Ligand Binding Kinetic Studies on the Hybrid Hemoglobin α(Carp):β(Human): A Hemoglobin with a Restricted Allosteric Range†

Dixie J. Goss and Lawrence J. Parkhurst*

ABSTRACT: The preparation of native α- and β-chains of carp hemoglobin and the preparation of the hybrid hemoglobin α(carp):β(human), hybrid I, are described. CO association and dissociation kinetics were determined for the hybrid hemoglobin α(carp):β(human) as a function of fractional saturation by stopped-flow and by flash and laser photolysis. Oxygen dissociation from the liganded distribution of conformers and overall oxygen association were studied by laser photolysis. An allosteric model was used to fit successfully all of the CO and oxygen kinetics and equilibria for this hybrid. In this model, it was assumed that the conformational changes were rapid with respect to ligand binding. A linear free-energy relationship relating rate constants and equilibrium constants was assumed in order to reduce the number of fitting parameters. In the allosteric model itself, it was assumed that L, but not c, varied with pH. For CO, the allosteric parameter c was 0.24; for oxygen, the value was 0.14. At pH 7, L was 80. In hybrid I, the calculated CO association rate constants for the R and T states differ by only a factor of 2. From the equilibrium data, with no assumptions as to the rate of conformational equilibrations, one can show that the allosteric model requires that the measured CO association constant from stopped-flow measurements be assigned to the T state. This state, however, is poised approximately midway between a carp–human T state and a carp hemoglobin R state. In this hybrid, neither chain shows normal R-state behavior; rather, the liganded state is also intermediate between R and T. In this hemoglobin, the low cooperativity appears to be associated with a very restricted range for the R–T conformational change.

Isolation of native α- and β-chains of human hemoglobin has allowed the study of properties of the individual subunits and how these properties are altered after combination to form hemoglobin. The availability of native α- and β-chains from other species allows one to extend such studies by comparing properties of diverse α- and β-chains and by forming hybrid hemoglobins that provide information on the effects of altered α–β chain contacts.

The first convenient preparation of native human α- and β-chains by Bucci & Fronticelli (1965) employed reaction with PMB [*p*-(hydroxymercuri)benzoate] at low pH. Various modifications of the procedure have been reported (DeRenzo et al., 1967; Geraci et al., 1969; Winterhalter & Colosimo, 1971). Noble (1971) isolated γ-chains from fetal hemoglobin. DeBruin et al. (1977) used linkage of the β-chains of beef hemoglobin to a disulfide column to isolate beef α-chains, which lack cysteine, but the β-chains were not recovered. It does not appear that native chains from other species have been isolated.

We report here the isolation of both α- and β-chains from carp hemoglobin and the ligand binding kinetics of the hybrid hemoglobin α(carp):β(human), hybrid I. The following paper (Parkhurst & Goss, 1984) will deal with the ligand binding kinetics of hybrid II, α(human):β(carp).

Materials and Methods

Human Hemoglobin, Chains, and Various Reagents. Human hemoglobin and chains were prepared as described elsewhere (Geraci et al., 1969). Human α- and β-chains and carp red blood cells were frozen in liquid nitrogen as small pellets and stored under liquid nitrogen. Carp hemoglobin was prepared according to Tan et al. (1972). Carp hemoglobin consists of at least three components (Yamanaka et al., 1965). Tan et al. (1972) found that the two major components had identical kinetics. For some of the kinetic experiments on the α(human):β(carp) hybrid (pH 6 and 7, see following paper) and the disc gel electrophoresis, the carp hemoglobin was fractionated by isoelectric focusing before the chains were separated, and the main component, pI = 6.3, was used. It was found that the carp β-chain sulfhydryls were readily oxidized even when the protein was stored at 4 °C. Therefore, carp hemoglobin was treated with dithiothreitol (2-fold molar excess, 12 h) before use. Carbon monoxide and argon were products of Matheson Scientific (East Rutherford, NJ). Glucose oxidase (type II), β-D-glucose, and catalase (from bovine liver, purified powder) were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex was the product of Pharmacia Fine Chemicals (St. Louis, MO). Dithionite was from Holden and Harding (Miles Platting, Manchester, England). Triethylamine hydrochloride was from Eastman Kodak (Rochester, NY), and *p*-aminophenyl mercuriacetate was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were reagent grade.

Preparation of the Mercuri Resin. The mercuri resin was either prepared from PAMBSYL[1] as described previously (Goss & Parkhurst, 1980) or prepared from *p*-aminophenylmercuriacetate and Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA). In the latter procedure, 25 mL of gel slurry was vacuum filtered to remove solvent and quickly added to a solution of 0.5 g of *p*-aminophenyl mercuriacetate in 50 mL of *N*,*N*-dimethylformamide (DMF) (Matheson Coleman and Bell, East Rutherford, NJ). The gel slurry was gently stirred for 18 h in the dark at room temperature. The gel was then gently vacuum filtered and washed successively with 100 mL of DMF and 200 mL of deionized water. The gel was suspended in deionized water and stored at 4 °C in the dark. The gel bound 1 μmol of hemoglobin/mL of resin.

