Cobalt(II)-substituted Horseshoe Crab Hemocyanins

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The horseshoe crab Co(II) hemocyanins were prepared by dialyses of *Tachypleus tridentatus*, *Tachypleus gigas*, *Limulus polyphemus*, and *Carcinoscorpius rotundicauda* apohemocyanins against Tris–HCl buffer (pH 8.0) containing Co(II) ion. The amounts of Co(II) introduced into apohemocyanins reached almost 100% of the total sites for copper ion in native hemocyanins. However, all the four Co(II) hemocyanins did not take up oxygen molecule at all. The extremely similar spectral data for the four species revealed that there is no essential difference in the structure of the respective active sites. Both the two Co(II) ions at an active site are in the high spin state and in an distorted tetrahedral geometry, having no such ligating groups as cysteinyl or methionyl sulfur, but at least three nitrogenous ligands, probably histidine imidazoles. On the basis of these data, the environment of active site in native horseshoe crab hemocyanin is discussed.

Hemocyanins(Hc) are known as respiratory proteins for arthropods and molluscs, binding one oxygen molecule per two adjacent copper atoms that constitute an active site unit.[1–4] The deoxy forms containing Cu(I) are colorless and diamagnetic. The deep blue color of the oxy form stems from the absorption band at 570—580 nm, of which the extinction coefficient (ε_{Cu}) was estimated to be *ca.* 500 M^{-1} cm^{-1}.† Resonance Raman studies of oxyhemocyanins established that the O–O stretching band for bound dioxygen is observed at 744—749 cm^{-1}, which is characteristic of peroxide, implying complete transfer of two electrons from each Cu(I) to O_2 on binding of an oxygen molecule (Cu(II) O_2^{2-} Cu(II)).[5,6] The oxyHc is not detectably paramagnetic, indicating that the copper(II) ions are arranged as an antiferromagnetically coupled pair.[7,8] A broad resonance Raman band was recently observed at 1075 cm^{-1}, which is plausibly assigned to the singlet→triplet transition of coupled Cu(II) ions.[9]

Various approaches have hitherto been carried out by many investigators to shed light on the structure and properties of the active site of hemocyanins. Mason and his coworkers prepared methemocyanin by treating *C. magister* hemocyanin with H_2O_2,[10] and reported that the resulting products of H_2O_2-oxidation consist of 80—95% of diamagnetic species, small amount of ESR-detectable Cu(II) and some unreacted native hemocyanin. The active site of the diamagnetic species was assumed to contain spin coupled binuclear Cu(II) cluster which is interpreted in terms of a relaxation of superexchange through one or more bridging ligands such as tyrosine, histidine, serine, or cysteine.

Recently a model for the oxygen binding site of hemocyanin was proposed, based on extended X-ray absorption fine structure studies.[11] The coordination number of copper was proposed as five for oxyHc (three imidazoles, O_2, and the putative extra bridging ligand) and three for deoxyHc (three imidazoles, presumably in a trigonal-like arrangement). This suggests that two ligand groups are detached from each copper upon deoxygenation. The Cu–Cu distance was estimated to be 0.37 nm for oxyHc and 0.34 nm for deoxyHc.

Solomon *et al.* prepared a series of active site derivatives of mollusc and arthropod hemocynains, namely half-apo, met-apo, half-met, ESR-detectable met, ESR-silent met forms.[12] Based on their spectroscopic data, they described that the binuclear copper site in oxyHc contains an endogenous ligand bridge together with the exogenous μ-peroxide bridge between the equatorial planes of two tetragonal coppers. They also pointed out that the active sites in both phyla are quite similar, although the active site of arthropod is distorted from that of mollusc as is indicated by their CD-spectral differences.

