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STERIC FACTORS MODERATE CONFORMATIONAL FLUIDITY AND CONTRIBUTE TO THE HIGH PROTON SENSITIVITY OF ROOT EFFECT HEMOGLOBINS

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Abstract

The structural basis of the extreme pH dependence of oxygen binding to Root effect Hbs is a long-standing puzzle in the field of protein chemistry. A previously unappreciated role of steric factors in the Root effect was revealed by a comparison of pH effects on oxygenation and oxidation processes in human Hb relative to Spot (*Leiostomus xanthurus*) and Carp (*Cyprinodon carpio*) Hbs. The Root effect confers five-fold increased pH sensitivity to oxygenation of Spot and Carp Hbs relative to Hb A₀ in the absence of anionic effectors, and even larger relative elevations of pH sensitivity of oxygenation in the presence of 0.2 M phosphate. Remarkably, the Root effect was not evident in the oxidation of the Root effect Hbs. This finding rules out pH-dependent alterations in the thermodynamic properties of the heme iron, measured in the anaerobic oxidation reaction, as the basis of the Root effect. The alternative explanation supported by these results is that the elevated pH sensitivity of oxygenation of Root effect Hbs is attributable to globin-dependent steric effects that alter oxygen affinity by constraining conformational fluidity, but which have little influence on electron exchange via the heme edge. This elegant mode of allosteric control can regulate oxygen affinity *within* a given quaternary state, in addition to modifying the T-R equilibrium. Evolution of Hb sequences that result in proton-linked steric barriers to heme oxygenation could provide a general mechanism to account for the appearance of the Root effect in the structurally diverse Hbs of many species.

Keywords

hemoglobin; Root effect; conformational fluidity; redox potential; allostery; steric controls

1. INTRODUCTION

1.1 Nature and physiological significance of the Root effect

The Root effect is perhaps the best known, but least understood, adaptation of the hemoglobin (Hb) tetramer. It is the remarkable pH sensitivity of Root effect Hbs that allows for proton-driven oxygen unloading to the swim bladders and eyes of fish even against high oxygen pressure gradients [1-5]. Air equilibrated samples of Root effect Hbs show progressively lower levels of oxygen saturation as the pH is decreased. The oxygen affinity of a Root effect Hb may be so low at low pH that full saturation may not be achieved even at oxygen pressures of 100 atm [2].

The Root effect has functional utility for the organisms in which it is found. It enables Hbs whose pH is altered by capillary networks to serve as elegant proton-driven molecular pumps for delivering oxygen to the swim bladders and eyes of fish, even at great depths. Organic phosphate anions, such as ATP and GTP, fine-tune oxygen unloading in the Root effect Hbs, and function *in vivo* as allosteric effectors that lower the oxygen affinity at any given pH to levels below that exhibited in their absence [6-8]

While far from a comprehensive listing, among the many Root effect fish Hbs that have been studied are Hbs of carp, marlin, menhaden, spot, tuna, several antarctic fish, and pH-sensitive Hb components isolated from trout and eels [8-17]. In these Hbs, oxygen is characteristically bound with low affinity at and below pH 6.5 and with high affinity at and above pH 8. Oxygen binding at high and low pH extremes becomes non-cooperative in some, but not all, Root effect Hbs [8, 12,17-20]. Most of the Root effect Hbs studied show appreciable functional heterogeneity between the chains that make up the Hb tetramers. In Carp Hb it appears that a single subunit type is primarily responsible for the pH dependent shifts associated with the Root effect, as evidenced by 50% deoxygenation of air-equilibrated solutions as the pH is decreased [21]. In other Root effect Hbs, such as Spot Hb and Trout IV Hb, however, significantly more than 50% of the binding sites in air-equilibrated samples become deoxygenated at low pH, indicative of more global pH-dependent alterations that affect oxygen binding [7,11].

1.2 Allosteric controls of hemoglobin function

Root effect Hbs, like Hbs of other vertebrates, are tetrameric proteins whose functions are subject to both homotropic and heterotropic allosteric controls. These types of controls have traditionally been viewed as operating on the quaternary equilibrium between conformations of low (T-state) or high (R-state) oxygen affinity. Homotropic allosteric controls underlie the cooperativity of oxygen binding, while heterotropic allosteric controls enable protons and other effectors to facilitate oxygen unloading to respiring tissues [22-24]. In the Root effect Hbs, where the pH dependence is greatly exaggerated, and in the Bohr effect Hbs of humans and most vertebrates, the greater protonation of globin residues as the pH is lowered is a heterotropic allosteric effect that regulates oxygen affinity and facilitates oxygen unloading.

Although proton binding generally favors the low affinity (T-state) of Hb, the behavior of Root effect Hbs poses considerable challenges to the two state model of Hb function [25]. Notably, at low pH the oxygen affinity of a Root effect Hb tends to decrease, rather than increase, as progressive oxygen molecules are bound. This apparent negative cooperativity of Root effect Hbs results in highly asymmetric Hill plots of oxygen binding and has been ascribed to significant differences in the pH dependence of oxygen binding to α and β chains of Root effect Hbs [7,10,26]. Moreover, it has been shown that allosteric effectors can not only shift the equilibrium between T- and R-states, but can also alter the intrinsic affinities of the apparent T- and R- states of Root effect Hbs [10]. As a result, the oxygen affinity of a

Root effect Hb at low pH can drop well below that of the T-state that is apparent at high pH, which is inconsistent with one of the key assumptions of the two-state model [25].

