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X-Ray Absorption Spectroscopic Studies of Metal Coordination in Zinc and Copper Proteins

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X-Ray Absorption Spectroscopic Studies of Metal Coordination in Zinc and Copper Proteins

X-ray absorption spectroscopic studies have contributed to the elucidation of the metal coordination in zinc and copper proteins, and to the refinement of structures of such proteins already known from crystallographic studies. Zinc proteins can be classified according to their X-ray absorption spectroscopic characteristics, and it is possible to discriminate between ligand environments with two sulfur ligands, as in the transcription factor, and with one sulfur ligand, as in the sorbitol dihydrogenase, in a comparative study. For phospholipase C, it has been shown in a substitution study that cobalt has a higher coordination number than the native zinc in the same size.

Comparisons of the copper–ligand interactions of various electron transfer or "blue-copper" proteins do not show a clear correlation with redox potential except perhaps for the relative rigidity of the Cu–S distances in that with the highest redox potential, rusticyanin. EXAFS (extended X-ray absorption fine structure) studies of type-2 copper enzymes are critically reexamined in view of the possibility that coordination by the recently discovered o-quinoline cofactors has been mistaken for imidazole coordination. Changes in the copper coordination of the dinuclear copper site in hemocyanin upon oxygen binding are interpreted in terms of the coordination of one additional imidazole ligand per copper.

Key Words: *X-ray absorption spectroscopy, extended X-ray absorption fine structure (EXAFS), zinc, cobalt, copper, NAD-dependent dehydrogenase, β-lactamase, phospholipase C, blue copper, type-2 copper, pyrrolo quinoline quinone (PQQ), dinuclear copper site*

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1. INTRODUCTION

a. Crystallographic and Spectroscopic Studies of Metalloproteins

Proteins are involved in all biological processes, and many proteins are in fact metalloproteins, including enzymes and proteins for the transport of electrons, dioxygen or other molecules or ions, or their storage. In the metalloprotein, the metal ion or ions are typically found at the active site, and the metal, usually selected because of properties distinguishing it from other metal ions, can be said to be further adapted for its specific task by the coordination chemistry and geometry forced upon it by the protein and its possible organic cofactors, putting it on the "rack"[1] or bringing it in the so-called "entatic state".[2] For these reasons, spectroscopic and magnetic studies of metals in metalloproteins, giving information on valence and spin states, coordination geometry, ligand identity, and changes in these aspects during catalysis or inhibition,[3] are particularly rewarding. However, while, to name a well-documented example, the crystal structure of the blue-copper protein plastocyanin[4] confirmed the predictions from spectroscopic studies in aspects of its copper coordination chemistry, it also refuted some of them, and showed unexpected features.[5] The interpretation of spectroscopic results can be made on more solid ground in the context of crystallographic data.[6] On the other hand, spectroscopy is considered to be important for the correction and refinement of crystal structures.[7]

b. X-Ray Absorption Spectroscopy

X-ray absorption spectroscopy (XAS) has made important contributions to metalloprotein studies in recent years.[8-14] With X-ray absorption near-edge spectroscopy (XANES), valencies, coordination geometries and average ligand distances have been probed, and extended X-ray absorption fine structure (EXAFS) studies have allowed significant refinements of the ligand geometry of metalloproteins of known crystal structure, as well as the elucidation of unknown metal coordinations. EXAFS refers to the oscillations observed in XAS over an energy range of a few hundreds of eV starting 50 eV above the edge step. When the X-ray energy is scanned, fluctuations in the XAS absorption coefficient occur, which are due to interference effects in the electron density on the absorber atom, caused by backscatterer by surrounding atoms of

the photoelectron wave leaving the absorber at X-ray energies above the edge. The oscillations can be extracted, expressed in the wave-vector k ($k^2 = 2m(E - E_0)/h$, with E the energy of the incident beam, and E_0 the threshold energy of the edge under consideration), and Fourier transformed to give a radial distribution function with peaks representing shells of atoms around the absorber atom.[15] When the peak positions in the Fourier transform are corrected for the phaseshifts that the electron undergoes while travelling between absorber and backscatterer, either by deriving them from reference compounds or by calculating them, the absorber–backscatterer distance is obtained with high accuracy (± 0.02 Å). For more experimental and theoretical backgrounds of XAS, which are only fragmentarily discussed here, the reader is referred elsewhere.[16] In this Comment, recent applications of XAS in the zinc and copper metalloprotein field will be critically reviewed, and an interpretation for some phenomena unexplained so far is given.

2. ZINC PROTEINS

a. Classification of Zinc Proteins Based on X-Ray Absorption Spectroscopy

Zinc proteins are interesting objects for EXAFS studies, as they are not accessible by other spectroscopic techniques because of the filled 3d-shell of the Zn(II) ion. Generally, in EXAFS, the backscatterer wave of sulfur is approximately π out of phase with those of nitrogen, oxygen or carbon, commonly referred to as "low-Z atoms." Therefore, it is possible by EXAFS to distinguish sulfur first shell ligands from nitrogen and/or oxygen. By analogy to the classical classification of copper proteins based on spectroscopic properties[17] (see below), a classification of zinc sites in proteins based on features distinguishable in EXAFS (cf. Table 1) has been proposed.[18] EXAFS is the only spectroscopic technique applicable to all zinc sites in proteins in their native states, i.e., without substitution, but it appears to have some shortcomings. One is that although, as stated above, sulfur is readily distinguished from nitrogen and oxygen,[19] discrimination between the latter two has always been considered more difficult. The number of nitrogen ligands in mixed-ligand first shells has, in recent studies on copper[20]

and iron[21] proteins, been estimated by means of quantification of the minor shells at 3–4 Å in the radial distribution function (Fourier transform). Biological EXAFS typically dies out after the first shell, or, in favorable cases, after a few shells, due to the limited mean free path of the electron, and to uncorrelated motion of absorber and remote potential backscatterer, the latter effect leading to a higher Debye–Waller-type factor for contributions of remote backscatterers. The occurrence of more than one shell in biological systems usually points to the presence of a relatively rigid system of ligand atoms. On the assumption that these shells arise from imidazole ligand coordination, and the imidazole ligands are the only possible nitrogen ligands in metalloproteins without additional cofactors, the number of imidazoles and hence the number of nitrogen donor ligands, can be quantified, if the multiple scattering effects of the electron wave in the imidazole rings are taken into account.[22–24]

Another problem arises if one investigates whether correlations exist between the structural features distinguished in Table I and the catalytic activity of the zinc in the protein. In enzymes, the Zn(II) ion can polarize bonds in the substrates by acting as a Lewis acid. One indication of such a role for Zn(II) in a protein is the presence in its coordination sphere of a water molecule which may be displaced by substrate. This is not possible in Type-A zinc sites. Another indication is the presence of negatively charged ligands, which can be considered to temper the Lewis acidity. In the type-A sites, with four negatively charged thiolate ligands, the Lewis acidity is expected to be considerably tempered, and therefore, structural rather than functional roles for these zinc sites are expected. Examples of type-A sites are metallothionein[28] and the structural sites of liver alcohol dehydrogenase (LADH)[25] and aspartate transcarbamoylase.[27] For the assessment of the catalytic activity of the other site types, it is a problem that it is not possible to discriminate by EXAFS between negatively charged (carboxylate, tyrosinate) and neutral (water) ligands to zinc. For this, crystallography or additional spectroscopic studies, involving zinc substitution, are needed. For example, the presence of exchanging water molecules can be probed by ^{1}H-NMR (Nuclear Magnetic Resonance) relaxation enhancement studies of Mn(II)-substituted enzyme, as done for human glyoxalase I.[37] Charged groups within

TABLE I

Classification of zinc sites in proteins (cf. Ref. 18).

Type A	
Horse LADH structural Zn	4 S (crystallography of native enzyme,[25] EXAFS with Co(II) in catalytic site[26])
Aspartate transcarbamoylase	4 S (EXAFS[27])
Metallothionein	4 S (EXAFS[28])
Type B	
Horse LADH catalytic Zn	2 S, 1 N, 1 OH_2 (crystallography[25])
Sheep liver sorbitol dehydrogenase	1 H_2O, 1 O, 1 N, 1 S (model based on alignment of amino acid sequence to homologous LADH[29])
	3–4 N/O, 1 S (EXAFS[30])
Human liver sorbitol dehydrogenase	1 H_2O, 1 O, 1 N, 1 S (UV-vis, Co(II) substitution[31])
Xenopus laevis transcription factor TF IIIA	2 S, 2 N (EXAFS,[32] ^{1}H-NMR[33])
Type B or C	
Bacillus cereus β-lactamase II	3 N, 1 H_2O, 1 S (crystallography of Cd(II) derivative,[34] UV-vis, Co(II) substitution[35])
	4–6 N/O, S only with low occupancy or high Debye–Waller-type factor (EXAFS of mono-Zn derivative[36])
Type C	
Glyoxalase	Octahedral (UV-vis, Co(II) substitution[37])
	2–4 N/O, possibly 7-coordinate (EXAFS and comparison of XANES, respectively[38])
Bacillus cereus phospholipase-C	
Zn-1, Zn-2, Zn-3 "catalytic" and "structural" site	2 N, 2 O, 1 OH_2 (crystallography[39,40])
	Octahedral (UV-vis, Co(II) substitution[41])
	5 N/O (EXAFS[36])

5 Å from the metal in ^{111}Cd-substituted enzymes can be probed by PAC (Perturbed Angular Correlation) spectroscopy, as done for carbonic anhydrase, carboxypeptidase, superoxide dismutase, and alcohol dehydrogenase.[42]

Another problem is the rather small spectral range in EXAFS

spectra so that in multi-zinc proteins, the contributions of zinc sites of possibly different coordination chemistry will not be resolved. For example, on the basis of the EXAFS study on the 8 zinc species of the tetrameric enzyme 5-aminolevulinate dyhydratase, showing 3 S and 1 N/O ligand per zinc,[19] one would be inclined to classify it as a type-B zinc enzyme. However, it is known that full enzymic activity is obtained with 4 Zn per tetramer,[43] and the EXAFS of the 8 Zn/tetramer species must therefore represent a mixture of catalytic and non-catalytic sites. This type of problem can be circumvented if selective removal of substitution of the metal in one of the sites is feasible, e.g., as described below for *Bacillus cereus* β-lactamase or phospholipase C, respectively,[36] although the question then arises, even if enzymic activity is retained, to what extent the metal environment in the non-substituted site is affected by the removal or substitution of the metal at the other site, especially if the sites are close.