We recently prepared a cobalt(II)-substituted Hc in order to disclose the environment of the active site of squid Hc.[13] Substitution of Co(II) ion for a native metal ion in metalloproteins has been an effective approach to the understanding of the structure of metal-site because of intensive and distinct visible absorption and magnetic circular dichroism (MCD) spectra given by Co(II) ion,[14] as clearly demonstrated through the studies of carbonic anhydrase, carboxypeptidase A, thermolysin, plastocyanin *etc.* The amount of Co(II) introduced into *S. lessoniana* apoHc reached 47% of the total sites for Cu in native form, being estimated as nearly complete formation of the half-apoCo(II) hemocyanin. The visible absorption and MCD spectra indicated that the Co(II) is in the tetrahedral geometry (high-spin state), and some histidine imidazoles are coordinated around Co(II). The binding of the imidazole ligands was also considered from the superhyperfine structure in the ESR spectrum of the purple hemocyanin formed on addition of ethylene glycol to oxyhemocyanin.[15–17]

This paper describes the preparation and the spectroscopic studies of Co(II)-substituted hemocyanins of all the four horseshoe crabs found in the world.

Experimental

Materials. Hemocyanins of the horseshoe crabs, *Tachypleus tridentatus*, *Tachypleus gigas*, *Limulus polyphemus*, and *Carcinoscorpius rotundicauda* were supplied by Professor K. Sekiguchi of the University of Tsukuba. These were purified from the respective hemolymphs according to the method of Omura *et al.*[18] The dark blue pellets of pure hemocyanins were dissolved in 50 mM Tris–HCl buffer (pH 8.0)†† and

† In this paper 1 M=1 mol dm^{-3}.

†† Tris: tris(hydroxymethyl)aminomethane.

Tabke 1. Electronic and CD absorption spectra of oxyhemocyanins

Hemocyanins	UV and VIS[a] λ_{max}/nm (ε_{Cu})	CD[a] λ_{max}/nm ($\Delta\varepsilon_{Cu}$)
T. tridentatus	340 (9320), 577 (514)	341 (−11.5), 495 (+0.752), 610 (+0.813)
T. gigas	340 (10300), 578 (500)	341 (−13.3), 495 (+0.886), 610 (+0.960)
C. rotundicauda	340 (11000), 577 (550)	341 (−14), 495 (+0.95), 610 (+1.0)
L. polyphemus	340 (10600), 578 (557)	341 (−14.0), 495 (+0.941), 610 (+1.05)

a) 50 mM Tris-HCl buffer (pH 8.0).

kept in a refrigerator at 4 °C except when the spectra were recorded. The purity of hemocyanin was checked by the ratio of the absorption coefficient at 280 nm to that at 340 nm. The ratios of the four horseshoe crab hemocyanins were in the range of 4.1—4.3.

Preparation of Co(II)-substituted Hemocyanin. First, apoHc was prepared by dialysis of oxyHc against pH 8.0 Tris-HCl buffer (50 mM) which contained 10 mM KCN at 4 °C for 3 d. The copper ion was completely removed from the active site of protein. The Co(II)-Hc was prepared by dialysis of the apoHc against the Tris-HCl buffer which contained about 1 mM $CoCl_2$ at 4 °C for 2 d under nitrogen. When a denatured protein was deposited by Co(II) ion, the dialysis bag was washed against Co(II)-free buffer to remove excess Co(II) ion bound to the protein until the turbid protein became transparent. After treating the apoHc with Co(II) ion, the Co(II)-Hc was dialyzed against Tris-HCl buffer of 2 l. Further the excess of Co(II) ion contained in the resulting crude sample was removed by treating with Chelex 100 resin (50% by volume, pre-equilibrated with the Tris-HCl buffer). The concentration of Co(II) in the final product was determined to be 85—100% of the total sites for Cu in native Hc by employing the method of standard addition, using an atomic absorption spectrophotometer (Nippon Jarrell-Ash AA-1 spectrometer).

In order to examine whether or not the Co(II) exists at the active site of Hc, reconstitution of holoHc was carried out by treating the Co(II)-Hc with $[Cu(CH_3CN)_4]ClO_4$ according to the method of Konings *et al.*[19] The introduction of Cu ion into Co(II)-Hc was observed to be inhibited by the substituted Co(II) ion. The recovery of oxyHc from Co(II)-Hc was thus limited to only 20% of that from apoHc. The extent of recovery of oxyHc was evaluated on the basis of CD intensity at 440 nm where oxyHc exhibited a maximum intensity, while Co(II)-Hc gave actually no CD absorption.

