Preparation, Spectroscopic Characterization and Anion Binding Studies of a Mononuclear Co(II) Derivative of *Carcinus maenas* Hemocyanin

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(Received March 19, 1986)

Abstract

The binuclear copper in the active site of *Carcinus maenas* hemocyanin has been substituted with one EDTA-resistant Co(II) per 75 000 M_r by reconstitution of the apo protein. Specific cobalt substitution at the copper binding site is demonstrated from the optical spectral changes directly correlated with the amount of Co(II) bound to the protein, the ellipticity in CD spectra in the near UV–Vis region, and the efficiency of tryptophan fluorescence quenching. The optical absorption spectrum of the cobalt-substituted protein is characterized by a band pattern attributable to d–d transitions of the metal ion. Both the position of the wavelength maximum (568 nm) and the molar extinction coefficient ($\cong$300 M^{-1} cm^{-1}) are typical of a four-coordinate, pseudo-tetrahedral Co(II) center.

Optical titrations indicate that Cl^-, Br^-, N_3^-, SCN^-, and CN^- bind to Co(II)–Hc, each with a stoichiometry of 1:1 per metal center. The apparent stability constants determined from Hill plots of titration data decrease in the order $CN^- >> N_3^- \cong SCN^- > Cl^- > Br^-$. Low temperature EPR studies demonstrate that at pH 7, the cobalt is high spin both in the presence and absence of anionic ligands. A low spin species is formed at pH 9 in the presence of cyanide. The spectrum of this latter complex exhibits superhyperfine structure indicative of metal ligation to ^{14}N supplied by the protein. Direct ligation of cyanide to cobalt is demonstrated by additional spectral splitting observed when this complex is formed using ^{13}C-labelled CN^-.

Introduction

Hemocyanins are high molecular weight copper proteins found in the hemolymph of molluscs and arthropods. They function physiologically as O_2 carriers, and the binuclear copper site they contain is essential for this process [1]. The minimal unit of these oligomeric proteins containing one active site has a molecular weight of approximately 50 000 for molluscan and 75 000 for arthropodan Hc* [1, 2]. A number of chemical and spectroscopic investigations are consistent with 4–8 histidines (2–4 per Cu) and probably one tyrosine serving as metal ligands [3–5]. The oxygenated metal center of oxy–Hc can be represented as a $Cu(II)\cdot O_2^{2-}\cdot Cu(II)$ complex, with the copper ions bridged by a peroxide dianion [6–8]. Despite the presence of two Cu(II) ions, oxy–Hc is EPR-silent because of strong antiferromagnetic coupling between the spins of the metal ions mediated by the bound peroxide and an endogenous bridging ligand [9].

An approach which has proven useful for the characterization of the metal sites in copper and zinc metalloenzymes involves substitution of the native metal with Co(II). Cobalt complexes exhibit characteristic optical absorption spectra and magnetic properties which are dependent on the geometry and the immediate environment of the Co(II)–ligand complex [10]. Also, changes in the coordination sphere of a protein-bound Co(II) caused either by conformational changes or by ligand addition or exchange may result in distinct modifications of the spectroscopic properties. In this way, Co(II) has been used to probe the metal binding sites of carbonic anhydrase [11], superoxide dismutase [12], stellacyanin [13], *Neurospora* tyrosinase [14], horseshoe crab and squid hemocyanins [15, 16], and several other metalloproteins.

The two copper ions in the active site of hemocyanin can be individually removed [17–21] and we have prepared various Co(II) derivatives, from both the molluscan and arthropodan proteins, that contain either a single Co(II) or a Co(II) paired with a single Cu(I) of half–apo–Hc [22]. In this paper we describe

*Abbreviations used: Hc, hemocyanin; CD, circular dichroism; EPR, electron paramagnetic resonance.

0020-1693/86/$3.50

the spectroscopic characterization of a derivative obtained from *Carcinus maenas* Hc which contains one Co(II) per 75 000 M_r (Co(II)–Hc). Optical and EPR spectroscopies were used to study the cobalt center and the anionic ligand binding reactions of this protein derivative, and to demonstrate that the specifically bound Co(II) most likely occupies the copper binding site.

