EXAFS Studies of Binuclear Copper Site of Oxy-, Deoxy-, Metaquo-, Metfluoro-, and Metazidohemocyanin from Arthropods and Molluscs

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Abstract: The extended X-ray absorption fine structure (EXAFS) beyond the Cu K edge has been analyzed for oxy-, deoxy-, and metaquohemocyanin (Hc) derivatives from a mollusc, *Busycon canaliculatum*, and arthropods representative of the two subphyla *Limulus Polyphemus* and *Cancer Irroratus* and also for the fluoride and azide metHc adducts for *Busycon* and *Limulus*. The oxyHc EXAFS Fourier transforms (FTs) show a prominent outer-shell peak due to scattering from the partner Cu, at a distance of 3.58–3.66 Å. The FTs for deoxy- and metHc show a pair of outer-shell peaks attributable to the distant atoms of bound imidazole, but imidazole alone does not account for the backtransformed outer-shell EXAFS. An additional scattering atom, at 3.39–3.48 Å, is required in all cases to reproduce the data in the beat-node region. Due to a phase ambiguity, the extra atom could be Cu, N, or O. As the antiferromagnetic behavior of the met derivative is associated with an endogenous protein bridge which requires a Cu–Cu distance of <4 Å (a group I ligand effect), the additional scattering atom is most logically assigned as the partner Cu; only a small decrease is observed in Cu–Cu distance upon deoxygenation. The results of the first-shell EXAFS fitting show the Cu coordination number to be four in oxy- and metHc and two in deoxyHc. Thus a significant change in ligation correlates with cooperativity in hemocyanin oxygenation, consistent with the strong involvement of the endogenous protein bridge indicated by recent spectral probe studies. Binding F^- to metHc produces very little change in the EXAFS pattern, while binding N_3^- increases the Cu–Cu separation to 3.66 Å. The three species studied show only minor differences in the EXAFS-derived structural parameters for any of the Hc forms. Hence, the strong catalase activity present only in mollusc hemocyanins must arise from variations in the active-site residues which do not significantly affect the Cu coordination number, ligand type, or bond lengths.

Hemocyanin (Hc), the cooperative O_2-carrying protein of mulluscs and arthropods,[1] has been of particular interest because of its coupled binuclear copper active site,[2] which appears to be extremely similar to the binuclear copper active site of the monooxygenase tyrosinase[3a] but significantly different[3b,c] from the O_2-binding binuclear copper site of the multi-Cu oxidase laccase.[4] The chemistry and spectroscopy of the binuclear site of Hc have been studied extensively,[5-7] and chemical differences between Hc from the two phyla have been noted,[5] which appear to correlate with the relatively higher catalase activity present in the mollusc Hc.

While no high-resolution protein crystal structure is presently available, X-ray absorption spectroscopy has provided important structural parameters of the Hc active site.[8,9] The energy and shapes of the K edges confirm Cu^I and Cu^{II} oxidation states for deoxy- and oxyHc, respectively. The postedge fine structure (EXAFS) established that the Cu nearest neighbors were first-row atoms only (i.e., N and O), hence excluding any sulfur ligation. The EXAFS modulation also showed contributions from the distant atoms of imidazole ligands and from the other Cu atom in the binuclear site. For oxyHc the Cu scattering contribution was unequivocal and determined Cu–Cu distances in the molluscs of 3.67 Å for *Busycon canaliculatum* and 3.55 Å for *Megathura crenulata*.[9a,10] For deoxyHc from *Busycon*, Brown et al.[8] located a distant scatterer at ~3.4 Å, a distance that made structural sense for Cu but not for a first-row atom (the phase of the modulation could not clearly distinguish between the two). Hodgson et al.,[9a] however, reported no distant scattering contribution for deoxyHc from *Megathura crenulata*, beyond that of imidazole, which they determined by a group fitting procedure.[9c]

Since the Cu–Cu distance in deoxyHc is of considerable importance in defining potential protein structure changes concomitant with oxygenation, we have reexamined the EXAFS modulation of deoxyHc not only for *Busycon* but also for two species of arthropods that belong to different subphyla, *Limulus Polyphemus* and *Cancer irroratus*. For all three we find clear evidence for a distant scatterer at 3.4–3.5 Å, which is most plausibly Cu; the data cannot be accounted for by imidazole scattering alone. The oxyHc EXAFS modulation for *Limulus* and *Cancer* is similar to that of *Busycon*; the Cu–Cu distances are all close to 3.6 Å. We have also examined metaquoHc from these species. The Cu–Cu distances are nearly the same as for deoxyHc and somewhat shorter than those of oxyHc; yet the coordination number of metHc, like that of oxyHc, is two units higher than that of deoxyHc. Thus, a change in ligation (perhaps involving the endogenous protein bridge) rather than a change in Cu–Cu distance appears to correlate with cooperativity in oxygen binding. Further, we find that binding azide to metHc of *Busycon* or *Limulus* increases the Cu–Cu distance to 3.66 Å, slightly larger than but within the uncertainty of that found in oxyHc, reflecting the bridging role of the azide ligand.[11] Finally, as the EXAFS of the met derivatives from both phyla appear to be extremely

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similar, the changes in the met site that correlate with catalase activity[5] cannot involve coordination number, type of ligand, or bond lengths but appear to relate to the coordination geometry of the exogenous ligand or its interaction with protein residues at the active site.