1.2 The elusive basis of the Root effect

The structural basis of the Root effect is an unresolved mystery in the field of protein chemistry. Most efforts to identify the structural basis of the Root effect have focused on amino acid differences between fish and mammalian Hbs and their potential role in T-state stabilization at low pH. Despite more than three decades of study, the sequences of these Hbs have not revealed a consistent structural explanation for the Root effect.

The Hbs that exhibit the Root effect typically have a serine residue at position $\beta 93$, instead of the cysteine residue that is highly conserved in this position in most vertebrate Hbs. It was predicted, based on the crystal structures of the T- and R-states of Hb A₀, that this replacement would allow new salt bridges to form with the C-terminal His, creating two additional hydrogen bonds in the T structure that would greatly stabilize the deoxy T-state of fish Hbs [20]. It was subsequently shown by site-directed mutagenesis that this substitution is not in itself sufficient to change human Hb into a Root effect Hb [27].

Enhanced oxygen unloading in Root effect Hbs at low pH has generally been attributed to proton-dependent stabilization of the T-state. The C-terminal residues help stabilize the T-state and contribute to the exaggerated pH sensitivity of some, but not all, Root effect Hbs. In Carp Hb for example, the pH sensitivity of oxygenation is decreased by a factor of two by removal of the C-terminal histidines of the β chains [21]. Stabilization of the T-state by C-terminal residues is not universal, however, since the β C-terminal His appears to make no salt bridges in either T or R structures of Tuna Hb [28].

Crystallographic and functional studies of Tuna (*Thunnus thynnus*) Hb identified residues that could contribute to pH-dependent stabilization of the T-state and lowering of T-state oxygen-binding affinity. In this Hb, pH-dependent Asp-Asp interactions in the deoxygenated structure have been implicated as a partial structural explanation for the Root effect [28]. A similar mode of T-state stabilization involving Asp-Asp interactions also seems apparent in the behavior of the Root effect Hb of two antarctic fish, *Pagothenia bernacchii* and *Trematomus bernacchii* [14]. However, some fish Hbs whose structures allow for similar Asp-Asp interactions do not exhibit the Root effect [29].

In Hb of the Spot fish (*Leiostomus xanthurus*), destabilization of the R-state is a major contributor to the Root effect. In Spot Hb, key residues strategically assemble positive-charge clusters across the allosteric subunit-interface in the R-state. At low pH, protonation of the N- and C-termini within these clusters destabilizes the R-state and thereby promotes oxygen release [19]. Precise oxygen binding equilibria obtained for the Root effect Hb of the Tench (*Tinca tinca*), also show strongly decreased R-state oxygen affinities at low pH [30].

As briefly reviewed above, the lack of structural and sequence consistency among the fish and amphibian Hbs that exhibit the Root effect suggests that Hbs having extreme pH sensitivity in oxygen binding have arisen via multiple evolutionary pathways.

1.3 Anaerobic oxidation of human and Root effect Hbs

In this report we consider the allosteric controls of Root effect Hbs and how they are manifest in oxygenation and oxidation processes. Although numerous studies have addressed proton and anion effects on Hb oxygenation, the technology to critically examine allosteric controls of the oxidation processes has only recently become available. Ferricyanide, the oxidant used in most studies of Hb oxidation, is itself a strong anionic effector of Hb function. Moreover, its oxidized and reduced forms bind with different

affinities to the T and R-states of Hb and thereby alter the protein's behavior [24]. As reported in previous publications from our laboratories, we overcame these difficulties by introducing use of a cationic redox mediator, ruthenium hexammine [31,32].

In studies of this report, we used spectroelectrochemistry with ruthenium hexammine as a cationic oxidation mediator to determine how pH and anions affect the redox behavior of human and Root effect Hbs. The redox potential of a heme protein ($E_{1/2}$), determined by plotting the ratio of oxidized and reduced species as a function of applied potential, is a measure of the thermodynamic ease of electron withdrawal from the redox active iron center. The $E_{1/2}$ of a given Hb, like its $P_{1/2}$, is typically pH- and anion-dependent and is sensitive to the equilibrium between the T- and R-states of Hb. Shifts toward the T-state make Hb more difficult to oxidize [24,32]. As in Hill plots of oxygen binding by Hb, the Nernst plots for anaerobic Hb oxidation reveal a sigmoidal relationship and cooperativity in the oxidation process. Mid-point slopes ($n_{1/2}$) with values above 1 in Nernst plots of the oxidation process reflect the homotropic allosteric controls (cooperative effects) operative when reduced Hb in the hard-to-oxidize T-state condition switches to the easy-to-oxidize R-state condition as an increasing fraction of the active site heme iron atoms becomes oxidized [32].

1.4 An alternative view of the Root effect

Data in this report on the oxygenation and oxidation processes of human and Root effect Hbs were measured under comparable experimental conditions. The results obtained make possible, for the first time, a direct comparison of pH and anion effects on Hill plots of oxygenation and Nernst plots of oxidation for these functionally distinct Hbs. In agreement with previous functional studies of Spot Hb [11] and Carp Hb [8,33], we observed a five-fold larger pH sensitivity of oxygenation of the Root effect Hbs relative to Hb A₀ in the absence of anionic effectors and even larger differences in pH sensitivity in the presence of phosphate. Remarkably, the Root effect of the two fish Hbs was not evident in their oxidation processes in either the absence or presence of phosphate.