Materials and Methods

Carcinus maenas Hc was purified from hemolymph collected by syringe from the dorsal lacunae of living animals. The clotted hemolymph was filtered through gauze and was collected by centrifugation. The clear supernatant was diluted 1:1 with 0.1 M tris-HCl buffer, pH 7.2, containing 20 mM $CaCl_2$, and the solution was brought to 52% saturation with $(NH_4)_2SO_4$. After a 1–2 h incubation, the bluish suspension was centrifuged and the precipitate was dissolved in the same buffer and reprecipitated as above. After dialysis, Hc was finally purified by two cycles of centrifugation at 160 000 $\times g$ for 5 h. The protein was finally dialyzed against 0.1 M tris-HCl buffer, pH 7.2, for 48 h. Sucrose (18% *w/v*) was added and this preparation was stored at −20 °C. All operations during protein purification were carried out at 4 °C.

Apo–Hc was prepared from Hc by CN^- treatment according to a published procedure [3]. Co(II)–Hc was reconstituted by dialysis of apo–Hc solutions (approximately 100 mg/ml) against 0.1 M tris-HCl, pH 7.0, containing 150 mM NaSCN, 10 mM $MgSO_4$, 0.5 mM $CoCl_2$, and 0.05% Tween 80, for 72 at 20 °C. The protein was then dialyzed against 20 mM phosphate buffer, pH 7.0, containing 20 mM EDTA, and finally against 20 mM phosphate buffer for 24 h.

Samples for ligand binding studies were prepared by dialysing protein solutions contained in 2 mm diameter Servapor dialysis tubing, against large volumes of anion solutions of the appropriate concentration. For titration experiments, small aliquots of concentrated ligand solutions were added to Co(II) Hc. Titration data were plotted using the Hill equation [23] in the following form:

$$\log(\mathrm{HcL})/((\mathrm{Hc_T}) - (\mathrm{HcL})) = n \log(\mathrm{L}) - \log K$$

where (HcL) = concentration of ligated Hc; (Hc_T) = total concentration of Hc; (L) = concentration of free ligand; n = Hill coefficient; K = equilibrium constant for the ligand association reaction. Protein concentrations were determined spectrophotometrically using $E_{278} = 1.24$ ml mg^{-1} cm^{-1} [24].

Optical absorption spectra were recorded on a Perkin-Elmer model 576 spectrophotometer and corrected for light scattering according to a published method [7]. Circular dichroism spectra were recorded on a Cary 61 spectropolarimeter. Fluorescence spectra were recorded at 21 °C with a Perkin-Elmer MPF 4 fluorimeter equipped with a thermostatted cell compartment. Solutions used for fluorescence measurements had an absorbance less than 0.08 at 295 nm, the excitation wavelength, to minimize the inner filter effect. Metal analyses were performed by atomic absorption spectroscopy using a Perkin-Elmer Model 300 flame spectrophotometer.

EPR spectra were recorded at X-band on a Varian E-112 spectrometer equipped with a Polytechnic Research and Development Co. wavemeter and a Varian NMR gaussmeter. Low temperature studies were performed using a Heli-Tran liquid helium transfer system. Some spectra were recorded at 77 K, using a liquid nitrogen cold finger fitted into the EPR cavity. Signal to noise for some EPR data was improved by averaging several scans under identical conditions. An Apple II Plus computer fitted with a data acquisition card (Computer Interface Technology, Inc., Wilmington, Del.) was used for the collection and processing of data.

For EPR studies, a sample of 1 mM Co(II)–Hc in 50 mM phosphate buffer, contained in a side-arm test tube fitted with rubber septum stoppers, was purged with argon for 10 min. Anaerobically prepared sodium dithionite was added by syringe to a final concentration of 18 mM. The Co(II)–Hc was incubated for 5 min to insure reduction of residual Cu(II), and exogenous ligand (either as solid or in concentrated solution) was added from the side arm. The sample was then transferred by syringe to an argon-purged EPR tube and immediately frozen in liquid N_2. The CN^- derivatives prepared with either 10 or 50 mM KCN had to be frozen within seconds after mixing because the excess anion quickly removes Co(II) from the protein, generating apo Hc.