Experimental Section

Proteins. Hemolymph was obtained from *Busycon canaliculatum* by foot puncture, from *Limulus polyphemus* by heart puncture, and from *Cancer irroratus* by removal of several legs.[5] The Hc was isolated and purified by ultracentrifugation and extensive dialysis at 4 °C in pH 6.3 phosphate buffer. The deoxy proteins were prepared by freeze–thaw–pumping or equilibration with water-saturated argon. *Busycon* metHc was prepared by incubation of oxyHc at 37 °C with a 100-fold excess of F^- for 48 h[12a] in pH 5.2 acetate buffer followed by extensive dialysis against pH 6.3 phosphate buffer. The arthropod met forms were obtained by oxidation of deoxyHc with a fivefold excess of H_2O_2.[12b] Dialysis into excess N_3^- or F^- solution was used to prepare the metHc azido and fluoro complexes. The proteins were concentrated to ~4–5 mM via ultrafiltration.

The following reference compounds were prepared according to the cited references: (1) copper(II) tetraphenylporphine (CuTPP);[13] (2) $[(ImH)_4Cu]SO_4$;[14] (3) aqueous copper(II) imidazole;[8] (4) $[(ImH)_3(H_2O)Cu]SO_4$;[14] (5) CuO (purchased from Matheson Coleman and Bell); (6) Cu metal; (7) [(*N,N',N'',N'''*-tetramethylethylenediamine)$Cu^{II}OH]_2Br_2$;[15] (8) [(2,2'-bipyridyl)$Cu^{II}OH]_2{\cdot}SO_4{\cdot}5H_2O$.[16] The solid samples were diluted by 90% with sucrose or boron nitride to allow X-ray fluorescence measurements to be made similarly to the protein samples.

X-ray Measurements. EXAFS data collection was conducted during dedicated operation of the SPEAR storage ring (3.0 GeV, 40–80 mA) at the Stanford Synchrotron Radiation Laboratory.[17-20] All data were collected on beam line I-5[17] using an array of plastic scintillation counters[19] together with an appropriate filter[20] to collect the Kα fluorescence. The samples were mounted in plexiglass holders with mylar windows and contained in a cryostat[19] whose temperature was maintained at ~−100 °C. Sample integrity was ensured by monitoring the Cu K edge and optical spectra immediately prior to and following EXAFS collection.

Analysis. K-edge and EXAFS data were analyzed as described previously.[8,19,20] (The equation for the EXAFS modulation is given in these references.) Approximately nine single scans were averaged together to enhance signal to noise. The free-atom contribution was subtracted by using a cubic B-spline, and the data were multiplied by k^3 to compensate for the $\sim k^{-3}$ dependence expected beyond $k \simeq 4$ Å^{-1}. The background-subtracted, k^3-weighted data were Fourier transformed and then backtransformed after single peaks or unresolved multiple peaks were isolated with a Fourier filter. The backtransformed data were then fit to well-characterized model compounds.[8] Both coordination number (N) and the difference in the Debye–Waller factor relative to the model ($\Delta\sigma^2$, model − sample) were allowed to vary separately and in all cases were found to be highly correlated. The parameter $\Delta\sigma^2$ contains information about both static structural disorder and thermal vibrations; a negative value indicates more disorder in the sample than in the model. The change in edge or threshold compared with model compounds was also a variable but was small (±3 eV) in all cases. The goodness of fit was described by χ^2, the sum of the residuals squared.

Results

The K-edge spectra of oxy- and deoxyHc from *Busycon*[8] and *Megathura*[9a,b] were analyzed previously and shown to be characteristic of imidazole-coordinated Cu^{II} and Cu^{I}, respectively. We found the edges to be essentially the same for *Limulus* and *Cancer* oxy- and deoxyHc as for *Busycon*. In addition, the metHc edge spectra were examined and found to be similar to those of oxyHc, consistent with their assignment as also containing antiferromagnetically coupled Cu^{II} ions.[2]

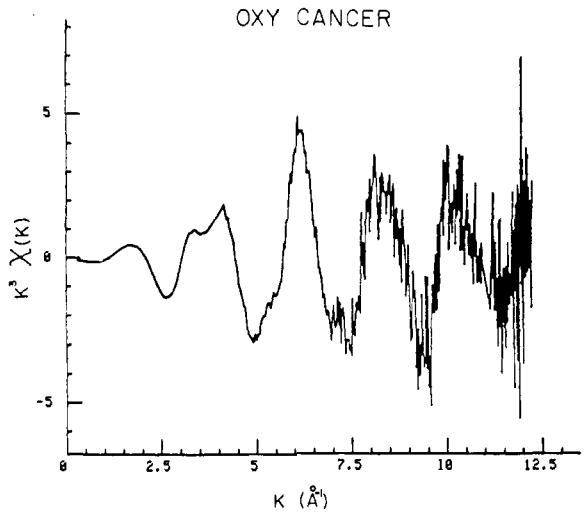

Figure 1. Background subtracted, k^3 weighted EXAFS modulation of oxyHc from *Cancer irroratus*.

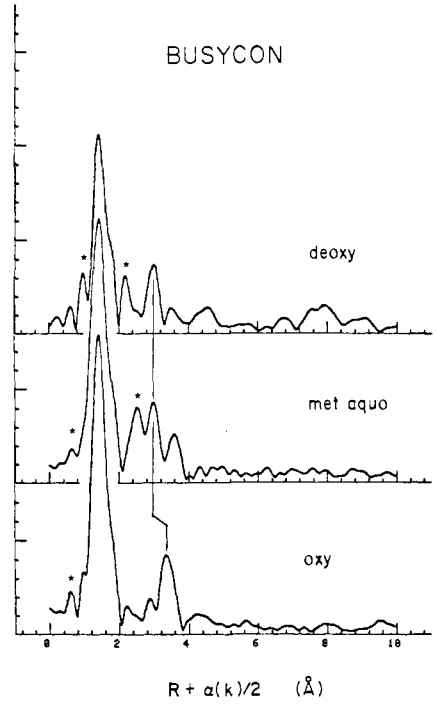

Figure 2. Fourier transforms of k^3 weighted EXAFS of deoxy-, met- and oxyHc from *Busycon Canaliculatum*. FT peaks marked with asterisks are side lobes due to truncation effects. The line connects peaks with Cu scattering contributions.