b. NAD-Dependent Dehydrogenases

Horse liver alcohol dehydrogenase (LADH, EC 1.1.1.1) has been crystallographically characterized[25] as a dimer with two zinc sites per monomer, one "structural" Type-A (see above) with 4 cysteine thiolate sulfur anion ligands, the other, close to the cofactor and substrate binding sites, a "catalytic" Type-B site with two cysteine thiolate sulfurs, one histidine imidazole nitrogen, and one non-protein, water oxygen ligand, which may be replaced by the substrate oxygen. Crystallographic studies on the binding of the NAD (nicotinamide adenine dinucleotide) cofactor binding near the catalytic site, and the associated "closing" of the protein structure, appear to show changes in the Zn–S distances of the structural site which are on the limit of crystallographic accuracy, viz. 0.1 Å,[44] but EXAFS studies on enzyme with cobalt substituted in the catalytic site have shown them to remain identical.[26] Sheep liver sorbitol dehydrogenase[45] (or L-iditol dehydrogenase, EC 1.1.1.14, L-iditol:NAD^+ 5-oxidoreductase) contains one zinc atom,[46] and shows considerable amino acid sequence homology with the crystallographically characterized horse LADH.[47] However, as the cysteines involved in the binding of the structural site are not conserved, it is concluded that only a catalytic zinc site is present in sorbitol dehydrogenase. A three-dimensional structure for sorbitol

dehydrogenase has been constructed by fitting the amino acid sequence to the crystal structure of LADH, in which one cysteine thiolate sulfur ligand to zinc is proposed to be substituted by a glutamate carboxylate oxygen.[29] The presence of a Glu rather than an additional thiolate ligand, and its hydrogen-bonding to the hydroxyl on C1 of the substrate, would account for the preference of the enzyme for sorbitol to primary mono-alcohols as substrates, and for its dehydrogenation to fructose.

This substitution has been investigated by EXAFS.[30] It has proved difficult to determine the number of sulfur and nitrogen and/or oxygen ligands in a mixed ligand system like the Type-B zinc site, for the very reason why a good discrimination between type-A, B and C sites is possible, viz. the backscatterer wave of sulfur is approximately π out of phase with those of the low-Z atoms, leading to destructive interference between the waves of sulfur and low-Z atoms at about the same distance. As a model compound study on a mixed-ligand Zn coordination compound had indicated a tendency for physically unreasonable, high coordination numbers if they were allowed to float in the refinement of the EXAFS simulations, occupancies of sulfur and low-Z contributions to the first shell were varied in steps and total coordination numbers of 4 and 5 were allowed in the analysis of sorbitol dehydrogenase data. In both cases, best fit indices were obtained with 1 rather than 2 sulfur ligands included in the simulation. The best fits were obtained with one low-Z ligand in addition to the 4 ligands deducted from the model, which may be an indication for coordination of an additional water molecule to zinc, or coordination by both Glu carboxylate oxygens. The most convincing demonstration of the substitution of one sulfur is the direct comparison with the EXAFS of the *Xenopus laevis* TF IIIA transcription factor, which has been shown to contain nine zinc atoms in so-called zinc fingers, with 2 Cys thiolate sulfur ligands and 2 His imidazole nitrogen ligands per zinc.[32] As can be seen from Fig. 1, although both proteins contain Type-B zinc sites, there is a clear difference in the amplitudes of the EXAFS at $k = 6\text{–}8$ Å^{-1} and of the Fourier transform, pointing to a lower number of sulfur ligands in the case of the sorbitol dehydrogenase. Independent evidence from UV-vis spectra of Co(II)-substituted human liver sorbitol dehydrogenase that here too only one sulfur ligand coordinates the metal has been reported.[31]

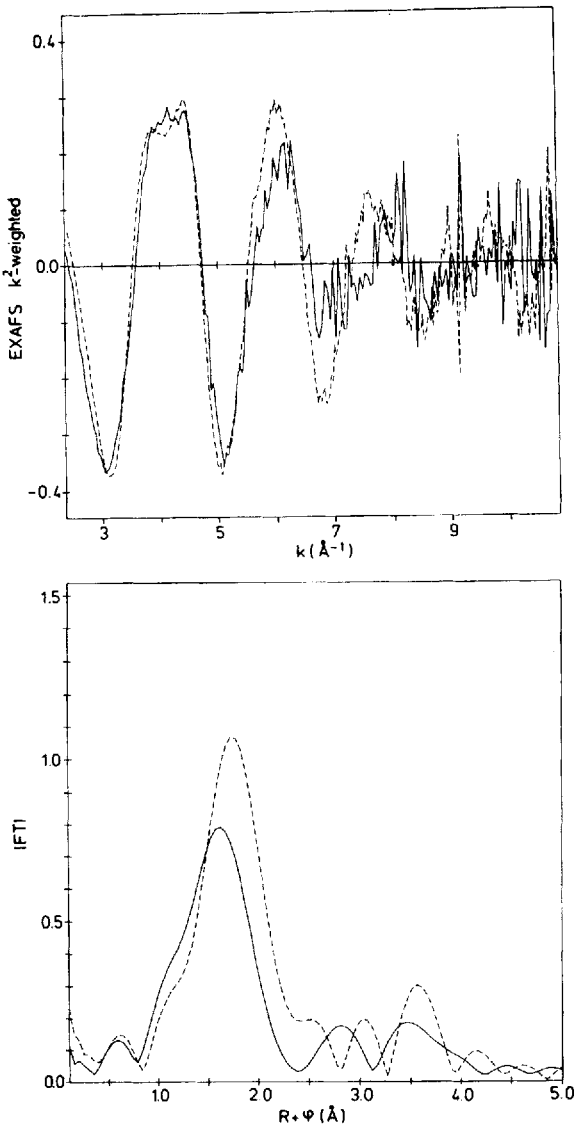


FIGURE 1 k^2-weighted Zn K-edge EXAFS (upper panel) and Fourier transform (bottom panel, not phase corrected) of sheep liver sorbitol dehydrogenase at 277 K, solid line (Ref. 30), and transcription factor TF IIIA at ambient temperature, dashed line (Ref. 32).

c. *Bacillus cereus* Exoenzymes

β-lactamase. From spectroscopic studies on cobalt-substituted enzyme species as well as (native) zinc enzyme, the catalytic zinc enzyme, the catalytic zinc site of β-lactamase II (EC 3.5.2.6, "penicillinase") from *Bacillus cereus* appears to have 3 His imidazole nitrogen ligands,[48] one water oxygen ligand,[35] and one Cys thiolate sulfur ligand,[49] and would be expected to have EXAFS characteristics of a Type-B site. However, upon investigation,[36] the EXAFS showed clear evidence for imidazole ligands, but the sulfur ligand could only be included in the simulations with a high Debye–Waller factor or a low occupancy, not significantly lowering the fit index. Crystallographic studies[34] of the cadmium derivative have indicated a possible reason for this discrepancy between spectroscopic and EXAFS results. The metal (cadmium)–thiolate distance is approx. 4.5 Å, a distance known to be too long to be detected by EXAFS, for example from the comparative cyrstallographic/EXAFS studies on the blue-copper proteins plastocyanin and azurin, with their long-distance (2.3–3.1 Å) methionine thioether sulfur coordination (see below). It should be noted that although the intensity of the 348 nm Co(II)–S charge transfer band in the UV-vis spectrum of Co(II)-substituted β-lactamase is rather low compared to that of other Co(II)-substituted zinc or copper proteins of known thiol coordination, it is enhanced upon substrate binding.[50] The question whether the sulfur comes closer to the metal during catalysis remains to be investigated by crystallography and EXAFS.

Bacillus cereus phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* requires two Zn(II) for enzymic activity.[51] One of these metal ions is exchangelabile, and is termed "catalytic," while the other is more tightly bound and termed "structural."[51,52] A detailed CD (circular dichroism), MCD (magnetic circular dichroism), EPR (electron paramagnetic resonance) and UV-vis absorption study[41] of enzyme species selectively substituted with Co(II) has shown that the two metal sites are similar but not identical, possess distorted octahedral ligand geometry, and are in close contact. In the spectroscopic study on phospholipase C and related studies on other proteins, e.g., glyoxalase,[38] the finding of distorted octahedral ligand geometry upon Co(II) substitution has been taken to imply that

the native Zn(II) has the same ligand geometry. Preliminary X-ray diffraction analysis of phospholipase C has shown two metal sites only 5.7 Å apart.[53]

EXAFS spectra at the zinc and cobalt K edges have been taken on the native (di-zinc) enzyme and the mono-zinc, mono-cobalt form (where the exchange-labile catalytic zinc has been substituted by cobalt).[36,54] The zinc EXAFS of the latter represents zinc in the structural site, while the EXAFS of zinc in the catalytic site is obtained from the difference spectrum of the native and substituted enzyme forms. As can be seen from a comparison of the EXAFS and Fourier transforms in Fig. 2, the data of structural and catalytic sites are very similar, in agreement with the result of the multi-spectroscopic study.[41]

In agreement with the absence of cysteine in the amino acid sequence of phospholipase C,[55] no indication of sulfur ligation is seen in the EXAFS. The chemical shifts observed in NMR for ^{113}Cd(II) bound to this enzyme are consistent with a mixture of nitrogen and oxygen liganding in both metal-binding sites.[56] Analysis of the Fourier-filtered main shells of the EXAFS gave coordination numbers of 5 ± 1 nitrogen and/or oxygen ligands. Chemical modification and ^{1}H-NMR studies have indicated the presence of only four His ligands, possibly one bridging the metals,[56,57] in agreement with the presence of shells at 3–4 Å in the EXAFS Fourier transforms, although their intensities do not permit an exact definition of the number of coordinating imidazoles; it is estimated at 2 or 3 for both sites. Since the presence of other nitrogen ligands is less likely, only 4–5 of the 6 nitrogen atoms implied in the first shells of the simulations for the two sites, presented in Table II, are likely to be correct. Splitting the main shell gives significantly better fits, and as the positions of the 3 Å imidazole shells indicate that the His nitrogen atoms coordinate at the shorter distances, oxygen atoms were included at the longer distances. Some or all of these oxygens could be water oxygens, as the Zn–O distances (Table II) are in good agreement with the values of 2.08–2.15 Å found by crystallographic analysis of octahedral Zn-nucleotide/water complexes.[58]

