Molecular Basis of the Bohr Effect in Arthropod Hemocyanin*

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Flash photolysis and K-edge x-ray absorption spectroscopy (XAS) were used to investigate the functional and structural effects of pH on the oxygen affinity of three homologous arthropod hemocyanins (Hcs). Flash photolysis measurements showed that the well-characterized pH dependence of oxygen affinity (Bohr effect) is attributable to changes in the oxygen binding rate constant, k_on, rather than changes in k_off. In parallel, coordination geometry of copper in Hc was evaluated as a function of pH by XAS. It was found that the geometry of copper in the oxygenated protein is unchanged at all pH values investigated, while significant changes were observed for the deoxygenated protein as a function of pH. The interpretation of these changes was based on previously described correlations between spectral lineshape and coordination geometry obtained for model compounds of known structure (Blackburn, N. J.; Strange, R. W.; Reedijk, J.; Vollbeda, A.; Farooq, A.; Karlin, K. D.; and Zubieta, J. (1989) Inorg. Chem., 28, 1349–1357). A pH-dependent change in the geometry of cuprous copper in the active site of deoxyHc from pseudotetrahedral toward trigonal was assigned from the observed intensity dependence of the 1s → 4p transition in x-ray absorption near edge structure (XANES) spectra. The structural alteration correlated well with increase in oxygen affinity at alkaline pH determined in flash photolysis experiments. These results suggest that the oxygen binding rate in deoxyHc depends on the coordination geometry of Cu(I) and suggests a structural origin for the Bohr effect in arthropod Hcs.

Hemocyanins (Hcs) are oxygen carrier and storage proteins found in molluscs and arthropods. Significant differences are observed between mollusc and arthropod Hcs in size of the functional units and in their tertiary and quaternary structures. Specifically, arthropod Hcs are structurally homogenous oligomeric proteins with a minimal functional subunit of 75 kDa. Under physiological conditions in the presence of calcium, the subunits are typically arranged in hexamers and dodecamers. Removal of Ca²⁺ with EDTA at neutral pH causes dissociation of the dodecamer into hexamers, which can be dissociated into monomers at alkaline pH (1). The different aggregation states are related to modified oxygen binding properties (2). As generally observed for respiratory proteins, a fundamental physiological property of Hc is its competence to bind oxygen with different affinity in response to allosteric effectors including hydrogen ions.

Detailed structural information concerning the active site of Hcs is derived mainly from a limited set of x-ray crystallographic investigations. The active site structures of the deoxy form have been described for two arthropod Hcs, Panulirus interruptus (3) and Limulus polyphemus (subunit II) (4). However, a comparison of the results is limited by the different solution conditions from which the proteins were crystallized. In both examples, each Cu(I) of the binuclear active site is coordinated by the ε-nitrogen of three histidine imidazole residues. In the P. interruptus structure obtained from protein crystallized at pH 4.8, two of the three histidine imidazole ligands and one copper atom lie nearly in a plane, with Cu-N bond lengths of 1.9 Å. In addition, each metal atom binds to a third histidyl imidazole nitrogen at a distance of 2.7 Å, perpendicular to the plane defined by the two equatorial histidine nitrogens and a copper atom. In contrast to this, the structure of L. polyphemus subunit II deoxyHc, obtained from crystals grown in 0.5 M chloride at pH 6.5–7.0, shows that each copper has approximately trigonal planar coordination geometry, with three histidyl imidazole nitrogens at a distance of 1.9–2.2 Å from the copper. These ligands define a plane from which the copper is displaced by 0.3 Å. An important additional difference between these two structures is the metal-metal distance, which is 3.6 Å in P. interruptus Hc (low pH and low ionic strength) and 4.6 Å for L. polyphemus Hc (physiological pH and higher ionic strength, Fig. 1).

The only example of an oxy structure for an arthropod Hc is that of subunit II of the L. polyphemus protein crystallized at pH 6.2 (5). In this example, the oxygen molecule is bound as a charge transfer; SOD, superoxide dismutase; MES, 4-morpholineethanesulfonic acid.
Bohr Effect in Hemocyanin

peroxide and forms a \( \mu\eta^2-\eta^2 \) bridge between the two Cu(II) atoms. Each copper atom is pentacoordinated in a square pyramidal geometry, where the equatorial plane is now defined by two histidyl imidazole nitrogens and the bound oxygen, while a third histidyl nitrogen is axially coordinated to copper. All three Cu-N bond lengths are 1.9 Å, and the metal-metal separation is 3.0–3.5 Å (6), a distance supported by EXAFS analysis for the closely related \textit{P. interruptus} oxyHc (7, 8).