Figure 1 is an example of the EXAFS modulation, for oxyHc of *Cancer*, after background subtraction, conversion of the X-ray energy of k, the photoelectron wave vector, and multiplication by k^3. The data are of excellent quality, and the modulation is well-defined out to $k = 12$ Å^{-1}. Figures 2–4 show the Fourier transforms (FTs) of these data for oxy-, met-, and deoxyHc of the three species included in this study.

The FTs are dominated by a peak at $R + \alpha(k)/2 \simeq 1.6$ Å, representing scattering from the nearest-neighbor atoms in the Cu coordination groups. At higher, and sometimes lower, $R + \alpha(k)/2$, are subsidiary peaks, marked with asterisks, whose intensity and position vary with the length of the data set; these are due, in part, to side lobes produced by the finite length of the data set. It is possible, however, that the peak at $R + \alpha(k)/2 \simeq 2.2$ Å also contains contributions from weakly bound (axial) ligands.

The outer shells are attributable to a superposition of contributions from the other Cu atom of the site and from distant atoms

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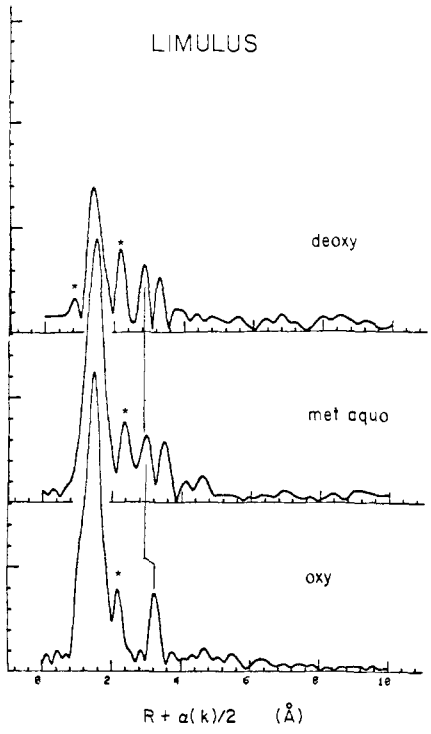

Figure 3. Fourier transforms for *Limulus Polyphemus*, as in Figure 2.

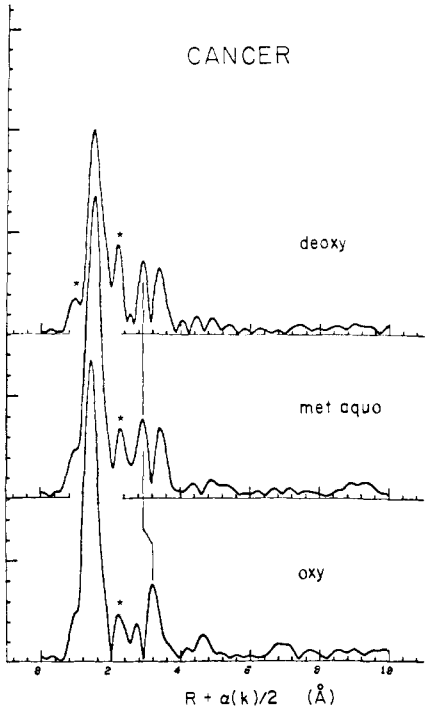

Figure 4. Fourier transforms for *Cancer irroratus*, as in Figure 2.

of the imidazole rings, which are known from resonance Raman,[21] nuclear modulate spin echo,[22] and chemical modification[23] studies to be coordinated to the Cu atoms. As noted previously[8,9] metal imidazole complexes characteristically show two outer-shell peaks (illustrated in Figure 5), which are attributable to the imidazole C_2,C_5, and N_3,C_4 atom pairs, which are held by the rigid rings at fixed distances (~3 and ~4 Å) from the Cu atom. Similar outer-shell peaks have been observed for Zn and Co carbonic

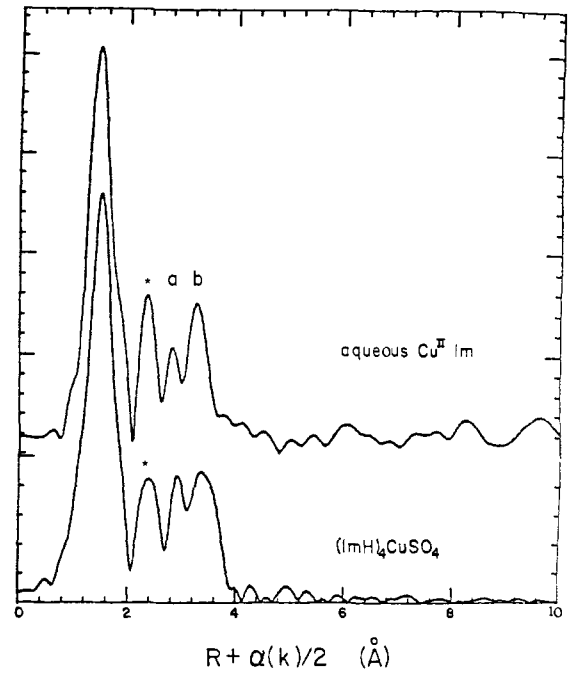

Figure 5. Fourier transforms of k^3 weighted EXAFS of Cu^{II} imidazole reference compounds; aqueous copper(II) imidazole and $[(ImH)_4Cu]SO_4$ solid. The peak marked with an asterisk is largely a truncation side lobe, while the peaks marked a and b are due to the imidazole outer shells (see text).

anhydrase,[24] in which the metal ion is known to be bound by three histidine side chains.[25] However, the amplitudes of the imidazole outer-shell peaks vary in a manner that is not currently understood. Note that their relative intensities are somewhat different in the FTs of the two Cu–imidazole compounds shown in Figure 5. In the case of Zn carbonic anhydrase,[24] the relative intensities of the two peaks reverse with changing pH, although their positions remain unaltered. Presumably, these intensity variations result from multiple-scattering phenomena, possibly associated with the relative orientation of the imidazole rings. The theory of multiple scattering[26] is insufficiently developed to pursue this hypothesis at present.

