Preparation and Spectroscopic Characterization of a Coupled Binuclear Center in Cobalt(II)-Substituted Hemocyanin[†]

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ABSTRACT: A binuclear cobalt derivative of arthropod hemocyanin (Hc) has been prepared by the reaction of apo-Hc with Co(II) in the presence of thiocyanate. The crude product of the reaction contains specifically and adventitiously bound metal, the latter being removable by EDTA treatment. The specifically bound Co(II) constitutes a binuclear metal center that exhibits optical and CD spectra typical in their absorption maxima and extinction coefficients of Co(II) complexes with near-tetrahedral geometry. The EPR spectrum of the binuclear Co(II) derivative contains a resonance at $g \sim 13$, which is characteristic of integer spin systems and indicates coupled metal ions; the excess Co(II) bound to crude products exhibits an EPR signal at $g \sim 4$. The time course of derivative formation was followed by EPR, optical and atomic absorption techniques, and by fluorimetry. The intensity of the optical absorption in the visible region due to Co(II) increases with increasing stoichiometry of specifically bound metal [up to 2 Co(II) per protein monomer], but the intensity of the Co(II) EPR signal increases only during the formation of a mononuclear derivative. As the reaction proceeds over approximately 100 h to the formation of the binuclear derivative, the EPR signal intensity decreases to 10% of the value expected for 2 mol of EPR-active Co(II)/mol of protein. The binuclear cobalt derivative cannot be reconstituted to native Hc with Cu(I), indicating the stable loading of Co(II) in the active site. EPR and optical spectroscopic evidence is presented showing that the binuclear derivative does not bind oxygen.

Hemocyanin (Hc)[1] is a copper protein found in the hemolymph of mollusks and arthropods. Its physiological function is the reversible association of dioxygen at a binuclear copper active site (Ellerton et al., 1983). The oxygenated site is currently formulated as $Cu(II)–O_2^{2-}–Cu(II)$, reflecting single-electron transfers from the cuprous copper of deoxy-Hc to the bound O_2 (Brown et al., 1980; Van Holde et al., 1967).

The blue color of the protein is related to the presence of bound O_2, the deoxy form of the protein being colorless. Despite the presence of what is formally Cu(II) in oxy-Hc, this form is EPR-silent due to antiferromagnetic coupling between the two Cu(II) ions mediated by the exogenous ligand (Van Holde, 1967; Freedman et al., 1976).

The X-ray crystal structure of deoxy *Panulirus interruptus* Hc (Volbeda & Hol, 1989) shows that the individual copper ions of the active site have similar ligands and coordination geometries, but one copper is more exposed to solvent. In fact, the kinetics of copper removal from apo-Hc with CN^- reveals a difference in reactivity for the two copper ions of the active site (Beltramini et al., 1984, 1986). Because of this difference, it is feasible to sequentially remove copper ions from a "fast reacting site" (FRS) and a "slow reacting site" (SRS), where these labels are assigned based on the relative rates of metal dissociation upon cyanide treatment. Cyanide treatment also provides a means to prepare a stable apo protein (Salvato et al., 1974), which is the starting material for the metal incorporation experiments described here.

In the present investigation, the characterization of the active site in hemocyanin is addressed by substitution of the native metal with Co(II). The rationale for this approach lies in the fact that Co(II) complexes, in contrast to those with copper, exhibit characteristic optical absorptions and magnetic properties that can often be directly correlated with geometry and ligand composition. Modifications of the spectroscopic properties of Co(II) are expected as a result of changes in the first coordination sphere caused either by ligand addition or ligand exchange (Cotton & Wilkinson, 1988). Co(II) substitution of Hc has been described in other reports (Lorosch & Haase, 1986; Suzuki et al., 1982a), though in those studies interferences due to adventitiously bound cobalt prevented characterization of the metal bound only at the active site.

A monocobalt derivative of *Carcinus maenas* Hc lacking adventitious metal was prepared and characterized by EPR and optical spectroscopy (Salvato et al., 1986). This paper reports the extension of that work to *binuclear* Co(II) derivatives. *C. maenas* and *Callinectes sapidus* Hcs are the major subjects of this investigation. *Limulus polyphemus* Hc was also used here to compare the optical and EPR spectroscopy of the cobalt derivatives produced by our methods to those reported elsewhere (Lorosch & Haase, 1986). *P. interruptus* Hc was used in order to obtain additional information on the active site since X-ray crystallographic structural information is available for this protein (Volbeda & Hol, 1989).

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[1] Abbreviations: Hc, hemocyanin; LMCT, ligand-to-metal charge-transfer transition; EPR, electron paramagnetic resonance; CD, circular dichroism; MCD, magnetic circular dichroism; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

On the basis of a kinetic analysis of Co(II) binding, a mechanism for the reconstitution reaction from apo-Hc to the binuclear cobalt derivative, $Co(II)_2$-Hc, is proposed. The optical, CD, and EPR spectra of $Co(II)_2$-Hc are also presented.

MATERIALS AND METHODS

Carcinus maenas and *Callinectes sapidus* Hcs were purified from hemolymph collected by syringe from the dorsal lacunae of live animals. The clotted hemolymph was filtered through gauze and centrifuged to eliminate debris and cells. The clear, dark green-brown supernatant was dialyzed overnight at 4 °C against 20 mM $CaCl_2$, pH 7.0, during which time an orange-brown precipitate formed. This precipitate was separated by low-speed centrifugation. The resulting clear blue-green supernatant was further purified by preparative ultracentrifugation at 160000*g* for 6 h. The pelleted protein was resuspended in 0.1 M Tris-HCl, pH 7.0, containing 10 mM $CaCl_2$. Sucrose was added to a final concentration of 18% (w/v), and the preparation was divided into aliquots for storage at −20 °C.

Limulus polyphemus Hc was collected and frozen at the Woods Hole Oceanographic Institute, Woods Hole, MA, and was purified as described above for *C. maenas* Hc. *Panulirus interruptus* Hc was collected at the Biomarine Lab, Venice, CA, and was purified according to a published procedure (Johnston et al., 1967). Apo-Hcs (containing less than 5% of the copper content of holo-Hc) were prepared from holo-Hc by dialysis against cyanide and EDTA, as described elsewhere (Salvato et al., 1974).

The monocobalt derivative of *C. maenas* Hc was prepared using a modification of the procedure previously described (Salvato et al., 1986) by dialyzing the apo protein (2.5 mL, approximately 75 mg/mL) for 96 h at 20 °C against 500 mL of 0.1 M Tris-HCl, containing 0.5 mM $CoCl_2$, 50 mM NaSCN, 10 mM $MgSO_4$, and 0.05% Tween 80, at pH 7.0.

$Co(II)_2$–Hcs were prepared from *C. maenas*, *C. sapidus*, and *P. interruptus* Hcs by dialysis of the apo proteins (2.5 mL of approximately 75 mg/mL) for 96 h at 20 °C against 500 mL of 0.1 M Tris-HCl containing 4 mM $CoCl_2$, 250 mM NaSCN, 10 mM $MgSO_4$, and 0.05% Tween 80, at pH 7.0. Each protein sample was then dialyzed for 48 h against four changes of 5 mM sodium phosphate buffer, pH 7.0, containing 2 mM EDTA, and finally, for 24 h against the same buffer without EDTA.

Treatment of *C. maenas* $Co(II)_2$-Hc with 10 mM NaCN and 50 mM NaSCN in 20 mM phosphate buffer, pH 8.0, for 20 h, followed by extensive dialysis against EDTA in the absence of CN^- and SCN^-, yielded a new monocobalt derivative different from that obtained by the dialysis of the apo protein against 0.5 mM Co(II), as described above and reported elsewhere (Salvato et al., 1986). A hybrid binuclear derivative was prepared by anaerobic dialysis (96 h) of holo-Hc (2.5 mL, approximately 75 mg/mL) against 500 mL of 0.1 M Tris-HCl, pH 7.0, containing 4 mM $CoCl_2$, 250 mM NaSCN, 10 mM $MgSO_4$, and 0.05% Tween 80, followd by dialysis against 5 mM sodium phosphate buffer, pH 7, containing 2 mM EDTA (48 h) and finally against phosphate buffer alone (24 h).