The findings of this report rule out pH-dependent alterations in the thermodynamic properties of the heme iron, measured in the oxidation reaction, as the basis of the Root effect. As will be further discussed, the elevated pH sensitivity of oxygenation of the Root effect Hbs is thus attributable to globin-dependent steric effects that have little influence on electron exchange via the heme edge. Sequence differences that introduce pH-dependent steric barriers to entry and exit of oxygen from the distal heme pocket could provide a general mechanism to account for the evolution of the Root effect in the structurally diverse Hbs of many species.

2. MATERIALS AND METHODS

2.1 Isolation and purification of Hbs

Hbs were stripped of effectors by chromatographic procedures. Anionic allosteric effectors were added as indicated in the text. Adult human hemoglobin (Hb A₀) was isolated and purified from human blood by ammonium sulfate precipitation, using stripped Hb and fast-phase liquid chromatography as the final purification step [34]. Spot and Carp Hbs were isolated as previously described [8,11]. Fish Hb samples were treated with sodium dithionite to reduce all oxidized heme sites and subjected to Sephadex G-25 chromatography to remove the reductant prior to functional analysis.

2.2 Oxygen binding studies

Oxygen equilibria were measured using tonometric methods and UV-Vis spectroscopy [35]. Deoxygenation of samples before air addition was achieved by repetitive cycles of exposure of 3 mL of Hb, held at 20°C in large volume tonometers, to N₂ and vacuum. A gastight syringe was used to inject measured volumes of room air through the rubber septum of the tonometer containing the Hb sample. After each addition the tonometers were rotated in a water bath for 10 min before an absorbance spectrum was measured from 400nm to 700nm. At each equilibration step the PO₂ was calculated and changes in the visible absorption spectrum, measured at three wavelengths on an HP-diode array spectrophotometer, were averaged and used to calculate the corresponding fractional O₂ saturation. The spectral characteristics of the fully oxygenated fish Hbs at known heme concentrations were determined at pH 8.5 under a full atmosphere of oxygen and these spectral standards were used to calculate the fractional O₂ saturation of the fish Hbs at low pH. To minimize effects associated with Hb oxidation at low pH, oxygen binding curves at a given low pH were obtained with only 4 or 5 injections and 3-4 of these curves were combined to generate a Hill plot for that condition. For data at and above pH 6.5, these procedures resulted in spectral transitions from deoxy to oxy with good isosbestic points and Hill plots with reproducible Log P_{1/2} values (± 0.1 Log units)

2.3 Anaerobic oxidation potential determinations

Anaerobic oxidation potentials of human and Root effect Hbs were determined under conditions like those used for oxygen binding determinations, making use of published spectroelectrochemical methods developed in our laboratories with ruthenium hexammine as a cationic mediator [31,32,36]. All potentials are reported versus NHE. UV-Vis spectra were taken on a Cary Bio 100 UV-Vis spectrophotometer. A cell temperature of 20°C was maintained using a circulating water bath. Applied potentials in spectroelectrochemical studies were controlled with an EG & G Princeton Applied Research model 363 potentiostat. Samples were allowed to equilibrate for at least 15 min at each applied potential. Full deoxygenation and oxidation was ensured through multiple pump-purge cycles with argon and exposure of samples to large positive applied potentials for at least 1 hr prior to analysis. Argon flushing was used to maintain an anaerobic environment throughout the experiments. Reproducibility of the spectroelectrochemical results was evaluated in two ways. First, for each experiment the Nernst plots and E_{1/2} values calculated from absorbance data at two different wavelengths were compared. Unless otherwise stated, E_{1/2} values reported here are the average of that calculated from the 405 nm data and the 430 nm data. Second, the method was calibrated using sw-Mb, and resulted in a reproducible E_{1/2} value ± 3 mV over multiple trials. In general, the same Nernst plot was generated whether the experiment was performed by increasing or decreasing the E_{app}. Reversibility of the redox reaction measurements was further confirmed by six clean isosbestic points between 350 nm and 700 nm and equivalent results obtained using met-Hb (405 nm) peak or the deoxy-Hb (430 nm) peak for analysis.

As described in our previous publications using these spectroelectrochemical techniques with ruthenium hexammine as a cationic mediator, a comparison of oxygenation and oxidation processes can be used to identify stereochemical contributions that do not have a large effect on electron transfer reactions of Hb but do affect oxygen entry and exit from the heme pocket [31,32,36,37].

3. RESULTS

3.1 Hill plots of oxygenation and Nernst plots of oxidation

Representative Hill plots of oxygen binding by anion-free solutions of Spot, Carp and human Hbs are shown in Figure 1A. Carp Hb and Hb A₀ bound oxygen with similar affinities at neutral pH at 20°C, while Spot Hb exhibited a lower intrinsic oxygen affinity. All three proteins showed cooperative interactions, with mid-point slopes ($n_{1/2}$) at neutral pH of ~2.7 for Hb A₀ and ~2 for the fish Hbs. As shown in Figure 1A, the slopes of the Hill plots for Spot Hb and Carp Hb strongly decreased above 50% saturation, as is characteristic of Root effect Hbs.