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[1] Abbreviations: PAMBSYL, *p*-mercuribenzenesulfonyl chloride; DMF, dimethylformamide; TEA, triethylamine; IHP, inositol hexaphosphate.

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Isolation of Carp α-Chains. Since carp α-chains contain no sulfhydryl groups (Hilse, & Braunitzer, 1968), β-chains and hemoglobin can be separated from the α-chains with a mercuri resin. Once the hemoglobin is split into chains, the β-chains and remaining hemoglobin are bound to a mercuri resin while α-chains remain in solution. For human hemoglobin, splitting occurs in 2 M triethylamine (Amiconi et al., 1971; Edelstein et al., 1970). Carp α-chains were isolated by diluting a concentrated stock hemoglobin solution into cold (4 °C) 0.1 M potassium phosphate buffer, pH 7.0, 2.5 M in triethylamine (TEA), so that the final heme concentration was 100 μM, and the concentration of TEA exceeded 2.2 M. The solution was either passed over a mercuri column (5 mL of resin/25 mL of solution), or resin was added dropwise directly to the solution, stirred, and allowed to settle. An excess of resin was added so that all of the sulfhydryl-containing β-chains and undissociated hemoglobin were bound to the resin. In the column procedure, sufficient resin was used so that only the top half of the column bound hemoglobin and/or β-chains. The supernatant, or column eluate, containing the α-chains was passed over a Sephadex G-25 column (2.2 × 35 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, 0.1 M in NaCl, at room temperature to remove triethylamine. As an additional test for the presence of β-chains or hemoglobin, mercuri resin was added to the eluate. The absence of further binding showed that neither β-chains nor hemoglobin was present.

Isolation of Carp β-Chains. Carp β-chains were isolated by diluting a concentrated stock hemoglobin solution into TEA as described above. The final heme concentration was 5 μM. At this concentration, the hemoglobin is largely dissociated into chains. The solution was passed over a mercuri column, or resin was added as described above except that 5 mL of resin was used for 100 mL of solution. The gel was washed with 0.1 M potassium phosphate buffer (about 25 mL in all) until no further protein was eluted. The β-chains were removed from the resin with 2 mM β-mercaptoethanol (10 mL) and then passed at once over a G-25 column as described above. Proteins were concentrated with an Amicon concentrator (Model 3, Amicon Corp., Lexington, MA) with a PM10 membrane under CO at 50–80 psi. The protein used for isolation was either cyanomet- or carbonmonoxyhemoglobin. In either case, care was taken to ensure that the protein was fully liganded at all times. Since the chains appear to have a rather low affinity for ligands, CO or sodium cyanide was always added after the chains were removed from, or passed over, the mercuri resin and again after the G-25 column, ensuring that the chains were always liganded. β-Chains were treated with 1 mM dithiothreitol before kinetic measurements were made.

Hybrid Hemoglobins. Hybrid hemoglobins were prepared by mixing equal amounts (assumming equal molar absorptivities at 540 nm for the CO forms) of the chains. Disc gel electrophoresis (Brewer & Ashworth, 1969) in polyacrylamide gels showed nearly complete (>95%) combination of the chains. For the α(carp):β(human) hybrid, approximately 1% of the material was uncombined. For the α(human):β(carp) hybrid, typically 1–3% of the chains failed to form a hemoglobin. These amounts were undetectable in kinetic measurements.

Removal of Triethylamine. The extent of removal of TEA was determined by flame-ionization gas chromatographic analysis on a Sigma 3 (Perkin-Elmer) gas chromatograph using a 2 mm i.d. × 1.524 m glass column packed with 4% Carbowax 20 M with 0.8% KOH on Carbopack B (Supelco, Inc., Bellefonte, PA). The flow rate of the carrier gas was 30 mL/min. Samples were prepared for analysis by mixing equal volumes of protein (20 μM in heme) with 2 N NaOH and letting the solutions stand at room temperature for 30 min. Samples of 1 μL were injected into the gas chromatograph. As little as 0.05 mol of TEA/mol of heme could be detected as determined from standard samples. α- and β-chains showed no detectable TEA.