L. polyphemus $Co(II)_2$-Hc was prepared from apo-Hc by dialysis for 48 h against 50 mM Tris-HCl buffer, containing 0.5 mM $CoCl_2$, 200 mM NaSCN, and 0.05% Tween 80, at pH 8. The protein was purified by dialysis for 48 h against 5 mM phosphate buffer, pH 7.0, containing 2 mM EDTA and then for 24 h against 5 mM phosphate buffer at the same pH.

The binding of EDTA-resistant Cu(I) to *C. maenas* $Co(II)_2$-Hc was examined by dialysis of the derivative against 1 mM CuCl and 50 mM NaSCN in 50 mM Tris-HCl buffer, pH 7, using a procedure previously described for the reconstitution of apo-Hc (Moriondo, 1984).

In order to determine whether thiocyanate remains bound to the various cobalt derivatives after their purification, an analytical determination was carried out following the procedure suggested by Newman (1975). Thiocyanate was determined by complexation with Fe(III) in acidic solution. Beer's law was obeyed for SCN^- standard solutions having concentrations between 20 and 200 μM, and the total SCN^- in samples containing protein was evaluated within this range. The internal standard method was used in order to eliminate interferences and to ensure the stability of thiocyanate under the conditions used for protein denaturation (pH $\sim$ 2).

Protein concentrations were determined spectrophotometrically using $\epsilon_{278} = 93\,000$ M^{-1} cm^{-1} for both *C. maenas* and *C. sapidus* Hcs (Tamburro et al., 1977), where the extinction coefficient refers to the 75-kDa monomeric subunit containing one active site; $\epsilon_{278} = 94\,400$ M^{-1} cm^{-1} (Ghiretti-Magaldi et al., 1966) for *P. interruptus* Hc and $\epsilon_{278} = 82\,400$ M^{-1} cm^{-1} for *L. polyphemus* Hc (Nickerson & van Holde, 1971), where the values are per monomeric subunit containing one active site. Optical absorption spectra were recorded on a Cary 14 spectrophotometer modified for computer control by Aviv Associates, Lakewood, NJ, or on a Perkin-Elmer Model LAMBDA 5 instrument. Molar extinction coefficients in the visible region for the various cobalt derivatives were calculated based on the molarity of Co(II) determined by atomic absorption spectroscopy. Co(II)/protein molar ratios were obtained by relating the Co(II) concentration in a sample (obtained by atomic absorption) to the protein concentration evaluated optically using the molar extinction coefficient at 278 nm. Metal analyses were performed by atomic absorption spectroscopy using a Perkin-Elmer Model 5000 spectrophotometer equipped with a graphite furnace or an acetylene/air burner for flame analyses. A standard addition method was applied using atomic absorption standard solutions of copper or cobalt (Aldrich) added to Hc solutions containing approximately 5–10 μM protein. No interferences were observed in the metal determination under these conditions.

Circular dichroism spectra were recorded on a Jasco Model J-500 spectropolarimeter. Fluorescence spectra were recorded at 20 °C using a Perkin-Elmer MPF 4 fluorimeter equipped with a thermostated cell compartment. Protein solutions used for fluorescence measurements had an absorbance less than 0.06 at 295 nm, the excitation wavelength, to minimize the inner filter effect (Lakowicz, 1986).

X-band EPR spectra were recorded on a Varian E-112 spectrometer equipped with a Systron-Donner frequency counter and a Varian NMR gaussmeter. Low-temperature studies were performed using a Heli-tran liquid helium transfer system. Co(II)–EDTA, used as primary standard for quantitative EPR of high spin Co(II), was prepared from an atomic absorption standard Co(II) solution (Aldrich) to which excess EDTA in 20 mM ammonia/ammonium sulfate, pH 9, was added. This solution, containing 1.39 mM Co(II), was diluted 1:1 in ethylene glycol to form a good glass. Data collection and double integration were executed using a personal computer interfaced to the EPR spectrometer and EPR Data Acquisition System software provided by P. D. Morse (University of Illinois). The $g = 6$ signal of the well-characterized monocobalt-Hc (Salvato et al., 1986) was also useful for EPR intensity calculations based on the Co(II)

Table I: Metal Binding Stoichiometry and Spectroscopic Properties during Preparation and Purification of Cobalt Derivatives of *C. maenas* Hc

sample	mol of Co/ mol of protein[a]	ϵ_{582} M^{-1} cm^{-1} [b]
(A) crude reconstitution product	8.1	1200[c]
(B) cobalt derivative after dialysis in Tris-HCl buffer	7.1	900[d]
(C) cobalt derivative after dialysis in 2 mM EDTA	2.0	850[d]
(D) cobalt derivative after additional dialysis (48 h)	2.0	800[e]
(E) D, plus 200 mM SCN^-	2.0	1200
(F) $Co(II)_2$-Hc after dialysis in 10 mM CN^-	1.2	350
(G) Co(II)–Cu(I)Hc	0.5	450[f]

[a] The metal was determined by atomic absorption spectroscopy, and the protein concentration was determined using the molar extinction coefficient of the monomeric subunit at 278 nm (see text). [b] Refers to protein concentration determined using the molar extinction coefficient of the monomeric subunit at 278 nm. [c] Includes the hyperchromic effect due to the presence of 250 mM thiocyanate. [d] Includes the hyperchromic effect due to the presence of bound thiocyanate in 10–20% of the sites (see text for explanation). [e] This value represents the extinction for the thiocyanate-free protein (less then 5% bound SCN^-). [f] The molar extinction coefficient is expressed per mol of bound Co(II).

concentration determined by atomic absorption. The intensity of the Co(II) signal for this derivative corresponded well with the intensity of signals for equivalent concentrations of Co(II)–EDTA prepared from standard Co(II) solutions.

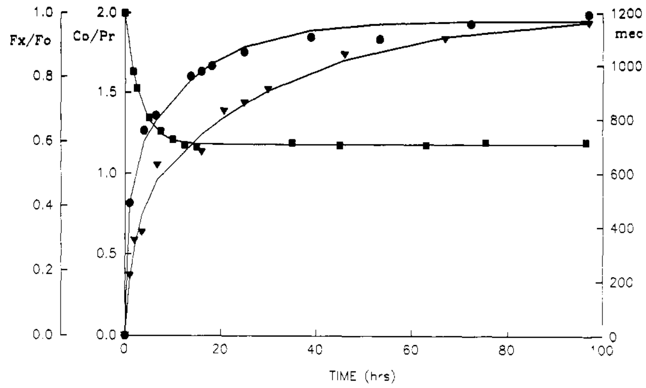

FIGURE 1: Time course of Co(II) binding to *C. maenas* apo-Hc. (●) Mol of Co(II) per mol of protein (after treatment with 2 mM EDTA in 5 mM phosphate buffer, pH = 7); (▼) molar extinction coefficient at 582 nm (the absorbance includes the hyperchromic effect due to the presence of 250 mM thiocyanate); (■) fluorescence quantum yield reported as a fraction of the apo-Hc quantum yield (emission measurement at 330 nm upon excitation at 295 nm). The continuous lines connecting the experimental points represent the best-fit curves obtained with the sequential model for Co(II) binding (see text). Reconstitution conditions: 2.5 mL of 75 mg/mL apo-Hc dialyzed against 500 mL of 0.1 M Tris-HCl, pH 7.0, containing 250 mM NaSCN, 10 mM $MgSO_4$, 4 mM $CoCl_2$, and 0.05% Tween 80 at 20 °C.

RESULTS

Preparation of Co(II)-Hc Derivatives. The preparation of cobalt-Hc from apo-Hc was accomplished in two steps: (1) dialysis of the apo protein in a buffer containing Co(II) and thiocyanate and (2) purification of the Co(II)-containing derivative by dialysis to remove excess reagents as well as nonspecifically bound Co(II).

