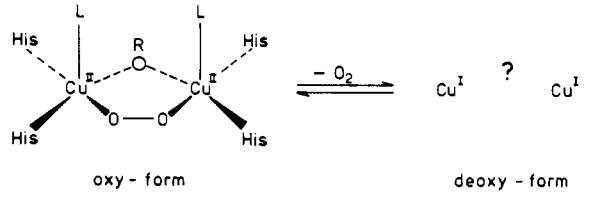
Cobalt(II)-Hemocyanin: A Model for the Cuprous Deoxy Protein Giving Evidence for a Bridging Ligand in the Active Site†

J. Lorösch and W. Haase*
Institut für Physikalische Chemie, Technische Hochschule, 6100 Darmstadt, West Germany
Received September 23, 1985; Revised Manuscript Received April 14, 1986

ABSTRACT: Substitution of the antiferromagnetically spin-coupled Cu(II) pair in *Limulus polyphemus* hemocyanin by cobalt(II) involves supplementary binding to peripheral sites that contribute to the aggregation process in hemocyanin. To separate the effects of the two different binding sites, an additional Co(II) derivative was prepared having still two Cu(II) in the active site. Co(II)-hemocyanin contains two Co(II) in the active site and about 4.5 mol of Co/mol of protein bound to the peripheral sites. Since the Co(II) pair in the active site is electron paramagnetic resonance silent and diamagnetic between 5.1 and 300 K, a spin coupling between the two Co(II) ions is assigned, indicating at least one bridging ligand between the two ions. The absorption spectrum of Co(II)-hemocyanin arises exclusively from the Co(II) ions in the active site. Intensities and energies of the d–d transitions in the visible (ϵ_{548} = 800 M^{-1} cm^{-1}, shoulders at 525 and 595 nm) and near-infrared region (ϵ_{1130} = 43 M^{-1} cm^{-1}) indicate tetrahedral coordination around this Co(II) center. Since the coordination geometry of this site is in contrast to the tetragonal cupric site in oxyhemocyanin, Co(II)-hemocyanin is discussed as a model for the cuprous deoxy form of the protein. Co(II) from the peripheral sites exhibits no detectable absorption spectrum. From magnetic susceptibility measurements, a zero-field splitting of 118 cm^{-1} was calculated, which is typical for octahedral ligand geometry of this Co(II) site.

Hemocyanins are copper-containing oxygen-carrying proteins in the hemolymph of molluscs and arthropods, binding one molecule of dioxygen per two copper atoms (Lontie & Vanguickenborne, 1974). Under physiological conditions, these are highly aggregated molecules with total molecular weights into the millions (Van Holde & van Bruggen, 1971). The level of aggregation depends on pH and on the concentration of divalent cations (Ca^{2+}, Mg^{2+}) (Bannister, 1977). The basic unit of hemocyanins contains one active site with two copper atoms having a molecular mass of 50–100 kDa; e.g., a mass of 66 kDa has been determined for the *Limulus polyphemus* protein (Sullivan et al., 1974).

EXAFS[1] measurements of different hemocyanins established Cu–Cu distances of 3.6 ± 0.2 Å for the oxy form as well as for the deoxy form (Brown et al., 1980; Woolery et al., 1984). Since the Cu(II) in the oxidized form was found EPR-nondetectable and showed diamagnetic behavior between 4 and 300 K (Dooley et al., 1978), the hemocyanin active site was assigned as an antiferromagnetically spin-coupled copper pair. Resonance Raman studies showed that the dioxygen is bound to the two copper atoms as peroxide in a μ-dioxo fashion (Loehr et al., 1974; Freedman et al., 1976). Detailed spectroscopic studies have demonstrated that the binuclear cupric site in oxyhemocyanin contains the exogenous peroxo and an unknown endogenous bridge between the equatorial planes of both tetragonal coppers (Eickman et al., 1979; Himmelwright et al., 1980a). The hemocyanin active site has been found extremly similar to that of the monooxygenase tyrosinase (Himmelwright et al., 1980b) but significantly different from the O_2-binding binuclear copper site of the multi-copper oxidase laccase (Spira et al., 1982; Winkler et al., 1982). The deoxyhemocyanin structure was rather unknown until the X-ray investigations of the cuprous protein form of *Panulirus interruptus* (Gaykema et al., 1984). From the crystal structure at 3.2-Å resolution, a 3-fold coordination of each Cu(I) by three histidine residues has been pointed out. No evidence for a bridging ligand could be given from this study.



For more information on the binuclear active site in hemocyanins, the copper of the native protein from the horseshoe crab *Limulus polyphemus* was chemically replaced by cobalt (Suzuki et al., 1982). This substitution is a widely used procedure because Co(II) complexes exhibit characteristic optical and magnetic properties that are highly dependent on the ligand geometry (Carlin, 1965; Makinen et al., 1984). Co(II) derivatives have been prepared of carboxypeptidase A, carbonic anhydrase, alkaline phosphatase (Vallee & Wacker, 1970), phosphoglucomutase (Ray et al., 1972), hemoglobin (Hoffman & Petering, 1970), horseradish peroxidase (Wang & Hoffman, 1977), horse liver alcohol dehydrogenase (Sytkowski & Vallee, 1976; Maret et al., 1979), superoxide dismutase (Calabrese et al., 1972), stellacyanin (McMillin et al., 1974), rubredoxin (May & Kuo, 1978), *Rhus* laccase type 1 (Larrabee & Spiro, 1979), and *Neurospora* tyrosinase (Rüegg & Lerch, 1981). In all the substituted proteins, monomeric Co(II) complexes are formed with exception of the tyrosinase derivative. The active site of Co(II)-tyrosinase is assigned containing an antiferromagnetically spin-coupled Co(II) pair, which is also expected for the hemocyanin derivative.

Since the metal substitution with Co(II) uses the metal-free

† This research was supported by the Deutsche Forschungsgemeinschaft. J.L. thanks the Fond der Chemischen Industrie, Frankfurt, for a scholarship. This paper is part of the Dr.-Ing. dissertation of J.L., D17, Technische Hochschule Darmstadt.

