Conditions Restricting Allosteric Transitions in Carp Hemoglobin

Anna L. Tan* and Robert W. Noble‡

From the Departments of Medicine and Biochemistry, State University of New York at Buffalo, Veterans Administration Hospital, Buffalo, New York 14215

Quentin H. Gibson§

From the Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, New York 14850

SUMMARY

Below pH 5.0, in the presence of endogenous polyphosphates, carp hemoglobin has a very low ligand affinity, and ligand binding is noncooperative, the values of n in the Hill equation being 0.75 and 1.0 for oxygen and carbon monoxide, respectively (Tan, A. L., De Young, A., and Noble, R. W. (1972) J. Biol. Chem. 247, 2493-2498). It has been postulated that under these conditions the molecule remains in the low affinity "deoxy" conformation even when liganded. Raising the pH above 5.0 or removing the organic phosphates converts carp hemoglobin to a molecule that exhibits the usual cooperative ligand binding. Ligand affinity and the degree of cooperativity are phosphate- and pH-dependent. Above pH 8.2 in the presence of organic phosphates and above pH 7.5 in their absence, the ligand-binding properties approach those characteristic of a molecule that again remains in a single conformation, only this time with a high ligand affinity.

Whenever ligand binding was cooperative, the rate of CO recombination with partially liganded hemoglobin produced by partial flash photolysis was faster than that with fully unliganded hemoglobin produced by full flash photolysis. Under conditions when the molecule was thought to remain in one structure, the rates of CO recombination upon full and partial flash photolysis were equal.

The time course of the conformation change in carp hemoglobin relative to ligand binding has been examined by measuring the rate of release of a fluorescent polyphosphate analogue during CO binding. At low pH there was a marked lag between dye release and CO binding. This lag decreased and was finally reversed with increased pH in agreement with our other results.

Kinetic studies of CO combination at wave lengths near the Soret isosbestic point revealed a difference in the rate of CO binding to α and β chains of carp hemoglobin. Although

* Henry M. Woodburn Graduate Fellow at the State University of New York at Buffalo.
† Recipient of Grant 5 RO1 HE12524 from the National Heart and Lung Institute, National Institutes of Health, and Designated Research Funds from the Veterans Administration.
§ Recipient of Grant GM 14276-08 from the United States Public Health Service.

our results for the most part can be explained on the basis of a simple two-state model of hemoglobin, computer fits of the time course of the CO combination reaction could not be achieved with such a simplified scheme, but rather required intra-dimer interactions.

At low pH, carp hemoglobin exhibits properties unlike those of most other hemoglobins (1, 2). All of the equilibrium and kinetic parameters which have been measured are pH-independent and are those of a hemoglobin with extremely low affinity. At 20°, it is half-saturated with oxygen when equilibrated with air at atmospheric pressure and has a rate of oxygen dissociation so rapid that it cannot readily be measured by conventional stopped flow kinetic techniques. In addition, the equilibrium curves are hyperbolic, indicating the absence of homotropic cooperative effects in the ligand-binding process. It was these properties that led Noble et al. (1) and Tan et al. (2) to postulate that at low pH, carp hemoglobin is frozen in the low affinity structure usually associated with deoxygenated hemoglobin and that the transition to the high affinity form, which results in cooperative effects in functionally normal hemoglobin, does not occur.

Merely raising the pH can convert carp hemoglobin to a molecule that exhibits the usual cooperative ligand binding that is observed in other hemoglobins. At still higher pH, its ligand-binding properties approach those characteristic of a molecule that is again frozen in a single conformation, only this time with a high ligand affinity.

Numerous observations have shown that the removal of organic phosphates from hemoglobin results in an increase in ligand affinity (3, 4). This has been shown to be true for carp hemoglobin by Tan et al. (5) and by Gillen and Riggs (6). Since the effect of phosphate removal on ligand affinity mimics the effect of increasing pH, it was of interest to determine if the effects of these two variables on the cooperativity of ligand binding would also be similar. For this reason we have studied the pH dependence of the binding of oxygen and carbon monoxide to carp hemoglobin stripped of organic phosphates. In addition
to providing us with a comparison to the system that was studied previously (1, 2), i.e. carp hemolysate with unidentified endogenous organic phosphates, these studies describe a more chemically defined system.