Dialysis of *C. maenas* apo-Hc against 4 mM Co(II) and 250 mM thiocyanate (plus other components) yields a protein derivative having a deep purple color. After 96 h (an incubation time long enough to achieve complete reaction), an aliquot of the protein solution in the dialysis bag contained a Co(II) concentration indicating 8 mol of cobalt bound per molecule of monomeric subunit. This was determined by subtracting the Co(II) concentration in the dialysis buffer from the total Co(II) in the protein solution and was based on the protein subunit concentration determined spectrophotometrically. During subsequent dialysis against Tris-HCl buffer for 5 h, the Co/protein molar ratio was reduced to 7 (Table I). Further dialysis against EDTA (48 h, 4 changes, vol. ratios 200:1) reduced the Co/protein molar ratio from 7 to 2. The species thus isolated is referred to as $Co(II)_2$-Hc. The 2 molar equiv of EDTA-resistant Co(II) could be removed by cyanide treatment in a reaction analogous to that which produces apo-Hc from holo-Hc (see below).

In order to localize the binding sites of the 2 mol of tightly bound Co(II), we evaluated the susceptibility of $Co(II)_2$-Hc to reconstitution with copper. The derivative was dialyzed against a saturated solution of CuSCN, a treatment that regenerates native Hc from apo-Hc (Moriondo, 1984). A negligible amount of EDTA-resistant copper was found by atomic absorption spectrophotometry, demonstrating that the tightly bound Co(II) renders the active site inaccessible to Cu(I).

Optical spectroscopy was used to monitor the products during purification after reconstitution. The first dialysis step in the purification scheme, which removes excess reagents and reduces the Co/protein molar ratio from 8 to 7, gave a notable reduction in the optical absorption intensity at 582 nm (Table I). This decrease is ascribed to the removal of thiocyanate from the cobalt derivative since the absorption intensity at 582 nm could be completely restored by adding excess thiocyanate to a sample of purified $Co(II)_2$-Hc (Table I). The observation that the full optical absorption could be restored in the derivative lacking excess Co(II) suggests that the optical change observed with thiocyanate occurs at that cobalt which is tightly bound and presumably in the active site. Additionally only a small absorbance arises from the EDTA-labile Co(II) since the optical spectrum of the derivative in buffer (without thiocyanate), before and subsequent to EDTA treatment, is very similar.

The high molar extinction coefficient found for the cobalt derivative, $\epsilon_{582} = 900\ M^{-1}\ cm^{-1}$ (Table I) suggested that some thiocyanate might remain bound to Co(II), even after 48-h dialysis, given that this anion causes a large hyperchromic effect both for mononuclear (Salvato et al., 1986) and binuclear cobalt derivatives [$\epsilon_{582} = 1200\ M^{-1}\ cm^{-1}$ for $Co(II)_2$-Hc in excess SCN^-, Table I]. A determination of SCN^- was made in a sample after reconstitution and dialysis for 12 h against Tris-HCl buffer to remove the reconstitution reagents. Approximately 20% of the binuclear centers contained a thiocyanate ligand. During subsequent dialysis of this sample against EDTA in phosphate buffer, a slow decrease in bound SCN^- was observed, and after 48 h, 10% of the binuclear centers still contained a bound thiocyanate ligand. Additional dialysis (48 h) in phosphate buffer decreased the bound thiocyanate to less than 5%. A sample of purified $Co(II)_2$-Hc containing less than 5% SCN^- was titrated with SCN^- to evaluate the molar extinction coefficient of the $Co(II)_2$-Hc–SCN^- complex and, by extrapolation to zero thiocyanate concentration, for thiocyanate-free $Co(II)_2$-Hc. These data are collected in Table I.

Kinetics of Formation of $Co(II)_2$-Hc. In order to probe the mechanism of protein reconstitution, the kinetics of formation of Co(II)-containing derivatives was monitored, following the change in three parameters (Figure 1): (1) the quenching of Trp fluorescence in the apo protein caused by Co(II) binding; (2) the molar ratio of EDTA-resistant Co(II) bound to the protein; and (3) the increase of optical absorption at 582 nm in samples removed from dialysis against Co(II) (but before removal of excess reagents).[2]

The fluorescence of Hc upon excitation at 295 nm arises from several tryptophans. The change in the fluorescence of apo-Hc during the reaction with metal ion is believed to arise from the quenching of fluorescence from a particular tryptophan when metal binds at the nearby active site (Bannister & Wood, 1971; Salvato et al., 1986). More specifically, X-ray crystallographic analysis shows that Trp197 in *P. interruptus* Hc (an arthropod Hc closely related to *C. maenas* Hc) (Linzen et al., 1989) is close to Cu_A in the active site. The presence of copper at this FRS is assumed to be responsible for a reduction of the tryptophan quantum yield (Beltramini et al., 1984). This reduction is not observed when Cu_B is bound at the other site of the binuclear center, the "slow reacting site" (Beltramini et al., 1984, 1986).

The binding of Co(II) to apo-Hc causes similar fluorescence changes in that the tryptophan quantum yield reaches a minimum when the Co(II)/protein molar ratio is near unity (Figure 1). Furthermore, negligible changes in fluorescence are observed when the Co(II)/protein ratio increases from 1 to 2, demonstrating that the second Co(II) binds to a site where the fluorescence of tryptophan is not quenched. The fluorescence behavior with Co(II), then, bears strong resemblance to that observed for copper in *C. maenas* Hc (Beltramini et al., 1984).

Figure 1 also shows that the molar ratio of cobalt bound to the protein after extensive dialysis against EDTA approaches a constant maximum value of 2 per monomeric subunit (~100 h), suggesting the binding of two Co(II) ions per active site. The absorbance reaches a maximum value of 1200 M^{-1} cm^{-1}, though the optical data does not follow the same curve as the data showing the stoichiometry of tightly bound cobalt. This result may be related to the incorporation of a small amount of Co(II) into sites that do not contribute significantly to the optical properties of the derivative but are detected by atomic absorption.

The data for ϵ_{582} and Co(II)/protein stoichiometry in Figure 1 do not fit a simple binding process for two equivalent independently loaded metal binding sites. Instead, a sequential binding scheme (eqs 1 and 2) from apo-Hc to Co(II)-Hc and then to $Co(II)_2$-Hc is required in which the binding of the second metal ion is significantly slower than the rate of incorporation of the first:

$$\text{apo-Hc} + \text{Co(II)} \rightarrow \text{Co(II)-Hc} \tag{1}$$

$$\text{Co(II)-Hc} + \text{Co(II)} \rightarrow \text{Co(II)}_2\text{-Hc} \tag{2}$$

The equations (eqs 3–5) that give the concentrations of the species [apo-Hc], monocobalt Hc ([Co(II)-Hc]), and dicobalt-Hc ($[Co(II)_2$-Hc]) at time *t* are

$$[\text{apo-Hc}] = [\text{Hc}]_T e^{-k_1 t} \tag{3}$$

$$[\text{Co(II)-Hc}] = [\text{Hc}]_T(k_1/k_1 - k_2)(e^{-k_2 t} - e^{-k_1 t}) \tag{4}$$

$$[\text{Co(II)}_2\text{-Hc}] = [\text{Hc}]_T(1 + (k_2/k_1 - k_2)\, e^{-k_1 t} - (k_1/k_1 - k_2)\, e^{-k_2 t}) \tag{5}$$

where k_1 and k_2 are the apparent rate constants for reactions 1 and 2, and $[Hc]_T$ is the total Hc concentration. At any time, *t*, during the reconstitution reaction, a mixutre of apo, mono-, and dicobalt protein is present, and the concentration of each species depends on the values of k_1 and k_2. (Neither nonspecific binding of cobalt nor direct formation of the $Co(II)_2$-Hc species from apo-Hc is considered in this scheme.) Three algorithms based on the concentration eqs 3–5 were used to fit the experimental data. The curves are generated from the sum of the contributions to a particular observable parameter arising from the different species present in solution (apo, mononuclear, and binuclear) as a function of time. Values of k_1 and k_2 were determined from the time dependence of an iterated least-squares fit of the atomic absorption data for the concentration of specifically bound cobalt. Values of k_1 and k_2 were also determined from the optical data using ϵ_{582} = 500 M^{-1} cm^{-1} for calculating the concentration of Co(II)-Hc and ϵ_{582} = 1200 M^{-1} cm^{-1} for the concentration of $Co(II)_2$-Hc. As noted above, these values for ϵ_{582} correspond to those obtained for the mononuclear (Salvato et al., 1986) and the binuclear (Table I) derivatives in the presence of excess thiocyanate. The sensitivity of the fitting curves to the choice of k_1 is greater in the early part of the experiment. After 10 h, the value of k_2 becomes more important in fitting the experimental data.