Instruments. The absorption, CD, and ESR spectra were recorded with a Hitachi 323 spectrophotometer, a JASCO J-500C circular dichroism spectrometer, and a JEOL JES-FEIX ESR spectrometer, respectively. The MCD spectra were recorded at 11500 G††† with a JASCO J-500C spectrometer equipped with an electromagnet. The measurements of spectra were carried out at 15—20 °C except for the ESR spectra which were recorded at 77 K.

Results and Discussion

Four Horseshoe Crab Oxyhemocyanins, and Effects of Addition of Ethylene Glycol to Them. The electronic absorption and CD spectra of oxyHc (*T. tridentatus*) are represented in Fig. 1. Other than the characteristic protein band at near 280 nm, two bands were

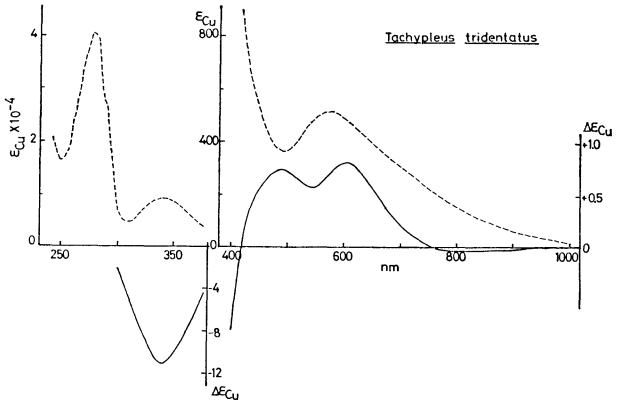

Fig. 1. Electronic absorption spectra (----) and CD spectra (——) of *T. tridentatus* oxyhemocyanin. Conditions: hemocyanin, 0.59 mM; 50 mM Tris-HCl buffer (pH 8.0). ε_{Cu}(M^{-1} cm^{-1}) and $\Delta\varepsilon_{Cu}$(M^{-1} cm^{-1}) are expressed per mol of Cu.

recorded in the near ultraviolet (340 nm), and visible (near 580 nm) regions. These two bands were absent in deoxy- and apo-Hc's. The electronic absorption band at 340 nm has been interpreted as arising from peroxide to copper charge transfer. However, the absorption band at 580 nm, which is responsible for the blue color is still controversial as to whether it should be assigned as peroxide to copper charge transfer[5,12] or as d-d transitions based on Cu(II).[20]

T. tridentatus Hc exhibited three CD bands in the visible region, namely two positive bands at 495 and 610 nm as observed in other arthropod oxyHc,[21] and a weak negative band at around 800 nm. On the other hand, molluscan oxyhemocyanins such as squid (*S. lessoniana*) oxyHc have three CD bands in the visible region; a positive band at 450 nm, a negative band at 570 nm and a positive band at around 710 nm.[21] Oxyhemocyanins of both the phyla display an intensive negative CD band at around 340 nm. The spectral data for the four horseshoe crab oxyhemocyanins bear a strong resemblance to each other as listed in Table 1. From these data it may be considered that the ligating groups and the coordination geometries around the Cu(II) ions are actually the same for the four hemocyanins.

It has been known that oxyhemocyanins exhibit no ESR signal on account of the spin-coupling of the two Cu(II) ions at the active site. However, addition of ethylene glycol (83 v/v% of the total volume) to the buffer solutions of the oxy forms produces a state containing ESR-detectable metHc. The X-band

††† 1 G=1×10^{-4} T.

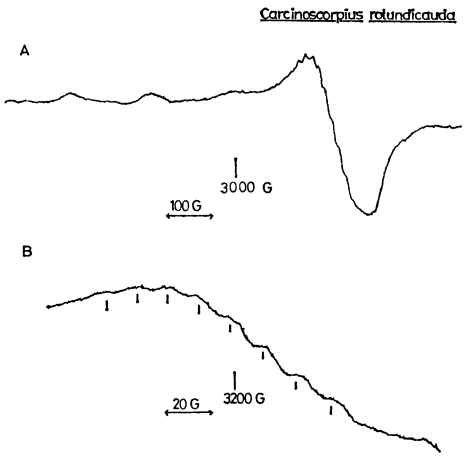

Fig. 2. (A) ESR spectrum of *C. rotundicauda* oxyhemocyanin treated with ethylene glycol (83 v/v%). $g_{//}$ 2.27, $A_{//}$ 178 G, $g_{\perp}$ 2.05. (B) Nitrogen nuclear superhyperfine structure of spectrum (A). Condition: hemocyanin, 0.25 mM.

