KINETICS OF THE BOHR EFFECT OF MENHADEN HEMOGLOBIN, BREVOORTIA TYRANNUS

Wilma A. Saffran and Quentin H. Gibson

Section of Biochemistry, Molecular and Cell Biology
Cornell University, Ithaca, New York 14853

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SUMMARY

The kinetics of proton release on ligation of menhaden hemoglobin was studied by flash photolysis over a range of pH. In contrast to all previous kinetic work with human hemoglobin, a nonlinear relationship between proton release and CO binding was found. Proton uptake was also observed in the course of O_2 replacement by CO at low pH. It follows that at least part of the proton release is associated with quaternary rather than tertiary conformational changes i.e. this result is consistent with a two-state model in which L is a function of pH.

The increasing oxygen affinity of hemoglobin with pH, the Bohr effect, is associated with a higher affinity of protons for the deoxy than the oxy conformation. In terms of the two-state model (1), at high proton concentration, or low pH, the conformational equilibrium is shifted toward the low affinity (T) state, and at high pH, toward the high affinity (R) state. If the affinity for protons is dependent on the quaternary conformation of hemoglobin rather than on its state of ligation, two predictions can be made (2). The shape of the oxygen equilibrium curve will vary with pH, and the number of protons released from hemoglobin at each step of ligation will be different. The shape does indeed vary with pH (3,4) but the results of proton release vs. saturation measurements have been contradictory. In kinetic studies with human hemoglobin, proton release was found to be proportional to percent saturation (5-7), while in equilibrium studies linearity of proton release with ligation has been reported, except for some observations at high pH (8,9). These results have presented a logical difficulty in accepting the two-state model for hemoglobin. The problem is difficult to investigate with human hemoglobin, which remains in the T state when deoxygenated and in the R state when liganded with CO or O_2 over its entire pH range of stability. Carp hemoglobin, on the other hand, re-

mains in the deoxy conformation at low pH, even when fully liganded, and in the liganded conformation at high pH, even when deoxygenated (10-12). In terms of the two-state model, protons are effectors strong enough to maintain carp hemoglobin in the T state at low pH, while at high pH it remains in the R state, with a region of conformational transition and cooperative ligand binding in between. The predictions of the two-state model can be tested in this system simply by changing the pH. Menhaden hemoglobin has properties similar to carp[1], but the pH range over which the Bohr effect occurs lies in the pH indicator region of phenol red, the most favorable indicator dye we have found. Since the tetramer-dimer dissociation constant of liganded menhaden hemoglobin is low, about 10^{-9} M,[2] we could use flash photolysis to study CO binding and the associated proton release without having to take into account any possible dimer Bohr effect.

Materials and Methods

Phenol red, water soluble, indicator grade (Aldrich) and inositol hexaphosphate (Sigma) were used without further purification.

Menhaden hemoglobin

Menhaden red blood cells were washed in 1% NaCl and stored in liquid nitrogen. The hemoglobin solution was prepared by mixing a portion of thawed cells with 10-20 volumes of distilled water and centrifuging down the nuclear material and red cell ghosts. The hemoglobin solution was then passed through a column of Sephadex G-25 equilibrated with 0.2 M NaCl to exchange out buffer components.

Flash Photolysis Experiments

The flash photolysis experiments were carried out using a tonometer consisting of a 500-ml round-bottom flask to the bottom of which a 1 cm square glass cuvette was fused. There were two openings at the sides, one fitted with a serum cap for addition of reagents during the experiment and one with a stopcock for evacuation and flushing with gas. The top was sealed with a rubber stopper, through which a Radiometer GK 2302 C electrode was inserted.

Menhaden HbCO[3] and phenol red were placed in the tonometer and enough 0.2 M NaCl added to bring the concentration of hemoglobin and dye to 40-50 μM. Then the contents of the tonometer were deoxygenated by alternate evacuation and flushing with N_2 gas, or O_2 in the case of the O_2 replacement experiments. CO gas was injected from a syringe into the tonometer to bring the partial pressure to 0.05 atmosphere (0.10 atmosphere in O_2 replacement experiments). A cuvette containing a similar concentration of HbCO, but no dye, to which

[1] F.G. Carey, unpublished experiments

[2] Q.H. Gibson and S.J. Edelstein, unpublished experiments

[3] Abbreviations: IHP, inositol hexaphosphate; Hb, hemoglobin.

sodium dithionite had been added to remove O_2, was used to find the Hb-HbCO isosbestic point. The solution in the tonometer was flashed and proton release, as measured by the dye absorbance excursion, followed at this isosbestic wavelength. Then the solution was flashed again and CO uptake followed at the phenol red isosbestic, 482 nm. The pH was changed by addition of 0.01 N HCl or NaOH, the new pH measured, and the procedure repeated.

