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Structure of the Active Site of Hemocyanin. Cobalt(II)-substituted Squid Hemocyanin

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*Cobalt(II)-substituted squid (*Sepioteuthis lessoniana*) hemocyanin was prepared by dialyzing the apohemocyanin against pH 8.0 Tris-HCl buffer containing 5 mM Co(II) ion. The electronic absorption, circular dichroism and magnetic circular dichroism spectra of Co(II)-substituted hemocyanins containing one mol of Co(II) and 2.6 mol of Co(II) per mol of protein were studied comparatively to characterize the coordination geometry and the ligating groups around the binuclear copper sites in native squid hemocyanin. The two copper sites at one active site in squid deoxyhemocyanin may differ from each other in coordination geometry, and may consist of a tetrahedral-like copper site and a tetragonal-like copper site. Three histidine imidazoles are expected to be coordinated around each copper(II). The structure of the active site of squid and horseshoe crab hemocyanins was comparatively discussed on the basis of spectral data of the respective cobalt(II)-substituted hemocyanins of the two phyla.*

Introduction

A number of studies have been carried out to investigate the coordination chemistry of the binuclear copper site in hemocyanin (Hc) [1]. Resonance Raman studies of oxyhemocyanins by Freedman *et al.* demonstrated that the O–O stretching vibration for bound dioxygen is characteristic of a peroxide bridge, hence one electron of each of the two Cu(I) ions transfers to O_2 on binding with an oxygen molecule ($Cu(II)O_2^{2-}Cu(II)$) [2].

Spiro *et al.* reported the result of an EXAFS (extended X-ray absorption fine structure) study on *Busycon canaliculatum* hemocyanin [3]. They proposed an active site model of oxyHc as consisting of two copper atoms which are separated by 0.367 nm. Further the two copper(II)'s were considered to be each bound to three imidazole nitrogens, and bridged by a peroxide and an endogenous protein ligand. Hodgson *et al.* also suggested from EXAFS analyses that two copper atoms in *Megathura crenulata* and *Helix pomatia* oxyhemocyanins are separated by 0.355 nm, being each bound to two imidazoles, and bridged by O_2^{2-} and a protein ligand in an approximately square-planar geometry [4].

Solomon *et al.* prepared a series of active site derivatives of mollusc and arthropod hemocyanins, namely half-apo, met-apo, half-met, ESR-detectable met, and ESR-silent met forms [5]. They described that the binuclear copper site in oxyHc contains an endogeneous bridging ligand, together with the exogenous μ-peroxide bridge between the equatorial planes of two square-pyramidal coppers which are each coordinated to two imidazoles and a water molecule as the axial ligand. Moreover, they pointed out the resemblance of the binuclear copper site of Hc to that of *Neurospora crassa* tyrosinase, from the results of a series of chemical and spectroscopic studies [6].

We have recently prepared cobalt(II)-substituted hemocyanin in order to disclose the structural geometry and the environment of the binuclear copper site of horseshoe crab Hc [7]. Replacement of Co(II) ion for a native metal ion in metalloproteins has proved to be useful in the structural elucidation of metal site, since Co(II) complexes exhibit characteristic visible absorption and magnetic circular dichroism (MCD) spectra due to the d–d transitions. Cobalt(II) derivatives have already been prepared for carbonic anhydrase [8], thermolysin [9, 10], carboxypeptidase A [9, 10], alkaline phosphatase [11, 12], creatine kinase [13], muscle pyruvate kinase [14], phosphoglucomutase [15], liver alcohol dehydrogenase [16, 17], superoxide dismutase [18, 19], plastocyanin [20], stellacyanin [21], serum amine oxidase [22], laccase [23], and tyrosinase [24]. The amounts of Co(II) incorporated into apohemocyanins (*Tachypleus tridentatus, Tachypleus gigas, Limulus polyphemus,* and *Carcinoscorpius rotundicauda*) reached almost 100% of the total sites for copper in native hemocyanins. The MCD spectra of Co(II) hemocyanins indicated both the Co(II) ions at the active site are in the high spin

state and in a distorted tetrahedral geometry, binding at least three nitrogenous ligands, probably imidazoles. The bindings of the imidazole groups were suggested from the nitrogen nuclear superhyperfine structures of ESR spectra of squid and horseshoe crab oxyhemocyanins treated with ethyleneglycol (80 V/V %) [7, 25, 26].

