Isolation and Stability of Partially Oxidized Intermediates of Carp Hemoglobin: Kinetics of CO Binding to the Mono- and Triferric Species†

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ABSTRACT: The monoliganded and triliganded forms of the asymmetric valency hybrids of carp hemoglobin were isolated using high-performance liquid chromatography. These partially oxidized hybrids were shown to be sufficiently stable to permit the measurement of the kinetics of CO binding. The effects of protons and inositol hexaphosphate on the rates of these reactions were examined. The kinetics of CO recombination with these partially oxidized derivatives were compared to the kinetics of CO binding to the fully ferrous molecule. To a first approximation, the kinetic behavior of the monoferric derivative was consistent with a small shift in the T↔R equilibrium in favor of the R state. The presence of three ferric ligands resulted in a still greater shift in the conformational equilibrium in favor of the R state. The kinetic behavior of the triferric molecule was similar, but not identical, to that of a fully ferrous molecule which is triliganded with CO. The properties of both asymmetric valency hybrids were responsive to the nature of the ligand; i.e., the rate of CO binding was increased more by the presence of cyanide on the ferric hemes than by water. Not all of the data could be accommodated within the two-state model. For example, there was evidence of an altered T state in the case of the tricyanomet derivative at low pH in the presence of inositol hexaphosphate.

The hemoglobin molecule exhibits a complex set of functional properties, among which is the cooperative binding of ligands to its four heme groups. Numerous models have been presented to account for the phenomenon of cooperativity. The most popular, in part due to its intrinsic simplicity, has been the two-state model of Monod, Wyman, and Changeux (1965). Although unable to account for every aspect of hemoglobin behavior, this model has appeared to offer a reasonable first approximation to the allosteric properties of most hemoglobin molecules, especially when modified to permit functional heterogeneity between the α and β subunits (Sharma, 1988; Mathews *et al.*, 1991) and pH dependencies of the properties of the two allowed structural states, the low affinity T state (Imai & Yonetoni, 1975) and high affinity R state (DeYoung *et al.*, 1976). However, a fundamental postulate of this model is that of molecular symmetry. Within the T state, all binding sites must have the ligand affinities associated with the T quaternary structure, and these affinities must be insensitive to the presence or absence of ligands on the other heme sites. The same must hold true for the R state. Other models, such as the sequential mechanism of Koshland *et al.* (1966), contain no such requirement.

Identification of the functional properties of the partially liganded intermediates of the reaction of hemoglobin with ligands is made difficult by the very behavior that makes these systems so interesting. Because of cooperativity, the concentrations of these intermediates at equilibrium are always low compared to those of the fully liganded and fully unliganded hemoglobin molecules. As a result the binding isotherm is relatively insensitive to the properties of these intermediates, as is indicated by the difficulty encountered in attempting to determine the four Adair constants from a single oxygen saturation curve. In general one can assign only the first and last dissociation constants along with the product of the second and third. In human hemoglobin, the situation is further complicated by the ligand-linked dissociation of the hemoglobin tetramer into high affinity $\alpha\beta$ dimers. Ackers and co-workers (Mills *et al.*, 1976; Smith & Ackers, 1985) have taken advantage of this complexity by measuring the effect of hemoglobin concentration on the oxygen binding isotherm. The data obtained at several different hemoglobin concentrations were simultaneously fitted to a model containing not only the four Adair constants but all of the parameters defining the dissociation into high affinity, noncooperative dimers. The Adair constants did not show the monotonic increase in affinity with increasing fractional saturation of the hemoglobin molecule predicted by a two-state model. Using the metcyanide heme as a model for a liganded heme group, the effects of number and topography of metcyanide hemes on the relative free energies of the dissociation of otherwise deoxygenated hemoglobins into $\alpha\beta$ dimers were examined (Daugherty *et al.*, 1991; Ackers *et al.*, 1992). The results confirmed the interpretation of the previous oxygen binding measurements and additionally demonstrated the energetic inequivalence of the four different diliganded topologies, directly contradicting the symmetry requirement of the two-state model.

The studies cited above indicate that hemoglobin function is critically dependent on the properties of the asymmetric intermediates of ligand binding, i.e., the mono- and triliganded derivatives. To date most studies of intermediate ligation states have been limited to the examination of symmetric, diliganded intermediates (Blough *et al.*, 1984; Cassoly & Gibson, 1972). This limitation is a consequence of the dynamic equilibrium between the hemoglobin tetramer and $\alpha\beta$ dimers which results in the rapid disproportionation of any asymmetric species into an equilibrium mixture of two symmetric and one asymmetric form. The difficulty in examining asymmetric

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structures has been overcome in part by the development of cryogenic techniques (Perella *et al.*, 1981, 1983) and double-mixing stopped-flow techniques (Sharma *et al.*, 1991). However, stable, homogeneous preparations of asymmetric species of human hemoglobin can only be achieved by preventing the dissociation of the hemoglobin tetramer through chemical cross-linking (Miura *et al.*, 1987; Fowler *et al.*, 1992; Shibayama *et al.*, 1993). The major objection to the use of cross-linked proteins is that they are chemically modified and such modifications appear to alter significantly functional properties (Larsen *et al.*, 1990; Shibayama *et al.*, 1991).

Hemoglobins which do not dissociate into dimers would permit the existence of stable asymmetric forms without the need for chemical alteration of the native molecule. Edelstein *et al.* (1976) reported that they were unable to detect the presence of dimers in carp hemoglobin solutions. Of course, a rapid, dynamic equilibrium between tetramers and a very small concentration of dimers is not precluded by this observation. Still, an examination of the potential stability of asymmetric forms of carp hemoglobin seemed warranted. The hemoglobin of the carp offers another potential advantage in the study of the functional properties of intermediates of ligand binding. Because of the unusual properties of this hemoglobin, its intermediates can be examined under conditions where the fully ferrous molecules are highly cooperative or predominately in either the R or T state (Noble *et al.*, 1970; Tan *et al.*, 1972, 1973).

From partially oxidized components of carp hemoglobin we have isolated the mono- and triferric derivatives and established their stability and purity. We have measured the effects of protons and IHP on the binding of CO to the ferrous hemes of these derivatives using flash photolysis and stopped-flow techniques. By replacing the water ligand on the aquomet hemes with cyanide, we have examined the influence of the spin state of the liganded iron atoms on the kinetics of CO binding to the unliganded, ferrous hemes. The properties of the two triferric derivatives are also compared to those of the triliganded, fully ferrous carp hemoglobin molecule as generated by partial flash photolysis of the CO derivative.

