The pH Dependence of the Affinity, Kinetics, and Cooperativity of Ligand Binding to Carp Hemoglobin, Cyprinus carpio

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SUMMARY

The pH dependence of the equilibrium and kinetics of ligand binding to carp hemolysate and the purified hemoglobin components of carp hemolysate with O2 and CO has been studied.

The affinity of carp hemoglobin for O2 at alkaline pH is 160 times that at acid pH. A large but significantly different pH effect on the affinity for CO is observed. pH also has different effects on the kinetics of the reactions with the two ligands. The change in the rate of O2 dissociation over the pH range studied is 10 times that found for the rate of CO replacement by NO; and as previously reported, the change in the rate of combination of CO is twice that of O2. The value of n in the Hill equation is pH-dependent for both ligands, being maximal at neutral pH and decreasing as the pH is shifted from neutrality. This decrease in the value of n is shown to be due not to heterogeneity in the carp hemoglobin but to be the result of an apparent decrease in the cooperativity of the ligand-binding reaction. At pH 5.6 and below, the value of n is 1.0 for CO and 0.75 for O2 indicating no cooperativity.

Below pH 5.6 and above pH 8.0, all equilibrium and kinetic constants are found to be unaffected by further pH changes. These results and the low, constant values of n are consistent with the hypothesis that at acid pH, carp hemoglobin, whether liganded or not, remains in a single low affinity conformation, perhaps that normally associated with the deoxygenated state. The hemoglobin appears to approach a similar situation at alkaline pH where the molecule has a high affinity similar to that of the ligand-saturated state.

The oxygen affinities of many fish hemoglobins have an unusually large pH dependence (1). Often the affinity at acid pH is so low that even when equilibrated with pure oxygen, the hemoglobin is only partially saturated. The equilibrium curves (percentage saturation plotted against log p(O2)) often become heterogeneous, leveling off at incomplete saturation as if a fraction of the heme groups were unable to bind ligand. This heterogeneous behavior has been shown by Hashimoto et al. (2) in the chum salmon, Onchorhyncus keta, and by Binotti et al. (3) in the trout, Salmo irideus, to be due to the presence of multiple hemoglobins with very different pH dependencies. For instance, the S component of the chum salmon has high oxygen affinity which is only slightly affected by pH. The F component has higher affinity than the S component at alkaline pH whereas at acid pH, its affinity is so low that the air-equilibrated hemoglobin is less than 20% saturated with oxygen (2). At neutral and alkaline pH, the equilibrium curves of most fish hemoglobins show cooperative oxygen binding with n values in the Hill equation similar to those found for mammalian hemoglobins.

The oxygen affinity of carp hemoglobin was shown by Noble et al. (4) to change by two orders of magnitude when the pH was decreased from 8.0 to 5.6. Although the carp hemolysate consisted of more than one fraction, they found that even at low pH the kinetics and equilibria of ligand binding did not suggest heterogeneity. Nevertheless, the shape of the equilibrium curve was pH-dependent indicating cooperative binding or heme-heme interaction at neutral and alkaline pH but apparently no cooperativity at low pH. To explain the low oxygen affinity and the lack of cooperativity at acid pH, they postulated that under these conditions the molecule was frozen in its low affinity, deoxygenated conformation (4). The results of their kinetic measurements were consistent with this hypothesis. In particular, they found the large change in oxygen affinity to be reflected in an equally large change in the rate of oxygen dissociation. The rate of O2 dissociation from carp hemoglobin at low pH was unusually rapid and similar to that of partially oxygenated hemoglobin as measured by Gibson (5).

Most of the data of Noble et al. (4) were obtained with carp hemoglobin which had been prepared by a toluene extraction method and which was rather unstable at low pH. Although they showed that the oxygen affinity actually reached a minimum value at low pH where it was no longer pH-dependent, they failed to demonstrate a similar leveling off of either the rate of oxygen dissociation or the rate of replacement of carbon monoxide by nitric oxide. In addition, although they found no gross heterogeneity in the oxygen equilibrium curve at low pH, the value which they found for n in the Hill equation was less than one. The possibility remained that the different components of carp hemoglobin might have somewhat different properties at the low pH range.

