to carbon and oxygen in the parent molecules.

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Supplementary Material Available: Tables of fractional atomic coordinates, thermal parameters, bond lengths, and bond angles for $Fe_5(CO)_{13}(CS)S_2$ (5 pages). Ordering information is given on any current masthead page.

Copper Site of Deoxyhemocyanin. Structural Evidence from X-Ray Absorption Spectroscopy

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Structural models for the copper site of oxyhemocyanin from *Megathura crenulata*,[1] *Helix pomatia*,[1] and *Busycon canaliculatum*[2] have recently been proposed using X-ray absorption spectroscopic (XAS) information. In this communication we report analogous XAS studies on the copper site of deoxyhemocyanin from *Megathura crenulata*. Extended X-ray absorption fine structure (EXAFS) has been analyzed as described previously in detail for the oxyprotein by using an imidazole group fitting technique.[1,3] This study on the deoxyprotein shows that each copper atom is coordinated to two imidazoles with an average Cu–N distance of 1.95 Å. No evidence of a copper–copper interaction within 4 Å is observed. A structural model for the binuclear binding site is presented from these EXAFS results. The model suggests two copper atoms each coordinated only to two histidines. Upon oxygenation, the two copper atoms are pulled together to 3.55 Å,[1] bridged by a low-Z atom, and the coordinated dioxygen with the dioxygen bound end-to-end to the binuclear copper site as a peroxide ion.[4]

Hemocyanin is a copper-containing protein found in molluscs and arthropods which functions as a dioxygen carrier. The smallest functional subunit contains two copper atoms and binds one molecule of dioxygen reversibly. The oxygenated form of the protein has been extensively studied over the past two decades.[5] However, studies on the other native form (deoxy) are relatively rare, since this derivative contains Cu(I) which is spectroscopically undetectable by both EPR and visible absorption techniques. Recently, an EXAFS study on hemocyanin from *Busycon canaliculatum* was reported.[2] The authors suggested a Cu–Cu interaction at 3.4 Å in deoxyhemocyanin. However, they also noted that the peak which was fitted with copper could also be fitted with low-Z scatterers.

Megathura crenulata hemocyanin was collected from live giant keyhole limpets (obtained from Pacific Biomarine, Venice, CA) as previously described.[6] The deoxygenated protein was prepared[7] by anaerobically dialyzing a purified sample at 4 °C for 3–4 days, in a 0.1 M phosphate buffer at pH 8.5 with 0.1 M $MgCl_2$. The buffer was kept deoxygenated by continuously bubbling N_2 through the solution. The colorless protein solution was finally centrifuged at 100000g for 3 h. The gel was transferred to an EXAFS cell which was kept in an anaerobic chamber continuously circulated with N_2. The protein remained colorless at the end of data collection period and turned blue on exposure to air. XAS data were collected both at room temperature and −70 °C as described previously[8] by using synchrotron radiation at the Stanford Synchrotron Radiation Laboratory. Radiation was monochromatized by using a Si[220] crystal monochromator on the wiggler beam line IV. Energy was calibrated relative to a copper foil by assigning 8980.3 eV to the first inflection point on the copper K-absorption edge.

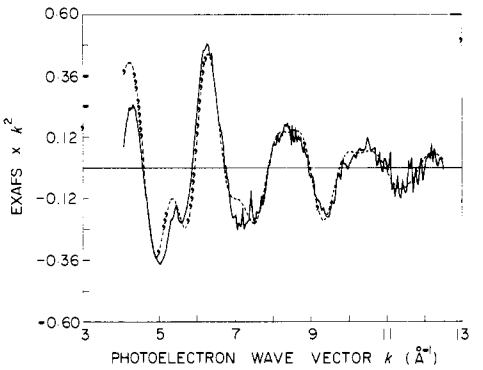

Figure 1. The normalized, background-subtracted EXAFS data (solid line) for deoxyhemocyanin from *M. crenulata*. The fit over a k range of 4–12 Å^{-1} (dashed line) is obtained by fitting with two imidazoles, giving a Cu–N distance of 1.95 Å.

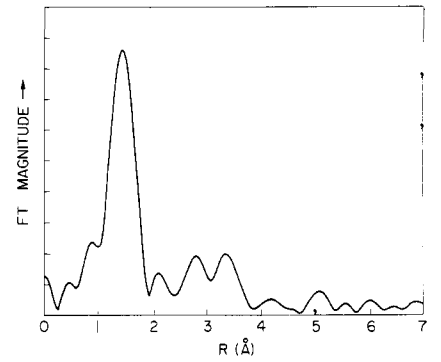

Figure 2. Fourier transform of the data in Figure 1, from a k range of 4–12 Å^{-1}, k^3 weighted. The first peak can be Fourier filtered and fit with two nitrogen atoms at a Cu–N distance of 1.95 Å.

The X-ray absorption edge spectrum of deoxyhemocyanin was examined for evidence concerning the oxidation state of copper. This method has been used previously in the investigation of the oxidation state of the coppers in cytochrome c oxidase[9] and hemocyanin.[1,2,6] Absorption edge spectra for deoxyhemocyanin indicate the absence of the small preedge feature at ca. 8979 eV, which is normally assigned to the 1s → 3d bound state transition for the Cu(II) state.[6] The transition occurring at ca. 8983 eV (assigned in the literature as "1s → 4s") is consistent with Cu(I) models, which average 8983 eV, 4 eV lower than the same transition generally assigned to Cu(II).[6] Hence, edge studies are consistent with the copper in deoxyhemocyanin being present in the reduced [Cu(I)] form.