Kinetic Measurements. Gas solutions were equilibrated as previously described (Steinmeier & Parkhurst, 1975). The buffers were 0.05 M in potassium phosphate except at pH 9, where the buffer was 0.05 M in borate. The buffer denoted "pH 6 (+1 mM IHP)" was 0.05 M in potassium phosphate and 1 mM in IHP. Since the hybrid hemoglobins were very sensitive to dithionite (see below), oxygen was removed from the gas solutions by addition of β-D-glucose, glucose oxidase, and catalase. The stopped-flow apparatus has been described elsewhere (Görisch et al., 1976). For absorbance measurements a Jarrel-Ash quarter meter monochromator and a 150 W Osram Xe lamp were used. The flash-photolysis sample chamber has been previously described (Boelts & Parkhurst, 1971). Photolysis experiments were carried out with a Phase-R 2100 BXH dye laser (0.5-μs pulse, 585 nm) with rhodamine 6G as the lasing dye. A Schoeffel (GM 100) monochrometer and Corning (CS3-66) filter were positioned in the sample chamber as described elsewhere (Goss et al., 1982). A Nova 2/10 minicomputer with 32K of memory was used for data acquisition as previously described (LaGow & Parkhurst, 1972).

Association of oxygen and carbon monoxide and dissociation of oxygen were measured by photolysis. The rate constants[2] were determined as follows.

(1) Oxygen association (k') was measured by observing the rapid phase of the reaction occurring after laser photolysis of HbCO or CO-containing valency hybrids in the presence of oxygen. The concentrations were as follows on a heme basis: O_2, 230–240 μM; CO, 160–180 μM; protein 35–50 μM. The observing wavelength was 430 nm. The fraction photolysis varied from 0.1 to essentially 1.

(2) Oxygen dissociation (k^*) was measured from the slow phase of the replacement reaction in experiment 1 observed at 425 nm. Since Hb can be assumed to be a steady-state intermediate, and $[O_2]$ and $[CO] >> [Hb]_{total}$, the first-order relaxation constant, R, can be obtained by inspection (Gilbert, 1977):

$$R = \frac{l'[CO]k^* + k'[O_2]l}{l'[CO] + k'[O_2]} \simeq \frac{l'[CO]k^*}{l'[CO] + k'[O_2]}$$

from which the oxygen dissociation constant, k^*, can be obtained.

(3) The association of CO (l') was measured by following the recombination reaction after photolysis of HbCO. Glucose, glucose oxidase, and catalase were added to the solution to remove oxygen. The complete removal of oxygen was determined by monitoring the amplitude of the relaxation reaction (experiment 2) at 425 nm. Approximately 5 min was required for removal of oxygen and the disappearance of the relaxation phase. The observing wavelength was 430 nm. The recombination reaction was also studied as a function of fraction photolysis from 10 to 100% photolysis. A rapid re-

[2] We follow the Roughton convention in which "l" denotes a rate constant for CO binding and "k" a rate constant for oxygen binding and where primed rate constants are for association reactions and unprimed rate constants are for dissociation reactions.

combination ($l^{*\prime}$) is indicative of the reaction of R-state sites.

(4) Carbon monoxide binding was also measured by stopped-flow. The protein was deoxygenated by dilution into argon-saturated buffer and addition of glucose, glucose oxidase, and catalase. Deoxyhemoglobin (10 μM in heme) was flowed against 90 μM CO and the reaction monitored at 430 nm. Both carp–human hybrid hemoglobins were very sensitive to dithionite below pH 7.0. There were no changes in the observed kinetics above pH 7.0 with the addition of small amounts of dithionite.

(5) Carbon monoxide dissociation (l) was measured by a modification of the procedure of Sharma et al. (1975, 1976). The carbonmonoxyhemoglobin (1 mL) was passed over a G-25 column (0.9 × 15 cm) equilibrated with argon-saturated buffer (pH 7.0, potassium phosphate 0.005 M) to remove excess CO. The protein was diluted into argon-saturated buffer (heme concentration 5 μM), and glucose, glucose oxidase, and catalase were added to remove oxygen. The protein was titrated with CO-saturated buffer until spectra showed that the hemoglobin was fully CO liganded (total CO concentration 6–7 μM). The protein was flowed against a microperoxidase solution (1 mg/20 mL in argon-saturated buffer to which a few crystals of dithionite had been added). The reaction was monitored at 419 nm. Dissociation of carbon monoxide from the partially saturated protein was measured in flow–flash experiments in the same stopped-flow apparatus. The protein was flowed against microperoxidase as described above. At 10 ms after flow ended, a photographic flash (Wabash electronic flash, type R-1140, 1-ms dead time) was fired to dissociate 70–85% of the CO. The dissociation reaction for the remaining bound CO was observed. In general, kinetic data were recorded over the first 87–95% of the reaction.