For hemoglobins which exhibit cooperative ligand binding, the rate of combination of carbon monoxide with a partially liganded molecule is greater than the rate of combination with a fully unliganded or deoxygenated molecule. This phenomenon can be studied by flash photolysis as shown by Gibson (7), since the extent of CO dissociation can be controlled by the intensity of the flash to which the hemoglobin is exposed. If a carboxyhemoglobin solution is fully photolyzed by an intense flash of light, the recombination of carbon monoxide with the hemoglobin under suitable conditions will be the same as that observed when deoxyhemoglobin is mixed with carbon monoxide. However, if only a small percentage of the bound carbon monoxide is dissociated, the rate of carbon monoxide recombination will be indicative of the behavior of the hemoglobin molecule in the liganded form. This can then be compared to the rate of carbon monoxide recombination upon full flash photolysis. If a hemoglobin is frozen in a single conformation, whether liganded or not, these two rates should of course be equal. We have used this method to study carp hemoglobin in the presence and absence of organic phosphates over a wide pH range.

Another method has been used to detect conformational changes kinetically. The time course of the release of 8-hydroxy-1,3,6-pyrene trisulfonic acid, a fluorescent organic phosphate analogue, from hemoglobin as the latter binds carbon monoxide was compared to the time course of carbon monoxide combination. Since PTS binds preferentially to the low affinity form of hemoglobin, its release is indicative of the transition to the high affinity structure. The time course of PTS release relative to that of ligand binding can give an indication of the relationship between the extent of ligand saturation and the conformational state of the hemoglobin molecule (8, 9).

In addition, we have detected differences in the α and β chains of carp hemoglobin by the method of Gray and Gibson (10). The reaction of the hemoglobin with carbon monoxide was monitored at a series of wave lengths near the isosbestic point for deoxy- and carboxyhemoglobin in the Soret region. The time course of the combination reaction was a function of wave length, demonstrating the presence of kinetically different components. These findings have been evaluated by computer simulation giving an approximation to the kinetic behavior of the two types of chains in carbon monoxide binding.

MATERIALS AND METHODS

Carp Hemoglobin—For the experiments done in the presence of phosphates, carp hemolysate and carp hemoglobin fractions were prepared as described previously (2). Carp hemoglobin stripped of organic phosphates was prepared by the method of Benesch et al. (4) after first being titrated to pH 7.5 to slow down metheglobin formation. The phosphate assay of Ames and Dubin (11) was used to confirm the removal of all organic phosphates.

Buffers—The buffers used for the series of experiments on unstripped carp hemoglobin were phosphate-citrate from pH 5 to 6.5, phosphate from pH 6.5 to 7.5, phosphate-borate from pH 7.5 to 8.5, and borate from pH 8.5 to 9. For the experiments on stripped heme, as described in detail previously (3). The pressure of half saturation of ligand, p50(O2) and p50(CO), and the n values were calculated from the Hill equation by the method of least squares.

Flash Photolysis—Flash photolysis experiments were performed using a 100 J flash with a time to 1/e of 0.27 ms. This was screened from the observation system by 1 cm of a saturated NaN3 solution. Observations were made at 372 nm using a Bausch and Lomb 200-mm grating monochromator backed by a 372-nm interference filter. Points were collected 1.5 ms after initiation of the flash using the data-processing system of DeSa and Gibson (14). The cell had an optical path of 1 mm and was surrounded by a jacket through which temperature-controlled water circulated. The hemoglobin concentration was 8.25 x 10⁻⁵ M in heme and the CO concentration was 3.72 x 10⁻³ M.

PTS Release and CO Combination Experiments—These were performed as described by MacQuarrie and Gibson (8, 9). The hemoglobin solution was 8.25 x 10⁻⁵ M in heme, 3.3 x 10⁻⁴ mg per ml in PTS (Eastman practical grade), approximately 5 x 10⁻⁴ M, and 2 x 10⁻⁵ M in dithionite. The carbon monoxide solution was 9.2 x 10⁻³ M in CO and 2 x 10⁻⁵ M in dithionite. The buffers used were the non-phosphate-like buffers described under "Buffers."

Kinetics of CO Binding to α and β Chains within the Tetramer—Stopped flow determinations of CO binding monitored at a series of wave lengths near the isosbestic point for deoxy- and carboxyhemoglobin in the Soret region were made with the apparatus of Gibson (15) using the data-collecting and processing equipment of DeSa and Gibson (14). The hemoglobin concentration was 1.8 x 10⁻⁴ M in heme and the CO concentration was 9.2 x 10⁻⁸ M. The cell had an optical path of 4 mm.