In this paper, we describe the preparation and the spectroscopic studies of Co(II)-substituted molluscan Hc (*Sepioteuthis lessoniana*). Comparison of the results on Co(II) hemocyanins from the two different phyla (squid and horseshoe crab) is also discussed. The preliminary data of Co(II) squid Hc have already been reported in a brief communication [27].

Experimental

Hemocyanin was obtained from hemolymph of squid (*Sepioteuthis lessoniana*), and purified according to the method of Omura *et al.* [28]. Dark blue pellets of pure oxyHc were obtained by ultracentrifuge, and then dissolved in 50 m*M* Tris-HCl buffer (pH 8.0). The purity of Hc was examined on the basis of the ratio of the absorption coefficients at 280 and 340 nm (the purity index A_{280}/A_{340} = 4.1) Determination of amino acid composition was performed by employing an amino acid analyzer A-3300. Protein samples were hydrolyzed for 20 and 40 h in 6 *M* HCl at 110 °C in sealed tubes. Both threonine and serine were estimated by extrapolation to be 0 h. Half-cystein was determined separately by oxidation with performic acid.

Preparation of Co(II) Hemocyanin

First of all, apoHc was prepared by dialysis of oxyHc against 50 m*M* Tris-HCl buffer (pH 8.0) which contained 10 m*M* KCN at 4 °C for 3 days. With this procedure 97% of the total copper was removed from the active site. The Co(II) Hc was prepared by dialysis of the apoHc against the Tris-HCl buffer containing 5 m*M* $CoCl_2$ at 4 °C for 2 days under argon atmosphere. 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) bound to the protein until the turbid solution became transparent. After treating the apoHc with Co(II) ion for 3 times, Co(II) Hc was dialyzed against a Tris-HCl buffer of 2 liter. The excess of Co(II) ion contained in the resulting crude sample was removed by treating with Chelex 100 resin (40% by volume, pre-equilibrated with Tris-HCl buffer) for 1 h. The concentration of Co(II) in the product was determined by use of an atomic absorption spectrophotometer (Nippon Jarrell Ash AA-1 spectrometer) and by employing the method of standard addition. In order to make clear whether or not Co(II) ion exists at the active site of Hc, the reconstitution of holoHc was carried out by treating Co(II) Hc with $[Cu(CH_3CN)_4]ClO_4$ according to the method of Konings *et al.* [29]. The introduction of Cu ion into the Co(II) Hc was inhibited by the substituted Co(II) ion. The recovery of oxyHc from the Co(II) Hc was evaluated on the basis of CD intensity at 440 nm where Co(II) Hc exhibited no CD band, being limited to only 20% of that from apoHc.

Instruments

The absorption, CD, and ESR spectra were recorded with a Hitachi 323 spectrophotometer, a JASCO J-40C circular dichroism spectrometer, and a JEOL JES-FE1X ESR spectrometer respectively. The MCD spectra were recorded at 1.14 T with a JASCO J-40C 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

Absorption and CD Spectra and Amino Acid Composition of Native Squid Hemocyanin

The electronic absorption and the CD spectra of oxyHc at room temperature are illustrated in Fig. 1, in which three absorption maxima are observed at