A simulation for the raw Co(II)-catalytic site data is presented in Figs. 3(a) and 3(c), the parameters used for this simulation being included in Table II. As in the case of the zinc protein EXAFS, the shells at 3–4 Å in the Fourier transform are assigned to im-

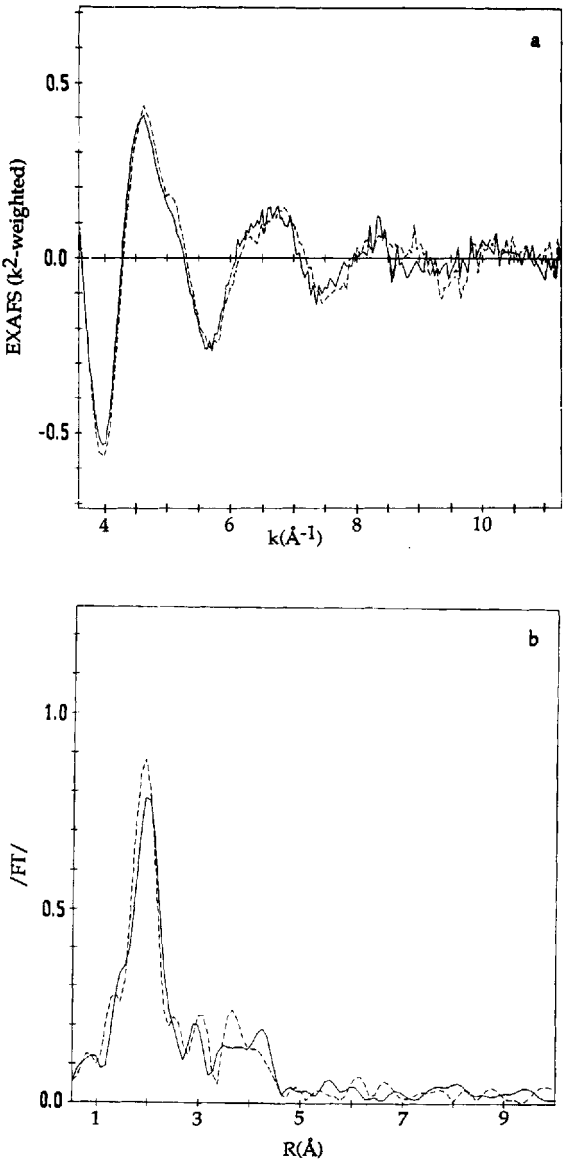


FIGURE 2 Zn K-edge EXAFS and Fourier transforms thereof of phospholipase C. k^2-weighted EXAFS (a) and their phaseshift-corrected Fourier transforms (b) are shown for the structural (solid line) and catalytic (dashed line) Zn of phospholipase C.

TABLE II

Parameters used to simulate the EXAFS associated with the Zn-edge of the structural and catalytic site Zn(II) of phospholipase C, and with the Co-edge of Zn(structural)–Co(catalytic) phospholipase C. N is the number of atoms, R the distance of atoms from the Zn atom and $2\sigma^2$ the Debye–Waller-type factor. FI is the fit index (k^2 weighting). The numbers shown in parentheses show the error expressed as the last significant figure of the parameter value determined.

Atom	Zn(II) in Structural site			Zn(II) in Catalytic site			Co(II) in Catalytic site		
	N	R (Å)	$2\sigma^2$ (Å^2)	N	R (Å)	$2\sigma^2$ (Å^2)	N	R (Å)	$2\sigma^2$ (Å^2)
N	3	2.01(3)	0.004(1)	3	2.02(3)	0.006(2)	–	–	–
O	2	2.14(3)	0.007(2)	2	2.15(4)	0.006(2)	7	2.08(3)	0.025(11)
O	1	2.70(4)	0.008(3)	–	–	–	–	–	–
C	2	2.98(4)	0.008(3)	2	2.91(4)	0.029(6)	3	2.97(4)	0.008(2)
C	4	3.74(4)	0.017(3)	4	3.73(4)	0.028(4)	2	3.78(5)	0.02(1)
N	2	4.30(4)	0.011(5)	3	4.33(4)	0.010(3)	4	4.33(4)	0.013(3)
FI		0.08667			0.08416			0.16337	

idazole ring atoms. Direct comparison of the EXAFS, and Fourier transform of the Co(II)-catalytic site data with the Zn(II)-catalytic site data (Figs. 3(b) and 3(d)) shows that the shapes of the first EXAFS oscillations are similar, but also that the amplitude of the cobalt EXAFS is larger and that the phases are different. This direct comparison is evidence that cobalt has a higher coordination number than zinc in this site, even though the error in the determination of the coordination numbers (7 ± 2 nitrogen/oxygen atoms for the enzyme-bound Co(II)) is large.

Comparing the Zn(II) and Co(II) catalytic site results, there is an increase in average ligand distance upon substitution by Co(II) (2.05 to 2.08 Å assuming oxygen coordination only). Crystal structure determinations of a range of hexa-coordinated cobalt and zinc compounds (cf. Ref. 59), e.g., imidazole[60–63] and pyridine N-oxide complexes,[64,65] indicate that the former have, on average, shorter ligand distances than the latter when similar complexes of the two metals are compared, a trend opposed to that found in the results for the phospholipase C catalytic site. This corroborates the conclusion from analysis and comparison that the coordination number for zinc in both sites is five and that for cobalt in the catalytic site is six or seven. Co(II) in the catalytic site may well be hexa-coordinated and have octahedral geometry, as indicated by the spectroscopic studies[41] but Zn(II) is most likely penta-coordinated. In general, it is perhaps too readily assumed that Co(II)–Zn(II) sub-

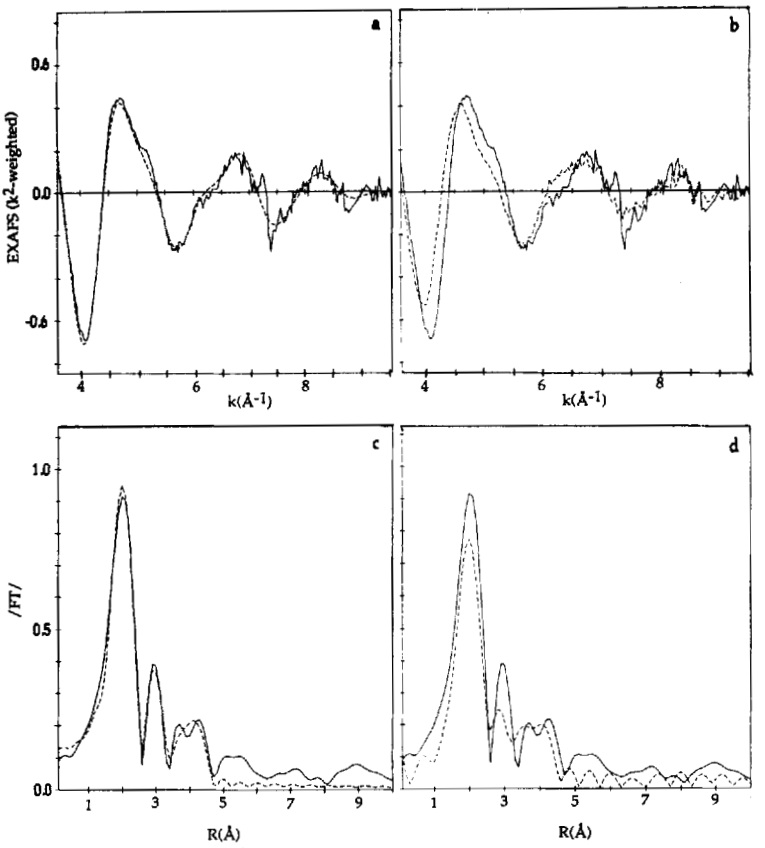


FIGURE 3 K-edge EXAFS of Co and Zn in the catalytic site of phospholipase C. Experimental (solid line) and simulated (dashed line) data are shown in (a) and (c), the latter representing a Fourier transform of the former. (b) and (d) show Co (solid line) and Zn (dashed line) EXAFS spectra from the catalytic site of the enzyme, the spectra shown in (d) being Fourier transforms of data in (b). The Zn data have been shifted by −5.1 eV relative to the Co data.

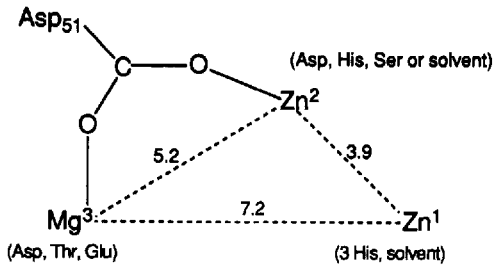
stitutions where a certain amount of catalytic activity is retained are perfectly isomorphous. However, in, e.g., carbonic anhydrase where, as in phospholipase C, the Co(II)-form is still catalytically active, Zn(II) is penta-coordinated whereas, depending on the pH or presence of certain inhibitors, Co(II) is tetra- or penta-coordinated.[59] Further investigations in which Co(II)-forms of Zn(II)-proteins are compared with their native counterparts may prove rewarding.

Very recently a detailed structure at 2.3 Å resolution of phospholipase C crystals grown in 10 μM (excess) zinc has been published.[40] The active site region in the crystal structure contains three zinc atoms. Such a zinc cluster is also found in the structure of *Escherichia coli* alakaline phosphatase crystals grown in the absence of Mg(II)[66] (Fig. 4). In the case of phospholipase C, the third zinc atom might represent a more loosely bound ion carried over on the enzyme from the zinc containing storage medium into the crystallization mixture. In the present EXAFS analysis, both zinc-binding sites analyzed contain two or three His rather than one. This would suggest that the Zn-1 and Zn-2 sites in the crystal structure are the sites studied in the present work, since in the crystal structure Zn-3 coordinates through a single His residue. Furthermore, Zn-1 and Zn-3 are only 3.27 Å apart (Fig. 4).[39] No evidence of Zn–Zn or Zn–Co backscattering was found in the EXAFS study. Studies on dinuclear iron proteins, with ligands bridging the metals, clearly show contributions of this close metal–metal location.[67–69] Its absence its additional evidence that Zn-3 is the zinc site not occupied in the di-zinc phospholipase species studied in this work, and also that it is still not occupied after substitution by cobalt. Further crystallographic studies are needed in order to establish which sites are occupied in the Co(II)-substituted enzyme species, and what is the additional ligand to cobalt.