[1] Abbreviations: EXAFS, extended X-ray absorbance fine structure; EPR, electron paramagnetic resonance; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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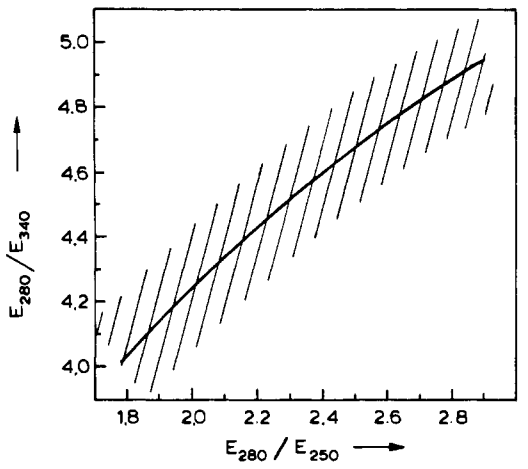


FIGURE 1: Correlation between ratios of absorption coefficients E_{280}/E_{340} and E_{280}/E_{250} resulting from superposed scattering effects $[I = f(\nu^4)]$ to the absorption spectrum of stripped hemocyanin. The marked region illustrates the maximum deviation tolerated for the used preparations.

form of hemocyanin, the process can be compared to the reconstitution of apohemocyanin with Cu(I), resulting in the deoxy form (Lontie et al., 1965). Thus, the Co(II) derivative can be assigned to monitor the properties of the cuprous site, which cannot be investigated by most common spectroscopic techniques because of the d^{10} system of Cu(I). Supplementary Co(II) binding to the sites occupied by the divalent cations that are involved in the aggregation process can give information about these sites in the native protein.

This paper describes the modification of a published preparation procedure for Co(II)-hemocyanin (Suzuki et al., 1982). Optical and magnetic properties of two different Co(II) binding sites are discussed by use of two Co(II) derivatives of hemocyanin. The results are related to low molecular weight complexes and other Co(II)-substituted proteins.

Materials and Methods

Preparation of Protein. Specimens of the horseshoe crabs *Limulus polyphemus* were obtained from the Supply Department of the Marine Biological Laboratory, Woods Hole, MA. The animals were kept in a large tank of circulating seawater. Hemolymph was obtained by cardiac puncture. Clotting agents and other debris were removed by centrifugation. The samples were dialyzed extensively against pH 8.0 Tris-HCl buffer (50 mM) and centrifuged again. Hemocyanin prepared by 72-h dialysis against 50 mM Tris-HCl and 10 mM EDTA at pH 8.9 is referred to as stripped hemocyanin (Sullivan et al., 1974).

The purity of the hemocyanin preparations was checked by correlating two ratios of absorption coefficients (E_{280}/E_{340} and E_{280}/E_{250}). This method takes into consideration changes of the normally used ratio E_{280}/E_{340} as a consequence of the variable superposing scattering due to the changing aggregation behavior of the protein. Basic values from absorption spectra without superposing scattering were taken with preparations of the nonaggregated stripped hemocyanin (E_{280}/E_{340} = 4.9 and E_{380}/E_{250} = 2.9). Superposition of scattering parts $[I = f(\nu^4)]$ leads to the dependence of the two ratios of absorption coefficients as shown in Figure 1. The purity of the preparations was verified to be within ±0.1 of the correlated ratios calculated.

Protein concentration of the native hemocyanin and derivatives was determined spectrophotometrically at 280 nm after correction of the superposed scattering part. The absorption coefficient used was $A^{1\%}_{1\,cm}$ = 13.9 (Nickerson & Van Holde, 1971).

Metal Substitution with Co(II). Two hemocyanin derivatives with Co(II) as the substituting metal ion have been prepared. The preparation scheme in Figure 2 illustrates the preparation process.

(A) Preparation of Co(II)-Hemocyanin. Co(II)-hemocyanin was prepared by a modification of the procedure described by Suzuki et al. (1982). Native or stripped hemocyanin (90–150 mg) was incubated with 50 mM potassium cyanide (molar excess over hemocyanin >1000) in 50 mM Tris-HCl, pH 8.0, for 3 days. The apoprotein was obtained by extensive dialysis against 50 mM Tris-HCl, pH 8.0, to remove low molecular weight components.

The Co(II)-hemocyanin was prepared by dialysis of the apoprotein (30–50 mg) against 0.25 mM $CoCl_2$ (20–30-fold excess over apohemocyanin) and 50 mM Tris-HCl, pH 8.0, for 72 h under nitrogen. Excess cobalt was removed by dialyzing the hemocyanin derivative against 50 mM Tris-HCl, pH 8.0, with a minimum of five buffer changes.

The last step of the published preparation procedure (Suzuki et al., 1982) has been omitted. Treatment of Co(II)-hemocyanin with ion exchangers like Chelex 100 results in a partial depletion of both Co(II) binding sites monitored by changes in the optical spectra (active site) and in the magnetic behavior (peripheral sites).

(B) Preparation of Co-b-Hemocyanin. Stripped hemocyanin (30–50 mg) was incubated with 0.25 mM $CoCl_2$ (20–30-fold excess over stripped hemocyanin) in 50 mM Tris-HCl, pH 8.0, for 72 h under nitrogen. Excess cobalt was removed in the same way as described for Co(II)-hemocyanin.

Spectroscopic and Magnetic Techniques. Absorption spectra in the ultraviolet, visible, and near-infrared region were recorded on a Varian Cary 17 spectrometer. Magnetic susceptibility measurements have been carried out on a Faraday system described previously (Merz, 1980). The samples for the magnetic measurements were concentrated in a Speed-Vac

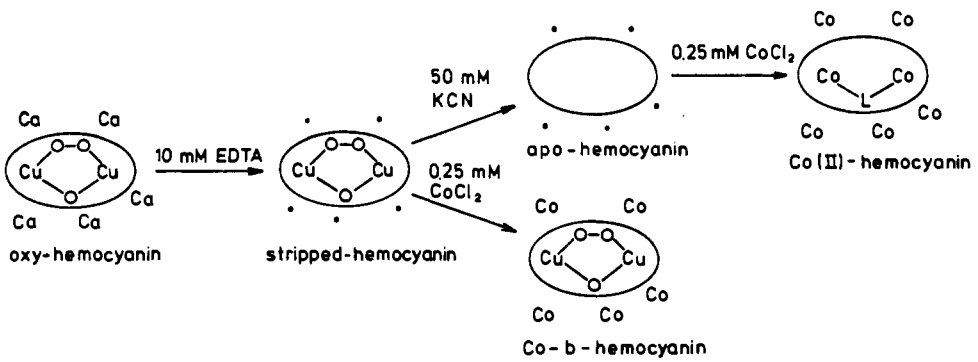


FIGURE 2: Illustration of different steps involved in preparation of two hemocyanin derivatives.