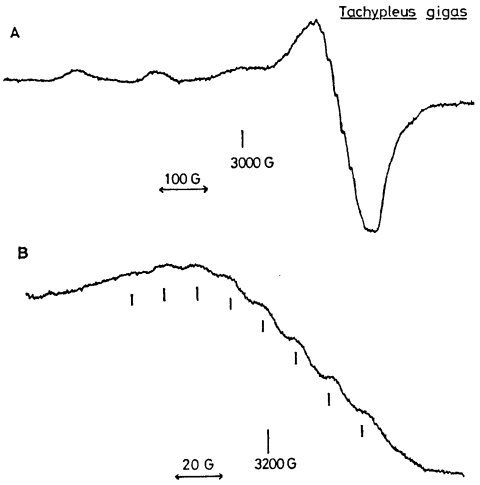

Fig. 3. (A) ESR spectrum of *T. gigas* oxyhemocyanin treated with ethylene glycol (83 v/v%). $g_{//}$ 2.27, $A_{//}$ 176 G, $g_{\perp}$ 2.05. (B) Nitrogen nuclear superhyperfine structure of spectrum (A). Condition: hemocyanin, 0.54 mM.

ESR spectra of *C. rotundicauda* and *T. gigas* are illustrated in Figs. 2 and 3, respectively. The periods between the preparations of the samples and the measurements of ESR spectra were 40 h at 4 °C. The amounts of ESR-active copper in the two samples were estimated to be *ca.* 20% of the total copper in the respective native Hc. The spectrum in Fig. 2A exhibited a signal at $g_{//}$=2.27 with spacings ($A_{//}$) between successive peaks of 178 G. The ESR parameters ($g_{//}$=2.27, $A_{//}$=176 G, $g_{\perp}$=2.05) of the spectrum in Fig. 3A are quite similar to those of *C. rotundicauda*. These values indicate that the Cu(II) ion has a tetragonal geometry. The patterns of the ESR spectra also suggested that there is no magnetic dipole-dipole interaction between the two coppers at the active sites. In other words, the ESR-active copper was supposed to be in a monomeric Cu(II) complex. The spin Hamiltonian parameters of these horseshoe crab hemocyanins were similar to those of

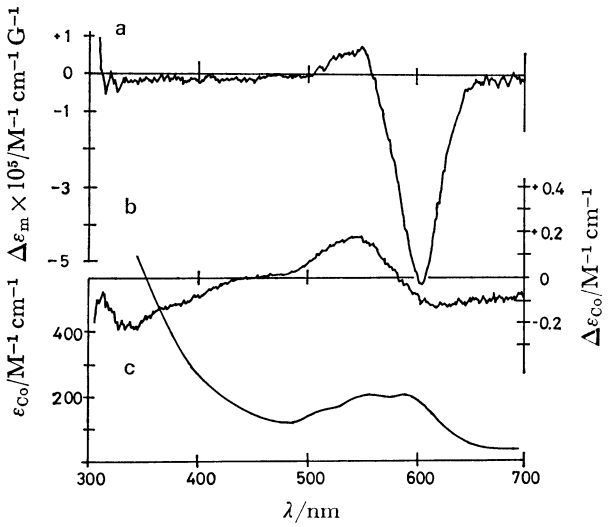

Fig. 4. MCD (a), CD (b), and electronic absorption (c) spectra of *T. gigas* Co(II) hemocyanin. Conditions: hemocyanin, 0.37 mM; 50 mM Tris-HCl buffer (pH 8.0). ε_{Co}, $\Delta\varepsilon_{Co}$, and $\Delta\varepsilon_m$ are expressed per mol of Co(II).