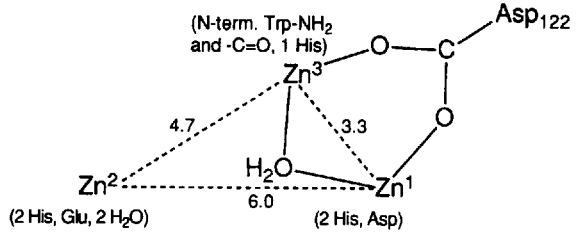
3. COPPER PROTEINS

a. Classification Based on Spectroscopy

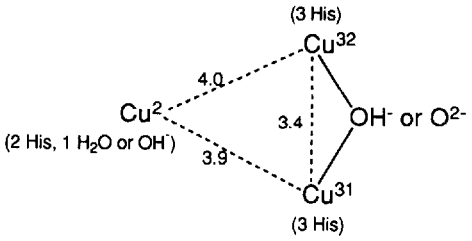
Copper sites in proteins are traditionally classified into three types based on spectroscopic studies.[17] They are the type-1 copper sites, also called "blue copper" (deep blue color due to visible light absorption around 600 nm with $\epsilon > 10{,}000\ M^{-1}\ cm^{-1}$, narrow hyperfine splitting in the g‖ region of the EPR spectrum, with $A_{\parallel} < 10$ mT,[17]), the type-2 copper sites, considered to be without typical spectroscopic features, and the type-3 sites, dinuclear copper sites with antiferromagnetic coupling of the coppers causing the absence of EPR-detectable copper in these sites even when the atoms are in the Cu(II) state. Type-1 copper sites are usually associated with electron transfer, whereas the binuclear copper



Alkaline phosphatase



Phospholipase C



Ascorbate oxidase

FIGURE 4 Trinuclear zinc and copper clusters and their bridging ligands in metalloproteins, distances in Å. From top to bottom: alkaline phosphatase from *Escherichia coli* (Ref. 66), phospholipase C from *Bacillus cereus* (Refs. 39 and 40) and ascorbate oxidase from green zucchini squash, *Cucurbita pepo medullosa* (Ref. 70).

sites are involved in oxygen binding. Multicopper proteins like laccase and ascorbate oxidase are considered to contain one "blue copper," one type-2 copper and one binuclear site per monomer, but spectroscopic studies[71-73] gave the first indications, now confirmed by crystallographic studies on ascorbate oxidase[70] combined with considerations of sequence analogy to laccase and ceruloplasmin,[74] that the 3 copper atoms considered to represent the type-2 and type-3 sites in these proteins are actually very close together. Studies on the multi-copper enzyme nitrous oxide reductase[75,76] have led to suggestions for the presence in this enzyme and the iron–copper enzyme cytochrome-C oxidase of copper sites related to blue copper in donor ligand set but somewhat different in spectroscopic properties, and of a mixed valence dinuclear copper site. The finding of novel organic cofactors in many type-2 copper proteins[77] may lead to new considerations in this area as well.

b. Blue Copper

The blue-copper spectroscopic features (see above) can to a large extent by mimicked by model compounds,[78] by substitution of zinc in the catalytic site of LADH by copper,[79,80] and by addition of copper and pentafluorothiophenolate to insulin.[81] In the crystal structures of *Populus nigra* plastocyanin,[4,82] azurin from *Pseudomonas aeruginosa*[83] and pseudoazurin from *Alcaligenes faecalis*,[84,85] all electron-transfer proteins containing blue copper, distant (2.7–3.1 Å) Met sulfur ligands to copper are found, in addition to 2 close His imidazole nitrogens and 1 close Cys thiolate sulfur ligand. In the structure of *Alcaligenes denitrificans* azurin[86] an additional distant peptide carbonyl ligand was found. The first EXAFS studies of blue copper proteins[87,88] reported only the two nitrogens and the close sulfur. The distant sulfur ligand has proved notoriously difficult to detect by EXAFS, even in plastocyanin crystals which were favorably oriented with respect to the plane of polarization of the synchrotron radiation[89] or at 4 K to minimize the temperature effect on the Debye–Waller-type factor.[90] Crystallographic studies of plastocyanin have revealed that the distant Met sulfur comes closer to the copper, viz. at 2.5 Å, in the reduced protein at low pH.[82] In oxidized *Pseudomonas aeruginosa* azurin, no interaction between copper and Met sulfur could be detected

in the EXAFS, but in reduced azurin, it could be detected at 2.7 Å[91] (Table III). Presumably, the bond between copper and Met sulfur in oxidized blue copper proteins is too long and weak to be detected. The fact that the distant Cu–S-interaction in reduced azurin was detected by EXAFS, but not in oxidized azurin, can be understood from the weakness of the non-detected interaction, and gives information about changes in it upon changes in redox state. Interestingly, Cu–S interactions of 2.6–2.7 Å, in addition to Cu–S interactions of 2.3 Å,[92] have recently also been detected in the multi-iron/multi-copper enzyme cytochrome C oxidase[93] and the multicopper enzyme nitrous oxide reductase.[76]

As for oxidized azurin, no sulfur ligand in addition to the close thiolate sulfur could be detected in oxidized *Rhus vernicifera* stellacyanin copper EXAFS.[87,94,95] In reduced stellacyanin, however, it could be detected at 2.66 Å[95] (Table III). Because of the absence of Met from the amino acid sequence of stellacyanin[96] the question is what this sulfur originates from, an additional but distant thiolate[97] or a disulfide bridge.[97,98] As the results of comparative ENDOR

TABLE III

"Blue"-copper ligand distances, in Å, as derived from recent EXAFS studies on azurin from *Pseudomonas aeruginosa*,[109] stellacyanin from *Rhus vernicifera*[95] and rusticyanin from *Thiobacillus ferrooxidans*.[110] Debye–Waller-type factors are geiven in parentheses as $2\sigma^2$, and can be considered equivalent to ΔR^2 r.m.s. (root mean square).

Protein/ Redox potential (mV)		2 N(His)	1 S(Cys)	1 Apical S
Azurin	oxidized, pH 9.1	1.95 (.0025)	2.22 (.012)	
+300[111]	pH 4.1	1.95 (.005)	2.18 (.006)	
	reduced, pH 9.2	1.96 (.024)	2.23 (.009)	2.73 (.035)
	pH 5.5	1.97 (.016)	2.25 (.0065)	2.70 (.016)
Stellacyanin	oxidized	1.93 (.003)	2.21 (.002)	
+180[112]	reduced	1.98 (.007)	2.25 (.014)	2.66 (.015)
	Ni(II)	3 @ 1.98 (.003)	2.24 (.009)	2.73 (.05)
	Co(II)	3 @ 1.94 (.007)	2.21 (.005)	3.12 (.007)
Rusticyanin	oxidized	1.99 (.006)	2.16 (.012)	2.84 (.004)
+680[113,114]	reduced	2.07 (.003)	2.17 (.009)	2.80 (.007)

studies of blue copper proteins[99] and the chemical shift in ^{113}Cd-NMR of Cd-substituted stellacyanin[100] are not consistent with two thiolate ligands, the possibility of disulfide coordination is favored.[95] The coordination by sulfur of Cys-59 in a disulfide bridge to Cys-93[101] also features in models of the stellacyanin structure based on comparison with plastocyanin[102] and ^{1}H-NMR studies of Co(II)-substituted stellacyanin.[103] On the other hand, in a model of the stellacyanin structure based on comparison to the X-ray diffraction structure of the related cucumber basic blue protein, additional sulfur ligands appear to be ruled out, and coordination of the amide oxygen of Gln-97 as a fourth ligand to copper is proposed instead.[104]

No evidence for an amide oxygen as a fourth or fifth ligand to copper as found in the crystal structure of *Alcaligenes denitrificans* azurin[86] or implied for stellacyanin on the basis of resonance Raman[105,106] and ^{113}Cd-NMR substitution studies[100] was found in oxidized *Pseudomonas aeruginosa* azurin[91] or oxidized *Rhus vernicifera* stellacyanin[95] EXAFS. It is possible that, like the long-distant Met or disulfide sulfur, the ligand is present but not observed in the EXAFS if it is at approx. 3.1 Å as in the *Alcaligenes denitrificans* azurin crystal structure.[86] EXAFS studies of substituted stellacyanin give a clearer indication of five-coordination, as a fifth, low-Z ligand appears to come closer to the metal.[95] For Ni(II) stellacyanin, the best fits (Table III) provide evidence for a low-Z ligand in addition to the 2 imidazoles, and ^{1}H-NMR studies[107,108] strongly suggest that it is the Asn-47 side chain oxygen rather than that of Gln-97,[104] although the elongated bonds found in that study are not reflected in the EXAFS. For Co(II) stellacyanin, the best fit was with 3 low-Z ligands and 1 sulfur (Table III), but in this case the EXAFS was relatively insensitive to the occupancies, as the possibilities of 2 N/O and 1 S, 4N/O and 1 S, 3 N/O and 2 S gave only slightly higher fit indices. The 3 N/O and 1 S result in consistent with the ^{1}H-NMR derived model for the structure of Co(II) stellacyanin[103] which features hydrogen bonding of the Asn-47 side chain amide-NH to the Cys-87 thiolate ligand and coordination to Co(II) of the amide oxygen. Differences between the native Cu(II) and the Co(II)-substituted protein can be explained by a reorientation of the Asn-47 side chain.

