Structural States and Transitions of Carp Hemoglobin*

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The wide ligand affinity range previously observed for carp hemoglobin is bounded at both extremes by regions of constant affinity. Within these regions, pH, organic phosphates, and the extent of ligand binding have no effect on the measured affinity and the cooperativity of ligand binding is greatly reduced or absent. The rates of CO recombination to fully and partially unliganded carp hemoglobin, under various organic phosphate and pH conditions, are shown to reflect this behavior. Constant kinetic rates are seen to directly correspond to the regions of constant affinity. Therefore, these are taken to be single protein conformational states, one of high and one of low ligand affinity. In the simplest view, these conformations represent the R and T states of a two-state model, and most of the properties of carp hemoglobin are explained quite well within this framework.

Increases in either hydrogen or phosphate ion concentrations favor the stabilization of the low affinity structure of even fully liganded carp hemoglobin. We have studied the structural transition from high to low affinity by monitoring the absorption spectra of carp hemoglobins at constant pH as a function of organic phosphate concentration. We find that different spectra are induced in both carp methemoglobin and cyanomethemoglobin by inositol hexaphosphate addition. Furthermore, the dependence of the magnitude of the spectral changes on pH and organic phosphate concentration is in close agreement with that predicted from studies of the ligand binding properties of the molecule.

Previous studies on carp hemoglobin (2-5) have established that to a good first approximation, the functional properties of this protein, including its enormous range of ligand affinity and large pH and organic phosphate effects, can be explained by a model which is based on the existence of two conformational states. Near neutral pH, carp hemoglobin exhibits cooperative ligand binding. Increasing the hydrogen ion or organic phosphate concentration can drive the protein into a state characterized by noncooperative ligand binding and the minimum obtainable ligand affinity. Raising the pH and removing organic phosphates also reduce the cooperativity of ligand binding but in this case ligand affinity is greatly increased. These properties, as reflected in both equilibrium and kinetic measurements, have led to the characterization of carp hemoglobin as having at one limit a conformational state with a low ligand affinity, which we shall label T in accordance with the notations of Monod et al. (6), and at the other limit, an R conformation with a high ligand affinity. The pH and phosphate effects on the ligand affinity are then explained by the preferential binding of these allosteric effectors to the T state of the carp hemoglobin molecule.

Although this model accounts for the major functional properties of carp hemoglobin, certain properties of the ligand-saturated and triliganded molecule, about which the model makes definite predictions, have been incompletely explored. Since according to this model the observed properties of carp hemoglobin, under any set of conditions, result from the prevailing equilibrium between the R and T states, the presence of ligands on three of the heme groups of the molecule should have a similar effect on ligand affinity or binding kinetics as a decrease in either the hydrogen ion or the organic phosphate concentration. Furthermore, since conditions of pH or organic phosphate concentration can be found in which even the fully liganded molecule remains in the T state, it must be possible to alter the conformational state of the ligand-saturated protein from R to T by modification of these two variables.

Tan et al. (4) have previously reported a comparison of the time course of CO recombination to unliganded and partially liganded carp hemoglobin generated by flash photolysis. They found that at both pH extremes, where the cooperativity of ligand binding to carp hemoglobin is reduced or absent, the recombination rates after full and partial photolysis were nearly identical. At intermediate pH values, the recombination of CO to partially liganded carp hemoglobin was faster than that to unliganded hemoglobin suggesting the presence of at least two affinity or conformational states. They also reported that removal of endogenous organic phosphates shifted this behavior toward lower pH. In their experiments partially

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liganded hemoglobin was generated by removal of 20% of the bound CO by flash photolysis. For comparison of the properties of partially liganded and unliganded hemoglobin this is quite satisfactory, but for the measurement of \( t_0 \), the rate of combination of CO to the triliganded molecule, photolysis levels greater than 10% are unsuitable since significant amounts of diligated hemoglobin are produced. Therefore, we have repeated and extended these measurements using only 10% photolysis for our partial dissociation. In addition, the effects on these rate constants of the addition of 1 mM IHP to stripped carp hemoglobin have been measured.