Data Fitting and Analysis. Data were fit to one- or two-exponential decay models by a Fletcher–Powell (1963) minimization algorithm. Estimates of the standard deviations in the parameters were obtained from the variance–covariance matrix (Draper & Smith, 1966; Bard, 1974). Sequential models were assumed for both CO association and CO dissociation kinetics. These models were then interpreted within the two-state allosteric framework, under the assumption that conformational equilibria were established at each stage of ligation. Thus, for CO association

$$\mathrm{Hb} \underset{l_1}{\overset{l_1'}{\rightleftharpoons}} \mathrm{Hb(CO)} \underset{l_2}{\overset{l_2'}{\rightleftharpoons}} \mathrm{Hb(CO)_2} \underset{l_3}{\overset{l_3'}{\rightleftharpoons}} \mathrm{Hb(CO)_3} \underset{l_4}{\overset{l_4'}{\rightleftharpoons}} \mathrm{Hb(CO)_4}$$

the rate constants were $l_i' = (Lc^{i-1}l'_T + l'_R)/(1 + Lc^{i-1})$. The CO dissociation model was simply the reverse of the above model, where, "i" denoting the initial number of bound ligands, the rate constant $l_i = (Lc^i l_T + l_R)/(Lc^i + 1)$. In laser-photolysis experiments, for fractional photolysis, F, the initial mole fraction (MF) of a given species for CO combination, or for dissociation in the presence of microperoxidase, is (setting $0^0 = 1$)

$$\mathrm{MF[Hb(CO)}_i] = [F^{4-i}(1 - F)^i]4!/[(4 - i)!i!]$$

Expression were derived for the concentrations of all species as a function of time in terms of the rate constants l'_T and l'_R and L, c, and F, and the appropriate linear combinations of terms were then fit to the observed absorbance changes as a function of time. The actual fitting proceeded as follows. It was first assumed that the only parameter that varied with pH was L. Since the overall variation in any one of the observed or apparent rate constants was quite small (just over 2-fold) and since the Hill number for oxygen binding was nearly 1 at both pH 6 and 9, we assumed a simple relationship between the observed extreme values of a rate constant and the values of that rate constant for the R and T states:

$$l'_R = fl^{*\prime}{}_{\mathrm{pH9}} \qquad l'_T = (1/f)l'_{\mathrm{pH6}}$$

We then assumed a linear free-energy relationship (Szabo, 1978) between the association rate constants and the equilibrium constants:

$$\log (l'_R/l'_T) = \log (K_R/K_T) = -\alpha \log c$$

We used a value for α of 0.53 (Parkhurst et al., 1983) and let c range from 0.1 to 0.24. From the above, $l'_R = (l^{*\prime}{}_{\mathrm{pH9}} l'_{\mathrm{pH6}} c^{-\alpha})^{1/2}$ and $l'_T = l'_R c\alpha$, where $l^{*\prime}{}_{\mathrm{pH9}}$ is the observed association constant for 10% photolysis at pH 9.

Similarly, $l_R = (l_{\mathrm{pH6,0.9}} l_{\mathrm{pH9}} c^{1-\alpha})^{1/2}$ and $l_T = l_R c^{\alpha-1}$, where $l_{\mathrm{pH6,0.9}}$ is the measured value for CO dissociation for 0.9 fractional photolysis (F). For pHs 6, 7, 8, and 9, we had CO association data for fractional photolysis and stopped-flow data. For pHs 6, 7, and 9, we had CO dissociation data for zero and fractional photolysis. For a given choice of c, we had only L as an adjustable parameter with which to fit all of the kinetic data at each pH (Table I). In addition, at pH 7, where the hybrid showed maximal cooperativity in oxygen binding, our value for L and the previous value for c of 0.18 ± 0.08 (Causgrove et al., 1984) should fit the oxygen equilibrium data, since L must be ligand independent.

Results and Discussion

Carp hemoglobin ligand binding kinetics can be interpreted as follows (Tan et al., 1972). At pH 5.6 and 6.0 in the presence of organic phosphates, carp hemoglobin is locked in the T state in both deoxy and liganded forms; at pH 9.0, the hemoglobin assumes an R-state conformation for both deoxy and liganded forms. At intermediate pHs, the hemoglobin can undergo an R–T transition as does human hemoglobin. The rate constants for CO recombination (l') and oxygen dissociation (k) vary dramatically over the pH range 6–9 (Noble et al., 1970). At the two extreme pHs, the flash-photolysis CO recombination reaction is the same at high and low breakdown. The rate constant is also the same when measured by stopped flow or flash photolysis. The Hill number is approximately 1 at both extreme pHs (Tan et al., 1972; Tan & Noble, 1973). The protein does show cooperativity at intermediate pHs, and the CO recombination rate varies with flash intensity (Tan et al., 1973). The ligand kinetics for carp hemoglobin cannot be fit in detail in terms of a two-state allosteric model in which the ligand binding is very much slower than conformational changes at pH 7 (Parkhurst et al., 1983).