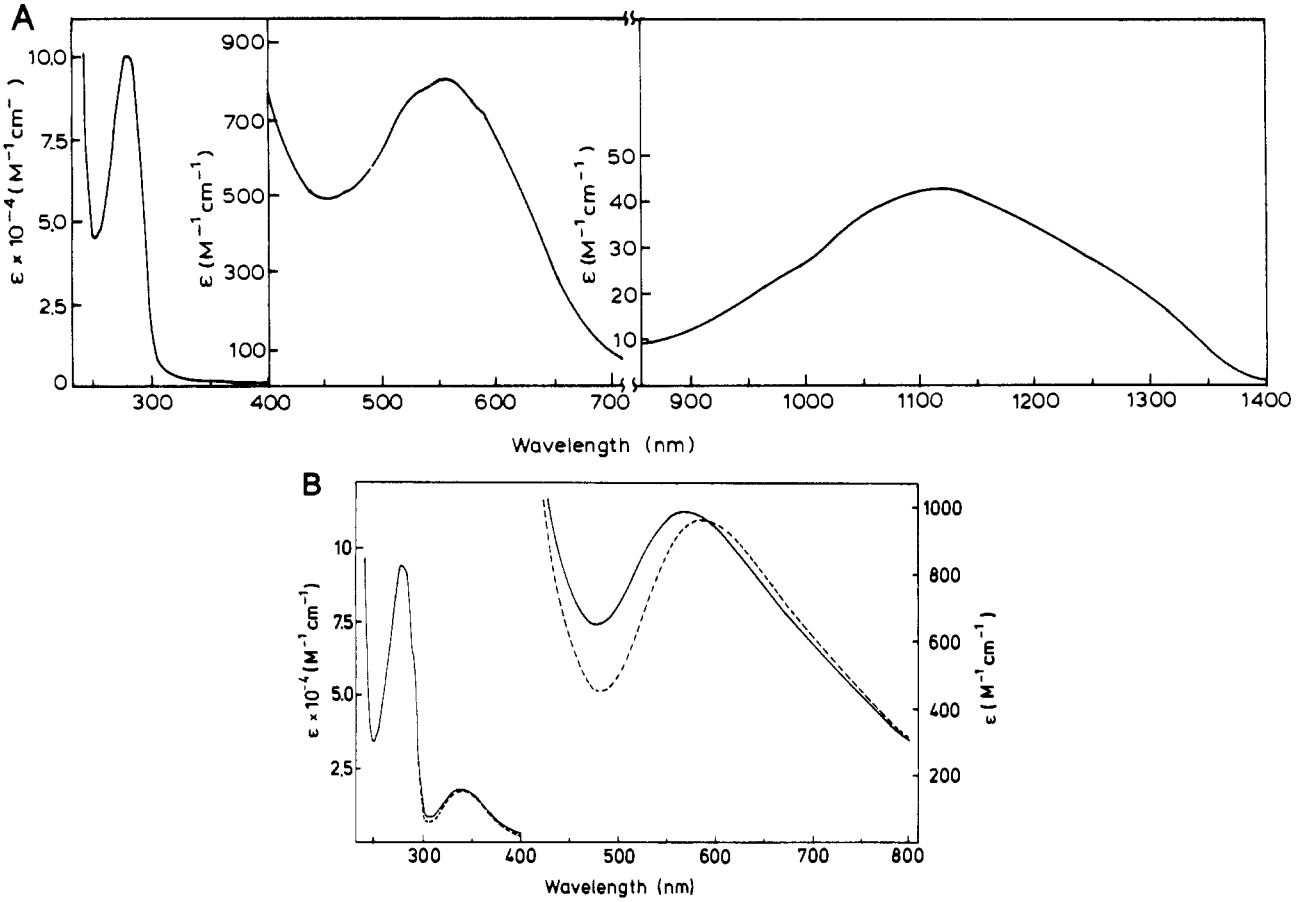


FIGURE 3: Optical spectra of Co(II)-hemocyanin and Co-b-hemocyanin at 25 °C. (A) Ultraviolet–visible and near-infrared absorption spectrum of 0.5 mM Co(II)-hemocyanin in 50 mM Tris-HCl, pH 8.0. (B) Ultraviolet–visible absorption spectrum of Co-b-hemocyanin compared to that of stripped hemocyanin (broken line). Protein concentration and buffer as in (A).

concentrator up to 165 mg/mL (~2.5 mM) upon addition of a 2.5-fold excess of sucrose relative to the protein (Heirwegh et al., 1959). EPR spectra in the X-band were run on a Bruker ER 420 spectrometer. Metal analysis was performed with an energy-dispersive X-ray fluorescence analysis system (Neff, 1976). The probes were measured by thin-layer techniques (Plesch, 1977) after 1.5–2.5 mg of the protein had been hydrolyzed in concentrated HNO_3 at 100 °C.

The glassware used for the preparation of the Co(II) derivatives of hemocyanin was soaked in concentrated nitric acid and detergents and extensively rinsed with bidistilled water. All chemicals used were of analytical grade.

Results

Metal Substitution with Co(II). During the analysis of the experimental results of Co(II)-hemocyanin, it became clear that there must be a second Co(II) binding site in addition to the active site. So we decided to prepare a second Co(II) derivative of hemocyanin having still two copper atoms in the active site. This derivative was labeled Co-b-hemocyanin (see the preparation scheme in Figure 2).