Calculations

Proton release is not proportional to absorbance excursion, especially at the ends of the phenol red indicator range. The concentrations of unionized and ionized dye, [HD] and [D] respectively, were calculated from the Henderson-Hasselbach equation, using the measured pH before flashing and a pK of phenol red of 7.90. ΔD, the decrease in [D], was calculated from the change in dye absorbance, using $\varepsilon_{546} = 46\ mM^{-1}cm^{-1}$ for the ionized form. Then the pH after flashing was calculated as:

$$\Delta pH = pK + \log \frac{[D] + \Delta D}{[HD] - \Delta D}$$

The ΔpH was converted to ΔH^+ by using the titration curve of hemoglobin plus dye. Since the pH changed over the course of the reaction, ΔH^+ was calculated in this way for each point in the time course.

Results and Discussion

Figure 1 shows the time courses of proton release and CO binding at two different pH values. At pH 6.22 proton release lags behind, while at 7.91 it leads CO uptake. In Figure 2 percent proton release is plotted against percent saturation. Over the pH range studied, 6.22 to 7.91, there is a transition, from lagging to leading proton release as the pH is increased. This non-linear relationship between proton release and CO uptake implies that at least part of the proton release is linked to quaternary rather than tertiary conformation changes in the hemoglobin.

The number of protons per heme released on ligation is plotted as a function of pH in Figure 3. The pH in each case is that of the fully saturated hemoglobin solution, measured before flash photolysis. Proton release first appears at pH 8, increases to a maximum at 6.5, then decreases as the pH is lowered further. Below pH 6, there is still a considerable Bohr effect, but it is so far from the pK of phenol red that the absorbance changes become too small to be measured accurately.

In terms of the two-state model, at low pH the conformational transition from T to R occurs very late in ligand binding and quaternary linked effectors

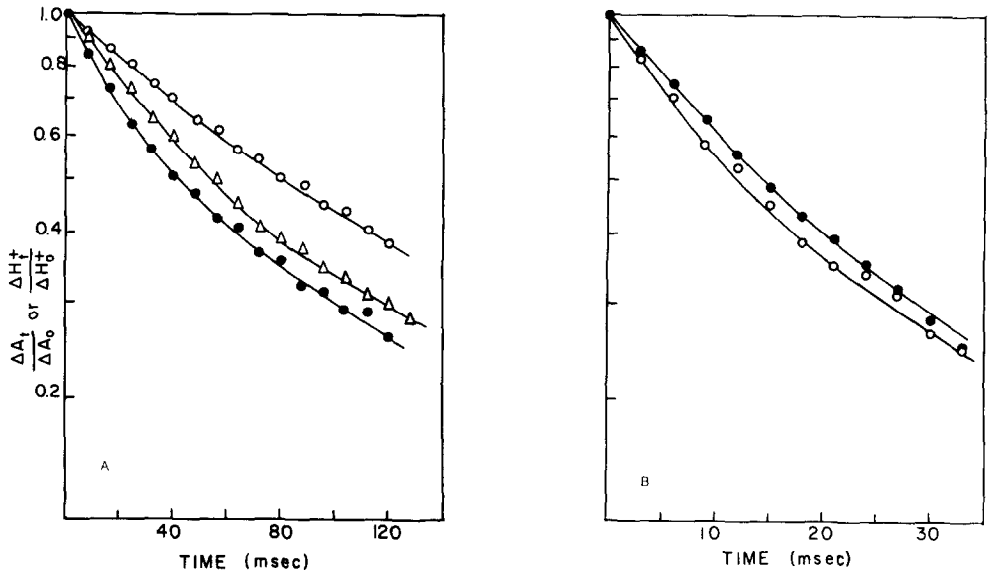

Fig. 1. Time courses of ● CO binding, followed at 482 nm, and proton release Δ as measured by absorbance excursion at 546 nm, O then calculated as described in Materials and Methods. Note the difference between the raw and corrected data. 52 μM heme 38 μM phenol red. A. pH 6.22 B. pH 7.91.

