; HbO_2, oxyhemoglobin; Hb^+, methemoglobin; IEF, isoelectric focusing; IHP, inositol hexaphosphate; Mb, deoxy (unliganded) myoglobin; MbCO, carboxymyoglobin; MbO_2, oxymyoglobin; Mb^+, metmyoglobin; MP, unliganded microperoxidase; MPCO, microperoxidase bound to CO; MP^+, oxidized microperoxidase.

from hemoglobin intermediates using the following reaction scheme,

$$Hb_4^{n+mCO} \xrightarrow{\text{Na dithionite}} Hb_4^{mCO} \quad (t_{1/2} = 0.3\ s) \qquad (1a)$$

$$Hb_4^{mCO} \rightarrow Hb_4 + mCO \quad (l = 0.03\text{–}0.2\ s^{-1}) \qquad (1b)$$

$$mCO + mMP \rightarrow mMPCO \quad (k = 10\ \mu M^{-1}\ s^{-1}) \qquad (1c)$$

$$mCO + Hb_4 \rightarrow Hb_4^{mCO} \quad (l' = 0.4\text{–}6\ \mu M^{-1}\ s^{-1}) \qquad (1d)$$

where $n + m = 4$, and the values for l' are from Ref. 15. In this scheme, the ligand exchange between hemes during the reaction is eliminated because microperoxidase is present in a 10-fold excess over deoxy hemes and has a faster combination rate with CO with respect to hemoglobin. Furthermore, the knowledge of the distribution of the partially oxidized species before the reduction of ferric iron by dithionite (routinely checked by IEF at −25 °C) avoided the need of assumptions regarding their distribution.

The approach reported herein for the preparation of nonequilibrium mixtures originates from the observation that all the products of the partial oxidation of $Hb(CO)_4$ can be obtained by hybridization of the species Hb^+, $Hb(CO)_4$, $(\alpha^+\ \beta\text{-CO})_2$, and $(\alpha\text{-CO}\ \beta^+)_2$ (20). These four species are stable with respect to both dimer and ligand exchange because of their symmetrical structure and the absence of free sites for ligand binding. The reduction of the ferric iron by dithionite yields the respective intermediates of the reaction of hemoglobin with CO, which can be studied with appropriate techniques. However, investigations into two-step reactions where the second step is faster than the first, as the dissociation of CO from diliganded intermediates, require starting solutions with large amounts of the intermediate species, as the monoliganded asymmetrical species. These species, when partially oxidized, are stable with respect to ligand exchange, but undergo rapid dimer rearrangement. Thus, they were obtained in ternary mixtures prepared by mixing equimolar amounts of any pair of symmetrical molecules. These partially oxidized mixtures were shown to contain roughly 50% of the asymmetrical hybrid (20) and were rapidly reduced by dithionite to yield the intermediates of the reaction with CO.

The aim of this work is to evaluate the cooperativity of the tetramer and to assess the functional heterogeneity of the α and β chains. We show that: (*a*) the dissociation of CO from the α chains was faster than the β chains; (*b*) the dissociation of CO was faster from the monoliganded than from the diliganded molecules; (*c*) organic phosphates and H^+ always enhanced a faster dissociation of the ligand; and (*d*) the symmetrical diliganded intermediates were functionally different from the asymmetrical diliganded intermediates.

EXPERIMENTAL PROCEDURES

Reagents—The buffer (20 mM KP_i, 50 mM KCl, 1 mM EDTA, pH 7.0, at 20 °C, unless otherwise stated) was deaerated with vacuum and stored in stoppered glass bottles under nitrogen. Fresh sodium dithionite (Carlo Erba, Milano, Italy) solutions (140 mg/ml) were prepared by dissolving anaerobically in the buffer, extracting residual gas with vacuum, and titrating to the selected pH in an IL237 tonometer (Instrumentation Laboratory, Paderno Dugnano, Italy). Stock anaerobic solutions of 18.5 mM inositol hexaphosphate, 0.37 mM sperm whale Mb^+, and 0.6 mM MP^+ (Sigma) were prepared daily.

Subzero IEF—Subzero (−25 °C) IEF was carried out loading 50–200 μg of protein onto the gel-containing tubes (20). At the end of the 22–24-h IEF, a color slide of the tubes was taken and the distribution of the intermediates was estimated by scanning the slide with a Cliniscan (Helena Laboratories, Beaumont, TX) integrator operating at 465 nm.

Hemoglobin—Oxygenated hemoglobin (HbO_2) was obtained from nonsmoker donors (HbCO < 2%), washing red cells with isotonic saline, hemolyzing with water and CCl_4 in the ratio 1:1:0.4, and freeing organic phosphates with Sephadex G-25 against 0.1 M KCl. The approximately 7.5 mM hemoglobin stock was stored in liquid nitrogen until use.