Stripped hemocyanin was found to be resistant toward direct substitution of the Cu(II) active site by Co(II), but 4.2 mol of cobalt/mol of protein (see Table I) was found after Co(II) incubation, forming Co-b-hemocyanin. On the other hand, apohemocyanin binds 6.7 mol of cobalt/mol of protein when it is exposed to Co(II) solutions. When Co(II)-hemocyanin is exposed to Cu(I), it binds only a small amount (<0.4 mol of Cu/mol of protein) (Suzuki et al., 1982) in contrast to apohemocyanin, which can be reconstituted with Cu(I). So both Cu(II) and Co(II) ions are tightly bound to the binuclear

Table I: Metal Binding Behavior of *L. polyphemus* Hemocyanin

sample	mol of Cu/mol of protein	mol of Co/mol of protein
oxyhemocyanin	1.8 ± 0.2	
apohemocyanin	<0.02	
Co(II)-hemocyanin	<0.02	6.7 ± 0.4
Co-b-hemocyanin	1.8 ± 0.2	4.2 ± 0.3

active site of hemocyanin and cannot be replaced directly by one another.

About 4.5 mol of cobalt/mol of protein is found in peripheral sites (see Table I), which are assumed to be normally occupied by Ca^{2+} or Mg^{2+} ions being responsible for the aggregation of native hemocyanin. Depletion of the peripheral sites without affecting the Co(II) within the active site by use of different ion exchangers is not possible. Co(II)/Ca(II) competition studies up to a 50-fold Ca(II) excess yield no changes in the Co(II) binding behavior of the hemocyanin derivatives.

Spectral Properties of Different Co(II) Binding Sites. Co(II)-hemocyanin is pink-violet; its absorption spectrum is shown in Figure 3A. In the visible region there is a broad absorption maximum at 548 nm (ϵ = 800 M^{-1} cm^{-1}). Shoulders at 595 and 525 nm indicate a splitting of the prominent absorption feature. The near-infrared region shows a weak absorption band at about 1130 cm (ϵ = 43 M^{-1} cm^{-1}). The charge-transfer band at 340 nm (ϵ = 20 000 M^{-1} cm^{-1}) typical for oxyhemocyanin (Himmelwright et al., 1980a) has completely vanished in Co(II)-hemocyanin.

The absorption features of Co-b-hemocyanin are identical with those from stripped hemocyanin (see Figure 3B). The

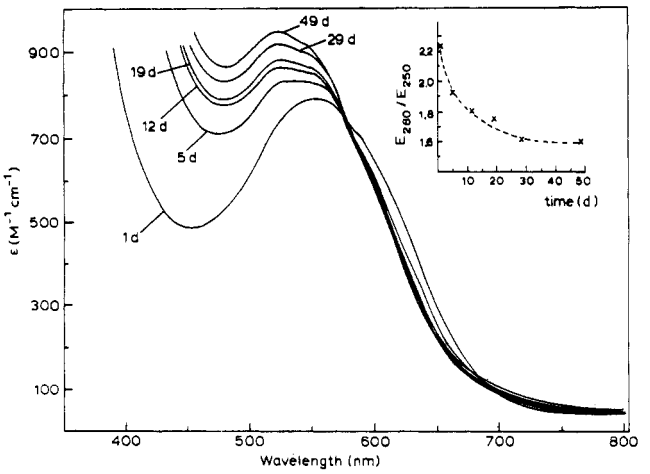


FIGURE 4: Variations of visible absorption spectrum of 0.5 mM Co(II)-hemocyanin during aerobic aging process at 4 °C. Changes in the UV protein absorption are monitored by the time-dependent variation of the ratio E_{280}/E_{250} after the end of the preparation procedure.

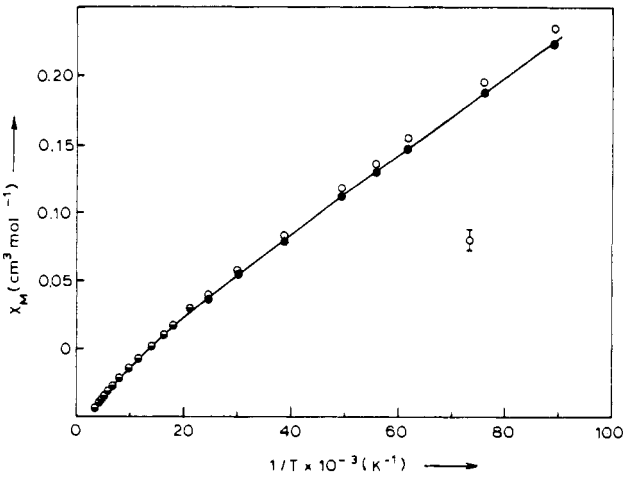


FIGURE 5: Temperature dependence of magnetic susceptibility of Co(II)-hemocyanin (○) and Co-b-hemocyanin (●) related to 1 mol of peripheral-bound Co(II). The full line represents the calcualted values.

blue shifts of the absorption bands of the Co(II) derivative arise from the superposed scattering part increased by a different aggregation behavior.

Aging of Co(II)-Hemocyanin. Co(II)-hemocyanin undergoes an aging process under aerobic conditions at 4 °C. The variation of the pink-violet color to light brown is monitored by changes of the absorption spectrum presented in Figure 4. We have included the changing ratio E_{280}/E_{250} as a function of time after the end of the incubation procedure, illustrating the influence of the superposing scattering part.

Ligand Binding Studies. Inhibitors of the native hemocyanin such as azide and fluoride and addition of peroxide do not affect the absorption properties of Co(II)-hemocyanin even at a 1000-fold molar excess over the protein derivative.

The addition of cyanide to Co(II)-hemocyanin in an equimolar amount causes no changes in the absorption spectrum. But with increasing cyanide concentration [molar excess over Co(II)-hemocyanin >10], the splitted absorption feature at 548 nm is decreased without affecting the spectral shape. Furthermore, a new absorption band is formed at 380 nm with a final absorption coefficient of about 700 M^{-1} cm^{-1}, very similar to the spectral feature reported for KCN-treated Co(II)-tyrosinase (Rüegg & Lerch, 1981).

Magnetic Susceptibility Measurements. Concentrated samples of Co(II)-hemocyanin and Co-b-hemocyanin (≈2.5 mM) have been measured in the temperature range 5.1–300 K. Protein concentration and metal content of each samle were checked by the methods described above.

