Ligand Binding Kinetic Studies on the Hybrid Hemoglobin α(Human):β(Carp): A Hemoglobin with Mixed Conformations and Sequential Conformational Changes[†]

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ABSTRACT: Oxygen and CO ligand binding kinetics have been studied for the hybrid hemoglobin (Hb) α(human):β(carp), hybrid II. Valency and half-saturated hybrids were used to aid in the assignment of the conformations of both chains. In hybrid II, an intermediate S state occurs, in which one chain has R- and the other T-state properties. In HbCO at pH 6 (plus 1 mM inositol hexaphosphate), the human α-chain is R state and the carp β-chain is T state. We have no evidence at this pH that the carp β-chain ever assumes the R conformation. At pH 6, the human α-chain shows human Hb R-state kinetics at low fractional photolysis and T-state rates for CO ligation by stopped flow. At pH 7, the human-chain R-state rate slows toward a carp hemoglobin rate. The carp β-chains, on the other hand, react 50% more rapidly in the liganded conformation than in carp hemoglobin, and while the human α-chains are in the R state, the two β-chains appear to function as a cooperative dimer. In this hemoglobin, the chains appear to be somewhat decoupled near pH 7, allowing a sequential conformational change from the R state in which the β-chains first assume T-state properties, followed by the α-chains. The rate of the R–T conformational change for the carp β-chains is at least 300 times greater than that for the human α-chains. At pH 9, the R → T conformational transition rate is at least 200 times slower than that for human hemoglobin. The carp β-chain R state at this pH reacts twice as rapidly with CO as in carp hemoglobin. Diagrams are presented showing all conformations supported by kinetic evidence. The ligand kinetics in the hybrid suggest that in carp hemoglobin, the α-chains prevent the carp β-chains from assuming full R-state properties and are themselves biased toward the T conformation. The marked tendency of the carp β-chains to assume the T state lends strong support to the model that assigns a central role to the β-chain in the Root effect [Perutz, M. F., & Brunori, M. (1982) *Nature* (*London*) *299*, 421–426]. From the data at hand, however, it does not appear possible to assign a definite number of Bohr protons to the four individual chains in carp and human hemoglobins.

Carp hemoglobin has been one of the most extensively studied of the fish hemoglobins (Noble et al., 1970; Tan et al., 1972, 1973; Tan & Noble, 1973; Messana et al., 1978; Perutz et al., 1978; Chien & Mayo, 1980). Both α- and β-chains have been sequenced (Hilse & Braunitzer, 1968; Grujic-Injac et al., 1979). It has been shown (Tan et al., 1973) that the hemoglobin can be "locked" in the T state at pHs below 6.4 in the presence of IHP[1] and in the R state at pHs greater than 8.5. It is therefore possible to determine the ligand kinetics for both the R and T states of the hemoglobin. A model has recently been proposed to account for the extreme Bohr effect shown by many of the fish hemoglobins (Perutz & Brunori, 1982). In that model, a central role is played by the β-F9 Ser and its interactions with the β-HC3 His and with the α-C5 Lys. We were therefore interested in studying the properties of the hybrid hemoglobin α(human):β(carp), hybrid II, in which these interactions should still obtain but where the $\alpha_1\beta_2$ contacts were changed from those in carp hemoglobin. We report here evidence demonstrating that hybrid II shows multiple conformations (more than one conformational state for the same degree of ligation) and "mixed conformations" in which one chain has R-state and the other chain has T-state properties.

Materials and Methods

Human hemoglobin and hybrid hemoglobins were prepared as described in the previous paper (Goss & Parkhurst, 1984). Kinetic measurements were performed as previously described with the following modifications. For the measurements of CO association for hybrids, such as α(CN)met:β(CO), dithionite was omitted, and the glucose–glucose oxidase–catalase system was used to deoxygenate the solutions. For CO dissociation, the microperoxidase was reduced by using an enzyme system (Asakura et al., 1972). To determine the rate constants for CO dissociation from the half-saturated hybrids, the following experiments were carried out. The deoxy α- or β-chains (10 μM in heme) were mixed with the reduced microperoxidase solution and flowed against the complementary carbonmonoxy chain (10 μM in heme). The absorbance change a 430 nm for chain combination was much faster ($t_{1/2}$ = 25 ms) than was the slow dissociation of carbon monoxide, showing that formation of the hemoglobin occurred well before the CO dissociation process. In general, all kinetic measurements were carried out on hybrids within 2 h of their preparation from the isolated chains.

Results and Discussion

In the figures (1, 7, 10, and 12) that follow, conformations are represented as follows: the R state is represented as a square and the T state as a circle. These conformations are assigned on the basis of ligand kinetics and hence refer to tertiary conformations. The four protein chains are shown as individual squares and circles so that the conformation of each chain can be represented. The α-chains are on the left and β-chains on the right half of each figure. The shaded areas

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[1] Abbreviations: IHP, inositol hexaphosphate; pH 6 (+IHP), a solution that contained 1 mM IHP in addition to the 0.05 M potassium phosphate buffer at pH 6. Other abbreviations have been defined in the preceding paper (Goss & Parkhurst, 1984).