EXPERIMENTAL PROCEDURES

Materials.[1] Carp hemoglobin is composed of two major components, A and B, which differ electrophoretically but have the same functional properties (Tan *et al.*, 1972). Carp hemolysate was prepared from carp red blood cells, equilibrated with CO, reduced to the fully ferrous state, and stripped of organic phosphates by passage through a Dintzis deionizing column (Dalvit *et al.*, 1984; DeYoung *et al.*, 1994). Fractionation of the components of carp hemolysate was accomplished by ion-exchange chromatography on a DEAE cellulose (DE 52, Whatman) column (2.5 × 20 cm), equilibrated with 10 mM Tris-HCl, pH 8.2. The hemolysate and buffer were saturated with CO during this separation procedure. The deionized hemolysate was applied to the column, and hemoglobin components A and B were eluted sequentially with the equilibrating buffer at a flow rate of 50 mL/h. If necessary, further purification of the components was achieved by HPLC with a glass TSK gel DEAE-5PW anion-exchange column (Toso-Haas, 22 × 150 mm) on a Waters Model 650 preparative apparatus maintained at 10 °C. Fractionation was accomplished with a 300-mL linear gradient at a flow rate of 5 mL/min. All buffers used in this separation were equilibrated with CO. The starting buffer for this gradient contained 3 mM HCl, 2 mM NaCl, 10 μM EDTA and was buffered to pH 8.15 with the basic form of Tris. The final buffer contained 10 mM HCl, 2.5 mM NaCl, 10 μM EDTA, and its pH was adjusted first to 7.0 with bis-Tris and then to a pH of 7.5 by the addition of Tris. The hemoglobins were concentrated and stored as the CO derivative in liquid nitrogen until needed.

Isolation of Partially Oxidized Intermediates. The CO derivative of component A or component B was converted to the oxygenated form by exposure to a bright light under a stream of oxygen at 0 °C as already described (Riggs, 1981; DeYoung *et al.*, 1994). A monoferric derivative was isolated from component A that had been partially oxidized with potassium ferricyanide. Both the monoferric and triferric valency hybrids were isolated from partially oxidized component B. The yield of the monoferric derivative could be maximized by limiting the oxidation to 25% of the heme groups while 60–75% oxidation increased the yield of the triferric derivative. Oxidation with ferricyanide was always carried out at room temperature for 15 min in 0.1 M Tris-HCl, pH 7.5. Each partially oxidized mixture was deionized by passage first through a G-25 Sephadex column equilibrated with 1 mM Tris-HCl, pH 8.2, and then through a Dintzis deionizing column. This last step removes all ions including any ferricyanide which might be bound to the protein. Separation of mixed valency hybrids was carried out by HPLC as described above except that the pH of the starting buffer was 8.25 for fractionation of oxidized component A and was usually 8.0 for fractionation of partially oxidized component B. All buffers were equilibrated with air so that the ferrous heme groups were saturated with oxygen. The partially oxidized derivatives were eluted in order from most to least oxidized as expected from their relative net charges. The identities of these derivatives eluted from the column were confirmed by their spectra. An aliquot of each was diluted into pH 7.0 buffer (0.1 M bis-Tris-HCl) to insure that the oxidized hemes were in the aquomet form. The percent of oxidized hemes was determined from the ratio of the absorbances at 414 and 405 nm. The central portion of each peak was harvested and rechromatographed until the combined contamination from other derivatives represented no more than 4% of the total hemoglobin. For the triferric derivative, somewhat larger amounts of the fully oxidized derivative were tolerated since fully ferric hemoglobin would not interfere with the subsequent functional studies. The purified derivatives were concentrated and stored in liquid nitrogen until needed.

Buffers. The ligand affinity of carp hemoglobin is sensitive to chloride ion concentration (Cerdonio *et al.*, 1983). For this reason all buffer concentrations were defined by the chloride ion component. Buffers were prepared by adjusting the pH of the appropriate amount of HCl with the basic form of bis-Tris (pH ≤7.2) or Tris (pH ≥7.2). For all kinetic experiments the final chloride concentration was 0.1 M. IHP (Sigma) was obtained in its sodium form. The pH of the IHP stock solution (0.2 M) was adjusted to 5.5 with the protonated form of Amberlite IR-120 resin. Stock solutions of KCN were always prepared at the time of use.

Flash Photolysis. Carbon monoxide recombination following photodissociation was measured at 20 °C with a flash-photolysis apparatus as described previously (Fowler *et al.*, 1992). The rates of CO recombination with deoxyferrous

[1] Abbreviations: bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane; HPLC, high-performance liquid chromatography; IHP, inositol hexaphosphate; Tris, tris(hydroxymethyl)aminomethane; aquomet, hemoglobin derivatives in which water is bound to the ferric hemes at the sixth coordinate position; cyanomet, hemoglobin derivatives in which cyanide is bound to the ferric hemes at the sixth coordinate position.

hemes of fully ferrous hemoglobin and the mixed valency hybrids were measured following full (100%) flash photolysis. In addition, the rate of CO recombination with triliganded fully ferrous carp hemoglobin, l'_4, was measured following partial (5–10%) flash photolysis. Reactions were followed at 420 and 435 nm. In our flash-photolysis apparatus the monochromator has been placed between the photomultiplier tube and the sample solution in order to protect the former from the photolytic flash. Interference filters transmitting maximally at the observation wavelength were placed between the lamp and the sample cuvette. This prevented photolysis of the CO ligand by the observation beam and minimized the photoreduction of the ferric hemes, which has been described by Bickar *et al.* (1984) and Fowler *et al.* (1992). The concentration of hemoglobin was generally 4 μM in heme equivalents, i.e., 1 μM in ferrous heme for the triferric derivative and 3 μM in ferrous heme for the monoferric derivative. The CO concentration was 38–40 μM. Anaerobic conditions were achieved with an enzyme system consisting of catalase, glucose oxidase, and D(+)-glucose (Fowler *et al.*, 1992). This enzyme system was chosen because it removes dissolved oxygen but does not reduce ferric heme groups. When examining fully ferrous hemoglobin derivatives either the enzyme system or dithionite could be used with identical results. For the cyanomet derivatives, experiments were performed in the presence of 10 mM KCN. When IHP was present the final concentration was 1 mM.

Stopped-Flow. The kinetics of CO combination with the triferric derivative of carp hemoglobin were measured by rapid mixing in a stopped-flow apparatus[2] (Gibson, 1959; Fowler *et al.*, 1992). To obtain anaerobic conditions, the stopped-flow apparatus was washed with an anaerobic solution of dithionite followed by thorough washing with large volumes of deoxygenated 1 mM Tris-HCl, pH 8.2. It was then washed with deoxygenated pH 7.0 buffered solutions of the catalase–glucose oxidase–glucose system. Anaerobic conditions were maintained in the stopped-flow by inclusion of this enzyme system in the hemoglobin and CO solutions. For all studies, a path length of either 1.7 or 2 cm was used and the reaction was monitored at 420 and 435 nm. The total heme concentration ranged from 2 to 11 μM and the CO concentration was 38 μM. When KCN was present, the final concentration was 5 mM.