Varied levels of protons and anionic effectors can exert large influences on the shapes of Hill plots of Root effect Hbs. This phenomenon is illustrated in Figure 1A by comparison of Spot Hb oxygenation at pH 7.0 in the absence of anions (filled triangles, solid line) to that at pH 7.0 in the presence of 0.2 M phosphate (open triangles, dotted line), and then to that at pH 6.5 in the presence of 0.2 M phosphate (inverted open triangles, dotted line). Large alterations in oxygen affinity occur for Spot Hb in the transition from pH 7 to pH 6.5 in the presence of phosphate.

Representative Nernst plots for oxidation of the three Hbs are shown in Figure 1B. The redox potentials ($E_{1/2}$ values) calculated at the mid-points of the oxidation curves of Spot Hb and Hb A₀ were similar at neutral pH at 20°C, while Carp Hb was more easily oxidized. This pattern differs from the relative oxygen-binding affinities of the three Hbs. Moreover, the Nernst plots for the fish Hbs do not show the characteristic change in shape (evident in Hill plots in Fig. 1A) that is a hallmark of the Root effect. *Significantly, there was no reduction in slope of the Nernst plots for the Root effect Hbs until above 90% oxidation.*

Figure 1B also compares Spot Hb oxidation at pH 7.0 in the absence of anions (filled triangles, solid line) to its oxidation behavior in the presence of phosphate at pH 7 (open triangles, dotted line) and at pH 6.5 (inverted open triangles, dotted line). Notably, the pH shift from 7.0 to 6.5 in the presence of 0.2 M phosphate that resulted in large alterations in oxygenation of Spot Hb (shown in Fig. 1A) resulted in almost negligible alterations in the oxidation process.

Figure 2 documents the cooperativity of anaerobic oxidation of human and Root effect Hbs in the absence of anionic effectors from pH 6 to pH 7. Across this pH range, the $n_{1/2}$ for all three Hbs was about 1.5, indicative of similar cooperativity in the oxidation of all three Hbs. The oxidation process in the absence of anionic effectors clearly reflects the cooperative transition between T- and R-states for all three Hbs, thereby excluding differences in quaternary transitions as a basis for differences in the oxygenation and oxidation behaviors of human and Root effect Hbs.

Oxidation processes for Root effect Hbs under anion-free conditions have not previously been reported. Figure 3 shows changes in the UV-VIS absorbance spectra as a function of Eapp for Carp Hb at pH 7 in 0.05 M Hepes buffer in the absence of added electrolyte. By use of a cationic mediator, we were able to obtain highly reproducible curves for the transition between oxidized and reduced forms of the fish Hbs of this study. The transition showed six clean isosbestic points between 350nm and 700 nm, and clean isosbestic points were also observed in the presence of anionic effectors. Equivalent Nernst plots were obtained whether the experiments were preformed by increasing or decreasing the Eapp. The data quality shown is representative of that exhibited for all hemoglobins and conditions of this study.

3.2 Effects of pH on oxygen and oxidation equilibria under anion-free conditions

To compare the pH sensitivities of oxygenation and oxidation processes for the Root effect and human Hbs, Hill and Nernst plots like those shown in Figure 1 were generated from data obtained in experiments conducted at various pH values at 20°C under anion-free conditions. Figure 4 shows the Log $P_{1/2}$ and $E_{1/2}$ values obtained as a function of pH. The large differences in the pH sensitivities of oxygen binding to the Root effect Hbs compared to human Hb in the absence of anionic effectors are reflected in the 5-fold steeper slopes of the curves for the Root effect Hbs in the low pH region. *The Root effect was not evident in oxidation of the Root effect Hbs, as evidenced by the remarkably similar slopes of the $E_{1/2}$ vs pH plots for the three Hbs.*

The comparative oxygenation and oxidation data illustrated in Figure 4 are summarized in Table I. The $E_{1/2}$ value for Hb A₀ at pH 6.5 reported in Table 1 was estimated from the trend line shown in Figure 4. *The trends depicted in Figure 4 show similar pH sensitivities for the anaerobic oxidations of Spot, Carp and human Hbs.*

3.3 Effects of phosphate on oxygenation and oxidation of human and Root effect Hbs

To compare the effects of pH on oxygenation and oxidation processes for human and Root effect Hbs in the presence of 0.2 M phosphate, Hill and Nernst plots were generated from data obtained in experiments conducted at pH 7 and pH 6.5 at 20°C. The bar graphs of Figure 5 show the varied effects of phosphate addition on the oxygenation and oxidation midpoints for human and Root effect Hbs at pH 7 and pH 6.5. All three Hbs showed phosphate-dependent shifts in Log $P_{1/2}$ and $E_{1/2}$ at both pH values, consistent with increased shifts toward a low affinity conformation that is less easily oxidized. The extent of the phosphate-induced shifts varied appreciably, being greater at pH 6.5 for shifts of oxygenation midpoints and at pH 7 for shifts of oxidation midpoints. The large shift toward a low affinity, less easily oxidized state brought about by phosphate was most pronounced for Spot Hb, whose oxidation curves had mid-point slopes ($n_{1/2}$ values) below 1 at both pH 7 and pH 6.5. Cooperative interactions in the oxidation of Carp Hb were unchanged at neutral pH by phosphate addition, but its $n_{1/2}$ values dropped to below 1 at pH 6.5 (data not shown).