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Table I: CO Binding Rate Constants[a]

(A) CO Association of Hybrid II

species (method)	pH 6 (+IHP)	pH 7	pH 9
(1) α_2:β_2 (SF)	0.15	0.25	0.7
(2) $\alpha_2(CO)_2$:$\beta_2(CO)_2$ (LP)	4.2 (50%) ($F = 1$) 0.15 (50%) ($F = 1$) 3.5 (60%) ($F = 0.2$) 0.14 (40%) ($F = 0.2$)	1.7 (50%) ($F = 1$) 0.25 (50%) ($F = 1$) 1.7 (90%) ($F = 0.15$) 0.25 (10%) ($F = 0.15$)	2.0 (80%) ($F = 1$) 2.0 (100%) ($F = 0.2$)
(3) α_2:$\beta_2(CN)_2$ (SF)	4.6 (65%) 0.16 (35%)	0.29	0.7
(4) $\alpha_2(CN)_2$:β_2 (SF)	0.17	0.28	0.65
(5) $\alpha_2(CO)_2$:$\beta_2(CN)_2$ (LP)	4.0 ($F = 1$) 3.6 ($F = 0.11$)	1.7 ($F = 1$) 1.7 ($F = 0.09$)	2.1 ($F = 1$)
(6) $\alpha_2(CN)_2$:$\beta_2(CO)_2$ (LP)	0.15 ($F = 1$) 0.15 ($F = 0.1$)	0.26 ($F = 1$) 1.7 ($F = 0.08$)	2.0 ($F = 1$)

(B) CO Dissociation of Hybrid II

species	pH 6 (+IHP)	pH 7	pH 9
(1) $\alpha_2(CO)_2$:$\beta_2(CO)_2$	0.10 (50%) 0.036 (50%)	0.05 0.08 ($F = 0.9$)	0.03 0.03 ($F = 0.8$)
(2) $\alpha_2(CN)_2$:$\beta_2(CO)_2$	0.095	0.065	0.036
(3) $\alpha_2(CO)_2$:$\beta_2(CN)_2$	0.036	0.044	0.034
(4) α_2:$\beta_2(CO)_2$		0.073	0.048
(5) $\alpha_2(CO)_2$:β_2		0.065	0.042

[a] Under the heading "species" is given the form of the protein mixed in the stopped-flow apparatus or the form of the protein subjected to laser photolysis. For part A, SF denotes a stopped-flow experiment and LP a laser-photolysis experiment. In part B, all experiments were carried out in the stopped-flow apparatus. All association rate constants have dimensions μM^{-1} s^{-1}; dissociation rate constants are s^{-1}. For biphasic reactions, the percentage figure following the value of the rate constant is the percent of the total absorbance change associated with that rate constant. The F value is the fraction of the bound CO that was photolyzed. Rate constants are for 20 °C. For part A, the concentrations of the various protein species in heme were (1) 5 μM, (2) 6-35 μM (all pHs), (3) 9 μM (total heme), (4) 8 μM (total heme), (5) 9 μM (total heme), and (6) 12 μM (total heme). For part B, all samples were 5 μM in heme.

indicate chains from the human parent hemoglobin. The unshaded squares or circles represent the carp component. A small circle represents a ligand molecule. The degree of ligation decreases from left to right, so that the fully liganded conformation appears on the left and the fully deoxy on the extreme right. The R state is shown at the top and T state at the bottom. Zeroes and Roman numeral prefixes denote the number of bound ligands. For the hybrid hemoglobins, only those species for which direct kinetics evidence exists are represented. There are presumably many other intermediate ligation states.

Figure 1 illustrates the proposed model at pH 7.0, where maximal cooperativity is observed. This model makes the following points: (1) for the same degree of ligation, the protein can be shown to assume at least two or three conformations (e.g., 0-B,0-C and II-B,II-D, and II-G); (2) the hemoglobin can assume a mixed conformational state in which half of the chains (α) are R state and half of the chains (β) are T state (e.g., II-D,II-J,0-B). The kinetic evidence for these conformations is presented below.

The CO recombination reaction following total photolysis is biphasic. As the percent photolysis decreases, the fraction of rapidly reacting material increases (Figure 2). This latter result suggests that at high ligand saturation the protein is mainly R state (conformations III-A,B and IV-A, Figure 1). If a mixed R and T conformation for the fully liganded protein were present, low breakdown should also give biphasic kinetics. This was not the case at pH 7.0. Consider first the photolysis experiments for which the protein is initially R-state, structure IV-A. [CO dissociation kinetics were homogeneous, with a rate constant consistent with R-state hemoglobin (see Figure 3), which summarizes all of the CO dissociation experiments.] Biphasic kinetics observed for CO recombination for total photolysis yielded two rate constants. The rapidly reacting component (50% of the total reaction amplitude) has a rate constant corresponding to that of the R state (Table I) while the slow phase rate is that of a T state. These results suggest

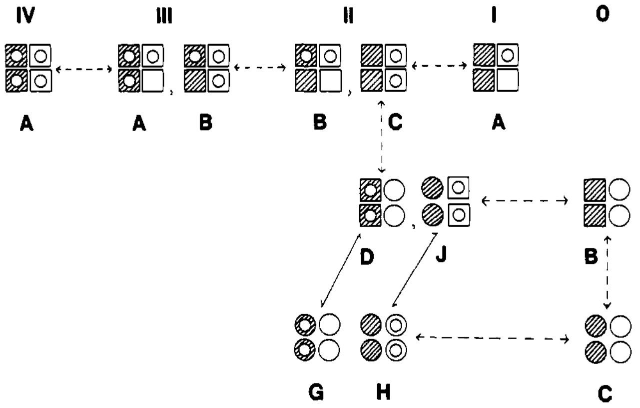

FIGURE 1: Diagram of the conformational equilibria for various degrees of ligation for α(human):β(carp) hemoglobin at pH 7.0.

