Concentration-dependent Effects of Anions on the Anaerobic Oxidation of Hemoglobin and Myoglobin*

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The redox potentials of hemoglobin and myoglobin and the shapes of their anaerobic oxidation curves are sensitive indicators of globin alterations surrounding the active site. This report documents concentration-dependent effects of anions on the ease of anaerobic oxidation of representative hemoglobins and myoglobins. Hemoglobin (Hb) oxidation curves reflect the cooperative transition from the T state of deoxyHb to the more readily oxidized R-like conformation of metHb. Shifts in the oxidation curves for Hb which as Cl\(^{-}\) concentrations are increased to 0.2 M at pH 7.1 indicate preferential anion binding to the T state and destabilization of the R-like state of metHb, leading to reduced cooperativity in the oxidation process. A dramatic reversal of trend occurs above 0.2 M as anions bind to lower affinity sites and shift the conformational equilibrium toward the R state. This pattern has been observed for various hemoglobins with a variety of small anions. Steric rather than electronic effects are invoked to explain the fact that no comparable reversal of oxygen affinity is observed under identical conditions. Evidence is presented to show that increases in hydrophilicity in the distal heme pocket can decrease oxygen affinity via steric hindrance effects while increasing the ease of anaerobic oxidation.

The globin structure controls the redox potential of the heme site of hemoglobins (Hbs) and myoglobins (Mbs), protects them from rapid oxidation, and thereby allows for reversible oxygen binding. Studying the redox properties of such systems allows us to gain insight on how anion-induced alterations fine tune the equilibrium between the oxidized and the reduced forms of Hbs. Our results extend earlier studies that have brought to light many possible modes of altering the electronic and ligand binding properties of the active site iron atoms (1–21).

Although a large body of literature describes mechanisms involved in controlling the oxygen affinities of Hbs and Mbs, there are still unanswered questions with regard to how globin structure controls the redox potential (\(E^{\text{m}}\)) of the active site, a parameter that describes the propensity of the site to donate or accept electrons. Evaluating and predicting trends in the redox potentials of Hbs and Mbs, as the composition of both the globin and the medium are changing, is a complex and challenging problem, but one that is fundamental to understanding the way nature is able to accomplish oxygen transport, oxygen storage, and electron transfer reactions in highly polar environments.

Oxidation and oxidation-reduction studies of Fe(II)/Fe(III) centers in diverse Hbs have uncovered parallels between these two processes (22). Notably, the shift from the T (deoxy Fe(II)) to the R (oxygenated) or R-like (met Fe(III)) conformation of the Hb tetramer underlies the cooperativity observed in both oxygenation and oxidation processes. Structural changes that stabilize either the T or the R conformation are typically reflected by parallel alterations of both oxidation and reduction processes (1, 3, 22). In many Hbs, heterotropic effectors such as protons, anions, and carbon dioxide, although bound at spatially remote sites, influence the oxygenation process and have been shown to affect the oxidation process as well (22–26). Some anionic effectors, known to bind at the \(\beta\)-\(\beta\) interface in the central cavity of Hb, are capable of influencing the oxygen affinity and the redox potential of the heme groups that are located several Å away.

Anion effects on Hb function have traditionally been explained in terms of preferential binding to the low affinity (T state) quaternary conformation (27, 28). Chiancone and co-workers (23, 29, 30) showed, using NMR techniques, that Cl\(^{-}\) binds with different affinities to deoxy and oxyHb and that the binding is proton-dependent. Their results indicated the presence of two major classes of binding sites, differing in Cl\(^{-}\) affinity. The high affinity sites of deoxyHb showed a 10-fold higher Cl\(^{-}\) affinity (about 100 mM\(^{-1}\)) than in oxyHb (about 10 mM\(^{-1}\)). These sites are distinctly different from the low affinity sites (about 0.1 mM\(^{-1}\)) that have approximately the same Cl\(^{-}\) affinity for both oxy and deoxyHb.