In addition to these relative intensity variations, the two outer-shell peaks can vary in intensity relative to the first-shell peak. This is illustrated most dramatically by the three oxyHc's (Figures 2–4) in whose FTs the outer-shell imidazole peaks can scarcely be seen. Rather, this region is dominated by a single peak, which has been unambiguously identified as due to Cu on the basis of the shape of the backtransformed wave.[8] In contrast, the FTs of both metHc and deoxyHc contain strong imidazole outer-shell peaks but, as demonstrated below, the inner of the two contains an additional scattering contribution from an atom at ~3.4 Å, again most plausibly Cu.

Outer-Shell Fits. In our previous study of *Busycon* Hc,[8] we fit single outer-shell peaks to single scatters and showed that the shape of the backtransformed wave identified the scatterer as Cu for oxyHc, at 3.67 Å, and either Cu or N for deoxyHc, at ~3.4 Å, the contribution being somewhat ambiguous at this distance. Comparison with model Cu dimers and Cu–N compounds suggested Cu, however, as the most likely assignment.[8] Since both oxy- and deoxyHc had FTs (Figure 2) that happened to be dominated by single outer-shell peaks, this was a reasonable procedure. However, deoxyHc from *Limulus* and *Cancer*, and metHc from all three species, showed unmistakable imidazole outer-shell doublets (Figures 2–4), clearly indicating that imidazole

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Table I. Filtered Outer-Shell EXAFS Analysis for Cu + ImH Scattering[a]

	Cu			ImH			
	R, Å	N	$\Delta\sigma^2 \times 10^3$	R,[b] Å	N	$\Delta\sigma^2 \times 10^3$	X^2
oxy							
Busycon	3.66	1.0	1.6	3.45	1.0	3.0	0.52
Limulus	3.62	1.0	−1.7	3.40	0.4	1.0	0.28
Cancer	3.58	1.0	−0.2	3.40	1.0	3.0	0.16
deoxy							
Busycon	3.43	1.0	−2.3	3.54	0.8	1.5	0.04
Limulus	3.46	1.0	−7.0	3.49	1.7	2.0	0.17
Cancer	3.48	1.0	−7.0	3.53	1.2	5.5	0.84
metaquo							
Busycon	3.45	1.0	−4.2	3.59	1.4	2.8	0.20
Limulus	3.45	1.0	−6.0	3.59	1.1	3.5	0.47
Cancer	3.39	1.0	−7.8	3.53	1.5	3.5	0.55
metazido							
Busycon	3.66	1.0	−6.4	3.50	1.9	−4.9	0.36
Limulus	3.66	1.0	−3.7	3.45	1.8	0.6	0.58
metfluoro							
Busycon	3.50	1.0	−4.0	3.59	1.0	4.0	0.30
Limulus	3.49	1.0	−2.0	3.59	1.1	4.0	0.29

[a] Cu scattering referenced to [(*N*,*N*′,*N*″,*N*‴-tetramethylethylenediamine)$Cu^{II}OH]_2Br_2$; ImH scattering referenced to aqueous copper(II) imidazole. R, N, and $\Delta\sigma^2$ are the distance, number of scatterers, and the change (model − sample) in the Debye-Waller factor, all referenced to the model compounds. (R (Å) ± 0.05; N ± 20%; $\Delta\sigma^2$ (Å^2) ± 1.5 × 10^3). X^2 is a goodness-of-fit parameter, representing the square of the residuals between model(s) and protein. [b] Average distance from Cu to the center of the ImH rings, taken as 3.50 Å for aqueous $Cu^{II}(ImH)_2$. On the assumption that the ImH rings retain the same orientations in the proteins, the Cu-N(ImH) bond distances can be obtained by subtracting 1.50 Å from R. These distances deviate from the Cu–ImH first-shell distances (Table II) by up to 0.12 Å. The differences are attributable to a combination of fitting uncertainties, multiple-scattering effects, and imidazole orientation changes.

contributions must be considered.

Our procedure was to filter the entire region of the imidazole outer-shell peaks ($R + \alpha(k)/2 \approx 2.4$–4.0 Å) and to fit this backtransformed EXAFS to two sets of scatters, Cu and ImH, using [(*N*,*N*′,*N*″,*N*‴-tetramethylethylenediamine)$Cu^{II}OH]_2Br_2$ and either $[(ImH)_4Cu]SO_4$ or aqueous $Cu^{II}(ImH)_2$ as reference compounds. The OH^- bridged Cu dimer was chosen over Cu metal as our model since it better represents the protein with respect to ligation and structure.[2,6,8] The ImH was given a single variable distance (3.5 Å, an approximate average of the second- and third-shell distances); i.e., it was allowed to move radially as a rigid body. This distance along with $\Delta\sigma^2$, N, and E_0, was allowed to vary (see below) until the best fit was obtained (with respect to χ^2 and physically reasonable parameter values). At this point one Cu atom was introduced, and the protein data were refit, allowing $\Delta\sigma^2$, E_0, and σ to vary for Cu as well. In the final fit the number of Cu atoms (which was previously held at one) was also varied, but it did not change significantly, nor did it appreciably affect χ^2. This procedure is similar to the group fitting analysis of Hodgson and co-workers,[9] with the critical difference that only the outer-shell scattering is being fit. When the entire EXAFS data set is included[9] the contributions from nearest-neighbor scattering dominate the fit. It is essential to filter out first-shell contributions if alternative distant scatters are to be discriminated.