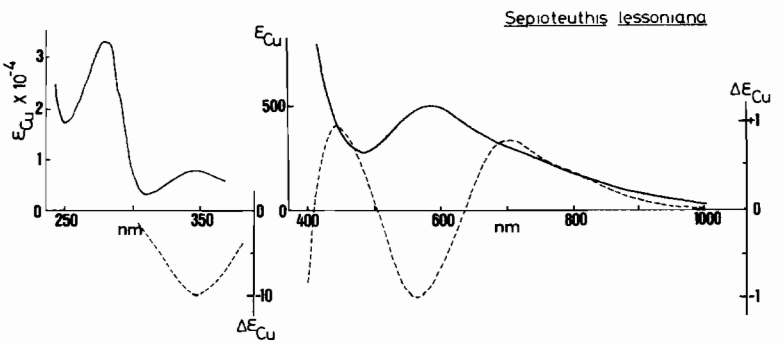

Fig. 1. Electronic absorption spectra (solid line) and CD spectra (broken line) of *S. lessoniana* oxyhemocyanin Conditions 50 m*M* Tris-HCl buffer (pH 8.0), room temperature $\epsilon_{Cu}(M^{-1}\,cm^{-1})$ and $\Delta\epsilon_{Cu}(M^{-1}\,cm^{-1})$ are expressed per mol of Cu.

near 280, 347, and 580 nm. The visible band at 580 nm is responsible for the blue color of oxyHc. Two bands at 347 and 580 nm were absent in colorless deoxy- and apo-forms. The absorption band at 280 nm is assigned to the characteristic band of protein. The CD spectrum of oxyHc exhibits two positive peaks at 446 and 702 nm and one negative peak at 565 nm in the visible region, which are in accordance with the spectra of the other molluscan oxy-hemocyanins [30].

The result of amino acid analysis of squid Hc is tabulated in Table I, which shows a high acidic amino acid content (aspartic and glutamic acid), being consistent with the isoelectric pH of 5.1–5.3. On comparison with the previous data on composition of molluscan hemocyanins [31, 32], *S. lessoniana*-hemocyanin also reveals a high content of serine and methionine, and a low content of proline.

TABLE I. Amino Acid Composition of *S. lessoniana* Hemocyanin.

Amino Acid	Mol %
Asp	10.4
Thr	6.1
Ser	7.1
Glu	10.3
Pro	3.3
Gly	6.4
Ala	8.1
Val	4.5
Met	3.5
Ile	4.9
Leu	8.7
Tyr	3.6
Phe	6.4
Lys	5.4
His	4.2
Arg	5.1
Cys	1.4

Preparation and Spectral Properties of Co(II)-substituted Hemocyanin

We have already described the preparation of Co(II)-substituted hemocyanins of four horseshoe crabs. The Co(II) Hc of squid was blue, whereas those of horseshoe crab were pink violet. Although Co(II) ion was almost completely introduced into the active site of horseshoe crab Hc, the active site of squid was limited to only half-filled with Co(II) ion. Namely, the reproducible amount through several runs of Co(II) incorporated into apoHc was *ca.* 50% of the total sites for copper in native Hc (1 mol of cobalt/mol of protein*). The half-filled Co(II) Hc which contains a [Co(II) (–)] active site can be defined as half apo Co(II) Hc, according to the mode of Solomon *et al.* [5]. In order to obtain the entirely Co(II)-substituted Hc, [Co(II) Co(II)], the Chelex 100 resin treatment was omitted from the preparation. However, the product contained Co(II) at more than 2 mol per mol of protein. The excess Co(II) ion bound to the protein was not removed by dialysis alone against pH 8.0 Tris-HCl buffer. The same sample was, however, converted into the half apo Co(II) Hc by treating with Chelex 100 resin. Neither half apo Co(II) Hc nor the excess Co(II) Hc(abbreviated hereafter as excess-Co(II) Hc) bound oxygen molecule at all.