Oxygen binding to arthropod HCs is very sensitive to pH, and the relevance of this Bohr effect resides in the capability of these proteins to respond to a pH variation in the medium with a variation in affinity for molecular oxygen to meet physiological needs. Although Hc has been studied extensively, detailed information concerning oxygen binding kinetics and the origins of both cooperativity and the Bohr effect are still limited (4–6). A useful approach for the study of oxygen binding kinetics is flash photolysis. OxyHc is blue and its optical spectrum is characterized by CT bands about 345 nm and at about 600 nm (9–12). DeoxyHc is colorless. Taking advantage of these optical differences, a photolysis approach was previously used to study the binding of oxygen to \textit{Streptomyces antibioticus} tyrosinase, a monomeric enzyme that like Hc contains a binuclear copper center with similar optical properties (13).

In the present study, flash photolysis was used to investigate oxygen binding to Hc allowing us to probe allosteric effects. A complementary approach to the kinetic studies is x-ray absorption spectroscopy (XAS), which allows for the investigation of the immediate coordination geometry of Cu(I) in deoxyHc. For Cu(I), a species with limited potential for other spectroscopic studies as it lacks color and paramagnetism, coordination geometry can be deduced from copper k-edge absorbance measurements. Previous studies on Cu(I) model complexes of known structure provide the background information used to define a correlation between k-edge features and coordination geometry (14, 15).

Three paradigmatic arthropod HCs that are structurally and functionally characterized were used here (2). Flash photolysis was used to obtain the kinetic parameters of oxygen binding, while XAS was used to define structural features of copper centers in the active site as a function of pH. The results suggest that the \( O_2 \) binding rate depends on the coordination geometry of Cu(I) in deoxyHc providing evidence for a structural origin of the Bohr effect.

EXPERIMENTAL PROCEDURES

Sample Preparation—\textit{P. interruptus} and \textit{Carcinus aestuarii} Hcs were purified and stored as previously described (1, 16). \textit{L. polyphemus} hemolymph was collected at the Marine Biological Laboratories, Woods Hole, MA. The clotted hemolymph was filtered through gauze and centrifuged to eliminate debris. The clear dark blue supernatant was dialyzed overnight at 4 °C against 20 mM CaCl\(_2\), pH 7.0. During dialysis, a small amount of precipitate was formed and was separated by low speed centrifugation. The individual subunits were purified according to a published method (17). Subunit II was in this study because of its well-characterized allosteric properties (18). The purity of the protein was verified by the electrophoretic mobility, while its aggregation state in the presence of Ca\(^{2+}\) was determined by analytical ultracentrifugation using Schlieren optics (18). Control oxygen binding experiments were carried out using a thin layer optical cell as previously described (19).

Flash Photolysis Measurements—Purified Hc of \textit{C. aestuarii} in 50 mM Tris-HCl buffer, pH 8.3, was degassed on a vacuum line and then flushed with nitrogen gas. Hydroxylamine was added to the sample solution anaerobically (final concentration, 1 mM) to reduce any oxidized protein. The solution was then dialyzed under aerobic conditions against 50 mM Tris-HCl, MES, or acetate buffer at the desired pH. After dialysis, the protein concentration was adjusted to 20 \( \mu \)M, and a sample was placed under an atmosphere of oxygen plus nitrogen such that the partial pressure of oxygen could be varied from 5 to 100% with a mixed gas generator (MX-3S, Crown, Tokyo, Japan). The sample solution was filtered under the appropriate oxygen concentration and was transferred into the sealed quartz cell, which was filled with the same gas mixture.