The results of the outer-shell fits are given in Table I and reveal a clear and consistent pattern. Oxy-, deoxy-, and metHc from all three species show contributions from one Cu and from ImH in small and apparently variable abundance, reflecting the variable intrinsic ImH outer-shell amplitudes discussed in the preceding section. As reported previously for *Busycon* deoxyHc[8] and some model compounds, larger negative Debye–Waller factors are observed for deoxyHc of arthropod Hc. However, the Cu–Cu distances are ~3.64 Å for oxyHc and ~3.45 Å for *both* deoxy- and metHc. There is a slight variation among the species, with oxyHc of *Busycon* having a larger Cu–Cu separation (3.66 Å) than *Limulus* (3.62 Å) or *Cancer* (3.58 Å) and deoxyHc or *Busycon* having a smaller Cu–Cu separation (3.43 vs. 3.46 and 3.48 Å); these differences are of the same order as the uncertainty (~ ± 0.05) of the multiparameter fits. There may, however, be a greater Cu–Cu expansion upon oxygenating deoxyHc from *Busycon* (0.23 Å) than *Limulus* (0.16 Å) or *Cancer* (0.10 Å) (±0.07 Å).

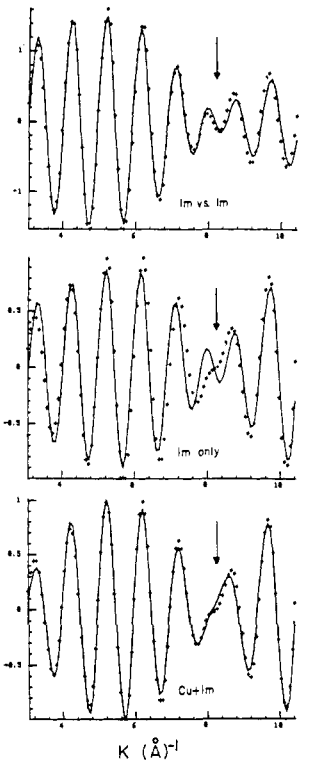

Figure 6. Comparison of backtransformed outer-shell Fourier transforms: top, data for $[(ImH)_4Cu]SO_4$ (+) fit to aqueous copper(II) imidazole (solid line); middle, data for *Cancer* metHc (+) fit to $[(ImH)_4Cu]SO_4$ (solid line); bottom, data for *Cancer* metHc (+) fit to $[(ImH)_4Cu]SO_4$ plus Cu.

To test the reality of the Cu–Cu scattering contribution we tried to fit the outer-shell EXAFS with imidazole only. For oxyHc this proved impossible, as expected from the dominance of the single Cu peak in the FT. For deoxyHc and metHc, which showed imidazole outer-shell FT doublets, the fit worsened significantly if Cu was omitted (χ^2 increased by a factor of 6 or more). More importantly, the shape of the filtered outer-shell EXAFS in the critical beat-node region could not be reproduced with ImH alone. This is illustrated by the *Cancer* metHc data, shown in Figure 6. The interference pattern at $k \simeq 8$ Å^{-1} is distinctly different for the protein and for Cu–ImH reference compounds and requires scattering by both Cu and ImH. Although different Cu–ImH reference compounds show variability in the outer-shell amplitudes (Figure 5), they can, nevertheless, be fitted to one another if N, $\Delta\sigma^2$, and E_0 are allowed to vary slightly (see the top of Figure 6). When this is done, the residuals show that the model-to-model fit is off slightly over *the entire k* range, whereas the model-to-protein fit without Cu is off primarily at the $k = 8$ Å^{-1} beat node, in the region where the heavy-atom scattering amplitude is expected to maximize. For the protein, the optimized ImH-only fit clearly fails; this was true even when the calculation allowed for two ImH groups with separately variable Cu–ImH distances.

An adequate fit can also be obtained, however, if the Cu is replaced by an additional N (or O) atom (not part of ImH). Strictly speaking, the EXAFS data for deoxy- and metHc require *either* Cu or N (or O) at ~3.4 Å, in addition to the bound ImH. A preference for Cu over N is, however, indicated by the k dependence of the data. Figure 7 shows that when the EXAFS modulation is multiplied by k^2 instead of k^3, the outer (Cu) peak

Table II. Filtered First-Shell Analysis for N and O Scattering[a]

	single scatterer "N"[b]				two scatters						
					"N"[b]			"O"[c]			
	R, Å	N	$\Delta\sigma^2 \times 10^3$	X^2	R, Å	N	$\Delta\sigma^2 \times 10^3$	R, Å	N	$\Delta\sigma^2 \times 10^3$	X^2
oxy											
Busycon	1.96	4.0	1.2	9.5	2.00	2.0	1.2	1.90	2.4	2.3	0.29
Limulus	1.96	4.0	1.7	6.0	1.98	2.0	4.0	1.86	2.5	−5.5	1.0
Cancer	1.95	3.8	1.0	10.0	2.02	2.0	−5.0	1.89	2.3	2.0	0.17
deoxy											
Busycon	1.95	1.9	3.5	0.6	1.95	1.0	4.3	1.92	1.3	−0.6	0.12
Limulus	1.94	1.6	2.6	3.2	2.00	1.0	−1.0	1.90	1.2	3.5	0.14
Cancer	1.96	2.0	3.5	6.5	1.98	0.9	3.5	1.95	1.0	3.4	0.50
metaquo											
Busycon	1.99	4.8	−2.0	1.6	2.02	2.0	0.7	1.91	3.5	−3.6	0.68
Limulus	1.99	4.0	0.2	10.0	2.02	2.0	3.5	1.91	3.5	−0.6	0.30
Cancer	1.98	3.2	2.8	8.0	2.02	1.9	1.5	1.94	2.1	3.5	0.30
metazido											
Busycon	2.00	3.5	1.5	7.0	2.04	2.0	0.8	1.94	2.0	2.6	0.67
Limulus	1.99	4.0	−5.0	1.2	2.02	2.0	−0.2	1.87	2.4		0.23
metfluoro											
Busycon	1.97	5.0	−1.2	4.8	2.03	3.0	−4.0	1.90[d]	2.2	0.2	0.29
Limulus	1.96	5.0	−0.8	4.1	2.02	3.5	−4.0	1.90[d]	2.0	1.3	0.50