We have extended the studies on carp hemoglobin. The separate components have been isolated and shown to behave in
a similar manner. The measurements of the rates of oxygen and carbon monoxide dissociation have been extended to lower pH values, and these rates level off at a maximum value as expected from the equilibrium behavior. Finally, equilibrium measurements have been obtained for the reaction of carp hemoglobin with carbon monoxide as a function of pH. As reported in this paper, all of our results are consistent with the original hypothesis that at acid pH, carp hemoglobin is frozen in a low affinity conformation, possibly that normally associated with deoxygenated hemoglobin.

EXPERIMENTAL PROCEDURE

Materials

Carp Hemoglobin—Blood was obtained from carp by cardiac puncture and mixed immediately with an equal volume of Alsever’s solution (6) at 4° to prevent coagulation. The red blood cells were separated from the plasma by centrifugation for 10 min at 3,020 x g at 4°, washed three times with Alsever’s solution, and the washed red blood cells were then stored in liquid nitrogen. Carp hemoglobin was obtained by lysing the red blood cells with a volume of distilled water equal to twice that of the packed red blood cells. The cell ghosts were removed by centrifugation at 48,200 x g for 20 min at 4°.

Buffers—The buffers used from pH 5 to 6.5 were phosphate-citrate; from pH 6.5 to 7.5, phosphate; from pH 7.5 to 8.5, phosphate-borate; and from pH 8.5 to 9, borate. All were 0.1 to 0.15 M in anion concentration. Buffers were deoxygenated by bubbling with hydrogen which had been washed with water after passage through two hot copper ovens placed in series.

DEAE-cellulose—The DEAE-cellulose used for separation of the fractions was Whatman DE-52.

Methods

Equilibrium Measurements—Oxygen equilibria were done at 10 and 20° with a modification (7) of the method of Allen, Guthe, and Wyman (8), using hemoglobin solutions that were 2.5 x 10^-4 M in heme. At low pH values it was impossible to saturate the hemoglobin completely even with one atmosphere of pure oxygen. In these cases, the spectrum of the fully saturated hemoglobin was obtained after raising the pH of the sample by adding a small amount of solid K2HPO4.

For carbon monoxide equilibria, a solution of hemoglobin was carefully deoxygenated and then titrated with a solution of dithionite in order to convert any trace of methemoglobin to the ferrous form. The dithionite solution was prepared by dissolving dithionite in carefully deoxygenated water in a closed system such as a syringe. The titration was carried out with an Agla micrometer syringe. The measurements were done by the same spectrophotometric method used to study oxygen equilibria. Anderson and Antonini (9) proved the validity of the assumption that the spectral changes are directly proportional to the fractional saturation of hemoglobin with carbon monoxide. Equilibration was done at 20° in the dark by covering the tonometer with aluminum foil. The time necessary to reach equilibrium was determined by taking successive spectral measurements until a constant level of saturation was reached. The time ranged from 40 min at acid pH to 100 min at neutral pH. At neutral pH, the affinity for carbon monoxide was so high that instead of adding pure carbon monoxide, small volumes of a carefully prepared mixture of carbon monoxide and hydrogen were introduced into the tonometer.

The pressure of half-saturation, p(02) and p(CO), and n values were calculated from the Hill equation by the method of least squares.

Partition Coefficients between Carbon Monoxide and Oxygen—
The partition coefficient, M, was measured essentially by the method of Rossi-Fanelli and Antonini (10). Aliquots of the same hemoglobin solution were equilibrated separately with oxygen, carbon monoxide, and a mixture of oxygen and carbon monoxide in 500-ml tonometers with spectrophotometric cuvettes attached. The spectra were recorded and the calculation of the percentage carboxyhemoglobin and oxyhemoglobin was based on the readings at 575, 555, and 537 nm. The equilibrations were done in the dark by covering the tonometers with aluminum foil. The minimum time found to be necessary to achieve equilibrium from both directions, i.e., using either oxyhemoglobin or carboxyhemoglobin at the start, was 15 min. When this was done at different pressure ratios of the two gases, the partition coefficient, M, was the slope of the line in a plot of the percentage carboxyhemoglobin to percentage oxyhemoglobin against p(CO):p(02). The slope was calculated by the method of least squares. When the experiment was done only at one carbon monoxide to oxygen ratio, the partition coefficient, M, was calculated with the equation

\[ M = \frac{(\text{HbCO})(\text{HbO}2)}{(\text{p}(\text{O}2)/\text{p}(\text{CO}))} \]

All spectral measurements were done with a Cary 14 recording spectrophotometer.