Photolysis of oxyHc samples was accomplished using the third harmonic of an Nd:YAG laser (Surelight I-10, Continuum, Santa Clara, pulse energy, 30 mJ; pulse width, 5 ns) for excitation. Time-resolved absorbance changes were measured at 20 °C with illumination from a Xe lamp orthogonal to the laser pulse and were recorded on a digital oscilloscope (TDS 3012B, Tektronix, Tokyo, Japan), which received voltage signals from the photomultiplier attached to a monochromator (RSP-601-03, Unisoku, Osaka, Japan). The traces were obtained as the average of 256 or 512 pulses, and least-squares exponential fits were performed for the time-resolved absorption data using Igor Pro ver. 4.0 (WaveMetrics).

X-ray Edge Measurements—Highly concentrated Hc (2 mM copper concentration) used in spectroscopic experiments was prepared using a Centricon filter (Amicon) and then transferred to Lucite\textsuperscript{c} sample holders designed for the x-ray measurements (20). DeoxyHc was obtained by passing a pre-humidified argon stream through an air tight, homemade chamber designed to host the loaded sample holders. Through the transparent faces (top and bottom) of the chamber, it was possible to
monitor the course of deoxygenation based on the disappearance of the blue color of oxyHc. It was estimated that no more than 2–3% oxyHc remained in the apparently colorless sample by visual inspection. After deoxygenation, the sealed chamber was cooled in liquid nitrogen and the frozen sample was removed and stored at 77 K until used. All samples were placed in a 25 × 2.5 × 2 mm Lucite® sample holder covered with Kapton® tape.

X-ray fluorescence data were collected on beam lines X-9A and X-10C at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, using double flat Si(111) (X-9A) and Si(220) (X-10C) crystal monochromators with fixed exit geometry. Beam harmonics were rejected using a nickel (X-9A) or rhodium- (X-10C) coated mirror positioned downstream of the monochromator. The sample temperature was maintained at ~120 K by flowing cooled nitrogen gas through a Lucite® cryostat as described previously (1). X-ray edge data having 3 eV resolution (X-9A) and 2 eV resolution (X-10C) were recorded by counting at single energy values for 3 s and incrementing the energy in 0.5 eV steps from 30 eV below the copper edge to 120 eV above the edge. Copper foil was used as an energy standard to account for any shifts in the monochromator calibration for any individual scan. Kapton® tape was mounted at a 45° angle to the incident x-ray beam to scatter x-ray photons through the copper foil. The photons were then counted with a photomultiplier tube positioned perpendicular to the x-ray beam as previously described (1). X-ray flux was 1.0 × 10¹⁰ (X-9A) and 1.3 × 10¹⁰ photons sec⁻¹ mm⁻² at 100 mA beam current (X-10C). X-ray edge data were generally taken in the range of 100–225 mA. K-copper fluorescence was detected with a 13-element solid-state energy-resolving germanium detector, and incident photon scattering was rejected by a nickel filter. Reference signals (incident beam intensity, I₀) were collected using a standard ion chamber. To monitor the condition of samples after x-ray exposure, optical spectra in the UV and visible regions were collected by visual inspection. After deoxygenation, the sealed chamber minus deoxyHc). The observed absorbance changes at 337 nm were fit successfully with single exponentials for all oxygen concentrations at pH 8.3. Although at this pH in the presence of Ca²⁺, C. aestuarii Hc is known to be a mixture of 90% dimer of hexamers and 10% hexamers (1), there was no observable heterogeneity in the kinetic data. There was likewise no evidence for bi-phasic character that might be expected if the Hc were switching between the R- and T-state upon photolysis.

The observed oxygen re-binding rate constant (k₉₀) increased upon increasing the pH from 6.5 to 8.7 (Fig. 3A). The k₉₀ values at pH 8.3 and 6.5 are plotted in Fig. 3B as a function of oxygen concentration; the dependence of k₉₀ on oxygen concentration was steeper for the measurements at pH 8.3 than that at pH 6.5. As will be discussed later, the ordinate intercept of the plots gave coincident k₉₀ values, demonstrating that only the k₉₀ values are pH-dependent. The shape of the optical spectrum of oxyHc is pH-independent, such that optical spectra at different pH values are superimposable. Therefore, our flash
photolysis approach does not require any correction for pH effects on the spectrum of oxyHc.4