For each estimate of the rates of CO binding to hemoglobin following full flash photolysis or mixing in the stopped-flow, three or four kinetic transients were recorded, each record being composed of 400 data points. Each estimate of l'_4 resulted from averaging data from 12 to 16 kinetic traces. The data were electronically digitized, averaged and stored on an OLIS[2] Model 4000 Data Acquisition and Instrument Control System complex. Kinetic transients were fitted to multiexponential functions using the fitting routines developed by OLIS. As will be reported the time courses of many of the reactions were best fitted to the sum of two exponential functions with rates differing by 3-fold or less. Extracting two rate constants differing by only 3-fold is associated with considerable error. In order to maximize reproducibility and optimize the comparisons of the properties of different derivatives, data were routinely obtained so that 400 data points were collected during the first 95% of the reaction.

[2] Two different stopped-flow instruments were used in these studies. One was the Dionex instrument based on the Gibson-Durrum design (Gibson & Milnes, 1964). The second was the OLIS-U.S.A. stopped-flow system of On Line Instrument Systems, Bogart, GA.

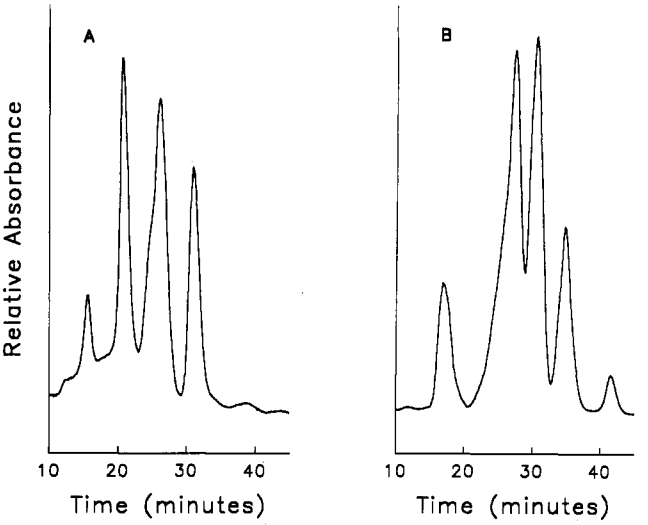

FIGURE 1: (A) Profile of the elution of 25% oxidized carp component A from a TSK gel DEAE-5PW anion-exchange HPLC column at 10 °C, monitored at 415 nm. A 5 mL/min, 60 min linear gradient from 3 mM Tris-HCl, 2 mM NaCl, pH 8.25, to 10 mM HCl bis-Tris-Tris (see Experimental Procedures), 2.5 mM NaCl, pH 7.5, was used. The third peak from the left was identified as the monoferric derivative. (B) Profile of the elution of 65% oxidized carp component B monitored at 540 nm. The second and fourth peaks from the left were identified as the triferric and monoferric derivatives, respectively. Experimental conditions were the same as in panel A except that the pH of the starting buffer was 8.15.

RESULTS

Isolation of Valency Hybrids of Carp Hemoglobin. Figure 1A shows a typical pattern of elution of 25% oxidized carp component A from a DEAE-5PW anion-exchange HPLC column monitored at 415 nm. About 90% of this sample was resolved into three major peaks which were identified spectrophotometrically from right to left as the fully ferrous, monoferric, and diferric derivatives. The triferric and fully ferric derivatives were not resolved in the elution. Although a peak appears at roughly 16 min, it is not a pure derivative. The monoferric derivative was rechromatographed as described in Experimental Procedures. Carp component A was oxidized to higher percentages of oxidized hemes in order to maximize the levels of the triferric derivative. Attempts to isolate and purify the triferric derivative using the same HPLC column while varying buffer conditions and other column parameters were not successful. Therefore, the asymmetric hybrids of carp component B were prepared. The β chains of component B differ from those of component A at four sequence positions, 13, 41, 122, and 143. For the β_A chains, the residues at these positions are Ala, Phe, Gly, and Lys, respectively. In the β_B chains, they are Gly, Tyr, Ala, and Cys (Grujic-Injac *et al.*, 1980). Therefore, under the conditions of the HPLC separation, the net charge on the component A molecule is roughly 3 units per tetramer more positive than that on component B. This explains the differences in the chromatographic behavior of the mixed valency hybrids of these two components. Figure 1B shows a typical elution pattern of 60% oxidized carp component B monitored at 540 nm. All five states of oxidation were resolved and identified from right to left, as the fully ferrous, monoferric, diferric, triferric, and fully ferric derivatives. The monoferric and triferric derivatives were rechromatographed to homogeneity as described in Experimental Procedures. However, it was not possible to distinguish differences in heme distribution within a given oxidation state. It should be noted that the HPLC separations were extremely sensitive to small

fluctuations in solution conditions which made perfect reproducibility of the elution patterns difficult. This sensitivity resulted in part from the necessity of working with buffers of very low ionic strength.

Stability of Asymmetric Valency Hybrids. The stabilities of the monoferric and triferric derivatives of carp hemoglobin were evaluated in the buffer solutions with which they had been eluted from the HPLC column. A sample of monoferric component B was thawed from liquid N_2 in which it had been placed following isolation. HPLC chromatography indicated that 96% of this material was in the monoferric form. After being stored on ice for 5 h, refrozen in liquid N_2, and thawed again the next day, HPLC chromatography showed the monoferric population to be approximately 91% of the total. Similar handling of a sample of triferric component B indicated that the proportion of the diferric derivative went from 2 to 2.5% during a 6-h incubation at 0 °C. Similar findings were obtained even when the triferric sample was stored overnight on ice. Taken together, these results demonstrate that valency hybrids of carp hemoglobin are quite stable with respect to autoxidation and disproportionation via the formation of $\alpha\beta$ dimers.

Fully Ferrous Hemoglobin. In order to determine the effect of one or three ferric hemes on the reactivity of the remaining ferrous heme groups, comparison must be made to the fully ferrous hemoglobin molecule, i.e. the overall kinetics of CO binding to deoxygenated carp hemoglobin, l'. In addition it is of interest to compare the effect of three ferric hemes, with water or cyanide bound, to the effect of three liganded ferrous heme groups on the reactivity of the one remaining unliganded ferrous heme. Therefore the kinetics of CO combination to the triferric molecule should be compared to the reaction of CO with a fully ferrous hemoglobin molecule to which three CO ligands are bound. The latter reaction, l'_4, is measured by partial flash photolysis as described under Experimental Procedures. Values of l' and l'_4 for carp hemoglobin have been determined previously (Pennelly *et al.*, 1975), but these measurements lacked the precision currently available. Therefore, redetermination of the values of these parameters as functions of pH and the addition of IHP was required in order to make meaningful comparisons with current measurements on partially oxidized intermediates.