In the presence of phosphate the pH sensitivity of oxygenation of Spot Hb was more than doubled, resulting in ~20-fold greater proton sensitivity than for oxygenation of Hb A₀ under these conditions. In contrast, the pH sensitivity of oxygenation of Carp Hb was relatively unaffected by the presence of phosphate, while that of Hb A₀ was greatly reduced (see Tables I and II). Remarkably, redox experiments done in the presence of phosphate showed increased $E_{1/2}$ values for all three Hbs with very little (< 15 mV) sensitivity to pH shifts from pH 7 to 6.5. *The fish Hbs demonstrated strong oxygenation Root effects in the presence and absence of phosphate, while no Root effect was observed in their anaerobic oxidations.*

3.4 Quantitation of pH effects on oxygenation and oxidation of human and Root effect Hbs

The thermodynamic effects of pH on the oxygenation and oxidation equilibria obtained in studies of this report were compared by calculating the change in free energy ($\Delta\Delta G$) at the midpoint of both reactions as a function of pH. Tables I and II show the results of these calculations for human and Root effect Hbs measured at pH 7 and pH 6.5 in the presence and absence of the phosphate anion. By calculating $\Delta\Delta G$ for oxidation and for oxygenation, the pH-sensitivities of the two processes can be compared quantitatively. Our data set made it possible to examine the effect of changing the pH from 7 to 6.5 on oxygen binding (Equation 1) where $T = 293^\circ\text{K}$ and $R = 8.314 \text{ JK}^{-1}\text{mol}^{-1}$) to the effect of the same pH shift on

oxidation (Equation 2). The values of ΔG and $\Delta\Delta G$ in Tables I and II for heme oxygenation were multiplied by four [24] to describe the pH sensitivity of the Hb tetramer.

$$\Delta\Delta G = RT (\ln P_{1/2}^{pH6.5} - \ln P_{1/2}^{pH7.0}) \quad (1)$$

In order to calculate $\Delta\Delta G$ for anaerobic oxidation, we used a modified form of the Nernst Equation (Equation 2), which quantifies the effect of changes in pH (from 7 to 6.5) on the anaerobic oxidation process. The $\Delta\Delta G$ values for the anaerobic oxidation of the Hb tetramer reported in Tables I and II were calculated using 4 as the value of n .

$$\Delta\Delta G = -nF (E_{1/2}^{pH6.5} - E_{1/2}^{pH7.0}) \quad (2)$$

In the absence of anionic effectors, the pH-dependent shifts in the mid-point $\Delta\Delta G$ values for *oxygenation* of the Root Effect Hbs were roughly 5-fold larger in magnitude than that of Hb A₀. This is a reflection of the exaggerated pH-sensitivity of oxygenation that is a hallmark of the Root effect. In contrast, the pH-dependent shifts in the mid-point $\Delta\Delta G$ values for the *oxidation* of the Root effect Hbs were found to be similar to those of human Hb (Table 1).

Similarly, the oxygenation process was found to be far more sensitive to changes in pH than the oxidation process when the pH-dependent shifts of $\Delta\Delta G$ values at the mid-points of oxygenation and oxidation were compared in the presence of phosphate (Table II). Said in another way, the values for $\Delta\Delta G$ for oxidation and oxygenation of the fish Hbs over the same pH range in the presence of phosphate also showed the presence of a strong oxygenation Root effect and absence of an oxidation Root effect.

4. DISCUSSION

4.1 Differences in the allosteric controls of Hb oxygenation and oxidation

Structurally distinct Hbs, such as those that exhibit the Root effect, have evolved to meet varied physiological and environmental needs. A general feature of these adaptations is that they take advantage of the functional flexibility, i.e. conformational fluidity, of the Hb tetramer and exploit allosteric controls of Hb function in varied ways. We show in this report that a major mode of allosteric regulation of Root effect Hbs involves steric effects that moderate the conformational fluidity of Hb and alter oxygen affinity *within* a given quaternary state. This mode of functional regulation, which is not widely appreciated, acts in addition to the commonly acknowledged mode of allosteric regulation that involves shifts of the equilibrium *between* the T- and R-states of Hb.

Although many studies have documented the effects of protons and anionic effectors on Hb oxygenation, our work is the first to directly compare the allosteric controls of oxygenation of human and Root effect Hbs with the allosteric controls of oxidation. The data summarized in Tables I and II quantifies the energetics of proton and phosphate effects on oxygenation and oxidation of human and Root effect Hbs and is drawn upon in the following discussion.

Our studies of oxygen binding by Spot and Carp Hb confirmed and extended previously published results on the nature of oxygen binding by these two Root effect Hbs [8,11,33]. We confined our oxygen binding studies to pH values of 6.5 and above. This strategy avoided most of the oxidative effects that introduce metHb as a substantial product at low pH, but did not bring the fish Hbs to affinities as low as have been reported at lower pH. As expected, the two fish Hbs exhibited about five-fold larger changes in oxygen affinity between pH 7 and pH 6.5 relative to human Hb when examined in the absence of anionic

effectors. Even higher sensitivity to pH was observed relative to human Hb when oxygenation of the Root effect Hbs was examined over this pH range in the presence of 0.2 M phosphate (see Tables I and II).