Precise pH-stat measurements of Cl\(^{-}\) effects on liganded and deoxyHb confirmed the fact that both forms of Hb bind Cl\(^{-}\) but with different affinities (31). These affinity differences have a direct bearing on the magnitude and often disputed origin of the Bohr effect (31–35). The N-terminal valine residues and positively charged residues of the \(\beta\) chain 2,3-diphosphoglycerate binding site have been implicated in preferential anion binding by deoxyHb, although recent x-ray and chemical studies of Perutz et al. (36) found no clear indications of specific Cl\(^{-}\) binding sites in either bovine or human hemoglobin. We recently showed that Cl\(^{-}\) effects on oxygen binding derive in part from steric hindrance effects that are not present in oxidation processes (22). The situation with regard to Cl\(^{-}\) effects on Hb function is thus more complex than can be explained by anion binding at a few specific sites. Lack of awareness of the rather dramatic changes in ease of anaerobic oxidation of the heme...
that occur as anion levels are altered between 0 and 0.4 M, as
shown previously (25). The absorbance of the fully oxidized (Ao) and fully reduced (Ar)
state of Mb/Hb at ca. 554 nm (3). The cell was kept anaerobic by capping the cuvette with a septum that allowed no air to enter but permitted a continuous flow of N2. A platinum wire connected to the platinum gauze working electrode was inserted through the septum. A Pasteur pipette salt bridge plugged at the bottom with an agar gel was prepared so as to connect the Ag/AgCl reference (Bioanalytical Systems Inc.) electrode to the working electrode. The salt bridge solution was composed of 0.2 M KCl in 0.05 M MOPS at pH 7.1 or 0.2 M KCl in 0.05 M HEPES at pH 7.5 and was degassed and then flushed with N2 for 1 h. The optically transparent thin layer electrode cell was purged with N2 for 15 min prior to injecting the protein solution.

Spectroelectrochemical experiments were carried out in an anaerobic spectroelectrochemical cell via a gas tight syringe. The cell was then placed in the temperature-controlled cell holder of a CARY 2300 UV-visible spectrophotometer held at 20 °C and linked to a PAR model 75 potentiotstat. Spectroelectrochemistry was carried out from 340 to 700 nm, with specific emphasis on the Soret region. Absorbance changes were monitored at 410 nm (metMb) or 406 nm (metHb) and 435 nm (deoxyMb) or 430 nm (deoxyHb). The absorbances of the fully oxidized (Ao) and fully reduced (Ar) Mb and Hb were obtained by applying a potential of +400 mV and −250 mV (versus NHE), respectively, and the absorbance was recorded when the system reached equilibrium (no more change in absorbance at the fixed potential and wavelength). For each experiment, the path length was determined using the Soret band absorbance. The concentration was determined independently by spectral analysis after addition of the oxygen scavenger sodium dithionite (2 mg/ml) to the unused sample solution.

A typical increment of 20 mV was applied to the system starting at approximately +300 mV down to −120 mV (versus NHE). At each applied potential, the absorbance was monitored until no change was detected. Although most experiments were performed going from fully oxidized to fully reduced Mb, the system was shown to be reversible under our experimental conditions (i.e., the Nernst plot can be generated in either the oxidation or reduction direction and equilibrium is achieved within 30–40 min at each applied potential). Nernst plots were then derived from the observed changes in absorbance as was described previously (25).

RESULTS

Fig. 1a sets the stage for comparative studies by illustrating a set of oxidation curves, presented as Nernst plots, for three distinct Mb systems. These plots illustrate redox differences associated with a first coordination shell effect. Monomeric Aplysia Mb exhibits an E1⁄2 value 75 mV more positive than that of sperm whale Mb and horse Mb. The Nernst plots have slopes of unity, as we previously reported for similar noncooperative one-electron transfer systems (3, 39). A valine residue at position E7 in Aplysia Mb replaces the more common distal histidine present in both sperm whale and horse Mbs (40). The crystal structures show the presence of a water molecule in the distal heme pocket of both horse and sperm whale Mbs, stabilized by the distal histidine residue (3, 40–43). The presence of this water molecule as a sixth ligand in the oxidized form of the protein is consistent with the negative shift in E1⁄2 observed for these two Mbs relative to Aplysia Mb, which does not possess this water ligand.