Interestingly, the amplitude of the copper EXAFS of various blue Cu proteins (azurin,[91] stellacyanin,[95] umecyanin[115]) decreases

upon reduction (Fig. 5(a)), which is reflected in the results of the analysis as a higher Debye–Waller-type factor of the Cu–N(His) interaction which is an indication of a larger spread in the distances in the Cu(I) protein (Table III). For *Populus nigra* plastocyanin, crystallographic results point to the loss of an imidazole ligand upon reduction at low pH[116] and there are indications from ^{1}H-NMR studies[117] that this also occurs in *Thiobacillus versutus* amicyanin. It is difficult to discriminate by EXAFS between the possibilities of 2 N with high Debye–Waller-type factor and 1 N with high Debye–Waller-type factor. For proteins where the copper is further from the surface than in plastocyanin, there is no reason to suppose that protonation and loss of one imidazole ligand can occur. Rusticyanin from *Thiobacillus ferrooxidans* shows no decrease in amplitude upon reduction (Fig. 5(b)), and there is no indication of a distant sulfur ligand coming significantly closer to copper, as the Cu–S distances are remarkably independent of the Cu oxidation state in this protein[110] (Table III). As there is no dramatic change in Debye–Waller-type factors upon reduction like in the other proteins (Table III), the copper site in rusticyanin must be relatively rigid, and this may be an explanation for the observed high redox potential,[113] because it would make it more difficult for the protein to accommodate both Cu(I) and Cu(II). The close sulfur ligands can be considered to destabilize the Cu(II) state as compared to the other blue-copper proteins.

c. Type-2 Copper, Interaction with Organic Cofactors

Bovine Cu,Zn superoxide dismutase has been extensively studied by EXAFS. The crystals structure shows that in each monomer, a Cu and a Zn ion are approx. 6 Å apart, bridged by an imidazolate.[118,119] Upon reduction, a significant decrease in Cu–N radii is observed, which points to a lower coordination number for Cu in the reduced enzyme and breaking of one of the Cu-imidazole bonds.[120] This is confirmed by ^{1}H-NMR studies[121] which indicate that the Cu-imidazole bond broken is that with the imidazolate bridging to Zn. Further EXAFS studies involving intensive application of multiple scattering simulations[20] point to different modes of coordination to Cu(II) for the inhibitor, azide anion (N_3^-), and the inhibitor/substrate analog, cyanide anion. Both coordinate in an equatorial position, but whereas in the azide complex one imidazole ligand has moved to an axial position, cyanide appears to

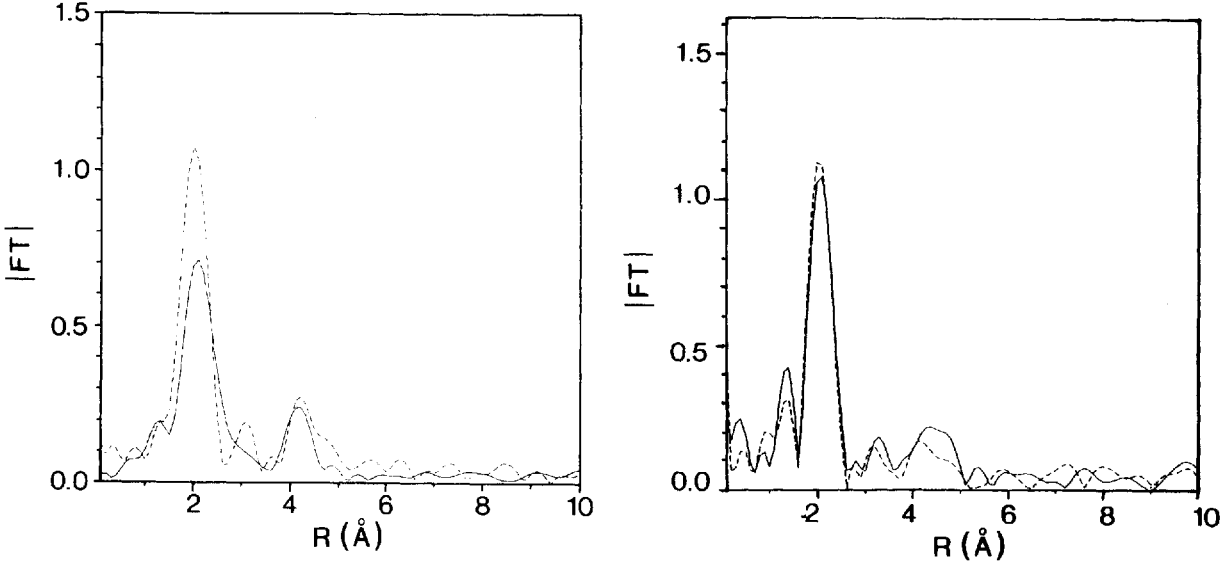


FIGURE 5 Phase-corrected Fourier transforms of reduced (solid line) and oxidized (dashed line) forms of *Rhus vernicifera* stellacyanin (left) and *Thiobacillus ferrooxidans* rusticyanin (right).

have displaced an imidazole ligand altogether. The NMR evidence on the possible displacement of an imidazole ligand is controversial.[122,123]

Most other type-2 copper proteins have in recent years been reported to be quinoproteins. The existence of the cofactor, pyrroloquinoline quinone[77] (PQQ, Fig. 6, left half), was established in bacterial methanol dehydrogenases (for a review, see Ref. 124). Bacterial apoenzymes can sometimes be reconstituted with free PQQ.[125] In mammalian quinoproteins, the cofactor is covalently bound and its presence can be demonstrated only after derivatization, as first shown for plasma amine oxidase.[126–128] The crystal structure of a bacterial (*Thiobacillus versutus*) methylamine dehydrogenase[129,130] showed that in the covalently bound cofactor, which can be thought to be biosynthesized from Tyr and Glu,[131,132] the Glu incorporated has not cyclisized to a pyridine ring (Fig. 6, right half), but rather is incorporated into the protein backbone, with the remaining Glu carboxyl group linked to an arginine side chain. This form of the cofactor has been called pro-PQQ,[133] TGA pro-PQQ[130] (Tyr-Glu-Arg pro-PQQ) or CHIQG[134] (4-(2-carboxy-6-hydroxyindolo-4,5-quinon-7-yl)glutamic acid). All proteins for which the presence of covalently linked PQQ was established by the so-called hydrazine method[135] probably contain, in fact, CHIQG.[133] The proposed presence of PQQ or CHIQG in a number of type-2 copper enzymes, amine oxidase,[126–128] dopamine-β-hydroxylase[136] (now disputed[137]), and galactose oxidase[138] as well as some non-heme iron enzymes, nitrile hydratase[139] and soybean lipoxygenase[140] raises questions about the possible metal-cofactor interactions, in view of the potential chelating capacities of the cofactors in their various ionization, redox and hydration states (Fig. 6). The cofactor pyrrole nitrogen can be deprotonated, with the negative charge delocalized on the quinone oxygens in the oxidized cofactor,[141] and chelate a metal together with the nearest carboxylate (Fig. 6, upper middle). In half-reduced and fully reduced cofactors, a metal can be coordinated to the ionized semiquinone[142] or dionized quinol (Fig. 6, lower middle). In hydrated, ionized PQQ, but not in CHIQG, a terdentate metal binding site exists[143] (Fig. 6, bottom), which has been proposed to coordinate iron and copper in soybean lipoxygenase[140] and galactose oxidase,[138] respectively. Spectroscopic evidence for soybean lipoxygenase is against the presence of PQQ, but consistent with

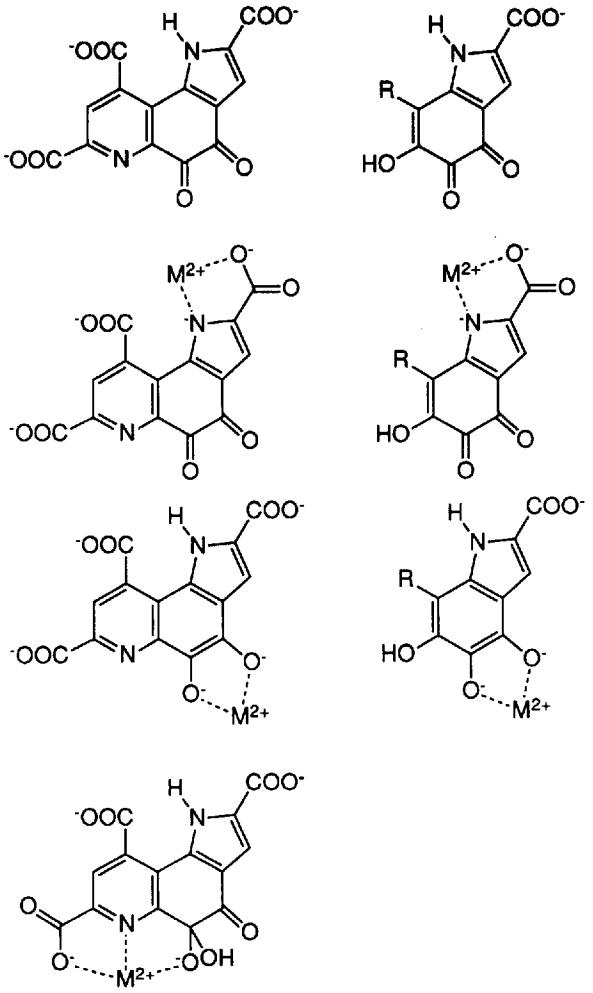


FIGURE 6 Structures and metal coordination modes of quino-cofactors, left: pyrroloquinoline quinone (PQQ), right: covalently bound CHIGQ or TGA-pro-PQQ cofactor (Ref. 130). Top structures, non-complexed oxidized cofactor, upper middle structures, coordination by oxidized cofactor pyrrole anion and nearest carboxylate proposed here for one of the coppers in amine oxidase, lower middle structures, coordination by fully reduced di-ionized cofactor as catecholate (similar structure for half-reduced, ionized semiquinone), lower structure, terdentate (Ref. 143) coordination by ionized hydrated oxidized cofactor, as proposed for iron in soybean lipoxygenase (Ref. 140) and copper in galactose oxidase (Ref. 138).

that of CHIQG, though not with its coordination to iron.[134] Interestingly, for the pterin-dependent iron or copper-containing amino acid hydroxylases, reaction mechanisms involving proximity of pterin cofactor and metal have been proposed,[144,145] and for bacterial (*Chromobacterium violaceum*) copper-containing phenylalanine hydroxylase, an EPR study of the interaction with native and ^{15}N-substituted pterin cofactor has proved that a nitrogen of a pterin pyrazine ring is a direct donor ligand to copper.[146]