RESULTS

Equilibrium Measurements—The pH dependence of carbon monoxide binding to stripped carp hemoglobin at 20°C was studied. The results are shown in Fig. 1 with the previously reported data on unstripped carp hemoglobin (2) which are included for comparison. From pH 5 to pH 6, the p50(CO) of stripped carp hemoglobin is 2.4- to 4-fold lower than that of unstripped carp hemoglobin. Whereas the value of n in the presence of phosphates below pH 5.6 is 1.0, this parameter in the absence of phosphates ranges from 1.4 to 1.5. This indicates that the removal of organic phosphates results in a pH-dependent increase in the affinity for carbon monoxide as well as the reappearance of cooperativity in ligand binding.

Since the affinity of stripped carp hemoglobin for carbon monoxide in the alkaline pH region is so high, the equilibrium studies were not carried further than pH 6. The full pH range was examined using oxygen as the ligand. The data for both stripped and unstripped hemoglobin at 20°C are presented in Fig. 2. The data for the unstripped hemoglobin include those previously reported (2) plus additional measurements in the
FIG. 1. The effect of pH on the carbon monoxide affinity of stripped and unstripped carp hemoglobin at 20°. In the lower part of the figure, the logarithm of the CO pressure for half-saturation, \( P_{50}(CO) \), is plotted against pH. In the upper part of the figure, the value of \( n \) in the Hill equation is plotted against pH. O, stripped carp Hb; ●, unstripped carp Hb.

FIG. 2. The effect of pH on the oxygen affinity of stripped and unstripped carp hemoglobin at 20°. The data are plotted as in Fig. 1. O, stripped carp Hb; ●, unstripped carp Hb.

FIG. 3. The effect of pH on the oxygen affinity of stripped and unstripped carp hemoglobin at 10°. The data are plotted as in Fig. 1. O, stripped carp Hb; ●, unstripped carp Hb.

At low pH and 20°, the removal of organic phosphates results in an increase in oxygen affinity and the appearance of heme-heme interaction just as seen in the binding of carbon monoxide. This difference in affinity continues to be seen as the pH is raised to 8.2, beyond which point the oxygen affinities of both stripped and unstripped carp hemoglobin are the same. In Fig. 2, the curve of log \( p_{50}(O_2) \) versus pH for stripped carp hemoglobin seems to be merely shifted toward lower pH. Although the maximum value of \( n \) attained is lower than when phosphates are present, the values of \( n \) follow the same pattern of rise and fall. In the alkaline pH region, \( n \) attains a minimum value of 1.0 at a lower pH than when organic phosphates are present. This correlates with the observation that stripped carp hemoglobin reaches the high affinity level at a lower pH than does unstripped carp hemoglobin.

In order to establish the reversibility of the stripping procedure, adenosine 5'-triphosphate, the predominant organic phosphate found in the red blood cell of many fishes (16, 17), was added to stripped carp hemoglobin. When ATP was added in the ratio of one ATP to one hemoglobin tetramer in phosphate buffer at pH 7, the log \( p_{50}(O_2) \) at 20° was 0.755 and the \( n \) value was 1.76, very similar to the values obtained with unstripped carp hemoglobin.

Oxygen equilibrium measurements in the absence of organic phosphates were also carried out at 10°. The data are shown in Fig. 3 together with the previously reported data for unstripped carp hemoglobin for comparison (2). In general, the behavior of stripped and unstripped carp hemoglobin is the same at 10° as at 20°. However, if the plots showing the pH effect on the value of \( n \) at 10° and 20° are superimposed, as in Fig. 4, they reveal a pH-dependent temperature effect or a temperature-dependent pH effect, whichever way one wishes to view it. The upper panel in Fig. 4 shows the data for unstripped carp hemoglobin; the lower panel shows the corresponding data for stripped hemoglobin. When phosphates are present, the \( n \) values at the two temperatures coincide only from pH 5 to pH 7. At pH 7, the \( n \) value at 20° is maximum with a value of 2.0 and decreases to 1.25 as the pH is raised further. The \( n \) values at 10° reach a higher maximum, with a value of 2.5 at pH 7.4, before decreasing to 1.5 with a further rise in pH. The same relationship between the \( n \) values at 20° and 10° is seen in the absence of phosphates although the values are different.

In Fig. 5, the plots of the logarithm of the \( p_{50}(O_2) \) as a function of pH at 10° and 20° in the presence of phosphates are superimposed by merely shifting the vertical axis by 0.2 units in the log scale. The corresponding data in the absence of phosphates can be superimposed by making the same shift in the vertical axis. Apparently, the temperature dependence of the oxygen...
affinity, unlike that of the $n$ values, is not pH-dependent. Moreover, the heat of reaction of oxygen binding to carp hemoglobin between 10° and 20° is the same whether organic phosphates are present or not. Conversely, the phosphate effect on oxygen affinity is the same at 10° and 20°.