The exchange-coupled Cu(II) pair in the active site of oxyhemocyanin, which is still present in Co-b-hemocyanin, is known to be diamagnetic in the entire temperature range treated (Dooley et al., 1978). So the measurable paramagnetic effect of Co-b-hemocyanin samples is related exclusively to the peripheral bound Co(II) ions. To monitor the magnetic effect resulting from the Co(II) ions in the active site of Co(II)-hemocyanin, we have compared the magnetic susceptibility per mole of peripheral Co(II) between the two derivatives (see Figure 5). The number of peripheral Co(II)'s in Co(II)-hemocyanin has been calculated by assuming the active site is occupied by two Co(II) ions. Figure 5 points out identical behavior for both hemocyanin derivatives without any contribution from the Co(II) pair in the active site. So it can be assumed that the active site of Co(II)-hemocyanin is formed by an antiferromagnetically spin-coupled Co(II) pair.

The mole susceptibility of the peripheral Co(II) ions show a significant deviation from Curie law behavior illustrated in the χ vs. T^{-1} plot in Figure 5. Quantitative description of the magnetic behavior was obtained with the following procedure.

The Hamiltonian $\hat{H}$ for the high-spin Co(II) becomes

$$\hat{H} = g\mu_B H\hat{S} + D[\hat{S}_z^2 - \hat{S}(\hat{S}+1)/3] + E(\hat{S}_x^2 - \hat{S}_y^2) \quad (1)$$

including the zero-field splitting parameters D and E. The energy values corresponding to this Hamiltonian can be obtained by standard procedures (Orton, 1968). The appropriate susceptibility equation per mole of Co(II) results in the van Vleck equation (eq 2), and all quantities have their usual

$$\chi_j^{mol} = \frac{N}{H} \frac{\sum_{i=1}^{4} -(\partial E_{ij}/\partial H) \exp[-E_{ij}/(kT)]}{\sum_{i=1}^{4} \exp[-E_{ij}/(kT)]}, \quad j = x, y, z \quad (2)$$

meaning. The mole susceptibility in the protein solution is obtained by averaging the calculated values over all three axis:

$$\chi_{av}^{mol} = (1/3)\sum_j \chi_j^{mol} \quad (3)$$

Temperature-independent magnetic effects resulting from the diamagnetism and the temperature-independent paramagnetism of the protein solution have been taken into consideration by the temperature-invariable susceptibility term χ_{ti}. So the experimental susceptibility values can be expressed as

$$\chi_{exp} = \chi_{av}^{mol} + \chi_{ti} \quad (4)$$

The fitting procedure using least-squares techniques has been applied assuming axial symmetry (floating parameters: $g_\parallel$, $g_\perp$, D, and χ_{ti}). The function minimized was $|\chi_{exp} - \chi_{calcl}|/\chi_{exp}$. The zero-field splitting δ has been calculated with

$$\delta = 2D \quad (5)$$

The resulting fit parameters point out an anisotropy in the g factors ($g_\parallel = 2.05 \pm 1.5$; $g_\perp = 3.35 \pm 0.1$) and a large zero-field splitting of $\delta = 118 \pm 4$ cm^{-1}.

From the calculated χ_{av}^{mol} values, the magnetic moment has been obtained with the relation

$$\mu_{exp}/\mu_B = 2.828(\chi_{av}^{mol} T)^{1/2} \quad (6)$$

Figure 6 illustrates the temperature dependence of the magnetic moment monitoring the low-lying $S = \pm^1/_2$ Kramers doublet.

Electron Paramagnetic Resonance Measurements. The EPR spectra of Co(II)-hemocyanin and Co-b-hemocyanin

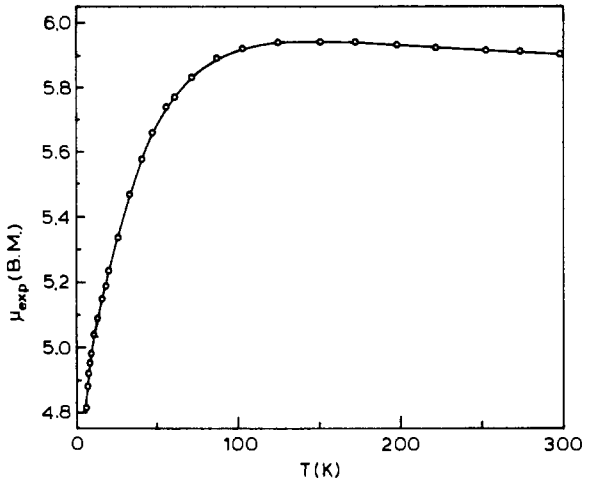


FIGURE 6: Temperature dependence of magnetic moment for peripheral-bound Co(II) calculated from χ_{av}^{mol} values of the applied theory.

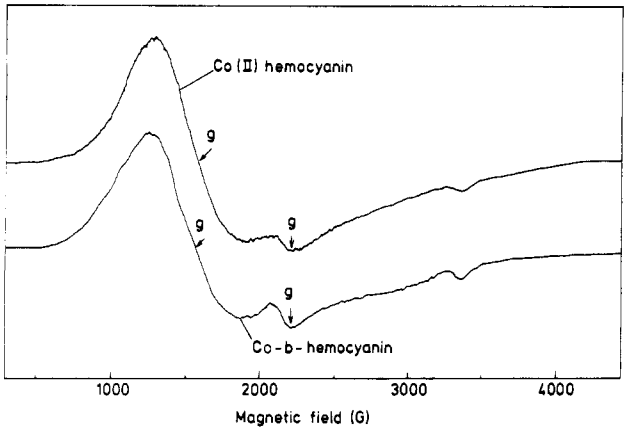


FIGURE 7: EPR spectra of 0.5 mM Co(II)-hemocyanin and Co-b-hemocyanin recorded in the X-band at 5 K.

(≈0.6 mM) were measured at 5 K and 9.46 GHz. Figure 7 reveals that the EPR spectra of both hemocyanin derivatives are identical. A broad resonance line is observed at 1570 G, whereas a second small signal has been found at about 2200 G. The weak resonance line at higher field strength (3350 G) is due to impurities of the sample holder. The EPR signals are broadened significantly with increasing temperature and disappeared at 76 K in the most concentrated sample (≈2.5 mM) measured.