To detect conformational transitions in ligand-saturated hemoglobin, difference spectroscopy has unique advantages. If the natural chromophores of the molecule are sensitive to the transition, then monitoring can be achieved without the introduction of any unnatural perturbation. There is considerable evidence that the spectral properties of the heme groups of hemoglobin are sensitive to the conformational state of the molecule. Spectral differences between normal low affinity deoxycyanohemoglobin and the deoxy state of numerous high affinity derivatives have been reported (7–9). More recently Perutz et al. (10–12) have demonstrated IHP-induced changes in the visible spectra of the high spin aquo- and fluoro-derivatives of human methemoglobin, which they attribute to the transition from the R to the T state. No such IHP-induced visible difference spectrum was reported for human cyanomethemoglobin, consistent with the expectation that this low spin derivative should be constrained in the R state independent of IHP addition, just as are the oxy and carbonmonoxy derivatives of the ferrous protein. The predicted properties of carp hemoglobin in this regard are very different. Both the oxy and carbonmonoxy derivatives of this hemoglobin can exist in the T state, and we report here IHP-induced spectral changes in both the met derivatives.

MATERIALS AND METHODS

Carp Hemoglobin—The hemoglobin used in these studies was collected and prepared in a method similar to that described by Tan et al. (3). Red blood cells were lysed in 4 times their volume of distilled water for 1 hour at 4°C. The procedure described by Benesch et al. (13) was used to strip the hemolysates of organic phosphate and the assay of Ames and Dubin (14) was used to test its completeness. The concentration of inositol hexaphosphate solutions was also determined by the latter procedure.

For the spectral studies, phosphate-free carp hemoglobin samples were oxidized to methemoglobin in the presence of potassium ferricyanide crystals. The metcyanide derivative was produced by addition of potassium cyanide to the methemoglobin. Excess inorganic reagents were removed by passage through a Sephadex G-25 coarse column (100 x 2.5 cm) equilibrated in 0.1 M Tris buffer, pH 8.15. These samples were used within 24 hours of their preparation.

Buffers—For the kinetic experiments, 0.05 M buffers of various compositions were used. Sodium cacodylate was used to buffer in the 5 to 6.5 pH range, bis(2-hydroxyethyl)amino-tris(hydroxy-methyl)-methane between pH 6.5 and 8, and tris(hydroxymethyl)aminomethane between pH 8 and 9. These buffers were deoxygenated by equilibration with moist, oxygen-free hydrogen.

For the spectral investigations, 0.05 M buffers were used to counteract any ionic effects due to IHP. The composition of these varied with pH in the same manner as described for the kinetic experiments.

CO Recombination Kinetics after Flash Photolysis—Kinetic measurements were made on a flash photolysis apparatus previously described by Lau et al. (15). Observations were made at 420 nm on a sample held in a 1-mm water-jacketed cell maintained at 20°C. Hemoglobin concentrations were approximately 5 x 10⁻⁴ M and solutions were about 3.2 x 10⁻⁴ M in CO. A small amount of dithionite was added to ensure anaerobic conditions and to prevent autooxidation of the hemoglobin. In those experiments involving IHP, its concentration was 1 mM. Second order kinetic plots were constructed and the slopes generally through the first 50% of the reaction were taken to represent the kinetic constants.

Spectral Studies—Difference spectra were recorded on a Cary-14 spectrophotometer. Solutions of either aquo- or cyanomethemoglobin (3 ml), approximately 10⁻⁴ M in hemoglobin, were placed into each of the two matched quartz cuvettes. Using the 0 to 0.1 optical density slide, a wavelength scan was made and the resulting spectrum was used as the baseline for the remainder of the experiment. The cuvettes were withdrawn and a small volume (3 to 30 μl) of 1.05 M IHP was added to the sample cuvette. An equivalent volume of buffer was added to the reference cuvette to compensate for dilutions made in adding IHP. The cuvettes were reinserted and the spectrum was recorded. The difference spectrum was then obtained by subtracting the baseline spectrum from this curve. The cuvettes were removed, more IHP was added, and the procedure was repeated. A pH determination at the end of each experiment showed that IHP had no effect on the pH.