Table II: Apparent Rate Constants for Incorporation of Cobalt into apo *Carcinus maenas* Hemocyanin

observed parameter	k_1, h^{-1}	k_2, h^{-1}
mol of Co(II)/protein	1.60	0.06
absorbance at 582 nm	0.48	0.04
fluorescence quenching	0.38	–[a]

[a] The value of k_2 cannot be evaluated from the fluorescence data because there is no change in this parameter associated with the binding of the second cobalt ion to the active site.

The tryptophan fluorescence data reveal that the apo protein provides the highest contribution to the emission intensity (this corresponds to $F_x/F_o = 1$), and the mono (Salvato et al., 1986) and binuclear cobalt derivatives have the same lower fluorescence value ($F_x/F_o = 0.6$). Therefore, the fluorescence data depend only on k_1. Fitting the three data sets gives the curves shown in Figure 1 and the values for k_1 and k_2 reported in Table II.

The value of k_1 obtained from fitting the Co(II) stoichiometry data was higher than the values obtained from fitting the optical absorption and fluorescence data (Table II). This difference may be related to the presence of a small amount of unspecifically bound cobalt, which leads to an overestimation of k_1.

The fluorescence data reaches a plateau after 15 h, beyond the time at which the Co(II)/protein stoichiometric ratio is equal to 1.0. This observation suggests that an exchange of cobalt from the Cu_A position (FRS) to the Cu_B site (SRS) occurs, restoring the fluorescence previously quenched. This exchange would be an intermediate reaction step before the binding of the second Co(II). However, in fitting the optical data and the Co(II) stoichiometric ratio data, no improvement was obtained when an intermediate exchange step was included in the reaction sequence. Therefore, in the absence of direct evidence for an exchange reaction the binding of the two Co(II) ions to apo-Hc is presented as a two-step process.

Optical Spectroscopy of $Co(II)_2$-Hc. The optical absorption and circular dichroism spectra of *C. maenas* $Co(II)_2$-Hc in the visible region contain characteristic d–d transition patterns (Figure 2, panels A and C) (Ballhausen & Jørgensen, 1955). The pattern of bands and their molar extinction coefficients

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[2] The presence of a large excess of thiocyanate eliminated the uncertainty in values for the molar extinction coefficient at 582 nm that would vary according to the thiocyanate bound.

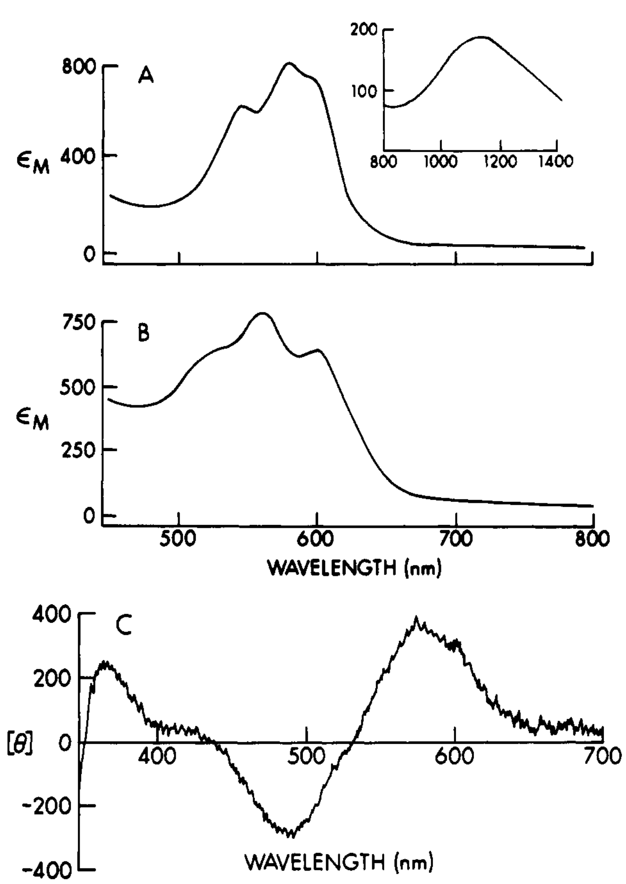

FIGURE 2: Optical absorption spectroscopy. (A) Absorption spectrum of *C. maenas* Co(II)$_2$-Hc in 5 mM phosphate buffer, pH = 7. (Insert) Absorption spectrum in the near-infrared region. (ϵ in M^{-1} cm^{-1}). (B) Absorption spectrum of *L. polyphemus* Co(II)$_2$-Hc in 5 mM phosphate buffer, pH = 7. (C) Circular dichroism spectrum of *C. maenas* Co(II)$_2$-Hc in 20 mM phosphate buffer, pH 7. ([θ] in deg decimol Co^{-1} cm^{-1}.)

are similar for the Co(II)$_2$-Hcs prepared from other decapod Hcs, *C. sapidus* and *P. interruptus* (ϵ_{602} = 540 M^{-1} cm^{-1}, ϵ_{582} = 800 M^{-1} cm^{-1}, ϵ_{562} = 650 M^{-1} cm^{-1}). In the infrared region, a broad band is found at 1150 nm for *C. maenas* Co(II)$_2$-Hc (insert, Figure 2A). The Co(II)$_2$-Hc derivative prepared from *L. polyphemus* (Chelicerata) has analogous spectroscopic features, but the position and intensity of the peaks is different from those for decapod Hcs. Three spectral bands at 600, 558, and 525 nm are seen with extinction coefficients of 605, 750, and 460 M^{-1} cm^{-1}, respectively (Figure 2B).[3] All features for these Co(II)$_2$-Hcs are characteristic of Co(II) in a geometry close to tetrahedral. Also the values for the extinction coefficients are consistent with two Co(II) ions per mole protein monomer.

The spectra of Co(II)$_2$-Hc from *C. maenas* and *C. sapidus* exhibit an absorption at 320 nm (Figure 3), and in CD spectra, a negative absorption band occurs at this wavelength (insert, Figure 3). The intensity of the absorption at 320 nm is proportional to the amount of thiocyanate tightly bound to the purified protein (data not shown). Moreover, both the absorption and the CD bands show a hyperchromic effect upon the addition of SCN$^-$ (insert, Figure 3).

EPR Spectroscopy of Co(II) Derivatives. Low-temperature EPR spectra were obtained for three different Co(II) derivatives of *C. maenas* Hc (Figure 4): (a) a monocobalt derivative exhibiting a spectrum like that reported in earlier work (Salvato et al., 1988); (b) a binuclear species presented here for the first time; (c) the Hc derivative containing excess Co(II), i.e., having a Co(II)/protein molar ratio of 7.

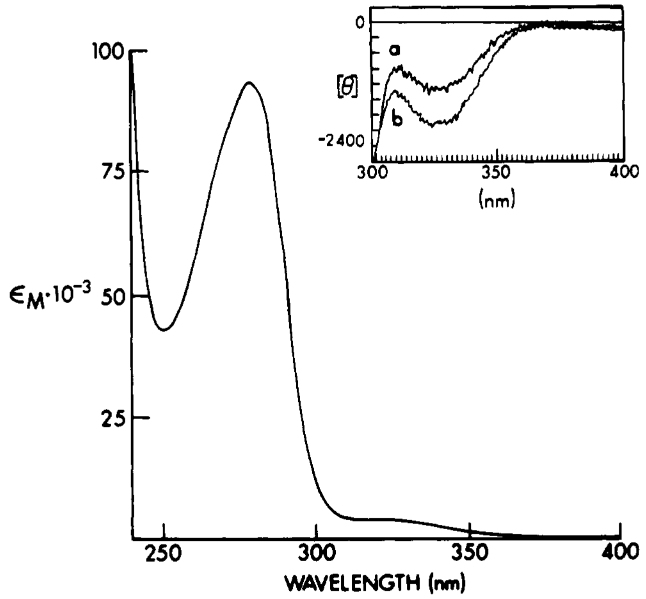

FIGURE 3: Optical spectra of *C. maenas* Co(II)$_2$-Hc. Absorption spectrum in the UV region, 5 mM phosphate buffer, pH = 7. (Insert) Circular dichroism spectrum in the near-UV. (a) In 5 mM phosphate buffer, pH = 7. (b) In 200 mM NaSCN and 5 mM phosphate buffer, pH = 7. ([θ] in deg decimol Co^{-1} cm^{-1}.)