Of the eukaryotic enzymes, the presence of the quinone cofactor has been longest established in independent studies in plasma amine oxidase.[126–128] The pig plasma enzyme is a dimer, with 2 slightly different copper sites, as judged from EPR,[147] and one highly reactive and one less reactive carbonyl cofactor moiety per dimer.[147] It could be that the finding of a biphasic reaction between pig plasma amine oxidase and the carbonyl reagent, 2-hydrazinepyridine, reflects subsequent reactions with the more reactive (C-5) and less reactive (C-4) carbonyls in the same cofactor moiety, rather than the presence of two completely distinct moieties of different reactivity. Although it was recognized that the coordination of the pyridine moiety of an organic cofactor was consistent with the data, early plasma oxidase EXAFS data was interpreted as copper coordination by three imidazoles, because of the similarity with copper-imidazole model compound EXAFS.[148] Imidazole coordination was confirmed in electron spin echo envelope modulation (ESEEM) studies,[149] indicating the presence of at least two types of magnetically distinct imidazoles, and completing the copper environment with two waters, one axially, one equatorially coordinated. More recently, an attempt was made to discriminate between the two copper sites in plasma amine oxidase by studying the EXAFS of apoenzyme reconstituted with one and two equivalents of copper per dimer, respectively.[150] The 1 Cu-reconstituted enzyme had regained 50% enzymic activity, and the EXAFS data of 1 Cu- and 2 Cu-reconstituted enzyme were identical. As these data point to a distribution of copper over two sites with equal affinity in both 1 Cu- and 2 Cu-reconstituted enzyme species, the EXAFS must represent a non-resolvable mixture of contributions of both Cu sites, and the conclusion from the EXAFS that differences between the Cu-sites are minimal[150] is totally unfounded. It was also concluded that the analysis provided no evidence for or against the presence of PQQ coordinated to copper, but, while an

elaborate curved wave multiple scattering approach was applied to show that small shells arose from imidazole coordination, a possible PQQ pyrrole, pyridine, catecholate or carboxylate coordination was not investigated, and only the possibility of PQQ phenolate coordination to copper was considered. Model compounds with pyridine coordination show features in the phase-corrected Fourier transform at approx. 4.7 Å[151] which could help distinguish pyridine from imidazole coordination. Any distinction between imidazole and cofactor pyrrole is difficult in EXAFS, although the finding that the inclusion of additional low-Z atoms in addition to the imidazole ring atoms improves the quality of the amine oxidase Cu EXAFS-simulations[150] is an indication for the cofactor pyrrole possibility. This type of coordination would leave the redox-active quinone group in the cofactor moiety free for interaction with the amine substrate,[152] account for the observed interactions between copper and quinone,[153] and put the copper in the right position to account for the ^{19}F-NMR measured distances to copper of fluorine atoms in an inhibitor presumably bound at the cofactor carbonyl.[154] Although the main shell EXAFS analyzes to only 4 low-Z atoms, the proposed model for copper coordination in the EXAFS study[150] is completed by an axial water ligand not observed in EXAFS, and a sulfur or chlorine at 2.38 Å. The peak in the EXAFS Fourier transform is almost certainly an artifact, not due to the multiple scattering approach itself, but to the inclusion of the low-*k* region in the EXAFS data range applied for Fourier transformation and multiple scattering simulation. Extension of the data range into the low-*k* region for multiple scattering simulations of the pospholipase-C Zn EXAFS led to the detection of sulfur/chlorine ligands,[155] which was not noted in single scattering simulations of a shorter data range[36] (see above) and not borne out by crystallographic studies (cf. Fig. 4, middle).[39,40]

Early EXAFS studies of dopamine-β-hydroxylase from bovine adrenal medulla identified 4 N(imidazole) and 2 O(H_2O) ligands.[156] The presence of 3 or 4 imidazole ligands was confirmed in ESEEM studies.[157] The enzyme requires 8 copper atoms per tetramer for full activity and only minor differences are detected by EPR between the 4 Cu- and 8 Cu-reconstituted enzyme species, in spite of a small difference in binding constants.[158] The EPR study suggests a coordination sphere of 2–3 imidazoles and 1–2

oxygen ligands per copper, and place a lower limit of 7 Å on the copper–copper distance, ruling out a type-3 dinuclear copper site for this enzyme. It has been noted that the binding of the two coppers to the pyrrole and pyridine nitrogens (or catecholate oxygens) of PQQ (or CHIQG) would keep them at a distance consistent with the EPR result.[77] With PQQ coordination mistaken for imidazole coordination, this is consistent with EXAFS results, but the presence of PQQ in dopamine-β-hydroxylase[136] is controversial,[137] unlike that in amine oxidase. Recent XAS studies of dopamine-β-hydroxylase have not considered the presence of PQQ or CHIQG nor possible differences between the species with 4 Cu or 8 Cu/tetramer, but focussed on the effects of ascorbate reduction, which is a necessary activation of the enzyme. The reduction leads to the appearance of a pre-edge feature at 8983 eV, which is considered an indication for three-coordinate Cu(I),[159] although the possibility of distorted higher-coordinate Cu(I) is left open, in view of the lower resolution of the dopamine-β-hydroxylase edge (XAES) region as compared to reduced Cu,Zn-superoxide dismutase.[160] One EXAFS study points to a change in coordination upon reduction from 4 ± 1 N/O ligands at 1.98 Å for Cu(II) to 2 or 3 N/O ligands at 1.93 Å and 1 S ligand at 2.3 Å. Although crystallographic and EXAFS studies of blue copper proteins[82,91] (see above) have shown some flexibility for Cu–S bonds in proteins depending on pH and Cu valency, the appearance of sulfur so close to copper upon reduction is unprecedented. It would have been expected to have a more profound effect on the XAES and it has therefore been argued that the observation of the Cu–S bond must be due to an EXAFS data collection artifact.[161] Another study shows the XAES and EXAFS of oxidized and reduced dopamine-β-hydroxylase to be similar to those of $Cu(II)(Imidazole)_4$ and $Cu(I)(imidazole)_4$ aqueous solutions, respectively. Analysis yields 4 low-Z ligands at 1.97 Å and 2.05 Å for oxidized and reduced enzyme, respectively.[161] Because of its correlation with the Debye–Waller-type factor, it is difficult to distinguish between the possibilities of 3- or 4-coordination on the basis of the EXAFS amplitude alone. However, the increase of the average ligand distance upon reduction is in line with the increase in ionic radius on going from Cu(II) to Cu(I) and argues against a decrease in coordination number, as observed for Cu,Zn-superoxide dismutase concomitantly with a decrease in average ligand distance[120,160] (see above).

d. Dinuclear Copper Sites

Hemocyanins, the oxygen-binding proteins of molluscs and arthropods, were among the first metalloproteins to be studied by EXAFS and are an interesting test case since a crystal structure is now available.[162] EXAFS studies on the dinuclear copper site of hemocyanins from various species point to coordination of 2 low-Z ligands per copper in the deoxy form,[163-166] whereas crystallographic studies on spiny lobster (*Panulirus interruptus*) hemocyanin, so far not studied by EXAFS (see below), reveal the presence of 3 imidazole ligands per copper, with a long Cu–N distance for 1 imidazole ligand per copper.[162,167,168] XAES (X-Ray Absorption Edge Spectroscopy) has been demonstrated to give spectra characteristic for the valence state of copper and hence the oxygenation state of the protein, oxygenation leading to the disappearance of a strong edge feature at approx. 8982 eV, and the appearance of a pre-edge feature in addition to an edge shift.[163] XAES has been applied to establish that the crystals in the crystallographic study contain hemocyanin in the deoxy state.[169] The same data were also interpreted to indicate that the copper atoms in deoxy hemocyanin are 3- or even 4-coordinated.[160] New EXAFS data[170] of the dinuclear copper site in *Panulirus interruptus* hemocyanin have been recorded and interpreted to reconcile the apparently conflicting X-ray spectroscopic and crystallographic data, and to provide a basis for an explanation of the cooperativity of oxygenation and its pH dependence. The Fourier transforms of k^3-weighted EXAFS of oxy- and deoxy-hemocyanin are presented in Fig. 7, showing an evident decrease in amplitude upon deoxygenation, clearly indicating a lower coordination number. This is borne out by analysis of the main shell, which gives 5 ± 1 nitrogen and/or oxygen ligands at 1.95 ± 0.02 Å for the oxy, and only 2 nitrogen and/or oxygen ligands at 1.97 ± 0.02 Å for the deoxy form (cf. Table IV). Analysis of the small shells reveals carbon atoms at approx. 4.2 Å indicative of imidazole coordination in both species, while a copper–copper distance of 3.5 ± 0.1 Å is unambiguously detectable only in the oxy-form.

The main shell distance results compare reasonably well with the results of earlier EXAFS studies on hemocyanin from a variety of sources, viz. *Busycon canaliculatum*, *Limulus polyphemus*, and *Cancer irroratus*.[165,166] Although a coordination number of only 4 for the oxyhemocyanins was found, the trend for a decrease in

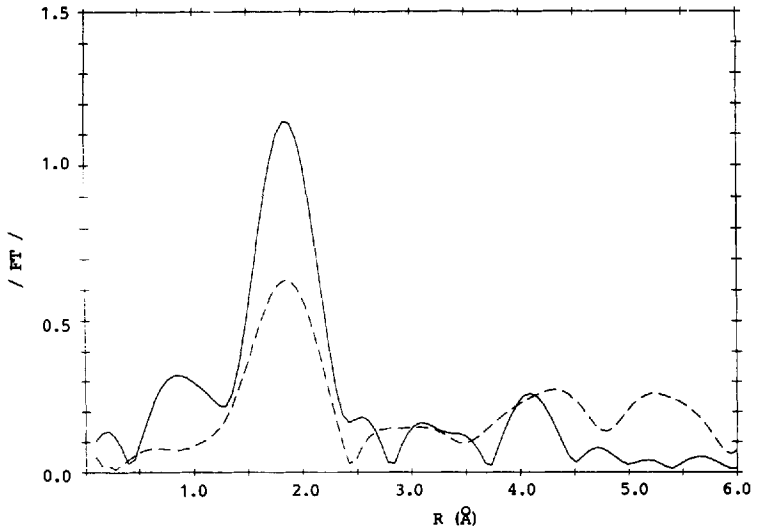


FIGURE 7 Fourier transform of Cu K-edge EXAFS of oxy (solid) and deoxy (dashed) hemocyanin. X-ray fluorescence spectra of concentrated aqueous solutions of *Panulirus interruptus* hemocyanin (pH 4.5 for deoxyhemocyanin, pH 8.75 for oxyhemocyanin) were taken at ambient temperature on the EXAFS station 7.1 of the SERC Daresbury Laboratory, using the experimental set-up with thallium-doped NaI fluorescence detectors described earlier (Refs. 171 and 172).