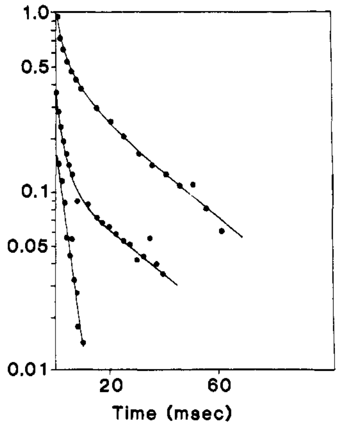

FIGURE 2: Absorbance changes for CO recombination following laser photolysis. The three curves represent varying degrees of photolysis for the α(human)CO:β(carp)CO hybrid hemoglobin at pH 7.0. The curves are normalized to the full photolysis reaction. Reaction conditions: [CO] 163 μM; 0.1 M potassium phosphate buffer; T = 22 °C; 430-nm observing wavelength. Heme concentration = 21 μM.

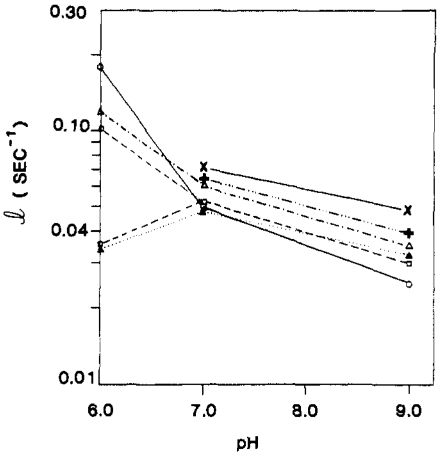

FIGURE 3: CO dissociation as a function of pH. Carp hemoglobin (open circles) is plotted for comparison. The open squares depict the values for α(human)CO:β(carp)CO hemoglobin. The open and solid triangles show respectively the α(human)CN:β(carp)CO and α(human)CO:β(carp)CN valency hybrids. The crosses represent CO dissociation for α(CO):β(deoxy); the plus signs refer to α(deoxy):β-(CO). The observing wavelength was 419 nm; T = 22 °C. Heme concentration = 5 μM.

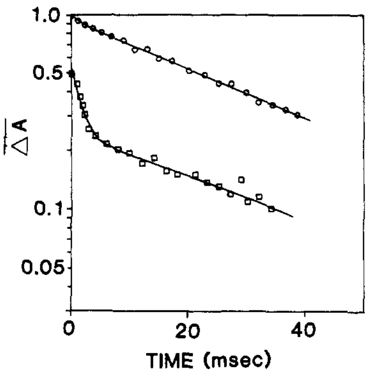

FIGURE 4: Normalized absorbance changes for CO recombination to α(human)CN:β(carp)CO hemoglobin measured by laser photolysis. The open circles and squares depict the experimental data points. The solid lines are the calculated curves. The upper curve is for full photolysis, and the lower curve is the 50% photolysis. The data are normalized to the absorbance change at full photolysis. The CO concentration was 110 μM, the buffer was 0.1 M potassium phosphate, pH 7.0, and the temperature was 22 °C. The reaction was observed at 430 nm. Heme concentration = 12 μM.

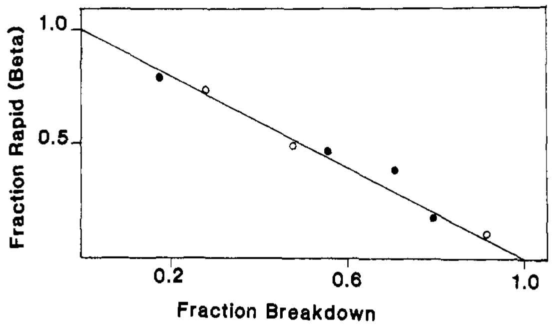

FIGURE 5: Fraction of the total absorbance change for the β-chains at pH 7 that is rapid vs. fraction breakdown (fraction photolysis). The solid points are for the $\alpha_2(CO)_2$:$\beta_2(CO)_2$ hemoglobin, and the open circles are from the α(human)CN:β(carp)CO valency hybrid data. See text for details.

either mixed conformations were present (a distribution of some R- and some T-state molecules in slow equilibrium) or that the protein molecule itself had both R- and T-state properties, which might occur, for instance, if the α-chain were in the R state and the β-chain in the T state. The fraction of rapidly reacting material at full photolysis did not change over a concentration range of 10–50 μM in heme, indicating that the biphasic kinetics were not due to a distribution of dimer and tetramer species in equilibrium. Large-zone gel filtration studies (J. Brinkman and L. J. Parkhurst, unpublished results) on this hybrid show that K_{TD} is less than 2 μM. To distinquish between the two possibilities (mixed conformations or both R and T properties in the same molecule), valency hybrids were made. Cyanide is assumed to be equivalent to CO in stabilizing the protein in the R state. Valency hybrids were made in which one chain was CN(met) and the other CO. Laser photolysis experiments could then probe the behavior of each chain of the hemoglobin. The α(CO):β(CNmet) hybrid showed homogeneous kinetics with a rate constant characteristic of R-state hemoglobin at *both* full and partial photolysis (structures II-C and III-B, Figure 1). The α(CNmet):β(CO) hybrid, however, showed homogeneous *slowly* reacting T-state kinetic at full photolysis (Figure 4)—evidence for structure II-D. (The assignment here of II-D rather than II-G is made because at full photolysis of HbCO, recombination is biphasic with 50% fast and 50% slow phase. Since 0-B is the assignment for full photolysis of HbCO, II-D must be the assignment for the valency hybrid.) For partial photolysis, a rapid recombination phase was observed (Figure 4) evidence for structure III-A. The rapid component observed for total photolysis of the fully CO hemoglobin can therefore be assigned as the α-chain and the slowly racting component as the β-chain. This is evidence for a hemoglobin molecule with both R- (α-chain) and T- (β-chain) state properties (conformation 0-B)!