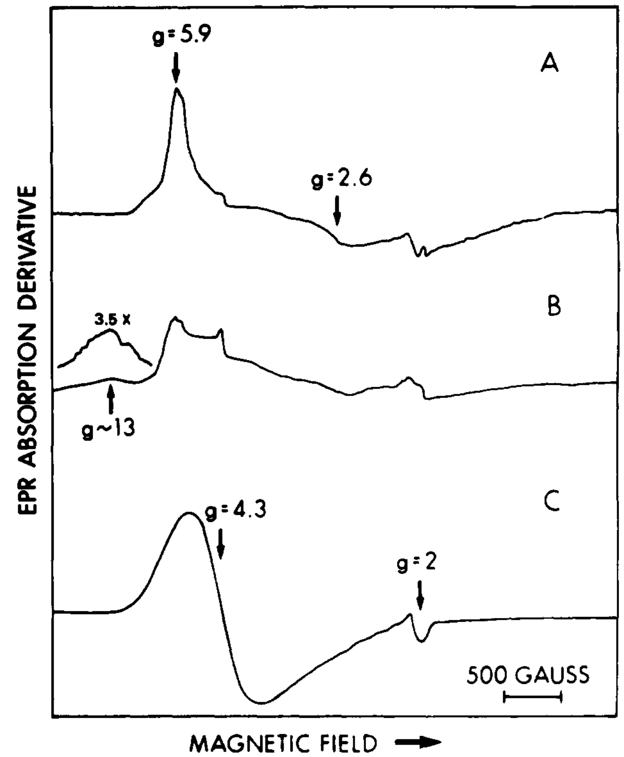

FIGURE 4: Comparison of EPR spectra of purified and crude *C. maenas* cobalt hemocyanin (A) 1.2 mM purified Co(II)-Hc in 20 mM Tris-HCl buffer, pH = 7; (B) 1.2 mM purified Co(II)$_2$-Hc in 20 mM Tris-HCl buffer, pH = 7; (C) 1.2 mM crude Co(II)$_2$-Hc at the end of the reconstitution reaction after dialysis in 20 mM Tris-HCl buffer, pH = 7, before EDTA treatment. EPR conditions: microwave frequency, 9.264 GHz; microwave power, 2 mW; modulation amplitude, 16 G; T = 8 K.

The derivative containing a single, EDTA-resistant cobalt per mol of protein subunit has a spectrum characteristic of high-spin Co(II) in a low-symmetry center, with g_{max} = 5.9;

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[3] For this Co(II)$_2$-Hc derivative as many as 10% of the active sites may have SCN$^-$ bound. Therefore, the values given for extinction coefficients may be too high.

$g_{mid} = 2.6$; (no g_{min} feature could be detected) (Figure 4A). A barely resolvable hyperfine splitting is seen in the low field feature ($A \approx 0.014$ cm^{-1}) arising from the ^{59}Co nuclear spin ($I = 7/2$). The spectrum is related to that of four-coordinate, nearly tetrahedral Co(II) complexes, including a model such as $Co(II)(C_2H_5COO^-)_2(imid)_2$ (Horrocks et al., 1980) and also resembles those of Co(II)-reconstituted zinc enzymes such as carbonic anhydrase (Grell & Bray., 1971), liver alcohol dehydrogenase (Makinen et al., 1981), and carboxypeptidase (Martinelli et al., 1989).

The EPR spectrum of Co(II)$_2$-Hc exhibits a new spectral feature with a very broad low-field absorption ($g \sim 13$) characteristic of integer spin systems (Figure 4B) (Banci et al., 1983; Hendrick & Debrunner, 1989; Hendrick et al., 1990). It also contains a residual absorption of the type shown in spectrum A of Figure 4, indicating the presence of some monocobalt derivative (on average less than 10%). The spectrum may also contain another signal centered near $g = 4$ that varies in intensity from one sample to another (see below). The low intensity signal near $g = 2$ results from residual Cu(II) that is not more than 5% of the protein subunit concentration, according to quantitation by atomic absorption.

The low-field signal suggesting the formation of a binuclear site led to the design of an experiment that followed metal incorporation into apo-Hc in two steps, the first being formation of a monocobalt derivative and the second, the formation of the binuclear derivative. Both optical spectroscopy and EPR were used here. Figure 5 shows the low-temperature EPR spectra of aliquots of Hc removed at the indicated times from dialysis against a reaction mixture used to prepare the monocobalt-Hc derivative (see Materials and Methods). Each sample was extensively dialyzed against EDTA/phosphate and then phosphate buffer alone, before freezing for EPR examination. This procedure, using 0.5 mM $CoCl_2$ (along with the other components), gave only the monocobalt derivative, even after extended incubation (96 h). The results of this first step are in agreement with previously published results (Salvato et al., 1986). Also shown in Figure 5B are the optical absorption spectra for these samples. The increasing absorbance was correlated with the increasing EPR intensity, though a complete quantitative analysis of the EPR data was not feasible due to the large uncertainty in double integration of the signals for samples with low concentrations of Co(II).

The sample whose spectrum is shown in e of Figure 5A, which contained nearly 1 mol of Co(II)/protein subunit, was again dialyzed against Co(II), but now against the buffer used to form Co(II)$_2$-Hc from apo-Hc (4 mM $CoCl_2$ plus 250 mM NaSCN and the other buffer components) in order to follow the formation of the derivative having higher Co(II)/protein ratios. Again, aliquots were removed from dialysis. These were extensively dialyzed against EDTA/phosphate and then against phosphate alone. During the secondary cobalt treatment, the optical absorption continued to increase with time, but the total EPR signal intensity decreased (Figure 5C). The appearance of the new signal at very low field ($g \sim 13$) became evident as the ratio of EDTA-resistant Co(II)/protein increased from 1 to 2. These results clearly demonstrate the formation of a coupled metal site given that the optical extinction attains a value consistent with the presence of 2 Co(II) ions per mol of protein. The loss of EPR intensity with increase in bound Co(II) could be a result of antiferromagnetic coupling, possibly mediated by spin exchange through a bridging ligand (Rohrs & Hatfield, 1989; Bonner et al., 1975).