Results

Preparation of Co(II) Hemocyanin

Native Hc was found to be resistant to cobalt substitution by direct addition of metal ion since the spectroscopic properties of oxy–Hc were unchanged after incubation in the buffer used for reconstitution. Apo–Hc, however, binds Co(II) efficiently as demonstrated by the appearance of an absorption in the visible with a maximum near 568 nm. The Co(II) binding process was monitored as a function of time by examining aliquots of the reconstitution mixture during a 72 h period. After dialysis against EDTA for the removal of non-specifically bound and excess Co(II), the optical spectrum of each aliquot was recorded and the amount of cobalt bound was determined by atomic absorption spectroscopy. The absorbance at 568 nm and the molar ratio of bound cobalt to Hc as functions of the incubation time reach a constant value after 72 h (Fig. 1a). The

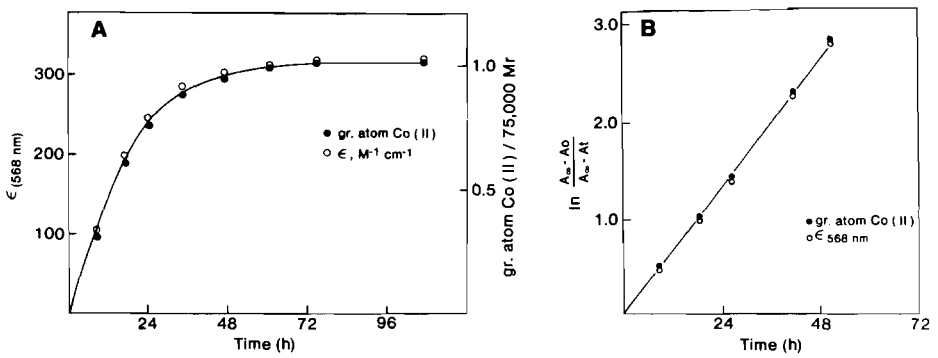

Fig. 1. (a) Kinetics of Co(II) binding to apo Hc. The figure shows both the increase of the 568 nm absorption (○—○) and the Co(II)-to-Hc stoichiometry (calculated per 75 000 M_r) determined by atomic absorption spectroscopy (●—●). Reconstitution conditions: apo Hc, 100 mg/ml; buffer solution: 0.1 M tris-HCl, pH = 7.0, containing 150 mM NaSCN, 10 mM $MgSO_4$, 0.5 mM $CoCl_2$, 0.05% Tween 80, T = 20 °C. (b) Semilogarithmic plot obtained from the data in (a). A_0, A_∞ and A_t indicate the initial absorbance at 568 nm, the final absorbance (72 h), and the absorbance of aliquots measured at time t, respectively. For details see 'Materials and Methods'.