To obtain methemoglobin (Hb^+), HbO_2 was oxidized by 1.2 eq of $K_3Fe(CN)_6$ for 1 h at room temperature, pH 6.8. The mixture was passed through Sephadex G-25, deaerated, and stored at 0 °C until use.

Hemoglobin concentrations are expressed on a molar heme basis (after mixing for stopped-flow experiments) and were obtained as met-cyanide derivatives using an extinction coefficient of 11.05 $mM^{-1}\ cm^{-1}$ at 540 nm (21). The other extinction coefficients at 592 nm for $Hb(CO)_4$, Hb, MP, and MPCO were 2.03, 5.96, 0.843, and 1.19 $mM^{-1}\ cm^{-1}$, respectively.

Preparation of $(\alpha^+\ \beta\text{-CO})_2$ and $(\alpha\text{-CO}\ \beta^+)_2$—Solutions containing $(\alpha^+\ \beta\text{-CO})_2$ and $(\alpha\text{-CO}\ \beta^+)_2$ were prepared from four different blood samples, using slight modifications of a method previously described to obtain the species $(\alpha^+\ \beta\text{-O}_2)_2$ and $(\alpha\text{-O}_2\ \beta^+)_2$ (22). Twelve ml of 7.5 mM HbO_2 was oxidized at pH 6.5 by 0.5 eq of $K_3Fe(CN)_6$ either for 2 h at room temperature or for 1 min at 0 °C to obtain solutions enriched in the former or the latter species, respectively. After replacement of O_2 with CO by gentle bubbling and passage through Sephadex G-25 at 0 °C, the solution was loaded to a 4 × 32-cm CM52 (Whatman Ltd., England) column previously equilibrated with a 5 mM KP_i, 0.5 mM EDTA, pH 6.8 at 25 °C buffer. All these operations were carried out anaerobically. An ionic strength gradient (5–15 mM KP_i, 1 mM EDTA, pH 7.5 at 25 °C) was then applied at a flow rate of 60 ml/h, and fractions were collected, bubbled with CO and then with N_2, concentrated to 1.2 mM, and stored under nitrogen at ice temperature. The purity of the solutions was in the 90–95% range, as checked by IEF at −25 °C.

Preparation of the Ternary Mixtures—Solutions with the species $(\alpha\text{-CO}\ \beta^+)(\alpha^+\ \beta^+)$ or $(\alpha^+\ \beta\text{-CO})(\alpha^+\ \beta^+)$ were obtained by mixing anaerobically (5 min at room temperature) equimolar amounts of Hb^+ with $(\alpha\text{-CO}\ \beta^+)_2$ or $(\alpha^+\ \beta\text{-CO})_2$, respectively, at a total protein concentration of 1.2 ± 0.1 mM. Solutions with the species $(\alpha\text{-CO}\ \beta\text{-CO})(\alpha^+\ \beta^+)$ or $(\alpha\text{-CO}\ \beta^+)(\alpha^+\ \beta\text{-CO})$ were prepared by mixing under the same conditions Hb^+ with $Hb(CO)_4$, or $(\alpha\text{-CO}\ \beta^+)_2$ with $(\alpha^+\ \beta\text{-CO})_2$, respectively. The mixtures were then refrigerated and used within 1 h. Isoelectric focusing at −25 °C showed that the amount of asymmetrical monoliganded and diliganded hybrids in the mixtures was 45 and 40%, respectively.

Kinetic Data Determinations—Kinetic progress curves were obtained at 20 °C with a Durrum-Gibson stopped-flow instrument (Dionex, Palo Alto, CA), equipped with a 2-cm path length cuvette (dead time, 0.003 s) and with a 1:3.8 reagents mixing ratio. Usually, 4 ml of the stock MP^+ solution was reduced by 0.5 ml of 140 mg/ml sodium dithionite and loaded into the larger stopped-flow syringe.

The time required to reduce 98% of the oxidized hemes (Reaction 1a) was <1 s for [dithionite] = 140 mg/ml. At the end of this reaction, the CO released by hemoglobin (Reaction 1b) reacts with MP (Reaction 1c) faster than with Hb (Reaction 1d) because of the higher values of the rate constants and of the concentration with respect to hemoglobin. Thus, the recombination of Hb with CO is inhibited, and the overall rate of the Reaction chain 1a–d is controlled by the rate of CO dissociation from hemoglobin. This measured rate was independent of slit width for values less than 0.2 mm, corresponding to a photomultiplier voltage of 500 V.