RESULTS

Studies of CO Recombination Reactions—The rates of carbon monoxide combination to deoxygenated hemoglobin (\( t_0 \)) and to Hb(CO) (\( t_0' \)), measured by flash photolysis, are shown in the lower and upper panels of Fig. 1, respectively. The pH dependencies of these rates are shown for carp hemolysate, phosphate-free carp hemoglobin, and stripped hemoglobin in the presence of 1 mM IHP.

The \( t_0' \) results are consistent with the equilibria and kinetic data described earlier (4). Very clearly, at high pH, there is an upper limit of the recombination rate which is both phosphate and pH invariant. Stripping the hemoglobin of endogenous phosphates results in generally faster rates which implies an increased affinity, but the fastest rate observed for hemolysate is not exceeded. The addition of IHP to stripped hemoglobin results in an overall lowering of the rate, undoubtedly due to this compound’s preferential binding to the low affinity structure. It is easier to compare the data for the different organic phosphate conditions if one observes that the curves are of similar shape and can be superimposed if shifted along the pH axis. Accordingly, as the pH is lowered, a minimum recombination rate is reached which is similar for all three phosphate

\(^1\) The abbreviation used is: IHP, inositol hexaphosphate.
conditions. These minimum recombination rates are indicative of the low affinity state of the hemoglobin. However, these minimum rate constants are not identical nor do the three curves come into perfect correspondence in the acid pH region. In this region the system is complicated by a reverse or acid Bohr effect which appears to be dependent upon the presence and nature of an organic phosphate. This acid Bohr effect was previously observed for carp hemoglobin in the presence of IHP (5) and we now see some indication of such an effect for the stripped hemoglobin, although this latter conclusion represents but a single data point. The complications created by this reverse Bohr effect are greatest below pH 5.5 where other properties of carp hemoglobin, i.e. its stability, are clearly pH-dependent.

When the data for \( l'_{4} \) is compared to that for \( l' \), important similarities and differences are observed. Again there is an upper limit to this rate constant which is pH- and phosphate-independent. This upper limit of \( l'_{4} \) is slightly greater than that observed for \( l' \), and this is as expected since it has been previously reported that at high pH cooperativity decreases but is never absent. The pH range over which the observed rate equals the upper limit is considerably greater for \( l'_{4} \) than for \( l' \). The pH and phosphate dependences of \( l'_{4} \) are very similar to those of \( l' \) except that the former is shifted toward lower pH by approximately 1 unit. This implies that the binding of three CO molecules has a similar effect on the structure of the hemoglobin molecule as does the removal of organic phosphates or an increase in the pH. This is consistent with the fact that CO binds preferentially to the high affinity structure just as organic phosphates and protons do to the low affinity state. At low pH, under all conditions where ligand binding has been shown to be noncooperative (3–5), \( l'_{4} \), \( l' \), and \( l'' \) are equal.

Effect of IHP on Spectral Properties of Methemoglobin—The addition of IHP to a sample of carp methemoglobin stripped of phosphates results in a different optical absorption in the 450 to 675 nm region. This is seen in the difference spectra in Fig. 2 between carp hemoglobin in the presence and absence of IHP. Measurements were made at three pH levels and at different IHP concentrations. Although the intensities of the difference bands change with both pH and phosphate concentration, their positions are relatively constant. Positive difference bands appear at 505 and 640 nm and negative peaks at 540, 575, and 610 nm. The accuracy of locating these bands and others reported here has not been tested by any type of deconvolution analysis.