The oxidation behavior of these Root effect Hbs has not previously been documented. A question posed by our studies was whether or not Spot and Carp Hbs would show a redox Root effect. The results of this report provide a definite negative answer to this question. *Neither Spot nor Carp Hb showed enhanced pH sensitivity of oxidation in either the presence or absence of phosphate compared to human Hb: i.e., they did not show a redox Root effect.*

Oxidation of Hb is sensitive to alterations in the T-R equilibrium, as evidenced by many previous studies. The redox potential of the heme iron atom changes due to electronic alterations that occur when it goes from the out-of-plane (T-state) condition of deoxygenated Hb to the in-plane (R-state) configuration. Conversely, the R-state configuration of the heme is favored when the heme is oxidized [31,32].

Hb oxidation, like Hb oxygenation, is sensitive to pH [24,32,38]. An earlier study of Trout IV Hb revealed a pH sensitivity for its oxidation that was remarkably similar to that of human Hb [39]. Moreover, it was reported that the greatly reduced cooperativity that characterizes oxygenation of Trout IV Hb at low pH was not evident in the oxidation process [39]. While these results suggest that this Root effect Hb, like Spot and Carp Hb, lacks a redox Root effect, this conclusion cannot be drawn with confidence because the study used ferricyanide as the oxidizing reagent. Ferricyanide is a strong anionic effector of Hb, and could have exerted different effects on the pH sensitivity of oxidation of human Hb and Trout IV Hb (see Section 1.3).

4.2 Is the Root effect due to a proton-driven shift in the T-R equilibrium toward the T-state?

Enhanced oxygen unloading in Root effect Hbs at low pH has often been attributed to proton-dependent shifts toward Hb's T-state. If this was the basis of the Root effect, enhanced proton sensitivity should be expressed in the oxidation of Root effect Hbs as well as in their oxygenation. As discussed here, the hypothesis that the Root effect is based entirely on proton-dependent shifts toward the T-state was explored and disproven by our comparisons of oxygenation and oxidation of human and Root effect fish Hbs.

The unexpected nature of our results lies not in the appearance of cooperativity or proton sensitivity in the process of Hb oxidation, but in the *remarkable similarity* of oxidation behavior exhibited by human and Root effect Hbs. Notably, Figure 4 shows that at neutral pH in the absence of anions, the proton sensitivity of oxidation of human Hb was like that of Spot and Carp Hb. Figures 1 and 2 show that under these conditions the *same* degree of cooperativity (with $n_{1/2}$ values of ~ 1.5) was expressed in the oxidation of human Hb as in Spot and Carp Hbs. Table I quantifies these results and shows that while the $\Delta\Delta G$ values for oxygenation of the Root effect Hbs between pH 7 and pH 6.5 were 5-fold larger than for human Hb, the $\Delta\Delta G$ values over this pH range for oxidation of human and Root effect Hbs were quite similar. Taken together, these results show that a proton-driven shift toward the T-state was *not* the sole basis of the oxygenation Root effect.

The data of Figure 1 show that the combined effects of low pH (pH 6.5) and anions (0.2 M phosphate) constrain Spot Hb in a low affinity, "T-like" conformation, with no evidence of cooperativity in either its oxygenation or oxidation. The heterogeneous Hill plots exhibited by Spot Hb in this condition reflect differences in the oxygen-binding affinities of α and β subunits. The Nernst plots of Spot Hb oxidation under these conditions also reflected differences in behavior of the protein's α and β subunits. What the oxidation process did *not*

show is the elevated pH sensitivity that was evident in the Hb's oxygen binding process in the presence of phosphate. In fact, Figure 5 and Table II show that Spot Hb oxidation in the presence of phosphate had *decreased* rather than increased pH sensitivity.

Absence of an oxidation Root effect for Spot and Carp Hbs in either the presence or absence of phosphate rules out the pH dependence of the thermodynamic properties of the heme group as the basis of the exaggerated pH-dependent alterations of oxygen binding to these Hbs. Something other than changes in the electronic state of the heme is required to explain those instances where allosteric effects on the oxygenation of the Root effect Hbs are greater than on their oxidation. Figure 5, which shows the relative changes in oxygen affinities and redox potentials of human, Spot and Carp Hbs in response to alterations of pH and anions, illustrates several instance of this situation. Tables 1 and II present quantitative evaluations of these results in terms of $\Delta\Delta G$ values for oxygenation and oxygenation for the transition between pH 7 and pH 6.5. Notably among these comparisons was the effect of pH in the presence of phosphate, where the $\Delta\Delta G$ for the shift from pH 7 to pH 6.5 in the oxygenation of the Root effect Hbs in the presence of 0.2 M phosphate consistently exceeded the $\Delta\Delta G$ for oxidation.

4.3 Oxygen affinity alterations within the T-state: a new perspective on the Root effect

Since the Root effect was not evident in the oxidation properties of Spot and Carp Hbs, the oxygenation Root effect in these Hbs must have resulted from pH-dependent changes in the heme environment that affected oxygenation more than oxidation. Moreover, as pointed out in the foregoing section, the pH-dependent alterations of the globin that resulted in lowered oxygen affinity of the Root effect Hbs must have been effective even when the heme was already constrained to be in the out-of-plane condition that is resistant to oxidation. This perspective requires a different mode of allosteric regulation of Hb oxygenation than can be accommodated by the two-state model of Hb function. The results presented in this report, focused on the equilibrium properties of Hb as expressed in Hill plots of oxygenation and Nernst plots of oxidation, provide insight into how steric factors can induce variations in conformational fluidity and thereby affect oxygen affinity *within* a given quaternary state. Similar steric allosteric controls in the kinetics of ligand binding to Root effect Hbs will be demonstrated in a companion paper (in preparation).