Flash Photolysis of Carp Hemoglobin—We have measured the rate of combination of carbon monoxide with unliganded and with partially liganded carp hemoglobin obtained by full and partial flash photolysis of carboxyhemoglobin. A high rate of CO combination is indicative of high ligand affinity of the hemoglobin molecule (1, 18-21). Therefore, these results offer a measure of the difference in the affinities of the liganded and unliganded forms of carp hemoglobin as a function of pH. The data for stripped and unstripped hemoglobin are shown in Fig. 6. The upper panel shows the second order rate plot for the recombination of carbon monoxide upon full and partial flash photolysis in the presence of phosphates at 20°. The corresponding data obtained with stripped hemoglobin are shown in the lower panel. On full flash photolysis, all of the carbon monoxide is dissociated, whereas on partial flash, only 20% of the ligand is removed. When conditions are such that the hemoglobin remains in the low affinity conformation even when liganded, partially liganded hemoglobin should react with carbon monoxide at the same rate as the unliganded form. This is what happens at pH 5.5 in the presence of organic phosphates. At pH 6.4 and 7.0, the rates of recombination with carbon monoxide upon partial flash photolysis are faster than upon full flash photolysis, the ratios of the rates being 2.4 and 3.5 at pH 6.4 and 7.0, respectively. This is indicative of the occurrence of at least two different conformations, the low affinity form...
that at pH 5.5.

The pattern of behavior of stripped carp hemoglobin appears to be shifted toward lower pH relative to that of the unstripped material. At pH 5.2, the rate of CO recombination on partial flash photolysis is somewhat greater than the rate of combination with unliganded protein. At pH 6.1, the ratio of the rates of carbon monoxide recombination on partial and full flash photolysis is 2.8. The situation in which the rates of carbon monoxide recombination on partial or full flash photolysis are equally rapid is reached at a lower pH than when phosphates are present. The carbon monoxide combination rates for both stripped and unstripped carp hemoglobin are the same at the high pH extreme.

Kinetics of PTS Release during CO Combination—We have studied the release of the fluorescent organic phosphate analogue, PTS, from stripped carp hemoglobin during carbon monoxide binding and compared the time courses of the two processes. MacQuarrie and Gibson have reported the stoichiometry of binding of PTS to human deoxy-Hb and CO-Hb to be 1.1 ± 0.1 moles per tetramer (8, 9). We have found approximately the same stoichiometry with carp hemoglobin. At the PTS concentrations used, the oxygen affinity of carp hemoglobin at pH 6.5 is the same as that of the stripped material and at pH 5.6 it is only 16% lower. Therefore, in these experiments, we are essentially observing the behavior of stripped carp hemoglobin. Fig. 7 shows the time course of carbon monoxide combination superimposed on the time course of PTS release at pH 5.2, 6.0, and 7.0. At pH 5.2, PTS release lags behind carbon monoxide binding. This lag is present but less pronounced at pH 6.0. At pH 7.0, PTS release occurs slightly faster than carbon monoxide combination. In addition, the fluorescence increase accompanying ligand binding is less at pH 5.2 than at pH 6.0 or 7.0, indicating that more PTS remains bound to the liganded hemoglobin at low pH. In Fig. 8, the percentage of liganded hemoglobin is plotted against the percentage of PTS release at the corresponding time. Here, the nonlinear relationship between ligand binding and PTS release is clearly seen. At pH 5.2, for instance, only 10% of the bound PTS is released when the hemoglobin is half-saturated with carbon monoxide. On the other hand, at pH 7.0, 60% of the bound PTS is released when the hemoglobin is only 50% saturated with carbon monoxide.

α and β Chain Differences within the Tetramer—Studies of the wave length dependence of the CO-binding reaction show that there are two spectrophotometrically distinct components with differing kinetic properties which can be visualized near the Soret isosbestic point at 424 nm, and which may plausibly be identified with the α and β chains. One example of the results obtained is given in Fig. 9 (experimental points). This shows the course of CO binding to unstripped hemoglobin at pH 7.0 at three wave lengths, 432 nm, where the over-all reaction is observed, and at 424 and 426 nm, near the isosbestic point.