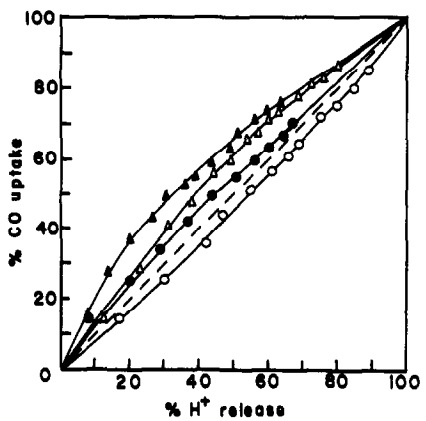

Fig. 2. Data of Fig. 1. plotted as % proton release vs. % of CO uptake; O pH 7.91, ● 7.71, Δ6.72, ▲6.22. The dashed line is the diagonal.

such as protons come off the hemoglobin only after most of it is liganded, giving rise to the observed lag at low pH. At high pH, where the conformational equilibrium constant L = [T]/[R] is small, the T to R transition occurs early in ligand binding, and proton release leads CO binding.

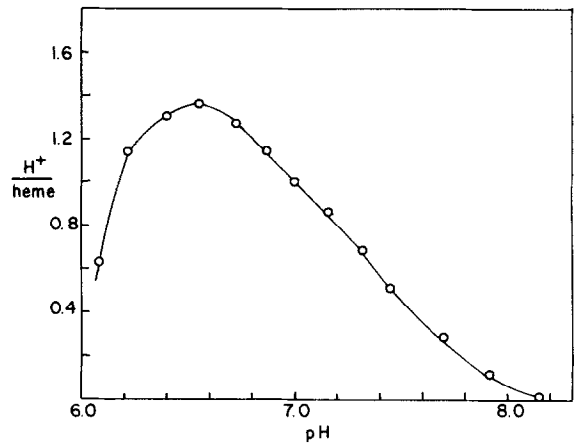

Fig. 3. Protons released per heme as a function of pH, calculated from the dye absorbance excursion after flashing. The plotted pH is the pH measured before flashing.

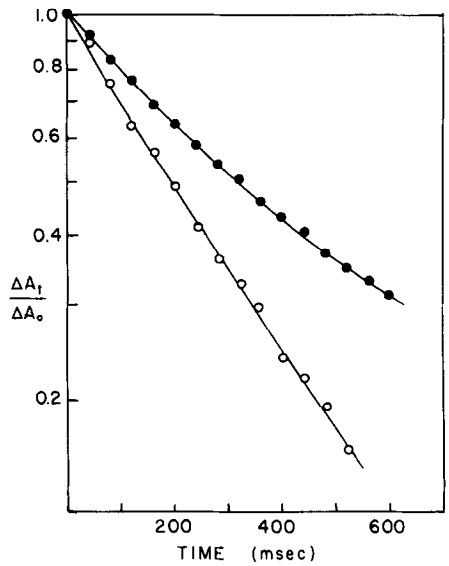

Fig. 4. Time courses of ● O_2 replacement by CO, followed at 450 nm, and O proton release from hemoglobin and IHP, followed at 547 nm. 52 μM heme, 38 μM phenol red, 100 μM IHP.

Salhany et al (13) have shown that the values of c (the ratio of the R and T ligand dissociation constants, K_R/K_T) for NO and CO are different. Assuming that c for O_2 is also different, the model predicts that at some pH HbO_2

may be predominantly in one conformation and HbCO in the other. We looked at O_2 replacement by CO and found a small but significant amount of proton uptake, but at a pH so low that the protein denatured rather readily. IHP shifts the T-R equilibrium curve to higher pH. In the presence of IHP proton release is seen as CO replaces O_2 at pH 6.52, where the hemoglobin is stable (Figure 4). The quaternary change leads the O_2 replacement, indicating that conformation change occurs early in the reaction.

The existence of proton release as hemoglobin goes from one fully liganded form to another, and the patterns of proton release observed during ligation, all follow the predictions of the two-state model and support the validity of its application to hemoglobin.

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