Discussion

The present study shows that the exchange-coupled copper pair of *L. polyphemus* hemocyanin can be substituted by cobalt(II) ions. In analogy to other Co-substituted metalloproteins (Larrabee & Spiro, 1979; McMillin et al., 1974) and because of its strong binding, the copper in the native protein cannot be replaced directly by cobalt. This is shown with the preparation of Co-b-hemocyanin. So in a prerequisite step for the metal substitution, the copper has to be removed first and then replaced by cobalt.

Since Co(II) occupies two different binding sites, we have prepared the Co-b-hemocyanin to distinguish between the effects of the different Co(II) sites. From the metal binding behavior, it seems to be obvious that the active site of Co(II)-hemocyanin is reconstituted by two Co(II) ions (see Table I), and about 4.5 Co(II) are found in the peripheral sites.

The peripheral-bound divalent cations, which are Ca^{2+} or Mg^{2+} in the native protein, are involved in the aggregation process of the protein subunits leading to a superposed scattering effect in the absorption spectrum. Since the Co(II) derivatives of hemocyanin show the same scattering effect, we assume the peripheral places substituted by Co(II) ions. Even excess Ca^{2+} concentrations do not affect the Co(II) binding behavior to the peripheral sites. So these Co(II) complexes seems to be stabilized by a larger complex formation constant in comparison to that of the same sites of the native protein.

The resulting aggregation behavior of hemocyanin and its derivatives has been shown to have a great influence on the ratios of the absorption coefficients. To check the quality of preparations of the native protein, it is important to correlate two ratios as shown in Figure 1. This method allows elimination of errors due to the superposed scattering.

Spectral Properties. A comparison between the optical spectra of Co-b-hemocyanin and stripped hemocyanin (see Figure 3B) reveals no contribution from the Co(II) bound to the peripheral places. Since octahedral Co(II) complexes are known to have small absorption coefficients ($\epsilon < 10$ M^{-1} cm^{-1}) (Carlin, 1965), the coordination of the peripheral-bound Co(II) ions can be assumed by an octahedral geometry. So the optical spectrum of Co(II)-hemocyanin arises exclusively from the Co(II) ions bound to the active site.

The visible absorption spectrum of Co(II)-hemocyanin shows three distinguishable transitions. The energies and intensities of the Co(II)-hemocyanin absorption bands are comparable to those of Co(II)-alkaline phosphatase (Simpson & Vallee, 1968), human Co(II)-carbonic anhydrase (Coleman & Coleman, 1972), Co(II)-phosphoglucomutase (Ray et al., 1972), the Co(II) derivatives of the blue copper proteins azurin, plastocyanin, and stellacyanin (McMillin et al., 1974; Solomon et al., 1976), and Co(II)-tyrosinase (Rüegg & Lerch, 1981). The Co(II) active sites of all these proteins are assigned as having tetrahedral or distorted tetrahedral coordination on the basis of visible absorption and circular dichroism (CD) data. X-ray analysis of the native carbonic anhydrase (Kannan et al., 1971) and native plastocyanin (Colman et al., 1978) demonstrated distorted tetrahedral geometry. All the mentioned proteins contain one metal ion in the active site of the native protein and one Co(II) ion in the derivative with the exeption of Co(II)-tyrosinase. The active site of the tyrosinase derivative contains a Co(II) pair in distorted tetrahedral environment established by its absorption and CD spectra (Rüegg & Lerch, 1981). The visible absorption spectrum shows very similiar band positions (ϵ_{526} = 465 M^{-1} cm^{-1}, ϵ_{564} = 630 M^{-1} cm^{-1}, ϵ_{607} = 670 M^{-1} cm^{-1}) as for Co(II)-hemocyanin but much better resolved.

Comparison of the energies and intensities of the Co(II)-hemocyanin d–d transitions with those of the mentioned proteins and with those of low molecular weight complexes (Cotton et al., 1961; Goodgame & Cotton, 1962) suggests tetrahedral coordination geometry for the Co(II) chromophore. The observed extinction coefficient per mole of Co(II) (ϵ_{Co} = 400 M^{-1} cm^{-1}) rules out octahedral and even 5-fold coordination. Examination of a selection of five-coordinate high-spin Co(II) complexes pointed out ϵ values smaller than 250 M^{-1} cm^{-1} for the principal d–d bands (Rosenberg et al., 1974).

A further suggestion for tetrahedral coordination is the near-infrared spectrum of Co(II)-hemocyanin, which establishes the ν_2 transition [$^4A_2 \rightarrow {}^4T_1(F)$] at 1130 nm ($\epsilon$ = 43 M^{-1} cm^{-1}). The low-intensity band is in agreement with related bands from low molecular weight complexes (Cotton et al., 1961; Goodgame & Cotton, 1962), with bands in Co(II)-substituted blue copper proteins (e.g., splitted transition bands at 1000 and 1449 nm for plastocyanin) (Solomon et al., 1976),

and with data from Co(II)-tyrosinase ($\epsilon_{960} \sim 15$ M^{-1} cm^{-1}, $\epsilon_{1180} \sim 30$ M^{-1} cm^{-1}) (Rüegg & Lerch, 1981).

An analysis of the d–d transitions of the Co(II) chromophore in the hemocyanin derivative assigned the three visible bands to be splitting components of the ν_3 transition [$^4A_2 \rightarrow {}^4T_1(P)$]. Since the separation of the splitting components is too large to be associated with spin–orbit coupling (800 and 1441 cm^{-1}), it may reflect distortions from tetrahedral symmetry. The average transition energy is estimated to be 18 200 ± 600 cm^{-1}. The near-infrared band shows no splitting, establishing a transition energy of 8900 ± 400 cm^{-1} for the ν_2 transition. The strength of the ligand field Δ_t has been calculated from (Lane et al., 1977)

$$\Delta_t^2 - 0.529(\nu_2 + \nu_3)\Delta_t + 0.29\nu_2\nu_3 = 0$$

by using the average term energies and neglecting spin-orbit coupling. Application of the above equation yields 5100 ± 300 cm^{-1} for Δ_t. Strong tetrahedral fields of about 5000 cm^{-1} are known from low molecular weight complexes containing mainly nitrogen ligands (Rosenberg et al., 1975). The obtained value is also comparable to the tetrahedral ligand fields of the Co(II) derivatives of the blue copper proteins (Δ_t = 4900 cm^{-1}) (Solomon et al., 1976) and tyrosinase (Δ_t = 5300 cm^{-1}) (Rüegg & Lerch, 1981), which are known to contain histidine ligands in the active site. X-ray analysis of the deoxyhemocyanin of *Panulirus interruptus* (Gaykema et al., 1984) shows each copper in the active site to be coordinated by three histidine ligands. This is in accordance with the calculated strength of the tetrahedral ligand field and confirms the Co(II) chromophore to be situated in the active site of hemocyanin.