Observations made at a number of wave lengths in addition to those shown in Fig. 9 revealed that there was no true isosbestic point, but instead a wave length at which the two components gave equal and opposite absorbance excursions so that the absorbance before and after reaction with CO was the same. Slightly to the shorter wave length side of this point, the more rapid component predominated (Fig. 9B) and slightly to the longer wave length side, the slower component predominated (Fig. 9C). At wave lengths where substantial absorbance changes accompany CO binding, the time course was intermediate (Fig. 9A). These findings are consistent with the presence of two components with difference spectra shifted by 1 to 2 nm with respect to one another, as might be expected for two types of chain, by analogy with the results for human chains which have been studied in detail (10, 22). Similar results were obtained under other pH conditions and in the absence of organic phosphate. The results have been summarized according to a scheme given in Fig. 10 and are presented in Tables I and II. The assignment of constants to the α and β chains is based on the assumption that as in human hemoglobin, the α chains of carp hemoglobin react more slowly than the β chains, and is not based on any direct chemical identification. The scheme in Fig. 10 is the kinetic equivalent of the Adair scheme (23) for

![Graph](http://www.jbc.org/)
Fig. 9. Time course of binding of CO (92 μM) to carp hemoglobin (18 μM) followed at 432, 424, and 426 nm. The reaction was followed at 20° in a 4-mm cuvette in 0.1 M phosphate buffer, pH 7.0. The lines were calculated using the scheme specified under “Discussion” with the rate constants and extinction coefficients of Tables I and II. The points are experimental. The dashed line represents the final absorbance change corrected for the 1.5-ms dead time of the stopped flow apparatus.

Table I
Rate constants in the combination of deoxygenated carp hemoglobin with carbon monoxide

| Condition | $k_a$ (μM⁻¹ s⁻¹) | $k_b$ (μM⁻¹ s⁻¹) | Enhancement factor | Mean residual |
|-----------|-----------------|-----------------|-------------------|--------------|
| Stripped Hb | 0.051 ± 0.002 | 0.286 ± 0.04 | 1.18 ± 0.04 | ±0.0013 |
| pH 5.6 | | | | |
| Unstripped Hb | 0.072 ± 0.003 | 0.347 ± 0.03 | 3.0 | ±0.0022 |
| pH 5.4 | | | | |
| pH 7.0 | 0.081 ± 0.003 | 0.266 ± 0.03 | 2.23 ± 0.09 | ±0.0017 |
| pH 8.14 | 0.111 ± 0.03 | 0.561 ± 0.05 | 4.21 ± 0.16 | ±0.0025 |

The reaction of tetrameric hemoglobin with a ligand, expanded to include α-β chain difference (24) and contains 10 species and 16 rate constants. In the most general case, such a scheme cannot be defined by a set of results such as those of Fig. 9, and the number of freely disposable constants must be reduced by making simplifying assumptions. The most radical of these is to assume as did Gray and Gibson (10) that the rate constants 1 to 14 in Fig. 10 are defined by two rate constants specific to α and β chains and by the appropriate statistical factors. In such a scheme, it is not necessary to take the species $αβx$ and $αβx$ separately into account, slightly reducing the arithmetical work involved. Constants 15 and 16 are assumed equal, and are taken from the results of the flash photolysis experiments. On applying this simplified scheme to the results of Fig. 9 and other analogous data, no satisfactory results were obtained. The $α-β$ difference required to reproduce Panels B and C of Fig. 9 gave a time course which failed to reproduce the acceleration observed during the early stages of the reaction seen in Panel A at 432 nm, where both chain contributions must be sensibly equal. The scheme was then modified to take account of the experience of Gibson and Roughton (25) and of MacQuarrie and Gibson (9), that the intrinsic rate constant for binding the second molecule of carbon monoxide is greater than that for binding of the first molecule, by multiplying Constants 3 through 8 by an arbitrary enhancement factor. Excellent fits, characterized by small and well distributed residuals, were then obtained as illustrated in the lines drawn through the experimental points in Fig. 9.

In these operations, the results obtained at three or four wave lengths were fitted simultaneously with a single set of rate constants. The relative contributions of the chains at different wave lengths at different pH and organic phosphate con-
concentration agree quite satisfactorily (Table I) when the difficulty of resetting the monochromator is taken into account.