At pH 5.56, the absence of isosbestic wavelengths and the parallel nature of the difference curves are indicative of protein denaturation. This is not unreasonable considering the fragility of carp hemoglobin at low pH, the length of time involved in the experiment, and the high ionic strength environment. In any case, the results at this pH indicate that no additional spectral changes occur when the IHP concentration is increased above 1 mM. At pH 6.96, the intensities of the difference bands increase with increasing IHP concentration. The nonlinear relationship between the magnitude of the difference spectrum and the concentration of IHP results from the relatively low apparent affinity of the system for this organic phosphate. This in turn is presumably the result of the dynamic equilibrium between the R and T structural states which at this pH, in the absence of organic phosphates, lies predominantly toward the R state. This effect is also shown in the pH 8.73 data. The different intensities seen for the three different pHs will be discussed later with respect to the equilibrium between hydroxy- and aquomethemoglobin.

Effect of pH on Spectrum of Carp Methemoglobin—The results of measuring the methemoglobin absorption spectrum between 450 and 675 nm as a function of pH are summarized in Fig. 3. These curves are pH-induced difference spectra. The hydroxymethemoglobin spectrum at pH 10 was used as a reference and, therefore, the increasing intensities of both the
The ligand affinity of carp hemoglobin is bounded at both extremes by regions of constant affinity associated with non-cooperative ligand binding. This suggests that each of these regions defines a separate single protein conformation. Kinetic and equilibrium experiments show that smooth transitions in functional properties occur between the minimum and maximum affinity states. Therefore, the simplest model proposes that the ligand affinity of carp hemoglobin is controlled by the equilibrium between these R and T conformations. The present data can also be dealt with quite nicely in terms of this two-state hypothesis.

Functionally, the fastest rate of CO combination to unliganded carp hemoglobin occurs within the same pH range as the maximum affinity as seen in the equilibrium results. The slowest combination rates relate to the minimum affinity in the same manner. Apparently, the magnitude of the rate of CO combination to unliganded carp hemoglobin is indicative of its conformational state. At high pH, although ligand binding to carp hemoglobin is slightly cooperative and the values for \( I' \) and \( I'' \) are not identical, they are very similar. According to the two-state scheme, this suggests that under these conditions the deoxygenated molecule is predominantly in the \( R \) state. However, \( I' \) is reduced relative to \( I'' \) because of a small but significant level of T state in the conformational equilibrium. Both hydrogen and phosphate ions act as allosteric effectors promoting the formation of the T conformation. Therefore, when carp hemoglobin is stripped of endogenous phosphates and is at a low hydrogen ion concentration, it is free of allosteric effectors and its properties represent the intrinsic behavior of the molecule. The properties of carp hemoglobin under these conditions are unlike those reported for any mammalian hemoglobin or for the hemoglobins of the trout \( Salmo gairdneri (15) \) and \( Salmo irideus (16) \). These latter hemoglobins have maximum ligand affinities at high pH but ligand binding remains highly cooperative and the deoxygenated proteins appear to be predominantly in \( T \) or low affinity states. At low pH, even though the carp hemoglobin data are somewhat complicated by an acid Bohr effect, the magnitude and the relative constancy of the minimum \( I' \) combination rates, when considered along with the previously reported constancy of their equilibrium and kinetic parameters (2-5), indicate a single, low affinity, T conformation. As with the R state, pH, phosphate concentration, and partial ligand-binding act only to modify the range of conditions over which this conformation exists. The existence of both R and T conformations whose properties are independent of the degree of liganding, of pH, and of organic phosphate concentration, clearly suggests a simple two-state protein whose affinity for ligand is dependent on the equilibrium between the two conformations. The extent to which the properties of carp hemoglobin can be explained by this two-state model is remarkable. No other hemoglobin behaves in so apparently simple a manner. However, even for carp hemoglobin some properties are found which are contrary to predictions. One of these is obviously the observed acid Bohr effect and the other is the detail of the time course of CO combination as examined by Tan et al. (4). Nevertheless, the vast majority of the pH and organic phosphate effects on the equilibrium and kinetics of ligand binding to this protein can be accounted for by invoking the existence of only two structural forms of the protein.