The values of l' and l'_4 are plotted as functions of pH in Figure 2. The data presented in Figure 2A were obtained in the absence of organic phosphates while those in Figure 2B were obtained in the presence of 1 mM IHP. In examining these complex results it is perhaps easiest to begin with the data obtained in the presence of IHP. Here one sees the most dramatic pH-dependent transition in functional properties. The functional properties of carp hemoglobin were first interpreted within the framework of the two-state model of Monod, Wyman, and Changeux (1965) by Noble *et al.* (1970) and subsequently by Tan *et al.* (1973). According to this model, the ligand affinity of hemoglobin is modulated by controlling the equilibrium between two quaternary states, the low affinity, T, state and the high affinity, R, state. Cooperativity results from the ligand-linked transition from the T to the R structure in the course of ligand binding. A corollary of the two-state hypothesis is that the ligand affinities of the T and R states define the minimum and maximum ligand affinities, respectively, of the hemoglobin molecule. Furthermore, these affinities can be achieved only with the loss of cooperative ligand binding, since they can be reached only if the protein remains in a single quaternary state throughout the process of binding ligand. With this in mind

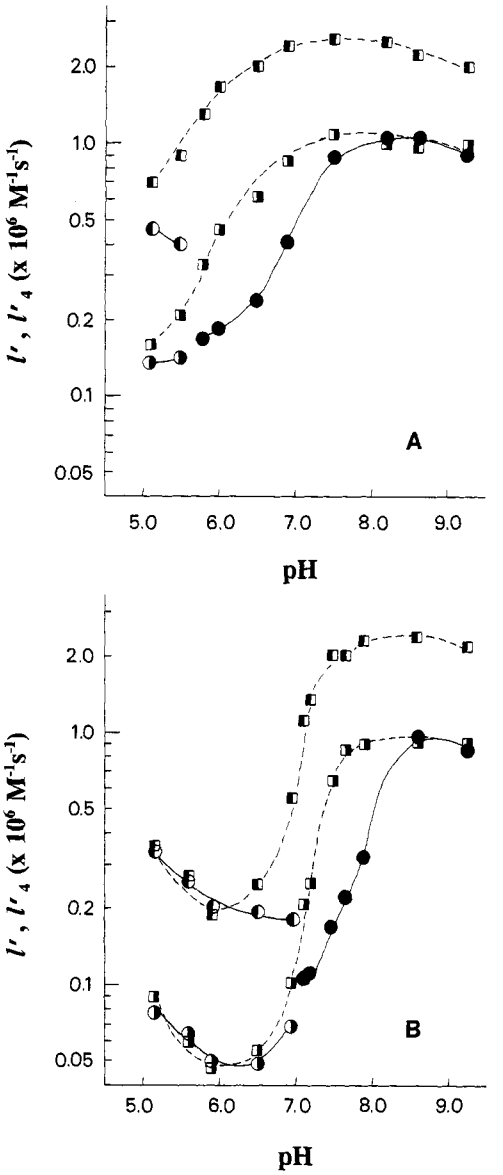

FIGURE 2: The pH dependence of l' and l'_4 of stripped, fully ferrous carp hemoglobin at 20 °C as measured at 420 and 435 nm by flash photolysis. Panel A presents data obtained in the absence of organic phosphates, and panel B that obtained in the presence of 1 mM IHP: (●) values of l' when a single kinetic process is observed, (◐, ◑) values of l' when two kinetic processes are observed, (□, ◨) values of l'_4. Buffers contained 0.1 M HCl to which bis-Tris (pH ≤7.2) or Tris (pH ≤7.2) was added for pH adjustment. The heme and CO concentrations were 5 and 38 μM, respectively.

the data in Figure 2B can be interpreted as a pH-dependent transition in the protein from being fixed in the T state at low pH to being almost fixed in the R state at high pH. Below pH 6, in the presence of IHP, carp hemoglobin binds ligands without cooperativity. This lack of cooperativity is evidenced by the kinetic identity of the reactions of CO with the fully unliganded hemoglobin tetramer and with the triliganded molecule. Under these conditions, CO recombination must be described by two second-order rate constants, a kinetic heterogeneity which is probably due in large measure to intrinsic differences between the α and β subunits. This heterogeneity is observed in l'_4 under all experimental conditions since partial photolysis can remove CO from either an α or β subunit. l' is also heterogeneous under conditions in which cooperativity is absent or low. However, the autocatalytic or accelerating nature of the cooperative binding of CO to an initially fully unliganded hemoglobin molecule

Table 1: Rate Constants, l'_M, As Determined by Flash Photolysis for the Binding of CO to the Monoferric Carp Derivative;[a] Effects of Different Ferric Ligands, IHP, and pH

			rapid process		slow process	
pH	ferric ligand	IHP	rate constant ($\times 10^6$ M^{-1} s^{-1})	% rxn[b]	rate constant ($\times 10^6$ M^{-1} s^{-1})	% rxn[b]
6.09–6.20	H_2O	–	0.18 ± 0.02	100		
	H_2O	+	0.16 ± 0.02	50 ± 6	0.05 ± 0.01	50 ± 6
	CN^-	–	0.30 ± 0.03	100		
	CN^-	+	0.17 ± 0.02	57 ± 5	0.05 ± 0.01	43 ± 5
6.36–6.46	H_2O	–	0.28 ± 0.02	100		
	H_2O	+	0.18 ± 0.01	50 ± 5	0.05 ± 0.01	50 ± 5
	CN^-	–	0.43 ± 0.05	75 ± 10	0.14 ± 0.03	25 ± 5
	CN^-	+	0.17 ± 0.02	55 ± 3	0.05 ± 0.01	45 ± 3
6.93–7.02	H_2O	–	0.87 ± 0.07	95 ± 3		
	H_2O	+	0.16 ± 0.02	50 ± 9	0.07 ± 0.01	50 ± 9
	CN^-	–	1.0 ± 0.10	93 ± 5		
(6.98)	CN^-	+	0.15 ± 0.02	60 ± 10	0.07 ± 0.01	40 ± 7
(7.02)	CN^-	+	0.13 ± 0.02	92 ± 6		
8.23–8.25	H_2O (OH^-)	–	1.4 ± 0.02	80 ± 5	0.50 ± 0.05	20 ± 3
	H_2O (OH^-)	+	N.D.			
	CN^-	–	1.2 ± 0.01	96.2		
	CN^-	+	N.D.			

[a] Experimental conditions are given in the legend of Figure 4. [b] Percentage of the total absorbance change attributed to the process.

is able to mask this heterogeneity. At pH 7 the two rates associated with l' coalesce into a single rate constant, and the reaction is detectably autocatalytic above this pH. Beginning at pH 6.5, the rates of l' and l'_4 diverge, CO reacting more rapidly with the triliganded than with the fully unliganded hemoglobin molecule. This difference in CO combination rate constants is another kinetic reflection of cooperative ligand binding, and the magnitude of the difference between l' and l'_4 correlates with the degree of cooperativity, increasing to a maximum at intermediate pH and falling once again above pH 8 as l' again approaches l'_4 (Tan *et al.*, 1973). At pH 8.5 and above, l' and l'_4 are relatively invariant. l' does not become biphasic nor does cooperativity disappear.