The absorbance change upon photolysis of oxyHc at pH 7.1 under O2 concentrations lower than 0.35 mM could not be successfully fit with a single exponential curve (supplemental Fig. S1, depicts the case for [O2] = 0.14 mM), although single exponential fits were adequate for Hc at pH 8.3 under all oxygen concentrations studied, and at pH 7.1 under higher oxygen concentrations ([O2] > 0.49 mM). For the samples at pH 7.1 under oxygen concentrations less than 0.35 mM, double exponential curves were required to fit the data, suggesting a second process is operative under these conditions. The cooperative homohexamers of CaeSS3 subunits of Hc (1), exhibited only a single phase at pH 7.5 and [O2] = 1.39 mM, with smaller kobs values than the heterohexameric native protein (CaeSS3 homohexamers, 61 ± 5 ms−1; the heterohexameric native protein 72.2 ± 3.2 ms−1).

To calculate thermodynamic parameters for the oxygen re-binding reaction, we studied the temperature dependence of kobs. In the tyrosinase case (13), the initial intensity change in absorbance in the photolysis experiments depended on temperature such that as the temperature increased, the intensity change increased, whereas for Hc, there was no observed temperature dependence of the optical features. These observations suggest that the structure of Hc at the active site is more rigid than that of tyrosinase (13) and that the increase in the oxygen-binding rate constant in Hc as a function of temperature is not due to protein structural changes. A linear Eyring plot was obtained based on the temperature dependence of kobs within the range 5 to 25 °C (Fig. 4). The activation enthalpy and entropy of oxygen binding to Hc calculated from the temperature dependence were as follows; ΔH‡ = 7.2 kcal/mol and ΔS‡ = −26 cal/(mol·K), respectively, based on the data in Fig. 4. These values compare reasonably well with those reported for oxygen binding to binuclear copper model complexes (21).

Copper K-edge Spectra of Arthropod Hemocyanin—Oxy and deoxyHc have readily distinguishable x-ray absorption spectra. For example, a comparison of the copper k-edge spectra of oxy and deoxy forms of P. interruptus Hc (Fig. 5), a very close homolog of C. aestuarii Hc, indicates a shift of 2–3 eV to higher energy expected from the increase in the 1s-electron binding energy for the metal ion in the oxy protein because of its cupric character (14). The difference spectrum between deoxy and oxyHc allows for an accurate determination of the peak positions in the edge spectra of the deoxy protein. The two largest absorbance difference peaks, at 8982.7 ± 0.5 and 8985.6 ± 0.5

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4 The kobs value also increased on the low pH side of the minimum at 6.5 (Fig. 3A) as has been noted before for an analogous arthropod Hc (23). This phenomenon is not analyzed further.
TABLE 1
Values of \( P_{50} \) for oxygen binding to Hcs as a function of pH in the presence of 10 mM CaCl$_2$

| Subunit | pH   | \( P_{50} \) O$_2$ mmHg |
|---------|------|-------------------------|
| P. interruptus | 5.25 | 172$^a$ |
|          | 7    | 42$^b$ |
|          | 9    | 5$^b$ |
| C. aestuarii | 7.1  | 44.3$^c$ |
|          | 7.5  | 33.7$^c$ |
|          | 7.9  | 13.6$^c$ |
| Subunit II L. polyphemus | 8.3  | 6.81$^d$ |
|          | 7    | 2.5$^d$ |
|          | 9    | 1.4$^d$ |

$^a$ In 100 mM Bis-Tris, pH 5.25 (30).
$^b$ In 50 mM Tris-HCl pH 7 and in 50 mM ethanolamine, pH 9 (31).
$^c$ In 50 mM Tris-HCl (1).
$^d$ In 100 mM Tris-HCl pH 7 and 9 (23).