For human hemoglobin, the protein undergoes an R–T transition at *all* pHs. The CO recombination rates as measured by total photolysis of HbCO and stopped flow are the same. At low fractional photolysis, the CO rate increases.

In contrast to the parent hemoglobins, the α(carp):β(human) hybrid hemoglobin shows little pH dependence either for CO combination (l') (measured by flash photolysis) or for oxygen dissociation (k^*) (measured by the replacement reaction). These two observed rate constants are plotted as a function of pH in Figures 1 and 2. These rate constants are intermediate between those characteristic of the R and T states observed for carp hemoglobin at pHs 9 and 5.6, respectively. The CO recombination reaction as a function of fraction of photolysis shows only a slight increase (1.5-fold or less) in the rate of CO recombination as the fraction of photolysis varies from 100 to 10%. CO combination by stopped flow gives the apparent rate constant for combination to the stable deoxy form of the protein and is the same as that obtained for 100%

Table I: Allosteric Fits to the CO Kinetic Data[a]

	pH 6	pH 7	pH 8	pH 9
association				
flow				
obsd	0.48 ± 0.03	0.52 ± 0.04	0.57 ± 0.04	0.62 ± 0.03
calcd	0.49	0.53	0.56	0.75
flash (F)				
obsd	0.60 ± 0.06 (0.15)	0.66 ± 0.07 (0.18)	0.71 ± 0.07 (0.1)	0.90 ± 0.08 (0.1)
calcd	0.55	0.65	0.74	0.91
dissociation				
flow				
obsd	0.10 ± 0.02	0.08 ± 0.01		0.06 ± 0.01
calcd	0.093	0.085		0.063
flash (F)				
obsd	0.11 ± 0.03 (0.85)	0.085 ± 0.008 (0.8)		0.05 ± 0.01 (0.84)
calcd	0.11	0.11		0.083
L	245	80	42	5
SSQ	0.06	0.26	0.019	0.55

$l'_R = 0.959\ \mu M^{-1}\ s^{-1}$ $l'_T = 0.450\ \mu M^{-1}\ s^{-1}$
$l_R = 0.053\ s^{-1}$ $l_T = 0.104\ s^{-1}$

[a] Kinetic data consisted of normalized absorbance changes represented by 20 points equally spaced in time over three half-lives. The association experiments were run under pseudo-first-order conditions. Units for association rate constants are $\mu M^{-1}\ s^{-1}$ and for dissociation constants are s^{-1}. The allosteric parameter c was 0.24 for the above fitting. The calculated rate constant was determined by fitting the theoretical progress curve for three half-lives to the best fitting single-exponential decay model. The data were collected at 20 °C. F is fraction photolysis, and the value is in parentheses beside the related rate constant.

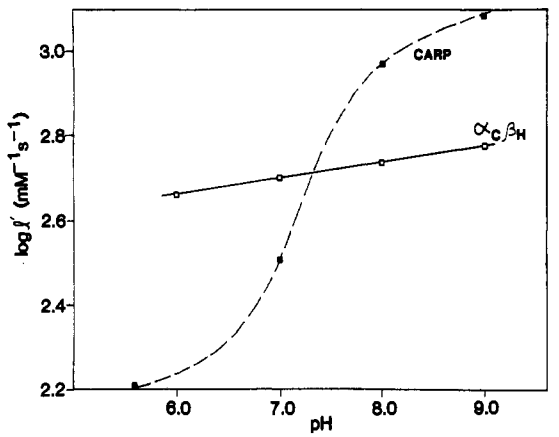

FIGURE 1: pH dependence of the rate constant (l') for CO recombination for 100% photolysis. The solid squares and dashed line show the data for carp hemoglobin. The open squares and solid line are for hybrid I. The observing wavelength was 430 nm, and the temperature was 20 °C.

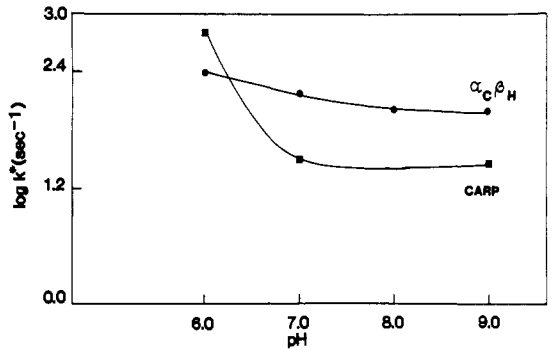

FIGURE 2: pH dependence of the rate constant (k^*) for the oxygen dissociation reaction. The data points are determined as described in the text. Carp hemoglobin (squares) is shown for comparison. The circles depict the data for hybrid I. The temperature was 20 °C for all measurements.