The removal of IHP from the system has the apparent effect of shifting the quaternary equilibrium in favor of the R state. Even at pH 5, l'_4 rate constants are larger than those of l', indicating that even at this pH the triliganded protein is not fully in the T state. l' ceases to be a biphasic reaction at pH 5.8 rather than pH 7, as in the presence of IHP. The transition to rapid CO combination rate constants occurs at lower pH, and these rapid rates are found over a broader pH range. However, once again l' never becomes biphasic at high pH and cooperativity persists. The effect of IHP on the T-R equilibrium is not uniform over the pH scale. Above pH 8.5 no effect of the organic phosphate is observed. The reason for this is unknown, but may result from a reduction in the affinity of the protein for the negatively charged effector molecule.

Monoferric Carp Hemoglobin. The second-order rate constants for CO recombination with deoxy monoferric carp hemoglobin, l'_M, as measured by full flash photolysis, are presented in Table 1. The percentage of the reaction attributed to each kinetic phase is also presented. Since the time courses for the reaction of CO with the monoferric carp component A and the monoferric component B were identical, the data for the two hemoglobin components were averaged together.

In the absence of IHP, the time courses for the recombination of CO with monoferric carp hemoglobin fall into three distinct patterns. Under three sets of conditions, the aquomet derivative at pH 6.1 and 6.4 and the cyanomet derivative at pH 6.1, the reaction with CO can be described by a single exponential function. However, these reactions are not as simple as their description by a single rate constant might imply. In every case the reactions are initially slightly autoaccelerating while displaying slight heterogeneity, i.e. deceleration toward their completion. A statistically significant second kinetic phase cannot be identified, and for this reason 100% of the reaction is attributed to the single rate constant. Under all other experimental conditions the kinetics are best fitted to a sum of two exponential functions. The aquomet derivative at pH 7 and the cyanomet derivative at pH 7 and 8.2 display the second pattern. In these cases over 90% of the reaction can be accounted for by a single rate constant. The remaining 10% or less is associated with a slower reaction for which the rate constant could not be measured with reasonable precision. In these cases only one rate constant is reported. For the aquomet derivative at pH 8.2 and the cyanomet at pH 6.4 the rate constants associated with the slow reactions could be determined.

The effect of adding IHP to the monoferric derivatives of carp hemoglobin is similar to that observed with the fully ferrous hemoglobin. At pH 7 and below, the overall rate of CO recombination is reduced and the reactions are composed of two kinetic phases of similar amplitude. At pH 7.02, 90% of the reaction with the metcyanide derivative is accounted for by the faster of the kinetic phases. This is reminiscent of the behavior of the fully ferrous hemoglobin for which the two kinetic phases observed below pH 7 merge into a single kinetic process above pH 7.1 as ligand binding becomes more cooperative. The reader may question the distinction between pH 6.98 and 7.02. Although the absolute values of our estimates of pH may be in error by more than the difference between these two values, the precision of our measurements is such that the difference between these values is significant. Furthermore, the difference in the kinetic properties observed under these two conditions is well outside our experimental error so that averaging these data would be inappropriate. Finally, in this pH region there are abrupt transitions in the functional properties of carp hemoglobin so that the exquisite sensitivity to pH suggested by our data is not unexpected.

A comparison of the second-order rate constants for CO recombination with monoferric and fully ferrous carp hemoglobin is present in Figure 3. The data points represent the rate constants for the monoferric derivatives while the lines represent the properties of the fully ferrous hemoglobin. In the absence of IHP (Figure 3A), one observes that, for both

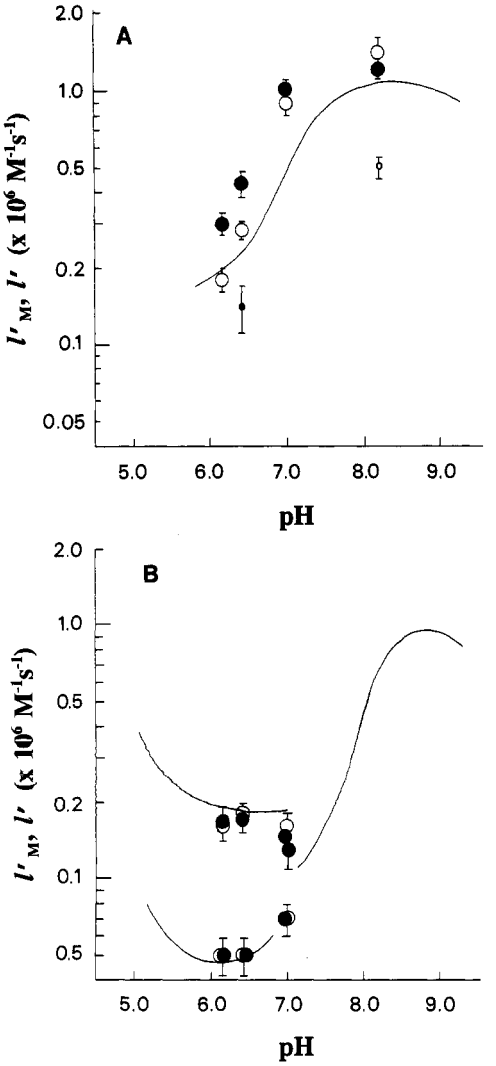

FIGURE 3: The pH dependence of the rate constant l'_m for the aquomet (○) and cyanomet (●) derivatives of monoferric carp hemoglobin as measured by flash photolysis at 20 °C. Panel A presents data obtained in the absence of organic phosphates, and panel B that obtained in the presence of 1 mM IHP. The lines represent the values of l' for fully ferrous carp hemoglobin. Buffers were the same as in Figure 3. The final CO and ferrous heme concentrations were 38 and 3 μM, respectively. The small data points at pH 6.4 and 8.2 in panel A represent rate constants that account for 25% and 20%, respectively, of the total reaction.

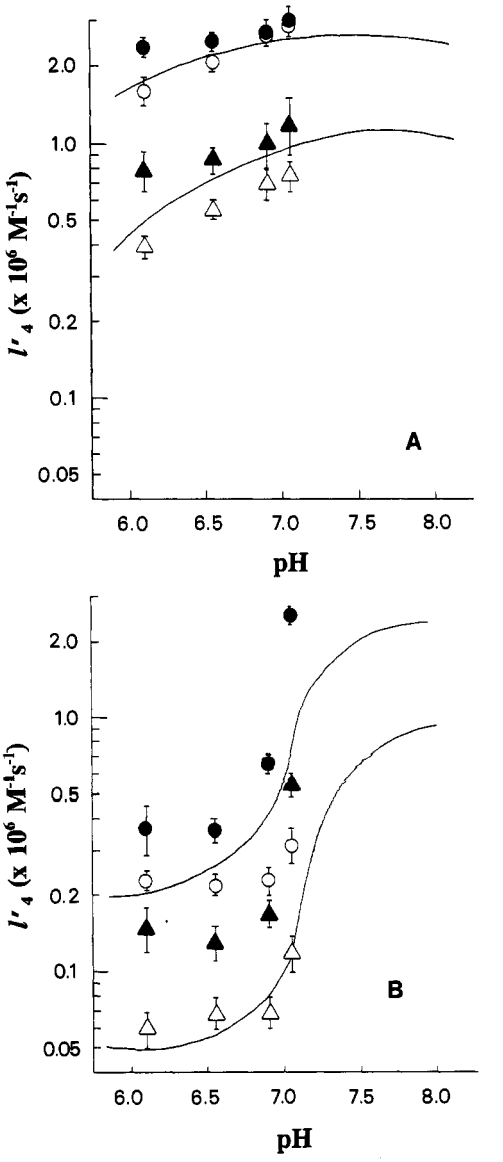

FIGURE 4: The pH dependence of the rate constants for the slow (△, ▲) and fast (○, ●) kinetic phases of CO recombination with the triaquomet (open symbols) and tricyanomet (closed symbols) derivatives of carp hemoglobin at 20 °C. The lines represent the rate constants, l'_4, for CO recombination with the triliganded CO derivative of fully ferrous carp hemoglobin. Panel A presents data obtained in the absence of organic phosphates, and panel B that obtained in the presence of 1 mM IHP. The ferrous heme concentration was 1 μM and the final CO concentration was 38 μM. Buffers were the same as in Figure 3.