Absorbance spectra of the species $Hb(CO)_4$, Hb, MPCO, and MP indicated that any wavelength in the range 450–650 nm could be used to monitor the CO dissociation kinetics. At 592 nm a large optical

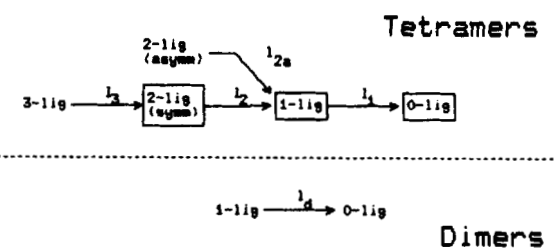

FIG. 1. **Scheme of the CO dissociation reaction that occurs in the presence of contaminants (diliganded asymmetrical and triliganded intermediates) and of the tetramer to dimer reaction.**

density change (about 0.4) was detected at high protein concentration with a 2-cm cuvette, and the spectral drift due to conformational changes that were observed using NO as ligand (10) is minimized. For the experiments at [heme] = 0.001 mM, we used a wavelength of 435 nm.

Kinetic data were collected by a Nicolet 3091 digital oscilloscope (Nicolet Instrument Corp., Madison, WI). The start of the reaction was set 1 s after the trigger signal to allow complete reduction of oxidized hemes, and data were transmitted to a PC-350 computer (Digital Equipment Corp., Maynard, MA) used for storage, retrieval, and computation.

Calculation of l_1 and l_2—Calculation of the rate constants was performed by two methods. The first is a standard first-order treatment of data in the 0–2.5 s range (about one-fifth of the reaction). The second is the application of a finite element-generating function (23) to the scheme of Fig. 1 and the full time courses of the reaction. The scheme accounts for the tetramer to dimer reaction and for the presence of contaminants of the symmetrical diliganded species, such as the asymmetrical diliganded and some triliganded species, that were detected in minor amounts (less than 3%) by IEF. Since several of these side reactions were never investigated before nor were studied here, we assumed that (unless otherwise stated): (*a*) the tetramer to dimer dissociation constants obtained for CN-liganded hemoglobin hold true also for CO-liganded hemoglobin; (*b*) the value for the CO dissociation from the triliganded species (l_3) is equal to l_4; (*c*) the value for the CO dissociation from the asymmetrical diliganded intermediate (l_{2a}) is equal to l_1; (*d*) the value for CO dissociation from dimers (l_d) is equal to l_4 (0.012 s^{-1}) as for noncooperative processes.

The rate constants referred to in this work are intrinsic microscopic constants.

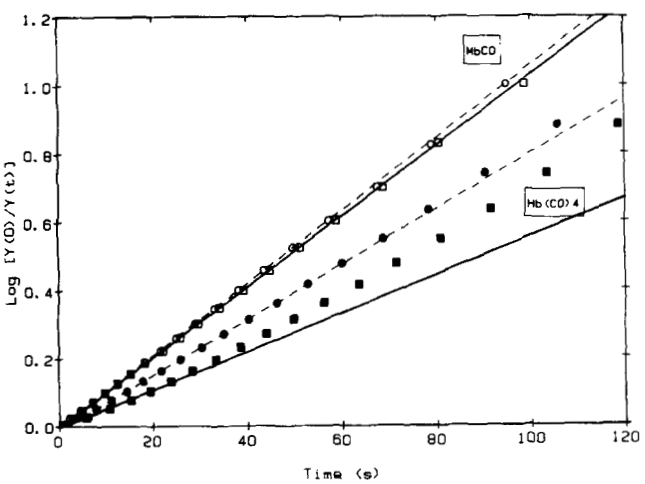

FIG. 2. **First order plot (Y = fractional CO saturation) for the CO dissociation from MbCO and Hb(CO)$_4$, without (——) and with (– – –) IHP ([IHP]/[Hb$_4$] = 2).** The *lines* are the linear regressions calculated in the 0–5 s range. Only one point out of 100 is drawn for clarity. Other conditions: pH 7.0, 20 °C, [heme] = 0.1 mM (after mixing).

RESULTS

CO Dissociation from MbCO and Hb(CO)$_4$—The kinetics of CO dissociation from 0.1 mM MbCO, pH 7.0, with and without added IHP (Fig. 2), were found to be first order up to 95% of the reaction, with an apparent $k = 0.024$ s^{-1}, to be compared to $k = 0.017$ s^{-1} in 3 μM MbCO (9). Under the same conditions, the dissociation of CO from Hb(CO)$_4$ is an accelerating process (Fig. 2). The acceleration is greater without added IHP. The value for l_4, calculated from the initial portion of the kinetics, is 0.011 s^{-1} (l_4 = 0.017 s^{-1}, when [IHP]/[Hb$_4$] = 2) to be compared with l_4 = 0.007 s^{-1} (19) and 0.009 s^{-1} (9), which were determined under slightly different conditions.