[a] Symbols as in Table I; R (Å) ± 0.02; N ± 20%; $\Delta\sigma^2$ (Å^2) ± 1.5×10^3. [b] N scattering referenced to $[(ImH)_4Cu]SO_4$ (Cu-ImH = 2.01 Å).[14] [c] O scattering referenced to CuO (Cu-O = 1.95 Å).[36] [d] F scattering referenced to CuF_2 (Cu-F = 1.93 Å).

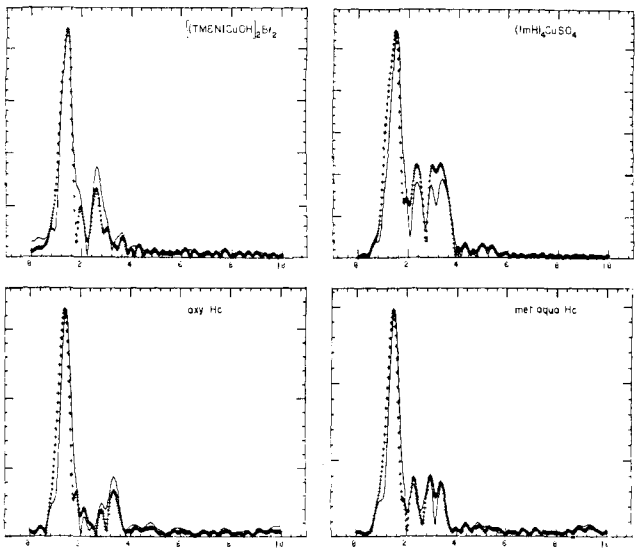

Figure 7. Variations in outer-shell FT peak intensities with k weighting (k^3, solid line; k^2, crosses) for Cu and ImH reference compounds (top) and oxy- and metaquoHc (bottom).

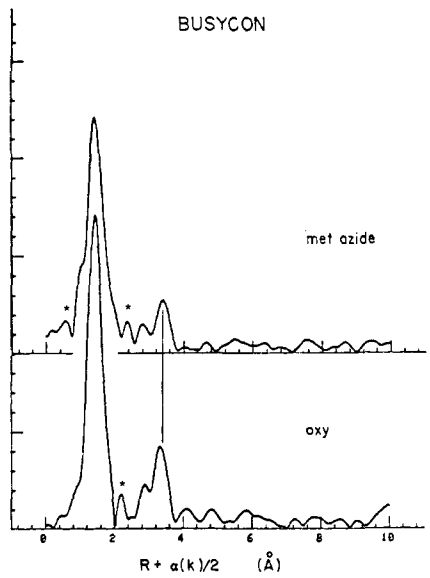

Figure 8. Fourier transforms of k^3 weighted EXAFS for *Busycon* oxy- and metazidoHc.

of the $[(N,N',N'',N'''$-tetramethylethylenediamine)$Cu^{II}OH)]_2Br_2$ FT decreases in relative amplitude, while that of the outer (N + C) peaks of the $[(ImH_4)Cu]SO_4$ FT increases. This is expected since heavy-atom scattering contributions maximize at higher k. The *Busycon* oxyHc outer peak also diminishes, confirming its assignment to Cu, while the outer peaks of met- and deoxyHc are essentially unchanged. Since the latter peaks contain a large ImH contribution, which is expected to be enhanced by the k^2 weighting, the lack of change implies a counterbalancing Cu contribution, which is expected to diminish with k^2 weighting. If the extra atom were from a first-row element, the outer peaks should have increased in relative amplitude.

First-Shell Fits. The FT first-shell peaks were filtered, back-transformed, and analyzed for nearest-neighbor scattering. As shown in Table II, the oxy-, deoxy-, and metHc data are all compatible with a single shell of N (or O) atoms, at 1.94–1.99 Å. As noted previously[8,9] for oxy- and deoxyHc, the data are incompatible with coordination by sulfur-containing ligands at any reasonable bond distance. The average single-shell coordination numbers are ~4 for both oxy- and metHc and ~2 for deoxyHc. These numbers show an appreciable range of variation, as expected, since N is significantly correlated with the Debye–Waller factor. The significant reduction in coordination number between oxy- or met- and deoxyHc is evident qualitatively in the FTs (Figures 2–4), which show a rougly twofold reduction in the deoxyHc first-shell peak heights.

Since Cu in oxyHc is bound to both N (ImH) and O (O_2 and, probably, an additional bridging O atom—see Discussion), the first-shell data were also fit to two sets of scatterers, N and O, using $[(ImH)_4Cu^{II}]SO_4$ and CuO as reference compounds (right side of Table II). The Cu–ImH distance is expected to be ~2.0 Å while Cu–O distances should be close to ~1.9 Å. A similar pattern was observed for metHc, in which bound O_2^{2-} is believed to be replaced by H_2O (or OH^-), although the Cu–O distance was slightly larger, and there was a tendency for the number of O's to exceed the number of N's by ~50%. For deoxyHc, the data were compatible with two different scatterers, with different distances for *Limulus*, 2.00 and 1.90 Å, but nearly the same distances for *Busycon* (1.95, 1.92 Å) and *Cancer* (1.98, 1.95 Å), all ± 0.02 Å.