Aging Process. The aerobic aging of Co(II)-hemocyanin turns the pink-violet color to light brown, increasing the intensity and changing the ratio between the three distinguishable d–d transitions. The spectral changes can be explained by aggregation of the hemocyanin subunits assuming responsible the Co(II) ions in the peripheral sites. The high molecular weight aggregates induce a scattering superposing the absorption spectra. Since the scattering is proportional to ν^4, transitions at higher energies are more affected in band positions and intensities than at lower frequencies.

Since the changing of the absorption spectra during the aging process can be related exclusively to the increasing scattering part, we assume that the active site of Co(II)-hemocyanin remains unchanged, contrary to the interpretation of Suzuki et al. (1982). Furthermore, no oxygen binding at the active site has been observed that could be taken as responsible for the observed spectral changes. Low molecular weight Co(II) complexes are known to bind dioxygen reversibly, displaying a strong charge-transfer band ($\epsilon \sim 10^3$–10^4 M^{-1} cm^{-1}) around 350 nm (Wilkins, 1971), never observed in Co(II)-hemocyanin. Possibly the tetrahedral coordination geometry is responsible for the lack of oxygen binding in Co(II)-hemocyanin because only octahedral Co(II) complexes show oxygen affinity (Wilkins, 1971).

Optical Perturbations. Absorption spectra of Co(II) complexes are highly sensitive to modifications of the nature, number, and coordination geometry of their ligands. Inhibitors of the native hemocyanin like azide and fluoride do not change the optical spectrum of Co(II)-hemocyanin. This indicates that these agents do not bind to the metal chromophore. Reaction with peroxide does not give rise to spectral changes, indicating that the formation of a peroxo-bridged Co(II) dimer is not possible in this way.

When Co(II)-hemocyanin is treated with excess KCN, the splitted transition at 548 nm decreases while a new band appears at 380 nm. The same UV transition ($\epsilon_{378} \approx 300$ M^{-1} cm^{-1}) is observed when a Co(II) solution (Tris-HCl, pH 8.0) is reacted with excess KCN, leading to a Co(III)–cyanide complex because of the instability of the analogous Co(II) complex (Kiss & Czeglédy, 1938). So reaction with cyanide oxidizes the Co(II) chromophore in the active site, which has been also observed for Co(II)-tyrosinase (Rüegg & Lerch, 1981).

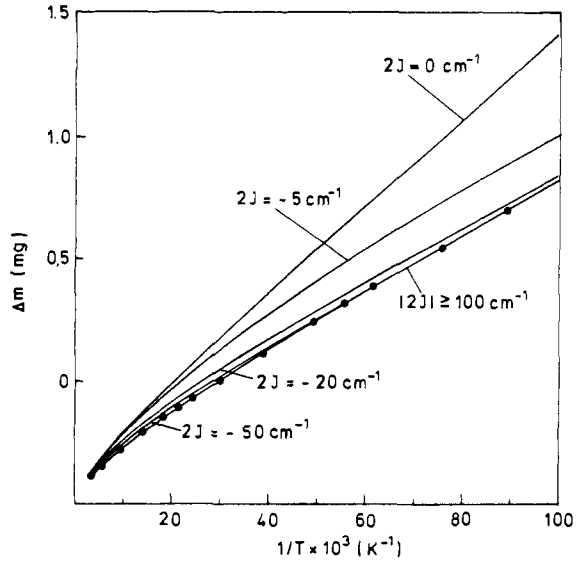


FIGURE 8: Simulation of magnetic behavior of Co(II)-hemocyanin assuming different exchange integrals for the Co(II) pair in the active site. Experimental values are included showing no effect from the spin-coupled ions. Susceptibility is expressed in weight differences ($\Delta m \sim \chi_M$) measured with the used Faraday equipment.

Magnetic Susceptibility Measurements. The identical magnetic behavior found for Co(II)- and Co-b-hemocyanin demonstrates that the paramagnetic susceptibility data are correlated exclusively with the Co(II) ions bound to the peripheral sites. No measurable effect could be attributed to the two Co(II) ions in the active site of Co(II)-hemocyanin. According to these experimental data, we have assumed the exchange integral $2J$ large for the fits, which has been verified by the calculation of a lower limit on $2J$.

The limit exchange integral has been obtained by assigning different values for $2J$. The resulting effects have been superposed on the susceptibility data due to the peripheral Co(II). Calculation of the susceptibility arising from the antiferromagnetically coupled site has been carried out with the Hamiltonian

$$\hat{H} = 2J\sum_{i>j}\hat{S}_i\hat{S}_j$$

and a Heisenberg–Dirac–van Vleck model for the appropriate susceptibility equation (van Vleck, 1932). The g factor used was 2.5. In this susceptibility data analysis, we have excluded the zero-field splitting of the tetrahedral Co(II)'s in the active site as a responsible effect for the diamagnetic behavior, because the resulting Kramers doublets never form a diamagnetic ground state ($S = 0$). Zero-field splitting was also neglected in the calculation of the limit exchange integral, because tetrahedral Co(II)'s are known to show only small values of <13 cm^{-1} (Makinen & Yim, 1981), leading to $2J \gg 2D$.

The resulting susceptibility data based on different $2J$ values are presented in Figure 8. To monitor the sensitivy of the used Faraday system (resolution limit at about 10 μg), the temperature dependence of the magnetic susceptibility is expressed in experimental weight changes ($\chi \sim \Delta m$). It becomes obvious that it is easy to distinguish whether the system is coupled or uncoupled. However, the effect connected with a

$2J$ value of -50 cm^{-1} cannot be separated from stronger antiferromagnetic coupling. Therefore, we estimate the lower limit for the exchange integral as $2J = -50$ cm^{-1} for the antiferromagnetically coupled binuclear Co(II) site in Co(II)-hemocyanin.