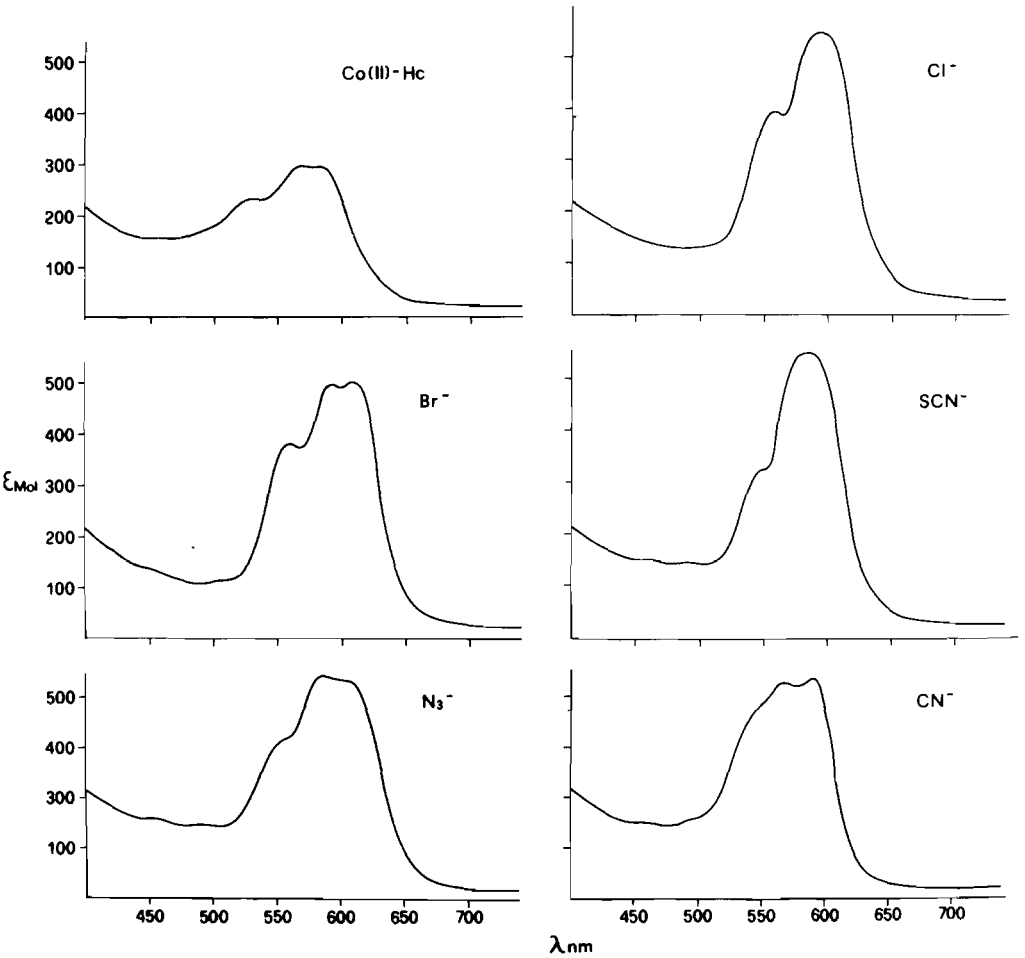

Fig. 2. Optical absorption spectrum of Co(II)–Hc in 20 mM phosphate buffer, pH = 7.0 and in buffer containing 1 M Cl^-, 1 M Br^-, 0.2 M SCN^-, 0.2 M N_3^-, or 5 mM CN^- as labelled. The spectrum of Co(II)–Hc is unaltered by the addition of 0.2 M F^-, I^-, SO_4^{2-}, HPO_4^{2-}, ClO_4^-, $HCOO^-$, or CH_3COO^- (data not shown).

TABLE I. Spectroscopic Properties of Co(II)–Hc, and Co(II)–Hc with Exogenous Ligands

Spectroscopic method	Buffer conditions											
	20 mM NaP_i, pH = 7.0		$+N_3^-$ (0.2 M)		$+CN^-$ (5 mM)		$+Br^-$ (1 M)		$+Cl^-$ (1 M)		$+SCN^-$ (0.2 M)	
	λ	ϵ[a]	λ	ϵ	λ	ϵ	λ	ϵ	λ	ϵ	λ	ϵ
Optical absorption	530	(230)	555	(315)	545	(375)	560	(580)	558	(390)	548	(323)
	568	(312)	587	(440)	565	(425)	592	(493)				
	585	(288)	605	(430)	588	(430)	608	(494)	596	(543)	583	(545)
	λ	$[\theta]$[b]	λ	$[\theta]$	λ	$[\theta]$	λ	$[\theta]$	λ	$[\theta]$	λ	$[\theta]$
Circular dichroism	327	(+850)	335	(−2700)	318	(+1360)	320	(+520)	320	(+650)	320	(−3300)
	355	(+900)	367	(+320)	355	(+620)	360	(+540)	362	(+670)	370	(+470)
	415	(+230)	432	(+120)	425	(+140)	425	(+60)	420	(+90)	425	(+150)
	485	(−680)	487	(−469)	495	(−730)[c]	492	(−780)	493	(−830)	485	(−900)
	567	(+550)	570	(+980)	585	(+400)	580	(−420)	570	(+510)	550	(+518)[d]
			622	(−90)			620	(−120)	610	(−260)	610	(+120)
	λ	ϕ/ϕ_{apo}[e]	λ	ϕ/ϕ_{apo}	λ	ϕ/ϕ_{apo}	λ	ϕ/ϕ_{apo}	λ	ϕ/ϕ_{apo}	λ	ϕ/ϕ_{apo}
Fluorescence	330	(0.69)	330	(0.50)	330	(0.69)	330	(0.69)	330	(0.69)	333	(0.37)