photolysis at all pHs (see Figure 3). The rate is intermediate between the rates observed for R- and T-state carp hemoglobin. The fact that partial breakdown by flash photolysis is not significantly biphasic implies a rapid equilibrium for the R–T

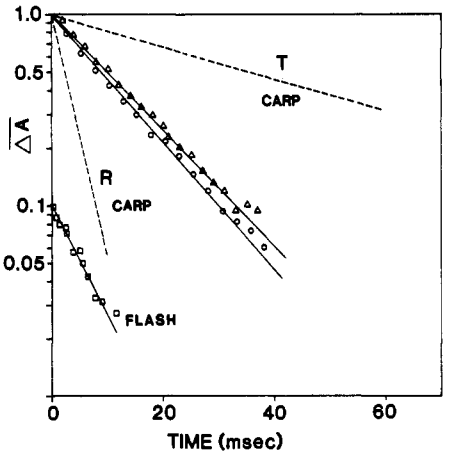

FIGURE 3: CO recombination reaction for hybrid I at pH 6.0 (+IHP). The triangles and squares show the data for CO recombination by flash photolysis at total and partial (10%) photolysis, respectively. The circles depict the data for CO combination by stopped flow. The reaction for R- and T-state carp hemoglobin (dashed lines) are shown for comparison. [CO] = 182 μM; pH 6.0; T = 20 °C; λ = 430 nm.

conformational change. Figure 4 compares hybrid I and carp kinetics for oxygen association and total CO dissociation. Data for all of the kinetics for hybrid I could be fit very well by single exponential decay models— there was never any indication of biexponential behavior.

The procedure described above under Materials and Methods was used to fit the CO association and dissociation data together as a function of pH where L, but not c, was allowed to vary with pH. The parameter c was varied between the limits 0.1 and 0.24. An upper limit on c of 0.242 is obtained from the appropriate ratio of the various observed rate constants. The sum of squared residuals, SSQ, was 2–5-fold larger for c = 0.1 than for c = 0.24. For CO binding, the overall fits according to the allosteric model are excellent (Table I). The CO data for hybrid I are consistent with a two-state allosteric model with conformational changes rapid with respect to ligation rates and with very little difference between the R and T states. We may use a linear free-energy relation approach to diagram the changes that occur when the hybrid is formed from the parent hemoglobins. From the

Table II: Allosteric Fitting of the Binding Data for Hybrid I[a]

pH	k^*(obsd)	k^*(calcd)	L	c	k'(obsd)	k'(calcd)	$\bar{O}_2$(obsd) (μM)	$\bar{O}_2$(calcd) (μM)	n^*(obsd)	n^*(calcd)
6	245 ± 15	193	1000	0.16	10 ± 0.5	9.33	42 ± 2	32.3	1.1 ± 0.1	1.18
		125	245[b]	0.16		11.32				
7	141 ± 8	141	375	0.16	11.5 ± 0.6	9.55	32.4 ± 1	27.4	1.4 ± 0.1	1.32
		129	375	0.14		10.2		28.1		1.43
		102	80[b]	0.16		12.5				
8	100 ± 9	95.9	42[b]	0.16		10.75				
9	82 ± 2	89.7	5[b]	0.16	13 ± 1	12.5	6.1 ± 0.5		1.0 ± 0.1	
		88.9	0.5	0.16		14.3		6.7		1.07

$k_R = 88.8\ s^{-1}$ $k_T = 351.3\ s^{-1}$ $k'_R = 14.33\ \mu M^{-1}\ s^{-1}$ $k'_T = 9.07\ \mu M^{-1}\ s^{-1}$

[a] Dimensions of k^* are s^{-1} and k' are $\mu M^{-1}\ s^{-1}$. All kinetic data are for 20 °C. The fitting for calculated rate constants was as described in the footnote to Table I. For the fitting, $c = 0.16$, unless otherwise noted. [b] Optimum values of L for fitting the CO kinetics. Any other listed value for L gave the best fit for O_2 kinetics consistent with the SSQ for the CO fitting being within the 95% confidence region for the F statistic.