Stopped Flow Kinetic Measurements—These were done with a stopped flow apparatus as described by Gibson and Milne (11).

In the usual measurements of the rate of O2 dissociation, air-equilibrated hemoglobin solutions, buffered to the desired pH are rapidly mixed with a deoxygenated dithionite solution in the stopped flow apparatus. At low pH, carp hemoglobin is only partially saturated with oxygen. Therefore one observes with this method the dissociation of oxygen from only those heme groups liganded under these conditions. Although it has been shown (4) that all heme groups in carp hemoglobin are similar with respect to O2 dissociation, it is desirable to use a technique in which the dissociation of oxygen from all heme groups is observed. This is achieved in the pH jump technique described by Noble et al. (4). Air-equilibrated hemoglobin solutions in 0.001 M potassium phosphate buffer at pH 7 which were 2.5 x 10^-4 M in heme were rapidly mixed with deoxygenated buffers of varying pH which were 0.1 to 0.15 M in anion concentration as described under "Materials". The reaction was followed at 478, 540, 560, and 578 nm at 2°. The pH recorded was the pH of the reaction mixture at 2° taken immediately after mixing.

At low pH, a secondary reaction occurs. At 540 and 578 nm, the secondary reaction has an absorbance change in a direction opposite to the absorbance change of the main reaction. The base line of the main reaction was obtained by extrapolating the secondary reaction to zero time on a stopped flow trace in which the primary reaction was complete within the first 10% of the trace.

When the reaction was followed at 478 and 560 nm, the secondary reaction had an absorbance change in the same direction as the main reaction. In this case it was more difficult to separate the main and the secondary reactions by inspection so the new base line was approximated by linear extrapolation of
Oxygen affinity is 1.6 times that at 20°C. This corresponds to a difference of 0.2 on the log scale, indicating that at 10°C, the comparison of the corresponding log p+(O2) at 10 and 20°C shows a that the curves would be superimposed as much as possible. A

Elution was by concave gradient, the starting buffer being 800 ml of 0.001 M sodium borate buffer and the final buffer being 1 liter of 0.008 M sodium borate buffer. The flow rate was 30 ml per hour. Inset shows starch gel electrophoresis of carp hemoglobin stained with Amido schwarz. From left to right: carp hemolysate, carp Fractions A, R, and C. All samples were 2 mg of protein/ml.

the portion of the stopped flow trace corresponding to that used at 540 and 578 nm. As will be seen, this method yielded identical rate constants for the dissociation reaction at all wave lengths.

The rate of replacement of carbon monoxide by nitric oxide, i4, was measured as described by Noble et al. (4). Carboxyhemoglobin in deoxygenated buffers of varying pH was mixed with aqueous solutions of 2.0 mm nitric oxide. For the carp hemoglobin fractions at low pH, these rates were also measured by the pH jump technique in which the nitric oxide solutions were made with buffers and the hemoglobin was in deoxygenated, 0.001 M potassium phosphate buffer at pH 7. The latter technique was used mainly for convenience and because it required less material. The rate constants, k and i4, were calculated as first order reactions by the method of least squares.

Fractions of Carp Hemoglobin—Carp hemoglobin was fractionated at 4°C by DEAE-cellulose chromatography in sodium borate buffer at pH 9. The fractions were eluted by a concave gradient of borate concentration. The elution pattern is given in Fig. 1. Separation and purity of the fractions were confirmed by starch gel electrophoresis at pH 8.6 as described by Poulik (12). The gels were stained with Amido schwarz. From left to right: carp hemolysate, carp Fractions A, B, and C. All samples were 2 mg of protein/ml.