If one examines the data for \( I'' \), it is clear that there are pH levels at which the addition of 1 mM IHP to the stripped,
liganded hemoglobin shifts the conformation from predominantly R state to predominantly T state. It is this conformational transition which has been confirmed by difference spectroscopy. If liganded ferrous carp hemoglobin was used to study this transition, the drastic reduction in ligand affinity and resultant ligand dissociation would impossibly complicate the difference spectra obtained and they would reflect both the R to T transition and the ligand dissociation. Unlike ferrous hemoglobin, methemoglobin has no unliganded state and the use of methemoglobin derivatives for difference spectroscopy eliminates the complications of ligand dissociation.

Perutz et al. (10-12) have published a series of papers in which they have studied hemoglobin systems where it is postulated that the structural R → T transition occurs without a corresponding change in ligand binding. In addition to using mutant and modified human hemoglobins, which are thought to remain locked in an R conformation even when totally unliganded, they have investigated the behavior of ferric hemoglobin derivatives. Their studies relate closely to those reported here on carp ferric hemoglobins. For both hemoglobins, IHP is seen to favor the low affinity conformation. In the R → T equilibrium view of carp hemoglobin, IHP at constant pH can trigger a shift favoring the T state. On the basis of x-ray crystallographic studies, Perutz (17, 18) postulated that in the R conformation the iron atom is found in the plane of the porphyrin while in the T state, the iron is out of the plane. Thus, when IHP is added to cause the R → T switch, it binds to the globin, which causes a change in the quaternary and the tertiary structure, and in doing so, the proximal histidine, which is coordinated to the iron, is repositioned. This movement in Fe²⁺ oxyhemoglobin pulls the iron out of the plane and favors its deoxygenation. However, when Fe³⁺ is strongly liganded there is an opposing force produced by the octahedral environment of the Fe³⁺ which tends to keep it in the porphyrin plane. Any movement of the Fe³⁺ would cause a disturbance of the electronic distribution, which is implied in the symmetry forced by the ligand environment. This distortion opposes the conformational R → T switch induced by the binding of IHP to globin. In addition, since the strength of this symmetry field is directed toward keeping the Fe³⁺ liganded while the conformational transition requires its distortion, the nature of the ligand which occupies the position opposite the proximal histidine is of extreme importance. A weak symmetry field ligand would allow the hemoglobin to relax into a T conformation more easily than a strong field (low spin) ligand which would tend to retain an R structure.

Such a dependence of the R to T transition on ligand strength is reported by Perutz et al. (10-12). They find that the addition of IHP to human aquomethemoglobin produces a significant change in the visible spectrum, while no such change is observed for cyanomethemoglobin. Furthermore, they have shown that the addition of IHP to human aquo- or fluoromethemoglobin produces an ultraviolet difference spectrum in which the peaks are identical to those in the difference spectrum between oxy- and deoxyhemoglobin. Both H₂O and F⁻ are low symmetry ligands and their conclusion is that in the presence of either of these ligands, IHP can induce a quaternary structure change. They have also found that the strong field cyanomethemoglobin derivative does not exhibit the same ultraviolet difference spectrum upon IHP binding, suggesting again that the R → T conversion is prevented.

The results obtained for carp hemoglobin can be well explained using these same considerations of ligand strength if it is realized that for this protein, IHP and protons are far more effective in inducing the R to T transition. Thus, unlike human hemoglobin, carp cyanomethemoglobin exhibits a distinct difference spectrum in the visible region as the result of IHP binding. This IHP-dependent difference spectrum of the cyanide derivative follows closely predictions based on the binding of the ferrous ligands, O₂ and CO. Thus, at pH 5.50, 1 mM IHP shifts the liganded molecule completely to the T state. At pH 6.96, 1 mM IHP is insufficient and 25 mM is required for a substantial conformational transition. At pH 8.73, the liganded molecule remains in the R state regardless of IHP concentration. IHP has advantages in such studies since its effects on the ligand affinities of hemoglobins are greater than those of other organic phosphates. However, it has been reported (19) that some of the effects of this compound on the properties of human hemoglobins are qualitatively different from the effects of other organic phosphates. Therefore, it is important to note that essentially the same difference spectrum resulted when ATP was added to carp cyanomethemoglobin.