FIGURE 6. K-edge x-ray absorption spectra of P. interruptus deoxyHc.
- a, pH 6 in 10 mM Tris-SO$_4$ buffer; b, pH 9 in 100 mM Tris-SO$_4$ buffer; c, pH 9 in 50 mM Tris-SO$_4$ buffer plus 500 mM NaCl; d, pH 6 in 50 mM Tris-SO$_4$ buffer plus 500 mM NaCl. CaCl$_2$ (20 mM) was present in all samples.
- Absorption peaks at 8982.7 eV, have been assigned to the 1s $\rightarrow$ 4pz and 1s $\rightarrow$ (s+p)$^*$ transitions, respectively (15). In a separate study, the intensities of these transitions were correlated with the coordination geometry of Cu(I) based on a systematic analysis of x-ray edge spectra for a collection of model complexes having different coordination geometries but the same tridentate nitrogen ligands (20, 22, 24). More precisely, the intensity of the 8982.7 eV feature, normalized to the edge jump, was found to be inversely proportional to the mean displacement along the z-axis of Cu(I) from the plane defined by the three directly coordinated nitrogen atoms. The intensity is significantly higher for the metal ion coordinated in a trigonal planar geometry compared with that for pseudotetrahedral geometry; high intensity corresponds to trigonal planar coordination (22, 27) and low intensity occurs for pseudotetrahedral coordination (22, 28). Therefore, the 8982.7 eV feature can serve as a spectroscopic probe of coordination geometry in Cu(I) sites with all nitrogen coordination as in deoxyHc and was used here to define the structural modifications induced by changes in pH.

The k-edge spectra for P. interruptus deoxyHc at different pH values are collected in Fig. 6. The oxygen affinity of this Hc increases $\sim$35-fold from pH 5 to 9 (Table 1) (25, 26). At pH 5.25, the intensity of the 8982.7 eV feature is low (Fig. 6a). As the pH is raised to 7, and finally to 9, the intensity increases (Fig. 6, b and c). This spectroscopic change is indicative of the alteration of Cu(I) geometry from pseudotetrahedral toward trigonal (22).

The spectral changes observed are not due to oxidation of Cu(I) to Cu(II) because the lineshape of the x-ray absorption edge spectra collected at different pH values overlay in the 9000 eV region, with no indication of an energy shift expected if Cu(I) were oxidized to Cu(II). A smaller increase in the intensity of the 8982.7 eV feature with increasing pH was noted for subunit II of L. polyphemus Hc (Fig. 7) compared with the data described for P. interruptus Hc. This protein also has a Bohr effect (18), and the structural modulation induced by pH is considered here to be analogous to that described for P. interruptus Hc. This change in structure is suggested to correlate with the Bohr effect known for the proteins investigated here. Pseudotetrahedral coordination geometry at low pH for both Hcs is interpreted as a change in coordination geometry from pseudotetrahedral at low pH toward trigonal planar at high pH. This change in structure is suggested to correlate with the Bohr effect known for the proteins investigated here. Pseudotetrahedral coordination geometry at low pH for Hc in solution is in agreement with the x-ray crystal structure of P. interruptus deoxyHc at pH 4.8 (3). The trigonal geometry of the high pH form of Hc cannot be corroborated by a comparison with an x-ray crystal structure as none is available at alkaline pH. It may be noted that the k-edge spectrum of Cu(I) in reduced SOD (28) closely resembles that of the high pH form of deoxyHc in terms of the intensity of the 8982.7 eV feature. This spectroscopic change is indicative of the alteration of Cu(I) geometry from pseudotetrahedral toward trigonal (22).
of the relative intensity of the 1s → 4p$_x$ band compared with the edge jump. The x-ray crystal structure of reduced SOD (Pdb file1ds) shows a trigonal coordination geometry for Cu(I), with three nearly equal Cu-N bond lengths and angles of 120° between these bonds, similar to the crystal structure for Hc at high pH. It is suggested that for deoxyHc, the changes in geometry as a function of pH and the resulting changes in oxygen affinity derive from constraints on the active site in response to changes in protein conformation.

Modulation of Oxygen Affinity in Hemocyanin—The interplay of many factors enters into the mechanism of oxygen binding and the modulation of oxygen affinity in Hcs. These include the coordination geometry of the copper, accessibility of the active site, the Cu-Cu distance and the redox potential of the copper. The latter is important since oxygenation involves electron transfer from each Cu(I) to oxygen to yield a Cu(II)-O$_2$-Cu(II) structure. Allosteric effectors may influence oxygen binding by producing alterations in the tertiary and quaternary structure of the multimeric proteins and their subunits that have effects on the active site. Here, we have focused on copper (I) coordination geometry as a parameter not recognized before as one having an impact on Hc function.