TABLE IV

Geometric parameters in Å of the dinuclear cooper site in hemocyanin (Fig. 8). Crystallographic values are the average of four (subunit 3–6) of the six dinuclear sites refined in the three dimers making up the unit cell.[167,168]

Bond	Crystallography[167,168] (deoxy)	EXAFS[170] (deoxy, pH 4.5)	EXAFS[170] (oxy, pH 8.75)
Cu^a–His 194 ε–N	1.96	1.97	1.95
Cu^a–His 198 ε–N	1.95	1.97	1.95
Cu^a–His 224 ε–N	2.76	n.d.	1.95
Cu^b–His 344 ε–N	1.95	1.97	1.95
Cu^b–His 348 ε–N	2.66	n.d.	1.95
Cu^b–His 384 ε–N	2.10	1.97	1.95
Cu–bound oxygen			1.95
or bridging ligand			1.95
Cu^a–Cu^b	3.54	n.d.	3.5

coordination number upon deoxygenation was also observed in all those hemocyanins,[165,166] although recent sequence studies indicate that the differences between molluscs and arthropods in the oxy-

gen-binding sites may not be insignificant.[173] An important conclusion from results presented here is that the crystallographically characterized *Panulirus interruptus* hemocyanin is not different in this respect. This appears to be in disagreement with the interpretation of the edge region spectrum leading to a coordination number of 3 to 4 for deoxy-hemocyanin.[160] The interpretation in that study was based on empirical correlations between positions and intensities of XAES features in a series of Cu-compounds, including only one, linear, two-coordinate Cu(I) compound, which is not sufficient to discriminate against 2-coordination in unknown Cu environments in general. For the determination of low coordination numbers, EXAFS, with an estimated error of 20%,[174] is the preferable technique. It is dangerous to infer coordination numbers from XAES features that are known to be more sensitive to other properties of the system, viz. ligand geometry,[175] average ligand distance,[176] and inter-ligand scattering.[177,178] In a more elaborate model compound XAES study,[179] the high intensity of the XAES feature in linear two-coordinate Cu(I) compounds has been described by transition from a 1s state to a doubly degenerate $4p_{xy}$ final state at lower energy than the $1s-4p_z$ transition, and any deviation from linear geometry is expected to perturb the $4p_{xy}$ degeneracy, as is proposed here to be the case in deoxyhemocyanin. In fact, it has been proposed[180] that the relatively high intensity of the 1s–4p transition in the deoxy hemocyanin XAES can be explained by a bent two-coordinate copper coordination geometry.

Just such a ligand arrangement is found at both copper atoms in the dinuclear site when the crystal structure is inspected in detail (Fig. 8, geometrical information from crystallography and EXAFS in Table IV). The thermal motion of the long-distance imidazoles (His 224 at 2.7 Å from Cu_a and His 345 at 2.7 Å from Cu_b) appears to be non-correlated with that of the Cu atoms to such an extent as to wipe out any possible contribution to the EXAFS due to a high value for its Debye–Waller-type factor, accounting for static (and thermal) disorder. In addition, contrary to the case of oxyhemocyanin, the absence of an unambiguous Cu–Cu backscattering in the deoxyhemocyanin EXAFS points to uncorrelated motion of the Cu atoms and argues against the presence of ligands, either exogenous or of protein nature, bridging the Cu atoms in this

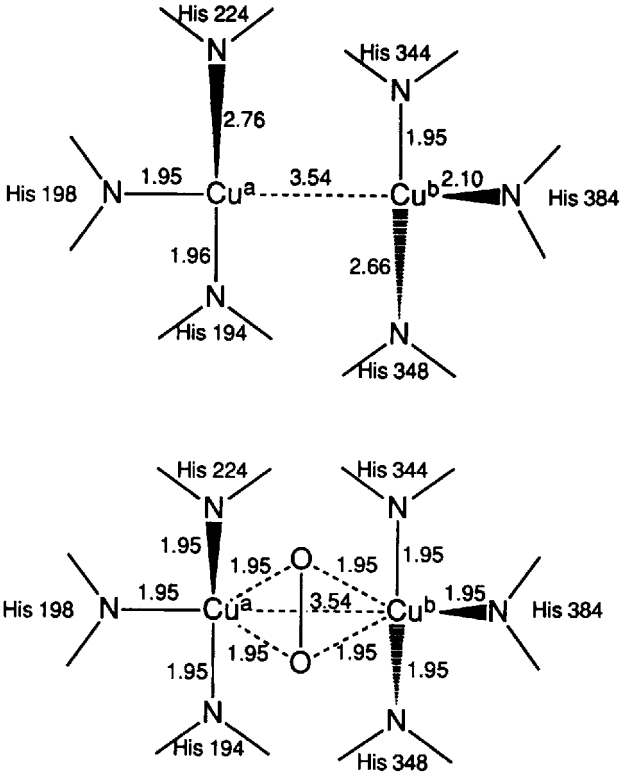


FIGURE 8 Simplified dinuclear copper site environment of deoxy and oxyhemocyanin. Top: Crystal structure derived geometry (Refs. 168 and 169) (Table IV) of dinuclear copper site in deoxyhemocyanin, with Cu^a–N(His 224) and Cu^b–N(His 348) distances consistent with loss of the ligand through protonation, bottom: oxyhemocyanin with oxygen bound as in the μ-η^2:η^2-peroxo dinuclear copper complex (Ref. 184) with EXAFS-derived distances (Ref. 170) (Table IV).

species. Such bridging ligands would have acted to reduce static disorder in the Cu–Cu interaction, facilitating detection in the EXAFS. In this respect, it is worth noting that the measurement and analysis of Cu_2-edtb $(ClO_4)_2$ EXAFS confirmed the results of others[181] who could not detect the Cu–Cu backscattering in this compound, which the crystal structure shows to have 2 Cu atoms 3.0 Å apart without a bridging ligand.[182] The problem of detection of Cu–Cu distances has been discussed earlier in relation to dopamine-β-hydroxylase.[183] Thus, the copper ions in deoxyhemo-

cyanin can be considered to be bent two-coordinate, with an additional imidazole nearby but not coordinating.

The increase in Cu coordination number upon oxygenation found in all EXAFS studies,[163-166] including the results presented here[170] (Table IV), can be explained by assuming that in oxyhemocyanin, in addition to the imidazoles also coordinating in deoxyhemocyanin and the atoms of the dioxygen molecule bridging the copper atoms, one additional imidazole is coordinating to each copper. The distances in Table IV are in good agreement with those of a recently reported crystal structure of a μ-η^2:η^2-peroxo dinculear copper complex[184] with a Cu–Cu distance of 3.56 Å, and two oxygens, two close nitrogens and one more distant nitrogen coordinating each copper at 1.91 Å (average), 2.00 Å (average), and 2.26 Å, respectively. The agreement with the first crystal structure of an oxygenated dinuclear copper model[185] with its 4.359 Å Cu–Cu distance is notably poorer. With both oxygen atoms of the dioxygen molecule bridging the copper atoms in the μ-η^2:η^2-peroxo model, there is no need to invoke additional bridging ligands in order to account for the magnetic coupling between them.[180] The extra coordination of one imidazole ligand per copper upon oxygenation of hemocyanin could be due to its being deprotonated, as the crystallographic Cu-long-distant imidazole distance (approx. 2.7 Å, cf. Table IV) is reminiscent of that found for a similar case in Cu(I) plastocyanin at pH 5.9, an imidazole coordinating Cu(I) at 2.25 Å at pH 7.8 moving away to 3.2 Å in that protein when the pH is lowered to 3.8.[116] In the present study, the deprotonation is probably due to the large difference between the pHs at which deoxy- and oxyhemocyanin were studied, viz. 4.5 and 8.75, respectively. It could also be driven by the uptake by bound dioxygen of one electron per Cu(I) ion, causing the formation of Cu(II) ions aspiring to a higher coordination number and different ligand geometry. The movement of the imidazoles, from 2.7 Å (crystallography) to 1.95 Å (EXAFS) to the copper ions upon oxygenation, would locate the change in copper coordination anticipated on the basis of the difference in ionic radius between Cu(I) and Cu(II).[186] Such changes in the Cu environment have been invoked as a mechanism of communicating the oxygen binding on one site in the protein to the others in order to explain the cooperativity of the oxygenation.[187] At low pH, the deprotonation would be more dif-

ficult, the distant imidazole would not approach the copper upon oxygenation, and possible oxygen binding would not be communicated to other oxygen binding sites, thus accounting for the absence of cooperativity at low pH[187] and the observation of a coordination number of 4 nitrogen and/or oxygen ligands per copper for oxyhemocyanin if the pH is kept at 6.3.[166] The long-distance imidazoles and changes in the oxidation state of copper upon oxygenation have been implicated in an earlier discussion of the cooperativity,[188] but with an emphasis on angular movement of the close His ligands as well as possible hydrogen bonding of the long-distance imidazoles to coordinated dioxygen.

It is interesting to compare these results with those of other enzymes known to contain dinuclear copper sites, tyrosinase and the multicopper oxidases (laccase, ascorbate oxidase and ceruloplasmin). For tyrosinase, the oxy enzyme shows 4–5 nitrogen and/or oxygen ligands per copper, with a Cu–Cu distance of 3.6 Å, and indications of imidazole coordination, but the deoxy enzyme has not been studied.[189] The four-copper enzyme laccase, with a type-1 Cu site, a type-2 copper site and an EPR-silent type-3 dinuclear copper site,[17] has been studied in different ways. It is possible to selectively remove the copper atom from the type-2 site.[190,191] It was established by XAES that the type-2 depleted laccase has its dinuclear copper site reduced[179,192,193] and can be oxidized by hydrogen peroxide only, not by dioxygen.[194,195] In one EXAFS study,[196] met-type-2 depleted *Rhus vernicifera* laccase, with all its remaining three coppers in the Cu(II) state, was compared to its azide complex, and the EXAFS of the dinuclear copper site isolated by correcting for the type-1 Cu by subtracting the EXAFS of the oxidized blue-copper protein, plastocyanin. The resulting difference EXAFS analyzes to 3.2 nitrogens per Cu (3.5 for azide complex), with no detectable copper contribution. In a comparative study of native and type-2 depleted laccase with oxidized and reduced type-3 copper,[197] it was confirmed that Cu–Cu backscattering is absent in type-2 depleted laccase, but it appeared to be detectable in both oxidized and reduced native laccase (0.5 Cu per 4 Cu at approx. 3.4 Å). It was concluded that the dinuclear site is significantly perturbed by type-2 depletion, involving either a substantial increase in the Cu–Cu distance of the type-3 site, or a substantial decrease in rigidity of the site, as

reflected in such an increase in the Debye–Waller-type factor as to wipe out the Cu–Cu backscattering contribution to the EXAFS, perhaps by disruption of a bridging group.