MetazidoHc and MetfluoroHc. The FT of the *Busycon* metHc azide complex is compared with that of oxyHc in Figure 8. The similarity is evident, with the outer-shell scattering being dominated by a single peak at about the same distance in both proteins.

The outer-shell fit (Table I) gives similar parameters for metazido- and oxyHc for both *Busycon* and *Limulus* (*Cancer* was not studied), with a somewhat greater ImH contribution to the metazidoHc scattering (N = 1.8–1.9 vs. 0.4–1.0) but essentially the same Cu–ImH and Cu–Cu distances. Thus, binding of N_3^- to metHc increases the Cu–Cu separation (3.45 → 3.66 Å). The first-shell parameters (Table II) are also quite similar for metazido and oxyHc.

The FT's of the metHc fluoride complexes (not shown) are, however, similar to those of metaquoHc for both *Busycon* and *Limulus*. Outer-shell fitting (Table I) shows the same extent of imidazole contribution and a Cu–Cu distance, 3.50 Å (±0.05), that is slightly longer than, although within the error of, that of metaquoHc, but distinctly shorter than that of oxy- or metazidoHc. First-shell fitting (Table I) shows a slightly shortened average distance, which resolves, in the two-scatterer analysis, into a normal (2.00 Å) Cu–N distance and a short (1.90 Å) Cu–F distance.

Discussion

A principal finding of the present study is that the outer-shell EXAFS of deoxyHc from *Busycon*, *Limulus*, and *Cancer* cannot be accounted for by imidazole alone but require an additional scatterer at ~3.45 Å. This is true also for metHc, which shows outer-shell EXAFS quite similar to that of deoxyHc. While the distant atom could be N, O, or Cu, the k dependence of the data favors Cu. Moreover, there is no chemical rationale for a light-atom scatterer held at 3.45 Å, while Cu at 3.45 Å is quite reasonable for a binuclear site. Indeed, metHc must have the two Cu's in close proximity to account for the strong antiferromagnetic coupling of the Cu^{II} ions. If the 3.45-Å scatterer were assigned to a light atom, there would remain no contribution attributable to the partner Cu.

The magnetic coupling[27] is believed to be mediated by a bridging ligand (RO^-), likely tyrosine[27b] or perhaps hydroxide.[28] When one of the two Cu^{II} ions is reduced (i.e., the half-met derivative), this bridge remains intact but can be broken (as evidenced by the opening of an additional coordination site) with group 2 ligands (N_3^-, SCN^-, and CN^-), which are capable of bridging the two copper atoms with a Cu–Cu distance of >5 Å. It is uncertain whether the bridge is still maintained in deoxyHc, with two Cu^{I} ions, and if not, whether the RO^- group still remains bound to one copper.

OxyHc is properly viewed as the peroxide adduct of metHc, as shown by the low frequency, ~750 cm^{-1}, of the O–O stretch in the resonance Raman spectra.[21,29,30] A variety of chemical and spectroscopic data on a series of hemocyanin derivatives has indicated that exogenous ligands bridge the two copper ions at the active site.[2] In the case of oxyHc, assignment of the O_2^{2-} → Cu(II) charge-transfer spectrum using a transition dipole vector coupling model (TDVC) strongly supported a μ-1,2 peroxy bridging geometry.[6] The EXAFS results show that O_2^{2-} binding to the metHc causes a small but significant increase of 0.10–0.23 Å in the Cu–Cu separation, depending on the species. An increase of this magnitude could be accommodated by an increase in the Cu–O–Cu angle of the endogenous bridging ligand, RO^-, and is consistent with the proposal that the endogenous bridge remains intact in oxyHc.[2]

If the Cu–OR distance for oxyHc is taken as 1.90 Å (Table II), then at Cu–Cu = 3.65 Å, the Cu–O–Cu angle should be 145°. Recently, Coughlin and Lippard[28a] reported a hydroxide-bridged binuclear macrocyclic Cu^{II} complex, with structure parameters very close to these: Cu–O = 1.92 Å, Cu–Cu = 3.64 Å, ∠Cu–O–Cu = 144°; an antiferromagnetic coupling constant, $-2J$ = 1000 cm^{-1}, was calculated from its magnetic susceptibility. These results support the assignment[21] of a broad resonance Raman band of oxyHc, at 1075 cm^{-1}, to the singlet → triplet transition of the coupled Cu^{II} pair, which is expected at $\nu = -2J$. The coupling constant is sensitive to the Cu–O–Cu bridging angle[31] and is therefore expected to be somewhat smaller for metHc, which has a shorter Cu–Cu distance but essentially the same Cu–OR distance. At Cu–Cu = 3.45 Å and Cu–OR = 1.90 Å, the Cu–O–Cu angle would be 130°. These parameters are close to those found by Burk et al.[28b] for another hydroxide-bridged complex with Cu–Cu = 3.38 Å, Cu–O = 1.85 Å, and Cu–O–Cu = 132°; $-2J$ was determined to be 850 cm^{-1}. While we expect a similar value for metHc, finding a Raman band associated with the singlet → triplet transition is much harder than for oxyHc, since there is no strong charge-transfer band[2] to provide resonance enhancement.