The flash photolysis results provide a quantitative approach to analyze the relationship between $k_{on}$ and oxygen affinity. The observed rate constant may be described according to Equation 1 (28).

$$k_{obs} = k_{on}[\text{deoxyHc} + [O_2]] + k_{off} \quad \text{(Eq. 1)}$$

Because close to 100% of Hc was oxygenated in all samples (Hc concentration is 1 μM and dissolved O$_2$ is 220 μM or greater) and less than 5% of the total protein is monitored in the re-binding experiment, the deoxyHc concentration term can be neglected in Equation 1. Least squares fitting of a plot of $k_{obs}$ against [O$_2$] at pH 8.3 (Fig. 3B, black curve) using Equation 1, gives $k_{on}$ and $k_{off} = (6.35 ± 0.05) \times 10^7 \text{M}^{-1} \text{s}^{-1}$ and 1,800 ± 500 s$^{-1}$, respectively. From these values, the oxygen dissociation constant ($K_D$) was calculated as $28 ± 8 \mu M$ at pH 8.3. This $K_D$ value was relatively close to that calculated (13 ± 1 μM) from the reported binding constant for $C. aestuarii$ Hc obtained from equilibrium measurements (1). The only other available kinetic parameters are $k_{on}$ and $k_{off}$ of oxygen binding in $P. interruptus$ Hc obtained by temperature-jump and stopped-flow measurements, respectively (29). Although the reported $K_D$ value for $P. interruptus$ Hc is smaller (2.0–9.1 μM), most likely because the measurements were carried out in the presence of 100 mM NaCl, which is known to act as a heterotropic effector increasing oxygen affinity (30). From the temperature-jump measurement, a $k_{on}$ value was estimated to be $3.2 \times 10^7 \text{M}^{-1} \text{s}^{-1}$, which is close to the value found here, but again was measured in the presence of 100 mM NaCl.

Here, the slope of the fitted curve (Fig. 3B, red curve) for oxygen rebinding rate was lower for the data at pH 6.5 than 8.3, with a calculated $k_{on} = (4.91 ± 0.06) \times 10^7 \text{M}^{-1} \text{s}^{-1}$ and $k_{off} = 1,800 ± 700 \text{s}^{-1}$. While the $k_{off}$ value remains the same at the two pH values within experimental error, the $k_{on}$ value decreases at low pH. The observed pH dependence of $k_{on}$ may be correlated with geometric flexibility of Cu(I), which can reasonably be assumed to govern the modulation of oxygen affinity. The pH-independent character of $k_{off}$ is consistent with the pH independence of both the optical spectra of oxyHc and the k-edge absorption properties of several oxyHcs (31). These observations are also consistent with the common square pyramidal structure for copper in the oxygenated Type III copper site found in tyrosinase (32), other Hcs (33) and catechol oxidase (34).

In the flash photolysis experiment, we are observing the behavior of a deoxygenated site in a nearly fully oxygenated R-state hexamer for the case at pH 8.3 and high oxygen concentration at pH 6.5. A plausible interpretation of the observed changes in $k_{on}$ is that the single deoxygenated site that is part of R-state hexamers, may in the time frame of the experiment, rearrange into different structures (R') in response to the pH of the medium such that oxygen binding is favored at higher pH. Furthermore, oxygen binding data (1, 35) for both $C. aestuarii$ and $P. monodon$ Hcs show that $K_T$ and $K_R$ both increase with pH (supplemental Fig. S2). The structural rearrangements of the deoxy site in the R multimer can then be the origin of the pH dependence of $K_{nu}$, this parameter being the oxygen dissociation constant from the R state. Therefore, it seems reasonable, and it is the simpler interpretation, that the parallel pH dependences of $K_T$ and $K_R$ reported earlier arise from the same phenomenon, i.e. a change in the coordination geometry of Cu(I) in deoxygenated sites. This allows us to assume that the deoxygen sites in the photolysis experiments are sensitive to pH in a similar way in a hexamer that is oxygenated or deoxygenated.