The finding that one of the oxygens originally bound to the type-3 coppers ends up in the coordination sphere of the type-2 copper was the first indication that type-2 and type-3 copper are close together in a hydrophobic cavity.[71] Since the EXAFS work, low temperature magnetic circular dichroism (MCD) studies of the laccase-azide complex have been interpreted to indicate that the type-3 and type-2 copper sites are actually very close in a novel trinuclear copper site.[72,73] This view has now received support from crystallographic studies[70] on another blue-copper oxidase, ascorbate oxidase from green zucchini squash (*Cucurbita pepo medullosa*), which is believed to have a structure (Fig. 4, bottom, Fig. 9) similar to that of laccase and ceruloplasmin because of amino acid homologies between cucumber (*Cucumis sativus*) ascorbate oxidase, fungal (*Neurospora crassa*) laccase and human ceruloplasmin.[74] With hindsight, one could reinterpret the differences between native and type-2 depleted laccase EXAFS by proposing that the Cu–Cu backscattering observed in native laccase is in fact not backscattering between the two copper atoms in the type-3 binuclear site, but between each of the type-3 copper atoms and the type-2 copper, which explains why the backscattering is extinguished upon removal of the latter. However, it could be argued that, as of the Cu–Cu distances in the crystal structure of the trinuclear site that between the two type-3 coppers is shorter (3.4 Å) than that between the type-2 copper and any of the type-3 coppers (3.9–4.0 Å, Fig. 4 and Fig. 9), the Cu–Cu backscattering observed is more likely to be that between the two type-3 coppers. On the other hand, as it has been shown that there is enough space for one of the imidazoles coordinating to each type-3 copper to turn and bridge between the type-3 and type-2 coppers,[70] which implies that these imidazoles may alternately coordinate the type-2 and type-3 coppers, the assignment may to some extent be arbitrary. An alternative interpretation for the disappearance of the Cu–Cu backscattering upon depletion of the type-2 copper is that the copper atoms of the trinuclear cluster are in fact held together by bridging imidazoles between the type-2 copper and the type-3 copper atoms, and that removal of the former results in a less rigid

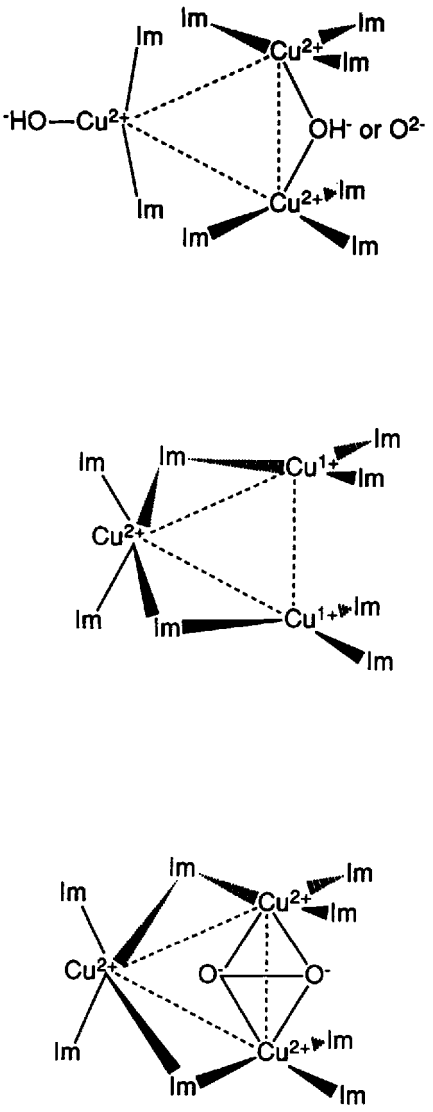


FIGURE 9 Possible mechanism of oxygen binding to trinuclear copper site of multicopper oxidases. Top: resting trinuclear copper site, with type-2 copper to the left and dinuclear type-3 copper to the right (Refs. 70 and 74) (cf. Fig. 4), middle: trinuclear copper site ready for oxygen binding, bottom: dioxygen is bound.

site with uncorrelated motion of the remaining type-3 copper atoms, leading to such a high Debye–Waller-type factor for the remaining Cu–Cu interaction as to wipe out its contribution to the EXAFS.

A model for oxygen binding in multicopper oxidases by analogy to that in hemocyanin can now be made (Fig. 9). The number of imidazoles coordinating to each type-3 copper is thought to vary from 2 in the deoxy enzyme to 3 in oxy enzyme, and the additional imidazole is not protonated in the deoxy state, as proposed above for hemocyanin, but coordinated to the type-2 copper, thereby modifying its redox potential. Resting laccase (Fig. 9, top) is inactive, presumably because it has OH^- coordinated to the type-2 Cu(II),[198] which, like the inhibitor F^-,[199,200] renders the type-2 site redox-inactive. Activation during the presteady state somehow results in dissociation of OH^- from the type-2 copper. The transfer of 2 electrons from the substrate to be oxidized to the type-3 copper atoms proceeds in an ordered way, i.e., subsequent 1 electron reduction of the type-1 and type-2 coppers, and then transfer of 2 electrons from the type-1 and type-2 coppers to the dinuclear site,[201] which is then ready for dioxygen binding (Fig. 9, middle). The "bridging" imidazoles are proposed to be close to the type-2 copper, and at a long distance from the type-3 coppers, like the long-distant imidazoles in the deoxyhemocyanin crystal structure[167,168] (Table IV, Fig. 8). It should be noted that the imidazole ligand should be deprotonated in order to be able to function as a bridging ligand, but coordination to either the type-2 (Fig. 9, middle) or type-3 coppers (Fig. 9, top and bottom) is more in line with the Cu–Cu distances (3.9–4.0 Å, Fig. 4, bottom), which are too short for imidazolate bridging (cf. the distance of 5.7 Å between imidazolate-bridged Cu and Zn in superoxide dismutase.[118,119] Upon dioxygen binding (Fig. 9, bottom) the "bridging" imidazoles move back to the type-3 copper atoms, facilitating their valence change to Cu(II) and leaving the type-2 copper in a coordination geometry more suitable for Cu(I), which makes it more inclined to accept from the substrate further electrons necessary for reduction, protonation, and splitting of the bound dioxygen to water. The type-2 copper can be thought to assist in the splitting of the dioxygen bound to the type-3 coppers, as one of the dioxygen atoms bound to the type-3 coppers ends up as water or hydroxyl anion in the type-2 copper coordination sphere after

reduction.[71] Recent progress in selective substitution methods for type-1 Cu in laccase[202] in addition to established methods for type-2 depletion[190,191] should allow further EXAFS investigations into the type-3 sites of multicopper oxidases, in particular multiple scattering analysis[20–24] in order to quantify the number of imidazoles per copper.

4. CONCLUSIONS

X-ray absorption studies have contributed to the understanding of structure–function relationships in zinc and copper metalloproteins, together with other spectroscopic techniques and crystallography. Care has to be taken to discern the structural features required by the data and those that are merely consistent with it,[13] in particular in analytical multiparameter approaches or multiple scattering simulations where it is not certain if the rigid ligand is an amino acid side chain or an organic cofactor. EXAFS is of limited use in probing distant ligands, like the distant sulfur in blue copper proteins, or metal–metal distances, if there are no structural features like bridging ligands that keep the atoms in the bond of interest at a fixed distance. The interpretation of XAES and EXAFS region of the X-ray absorption spectrum should be in agreement with respect to coordination numbers. Multi-metal proteins can be studied by selective substitution, but results for substituted sites cannot always be extrapolated to the native protein. Several examples have been presented where structural evidence was obtained from comparison of data sets rather than independent analysis, viz. sorbitol dehydrogenase vs. transcription factor, Zn(II)- and Co(II)-substituted phospholipase, various oxidized and reduced blue copper proteins, and oxy and deoxy hemocyanin.

Note Added in Proof

The fungal laccase from *Phlebia radiata* was recently reported to contain a type 1 and a type 2 Cu atom in addition to PQQ.[203] Soybean lipoxygenase is now established not to contain PQQ or any other quinonoid compound[204] while the organic cofactors in pig and bovine serum amine oxidase are reported to be pyridoxal phosphate[205] and 3,4,6-trihydroxy phenylalanine,[206] respectively.

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List of Abbreviations

Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
CD	Circular Dichroism
CHIQG	4-(2-carboxy-4,5-dihydro-6-hydroxy-4,5-dioxo-1*H*-indol-7-yl)glutamic acid ("pro-PPQ,"4-(2-carboxy-6-hydroxyindolo-4,5-quinon-7-yl) glutamic acid)
Cys	Cysteine
edtb	N,N,N',N'-tetrakis[(1*H*-benzimidazol-2-yl)-methyl]-1,2-diamine
ENDOR	Electron Nuclear Double Resonance spectroscopy
EPR	Electron Paramagnetic Resonance
ESEEM	Electron Spin Echo Envelope Modulation spectroscopy
EXAFS	Extended X-ray Absorption Fine Structure
Gln	Glutamine
Glu	Glutamic acid
His	Histidine
Im	Imidazole
LADH	Liver Alcohol Dehydrogenase
MCD	Magnetic Circular Dichroism
Met	Methionine
NAD	Nicotinamide Adenine Dinucleotide
NRM	Nuclear Magnetic Resonance
PAC	Perturbed Angular Correlation spectroscopy
PQQ	4,5-dihydro-4,5-dioxo-1*H*-pyrrolo[2,3-*f*]-quinoline-2,7,9-tricarboxylic acid (methoxatin or Pyrrolo Quinoline Quinone)
TF	Transcription Factor
TGA pro-PQQ	Tyrosine-glutamic acid-arginine pro-PQQ (cf. CHIQG)
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
UV-vis	Ultraviolet and visible light
XAES	X-ray Absorption Edge Spectroscopy
XANES	X-ray Absorption Near-Edge Spectroscopy
XAS	X-ray Absorption Spectroscopy

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