CO Dissociation from the Intermediates at [Heme] = 0.1 mM—Table I shows the values for $l_{1\alpha}$, $l_{1\beta}$, $l_{2\alpha}$, and $l_{2\beta}$ that were calculated as explained under "Experimental Procedures." Fig. 3, *left panel*, shows the initial portion of the kinetics of CO dissociation from (α-CO β)$_2$ and the ternary mixture (α-CO β)$_2$ + (α-CO β)(α β) + (α β)$_2$, pH 7.0. Fig. 3, *right panel*, shows similar data obtained on (α β-CO)$_2$ and the ternary

TABLE I

Values of the kinetic rate constants (s^{-1}, values accurate to ±14%) for the dissociation of CO from symmetrical diliganded ($l_{2\alpha}$ and $l_{2\beta}$) and monoliganded ($l_{1\alpha}$ and $l_{1\beta}$) intermediates, [heme] = 0.1 mM

Rate constants are intrinsic and were calculated using the first order plot and the scheme reported in Fig. 1.

	pH	IHP	$l_{1\alpha}$	$l_{1\beta}$	$l_{2\alpha}$	$l_{2\beta}$
1st order plot	7.0	No	0.16	0.10	0.10	0.049
	7.0	Yes	0.16	0.11	0.15	0.10
	6.0	No	0.12	0.12	0.10	0.12
	6.0	Yes	0.096	0.08	0.064	0.060
	8.0	No	0.009		0.005	
	8.0	Yes	0.006	0.004	0.006	0.003
Scheme of Fig. 1	7.0	No	0.22	0.15	0.085	0.035
	7.0	Yes	0.22	0.15	0.17	0.10

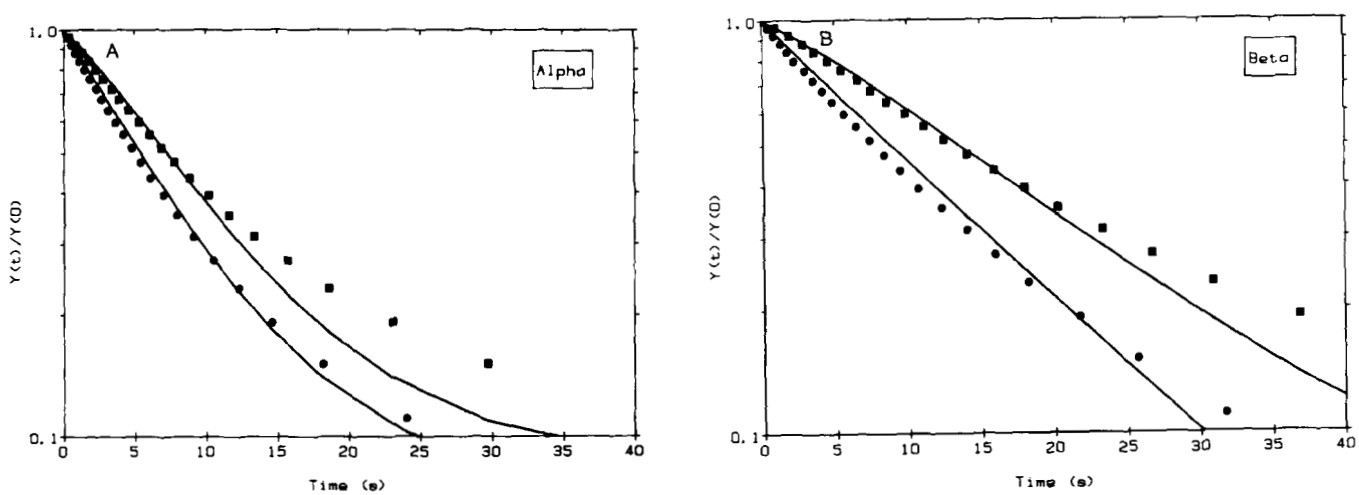

FIG. 3. **Dissociation of CO from the α and β chains of hemoglobin at pH 7.0, 20 °C, and [heme] = 0.1 mM for the symmetrical diliganded intermediate (■) and the ternary mixtures (●).** Displayed data (1 point out of 100) are the average of 6–10 progress curves for each solution, obtained from 4 different preparations. The *lines* are the results of the fits of the data to the scheme in Fig. 1, assuming $l_3 = l_4$, $l_{2a} = l_1$, $l_d = l_4$; final root mean square = 0.0097 and 0.0120 for the α and β chains, respectively.