Apart from studies of Root effect Hbs, the two-state model of allosteric control via T-R shifts has previously been challenged by reports on Hbs that exhibit the traditional structural markers of T- or R- quaternary states, but show widely varying oxygen affinities [40]. A wide range of tertiary conformations are possible within the T-state of human Hb, as evidenced by Raman spectra and ligand binding kinetics [41-47]. The results of this report provide an explanation for these observations, and show that steric factors can affect oxygen affinity *within* a given quaternary conformation. The results of this study show that steric factors introduced by the interplay of the Hb molecule with protons and anions can, in addition to affects on the T-R equilibrium, moderate the tertiary conformations *within* the T-state and thus play an important role in the allosteric regulation of function in human as well as Root effect Hbs.

Constraints on the conformational fluidity of the Hb tetramer imposed by low pH and anionic effectors can result in a distribution of very low affinity T-state tertiary conformations that underlie Hb's equilibrium properties. Evidence for such a mechanism was seen for the extreme low affinity Root effect Hb of *Corphaenoides armatus*, where time resolved Raman showed that under low pH conditions the tertiary relaxation back to the deoxy T-state conformation upon ligand dissociation is extremely fast compared to high pH conditions [48].

4.4 Steric factors contribute to the allosteric control of oxygen affinity in Root effect Hbs

The foregoing comparison of oxygenation and oxidation behavior of Hb shows that steric factors play a previously unappreciated role in the allosteric control of oxygen affinity of Root effect Hbs. Oxidation, by monitoring the electronic changes of the heme, provides an indication of allosteric effects associated with shifts of the T-R equilibrium. In those cases where oxygenation is much more affected than can be accounted for the changes in the electronic state of the heme, the difference can be attributed to globin-induced steric effects. The steric factor was exaggerated in the two Root effect Hbs of this study, and imposed a mode of allosteric control of heme oxygenation that acted in addition to the affinity-linked shifts between T- and R-state conformations. This result showed that steric constraints that affect the intrinsic oxygen affinity of a Hb can come into being via a wide range of sequence alterations, and be further modulated by steric constraints imposed by binding of protons or anions. With many possible routes to accomplish the end result, widely different sequence modifications that introduce proton-linked steric constraints could have been exploited in evolution of the Root effect.

Although many fish and amphibian Hbs exhibit the Root effect, the details of how their structures allow steric constraints to be imposed can differ significantly (see Section 1.2). In this regard it is relevant that alterations in the positions of residues of the distal pocket are typically required for entry of oxygen into the distal heme pocket of deoxygenated Hb [22,23]. Alterations of the conformational fluidity of these gating residues could alter oxygen affinity without a concomitant change in heme redox potential. Steric constraints could also induce shifts of the T-R equilibrium, but these would be evident as changes in heme redox potential as well as changes in oxygen affinity.

Minor differences in Hb structure could determine whether or not formation of a specific proton-linked salt bridge will affect the mobility of gating residues in the distal heme pocket. As an example of this, the Root effects of Tuna Hb and *P. bernacchii* Hbs are attributed to the formation of Asp-Asp linkages that introduce additional salt bridges *within* the T-state of these Hbs [14,28]. However, these same linkages can form in *Trematomus newnesi*, an Antarctic fish that lacks the Root effect [49]. Since the T-state in *T. newnesi* Hb is characterized by an atypical flexibility of two α chains within the tetramer, it was concluded that an order-disorder transition plays a role in switching off the Root effect in this Hb [29]. This result supports the notion that the transmission of proton-induced steric constraints to Hb's distal pocket residues is itself subject to evolutionary modification.

In summary, this paper makes the point that structurally distinct Hbs, such as those that exhibit the Root effect, have evolved to meet varied physiological and environmental needs by taking advantage of the conformational fluidity of the Hb tetramer. By comparing the pH dependence of oxygenation and oxidation of Hb under the same conditions, we have shown that the conformational fluidity of Hb tetramer can be altered by steric factors introduced by the interplay of the Hb molecule with protons and anions. These alterations of conformational fluidity can, in addition to affects on the T-R equilibrium, moderate oxygen affinity within the T-state and thus play an important role in the allosteric regulation of function in human as well as Root effect Hbs. The paradigm of Hb function as reflecting combined electronic and steric controls provides a general mechanism to account for the evolution of the Root effect via different sequence alterations in the structurally diverse Hbs of many fish and amphibian species.

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HIGHLIGHTS

We sought insight into the allosteric controls of Hb oxygenation and oxidation. Human and Root effect Hbs differ in pH-sensitivity of oxygenation, not of oxidation.

Conformational fluidity of key residues affects oxygenation more than oxidation.

Conformational fluidity moderates O₂ binding within T-states of human and fish Hbs.

Allosteric controls of conformational fluidity underlie Root effect Hb adaptations.

Figure 1.

Comparison of oxygenation (A) and oxidation (B) processes for Carp Hb (squares), Spot Hb (triangles), and Hb A₀ (open circles). Hill plots of oxygen binding (A) were determined with samples 0.03 mM - 0.09 mM in heme in the absence of anionic effectors in 0.05 M Hepes buffer at pH 7, 20°C. Data for Spot Hb in the presence of 0.2 M phosphate at pH 7 (open triangles) and at pH 6.5 (inverted triangles) are also shown. Nernst plots of oxidation (B) were measured under the same conditions, with no added electrolyte, using 1 mM Ru(NH₃)₆Cl₃ as an electron transfer mediator. E_{app} is reported as mV vs. NHE and absorbance values at 430 nm were used to calculate the Nernst plot.