FIGURE 8. Reaction scheme for oxygenation of arthropod hemocyanin including a change in geometry of copper in the deoxygenated active site with pH. A. Square pyramidal, oxyHc active site structure drawn from the x-ray crystal structure coordinates of L. polyphemus Hc (1OXY.pdb); B. trigonal, active site of P. interruptus deoxyHc (1HCY.pdb) assumed here to be representative of the low pH form. C. pseudotetrahedral, active site of L. polyphemus deoxyHc (1LLA.pdb) assumed to closely represent the alkaline pH form. The software PDBeviewer (40) was used to illustrate the structures. The $k_{on}$ and $k_{off}$ represent the on-rates discussed in the text for the high and low pH conditions.
Central to this idea is the fact that all oxygenated type III structures reported from x-ray crystal data are superimposable, despite the fact that the Hcs are from organisms that belong to different phyla, and the proteins may have been crystallized at different pH values (3–4). Oxygen dissociation occurs from this unique structure, which would explain the pH independence of \( k_{\text{off}} \) values found here. It is reasonable then to suggest that a general mechanism for arthropod Hcs to modulate oxygen affinity involves a structural rearrangement in the deoxygenated protein that produces a change in \( k_{\text{on}} \). This conclusion is substantiated by the comparison of Figs. 6 and 7 where a greater magnitude of spectral change was observed for \( P. \) interruptus deoxyHc compared with \( L. \) polyphemus Hc. The former protein has a greater difference in oxygen affinity per pH unit change than does \( L. \) polyphemus Hc. In further support of this concept, it should be noted that the presence of a Bohr effect for the binding of carbon monoxide to \( P. \) interruptus deoxyHc (36), a ligand that binds to only one copper, can be rationalized by a change in coordination geometry of a single Cu(I) with pH. Previous suggestions have focused instead on a change in copper–copper distance to explain the phenomenon (4, 6).

During oxygen coordination, ligand reorientations must occur as the copper coordination geometry is altered from distorted trigonal planar (or pseudotetrahedral) to square pyramidal in the Cu(II)-oxy form. A calculated reaction coordinate for reversible oxygen binding was investigated for Hc (24), and it was proposed that the protein matrix holds the Cu(I) ions in close proximity and therefore enables a simultaneous 2-electrons transfer. The structural effect of pH on the individual Cu(I) sites of the binuclear center proposed here may result in a modulation of both the redox potential of the individual copper ions and also the Cu-Cu distance.

The thermodynamic data reported here show that the \( \Delta H^\circ \) and \( \Delta S^\circ \) values for oxygenation of Hc are comparable to those reported for the \( \mu \eta^2-\eta^2 \)-peroxodicopper(II) and superoxo model compounds (\( \Delta H^\circ = 8.9 \) and \( 7.2 \) kcal/mol and \( \Delta S^\circ = -15 \) and \(-13 \) cal/(molK), respectively) (21). The similarity between the parameters for superoxo binding and peroxo binding and the overall similarities between the models and the results for Hc suggest that superoxo complex formation may be the rate-limiting step, and the peroxo species could be produced relatively easily from the superoxo species during the \( \mu-\eta^2:\eta^2 \)-peroxodicopper(II) model complex formation and during Hc oxygenation as well. The latter is in agreement with a nearly simultaneous 2-electron transfer to O\(_2\) as previously suggested (24). Also, the similarity in the parameters for Hc and the models suggests that despite the fact that the ligand must diffuse through the protein matrix to the active site, the interactions do not have an impact on the thermodynamics of oxygen binding.

It should be mentioned that the molecular basis of the Bohr effect proposed here implicates a structural change in coordination geometry for the copper only, leaving the question of identifying the role of individual amino acids to future studies. The ionization state of amino acids throughout the Hc molecule and changes in conformation of the polypeptide involved in the Bohr effect are expected to also affect the R/T equilibrium and the Hill coefficient, which combined produce the pH dependence of the oxygen binding parameters. Salt bridges between some conserved histidine and glutamate residues on the contact surface between monomers of \( E. \) urypelma californicum Hc (37) are proposed to be important. Possible mediators of allostery signals have been recently described for \( L. \) polyphemus Hc (38). The deoxy protein is known to exhibit different conformations, which are evident in changes in the binding of small molecule allosteric effectors. Whether pH effects on the binding of these molecules and the Bohr effect are related remain to be demonstrated (39). However, unraveling the details of the mechanism that links the active sites in the oligomers leading to conformational changes, will require further study. In conclusion, the analysis presented here provides a reasonable general explanation of the structural relationship between metal geometry and the Bohr effect in arthropod Hcs.

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