Fig. 1b and results shown in Table I illustrate how globin differences in Hb can alter the shape and position of the Nernst plots. Nernst plots for horse Hb, bottlenose dolphin Hb, and human Hb (Hp A0) have mid-point (E1⁄2) slopes greater than 1,
Fig. 1. Nernst Plots of (a) sperm whale (swMb, O), horse (hMb,●), and Aplysia (aMb, ▲) myoglobins in 0.05 M MOPS at pH 7.1 and (b) bottlenose dolphin (dMb, ○), horse (hHb, ◦), and human (Hb A0, ▲) hemoglobins in 0.05 M HEPES at pH 7.5. The reduction potential (E1⁄2 versus NHE) and Nernst coefficient (n1⁄2) for each species are as follows: E1⁄2 (swMb and hMb) = 29 mV with n1⁄2 = 1.0, E1⁄2 (aMb) = 103 mV with n1⁄2 = 1.0, E1⁄2 (dMb and hHb) = 52 mV with n1⁄2 = 1.6, and E1⁄2 (Hb A0) = 106 mV with n1⁄2 = 1.7. [hem] = 0.06—0.08 mM.

consistent with a cooperative redox process. The redox curves of dolphin Hb and horse Hb are equivalent and shifted to lower potentials relative to Hb A0, indicating their increased ease of anaerobic oxidation reversal. The crystal structures and primary sequences of representative Mb and Hbs as a function of varied levels of three anionic effectors (Cl−, NO3−, and ClO4−). We show that the concentration-dependent reversal of E1⁄2 observed for Cl− interactions with Hb also occurs for other small anionic effectors. The same trends are evident for all three effectors, with sensitivity to anion concentration decreasing in the order NO3− > Cl− >> ClO4−. This ease of anaerobic oxidation reversal is clearly associated with the cooperative Hb system, since, as also shown in Fig. 4a, this trend is not observed in the case of various nonmonomeric Mbs. The relatively small and progressive decrease in E1⁄2 for these Mbs is like that shown in Fig. 3c for Hb exposed to Cl− concentrations of 0.4 to 2.0 mM, indicating that the larger anion-induced shifts observed in tetrameric Hbs are superimposed on the background effects seen with the noncooperative Mb systems.

The inset of Fig. 4a shows that there is a competition between IHP and Cl− for sites on Hb A0 as Cl− levels are raised above 0.2 mM. This is a significant result with respect to the globin binding site responsible for the reversal of redox potential at the active site, because the dominant IHP binding site is known to be in the 2,3-diphosphoglycerate binding region, in the central cavity between the two β chains (47). Although IHP is a much stronger allosteric effector (and produces larger redox shifts), the presence of Cl− gradually attenuates the IHP effect until the system returns to the behavior observed when
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**TABLE I**