EXAFS data for oxy- and deoxyhemocyanin show Cu–Cu distances of 3.6 ± 0.2 Å (Brown et al., 1980; Woolery et al., 1984). If the Co(II) ions are assumed to replace the copper ions without drastic changes in the active site of hemocyanin, the separation of the two ions is too large to involve a direct magnetic exchange mechanism through space. So the antiferromagnetic coupling within the binuclear active site of Co(II)-hemocyanin can be explained by the assumption of a superexchange mechanism involving at least one bridging ligand between the two Co(II) ions. The magnetic molecular orbital contains contributions from the molecular orbitals of the bridging ligands, which render possible magnetic exchange coupling over larger distances. A bridging ligand has also been postulated by interpreting the spin coupling in Co(II)-tyrosinase (Rüegg & Lerch, 1981) and in bovine superoxide dismutase upon Co(II) substitution of Zn(II) (Fee, 1973; Rotilio et al., 1974).

The magnetic behavior of the peripherally bound Co(II) ions, deviating from Curie law paramagnetism, will be discussed in terms of the model described above. The calculated susceptibility values are in very good agreement with experimental data (see Figure 5), confirming the assumption of axial symmetry ($g_x = g_y = g_\perp$; $g_z = g_\parallel$; $E = 0$). To discuss the g factors and the zero-field splitting parameter D obtained in the fitting procedure, the uncertainty in the metal analysis must be considered. Assuming two tetrahedral Co(II)'s in the active site, 4.7 ± 0.4 Co(II) remain for the peripheral sites in Co(II)-hemocyanin. So we have applied the fitting procedure to susceptibility data that were calculated on the basis of 4.3 and 5.1 mol of peripheral Co(II)/mol of protein, respectively. Only small variations were found for D ($D = 59 \pm 2$ cm^{-1}) and $g_\perp$ ($g_\perp = 3.4 \pm 0.1$) while the obtained value for $g_\parallel$ ($g_\parallel = 2.0 \pm 1.5$) changes considerably with the metal content used. This indicates that the numeric values of the g factors and the related magnetic moments are not significant. So only the temperature dependence of the magnetic moment can be used for interpretation, pointing out a low-lying $S = \pm^1/_2$ Kramers doublet. The increase of the magnetic moment with increasing temperature (see Figure 6) illustrates the temperature-dependent population of the $S = \pm^3/_2$ doublet. Despite the uncertainty of the g factors, an anisotropy of the g factors $g_\perp > g_\parallel$ can be assigned, which has been found in tetragonal distorted octahedral Co(II) complexes such as $CoCl_2(H_2O)_4 \cdot 2H_2O$ (Gerloch et al., 1971).

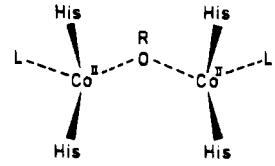
The zero-field splitting in high-spin Co(II) complexes has been shown to be an useful tool to identify the coordination geometry (Makinen et al., 1984). By use of saturation experiments on EPR signals, a zero-field splitting of 0–13 cm^{-1} has been attributed to tetrahedral complexes, of 20–60 cm^{-1} to 5-fold coordination, and of 90–310 cm^{-1} to octahedral Co(II) complexes (Makinen & Yim, 1981). The calculated value of 118 cm^{-1} has the same order of magnitude as data found for octahedral coordination. So interpretation of the zero-field splitting and of the anisotropy in the g factors suggests a distorted octahedral coordination geometry for the Co(II) ions bound to the peripheral places.

Electron Paramagnetic Resonance. The EPR signals are related to the peripherically bound Co(II) ions only, whereas the contiguous Co(II) pair in the active site is EPR-silent. This is a confirmation of the magnetic susceptibility measurements characterizing the Co(II) in the active site as a pair of antiferromagnetically spin-coupled ions.

The EPR spectra are described by g factors of $g = 3.1$ and $g = 4.3$. Any interpretation of the extremely broad and featureless EPR signal remains difficult. We assume that differences in the peripheral sites are responsible for the resonance signal of Co(II)-hemocyanin. So the values of the g factors are not significant for one site resulting from a superposition of several signals. In contrast, it can be assumed that the results from the magnetic susceptibility measurements describe average values for the peripheral Co(II) sites. The significant broadening of the EPR signal with increasing temperature is related to the rapid spin–lattice relaxation well-known for high-spin Co(II) (Banci et al., 1982).

Conclusions

The binuclear copper site in met- and oxyhemocyanin has been characterized as containing two tetragonal Cu(II) atoms (Eickman et al., 1979; Himmelwright et al., 1980a), which is in contrast to the tetrahedral structure in the Co(II) derivative of hemocyanin. Since Cu(I) ions often show tetrahedral coordination (Cotton & Wilkinson, 1972), the active site structure in Co(II)-hemocyanin is assigned to reflect the coordination geometry of deoxyhemocyanin. X-ray analysis



of deoxyhemocyanin from *P. interruptus* (Gaykema et al., 1984) points out that each Cu(I) ion is coordinated by three histidine ligands, but no bridging group between the metal centers has been detected. So we assume that the bridging ligand, which has been established in our study, is a small molecule, e.g., hydroxide or water, which is not detectable with a resolution of 3.2 Å obtained in the X-ray experiment. This assumption is in agreement with a resonance Raman study on phenolate-bridged binuclear Cu(II) complexes (Lorösch et al., 1986). In comparison to experiments on native hemocyanins (Loehr et al., 1974; Freedman et al., 1976; Larrabee & Spiro, 1980), it has been established that a tyrosine residue, often mentioned as an endogenous bridge (Eickman et al., 1979; Wilcox et al., 1984), cannot be detected in the active site of oxyhemocyanin. Summarizing these new aspects about the active site strucutre, we have to describe the so-called endogenous bridge as a strongly bound small molecule in oxy- as well as in deoxyhemocyanin.

Acknowledgments

We thank Prof. M. Zeppezauer for instruction in metal substitution techniques, for stimulating discussions, and critical reading of the manuscript. We are grateful to Dr. W. Maret for running the EPR spectra and helpful discussions. We found some suggestions from Dr. L. Petterson very useful for the interpretation of the magnetic part. We are also indebted to Prof. Bächmann and H. Klenk for the metal analysis.

Registry No. Co, 7440-48-4; Cu, 7440-50-8.

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