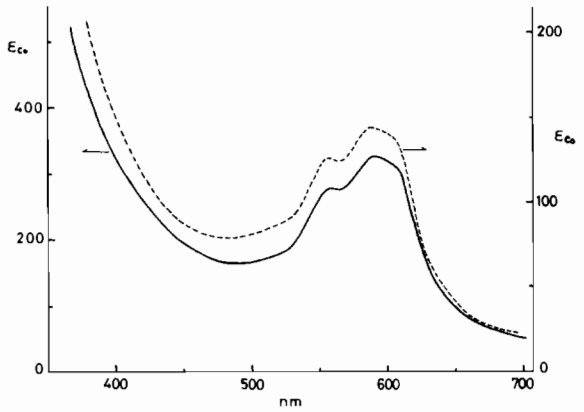

Fig. 2. Electronic absorption spectra of half apo Co(II) Hc (solid line) and excess-Co(II) Hc (broken line). Conditions: protein, 1.9 m*M*; 50 m*M* Tris-HCl buffer (pH 8.0); room temperature. ϵ_{Co} (M^{-1} cm^{-1}) is expressed per mol of Co.

The visible absorption spectra of the half apo Co(II) Hc and the excess-Co(II) Hc are shown in Fig. 2. The latter contained 2.6 mol of cobalt per mol of protein, being blue violet. In the visible region, both the cobalt(II)-substituted hemocyanins exhibit two prominent maxima at 558 and 590 nm and a shoulder at around 610 nm,* all of which are attributed to the d–d transitions.

The CD spectra of the excess-Co(II) Hc and the half apo Co(II) Hc were represented in Fig. 3a and 3b, respectively. The appearances of CD bands in the region of d–d transitions are indicative of the binding of Co(II) with the protein. The CD spectrum of the excess-Co(II) Hc is quite similar to that of half apo Co(II) Hc, except for a negative band at around 630 nm.

*The mol of protein is expressed as the half value of total copper concentration in Hc

*In a previous communication [27], we reported the absorptivities of the maxima at 558 and 590 nm as 540 and 620 M^{-1} cm^{-1}, respectively, whereas corresponding values in this work were estimated as 274 and 324 M^{-1} cm^{-1}, respectively. Although the difference of these absorptivities seems equivocal, both values are considered to be acceptable as those of other tetrahedral Co(II) complexes.

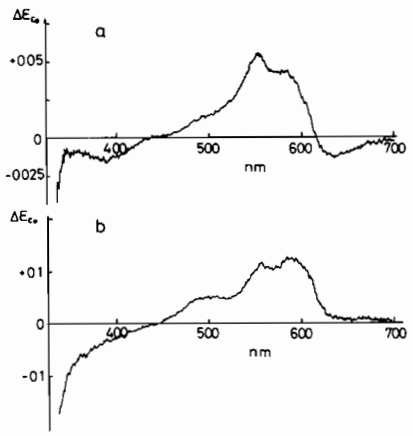

Fig. 3. CD spectra of excess-Co(II) Hc (a) and half apo Co(II) Hc (b). Conditions: protein, a 1.9 m*M*, b 1.7 m*M*, 50 m*M* Tris-HCl buffer (pH 8.0), room temperature. $\Delta\epsilon_{Co}$ (M^{-1} cm^{-1}) is expressed per mol of Co

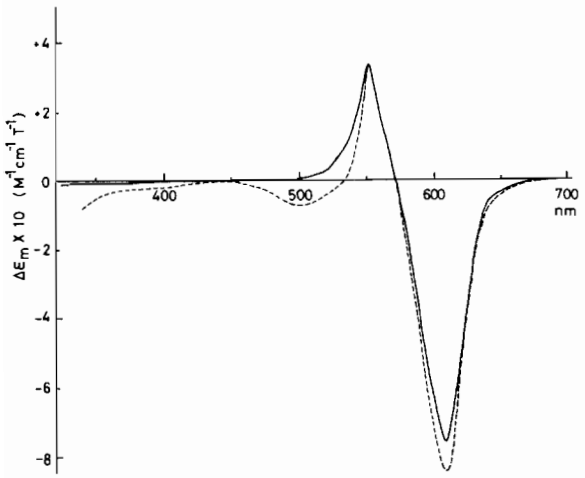

Fig. 4. MCD spectra of half apo Co(II) Hc (solid line) and excess-Co(II) Hc (broken line). Conditions: protein, 1.7 m*M*; 50 m*M* Tris-HCl buffer (pH 8.0), room temperature. $\Delta\epsilon_m$ is expressed per mol of protein