The conformations II-C and II-D are assigned from the above valency hybrid data for CO association and dissociation, assuming that CN and CO are equivalent ligands. We refer to the mixed state having α "R" and β "T" properties as an "S" state. For the CO hemoglobin, the biphasic data for fractional photlysis were analyzed in the following way. We assumed that the α- and β-chains have equal quantum yields and equal absorbance changes for the reaction. The fact that at full photolysis the fraction of rapidly reacting material is 50% indicated this is approximately correct. Compensating changes such as a higher quantum yield but lower absorbance change do not affect the analysis. The fraction of fast and slowly reacting material was analyzed as a function of fraction photolysis. At least half of the reaction is rapid for any degree of photolysis, and from the CO–CN hybrid studies, the rapid phase is assigned to the α-chains. The α-component can then be subtracted and the amount of rapidly reacting material due to the β-hemes determined. If one considers the β-chains as an independent unit acting as a simple cooperative dimer, then the β-chains are T state for zero ligation and R state for single or double ligation. For the β-chain, total photolysis should result in slow CO recombination, and the rapid component for fractional β-chain photolysis would follow:

$$\Phi = pq/(p^2 + pq) = 1 - p$$

(since $p + q = 1$), where ϕ is the fraction of the β-chain absorbance change due to the rapid phase, p is the fraction of β-heme sites that are unliganded (or the fraction β-chain photolysis), and q is the fraction of β-sites that are liganded. Figure 5 shows a plot of the β-chain rapid phase (as a fraction, ϕ) vs. the fraction photolysis (p). The straight line is calculated from the above model that regards the two β-chains as comprising a cooperative dimer within the hemoglobin tetramer. The data are in very good agreement with this simple model and support the conformational assignments for structures

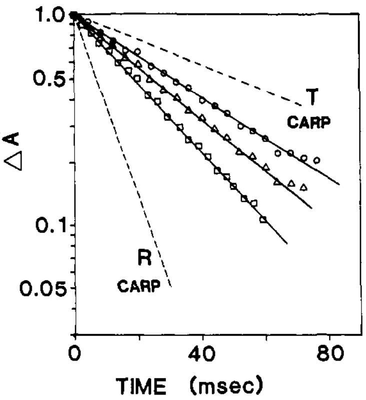

FIGURE 6: CO combination at pH 7 measured by stopped-flow. The upper curve (open circles) depicts the reaction for CO combination to α(human):β(carp) hemoglobin. The second (triangles) and third curves (squares) represent the reaction for α(human)CN:β(carp)deoxy and α(human)deoxy:β(carp)CN, respectively. Note: for all except α(human)deoxy:β(carp)CN, for which the CO concentration was 126 μM, the CO concentration was 90 μM. The observing wavelength was 430 nm, and the temperature was 20 °C. Heme concentrations are in the footnote to Table I.

III-A, II-B, and II-D. The α-chains remain in the R state during the time for β-chain conformational changes and ligation. We do not know the equilibrium distribution of conformers for $\alpha_2{}^R(CN)_2$:β_2(CO) and cannot therefore determine a Hill number or interaction free energy for the β-chains. Suppose, however, that the singly liganded β-chain form has the β-chains in the R conformation while the α-chains are also R, as is the case during CO recombination. Then, from the data in Table I for l', we can calculate an apparent Hill number of 1.42 and an interaction free energy of 1.05 kcal/mol of β-sites for β-chain ligation at pH 7 when the α-chain are in the R state. Weber (1982) has recently discussed interactions in the β–β dimer in human hemoglobin, and multiple cooperative domains have been proposed for a high molecular weight hemoglobin (Schreiber, 1983). These photolysis results point to a cooperative domain in this hybrid.

Stopped-flow measurements of CO combination (Figure 6) to the deoxy protein gave T-state-like rates that were slightly faster than those for T-state human and carp hemoglobin (evidence for conformation 0-C), which, with the photolysis results, show that the fully deoxy protein can have at least two conformations. The flash photolysis data gave evidence for 0-B, and stopped-flow combination allows us to assign 0-C. At very short times after photolysis, the protein presumably has yet another conformation: the deoxy R state. This is quite likely, since assignment of I-A shows that R → T for the β-chain does not occur simultaneously with loss of ligand. However, since we are depicting only those conformations for which we have direct evidence, the fully deoxy R state is not shown in the model. The β-chains must undergo a rapid R → T conformational change since the fully liganded protein is R state. The β-chains assume a T state much more rapidly than CO recombination occurs. This is in accord with the observations at pH 7.0 for most cooperative hemoglobins (Parkhurst, 1979). Detailed analyses of the reaction traces allow limits to be assigned to the various rate constants. Let the superscript denote the number of ligands in the tetramer immediately following photolysis. Then, $k^0(R \rightarrow T)$ for the β-chain at pH 7 is >3000 s^{-1}; $k^{II}(R \rightarrow T)$ for the β-chain in $\alpha_2(CN)_2$:β_2 is >2100 s^{-1}. The α-chains, however, must have a very much slower rate for the R → T conformational change. Within the CO recombination time, the chains have not changed to the T state, and only in stopped-flow experiments, where the protein is initially in the unliganded form, do we observe T-state kinetics. Thus, for the α-chain, $k^0(R \rightarrow T)$ at pH 7 is <13 s^{-1}.