derivatives of monoferric hemoglobin, the pH-dependent transition in reaction rates is shifted to lower pH. This shift is consistent with the presence of a single ligand slightly altering the T↔R equilibrium in favor of the R state. The difference in the properties of the aquo- and cyanomet derivatives at pH 6.1 and 6.4 suggest that cyanide is more effective than water in altering the T↔R equilibrium. As will be recalled, above pH 6.1 most of these reactions are biphasic. However, in all but two cases the slow phase was too small to permit the determination of its rate. As can be seen in Figure 3A, the two slow phases for which rate constants could be assigned exhibit unexpected behavior. In each case the rate constant of the reaction is even lower than that for CO binding to the fully ferrous hemoglobin under the same solution conditions.

The kinetics of CO recombination with the monoferric derivatives and fully ferrous carp hemoglobin in the presence of IHP are compared in Figure 3B. At pH 6.1 and 6.4, the rates for the monoferric derivatives correspond closely with those for the fully ferrous molecule. For the aquomet derivative this is also true at pH 7. However, as previously mentioned, there is a transition in the properties of the cyanide derivative at this pH so that at pH 7.02 this derivative displays a behavior similar to that of the fully ferrous molecule at pH 7.3.

Triferric Carp Hemoglobin. The second-order rate constants for CO recombination with the deoxy ferrous heme of the triferric derivative following flash photolysis are given in Table 2 as functions of pH and of the absence and presence of IHP and cyanide. Under all conditions examined, the kinetics are heterogeneous, requiring a sum of two exponential functions in order to be fitted. The percent of the total absorbance change contributed by each kinetic phase is also shown.

In Figure 4 the pH dependencies of the rate constants for CO recombination with the triferric derivatives of carp hemoglobin are compared to l'_4, the rate constants for CO recombination with the fully ferrous triliganded CO derivative. In the absence of IHP (Figure 4A), the kinetic properties of

Table 2: Rate Constants As Determined by Flash Photolysis for the Binding of CO to Triferric Carp B;[a] Effects of Different Ferric Ligands, IHP, and pH

			rapid process		slow process	
pH	ferric ligand	IHP	rate constant ($\times 10^6$ M^{-1} s^{-1})	% rxn[b]	rate constant ($\times 10^6$ M^{-1} s^{-1})	% rxn[b]
6.08–6.18	H_2O	–	1.6 ± 0.20	60 ± 5	0.39 ± 0.04	40 ± 5
	H_2O	+	0.23 ± 0.02	60 ± 7	0.06 ± 0.01	40 ± 7
	CN^-	–	2.4 ± 0.20	80 ± 6	0.80 ± 0.15	20 ± 5
	CN^-	+	0.37 ± 0.08	50 ± 10	0.15 ± 0.03	50 ± 10
6.53–6.61	H_2O	–	2.1 ± 0.10	70 ± 5	0.55 ± 0.05	30 ± 5
	H_2O	+	0.22 ± 0.02	70 ± 7	0.07 ± 0.01	30 ± 3
	CN^-	–	2.5 ± 0.20	80 ± 5	0.87 ± 0.10	20 ± 5
	CN^-	+	0.36 ± 0.04	60 ± 5	0.13 ± 0.02	40 ± 5
6.87–6.92	H_2O	–	2.7 ± 0.2	80 ± 7	0.70 ± 0.10	20 ± 7
	H_2O	+	0.23 ± 0.03	60 ± 6	0.07 ± 0.01	40 ± 5
	CN^-	–	2.7 ± 0.20	70 ± 10	1.0 ± 0.20	30 ± 10
	CN^-	+	0.66 ± 0.20	70 ± 5	0.17 ± 0.02	30 ± 4
7.03–7.08	H_2O	–	2.8 ± 0.20	80 ± 4	0.75 ± 0.10	20 ± 4
	H_2O	+	0.32 ± 0.05	60 ± 8	0.12 ± 0.02	40 ± 5
	CN^-	–	3.0 ± 0.40	70 ± 10	1.2 ± 0.30	30 ± 10
	CN^-	+	2.5 ± 0.20	80 ± 5	0.55 ± 0.06	20 ± 5

[a] Experimental conditions are given in the legend of Figure 5. [b] Percentage of the total absorbance change attributed to the process.

both the aquomet and cyanomet derivatives of triferric hemoglobin parallel the pH dependence of l'_4. Of the three triliganded derivatives it is the tricyanide form with which CO reacts most rapidly, although under some conditions the differences are not significant. In addition, the slow kinetic phase of the aquomet derivative seems to be uniformly slower than that of the triliganded CO derivative.

In examining the effect of IHP addition (Figure 4B), one observes larger differences among the three triliganded derivatives. These differences are most clearly seen at pH 7.1. Here the addition of IHP to the tricyanide derivative results in only a relatively small change in the two rates of CO recombination. In the case of the triaquomet derivative, IHP addition lowers the rates of CO recombination to values that approach those which we would typically assign to the T state. At this same pH, the triliganded CO derivative exhibits an intermediate response to IHP with values of l'_4 which lie between those of the R state and those of the T state. As the pH is decreased in the presence of IHP, the properties of the triaquomet derivative change very little, consistent with it being nearly in the T state at pH 7.1. As previously mentioned, the values of l'_4 decrease with decreasing pH until they reach those of the T state at pH 6.1 or below. The tricyanide derivative behaves very differently. Its rates of CO recombination decrease going from pH 7.1 to 6.5 but are virtually unchanged in going from pH 6.5 to 6.1. However, these pH invariant rates are significantly greater than those for the triaquomet and triliganded CO derivatives under these same conditions. This raises the serious possibility that the presence of three metcyanide hemes alters the functional properties of the remaining ferrous heme within the T state. If the greater rate of CO binding to the tricyanomet derivative were due to the T↔R equilibrium, then the pH dependence of this rate should persist.