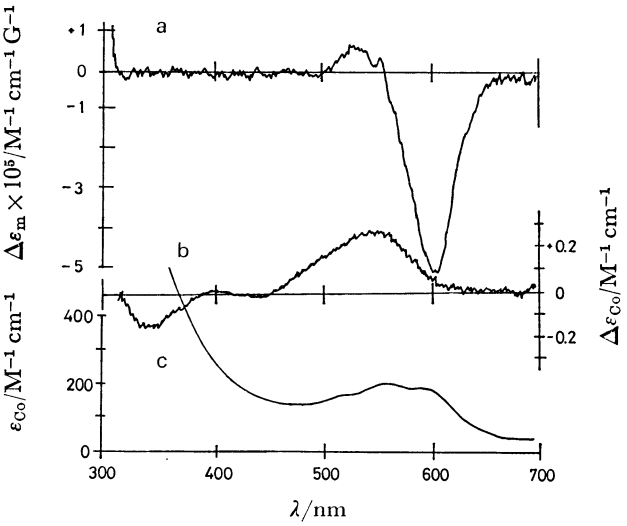

Fig. 5. MCD (a), CD (b), and electronic absorption (c) spectra of *L. polyphemus* Co(II) hemocyanin. Conditions: hemocyanin, 0.34 mM; 50 mM Tris-HCl buffer (pH 8.0).

the squid Hc treated with ethylene glycol (80 v/v%), where the values of $g_{//}$, $g_{\perp}$, and $A_{//}$ were 2.261, 2.059, and 173.8 G, respectively.[16] Further it is noteworthy that at least eight superhyperfine lines due to nitrogens with spacing (A_N) of about 14 G were observed in the region of $g_{\perp}$ (Figs. 2B and 3B), suggesting that at least three nitrogenous ligands are coordinated around Cu(II). The same kind of ESR signals and the superhyperfine structure were also observed for the other two hemocyanins (*T. tridentatus* and *L. polyphemus*) upon addition of ethylene glycol.

Preparation and Spectral Properties of Co(II)-substituted Horseshoe Crab Hemocyanins. We have already succeeded in preparing Co(II) substituted squid hemocyanin.[13] By employing the same procedure, Co(II) hemocyanins of the four horseshoe crabs were prepared. All the Co(II) hemocyanins were pink-violet.

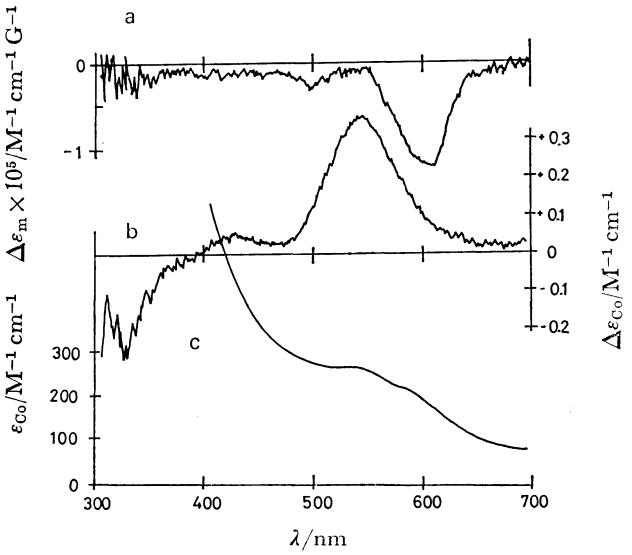

Fig. 6. MCD (a), CD (b), and electronic absorption (c) spectra of aged *T. gigas* Co(II) hemocyanin. Conditions: hemocyanin, 0.55 mM, 50 mM Tris-HCl buffer (pH 8.0). The period between the preparation and the measurement was 20 d at 4 °C.

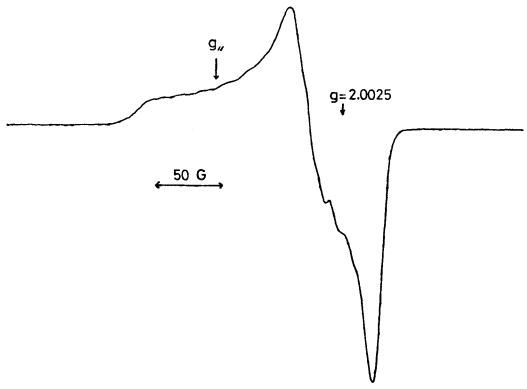

Fig. 7. ESR spectrum of mixture of *T. gigas* Co(II) hemocyanin and cyanide. Conditions: hemocyanin (Co(II), 5.5 mM) plus cyanide(22 mM); 50 mM Tris-HCl buffer (pH 8.0); 77 K.