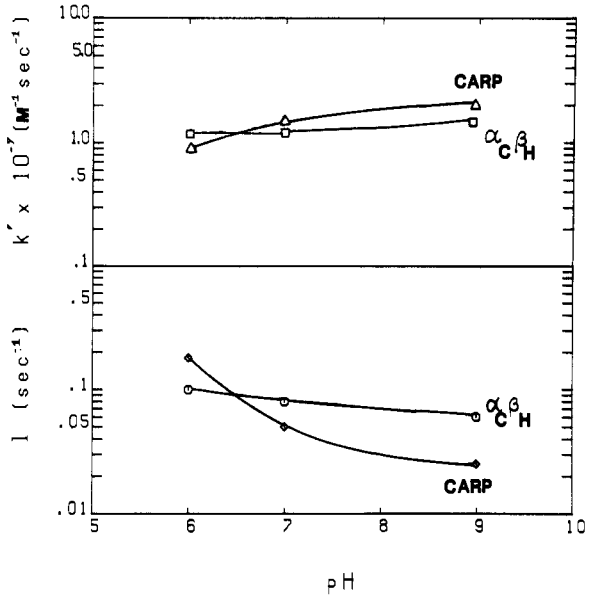

FIGURE 4: Rate constants for CO dissociation (l) and oxygen association (k') vs. pH. The diamonds and triangles represent the CO dissociation and oxygen association rate constants, respectively, for carp hemoglobin. The circles and squares depict CO dissociation and oxygen association rate constants for the α(carp):β(human) hybrid hemoglobin (T = 20 °C).

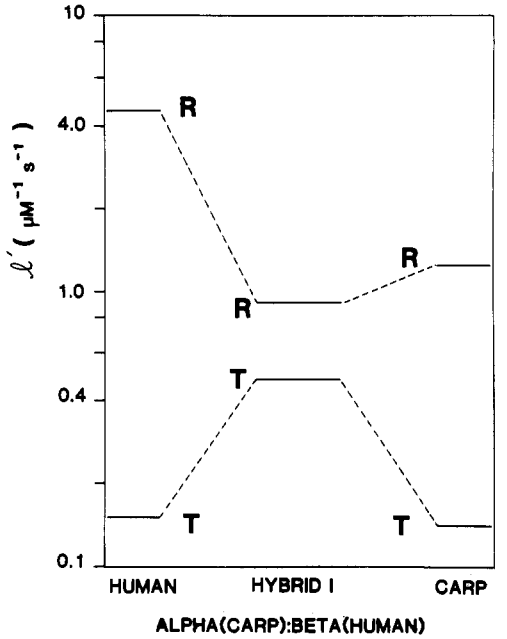

FIGURE 5: Plot of l' for the R and T states of three hemoglobins. Differences between R and T reflect differences in $\Delta G°$ for ligation of CO to the two states as described in the text.

equations above, $\log l'_R - \log l'_T = \alpha \log c$. For a set of hemoglobins having the same α, $\log l'_R - \log l'_T$ is proportional to the difference in free energy ($\Delta G_R° - \Delta G_T°$) for ligation of CO to the R and T states. Thus, in Figure 5, the difference between T and R for each of the hemoglobins is proportional to this difference in $\Delta G°$, and we can see how hybrid formation alters this quantity.

The oxygen equilibrium data at pH 7, where cooperativity is maximal, are not quite in agreement with the optimum value of L for the CO fitting. That is, since L must be ligand independent, the same value of L should fit the CO and oxygen data. If $L = 80$ is used to fit the oxygen equilibrium data, however, the optimum value of K_R is twice the maximum allowable value, calculated from k^*_{pH9}/k'. The following strategy was employed in seeking agreement for CO and oxygen fitting. For the allosteric model, let $A = [O_2]/K_R$. Then, $\bar{A}$ is A for $\bar{Y} = 0.5$ and is the root of the polynomial $(1 + Lc^4)A^4 + 2(1 + Lc^3)A^3 - 2(1 + Lc)A - (1 + L)$. The solution $\bar{A}$ is constrained, however, to be greater than 4.5, obtained from a lower bound for $\bar{O}_2$ and the upper bound for K_R. The Hill number, n^*, can be readily calculated from $n^* = \bar{A}\dot{D}/D + \bar{A}^2\ddot{D}/D - 4$, where $D = L(1 + cA)^4 + (1 + A)^4$, the binding polynomial for the allosteric model and D, $\dot{D}$, and $\ddot{D}$ are all evaluated at $\bar{A}$, and $\dot{D} = dD/dA$, and $\ddot{D} = d^2D/dA^2$. An upper limit for c was 0.257, calculated from the ratio of the appropriate observed rate constants. The parameters L and c were thus varied in a grid search such that $\bar{A}$ was greater than 4.5, and the Hill number was between the limits of 1.3 and 1.5. The parameter c was required to be less than 0.257, and L was such that the SSQ for the CO data fitting at that L value was less than twice that for $L = 80$, the optimum value for CO at pH 7. The factor of 2 variation in the SSQ coincided with the 95% confidence limit for the F statistic (Draper & Smith, 1966). In practice, the problem was to find a lower bound for L and a minimum value for c, subject to the above constraints. At pH 7, the optimum parameters were $L = 375$ and $c = 0.14$, for which $\bar{A} = 4.53$, $K_R = 6.2\ \mu M$, and $n^* = 1.42$. The calculated CO rate constants changed by 3–26% for this value of L compared to those at the optimum value, $L = 80$. Both c and K_R are in excellent agreement with the results reported in the previous paper ($c = 0.18 \pm 0.08$ and $K_R = 6.7 \pm 2.2\ \mu M$). A value of L has thus been found that gives reasonable agreement at pH 7 for the CO kinetics, for the oxygen equilibria, and, as will be shown below, for the available oxygen kinetics data.