The binding of N_3^- to metHc likewise increases the Cu–Cu separation to 3.66 Å. This expansion can be attributed to N_3^- bridging, since the presence of four charge-transfer transitions for metazidoHc implies coupled transitions to both Cu^{II} ions[2] (only two charge-transfer transitions are possible for N_3^- bound to a single Cu^{II}). Two bridging modes are available to N_3^-, 1,3 (sideways) or 1,1 (end on). The Cu–Cu distance is compatible with the 1,3 geometry, as shown by a recent crystal structure of a binuclear Cu^{II} complex with a 1,3 N_3^- bridge and Cu–Cu = 3.61 Å.[32] It is not compatible with 1,1 binding, provided that the endogenous bridge is intact, since the bridging atoms would then come into much too close a contact (1.0 to 1.6 Å if the Cu–bridge bond distances are 1.90–2.00 Å). Detailed temperature-dependent EPR studies on a number of met-X derivatives (X = aquo, F^-, Cl^-, Br^-, N_3^-) have demonstrated[33a] that they all contain antiferromagnetically coupled Cu^{II} ions, with the intact endogenous bridge providing the pathway for superexchange. A μ-1,3 azido geometry is also strongly suggested[33b] by the result of a TDVC analysis of the N_3^- → Cu^{II} charge-transfer spectrum with respect to the absorption and CD selection rules and the quantitiative splittings of the transitions due to coupling of the two metal centers. Resonance raman spectroscopy reveals two azide asymmetric stretches for the $^{15}N^{14}N^{14}N$ isotopic species, and a normal coordinate analysis indicates that the splitting is due to unequal N–N bond lengths. While this result is compatible with a μ-1,1 binding geometry, it could also arise from asymmetric H bonding or electrostatic interactions with protein residues in the vicinity of the active site.

The Cu–Cu distance, ~3.45 Å, determined for metaquo- and metfluoroHc is also too long to permit double monoatomic bridging (exogenous and endogenous) of the Cu^{II} ions. The cross-bridge contact would be 1.6–2.0 Å for Cu–bridge bonds of 1.90–2.00 Å. Consequently bridging by the exogenous F^- or H_2O (or OH^-) can be ruled out in favor of a structure maintaining the endogenous bridge and coordinating one F^- or H_2O to each Cu^{II} (Figure 9).

While the EXAFS-derived coordination numbers show some variability, they point toward four-coordination for all the Cu^{II} forms and two-coordination for deoxyHc. It was previously suggested[8] that *Busycon* oxyHc is five-coordinate (O_2, OR^-, and three ImH) based primarily on the complexity of the resonance Raman spectra,[21] which suggests three ImH ligands. However, the optical spectra of oxy- and metHc indicate normal tetragonal Cu^{II},[2] whose axial ligands should have appreciably longer bonds and are not expected to contribute significantly to the first-shell EXAFS. Consistent with this, the first-shell fits for oxy- and metaquo, metfluoro-, and metazidoHc all show ~2 N atoms at ~2.0 Å (ImH) when analyzed for two sets of scatters.

The loss of ~2 ligand atoms per Cu ion upon deoxygenation implies that one of the imidazole ligands or the endogenous bridge

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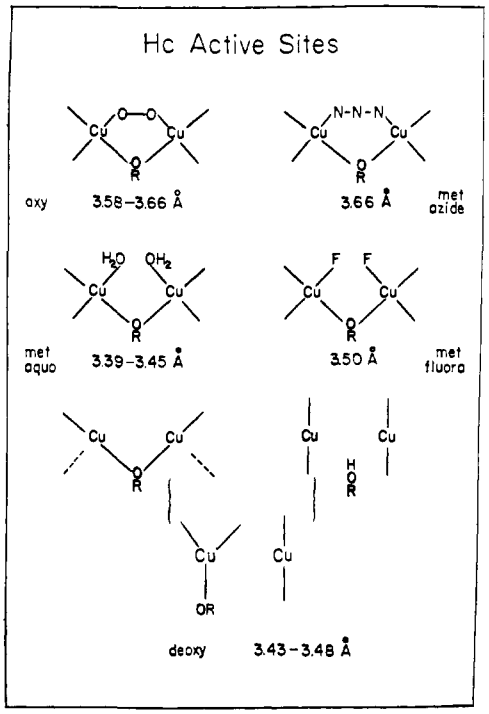

Figure 9. Models of the Hc active sites, showing the EXAFS-derived Cu–Cu distances and bonds to exogenous ligands, the endogenous bridge, OR^-, and, where not otherwise identified, endogenous imidazoles. Alternative possibilities are shown for deoxyHc.

also dissociates, presumably leaving two-coordinate Cu^I. Linear two-coordination is well-known in Cu^I chemistry.[34] Given the EXAFS error limits, it is also possible that the endogenous bridge dissociates from one of the Cu^I ions but remains bound to the other one. These alternative structures are illustrated in Figure 9. Since the Cu–Cu separation does not appear to change significantly, it is the change in ligation that must produce the protein conformation change that is implied by the cooperativity in O_2 binding displayed by Hc.[35] Indeed, EPR studies of a spectral probe derivative of Hc (containing a small fraction of EPR-detectable half-met set) have demonstrated ligation changes associated with the transition from the relaxed (oxy) to the tense (deoxy) quaternary structures.[36] The results are consistent with greater steric competition between exogenous liquids and the endogenous bridge in the tense structure.

Finally, a chemical and spectroscopic comparison[5] of the active-site derivatives of five mollusc and five arthropod hemocyanins has indicated that while all have qualitatively similar coupled binuclear copper active sites, there are quantitative differences in the spectra of these derivatives that reflect a distortion of the arthropod active site relative to that of the mollusc Hc. For the met derivative, this distortion appears to inhibit exogenous ligand binding and in the case of exogenous peroxide, regeneration to oxy, which is required for catalase activity. While the EXAFS parameters for *Busycon*, *Limulus*, and *Cancer* Hcs show some variability, the differences are small and are insufficient to account for these differences. Thus, this distortion cannot reflect a change in ligand number or type or in Cu–L or Cu–Cu bond lengths but appears to relate to the coordination geometry of exogenous ligand binding or its interaction with protein residues at the active site.

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Registry No. $[(InH)_4Cu]SO_4$, 76633-47-1; copper, 7440-50-8; oxygen, 7782-44-7; fluoride, 16984-48-8; azide, 14343-69-2; water, 7732-18-5; copper(II) imidazole, 14489-15-7.

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