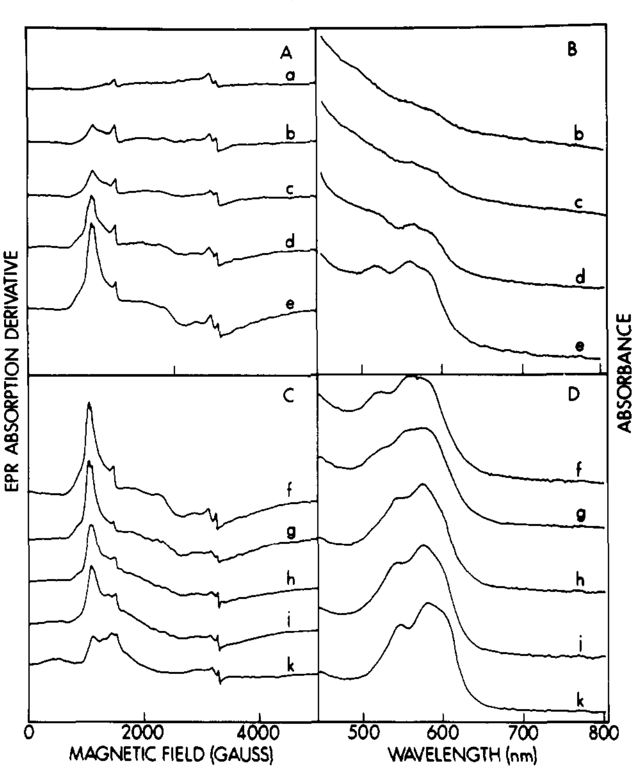

FIGURE 5: (A) EPR spectra and (B) optical absorption spectra of *C. maenas* cobalt Hc, at different times during the reconstitution of apo-Hc. T = (a) 0 h, (b) 14 h, (c) 28 h, (d) 47 h, (e) 71 h. Reaction conditions: 2 mL of 75 mg/mL apo-Hc dialyzed for the indicated times against 500 mL of 0.1 M Tris-HCl, pH 7.0, containing 50 mM NaSCN, 10 mM $MgSO_4$, 0.5 mM $CoCl_2$, and 0.05% Tween 80 at 20 °C. Each sample was dialyzed for 48 h (4 changes) against 2 mM EDTA. (C) EPR spectra of *C. maenas* cobalt Hc, starting from the 71-h sample, at different times during the second Co(II) dialysis. (D) Absorption spectra in the visible region. T = (f) 4 h, (g) 8 h, (h) 16 h, (j) 24 h, (k) 48 h. Reaction conditions: 2.5 mL of 75 mg/mL Co(II)-Hc dialyzed against 500 mL of 0.1 M Tris-HCl, pH 7.0, containing 250 mM NaSCN, 10 mM $MgSO_4$, 4.0 mM $CoCl_2$, and 0.05% Tween 80 at 20 °C. Each sample was dialyzed for 48 h (4 changes) against 2 mM EDTA.

The low-field signal at $g \sim 13$ was narrowed by the addition of azide, but not by addition of thiocyanate, though according to changes in the CD and optical spectra, both these anions bind to the Co(II) site (data not shown).

A sample of a *C. maenas* Hc derivative containing Co(II) in excess of 2 mol/protein subunit had an intense, broad EPR signal at $g = 4$ (Figure 4C), but the intensity of the optical spectrum was not significantly greater than that observed for Co(II)$_2$-Hc. The $g = 4$ EPR signal is similar to that reported by Lorosch and Haase (1986) for *L. polyphemus* Hc in the presence of excess Co(II). Since the $g = 4$ signal disappeared after EDTA treatment, it is believed to arise from adventitiously bound Co(II). As there is essentially no change in optical absorption upon the removal of this adventitious cobalt with EDTA, it is suggested that peripheral sites in the protein bind Co(II) in an octahedral complex (Cotton & Wilkinson, 1988).

Reaction of Co(II)$_2$-Hc with CN$^-$. The reaction of *C. maenas* holo-Hc with CN$^-$ yields selective and sequential removal of copper (Beltramini et al., 1984). Similar behavior was expected for cobalt in Co(II)$_2$-Hc since the metal bound at the binuclear active site should require high-affinity ligands for removal. In the presence of low concentrations of CN$^-$, Co(II)$_2$-Hc exhibits a small hyperchromic effect at 582 nm. Upon dialysis against higher CN$^-$ concentrations ([CN$^-$] > 1 mM, in the presence of SCN$^-$) a decrease of the Co(II)/

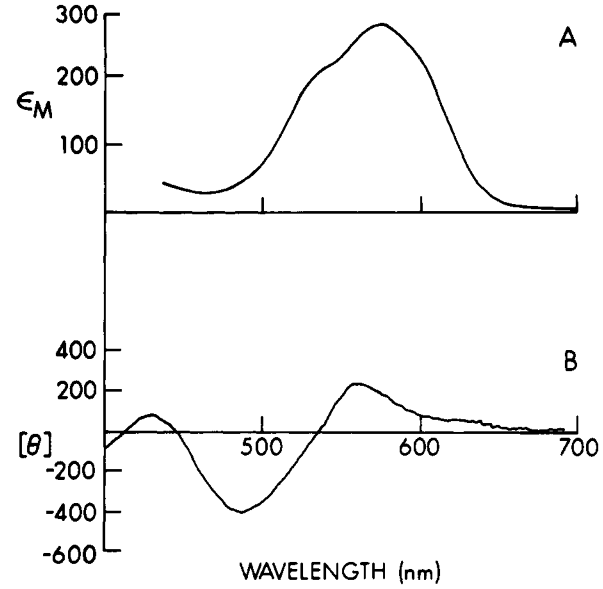

FIGURE 6: Optical spectra of Co(II)-Hc obtained from the reaction of *C. maenas* Co(II)$_2$-Hc with CN$^-$ (reaction conditions: 2.5 mL of 75 mg/mL Co(II)$_2$-Hc dialyzed against 500 mL of 20 mM phosphate buffer, pH 8.0, containing 10 mM NaCN and 50 mM NaSCN for 12 h, at 20 °C). (A) Absorption spectrum in 20 mM phosphate buffer, pH = 7. (B) Circular dichroism spectrum in 20 mM phosphate buffer, pH 7. ([θ] in deg decimol Co^{-1} cm^{-1}.)

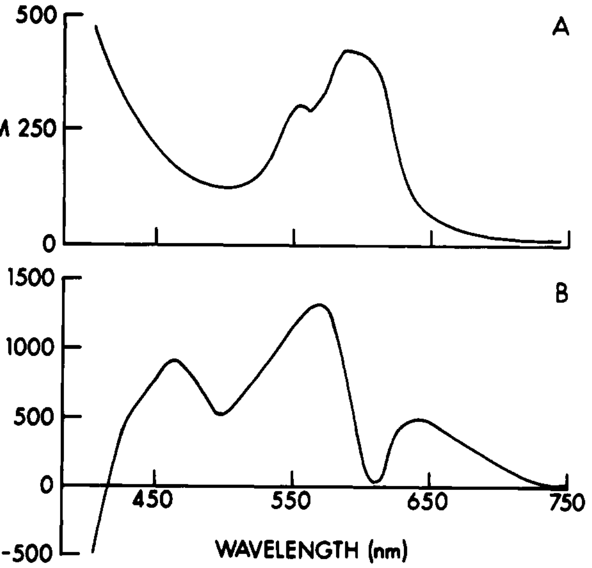

FIGURE 7: Optical spectra of Co(II)–Cu(I)-Hc obtained from native Hc by exchange reaction (reconstitution conditions: 2.5 mL of 75 mg/mL native Hc dialyzed against 500 mL of 0.1 M Tris-HCl, pH 7.0, containing 250 mM NaSCN, 10 mM MgSO$_4$, 4 mM CoCl$_2$, and 0.05% Tween 80 at 20 °C). (A) Absorption spectrum in 20 mM phosphate buffer, pH = 7. (The molar extinction coefficient is expressed per mol of Co(II).) (B) Circular dichroism spectrum in the visible region in 20 mM phosphate buffer, pH 7. ([θ] in deg decimol Co^{-1} cm^{-1}.)

protein ratio was found by atomic absorption spectroscopy after dialysis of the protein in EDTA/phosphate buffer. A sample isolated from the reaction with CN$^-$ after 12 h contained approximately 1.2 mol of Co(II)/protein subunit. The optical and CD spectra of this EDTA-stable monocobalt derivative were different in shape but not in intensity from those of the monocobalt derivative obtained by reconstitution of Co(II)-Hc from the apo protein (Salvato et al., 1986) (Figure 6). Furthermore, the fluorescence quenching was less than 50% of the value observed for the monocobalt derivative obtained by reconstitution of apo-Hc and the value observed for the binuclear cobalt derivative. These results suggest that the cobalt remaining after CN$^-$ treatment is bound in a site different from the one formed upon reconstitution of the apo protein to Co(II)-Hc (Salvato et al., 1986).

The formation of apo-Hc from Co(II)$_2$-Hc was complete after dialysis in the presence of cyanide for 24 h.