The absorption, CD, and MCD spectra of *T. gigas* and *L. polyphemus* Co(II) hemocyanins measured at room temperature are represented in Figs. 4 and 5, which display that all the spectra of the two species are remarkably similar to each other. The appearance of CD band at 500—600 nm is indicative of the binding of Co(II) ion with the protein. The absorption and MCD bands of the Co(II)-chromophore at 500—650 nm are attributed to the d-d transition ($^4A_2(F) \rightarrow {}^4T_1(P)$) arising from a tetrahedral Co(II) in the high spin state. In general, Co(II) complexes with tetrahedral geometry are known to show a negative MCD band at a longer wavelength region and one or two weaker positive bands at a shorter wavelength region.[14] The patterns of MCD spectra in Figs. 4 and 5 further reveal that there is no MCD band arising from S→Co(II) charge transfer transition[22-24] in the range of 300—400 nm, suggesting that there is no thiol-coordination of cysteinyl residue around Co(II).[14,25] Cysteinyl sulfur (also probably methionyl sulfur) might not be a ligating atom around the two copper ions in native horseshoe crab hemocyanins. The negative CD band at around 350 nm is not assigned to charge transfer transition of the Co(II)-chromophore because of the absence of MCD band in the range of 300—400 nm. It is suggested at present that the negative CD band might be attributed to the protein structure and/or the amino acid residues (*e.g.* disulfide group) in the protein.

The active sites of the four horseshoe crab hemocyanins were almost completely filled with Co(II) ion, being contrasted to the case of the squid hemocyanin, from which the half-filled Co(II)-Hc was obtained, as already communicated.[13] However, the horseshoe crab Co(II) hemocyanins did not take up oxygen molecule either. With storage at 4 °C for several weeks pink-violet Co(II) hemocyanins turned light brown. This may be due to a kind of aging of hemocyanins, and is visuallized through absorption, CD, and MCD spectra of *T. gigas* Co(II)-Hc as illustrated in Figs. 4 and 6. Comparative inspection of these Figures apparently indicates that the intensity of the ultraviolet absorption band increased significantly and patterns of CD and MCD spectra changed considerably with aging of Co(II)-Hc. The change in 500—600 nm MCD band suggests that the distorted tetrahedral geometry of Co(II) center is converted into another geometry, probably to a five- or six-coordinate structure. It was also confirmed that the oxidation state of Co ion in Co(II)-Hc does not change with aging of the sample. This was proved through an ESR study of aged Co(II)-Hc (*vide infra*).

At liquid nitrogen temperature (77 K) we could not observe the ESR peak for the high spin Co(II) center in the hemocyanin because of the extremely broadening and low amplitude of signal. Addition of one-equivalent cyanide to Co(II) ion gave no ESR-signal either. However, the ESR signal was observed at $g=2$, upon addition of above 2-equivalent cyanide, as shown in Fig. 7 (*T. gigas* Co(II) hemocyanin). The signal did not increase any more with further addition of cyanide, whereas the signal ultimately disappeared by allowing the sample to stand for a day at 4 °C. The ESR signal having the spin Hamiltonian parameters of $g_{//}=2.06$, $A_{//}^{Co}=16$ G, and $A_{\perp}=11$ G,[26] is suggestive of the formation of 1:1 O_2:low spin Co(II) complex with CN^- donor groups and probably endogenous ligands. The characteristic feature of ESR spectrum ($g_{//}>g_{\perp}\simeq 2.00$) closely resembles those of various monomeric Co(II)-dioxygen complexes in literatures.[27-31] The result of evaluation according to the method of Hoffman *et al.* indicated that above 90% of unpaired spin density resides on the dioxygen moiety and below 10% resides on Co(II) ion.[27] The aged *T. gigas* Co(II)-Hc also quantitatively afforded the 1:1 dioxygen adduct with the addition of above 2-equivalent cyanide. This finding implies that no oxidation of the cobalt ion (from Co(II) to Co(III)) took place in the course of the aging.