RESULTS

Oxygen Equilibrium—Oxygen equilibrium measurements were carried out at 10°C on the total carp hemolysate and Fractions A and B. At this temperature carp hemoglobin has less tendency to denature or to be oxidized to methemoglobin than at 20°C. In Fig. 2, log p+(O2), the O2 pressure required for half-saturation, is plotted as a function of pH for carp hemolysate and Fractions A and B at 10°C. It is clear that the fractions and the total hemolysate have essentially the same behavior. The data of Noble et al. (4) for the O2 affinity of carp hemolysate at 20°C is included for comparison. The vertical axis for the 20°C data was shifted such that the curves would be superimposed as much as possible. A comparison of the corresponding log p+(O2) at 10 and 20°C shows a difference of 0.2 on the log scale, indicating that at 10°C, the oxygen affinity is 1.6 times that at 20°C. This corresponds to a

\[ \Delta H^o = 7.7 \text{ kcal per mole of ligand} \]

Since the two curves can be superimposed, this \( \Delta H^o \) is constant within our experimental error throughout the pH range studied.

Fig. 2 also includes a plot of the value of n in the Hill equation as a function of pH for carp hemolysate and its fractions at 10°C. The extent of cooperativity, as indicated by this parameter, is pH-dependent. The value of n is below 1.0 at acid pH, rises gradually to a maximum at neutral pH, then falls again as the pH is raised to 9. This indicates that the oxygen-binding process is not cooperative at acid pH, somewhat so at alkaline pH and highly cooperative at neutral pH. There is a large experimental error in the values of n above pH 7.5 and these should be considered preliminary. Nevertheless, as the pH is increased beyond 7.5, a trend to lower values of n is clear.

Since a homogeneous noncooperative hemoglobin is not expected to have a value of n below 1.0, extensive measurements were made on the carp hemolysate at acid pH. We find that at oxygen pressures below 10 mm Hg, where the hemoglobin is less than 20% saturated, the slope of the Hill plot is 1.0, but all other points from 20 to 85% saturation fall on a straight line with a slope of 0.75. This suggests the presence of functionally different heme groups. This heterogeneity does not arise from a difference in the fractions since these have oxygen equilibrium curves that are identical with those of the total hemolysate.

Rate of O2 Dissociation, k—The rates of O2 dissociation from carp hemolysate and from Fractions A and B were measured at 20°C by the pH jump technique. The results for the carp hemolysate are shown in Fig. 3A. At pH 5.6 and higher, the values of k agree with the previously published results of Noble et al. (4). Below pH 5.6 and above pH 8.0, our results show a leveling off of the value of k as would be expected from the pH dependence of the oxygen affinity. The O2 dissociation rate in the acid pH region is 90 times that in the alkaline region whereas there is a 100-fold change in oxygen affinity.

In Fig. 3B, k is plotted as a function of pH for Fractions A and B. As in the case of the oxygen equilibrium constant, K, the

\[ V = 1.80 \text{ mL per min per mg of protein} \]

\[ A = 2.40 \text{ mL per min per mg of protein} \]
**Fig. 3.** A, the effect of pH on the rate of oxygen dissociation, $k$, of carp hemolysate at 20°. The heme concentration was $2.5 \times 10^{-9}$ M. The reaction was followed at 578 nm, ○; 540 nm, ■; 478 nm, □; and 560 nm, ▶. Each experimental point is the average of three separate determinations. B, the effect of pH on $k$ of carp Fractions A and B under the same conditions as in A. The reaction was followed at 578 nm, ■; and 478 nm, □, for Fraction A; and 578 nm, ○, and 478 nm, △, for Fraction B.

**Fig. 4.** The effect of pH on the carbon monoxide affinity of carp hemoglobin as compared to the oxygen affinity. In the lower part of the figure, the logarithm of the ligand pressure for half-saturation is plotted against pH and in the upper part of the figure, the value of $n$ in the Hill equation is plotted against pH. The heme concentration was $2.5 \times 10^{-9}$ M. The vertical axis on the left is for the CO data (O-O), the axis on the right for O$_2$ data (---), the latter being reproduced from Fig. 2. A comparison of the vertical axes for oxygen and carbon monoxide equilibrium at 20° shows that carbon monoxide has about 100 times higher affinity than oxygen. Both curves show a level region at acid pH and a sharp decrease in log $p_l$ as the pH is raised to 7. However, the change in affinity over this pH range is less for CO than for O$_2$.