Co(II)–Cu(I)-Hc Derivative. Treatment of holo-Hc under the conditions used for the reconstitution of Co(II)$_2$-Hc from apo-Hc resulted in the binding of about 7 mol of cobalt per mol of protein monomer. Of these, a single cobalt could be removed by dialysis against Tris-HCl buffer and 5.5 additional mol could be removed by EDTA treatment. The purified product of this reaction contained 0.5 mol of EDTA-resistant cobalt per protein monomer. During the course of the reaction, the Cu/protein molar ratio decreased from 2 to 1.5, while the Co(II)/protein molar ratio increased from 0 to 0.5. Longer incubation did not yield products with a Co(II)/protein ratio higher than 0.5. The stability of the product in the presence of EDTA is taken as evidence that a hybrid active site was formed, since metal ions bound elsewhere are EDTA-labile.

The change in the intensity of the 340-nm band, characteristic of oxy-Hc was also monitored during metal exchange. As the substitution of cobalt for copper proceeded, the intensity at 340 nm decreased, consistent with the loss of capability to bind O$_2$ and with the formation of a hybrid Cu(I)–Co(II) derivative lacking an intense absorption in this region.

The optical spectrum of the hybrid derivative, Cu(I)–Co(II)-Hc (Figure 7) is similar to that of Co(II)$_2$-Hc. However, the molar extinction coefficient per cobalt (ϵ_{582} = 450 M^{-1} cm^{-1}) is 50% greater than the value obtained for the monocobalt derivatives produced either by reconstitution of apo-Hc (Salvato et al., 1986) or from the reaction of Co(II)$_2$-Hc with CN$^-$ and is comparable to that observed per Co(II) in Co(II)$_2$-Hc. This result suggests that the extinction coefficient for Co(II), and therefore its geometry in a binuclear site, is dependent on a structural feature only present when a second metal ion is bound.

Oxygen Binding. The presence of binuclear cobalt in the active site of Co(II)$_2$-Hc suggested that the derivative might bind O$_2$, in analogy with the native protein. Optical evidence for oxygen binding in holo-Hc is based on the 340-nm absorption (Eickman et al., 1979). *C. maenas* Co(II)$_2$-Hc prepared in air shows an absorption maximum at shorter wavelength (320 nm), whose extinction coefficient is too low ($\epsilon_{320} \approx$ 1000 M^{-1} cm^{-1}) to be a charge-transfer transition, given that peroxide to Co(III) LMCT in oxygenated cobalt complexes normally exhibits extinction coefficients greater than 7000 M^{-1} cm^{-1} (Bagger & Gibson, 1972). The intensity of the 320 band in Co(II)$_2$-Hc is the same under aerobic and anaerobic conditions, even after incubation for several hours in the absence of oxygen (data not shown).

The O$_2$ affinity of holo-Hc is known to increase at high pH, and oxygen binding to a cobalt derivative of *L. polyphemus* Hc has been suggested to also occur at high pH (Dutton et al., 1989). A sample of our *C. maenas* Co(II)$_2$-Hc was incubated overnight at pH 9 under an atmosphere of O$_2$. The optical spectrum in the near-ultraviolet and in the visible region was unchanged by this treatment (data not shown), suggesting that neither oxidation of Co(II) nor oxygen binding occurred.

The EPR spectrum of Co(II)$_2$-Hc was unchanged under anaerobic conditions before and after the addition of excess dithionite. The expected increase in intensity of the EPR signal was not observed after reduction. Therefore, the low intensity of the EPR signal of Co(II)$_2$-Hc is due to coupling phenomena and not to the presence of EPR-silent Co(III).

Dithionite reduction abolished the signal around $g = 2$ arising from residual Cu(II) and gave in some samples an increase in intensity of the signal near $g = 4$. This increase likely comes about from the reduction of small amounts (<5%) of Co(III) bound to peripheral sites in the protein.

DISCUSSION

Optical and EPR Spectroscopy of Co(II)₂-Hc. The rationale for metal substitution studies lies in the potential for a deeper understanding of the structure and function of a metalloprotein. To this end, the spectroscopically uninformative Cu(I) in the binuclear active site of Hc has been substituted with Co(II), a d^7 metal ion with more instructive spectroscopic features (Salvato et al., 1986; Lorosch & Haase, 1986; Suzuki et al., 1982a; Dutton et al., 1989). The value of the cobalt derivative of Hc as a model for the structure and function of holo-Hc depends on how closely it resembles the native form. In previous studies, the endeavor to use Co(II) to characterize the binuclear active site of Hc was precluded by the presence of adventitiously bound metal ions, which interfered with the use of magnetic resonance techniques.

We have prepared a binuclear cobalt derivative of a number of Hcs from various species and fully substantiate the integrity of one example, that from *C. maenas*, by noting the following features: (1) It contains 2 mol of Co(II) that are resistant to removal by dialysis against EDTA but are, however, removed by CN^-, much like the copper in the holo protein; (2) A reaction of the cobalt derivative with CuSCN, known to reconstitute holo-Hc from apo-Hc, does not yield a protein containing tightly bound copper; (3) The binuclear cobalt derivative exhibits an EPR signal characteristic of integer spin systems, indicating magnetic coupling between Co(II) ions; (4) Preliminary results of an EXAFS study are consistent with a metal site containing 2 Co atoms separated by 3.56 ± 0.06 Å (L. Powers and L. Bubacco, unpublished observation), in good agreement with the distance of 3.58 ± 0.05 Å observed for arthropod deoxy-Hc (Woolery et al., 1984); (5) The optical properties of $Co(II)_2$-Hc indicate that some feature of the doubly reconstituted metal binding site gives rise to an extinction coefficient per mole of cobalt that is greater than the value for monocobalt derivatives.

Optical and EPR spectroscopy allowed for some distinction to be made between the Co(II) in the active site and that in peripheral sites. The Co(II) bound to Hc in excess of 2 mol/mol of monomer makes little contribution to the optical absorption, and this peripherally bound population is characterized by its $g = 4$ EPR signal. A broad $g = 4$ signal (unpublished observations) was found for other Co(II) species known or expected to be octahedral, such as Co(II)–EDTA in ammonia/ammonium chloride buffer or Co(II) in the reconstitution buffer containing SCN^- [in which the majority of Co(II) species have octahedral coordination (Silber & Murguia, 1984)], and was also reported for *L. polyphemus* Hc in the presence of excess Co(II) (Lorosch & Haase, 1986). The low optical absorbance for the adventitious or excess Co(II) in our preparations is also consistent with an octahedral geometry (Cotton & Wilkinson, 1988).

The low-field absorption near $g = 13$ in EPR spectra of $Co(II)_2$-Hc presented here was not observed for a binuclear cobalt derivative of tyrosinase, which was EPR silent (Rüegg & Lerch, 1981). Strong magnetic coupling between metal ions was suggested to be responsible for the lack of EPR.

Optical spectra of *C. maenas* $Co(II)_2$-Hc are characteristic of tetrahedral or weakly distorted tetrahedral coordination geometry, according to the intensity and the positions of the maxima (Cotton & Wilkinson, 1988). The energies of the $\nu_3(^4A_2 \rightarrow {}^4T_1(P))$ transition, 17 200 ± 200 cm^{-1} (582 nm), and the $\nu_2(^4A_2 \rightarrow {}^4T_1(F))$ transition, 8700 ± 200 cm^{-1} (1150 nm), are in the range observed for inorganic Co(II) complexes with a distorted tetrahedral geometry (Rosenberg et al., 1975). The value of the extinction coefficient for the ν_3 transition is 450 $M^{-1}\,cm^{-1}$ per mol of cobalt for the binuclear derivative and 300 $M^{-1}\,cm^{-1}$ per mol of cobalt for the mononuclear derivative (Salvato et al., 1986), confirmed here. In both cases, the extinction coefficient is larger than 250 $M^{-1}\,cm^{-1}$, a value considered to be a lower limit for Co(II) complexes having distorted tetrahedral coordination geometry (Rosenberg et al., 1975). Further evidence of a distorted tetrahedral coordination geometry is found in the circular dichroism spectrum of $Co(II)_2$-Hc (Figure 2C), which shows bands typical of distorted tetrahedral Co(II) complexes (Kaden et al., 1974).