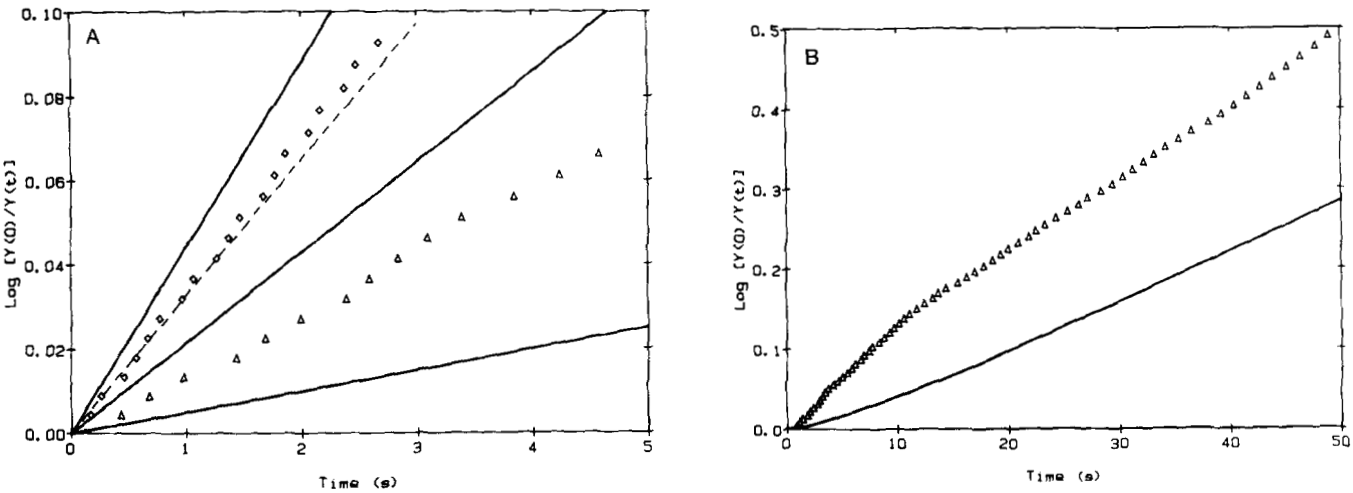

FIG. 4. The *left panel* shows the CO dissociation from the two ternary mixtures, $(\alpha\text{-CO}\ \beta\text{-CO})_2 + (\alpha\ \beta)(\alpha\text{-CO}\ \beta\text{-CO}) + (\alpha\ \beta)_2$ (△) and $(\alpha\text{-CO}\ \beta)_2 + (\alpha\text{-CO}\ \beta)(\alpha\ \beta\text{-CO}) + (\alpha\ \beta\text{-CO})_2$ (◇). Some of the kinetics already reported in Figs. 2 and 3 are drawn here to help comparison (——): from *left* to *right*, $(\alpha\text{-CO}\ \beta)_2$, $(\alpha\ \beta\text{-CO})_2$, and $Hb(CO)_4$. - - -, calculated kinetics for the CO dissociation from equimolar amounts of $(\alpha\text{-CO}\ \beta)_2$ and $(\alpha\ \beta\text{-CO})_2$ without their asymmetrical hybrid. The *right panel* shows the kinetics of the mixture $(\alpha\text{-CO}\ \beta\text{-CO})_2 + (\alpha\ \beta)(\alpha\text{-CO}\ \beta\text{-CO}) + (\alpha\ \beta)_2$ (△) and of $Hb(CO)_4$ (——) as in the *left panel*, but on a different time scale.

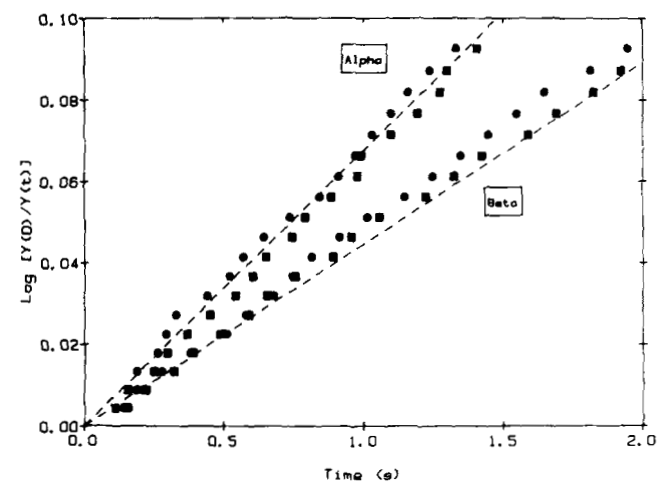

FIG. 5. **Same as in Fig. 3, but with IHP ([IHP]/[Hb_4] = 2), pH 7.0, [heme] = 0.1 mM, 20 °C.** Symmetrical diliganded intermediate (■), ternary mixtures (●), and (- - -) CO dissociation from the ternary mixtures without IHP as in Fig. 3, added here to help comparison.

mixture $(\alpha\ \beta\text{-CO})_2 + (\alpha\ \beta\text{-CO})(\alpha\ \beta) + (\alpha\ \beta)_2$. All curves are first order in the 0–2.5 s range (not shown).