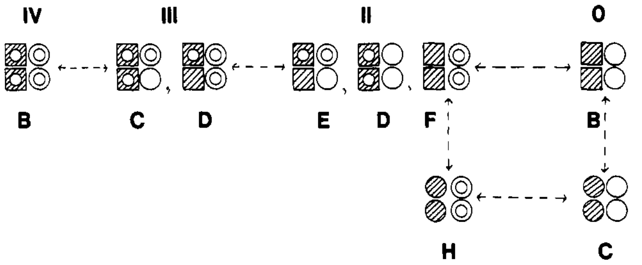

FIGURE 7: Diagram of the conformational equilibria for various degrees of ligation for α(human):β(carp) hemoglobin at pH 6.0.

In order to investigate the properties of the intermediate half-saturated species after sufficient time had elapsed to allow conformational equilibration, stopped-flow experiments were carried out. Human α- and β-chains combine very rapidly (Antonini et al., 1966; Brunori et al., 1968) to form hemoglobin. This reaction is accompanied by an absorbance change at 430 nm when the R-state chains form a T-state deoxy hemoglobin. In order to obtain half-saturated intermediates of the type α(deoxy):β(CO) and α(CO):β(deoxy), the deoxy chain in the presence of microperoxidase was flowed against the CO chain. The rate of CO dissociation from only one chain could then be measured. The small absorbance change at 430 nm was much faster ($t_{1/2}$ = 25 ms) than CO dissociation, showing that the tetrameric hemoglobin was formed *before* release of the ligand. These experiments showed that for *both* hybrids the CO dissociation rate was intermediate between that for R- and T-state rates (Table IB). We have a choice: either represent the liganded chain as an intermediate conformation between R and T or assume that we are measuring an average property of two structures, shown as the pairs II-H:II-J and II-G:II-D. We chose the latter option since we then need only two conformations, R and T, for each chain to describe all the ligand kinetics. Of all the structures shown, only II-J is based on this equilibrium assumption. The conformation of the *deoxy* chain in the half-saturated hybrids was determined from studies on valency hybrids by assuming CN and CO were equivalent ligands. CO combination by stopped-flow (Figure 6) gave a slow CO binding rate about twice that of carp T-state hemoglobin for both valency hybrids, suggesting some shift in conformational equilibria toward R. [As a control, flash photolysis on the resulting product hybrids, α(CNmet):β(CO) and α(CO):β(CNmet), gave identical results with those obtained when the hybrids were prepared by mixing the corresponding CNmet and carbonmonoxy chains.] Conformations II-G and II-H are thus established, in which at half-saturation the molecule is in the T-state conformation. At pH 7, we therefore have at least *three* distinct conformations for the half-liganded protein.

At pH 6.0 in the presence of IHP, the hybrid exhibits different behavior, summarized in Figure 7. CO recombination at full photolysis gives a biphasic reaction. Again, the slowly reacting phase (50% of the reaction) has T-state-like rates. The fractions fast and slow, in contrast to the pH 7 behavior, do not change with fraction photolysis. Even at 15% breakdown, half of the material is slowly reacting (Figure 8). This again suggests either mixed (half R and T state in the same molecule) or multiple conformations (some R- and some T-state molecules in equilibrium). Valency hybrids were studied at this pH as well. Studies of CO recombination to the α(CN):β(CO) and α(CO):β(CN) hybrids showed that the carp component is responsible for the slow CO recombination rate and the human α-chains give rise to the fast recombination

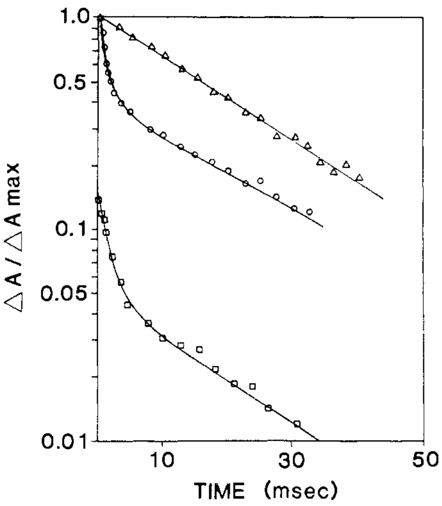

FIGURE 8: CO recombination reaction following laser photolysis at pH 6.0. The open circles and squares show the reaction at full and partial (15%) photolysis, respectively, for α(human)CO:β(carp)CO hemoglobin. The triangles depict the reaction for α(human)CN:β-(carp)CO hemoglobin. Reaction conditions: CO concentration 265 μM; heme concentration 35 μM; T = 21 °C; observing wavelength = 430 nm.