**DISCUSSION**

The simplest model to explain the properties of hemoglobin is one which assumes the existence of only two structural forms of the molecule—the low affinity form normally associated with deoxygenated hemoglobin and the high affinity form associated with liganded hemoglobin. In this model, originally suggested by Forbes and Roughton (26) and formalized by Monod et al. (27), heme-heme interaction arises from the transition from the low to the high affinity form in the course of ligand binding. If the properties of the two states were invariant, an alteration of ligand affinity by an outside agent such as hydrogen ions or polyphosphates could be achieved by preferential binding to one of the structures, thereby changing the free energy of the transition from the low to the high affinity form. In order for the over-all ligand affinity to approach that of either the high or the low affinity forms, the structural transition must be inhibited and the molecule must remain for the most part in a single form both in the presence and absence of ligand. Elimination of the structural transition will eliminate heme-heme interaction and, therefore, as pointed out by Edelstein (28), one would predict a relationship between ligand affinity and heme-heme interaction similar to that observed in carp hemoglobin. Although this model is clearly an oversimplification, a surprisingly large amount of the data obtained for carp hemoglobin can be understood in these terms.

At pH 6.1 at 20°, the value of the Hill parameter, n, in the oxygen equilibrium curve of stripped carp hemoglobin is higher than at any other pH. At this point, oxygen binding is highly cooperative; that is, the hemoglobin undergoes a change in conformation during the ligand-binding process. As the pH is lowered, the ligand affinity and the value of n decreases. Apparently an increase in the hydrogen ion concentration shifts the equilibrium toward the low affinity form. In this pH range, the presence of organic phosphates achieves a similar effect. Below pH 6.1 at 20°, the oxygen affinity and the values of the Hill parameter, n, for unstripped carp hemoglobin are lower than the values for the stripped material. Below pH 5.6, in the presence of organic phosphates, ligand binding is noncooperative, and the equilibrium and kinetic parameters were found to be pH-invariant. Under these conditions, the ligand affinity is very low (at 20° $p_{O_2} = 123$ mm Hg and $p_{CO}(CO) = 1$ mm Hg), and the molecule appears to be frozen in its low affinity conformation whether liganded or not.

Increasing the pH from pH 6.1 in the absence of organic phosphates increases the oxygen affinity and decreases the extent of heme-heme interaction for stripped carp hemoglobin. In this pH range, the high affinity form of the hemoglobin is favored. Above pH 7.5, the oxygen affinity of carp hemoglobin reaches what seems to be the maximum attainable affinity at a given temperature. The value of n in the Hill equation is pH-invariant, and at 20° is near 1.0. None of the kinetic parameters was found to be pH-dependent. In this pH range, therefore, carp hemoglobin seems to be approaching the situation in which the hemoglobin molecule is frozen in its high affinity conformation even in the absence of ligand. When organic phosphates are present, this situation is also reached but only at higher pH. Again, phosphates and hydrogen ions affect the hemoglobin molecule in the same way and their absence favors the high affinity form.

Although the absolute values are not the same, this spectrum of behavior in all respects is observed when another ligand, carbon monoxide, is used, or when the experiments were conducted at a different temperature, $10^°$.

Although we have not proven that the properties of the high and low affinity states are invariant, in the pH ranges in which they have been examined they appeared to be unaffected by either pH or polyphosphates. In fact, if the hemoglobin were to remain stable below pH 5, we postulate that we would find that the oxygen affinity in the absence of phosphates would eventually reach the same low affinity limit as when phosphates are present. If such were the case, it would mean that organic phosphates cause a shift in the pH scale but do not change the magnitude of the pH effect. This would be contrary to the conclusion we would make if we were to consider only the slope of the plot of log $p_{O_2}$ (liganded) versus pH at a particular pH value, as did Gillen and Riggs (6).

An n value of 1.0 cannot prove the lack of a conformational transition. Even with cooperative ligand binding, heterogeneity, perhaps resulting from $α−β$ chain differences, can lower the apparent value of n to unity. Flash photolysis experiments provide an independent parameter to study the occurrence of conformational differences between the unliganded and ligand-saturated molecule. Full flash photolysis dissociates all the ligand; therefore, the rate of carbon monoxide recombination after full flash photolysis, $P_6$, would reflect the behavior of hemoglobin in all of the states of liganding. On partial flash photolysis, only a small percentage of the carbon monoxide is dissociated so that the rate of carbon monoxide recombination would involve primarily only the final step in liganding, $P_4$ (7). When hemoglobin undergoes a low to a high affinity conformational change on ligand binding, $P_4$ should be higher than $P_6$. When hemoglobin remains in a single conformation, $P_4$ should be equal to $P_6$. Although the flash photolysis experiments involve the carbon monoxide derivative of hemoglobin, the pH and phosphate dependence of the equilibrium parameters on binding oxygen and carbon monoxide are similar enough (2).
for us to correlate the results with the oxygen equilibrium data at 20°. In every situation, the results of the flash photolysis experiments agreed with our interpretation of the equilibrium measurements. When the state of the molecule is changed upon ligand binding as indicated by a high n value, the rate of carbon monoxide recombination on partial flash photolysis was found to be faster than on full flash photolysis. There was no difference in the rates of carbon monoxide recombination after full and partial flash photolysis under conditions where equilibrium studies indicated that the hemoglobin remains in a single conformation. Both rates were slow when the hemoglobin was expected to remain in a low affinity conformation and rapid when the hemoglobin was expected to remain in a high affinity conformation.