The degree of cooperativity, indicated by the value of $n$ of the carbon monoxide equilibrium curves, is pH-dependent, being high at neutral pH and gradually decreasing until the cooperativity disappears at acid pH where the value of $n$ is 1.0. This follows the same trend as the $n$ values in the oxygen equilibrium measurements although the values for oxygen equilibrium are consistently lower. In both oxygen and carbon monoxide equilibrium studies, the pH at which the value of $n$ reaches a minimum value coincides with the pH at which the affinities for both ligands reach a constant level.

Because of the very high affinity of carp hemoglobin for carbon monoxide at pH values above 7, direct measurements of CO-binding equilibria could be obtained only with considerable difficulty. Therefore in this pH range, the relative affinities of the hemoglobin for oxygen and carbon monoxide were assayed by measuring the partition coefficient.

**Fig. 5.** Determination of the partition coefficient, $M$, of carp hemoglobin at 20° and pH 7. The experimental points were obtained by starting with oxyhemoglobin, ○, and carboxyhemoglobin, △.

**Fig. 6.** The log of the partition coefficient is plotted as a function of the ratio of the pressure of CO to that of O$_2$ at pH 7. The open circles represent the values obtained when equilibration was begun with the hemoglobin in the oxy form and the filled circles represent the values obtained with carboxyhemoglobin at the start. Similar plots were obtained at pH 6.5, 8, and 9. At other pH values, $M$ was determined at one $p(CO) : p(O_2)$ ratio.

In Fig. 6, the log of the partition coefficient is plotted as a
The pH dependence of $\Delta$ is similar for Fractions A and B.

At low pH, the value of $n$ for oxygen binding is less than 1.0. This indicates heterogeneity of the heme groups in the system, but again, it cannot be the result of the heterogeneity of carp hemolysate since the isolated fractions have the same behavior. There remains the possibility that the $\alpha$ and $\beta$ chains of the carp hemoglobin have different oxygen affinities. This is not unexpected. Perutz (13) has pointed out that in deoxygenated hemoglobin, the regions around the heme groups of the $\alpha$ and $\beta$ chain have very different structures. It would be most unlikely that heterogeneity of the chains in carp hemoglobin could explain the drop in the $n$ value from 3.0 to 0.75 but it may explain the drop from the expected lower limit of 1.0 to that of 0.75.

The measurements of carbon monoxide-binding equilibrium have a number of advantages over those of oxygen affinity, especially at low pH. The CO-binding measurements were carried out anaerobically, and therefore, no auto-oxidation of the hemoglobin occurred. Furthermore, although the affinity for CO is much reduced at low pH, it was sufficiently high to allow complete saturation of the hemoglobin without having to raise the pH of the sample.

We can compare the affinities for oxygen and carbon monoxide from pH 5.1 to 7.1 directly. Between pH 5.1 and 5.6, both affinities show no pH dependence. From pH 5.6 to 7.1, there is a large pH effect on the affinities for the two ligands. The carbon monoxide affinity increases by a factor of 10, as compared to a 20-fold increase in the oxygen affinity in this pH range.

The pH dependence of the value of $n$ for the carbon monoxide equilibrium parallels the values from the oxygen-binding data. However, they are consistently higher, and at acid pH, the minimum value of $n$ is 1.0. If cooperativity or heme-heme interaction is truly absent at low pH, this would indicate that all the heme groups are homogeneous with respect to carbon monoxide-binding affinity.

Above pH 7.1, the relative affinity of carp hemoglobin for oxygen and carbon monoxide was assayed by means of the partition coefficient. This was found to equal 92 and to be invariant with pH from 6.5 to 9. However, the partition coefficient is not directly related to the relative affinity of the molecule for the two ligands as computed from the ligand pressures for half-saturation. In measuring the partition coefficient, one always deals with a fully liganded hemoglobin molecule and it is the relative affinity of this form of the molecule for the two ligands which is obtained. In measuring a ligand-binding equilibrium curve, one begins with fully unliganded hemoglobin and ends the experiment with a ligand-saturated molecule. Therefore the affi-
finity one measures is a function of these two forms and the transition between them in the course of ligand saturation. There is no a priori reason to assume that these two forms and the intermediate partially liganded structures will all have the same relative affinity for CO and O₂, nor should it be assumed that the pH dependence of the transition between the various hemoglobin structures will be the same for both ligands. In fact, as shown in Fig. 4, the ratio of the pK₅(O₂) to that of pK₅(CO) actually is pH-dependent, decreasing from a value of 100 at low pH to a value near 40 from pH 6.7 to 7.1. Nevertheless, if we can consider our carbon monoxide equilibrium and partition coefficients data together, it is clear that over the pH range from 5 to 9 there is a large change in the ligand affinity of carp hemoglobin for both oxygen and carbon monoxide.