When CO recombination following flash photolysis was compared under the same experimental conditions with CO combination as measured by stopped-flow techniques at pH 6.1, the patterns of the dependence of the overall reaction on IHP and the nature of the ferric ligand were remarkedly similar. This is clearly shown in Figure 5. This result is unlike earlier findings with the triferric derivative of the cross-linked human hemoglobin A (Fowler *et al.*, 1992), where differences in kinetic properties observed by flash photolysis and stopped-flow techniques prompted the hypothesis of the occurrence of slow structural relaxations in the protein. Given the limited stopped-flow data obtained in the present study, it is premature to conclude that results obtained by stopped-flow and flash photolysis will be identical under all experimental conditions.

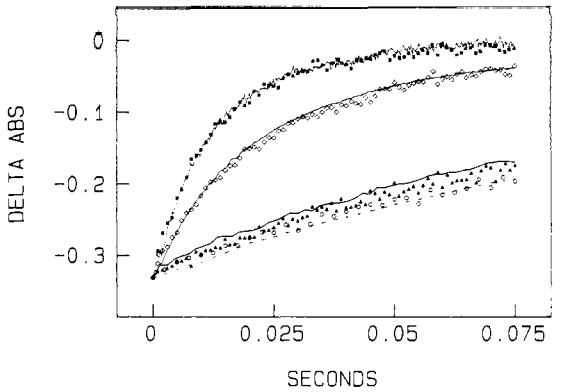

FIGURE 5: A comparison of the time courses of CO binding with the triferric derivative following flash photolysis (symbols) and rapid mixing in a stopped-flow (lines) at pH 6.1, monitored at 420 nm: (■) tricyanomet, (◇) triaquomet, (▲) tricyanomet + 1 mM IHP, (○) triaquomet + 1 mM IHP. The change in absorbance is plotted as a function of time in seconds. The change in absorbance at time zero was normalized to –0.33. Buffers were the same as in Figure 3.

DISCUSSION

We have isolated the asymmetric monoferric and triferric derivatives of carp hemoglobin in their aquomet form and demonstrated their stability in solution at 0 °C for 6 h or more. This stability requires that the dissociation of the tetrameric hemoglobin molecule into $\alpha\beta$ dimers is not only energetically unfavorable but kinetically hindered, since reequilibration between these structure forms results in the disproportionation of asymmetric states. Brunori and co-workers reported the isolation of partially oxidized derivatives of a trout hemoglobin by isoelectric focusing (Brunori *et al.*, 1974; Giardina *et al.*, 1982). However, they never subjected their isolated derivatives to refocusing to establish purity or stability in solution. Nevertheless, the fact that asymmetric valency hybrids would band in isoelectric focusing indicates that the dissociation of trout hemoglobin into $\alpha\beta$ dimers also occurs very slowly, if at all.

In attempting to interpret the kinetic properties reported here, it is important to remember that the monoferric derivative consists of a mixture of $\alpha^+\alpha^0\beta^0\beta^0$ and $\alpha^0\alpha^0\beta^0\beta^+$; i.e. the ferric heme can be on either an α or a β chain. The triferric derivative consists of a mixture of $\alpha^+\alpha^+\beta^+\beta^0$ and $\alpha^0\alpha^+\beta^+\beta^+$. The relative amounts of the two species in either derivative depend on the relative rates of oxidation of the α and β chains. We have been unable to isolate any of these species.

A priori one would anticipate that each of the two triferric species would be kinetically homogeneous since each contains a single CO binding site. When reactions of CO binding to a triferric derivative of hemoglobin are characterized by two kinetic phases, each phase should represent the properties of one of the species. If this were the case, then the percentages of the two kinetic phases should remain constant within experimental error regardless of the solution conditions. In Table 2 we see that the percentage of the reaction contributed by each phase is not constant. The percentage of the slow phase, for example, varies from 20% to 50%. The distribution of these percentages is not random. Under conditions which result in the most rapid rates of CO recombination the percentages of the fast and slow phases are, within experimental error, 80% and 20%, respectively. Under conditions resulting in the slowest rates, percentages of the fast and slow phases are 60% and 40%, respectively. As previously mentioned, the estimation of the percentages of kinetic phases whose rates differ by less than 3-fold is associated with significant error, but such errors should be random.

The origin of this variation in distribution between fast and slow kinetic phases is unknown. If the two kinetic phases are actually associated with the two different triferric species, the relative amounts of the two species must vary with solution conditions requiring electron transfer between heme groups. This seems to be inconsistent with the stability of the asymmetric valency hybrids. Alternatively, a single triferric species might exhibit more than one reaction rate. This would require the existence of functionally distinct conformers which reequilibrate with one another at rates which are slow compared to the rate of CO binding. These could be protein conformers or substrates as hypothesized by Austin *et al.* (1975), or even hemoglobins differing in the orientations of their heme groups. La Mar *et al.* (1984) interpreted the NMR spectra of carp hemoglobin to indicate conformational heterogeneity of the heme groups of the native protein. Light *et al.* (1987) demonstrated that heme orientation has no effect on the functional properties of myoglobin, but no evidence is available on the potential effect of heme orientation on the functional properties of hemoglobin. At present there is no basis for choice among these possibilities, nor is the list necessarily complete.

The properties of the triliganded derivatives of carp hemoglobin cannot distinguish between different sequences of functional changes associated with ligand binding. However, they do permit one to evaluate the extent to which ferric hemes are appropriate models for liganded ferrous hemes *vis a vis* their effects on conformational equilibria and on the functional properties of neighboring heme groups. The kinetic differences among the three triliganded hemoglobins can be attributed in part to differences in the effects of the three different ligands, water, cyanide and CO, on the T↔R quaternary equilibrium. The effects of pH and IHP on the kinetic properties of the three derivatives appear to be similar. However, in the presence of IHP, the pH-dependent transition from minimum to maximum rate constants occurs over a lower pH range in the case of the tricyanide derivative and over a higher pH range in the case of the triaquomet molecule as compared to the properties of the triliganded CO derivative.

The two-state model can accommodate a shift in the pH dependence of the transition from the T to R state by permitting different ligands to have differing effects on the equilibrium between these two quaternary states. However, the model predicts that the functional properties of the R and T states themselves will be invariant. Although the data fail to establish that the R and T state kinetic properties of the triaquomet derivative are different from those of the triferrous CO derivative, they also fail to demonstrate identity. However, at low pH in the presence of IHP, one observes T-state properties for the tricyanide derivative which are distinctly different from those of the other triliganded forms. As observed with the other triliganded derivatives, the rates of CO recombination with the tricyanide derivative are virtually unchanged from pH 6 to 6.5, but are roughly 2-fold greater than the rates of CO recombination with the other two triliganded forms. From the properties of the triliganded derivatives in the absence of IHP, we know that the T↔R equilibrium is strongly pH dependent between pH 6 and 6.5. Therefore, the greater rates of CO binding to the tricyanide derivative suggest a significant sensitivity of the T state of carp hemoglobin to the presence of cyanide ions on three heme groups.