EXAFS data were processed as previously described.[8] The normalized, background-subtracted data, presented in Figure 1, are averages of 15 room temperature scans, each scan comprising data collected by 20 NaI scintillation detectors. The signal-to-noise level of the data was among the best of any data that we have

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ever collected for proteins. A Fourier transform of the data over a range of $k = 4$–12 Å^{-1} is presented in Figure 2. This transform shows striking similarity with those for blue copper proteins.[10,11] The features above 2 Å (not corrected for phase shift in the Fourier transform) have been previously suggested to provide evidence for the presence of imidazole ligands.[11] Another obvious feature in this transform is the absence of the large peak at 3.3 Å present in the oxyprotein, which was assigned as a backscattering peak from a copper atom at about 3.6 Å from the absorbing copper.[1,2] The first major peak in the transform was filtered and found to fit two nitrogen (or oxygen) atoms at 1.95 Å. A similar result is obtained for deoxyhemocyanin from *Busycon canaliculatum*.[2] For the obtainment of a more detailed view of the copper coordination sphere, the new technique of "group" curve fitting was applied.[3] The group fitting approach allows more accurate modeling of the phase and amplitude effects of second and third shell atoms of imidazole groups. This technique applied to oxyhemocyanin allowed us to suggest a model for the binding site which consists of two copper(II) atoms separated by 3.55 Å, each bound to two histidines and two oxygen atoms in an approximately square-planar geometry.[1]

Using the imidazole group fitting technique on the EXAFS data for deoxyhemocyanin reveals 2.0 imidazoles at 1.95 Å. The calculated fit, as shown in Figure 1, matches the data extremely well. For investigation of the possibility of a copper scatterer in the copper environment, a copper atom at various distances less than 4.5 Å was fitted to the data. Under no circumstances did copper ever contribute significantly to the overall fit, in most cases giving rise to less than 0.2 atom or a negative number of atoms. Similar results were observed when the same procedures were applied to the half-apo derivative of the protein[12,13] or to the low-temperature data set. Fitting atoms at longer distances becomes fruitless because of the insignificant contribution of scattered waves by long-distance scatterers to the total EXAFS which is dominated by nearest-neighbor scatterers. However, a peak significantly above the noise level was observed at 5.1 Å in the transform (see Figure 2). This peak, Fourier filtered from the *R*-space data, can be fit well with 0.6 copper atom at 5.6 Å. This could be interpreted to mean that, in deoxyhemocyanin, the two copper atoms are separated by a large distance, in contrast to the 3.55-Å distance found in oxyhemocyanin. EXAFS analysis for such a long-distance single-atom scatterer, however, must be taken with extreme caution. It is interesting to note that computer simulations of EPR spectra have calculated Cu(II)–Cu(II) distances to be ca. 6 Å for nitrite-treated hemocyanin.[14]

The EXAFS results presented here for the deoxyhemocyanin suggest that only two ligands are found coordinated to each of the copper sites of the protein. Although such a low coordination number has not previously been found in a metalloprotein or metalloenzyme,[15] this coordination geometry is, nevertheless, quite common among Cu(I) compounds.[16] Crystals of $[Cu(Im)_2]ClO_4$, obtained in a size suitable for powder diffraction, give intensity values comparable to those of linear $[Ag(Im)_2]NO_3$, suggesting that the two structures are essentially isomorphous.[17] A two-coordinate geometry of Cu(I) in a solution of 2:1 complexes with monodentate ligands like NH_3 or imidazole is also strongly supported by the properties of mixed systems containing two different ligands.[16] Hence the copper site in the deoxyhemocyanin can be postulated to consist of two Cu(I) atoms, each coordinated to two histidines, with the facial sides open for attachment of dioxygen. Such an arrangement should also facilitate the removal of dioxygen from the oxyprotein. However, it has been shown that for some Cu(I) models, the low coordination number is sometimes supplemented by one or two additional ligands at considerably larger distances. EXAFS is not very sensitive for detecting a low-Z atom at a large distance from the metal site, and hence the analysis reported herein could have missed a weakly bound ligand at a long copper–ligand distance. Such weak interactions, however, are probably of minor significance in contributing to the ligand field at the copper site.

EXAFS studies on oxy- and deoxyhemocyanin both indicate the presence of two histidines in the copper coordination sphere. However, the oxygenation process involves several significant changes in the copper site. Upon binding dioxygen, the copper oxidation state changes from Cu(I) to Cu(II), the coordination number increases from 2 to 4, and the Cu–Cu distance is shortened to 3.55 Å. These processes must certainly be accompanied by a substantial rearrangement in the protein structure. Such a conformational change can provide explanations for numerous observations on oxygen binding, such as the dissociation of subunits,[18,19] changes in the 250-nm band in circular dichroism,[18,20] and a slow relaxation time in kinetics studies.[21] A comparative study on four different states of hemocyanin (oxy, deoxy, half-apo, and dimer) will be presented in detail in a forthcoming paper.[12]

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Magnetic Circular Dichroism Studies. 59.[1] Substituent-Induced Sign Variation in the Magnetic Circular Dichroism Spectra of Chlorins

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The occurrence of sign inversions in the magnetic circular dichroism (MCD) of porphyrin derivatives has important analytical applications as well as theoretical implications; however, only a few studies have been made of chlorophylls and chlorin derivatives[2-8] compared to the number dealing with porphyrins[9a]

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