Fig. 4, *left panel*, shows data obtained with the ternary mixtures Hb + $(\alpha\ \beta)(\alpha\text{-CO}\ \beta\text{-CO})$ + $Hb(CO)_4$ and $(\alpha\text{-CO}\ \beta)_2$ + $(\alpha\text{-CO}\ \beta)(\alpha\ \beta\text{-CO})$ + $(\alpha\ \beta\text{-CO})_2$ at pH 7.0. For comparison, we added in the figure the kinetics of $(\alpha\text{-CO}\ \beta)_2$, $(\alpha\ \beta\text{-CO})_2$, and $Hb(CO)_4$ and the kinetics for a mixture of equimolar amounts of $(\alpha\text{-CO}\ \beta)_2$ and $(\alpha\ \beta\text{-CO})_2$, without their hybrid $(\alpha\text{-CO}\ \beta)(\alpha\ \beta\text{-CO})$, calculated from the data reported in Table I, top section.

Additional experiments were performed at pH 6.0 and 8.0. At pH 8.0 the dissociation of CO from the β chains in the absence of IHP was found to be too slow for the stopped-flow apparatus and could not be measured.

Effect of the Protein Concentration—We evaluated the effect of dimers on the CO dissociation progress curves of the symmetrical diliganded species, performing experiments at [heme] = 0.02 and 0.001 mM. At [heme] = 0.02 mM, the first-order rate constants were comparable within experimental error (±20%) with those obtained at [heme] = 0.1 mM. By contrast, at [heme] = 0.001 mM, the rate constants for $(\alpha\text{-CO}\ \beta)_2$ (0.032 s^{-1}, at pH 7.0, independent of IHP) were about one-third to one-fifth of the values obtained at [heme] = 0.1 mM.

Effect of IHP—Fig. 5 shows the CO dissociation kinetics of the symmetrical diliganded intermediates and the ternary mixtures containing the monoliganded intermediates in the presence of IHP ([IHP]/[Hb_4] = 2), pH 7.0, and [heme] = 0.1 mM. The kinetics of the ternary mixtures without IHP was added in the figure for comparison.

DISCUSSION

Distribution of the Partially Oxidized Species and of the Intermediates—The concentration of the intermediates before the reaction with MP is of critical importance for the interpretation of the kinetics. We must consider the system at the two stages in our protocol: (*a*) initially the hemoglobin mixture was in the CO/met form at equilibrium with respect to dimer and ligand exchange; and (*b*) the addition of dithionite and MP perturbed the pre-existing dimer and ligand equilibrium.

Subzero IEF provided information on the concentrations of all the species present in the partially oxidized mixtures. The purity of $(\alpha\text{-CO}\ \beta^+)_2$ and $(\alpha^+\ \beta\text{-CO})_2$ obtained by chromatography was >90%. Focusing of the ternary mixtures showed that the asymmetrical hybrid formed by the recombination of the dimers from the parent species was 45% of the total. This value was consistent with the distribution at equilibrium of dimers, parent species, and their hybrids, calculated at the highest heme concentration used here (Table II). For this calculation, the tetramer to dimer dissociation constants of each of the partially oxidized species were assumed to have the same value of 1 μM reported for $Hb(CO)_4$ (24).

This equilibrium was perturbed by the ideally instantaneous reduction of ferric iron. To predict the extent of the changes due to dimer rearrangement, we used the equilibrium constants for the tetramer-dimer reactions reported for the partially CN-liganded intermediates under comparable conditions (14), since similar data on CO intermediates are not available. Even if the ligand is different, there are good reasons to believe that the values of the constants will not be too different when using CO instead of CN^-, as pointed out

TABLE II

Fractional distribution at equilibrium in a mixture containing the diliganded symmetrical intermediate (α-CO β)₂, unliganded hemoglobin, and the monoliganded intermediate (α β)(α-CO β) before and after the reduction with dithionite at the three heme concentrations used in this work

The same pattern occurs for the other intermediates with the β chains bound to CO.

Before reduction[a]					
[Heme]	(α-CO β^+)₂	(α-CO β^+)	(α^+ β^+)₂	(α^+ β^+)	(α-CO β^+)(α^+ β^+)
mM					
0.1	0.232	0.035	0.234	0.035	0.466
0.02	0.209	0.083	0.208	0.083	0.417
0.001	0.122	0.254	0.123	0.254	0.246
After reduction[b]					
[Heme]	(α-CO β)₂	(α-CO β)	(α β)₂	(α β)	(α-CO β)(α β)
mM					
0.1	0.234	0.037	0.259	0.001	0.463
0.02	0.199	0.086	0.270	0.001	0.437
0.001	0.096	0.237	0.329	0.002	0.334

[a] Calculated using the same value for the dissociation constant for the three tetramers (1×10^{-6} M).

[b] Calculated using the following values for the dissociation constants: 1.7×10^{-6}, 2.8×10^{-8}, 5.2×10^{-10} M for (α-CO β)₂, (α-CO β)(α β), and (α β)₂, respectively.

by the same authors (14). With this assumption, we calculated the distribution of the components in the ternary mixture (including also free dimers) at the three heme concentrations employed in this work (Table II). At equilibrium, the concentration of the asymmetrical hybrid is similar to that found in the partially oxidized solution when [heme] = 0.1 mM in spite of the change of the equilibrium constants. Thus, the distribution of the partially oxidized species obtained by IEF is reasonably representative of the distribution of the intermediates at the start of the reaction with MP.