The MCD spectra of the half apo Co(II) Hc and the excess-Co(II) Hc are shown in Fig. 4, in which $\Delta\epsilon_m$ is expressed per mol of protein.* The two curves are almost superposed upon each other, except for the region of 450–550 nm corresponding to the absorption bands (Fig. 2) in the region of 500–650 nm.

*In the case of half apo Co(II) Hc, the value of $\Delta\epsilon_m$ is equal to that expressed per mol of Co(II).

Discussion

Spectral Properties of Squid Oxyhemocyanin

The intense CD band at 346 nm in Fig. 1 has been interpreted as $O_2^{2-} \rightarrow$ Cu(II) charge transfer transitions [5, 33]. The positive CD maximum at 446 nm was previously shown to consist of two peaks (430 and 490 nm) based on the temperature dependence of the CD spectrum [34], and has been assigned as the CT transition associated with a protein ligand (phenolate or oxo) → Cu(II) and $O_2^{2-} \rightarrow$ Cu(II) CT transitions, respectively [5, 33]. However, the negative CD band at 565 nm and the absorption band at 580 nm which are responsible for the blue color are still controversial as to whether they should be assigned as the $O_2^{2-} \rightarrow$ Cu(II) CT transition [5, 33], or as the d–d transitions of Cu(II)-chromophore [35]. The d–d band of Cu(II) should be observed at least in the wavelength region of 700–900 nm, as is clear from a positive CD band at 702 nm. In fact, one of the d–d bands of horseshoe crab Hc was observed at near 800 nm [7].

Characterization of Co(II)-substituted Squid Hemocyanin

The absorption and MCD bands of Co(II)-chromophore of half apo Co(II) Hc in Figs. 2 and 4 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, since the MCD pattern in the visible region of Co(II) complexes with tetrahedral geometry has been known to reveal a negative band at a longer wavelength region, and one or two weaker positive bands at a shorter wavelength region [36], as depicted by the solid line in Fig. 4. The pattern of MCD spectrum for half apo Co(II) Hc is almost identical with that of the 1:1 Co(II) HCAB:CN^- complex* which has previously been established as tetrahedral band pattern, although the band for the former occurs at *ca.* 10 nm longer wavelength region than that for the latter. The Co(II) in half apo Co(II) Hc is considered to have an extremely similar coordination geometry to that in Co(II) HCAB·CN^-. The same kind of similarity was also recognized through their visible absorption spectra, hence the environment around the Co(II) in half apo Co(II) Hc is considered to be nearly regular tetrahedral, being bound by three protein ligands (histidine imidazoles) and probably by an additional ligating group. The fourth coordination site might be occupied by a water molecule because the visible absorption spectrum of half apo Co(II) Hc bears also a resemblance to the spectrum of

*HCAB represents human carbonic anhydrase B. The MCD spectrum of Co(II) HCAB·CN^- has been characterized as arising predominantly from the C term due to the Zeeman splitting of the ground state [8]

the H_2O-occupied Co(II) HCAB at pH 6.0 [37]. The MCD spectrum of half apo Co(II) Hc further reveals that there is no band arising from S → Co(II) [20, 36]. Cysteinyl sulfur (also probably methionyl sulfur) might not be a ligating atom at least around one of the two copper ions in native Hc. Both the Co(II) ions at the active site of horseshoe crab hemocyanins had also no sulfur ligating group [7].