At equilibrium, organic phosphates bind preferentially to the low affinity form of hemoglobin (29, 30). Therefore, when ligand binding is accompanied by a change from the low to the high affinity conformation, organic phosphates are released. MacQuarrie and Gibson have shown that PTS competes with 2,3-diphosphoglyceral for the organic phosphate binding site in human Hb A (8, 9). PTS fluoresces as the free compound but its fluorescence is quenched when bound to hemoglobin. Therefore, its release from hemoglobin upon ligand binding can be monitored. These kinetic studies provide a close look at the time course of the conformation changes which occur during the course of ligand binding. At pH 5.2 and 6.0, PTS release lags behind CO binding, indicating that partially liganded intermediates which bind PTS are formed during the reaction with carbon monoxide. We know that the PTS-bound hemoglobin used in these studies behaves like stripped carp hemoglobin. At pH 6.0, stripped carp hemoglobin undergoes a conformation change upon ligand binding as indicated by a high n value. It is interesting to note that the behavior of carp hemoglobin at this pH, where it exhibits cooperative oxygen binding, is almost identical to that of human Hb A at pH 6 (8). At pH 5.2, CO binding to stripped carp hemoglobin is cooperative, n being 1.4, but the affinity is much lower than at pH 6.0. Therefore, a switch to the high affinity conformation upon ligand binding is expected, but, as observed, it occurs later in the liganding process than at pH 6.0 and is less complete. At pH 7.0, the value of n for CO binding is unknown, but O2 binding is still slightly cooperative. Since the affinity is high, the structural transition from the low to the high affinity form should occur earlier in the CO-binding process than at pH 6.0. This is consistent with the observed lag in CO binding relative to PTS release.

The results discussed so far fit into our simple two-state scheme. However, not all observations made on carp hemoglobin do so. For example, the value of n in the O2 equilibrium experiments at low pH was found to be below 1.0, not only in the homoleptic but in the isolated carp hemoglobin components as well (1, 2). The second order kinetic plots for the CO combination reaction at pH 5.6 showed a decelerating reaction when a homogeneous reaction would be expected (1). Now we have found lower values of n in the O2 equilibrium measurements at 20° compared to that at 10° only at high pH, whereas the temperature dependence of the pK2(ligand) is not pH-dependent. The key to these unexplained observations may be in the α-β chain differences which we have shown in the carbon monoxide combination experiments. The rather significant chain difference of some 5-fold in CO-combining rates, if carried over into equilibrium constants, is sufficient to account for the values of n below 1.0 in Hill’s equation. Furthermore, changes in temperature may affect the magnitude of the difference in the ligand affinities of the α and β chains.

The computer simulation of the carbon monoxide combination reactions show that the carp hemoglobin system, although apparently simple when only the two end states, i.e., unliganded and liganded forms, are considered as in equilibrium experiments, is not so simple when the reaction path from the unliganded to the liganded form is studied closely. The scheme on the basis of which the kinetic curves were fitted excludes the representation of the carp hemoglobin ligand reaction by the Monod-Wyman-Changexus model (27) either in its original form which postulates two species, the R and T forms, each containing four identical chains, or in its extension to admit α—β chain differences (24). Whereas both models prescribe a monotonic change in the intrinsic rate and equilibrium constants for ligand binding as saturation proceeds, the intrinsic rate constant for binding the second molecule of CO to carp hemoglobin had to be made greater than the first rate constant by an arbitrary enhancement factor in order to arrive at a good curve fitting. The results with carp hemoglobin, like those with mammalian hemoglobin, require that intra-dimer interactions be taken into account, and therefore exclude kinetic models admitting only two conformations.