| Protein | Anion | 0.0 M | 0.02 M | 0.05 M | 0.1 M | 0.2 M | 0.5 M | 1.0 M | 2.0 M | Buffer |
|---------|-------|-------|--------|--------|-------|-------|-------|-------|-------|--------|
| Hb A0  | Cl−   | 98 (2) | 104 (9) | 113 (2) | 122 (2) | 94 (2) | 82 (2) | 80 (2) | MOPS  |
|        | NO−   | 98 (2) | 126 (3) | 133 (2) | 114 (2) | 89 (4) |       |       | MOPS  |
|        | ClO4− | 83 (2) | 86 (2)  | 84 (2)  | 67 (2)  |       |       |       | HEPES |
|        | Cl− / 6 | 143 (3) | 141 (2) | 137 (5) | 124 (3) | 116 (2) | 89 (6) |       | HEPES |
| sHb    | Cl−   | 104 (3) | 117 (3) | 110 (3) | 128 (2) |       |       |       | MOPS  |
|        | P−    | 104 (3) | 131 (3) | 133 (3) | 128 (2) |       |       |       | MOPS  |
| pHb    | Cl−   | 103 (13) | 117 (2) | 131 (4) | 103 (8) |       |       |       | MOPS  |
| hHb    | Cl−   | 52 (2)  |       |       |       |       |       |       | HEPES |
| dHb    | Cl−   | 52 (2)  |       |       |       |       |       |       | HEPES |
| fHb    | Cl−   | 61 (6)  |       |       |       |       |       |       | HEPES |

**TABLE II**

| Protein | [HPI: Heme] | 0:1 | 1.8:1 | 2.5:1 | 4.5:1 | 27:1 | 35:1 | 40:1 | 41:1 | 53:1 | 80:1 | 175:1 | 180:1 | 210:1 |
|---------|-------------|-----|-------|-------|-------|------|------|------|------|------|------|-------|-------|-------|
| sw-Mb  |             | 33 (3) | 33 (3) | 137 (3) | 137 (3) | 125 (2) | 131 (5) | 138 (6) |
| c-swMb |             | 67 (2) | 93 (2)  | 143 (4) |       |       |       |       |       |       |       |       |       |
| aMb    |             | 86 (3) | 133 (2) | 143 (4) |       |       |       |       |       |       |       |       |       |

*Hb A0, human hemoglobin; sHb, spot fish hemoglobin; fHb, fetal (human) hemoglobin; pHb, hemoglobin Presbyterian; hHb, horse hemoglobin; dHb, bottlenose dolphin hemoglobin; swMb, sperm whale myoglobin; c-swMb, carbamylated sperm whale myoglobin; hMb, horsemyoglobin; c-hMb, carbamylated horse myoglobin; aMb, Aplysia myoglobin.

**DISCUSSION**

This study of anion effects on the redox behavior of representative Hbs and Mbs provides further insight into the steric and electronic consequences of anion-globin interactions. The concentration-dependent effects observed underscore the need for care in comparing the functional properties of heme proteins, because the nature and type of anions in the experimental medium can have a dramatic effect on the redox process and other measures that probe the electronic properties of the active site iron atoms of these proteins.

We show in Fig. 5a that the extra positive charges in the central cavity of Hb Presbyterian has the effect of strengthening the Cl− effect on its redox properties, as well as increasing the level at which the reversal of electron affinity occurs. The
reverse trend in $E_{1/2}$ with anion concentrations above 0.2 M is unequivocally a result of the tetrameric structure of Hb because this phenomenon is not observed for the Mbs. Because low anion concentrations stabilize the T structure and shift the $E_{1/2}$ positive, it follows that a reversal of this trend at higher anion concentrations is either due to filling lower affinity anion binding sites or to an increase in the hydrophilicity of the heme pocket (e.g. through greater solvent exposure) or both.

We have drawn attention to a reversal of the trend in active site $E_{1/2}$ that occurs in Hb A0 when Cl$^-$ levels are raised above 0.2 M. Very similar patterns, with maxima near 0.2 m Cl$^-$ at neutral pH, were observed in earlier studies of the influence of Cl$^-$ on the pH dependence of Hb oxygenation. To explain these earlier results, Chiancone and co-workers (23, 29, 30) showed, using NMR techniques, that Cl$^-$ binds differently to deoxyHb and oxyHb and that the binding is proton-linked. The NMR studies showed the presence of binding sites on deoxyHb that have a 10-fold higher Cl$^-$ affinity (about 100 M$^{-1}$) than in oxyHb (about 10 M$^{-1}$). Some, but not all, high affinity Cl$^-$ binding observable by NMR was lost in the presence of the competitive anion IHP that binds in the central cavity of the Hb tetramer.