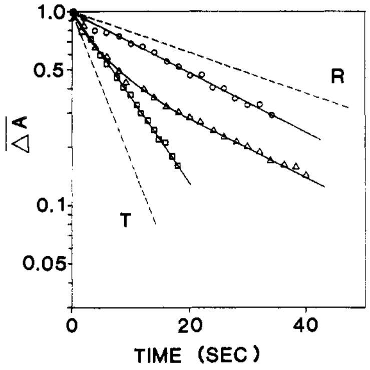

FIGURE 9: CO dissociation reaction at pH 6. The circles and squares represent the CO dissociation reaction for the two valency hybrids, α(human)CO:β(carp)CN and α(human)CN:β(carp)CO, respectively. The triangles depict the data points for the α(CO):β(CO) protein. The data are normalized for each curve. The reaction was measured by flowing the protein against microperoxidase (see text for details). The observing wavelength was 418 nm, and the temperature was 18 °C. Heme concentration = 5 μM. The time courses for R- and T-state carp hemoglobin are shown as dashed lines for comparison.

phase. These experiments give evidence for conformations II-D, II-F, and 0-B. Since the rate of CO recombination does not change at low photolysis for either valency hybrid, conformations III-C and III-D are appropriate representations. Partial photolysis of the fully liganded molecule, which gives a biphasic reaction, suggests the assignment of conformation II-E. Since the triply liganded conformations (III-C and III-D) are half R and half T state, what is the conformation of the fully liganded species? CO dissociation was measured for both the fully liganded molecule and the valency hybrids to address this question. The CO dissociation from the fully liganded molecule gave biphasic kinetics (Figure 9). CO dissociation from the α(CN):β(CO) valency hybrid showed monophasic T-state kinetics. The complementary α(CO):β-(CN) hybrid gave monophasic R-state CO dissociation rates. This completes the evidence for a molecule with properties that are half R state (human α-chains) and half T state (carp β-chains) in the *fully liganded* conformation. There is no evidence that the molecule can assume a full R state; rather, it appears to be in a mixed R and T conformation, or S state, when liganded.

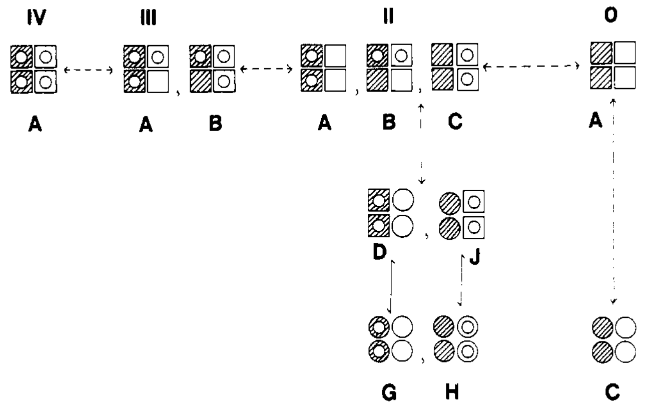

FIGURE 10: Diagram of the conformational equilibria for various degrees of ligation for α(human):β(carp) hemoglobin at pH 9.0.

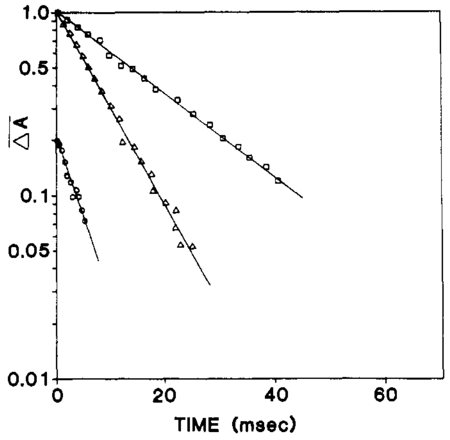

FIGURE 11: Normalized absorbance change for the CO association reaction at pH 9 measured by stopped flow and laser photolysis. The CO recombination reaction to α(human):β(carp) hemoglobin by laser photolysis is shown by the triangles (full photolysis) and circles (partial photolysis). The stopped-flow reaction is depicted by the squares. Reaction conditions: CO concentration = 70 μM; heme concentration = 10 μM and 5 μM for the stopped-flow reaction; T = 22 °C; observing wavelength = 430 nm.

Species II-D and II-F were assigned from the CO recombination experiments for the α(CN):β(CO) and α(CO):β(CN) hybrids, respectively. The fully deoxy conformations (0-B and 0-C) are suggested by the fact that (1) CO recombination after full photolysis is biphasic and (2) CO combination by stopped flow gives a monophasic slow reaction characteristic of a T state. The rate of the conformational change for the α-chains must be much slower than CO recombination. From detailed fitting, $k^0(\mathrm{R}\rightarrow\mathrm{T})$ for the α-chains at pH 6 must be $<10\ \mathrm{s}^{-1}$. In order to examine the properties of the α-chains in the half saturated hybrid, CO combination to the valency hybrid α-(deoxy):β(CNmet) was measured by stopped flow. This hybrid shows biphasic CO combination by stopped flow. These results can be interpreted in terms of a slow equilibrium between conformations II-F and II-H. The carp component at this pH appears to be locked in a T-state conformation. The human α-chains very readily assume an R state and only slowly revert to T after full photolysis. The model (Figure 7) for pH 6 differs from that for pH 7 (Figure 1) in one very important way—we have *no* evidence that the β-chain is ever R state, whereas the α-chain can assume both conformations. The behavior of the β-chain is that of carp itself, which is locked in the T state at low pH.