Carp hemoglobin is not unique in the sense that we have so far been able to explain its behavior on the basis of generally accepted theories of hemoglobin function. Heme-heme interaction and pH effects on ligand affinity have been seen in many hemoglobins. Furthermore, a relationship between ligand affinity and cooperativity in mammalian hemoglobins has been suggested by Edelman (25) and shown for human Hb A by Bunn and Guidotti (31). Even the situation of being constrained in a high affinity conformation is not unknown. For instance, the hemoglobin-lacto-globin complex (12), carboxypeptidase A-digested human Hb A (32), and the bis(N-maleimidomethyl)ether derivative of horse hemoglobin (33) have high affinity and exhibit no heme-heme interaction. However, carp hemoglobin is the only case known in which a mere change of pH or organic phosphate concentration can reversibly transform the molecule from one that shows highly cooperative ligand binding to one that is constrained in a single conformation of either high or low ligand affinity.

REFERENCES

1. Noble, R. W., Parkhurst, L. J., and Gibson, Q. H. (1970) J. Biol. Chem. 245, 6628-6633
2. Tan, A. L., De Young, A., and Noble, R. W. (1972) J. Biol. Chem. 247, 2403-2408
3. Chanutin, A., and Curnish, R. R. (1967) Arch. Biochem. Biophys. 121, 96-102
4. Bensche, R., Bensche, R. E., and Yu, C. I. (1968) Proc. Nat. Acad. Sci. U. S. A. 59, 596-597
5. Tan, A. L., De Young, A., and Noble, R. W. (1972) Fed. Proc. 31, 3069
6. Gillen, R. G., and Higgs, A. (1972) J. Biol. Chem. 247, 6939-6946
7. Gibson, Q. H. (1956) J. Physiol. 134, 123-134
8. MacQuarrie, R., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 5332-5335
9. MacQuarrie, R., and Gibson, Q. H. (1972) J. Biol. Chem. 247, 5080-5084
10. Gray, R. D., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 5176-5178
11. Ames, B. N., and Dubin, D. T. (1960) J. Biol. Chem. 235, 769-777
12. Nagel, R. L., Wittenberg, J. B., and Ranney, H. M. (1950) Biochim. Biophys. Acta 100, 286-289
13. Allen, D. W., Guthe, K. F., and Wyman, J., Jr. (1950) J. Biol. Chem. 187, 393-410
14. De Sa, R. J., and Gibson, Q. H. (1969) Comput. Biomed. Res. 2, 494
15. Gibson, Q. H. (1970) Meth. Enzymol. 16, 187-228
16. Rapoport, S., and Gudes, G. M. (1941) J. Biol. Chem. 136, 269-282
17. Gillen, R. G., and Riggs, A. (1971) Comp. Biochem. Physiol. 38B, 585-595
18. Gibson, Q. H. (1959) Biochem. J. 71, 293-303
19. Antonini, E., Brunoii, M., and Anderson, S. (1968) J. Biol. Chem. 243, 1816-1822
20. Antonini, E., Bucci, E., Francicelli, C., Wyman, J., and Rossi-Fanelli, A. (1965) J. Mol. Biol. 12, 375-384
21. Nagel, R. L., and Gibson, Q. H. (1966) J. Mol. Biol. 22, 249-255
22. Olson, J. S., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 5241-5253
23. Adair, G. S. (1925) J. Biol. Chem. 65, 529-545
24. Ogata, R. T., and McConnell, H. M. (1972) Proc. Nat. Acad. Sci. U. S. A. 69, 333-339
25. Gibson, Q. H., and Roughton, F. J. W. (1957) Proc. Roy. Soc. Ser. B. 146, 206-224
26. Forbes, W. H., and Roughton, F. J. W. (1931) J. Physiol. 61, 230-237
27. Monod, J., Wyman, J., and Changeux, J.-P. (1965) J. Mol. Biol. 12, 88-118
28. Edelstein, S. J. (1971) Nature 230, 224-227
29. Chanutin, A., and Hermann, E. (1969) Arch. Biochem. Biophys. 131, 180-184
30. Garry, L., Gerber, G., and de Verdier, C. H. (1969) Eur. J. Biochem. 10, 110-115
31. Bunn, F. H., and Guidotti, G. (1972) J. Biol. Chem. 247, 2345-2350
32. Antonini, E., Wyman, J., Zito, R., Rossi-Fanelli, A., and Caputo, A. (1961) J. Biol. Chem. 236, PC60-PC63
33. Simon, S. R., Arndt, D. J., and Konigsberg, W. H. (1971) J. Mol. Biol. 58, 69-77
Conditions Restricting Allosteric Transitions in Carp Hemoglobin
Anna L. Tan, Robert W. Noble and Quentin H. Gibson

J. Biol. Chem. 1973, 248:2880-2888.

Access the most updated version of this article at http://www.jbc.org/content/248/8/2880

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/248/8/2880.full.html#ref-list-1