Metal Binding Site of Hemocyanin. The ESR spectra of oxyhemocyanins treated with ethylene glycol are considered from g and A values to be characteristic

of many square-planar or tetragonally-distorted octahedral Cu(II) complexes with small ligands (Figs. 2 and 3). The copper nuclear hyperfine splitting constants, $A_{//}$, were 176—178 G. These values are more compatible with those observed for Type II copper proteins (150 to 190 G[32,33]) than those for Type I copper proteins (30 to 100 G[32]). The spin Hamiltonian parameters of these denatured hemocyanins are remarkably similar to that of $[Cu(imidazole)_4]^{2+}$ complex ($g_{//}=2.26$, $A_{//}=179$ G, $g_{\perp}=2.05$ at pH 8). The superhyperfine structures on the $g_{\perp}$ region are probably attributable imidazole nitrogens of histidine residues judging from the spacing of about 14 G. The seven or nine superhyperfine lines are theoretically an indicative of coordination of three or four equivalent nitrogens around Cu(II), respectively. In Figs. 2B and 3B, eight nitrogen superhyperfine lines are observed. Thus the present findings do not permit us to conclude which of $(N)_3$- or $(N)_4$-coordination is valid. However, on the basis of these superhyperfine structures, we can actually understand that copper ions at the active site in all horseshoe crab hemocyanins are surrounded by at least three nitrogens, probably histidine imidazoles.

Contrary to the case of squid Co(II)–Hc,[13] the amount of Co(II) introduced into horseshoe crab apohemocyanins essentially reached 100% of the total sites for copper. In the light of the absorption and MCD spectra illustrated in Figs. 4 and 5, coordination geometries around the two Co(II) ions at the active site were proved to be tetrahedral and almost equal to each other. Horseshoe crab Co(II) hemocyanins did not take up molecular oxygen either, although they have two adjacent Co(II) ions contrary to the case of squid Hc. This may be understood by taking into account the structural restriction of the Co(II)-binding site that favors the tetrahedral but not the octahedral geometry which enables binding of dioxygen as exhibited by many Co(II)–dioxygen complexes of low molecular weights.[29,34] The previous experimental results of squid Co(II)–Hc revealed that at least one of the two adjacent copper sites in deoxyhemocyanin provides copper with a tetrahedral-like environment, where some histidine–imidazoles are arranged as ligating groups. In the case of horseshoe crab hemocyanins, the environment of the two adjacent metal sites in deoxy form is considered to be a little more seriously distorted from tetrahedral geometry than that of the active site of squid deoxyhemocyanin, as displayed by the broad splitting of the spin allowed transition ($^4A_2(F)\rightarrow{}^4T_1(P)$) band. The amino acid residues around the metal sites in deoxy-form might be at least three histidyl imidazoles in the light of the ESR measurements of oxyhemocyanins treated with ethylene glycol. Furthermore the fourth coordination site of tetrahedral Co(II) might be occupied by a water molecule.

Recently Spiro *et al.* described a trigonal-like geometry of Cu(I) center bound by three imidazoles in deoxyhemocyanin.[9] Their conclusion does not necessarily contradict our present data, since the Cu(I) sites in deoxy form are supposed to undergo a geometrical rearrangement from a trigonal-like coordination geometry into a tetrahedral-like geometry upon substitution of Co(II) for Cu(I). Thus the active site of native oxyhemocyanin is considered as constituted from a pair of five-coordinate Cu(II) ions (distorted trigonal bipyramidal or square pyramidal geometries) which are bridged by the bound O_2^{2-} and by a putative extra bridging ligand. Although the existence of a bridging protein ligand such as tyrosine phenol was proposed by many investigators,[9,20,35] based on ESR-silent character of metHc, there has been no direct evidence for the presence of phenolate residue.[36] From the spectral investigations (MCD) of the Co(II)–substituted hemocyanins, possibility of presence of tyrosine bridge still remains unknown.

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