The Co(II) derivatives of squid Hc did not bind molecular oxygen at all. This finding is better understood when one takes into account the structural restriction of the Co(II)-binding site that favors the tetrahedral coordination but not the octahedral coordination, which is capable of binding dioxygen as observed in many Co(II)-dioxygen complexes of low molecular weight [38].

The MCD spectrum of the excess-Co(II) Hc (represented by a broken line in Fig. 4) indicates that the Co(II)-chromophore consists of the tetrahedral species, the amount of which is practically equal to that of half apo Co(II) Hc, and some extra species which has a weak negative MCD band at around 500 nm. At a comparable inducing field, the MCD spectra of tetrahedral complexes are known to exhibit 10 to 100 times stronger bands than those of the octahedral ones [36]. The MCD peaks for octahedral Co(II) complexes, namely $Co(H_2O)_6^{2+}$, Co(II) (glycylglycine)$_2$, and Co(II)-substituted muscle pyruvate kinase [14], were reported to exhibit an explicit negative band at around 500 nm, which was assigned to the $^4T_{1g} \rightarrow {}^4T_{1g}(P)$, $^2T_{1g}$ transition [36]. The extra band of the excess-Co(II) Hc at around 500 nm is thus probably due to the octahedral Co(II) chromophore. In the excess-Co(II) Hc (2.6 mol of cobalt per mol of protein), nearly 1 mol of cobalt per mol of protein is thus considered to locate in the tetrahedral environment (one half of two sites) provided with a N_3X donor set. On the other hand, the residual Co(II) (1.6 mol/mol of protein) is supposed to reside in octahedral geometries and in a less stable complexation compared with the species in tetrahedral environment, in the light of the fact that the octahedral Co(II) is removed preferentially by Chelex 100 resin. The difference between the absorption of excess-Co(II) Hc and half apo Co(II) Hc in the region of 450–600 nm in Fig. 2 may be attributed to the d–d band of octahedral Co(II) chromophore which is estimated as below 50 in ϵ_{Co}. It is not clear whether the octahedral Co(II) ion resides at the other side of the active sites and/or the outer sphere of the protein.

The present experimental results on the half apo Co(II) Hc imply that the active site of squid Hc consists of two non-equivalent environments, contrary to the case of horseshoe crab hemocyanins, in which the active site was completely filled by two tetrahedral Co(II) [7]. A comparison of the MCD spectrum of squid half apo Co(II) Hc with that of

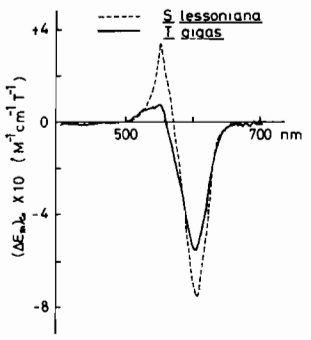

Fig. 5. MCD spectra of squid half apo Co(II) Hc (broken line) and horseshoe crab Co(II) Hc (*T. gigas*, solid line). Conditions: protein, 1.7 m*M*; 50 m*M* Tris-HCl buffer (pH 8.0); room temperature. $(\Delta\epsilon_m)_{Co}$ is expressed per mol of Co.

horseshoe crab Co(II) Hc is given in Fig. 5. The close similarity between the two spectra suggests that the donor groups of the tetrahedral Co(II) site of squid half apo Co(II) Hc coincide with those of horseshoe crab Hc, although there might be a difference in the extent of distortion from the typical tetrahedral geometry in each phylum.

More recently, an incorporation of Co(II) in *Neurospora crassa* tyrosinase has been described by Lerch *et al.* [24]. The copper pair in the active site of tyrosinase was stoichiometrically substituted by Co(II) (2 mol of cobalt/mol protein with a molecular weight of 46000). The absorption spectrum of Co(II) tyrosinase with four transitions at 526, 564, 607, and 635 nm bears more resemblance to that of horseshoe crab Co(II) Hc rather than that of squid half apo Co(II).