By extrapolation and comparison with other hemoglobins, we would predict a slower R → T transition for the carp β-chains at pH 9 and a bias in conformational equilibria toward the R state. At pH 9.0, the ligand kinetics can be interpreted in terms of a model (Figure 10) where $Hb_R \rightarrow Hb_T$ is very slow. CO recombination rates by stopped flow and laser photolysis differ by less than a factor of 3 (Figure 11). The CO recombination rate measured by flash photolysis is rapid and does not appear to vary with flash intensity. This suggests that laser photolysis reactions are mainly a probe of the R state and the protein does not have time to switch to a more slowly reacting form before CO recombination occurs. The problem is to describe the rapid CO binding measured by stopped flow to the deoxy form. The photolysis data show that we cannot consider the stable deoxy form to be represented by a rapid equilibrium between R and T states. We can either regard the alkaline deoxy T state to be distorted toward R, or, in terms of previous forms, we can describe the results in the following way. The stable deoxy form of the protein is an equilibrium mixture of T state (0-C) and S state (unliganded analogues of forms D and J) that equilibrate rapidly with respect to CO binding. The transition from the R state to this equilibrium mixture of S and T states is, however, slow. From the data, we can conclude that the rate for this latter process is <30 s^{-1}. As described above, flash photolysis data on both CNmet:CO valency hybrids showed rapid CO recombination rates for both full and partial photolysis of the CO chain. These results indicate (Figure 10) conformations III-A, III-B, II-A,II-B,II-C, and 0-A are appropriate representations, again under the assumption that CO and CN are equivalent ligands. Conformation IV is assigned both from the CO combination data (if conformations III-A and III-B are R state, IV is very unlikely to be other than R state) and from CO dissociation, which gave monophasic R-state rates. The α(deoxy):β(CO) and α(CO):β(deoxy) hybrids both give CO dissociation rates and CO association rates (Table I) characteristic of mixed S and T states in rapid equilibrium, allowing us to assign conformations II-G, II-D, II-J, and II-H. At this pH, the α(human):β(carp) hybrid behaves more like carp hemoglobin in that it reacts like R-state hemoglobin by flash photolysis. The differences between stopped-flow and photolysis results show that some cooperativity is present. At pH 9, as at pH 7, the carp β-chains can assume an R conformation. At pH 9, the rate of the conformational transition from the R state (<30 s^{-1}) is much slower for both chains than that observed for human hemoglobin (Sawicki & Gibson, 1977), 6400 s^{-1} at 20 °C.

This hybrid has several intersting properties. (1) There are multiple conformations for the same degree of ligation present (e.g., conformations II-B, II-D, and II-G). (2) The tetrameric molecule exhibits mixed conformational states at pH 6, the CO form has α-chains with R-state but β-chains with T-state properties. (3) The rate of the R → T conformational change has a large pH dependence for the β-chain. At pH 6, the β-chains appear locked in the T state, whereas at pH 9, R → T is slow. (4) Sequential conformational changes can be observed at pH 7 in which the β-chains change from R to T at least 300 times faster than do the α-chains. These results show that a simple two-state allosteric model will not fit the data for this protein. One must assume a variety of intermediate states, such as S states.

An overall model for this protein at all pHs is shown in Figure 12. Here we assume that the effect of pH is simply to make certain conformations more evident than others in our experiments but that all species are present at each pH, and again, only forms for which we have kinetic evidence are shown. At pH 6, the conformational equilibria for the protein do not favor a fully R-state conformation (top row). The protein follows the second and bottom rows for changes in ligation. The conformational equilibria are shifted toward the T state. At low pH, a rapid R → T transition occurs for the β-chains at all stages of ligation. At pH 6, the β-chains in the liganded protein appear to be T state. The α-chains switch to the T state only very slowly and may be acting more or less independently of the β-chains. The protein at pH 7 can attain an R state (top row) for the liganded species; however, the deoxy conformation is at least partially T state at short times after removal of ligand (middle row, right side). At half-saturation, the equilibria at pH 7 are poised between T and S, with the unliganded chain T state. Transient S-state (II-D, E, F) species appear in photolysis experiments. The rate of the conformational transition from 0-A to 0-B for the β-chains exceeds 3000 s^{-1}; that from 0-B to 0-C, for the α-chain, is less than 13 s^{-1}. At pH 9, the equilibria are shifted toward the R state. At short times after dissociation of ligand, the protein maintains the R-state conformation (top row). The deoxy protein is an equilibrium mixture of T and S states. The R → T transition at pH 9 is slow for both chains. The conformational equilibria are shifted progressively more toward the R state with increasing pH.

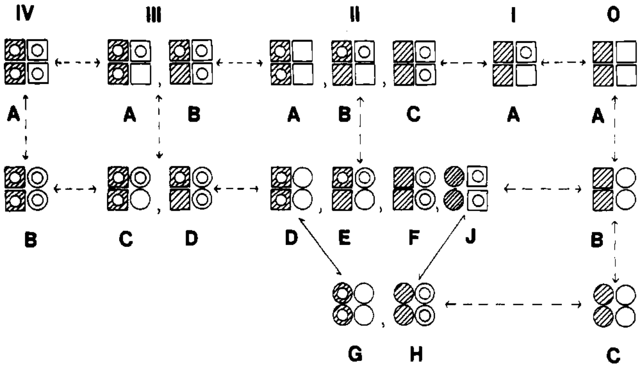

FIGURE 12: Diagram of overall conformational changes on ligand binding for α(human):β(carp) hybrid hemoglobin.