By examining the effects of the number and distribution of metcyanide hemes on the energetics of dissociation of the human hemoglobin molecule into $\alpha\beta$ dimers, Ackers and coworkers have demonstrated a high degree of cooperativity between the conversion of the first and second unliganded heme to this liganded form so long as both metcyanide hemes lie within the same $\alpha\beta$ dimer in the hemoglobin tetramer (Daugherty *et al.*, 1991). By examining the effects of single amino acid mutations on the dimer–tetramer equilibria of hemoglobins with different distributions of metcyanide hemes, it was possible to demonstrate that this cooperativity between the hemes of an $\alpha\beta$ dimer occurs within the quaternary T state (LiCata *et al.*, 1993). Although this phenomenon is not the same as that observed with the tricyanide T state of carp hemoglobin, in both cases heme groups within a quaternary T state appear to respond to the presence of metcyanide hemes elsewhere in the molecule.

It is in the kinetics of CO binding to the monoliganded derivatives monoaquomet and monometcyanide that one might detect behavior which distinguishes between different patterns or mechanisms of allosteric interaction. Many of the effects reported here of the oxidation of a single heme group on the kinetics of CO recombination fall within the expectations of the two-state model. The effects observed would suggest that the presence of a ligand on one heme group shifts the conformational equilibrium in favor of the R state, with the cyanide ion having a greater effect than the water molecule. However, there are details of our results which are not easily accommodated within the framework of this model.

Slow kinetic phases are observed in the recombination of CO with monoferric carp hemoglobin in the absence of IHP. In two cases the rate constant associated with the slow phase could be measured. These rate constants are slower than those of the reaction of fully ferrous, deoxygenated carp hemoglobin with CO under the same solution conditions. Taken at face value this suggests that the presence of a single ligand on an otherwise deoxygenated tetramer can reduce the rate at which CO binds to some of the remaining heme groups, a phenomenon for which we can offer no explanation. In addition it was expected that, at pH 8 and above in the absence of IHP, the

kinetic properties of the monoferric derivative would more closely resemble those of the R state as estimated from measurements of l'_4. Under these conditions both the Soret spectrum (McDonald *et al.*, 1976) and the NMR spectrum of exchangeable proton resonances (Dalvit *et al.*, 1984) of deoxygenated carp hemoglobin suggest that the molecule is at least partially in the R state. The rates of ligand binding to the deoxygenated molecule are consistent with this interpretation. If the fully unliganded molecule were partially in the R state, the two-state model would predict that the binding of a single ligand molecule would result in a major increase in the proportion of R state molecules exhibiting the appropriate kinetic properties for this quaternary state. However, no binding reaction corresponding to the rapid kinetic phase of l'_4 is actually observed. A number of modifications to the two-state model might account for this finding. The simplest may be to invoke an exaggerated T state Bohr effect in carp hemoglobin. The ligand affinity of the T state at high pH would then be much greater than at pH 6, and the difference between the ligand affinities of the T and R states would become relatively small, minimizing the conformational effect of binding a single ligand.

It is quite possible that other kinetic complexities exist that have not been identified in the binding of CO to mixed valency hybrids of carp hemoglobin. The intrinsic heterogeneity of the α and β chains limits our sensitivity to the additional heterogeneity that might result from alternative pathways of ligand binding. The properties of the metcyanide derivatives at low pH in the presence of IHP serve as an example of this limitation. The studies of Ackers and co-workers (1992) on metcyanide valency hybrids of human hemoglobin have identified cooperativity within the $\alpha\beta$ dimer of the T state of this protein. The kinetics of CO binding to the tricyanide derivative of carp hemoglobin at low pH in the presence of IHP suggest that the properties of the deoxy ferrous heme groups within the T state of this hemoglobin are also sensitive to the presence of metcyanide hemes on neighboring subunits. If the metcyanide heme within the carp hemoglobin T state exerts its effect on the neighboring heme of the $\alpha\beta$ dimer, then the kinetics of CO binding to the monocyanide derivative at low pH in the presence of IHP should be complex. Under these conditions, one-third of the hemes should exhibit the kinetic behavior of the tricyanide derivative with its two rate constants while two-thirds should be kinetically similar to the fully ferrous hemoglobin with its two rate constants. We have constructed an artificial data set by summing the four appropriate exponential functions and superimposing the level of random noise commonly observed in our experiments, 1% of the total optical density change. As shown in Figure 6, this data set can be satisfactorily fitted to a sum of two exponential functions. A third exponential transient cannot be identified with statistical significance. Therefore one cannot exclude the possibility that additional kinetic complexity is hidden within our experimental results. The reported pairs of rate constants represent the best fit of the data with the smallest possible set of adjustable parameters. Although these rate constants reproduce the data within experimental error, they do not necessarily represent the actual set of kinetic transients which combine to yield the data we observe.

In summary, we have isolated asymmetric mixed-valency hybrids of native carp hemoglobin and shown that they are stable over a sufficient period of time to permit the measurement of their functional properties. We have measured their rates of CO binding and compared them to the rates of CO binding to fully ferrous deoxy carp hemoglobin and to the triliganded CO derivative. The data are consistent with the two-state model only if the model is modified to permit considerable plasticity in the properties of the T quaternary state. Properties of unoccupied heme groups must be sensitive to the presence and nature of ligands on the hemes of neighboring subunits. In addition, T-state properties may be sensitive to pH. Similar phenomena have already been identified in human hemoglobin. The Bohr effect, associated with the binding of ligand to the T state, is well documented (Imai & Yonetoni, 1975). The studies of Ackers and co-workers already referred to amply document communication among the heme groups within the T state of human hemoglobin. It seems that the properties of both carp and human hemoglobin deviate from the predictions of the simple two-state model in similar ways.

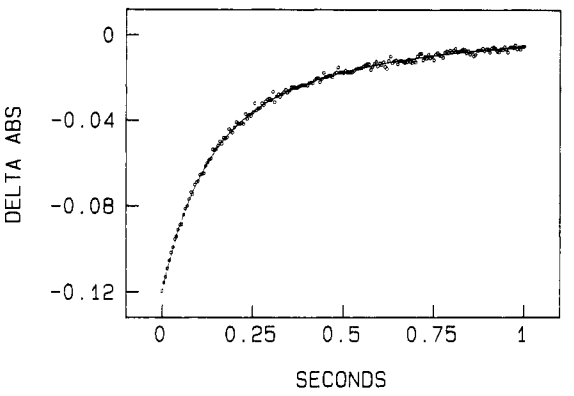

FIGURE 6: Application of fitting routines to synthetic data. The open circles represent a synthetic data set which was obtained by summing four exponential functions. Two of these functions represent the time courses for the reaction of 38 μM CO with the tricyanomet derivative at pH 6.1 in the presence of IHP with rates of 14.1 and 5.7 s^{-1}. The other two functions represent the binding of CO to the fully ferrous molecule under the same conditions with rates of 7.6 and 1.9 s^{-1}. The contribution of each function to the total optical density change was $^1/_6$, $^1/_6$, $^1/_3$, and $^1/_3$, respectively. The line is the result of fitting this data set to the sum of two exponential functions using the OLIS routines.

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