Figure 2.

Cooperativity at half oxidation ($n_{1/2}$) as a function of pH for Carp Hb (squares), Spot Hb (triangles), and Hb A₀ (open circles). Conditions as in Figure 1. Error bars indicate the difference between the $n_{1/2}$ value calculated from different wavelengths in one experiment. Dashed horizontal line indicates the $n_{1/2}$ value of 1 expected for a non-cooperative process.

Figure 3.

UV-vis absorbance spectra as a function of E_{app} for Carp Hb. Quality of data is representative of all hemoglobins and conditions studied. Conditions: 0.07 mM Hb, 0.05 M Hepes buffer, pH 7, no added electrolyte, 1 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$, and 20°C. Potentials reported vs. NHE.

Figure 4.

Comparison of pH effects on oxygenation (A) and oxidation (B) of Carp Hb (squares), Spot Hb (triangles), and Hb A₀ (open circles) in the absence of anionic effectors. Samples contained 0.03 mM - 0.09 mM in heme in 0.05 M Hepes buffer at 20°C. Oxygen binding data (A) are averages of $\text{Log } P_{1/2}$ values calculated at three wavelengths. Error associated with each data point is ~0.1 Log units for these samples. Redox potentials (B) are values of $E_{1/2}$ reported as mV vs. NHE, determined with no added electrolyte using 1 mM $\text{Ru}(\text{NH}_3)_6\text{Cl}_3$ as a mediator. Each data point is the average $E_{1/2}$ value calculated from the oxidized species (405 nm) and reduced species (430 nm) in the same trial. Error associated with each data point calculated using this method is ~3 mV for these samples.

Figure 5.

Bar graphs are presented that illustrate the effects of protons (pH 6.5 and pH 7) and anions (Anion-free Hepes, solid bars; Phosphate, stippled bars) on oxygenation and oxidation of human and Root effect Hbs.

Thermodynamic parameters of Carp Hb, Spot Hb, and Hb A₀ in Hepes buffer. E_{1/2} value for Hb A₀ in Hepes buffer at pH 6.5 is estimated from trend line shown in Figure 4.

Table 1

	Anaerobic Oxidation			
	Hbs in Hepes	E _{1/2} (V) pH 6.5 ^a	ΔG (kcal) pH 6.5 ^b	ΔΔG (kcal) pH 7 ^b
Spot		131	12.1	9.4
Carp		89	8.2	6.0
HbA ₀		133	12.3	9.5
				2.7
				2.2
				2.8

	Oxygenation			
	Hbs in Hepes	Log P _{1/2} pH 6.5 ^d	ΔG (kcal) pH 6.5 ^e	ΔΔG (kcal) pH 7 ^e
Spot		1.05	5.6	3.2
Carp		0.35	1.9	-0.5
HbA ₀		0.2	1.0	0.5
				2.4
				2.4
				0.5

^a Half potential for the reduction reaction vs. NHE (estimated reproducibility ± 3mV).

^b Gibbs free energy calculated for the conversion of deoxy- to met-Hb (the oxidation reaction) - estimated reproducibility ± 0.3 kcal.

^c The ΔΔG value calculated as the difference between the ΔG values of oxidation at pH 6.5 and pH 7.

^d Half pressure for the oxygenation reaction – estimated reproducibility ± 0.1 Log units.

^e Gibbs free energy for the oxygenation reaction.

^f The ΔΔG value calculated as the difference between the ΔG values at pH 6.5 and pH 7 as illustrated in Equation 1.

Table II

Thermodynamic parameters of Carp Hb, Spot Hb, and HbA₀ in phosphate buffer.

Anaerobic Oxidation					
Hbs in Phosphate	E _{1/2} pH 6.5 ^a	ΔG (kcal) pH 6.5 ^b	E _{1/2} pH 7.0 ^a	ΔG (kcal) pH 7 ^b	ΔΔG (kcal) ^c
Spot	152	14.0	149	13.7	0.3
Carp	133	12.3	120	11.1	1.2
HbA ₀	152	14.0	144	13.3	0.7

Oxygenation					
Hbs in Phosphate	Log P _{1/2} pH 6.5 ^d	ΔG (kcal) pH 6.5 ^e	Log P _{1/2} pH 7 ^d	ΔG (kcal) pH 7 ^e	ΔΔG (kcal) ^f
Spot	2.5	13.4	1.5	8.0	5.4
Carp	1.37	7.3	0.85	4.6	2.8
HbA ₀	1	5.4	0.87	4.7	0.7

^aHalf potential for the reduction reaction vs. NHE (estimated reproducibility ± 3mV).

^bGibbs free energy calculated for the conversion of deoxy- to met-Hb (the oxidation reaction) - estimated reproducibility ± 0.3 kcal.

^cThe ΔΔG value calculated as the difference between the ΔG values of oxidation at pH 6.5 and pH 7.

^dHalf pressure for the oxygenation reaction - estimated reproducibility ± 0.1 Log units.

^eGibbs free energy for the oxygenation reaction.

^fThe ΔΔG value calculated as the difference between the ΔG values at pH 6.5 and pH 7 as illustrated in Equation 1.