The calculation of meaningful equilibrium constants from over-all rates of ligand binding and dissociation for an allosteric protein is not possible. However, we can legitimately ask whether or not the magnitude of the changes in the over-all rate constants are consistent with an observed change in ligand affinity. For the oxygen reactions, a total affinity increase of 160 fold is observed in going from pH 5 to 0. Over this same pH range, k' increases by 34-fold (4) while k decreases by 90-fold. These changes in rate constants can accommodate an affinity change as large as 300-fold. For the carbon monoxide reactions, l increases by 7-fold while l decreases by a factor of 10. Although the relation of Z₄ to l and its dependence on pH range, k' increases by 3.5-fold (4) while k decreases by 90-fold. Over this same pH range, k' increases by 7-fold while k decreases by a factor of 10. Therefore, the rate of oxygen combination is less affected but the effect on the kinetic constants is very different.

We have shown that the reactions of oxygen and carbon monoxide with carp hemoglobin have very different pH dependencies. The effect of pH on the rate of oxygen dissociation is much greater than its effect on the rate of carbon monoxide dissociation. In contrast, the rate of oxygen combination is less affected by pH than is the rate of combination of carbon monoxide. Finally, the magnitude of the change in ligand affinity over the pH range studied is greater for oxygen than for carbon monoxide. It is interesting to note that in the comparison of the β chain of human hemoglobin and its p-HMB¹ derivative we find another example of a large modification of the ligand affinity of a heme protein which affects the oxygen and carbon monoxide reactions differentially. Thus, at pH 7, βSH has higher oxygen and carbon monoxide affinities than β₆₋₇ scrambl. The rate of oxygen dissociation of β₆₋₇ scrambl is 10 times that of βSH while the rate of carbon monoxide dissociation of β₆₋₇ scrambl is only 34 times that of βSH. The rates of oxygen combination for both are nearly the same while the rate of carbon monoxide combination of βSH is 2 times that of β₆₋₇ scrambl. The effect on the ligand affinities of introducing p-MB into βSH subunits is nearly the same for both ligands but the effect on the kinetic constants is very different (14, 15).

It appears that cooperative ligand binding of hemoglobin is a result of a transition between a low affinity form of hemoglobin, the deoxy or unliganded hemoglobin structure, and a high affinity form, the oxy or liganded hemoglobin structure. Although it has never been proven, it is possible that variables, such as pH and specific ions, alter the over-all ligand affinity of hemoglobin by affecting the transition between these two forms without modifying their properties. Then the ligand affinities of these two structures would be the minimum and maximum affinities that the hemoglobin molecule could have. If conditions were found in which the over-all ligand affinity of the hemoglobin molecule equaled one or the other of the limiting values, then cooperativity would necessarily be lost since under these conditions, the molecule would exist in the same structure both when deoxygenated and when liganded.

Below pH 5.6, all of the equilibrium and kinetic constants for the reactions of oxygen and carbon monoxide with carp hemoglobin are pH-independent. On the basis of the low ligand affinity, lack of cooperativity of oxygen binding, and the high value of k, Noble et al. (4) postulated that at low pH, carp hemoglobin is frozen in its low affinity conformation. The data presented in this paper further support this hypothesis.

Above pH 8, the equilibrium and kinetic constants for the reactions of carp hemoglobin with oxygen and carbon monoxide are again pH-independent. The ligand affinity is very high, the pK₅(O₂) being 0.63 mm Hg at 20° and although oxygen binding is cooperative, the value of n is only 1.5. It may be that at high pH, the ligand affinity of carp hemoglobin is approaching the other limit, the maximum ligand affinity.

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