As for the effect of ligand exchange, we evaluated the rate of CO dissociation from the partially oxidized species during the time required to reduce oxidized hemes, assuming that the values of l_1 and l_2 reported in Table I hold true. This assumption may represent the most unfavorable condition since the partially oxidized species are likely to have a stronger affinity for CO than the respective intermediates. Nevertheless, we found that CO dissociation during this time was <2% of the total.

To use the scheme of Fig. 1 as the generating function to fit the data, the assumptions listed under "Experimental Procedures" had to be made. As shown in Table III, these assumptions gave the best results in term of residual errors, as compared with the results obtained using other arbitrary sets of assumptions.

Dissociation of CO from the Hemoglobin Intermediates—The CO dissociation from Hb(CO)₄ was an accelerating process almost unaffected by IHP in the initial portion, in agreement with previous results that l_4 is a rate-limiting step (19). The acceleration is likely due to the disappearance of the rate-limiting species Hb(CO)₄, with increasing amounts of intermediates with faster dissociation rates than Hb(CO)₄. IHP increases the rate of CO dissociation from at least some of the intermediates, leading to lower amounts of intermediates, and, therefore, the acceleration, not the rate, of the reaction is apparently less in the presence of IHP than in its absence.

The dissociation of CO from the α chains of the intermediates was faster than from the β chains at any pH, [IHP], ligation state, and [heme] in the 0.1–0.02 mM range. At pH 7.0, the dissociation of CO from the monoliganded intermediates was faster than from the diliganded intermediates, implying a conformational change of the protein when dissociating the first CO from (α-CO β)₂ or (α β-CO)₂. The species (α β-CO)₂ exhibits more cooperativity in the kinetics than (α-CO β)₂, since $l_{1\beta}/l_{2\beta} > l_{1\alpha}/l_{2\alpha}$.

The kinetics of the monoliganded intermediates was unaffected by IHP, while IHP increased the rate of CO dissociation from the symmetrical diliganded intermediates. In the presence of IHP, the l_1/l_2 ratio approached unity, both for the α and the β chains, implying that the quaternary conformation of the symmetrical diliganded intermediates with IHP is very similar to that of the monoliganded intermediates.

The difficulties in studying the asymmetrical diliganded species in comparison to the symmetrical diliganded intermediates are: (*a*) they cannot be studied as pure species; (*b*) the dissociation of CO yields two different monoliganded species because the reaction of the α chains is indistinguishable from that of the β chains; and (*c*) the asymmetrical diliganded species may exchange dimers at a rate comparable with the rate of CO dissociation. We calculated from the first-order plot of the CO dissociation from the ternary mixture Hb + (α-CO β-CO)(α β) + Hb(CO)₄ that apparent $k = 0.033$ s^{-1} in the 0–2.5 s range. This value could be assigned to (α-CO β-CO)(α β), since the dissociation of CO from Hb(CO)₄ should be negligible in this time range. The possible disappearance of the asymmetrical diliganded intermediate to yield the parent species Hb and Hb(CO)₄ following dimer rearrangement is consistent with the kinetics of this ternary mixture which slows down and becomes nearly parallel to that of Hb(CO)₄ after 15–20 s (about 20–30% of the reaction, Fig. 4, *bottom panel*). This indicates that the only CO-dissociating species present here is Hb(CO)₄.

The interpretation of the CO dissociation kinetics from the mixture (α β-CO)₂ + (α-CO β)(α β-CO) + (α-CO β)₂ is even more complex because the progress curve lies between those of the two parent species. The observed kinetics is indistinguishable from that calculated for a 1:1 mixture of the two parent species (*dashed line* in Fig. 4, *top panel*), suggesting that the hybrid dissociates CO at a rate comparable to (α-CO β)₂ or slower.

CONCLUSIONS

The main innovative features of this work were the characterization of the functional heterogeneity of the chains of hemoglobin and the measurement of l_1 and l_2 under conditions closer to the environment of the red cell than in earlier studies. The use of a higher protein concentration was limited in this approach by the solubility of MP and its loss of activity due

TABLE III

Results of fits of the same sets of data ([heme] = 0.1 mM, pH 7.0) to the scheme of Fig. 1 at different arbitrary values for some of the parameters

The rate constants are intrinsic microscopic constants, and RMS is the residual mean square error, expressed in fractional saturation units.