The results obtained with carp methemoglobin differ from those for cyanomethemoglobin and reflect the weak nature of the water ligand. Thus at pH 5.56, stripped aquomethemoglobin is not predominantly in the R state, and with a smaller population of R state, IHP addition results in a relatively small spectral change. At pH 6.96 the observed difference spectrum is similar to those reported for human aquomethemoglobin. At pH 8.7, where no difference spectrum was obtained with cyanomethemoglobin, the equilibrium mixture of aquo- and hydroxymethemoglobin exhibits a small but significant one. There are two possible origins of this difference spectrum. At this pH there is a significant amount of aquomethemoglobin present and IHP might induce an R to T transition in this when it cannot in the cyanomethemoglobin. Alternatively, if the pK shift observed is real, this difference spectrum could reflect a shift in the equilibrium from hydroxy- to aquomethemoglobin. This possibility is enhanced by the similarity between this IHP-induced difference spectrum and the pH-dependent difference spectra in Fig. 3.

While the heme stress can be observed spectrally in the visible region, a probe of the globin structure can be made by monitoring the aromatic amino acid absorptions in the ultraviolet region. The IHP-induced difference spectra for both carp aquo- and cyanomethemoglobin have been measured in the 350- to 270-nm region and are qualitatively the same, indicating that the globin undergoes a conformational transition in the case of both ligands.

Although a relationship between ligand strength and the facility of the R to T transition is observed in carp hemoglobin, our results offer no confirmation of the postulated relationship between the spin state of the iron atoms and the conformational state of the protein. When an R to T transition is observed for aquomethemoglobin, one begins with a predominantly high spin R state. One expects only a small spin state change toward still higher spin to accompany the additional movement of the iron out of the heme plane as the protein shifts to the T state. In contrast, with cyanomethemoglobin, the R state is predominantly low spin, yet the transition to the T state produces a spectral change that is no larger than that observed for the aquomet derivative. True, this spectral change is consistent with a shift toward higher spin, but the magnitude of this change is only 5% of that seen between the spectra of aquo- and hydroxymethemoglobin. Therefore, the iron of the
cyanomethemoglobin derivative of carp hemoglobin remains predominantly low spin even when in the T state. This is also true of the ferrous carbon monoxide derivative, which undergoes no obvious spectral changes when converted to the T state as a result of lowering the pH or adding organic phosphates or both. To this extent we are in agreement with the recent conclusions of Hensley et al. (20). Indeed, one must be cautious in attributing such small changes in spectrum to changes in the spin state of the iron atom. The visible spectrum which one observes is the porphyrin spectrum. This spectrum is perturbed by the iron atom and it is true that low and high spin derivatives have distinctive spectral properties. However, it is also true that the porphyrin spectrum is perturbed by the interaction of the porphyrin with the protein, and changes in this interaction resulting from changes in protein conformation could significantly modify the spectrum independent of any change in the state of the iron.

From the results of our studies of carp hemoglobin, we would suggest that the transition from the R to the T conformation is not unalterably linked to or controlled by any one parameter, but is instead modulated by a number of variables. Among these is the presence or absence of ligand at the sixth coordinate position of the iron atoms, the strength of the ligand field, the intrinsic R → T equilibrium of the hemoglobin itself, and the preferential binding of heterotropic allosteric ligands such as protons or polyphosphates. The presence or absence of ligands on the iron atoms and the strength of the ligand field are related to the spin state of the iron atoms and to the positioning of these atoms with respect to the planes of the porphyrin rings. However, one cannot go from this observation to a definite requirement of spin state or iron position for existence of a conversion to the T state. It is very doubtful that the iron atoms of carp cyanomethemoglobin are significantly out of the planes of the porphyrins, even in the T conformation, although the effect that such movement would have on the spectrum of this derivative is unknown.

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