It is reasonable to conclude that our redox studies show the similar pattern of Cl$^-$ concentration dependence as found by the workers cited above because deoxyHb has a higher Cl$^-$ affinity than R-like metHb. The shifts of $E_{1/2}$ as Cl$^-$ levels increase from 0 to 0.2 m (as shown in Fig. 3a) are classic representations of allosteric effects brought about by preferential binding of anions to deoxyHb. As Cl$^-$ levels are raised from 0.2 to 0.4 m at neutral pH, the occupancy of lower affinity Cl$^-$ binding sites on metHb (possibly aided by increased hydrophilicity of the heme pocket) reverses the trend in $E_{1/2}$ of the heme (Fig. 3b). This effect is seen with other small anions, with sensitivity to anion concentration decreasing in the order NO$_3^-$ .. Cl$^-$ .. ClO$_4^-$.

Both Hb and Mb show small, progressive changes in redox potential as Cl$^-$ or NO$_3^-$ levels are raised above 0.4 M. These redox changes are thus not unique to Hb tetramers. Redox changes in this range of anion concentrations are expected to accompany tertiary level changes in subunits of the Hb tetramer. The anion binding responsible for these redox shifts appears to be correlated with the low affinity class of Cl$^-$ binding sites, documented by NMR techniques as having about 100-fold lower Cl$^-$ affinity than the relatively high affinity sites on R state Hb and 1000-fold lower Cl$^-$ affinity than even higher affinity sites on deoxyHb (23).

It is significant that the redox shifts observed for Mbs and Hbs at high anion levels (Figs. 3b and 4a) are like those associated with more hydrophilic heme pockets (Fig. 1). This similarity supports the concept that anion-globin interactions are capable of creating a more hydrophilic environment for the heme group. This was hypothesized by Caughy and co-workers (53, 54) in previous studies on anion effects on auto-oxidation of air-equilibrated Hb. These workers proposed that Cl$^-$ is a weak...
nucleophile that mediates the release of superoxide and could be responsible for metHb and superoxide formation under physiological conditions. A general (weaker) electrolyte effect on the autoxidation process documented by Caughy and co-workers (53) was proposed to have its origin in an electrolyte induced relaxation of the heme pocket allowing freer access of water to the oxygen binding site. Elegant studies using site-directed mutagenesis methods to modify the heme pocket of Mb led Brantley and co-workers (55) to a similar conclusion, i.e. that the rate of auto-oxidation could be dramatically increased by increasing the polarity of the heme pocket or by increasing the net anionic charge at the protein surface in the vicinity of the heme.

The anion-induced reversal of Hb \(E_{1/2}\) trends as \(Cl^–\) levels are raised above 0.2 M is not seen in oxygen binding curves, where the dominating consequence of increased anion concentration can be inferred to be largely a steric rather than an electronic effect. The sharp contrast between anion effects on oxidation and oxygenation processes lends additional support to our hypothesis (26) that anion-induced restrictions of the frequency or extent of conformational fluctuations of the Hb molecule play a large role in control of its oxygen affinity. In light of the work cited above, it is tempting to speculate that \(Cl^–\) levels above 0.2 M make water more accessible or more tightly held in the heme pocket, thereby accounting for the greater ease of oxidation. The resulting increase in hydrophilicity in the distal heme pocket could decrease oxygen affinity via steric hindrance effects, while increasing the ease of oxidation, as is experimentally observed. Further studies are underway to explore this possible explanation for the concentration-dependent anion effects observed.

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- **385–390**
- **33, 56–65**
- **18, 91–94**
- **561–568**
- **83, 13383–13388**
- **1303–1307**
- **33, 536–545**
- **253, 536–545**
- **1928–1936**
- **105–113**
- **143–152**
- **90–93**
- **1026–1030**
- **13604–13612**
- **116–119**