The CO association and dissociation rate constants are shown in Table I.[2] These constants were used to assign the conformations of both chains in the various liganded forms as R or T. The T-state rates at pH 6 and 7 are essentially those of the parent hemoglobins. At pH 6, the R state for the human α-chain is that expected for human hemoglobin; at pH 7, the rate constant has decreased by more than a factor of 2 and approaches the value (1.2 μM^{-1} s^{-1}) characteristic of *carp* Hb R state. Recall that at pH 6, the CO dissociation data show that the liganded chains can be considered functionally "decoupled", α-chain being R state and the β-chain T state. The carp β-chains at pH 7 can react with a *faster* rate than in carp Hb (1.7 vs. 1.2 μM^{-1} s^{-1}), and at pH 9, the β-carp chain R state reacts nearly *twice* as rapidly as in carp itself! This behavior is consistent with a picture in which the carp α-chain is relatively noncompliant, and in carp Hb prevents the β-chain from assuming full R-state properties. In hybrid I, the human β-chains, when combined with the carp α-chains, have CO binding rates that are considerably lowered from values for a human R state. In hybrid II, the carp

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[2] Of the rate constants reported in Table I, part A, only the results of experiment 2 could have been influenced by the presence of dimers, since for the other experiments, either the dimer concentration was insignificant or the kinetics were monophasic. Since the kinetics in experiment 2 did not vary significantly with concentration, the various tertiary assignments of R and T behavior stand as reported in the text. In part B of Table I, dimers must be considered only for the pH 9 results, but here again, the monophasic kinetics allow us to conclude, in agreement with CO association data, that the liganded forms are essentially in the R state.

Table II: O_2 Kinetics for Hybrid II[a]

rate constant	pH 6 (+IHP)	pH 7	pH 9
k' (μM^{-1} s^{-1})	12	14	21
k^* (s^{-1})	18 (α-chain) 520 (β-chain)	40	31

[a] All rate constants were measured for protein 10 μM in heme; at pH 6, measurements were also made at 35 μM heme. For all pHs, the relaxation reaction was monophasic (relaxation constant R). At pH 6, the two values for k^* (rate constant for oxygen release from the liganded conformation or distribution of conformations) were determined from R, k', and the two values measured for l' (see Table IA, species 2). T = 20 °C.

β-chains, in association with human α-chains, can attain a state that is "more R" than that in carp hemoglobin. Our results are consistent with the model for the Root effect (Perutz & Brunori, 1982), where the interaction of β-F9 Ser with HC3 His and the COO^- of the FG1 residues (Glu in carp; Asp in human) would be sufficient to force the α-chain toward T at low pH. For human β-chains in hybrid I, which lack the F9 Ser, the interactions are insufficient.

The oxygen kinetics data are summarized in Table II. Two values appear for k^* at pH 6. Although the replacement kinetics were monophasic, we used two values of l' to calculate k^* since the CO data showed clearly that the S state was the ligand-bound conformation. The k^* rate constants are those expected for R-state human α-chains and for T-state carp chains (Parkhurst, 1979; Parkhurst et al., 1983). At pH 7, the value of 40 s^{-1} is consistent with an altering of the human component toward a carp R state, as discussed above.

In the first paper in this series (Causgrove et al., 1984), we showed that a simple model of the Bohr effect was insufficient to allow contributions to be assigned to the four types of chains. The model can be extended, still excluding chain–chain interactions, by writing

$$n_H(\text{human}) = n_H(\alpha\text{-human}) + n_H(\beta\text{-human})$$

$$n_H(\text{carp}) = n_H(\alpha\text{-carp}) + n_H(\beta\text{-carp})$$

$$n_H(\text{I}) = n_H(\alpha\text{-carp})f^{\text{I}}(\alpha) + n_H(\beta\text{-human})f^{\text{I}}(\beta)$$

$$n_H(\text{II}) = n_H(\alpha\text{-human})f^{\text{II}}(\alpha) + n_H(\beta\text{-carp})f^{\text{II}}(\beta)$$

where the n_H quantities on the left are measured values, given in the first paper, and we assign full R- and T-state properties to each chain in the parent hemoglobins. The fs allow fractional contributions from each chain, depending on the extent to which a given chain can assume the parent R- or T-state properties in oxy and deoxy states, respectively. Attempts to assign values to the fs from the ligand kinetics were uniformly unsuccessful in that negative n_H's were often calculated or differences between carp and human chains were found that were not in accord with other chemical data. The attempts showed the extreme sensitivity of a calculated n_H for a given chain on the fs. If interchain interactions are large, it will be misleading to assign an invariant number of Bohr protons to a given chain and erroneous to use such simple linear formalisms as given above for the Bohr effect.

In human hemoglobin, 60% of the alkaline Bohr effect has been assigned (Perutz, 1976) to the N-terminal NH_3^+'s of the α-chains and to the β-C-terminal histidines. The remainder of the Bohr effect was assigned to the α-122 histidines. In carp hemoglobin, the α-chain N-terminal groups are acetylated. In human α-chain, there are 10 histidines; in carp, there are five, all of which occur at the same positions as in human. If the Bohr effect contribution from the carp α-chains is derived from histidines, then the number of Bohr protons assigned to carp α-chains should be less than that for human α-chains. Since n_H for carp is $> n_H$ for human hemoglobin, we would conclude that $n_H(\beta)$ for carp $> n_H(\beta)$ for human hemoglobin from the above assignments. On the other hand, human β-chain has nine histidines, and carp β-chain has only four, all of which are in the same locations as in the human chain. This suggests that groups other than histidine must be considered in the Bohr effect for carp hemoglobin or that one must consider in considerable detail the electrostatic interactions of histidines with various charged groups in the protein.

Registry No. CO, 630-08-0; CN, 57-12-5; oxygen, 7782-44-7.

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