Metal Binding Site of Native Squid Hemocyanin

It is perhaps not too speculative to consider that the tetrahedral Co(II) site of the squid Hc corresponds to the one of the copper sites in deoxyHc (represented as 'site A'), though there may exist minor differences. Thus the present data for squid Hc reveal that at least one active site in deoxyHc provides copper with a tetrahedral-like environment, where three histidine imidazoles are arranged as ligating groups, as presented in Fig. 6 (site A).* The fourth site might be occupied by water molecule or the putative bridging ligand, X. Recently Spiro *et al.* proposed a trigonal-like Cu(I) center, bound by three imidazoles for the structure of deoxyHc [3]. Although their model involves no bridging ligand, it

*More recently Co and Hodgson described that EXAFS study on deoxyHc indicates the presence of two histidine ligands in the copper(I) coordination sphere [39]. Our present data for Co(II) Hc however support the presence of at least three endogenous protein ligands in the active site to allow the tetrahedral geometry of Co(II) center.

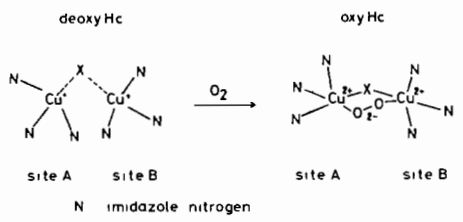

Fig. 6 Proposed active site models of squid deoxy- and oxy-hemocyanins.

is not inconsistent with our result obtained through the study of Co(II) Hc, since the Cu(I) site is supposed to easily undergo a geometrical rearrangement from a trigonal-like coordination into a tetrahedral-like coordination upon substitution of Co(II) for Cu(I). Though in Fig. 6 we tentatively depicted the additional fourth ligand (X) as the bridging ligand for the structure of deoxyHc, this may not be the actual situation, and this is why the bindings between the two Cu(I)'s and X were expressed in Fig. 6 by broken lines.

As for the other site (site B), the coordination geometry was suggested to be in a distorted tetragonal form because of our previous results. In previous papers we have described the purple Hc which was produced upon addition of a large quantity of ethyleneglycol to squid oxyHc [25, 26]. The purple Hc was considered to be an equilibrium mixture between about 60% of ESR-inactive species, which is responsible for the purple color, and about 40% of ESR-active species. The ESR signal ($g_{\parallel}$ 2.261, $g_{\perp}$ 2.059, $A_{\parallel}$ 17.38 mT) of the latter species was characteristic of mononuclear Cu(II) complexes and of tetragonal, square planar structures. The observation of this kind of ESR spectrum upon addition of ethylene glycol suggested the presence of a copper site which readily transforms into a tetragonal-like structure. This site is apparently different from the site A, which has a tetrahedral-like environment as described above. The ESR-active site in the purple Hc (site B) and the tetrahedral Co(II) site in the half apo Co(II) Hc (site A) seem to provide useful information on the complete feature of an active site in squid Hc.

The active center of native oxyHc is proposed to consist of a pair of five-coordinate Cu(II) ions which are bridged by the peroxide ion and a putative ligand. The two copper ions were inferred to be Cu(II) ions each in a non-equivalent coordination geometry of very low symmetry [34]. In Fig. 6 the dotted lines of oxy form presented the original geometry of deoxy form. Contrary to the case of squid Hc, the coordination geometries around the two copper ions at the active site of horseshoe crab Hc were observed to be nearly equivalent [7].

Although the existence of a bridging protein ligand such as tyrosine phenol was proposed by many investigators [33, 35, 40, 41], there has been no direct evidence for the presence of phenolate residue [4, 42]. Oxide (O^{2-}) or hydroxide (OH^{-}) bridge might be the most possible candidate for the bridging ligand at the binuclear copper site of oxyHc [42].

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