The crucial remaining problem was to see if all of the oxygen kinetics could also be described by allosteric parameters.

Sensitivity of the hybrid to dithionite only allowed measurements of k^* to be made. In terms of the allosteric model, under the assumption that CO and oxygen are equivalent ligands for establishment of R and T conformational equilibria, $k^* = (Lc^4k_T + k_R)/(Lc^4 + 1)$. To fit the k^* data, c was allowed to vary slightly but was found to be optimal at 0.16. L was allowed to vary as before so that the SSQ for the CO data could not exceed the 95% F statistic contours but also had to fit well the oxygen equilibrium results. A scheme, similar to that employed for CO data fitting, was also adopted for oxygen data fitting, since c also appeared to be rather large for oxygen binding. A value for α of 0.25 was found for the oxygen data of des-His carp hemoglobin (Parkhurst et al., 1983) and was assumed here, since its kinetic properties are very similar to those of hybrid I. (A value for α of 0.23, appropriate for carp Hb, gives rate constants that differ by 10–20% from those calculated for $\alpha = 0.25$.) Values for the four rate constants were calculated from $k'_R = (k'_{pH6}k'_{pH9}c^{-\alpha})^{1/2}$, and $k'_T = k'_Rc^{\alpha}$. Since K_R was found to be 6.2 μM, $k_R = 6.2k'_R$ and $k_T = k_Rc^{\alpha-1}$. This left only a small region of $L - c$ space to search for a good fit to the k^* and k' data. The apparent constant k' that is reported was for total photolysis, $F = 1$, although k' showed little variation with F. As for CO, a progress curve for the absorbance change for $Hb \rightarrow Hb(O_2)_4$ was calculated in terms of k'_R, k'_T, c, and L. The results of the oxygen kinetics fitting are in Table II. The data were fit quite well, except at pH 6 for k^*, by using only a constrained variation in L and a small adjustment in c (0.14–0.16). At pH 6, L was allowed to increase from 245, found from fits to the CO data, to 1000, which increased the SSQ for the CO fits by a factor of 2 (the limits of the 95% F contour). The constant k_R was slightly larger than k^*_{pH9}, whereas an ideal fit would result in a smaller value. The oxygen equilibrium data at pH 6 and 9 showed very low cooperativity (n^* approximately 1.1), and thus, L would be poorly defined. Therefore, the kinetics were first fitted for $c = 0.16$ and variable L, and the corresponding values for $\bar{O}_2$ and n^* were calculated and are listed in Table II. No attempt was made to optimize the fitting for *both* the kinetics and the equilibria. Altogether, however, the equilibria and the ligand kinetics for oxygen and for CO can be fit quite well in terms of the allosteric model.

The above procedures showed that consistent allosteric parameters and rate constants could be found that would fit the equilibria and kinetics for CO and oxygen binding to hybrid I. At this point, however, we had not shown that c was necessarily large. The kinetic data did not allow a critical test of the range of c because of various extraallosteric assumptions, such as rapid equilibrations and linear free-energy assumptions. For oxygen equilibria at pH 7, we could conclude, as above, that $\bar{A} > 4.5$ and explore, for a given values of c, the allowed values of L such that n^* was between 1.3 and 1.5. For $c = 0.05$, L was between 25 000 and 61 000. For smaller values of c, L increased rapidly. For instance, for $c = 0.02$, L was between 1.1×10^6 and 2.5×10^6. If we examine the equilibrium populations for various liganded intermediates for the $c \leq 0.05$ values, we find that the measured l' at pH 7 must correspond to l'_T, quite independent of any assumptions as to the rates of conformational transitions. Thus, within the framework of the allosteric model, we must conclude that the T-state properties of this protein are altered from carp or human T-state toward R-state properties as indicated in Figure 5. We cannot in a similar fashion conclude, however, that the R state is necessarily depressed as indicated in Figure 5. If we adopt a "principle of parsimony" toward free-energy differences between R and T, arguing that we should take the minimum difference that accounts for the data, then R can indeed be considered to be located as indicated, and the allosteric range is severely restricted for this hybrid hemoglobin.

Registry No. CO, 630-08-0; oxygen, 7782-44-7.

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