Assumptions	α/β	No IHP		With IHP		RMS
		l_1	l_2	l_1	l_2	
See "Experimental Procedures"	α	0.22	0.085	0.22	0.17	0.0097
	β	0.15	0.035	0.15	0.10	0.0120
$l_{2a} = l_2$	α	0.13	0.085	0.13	0.16	0.0166
	β	0.11	0.039	0.11	0.095	0.0146
$l_3 = l_2$	α	0.15	0.082	0.15	0.16	0.0168
	β	0.20	0.025	0.20	0.072	0.0148
$l_d = 0$	α	0.15	0.082	0.15	0.16	0.0168
	β	0.20	0.029	0.20	0.072	0.0148
No tetramer to dimer reaction	α	0.13	0.095	0.13	0.16	0.0165
	β	0.13	0.037	0.13	0.091	0.0148

to polymerization phenomena (25). Data shown in Table II and the kinetics obtained at the lowest heme concentration point out that previous work (9) performed at [heme] = 0.001 mM suffered from significant tetramer to dimer reaction, thus giving little insight into the cooperativity of the molecule. A recent study on the distribution of the intermediates of hemoglobin half-saturated with CO (26) suggests that the asymmetrical and symmetrical diliganded intermediates are functionally different. This result, consistent with the findings on CN-met intermediates (14), reinforces our observation on the CO dissociation from the diliganded intermediates, suggesting that there are preferred pathways in the ligand dissociation reaction from hemoglobin.

Acknowledgments—We acknowledge the great help kindly provided by Drs. Louise Benazzi in the preparation of this manuscript, Andrea Mosca for performing part of the subzero IEF work, and Gary K. Ackers for helpful criticism of this paper and discussions.

REFERENCES

1. Perrella, M., Benazzi, L., Cremonesi, L., Vesely, S., Viggiano, G., and Rossi-Bernardi, L. (1983) *J. Biol. Chem.* **258,** 4511–4517
2. Makino, N., Sugita, Y., and Nakamura, T. (1979) *J. Biol. Chem.* **254,** 10862–10867
3. Nigen, A. M., Manning, J. M., and Alben, J. O. (1980) *J. Biol. Chem.* **255,** 5525–5529
4. Imai, K., Ikeda-Saito, M., Yamamoto, H., and Yonetani, T. (1980) *J. Mol. Biol.* **138,** 635–648
5. Blough, N. V., and Hoffman, B. M. (1982) *J. Am. Chem. Soc.* **104,** 4247–4250
6. Simolo, K., Stucky, G., Chen, S., Bailey, M., Scholes, C., and McLendon, G. (1985) *J. Am. Chem. Soc.* **107,** 2865–2872
7. Hofrichter, J., Henry, E. R., Sommer, J. H., Deutsch, R., Ikeda-Saito, M., Yonetani, T., and Eaton, W. A. (1985) *Biochemistry* **24,** 2667–2679
8. Miura, S., and Ho, C. (1982) *Biochemistry* **21,** 6280–6287
9. Sharma, V. S., Schmidt, M. R., and Ranney, H. M. (1976) *J. Biol. Chem.* **251,** 4267–4272
10. Cassoly, R. (1978) *J. Biol. Chem.* **253,** 3602–3606
11. Sharma, V. S., and Ranney, H. M. (1982) *J. Mol. Biol.* **158,** 551–558
12. Sharma, V. S. (1983) *J. Mol. Biol.* **166,** 677–684
13. Baldwin, J. M. (1975) *Proc. Biophys. Mol. Biol.* **29,** 225–320
14. Smith, F. R., and Ackers, G. K. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 5347–5351
15. MacQuarrie, R., and Gibson, Q. H. (1971) *J. Biol. Chem.* **246,** 5832–5835
16. Gibson, Q. H. (1970) *J. Biol. Chem.* **245,** 3285–3288
17. Cassoly, R., and Gibson, Q. H. (1975) *J. Mol. Biol.* **91,** 301–313
18. Antonini, E., and Gibson, Q. H. (1960) *Biochem. J.* **76,** 534–538
19. Salhany, J. M., Ogawa, S., and Shulman, R. G. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 3359–3362
20. Perrella, M., Cremonesi, L., Benazzi, L., and Rossi-Bernardi, L. (1981) *J. Biol. Chem.* **256,** 11098–11103
21. Van Kampen, E. J., and Zijlstra, W. G. (1965) *Adv. Clin. Chem.* **8,** 141–186
22. Tomoda, A., and Yoneyama, Y. (1981) *Anal. Biochem.* **110,** 431–436
23. Samaja, M., Niggeler, M., and Rovida, E. (1985) *J. Protein Chem.* **4,** 319–331
24. Barksdale, A. D., and Rosenberg, A. (1978) *J. Biol. Chem.* **253,** 4881–4885
25. Wilson, M. T., Ranson, R. J., Masiakowski, P., Czarnecka, E., and Brunori, M. (1977) *Eur. J. Biochem.* **77,** 193–199
26. Perrella, M., Sabbioneda, L., Samaja, M., and Rossi-Bernardi, L. (1986) *J. Biol. Chem.* **261,** 8391–8396