The sum of spectroscopic data indicates that both Co(II) ions in $Co(II)_2$-Hc are bound in an approximately tetrahedral geometry, a structure closely related to the active site described by X-ray diffraction data for the deoxy, holo protein (Volbeda & Hol, 1989).

Cu(I)–Co(II)-Hc Derivative. The use of SCN^- in a reaction with holo-Hc and Co(II) provided a means to obtain a hybrid Cu(I)–Co(II) binuclear derivative. In the absence of thiocyanate, exchange of copper for cobalt at low concentrations of Co(II) does not occur (Lorosch & Haase, 1986). In its presence, the reaction is probably facilitated by mobilization of Cu(I) through complexation by thiocyanate and may be further driven by precipitation of CuSCN ($K_{sp} = 1.6 \times 10^{-11}$) (Weast, 1974). Though the latter process leads to removal of copper, the incorporation of only 0.5 mol of Co(II)/protein to form a hybrid site suggests that apo-Hc is not formed in this reaction. Whether the mechanism involves a random substitution of either metal ion in the binuclear site or a specific substitution of one copper in particular could not be determined from the spectroscopic techniques at our disposal. However, the exchange is likely to occur at the FRS, in analogy with the exchange of ^{64}Cu(I) in solution for copper in holo-Hc in the presence of CN^- (Cox & Elliot, 1974).[4]

The optical spectrum of the Cu–Co hybrid Hc is dominated by features characteristic of a low-symmetry Co(II) site, similar to that of monocobalt Hc.[5] This result indicates that the Co(II) is not bound in a peripheral octahedral site. The extinction coefficient of the hybrid derivative ($\epsilon_{582} = 450\ M^{-1}\,cm^{-1}$), expressed per mol of cobalt, is higher than that of the monocobalt derivatives described above. The difference in extinction and also in the shape of the visible absorption spectrum for the Cu–Co hybrid compared to the monocobalt derivatives indicates that the presence of the pair of ions imposes a distortion of the coordination sphere not present when only one ion is bound. Further evidence of some change in coordination geometry of the site in the binuclear derivative is the lower ellipticity observed in the visible region for $Co(II)_2$-Hc compared to the mononuclear cobalt derivative (Salvato et al., 1986). Furthermore, the extinction coefficient for the binuclear derivative is larger than two times the value for the mononuclear derivatives, whether the latter were obtained by reconstitution of apo-Hc to Co(II)-Hc or by CN^- treatment of $Co(II)_2$-Hc. It is, however, twice the value of

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[4] Metal ion exchange between Cu(I) in the active site of *Pila leopolvillensis* Hc and $K_3{}^{64}Cu(CN)_4$ in solution has been observed (Cox & Elliott, 1974).

[5] The hybrid derivative exhibits an EPR signal due to Co(II) and a signal due to a small amount of Cu(II) (<10%).

the extinction coefficient attributed to Co(II) in the Cu–Co hybrid derivative. These findings are taken as evidence for different structural features for Co(II) when bound alone or in a binuclear site, possibly indicating a closer approximation to a tetrahedral coordination geometry for the ions in a binuclear center.

Reconstitution Conditions. The successful preparation with high yield of the cobalt derivatives having the metal-to-protein stoichiometry of native Hc resulted from the addition of thiocyanate to the reconstitution buffer. The high $SCN^-/Co(II)$ ratio in the buffer, according to the equilibria discussed by Silber and Murguia (1984), would favor formation of Co(II) bis- and tris(thiocyanate) complexes that contain H_2O to complete the octahedral coordination. The presence of the negatively charged species $Co(II)(SCN)_3(H_2O)_3^-$ could facilitate metal transfer to the active site in our experiments by providing for a favorable electrostatic interaction between the cobalt complex in solution and the imidazoles at the active site, some of which would be positively charged at neutral pH.

In the past, the reconstitution of Hc with Co(II) was often impeded by protein precipitation occurring at Co(II) concentrations higher than 0.5 mM. This precipitation also occurs with other heavy metals and cations (Brouwer et al., 1982; Suzuki et al., 1982b; Dutton et al., 1989). The process, reversed by EDTA treatment, presumably involves the binding of metal to negative sites on the protein surface, resulting in the neutralization of charge (Suzuki et al., 1982b). The use of SCN^- in the reconstitution buffer prevents the precipitation of Hc that would otherwise occur in 4 mM aqueous Co(II). The reconstitution buffer, as described above, contains a majority of cobalt species having two or three thiocyanate ligands and is therefore neutral or negatively charged. Thus, interactions between cobalt and negatively charged peripheral metal binding sites are inhibited, and the subsequent precipitation is avoided.

Oxygen Binding. The binding of O_2 to a cobalt derivative of *L. polyphemus* Hc has been reported to occur at high pH (Dutton et al., 1989). For that derivative, an absorption band at 320 nm, appearing when a deoxygenated sample was exposed to air, was assigned to a LMCT between bound oxygen and metal ion, in a structure written by analogy with oxy-Hc as $Co(III)–O_2^{2-}–Co(III)$, and was therefore taken as evidence for O_2 binding. Similar absorption bands are found in the optical spectra of oxygenated dimeric Co(II) complexes (Morris & Martin, 1969; Michailidis & Martin, 1969). The 320-nm band in the optical spectrum of our $Co(II)_2$-Hc preparation, however, is not changed by exhaustive deoxygenation nor is any change observed in the visible region when dithionite reduction is performed.[6] Dithionite would be expected to reduce bound oxygen and also reduce Co(III) to Co(II) in the oxygenated binuclear center. Reduction of cobalt should lead to an increased absorption intensity in the visible region due to the formation of Co(II). These results are consistent with the lack of oxygen binding reported for the cobalt derivative of *L. polyphemus* Hc (Lorosch & Haase, 1986) and for cobalt-substituted tyrosinase (Rüegg & Lerch, 1981). It should be noted that Co(III) complexes also exhibit absorptions in the spectral region 300–350 nm (Simplicio & Wilkins, 1967; Bagger & Gibson, 1972; Watters & Wilkins, 1974). Therefore, the presence of a 320-nm absorbance does not necessarily indicate O_2 binding.

It is noteworthy that the 320-nm absorption band was first observed in the cobalt derivatives of *L. polyphemus*, *Tachypleus tridentatus*, and *Tachypleus gigas* Hcs prepared by Suzuki et al. (1982b) using a procedure for the preparation similar to the one used by Dutton et al. (1989). The 320-nm band, found also in the CD spectrum of those Co(II)-Hcs, could not be ascribed to a LMCT such as occurs in $O_2^{2-}–Co(III)$ complexes because no contribution in the MCD spectra was found (Suzuki et al., 1982b). Also, the presence of excess cobalt in solutions of the derivative claimed to bind O_2 (Dutton et al., 1989) may have interferred with the spectroscopic measurements, since at the high pH used in the oxygenation/deoxygenation experiments, Co(II) is readily oxidized to Co(III). Therefore, an absorption at 320 nm may arise from the presence of free or adventitious Co(III). This assignment is consistent with the absence of an MCD band for the 320-nm absorption (Suzuki et al., 1982b).

We note in conclusion that the binding of oxygen to simple cobalt complexes having either tetrahedral or octahedral geometry yields an octahedral oxygenated complex (Morris & Martin, 1969; Michailidis & Martin, 1969). For this reason, oxygen binding is not expected to occur at a binuclear cobalt site because the large alteration in geometry and ligand composition required for formation of an octahedral species is unlikely to occur in the protein.

Conclusions. In summary, we have prepared a binuclear derivative of Hc in which cobalt occupies the same site as copper in the native protein. Both Co(II) ions are bound in sites having distorted tetrahedral geometry. The optical spectroscopic properties of Co(II) in the binuclear center are not equivalent to the sum of contributions from individual cobalts, suggesting that when two metals are bound, a more tetrahedral geometry is produced. A new feature, perhaps the presence of a bridging ligand between the two cobalt ions in the binuclear active site leading to spin coupling, is suggested by EPR spectroscopy. Finally, even though the metal site of the binuclear cobalt derivative is structurally similar to that of the holo protein, no evidence for oxygen binding was found.

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[6] Changes in the near-UV could not be investigated because dithionite has an absorption maximum at 320 nm.

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