[a] ϵ, M^{-1} cm^{-1}. [b] θ, deg decimol Co^{-1} cm^{-2}. [c] A shoulder is present at 520 nm ($[\theta]$ = −550). [d] A shoulder is present at 570 nm ($[\theta]$ = +550). [e] Measured upon excitation at 295 nm. ϕ and ϕ_{apo} indicate the quantum yield of Co(II)–Hc and of apo–hemocyanin, respectively.

limiting molar extinction coefficient is 312 M^{-1} cm^{-1}, which corresponds to a stoichiometry of a single Co(II) (1.0 ± 0.02) per 75 000 M_r. A semilogarithmic plot of these data indicates that the binding of Co(II) follows pseudo-first order kinetics, with a $t_{1/2}$ of approximately 13 h and an apparent rate constant of about 0.05 h^{-1} (Fig. 1b). The correspondence between the absorbance change at 568 nm and the amount of metal bound demonstrates that Co(II) binding is specific, yielding a mononuclear derivative containing one Co(II) per 75 000 M_r.

Optical Spectroscopy of Co(II)–Hemocyanin

Co(II)–Hc has an absorption spectrum characterized by a resolved d–d transition pattern with maxima at 530, 568, 585 nm (Fig. 2, Table I). No differences in the optical spectrum are observed from pH 6 to 10. The CD spectrum of Co(II)–Hc shows two Cotton extrema in the visible with maxima at 567 nm and 485 nm, and a shoulder near 510 nm (Fig. 3, Table I).

Addition of up to 0.2 M F^-, I^-, ClO_4^-, $HCOO^-$, CH_3COO^-, SO_4^{2-}, or HPO_4^{2-} to Co(II)–Hc does not affect the absorption or the CD spectrum of the protein. In contrast, Cl^-, Br^-, N_3^-, SCN^- or CN^- anions each cause a strong hyperchromic effect and a red-shift of the absorption spectrum (Fig. 2) which suggests that these anions bind directly to Co(II). The pK for the binding, determined from titration data and Hill plots (data not shown) range from 6 for CN^- to approximately 1 for Cl^- and Br^- (Table II). The stoichiometric ratio of bound ligand to cobalt was unity in all cases. Ligand binding is reversible since dialysis against phosphate buffer restores the original optical spectrum.

TABLE II. Stability Constants for Anion Complexes of Co(II)–Hc

Anion	pK[a]
CN^-	6.0[b]
N_3^-	1.83
SCN^-	1.74
Cl^-	1.01
Br^-	0.64

[a] The pK values were calculated from Hill plots of optical titration data as described in 'Materials and Methods'. [b] The data in the cyanide binding experiment were corrected for the dissociation of HCN (pK = 9.35, 25 °C).

CD spectra also exhibit exogenous ligand-dependent changes (Fig. 3). An optically active component appears near 610–620 nm with addition of Cl^-, Br^-, SCN^-, or N_3^-. The binding of N_3^- or SCN^- also causes changes in the near ultraviolet region of the CD spectrum (300–360 nm) (Fig. 4, right). No differences are observed in the aromatic residue region (250–300 nm) in the presence of the anions, suggesting that Co(II)–Hc does not undergo conformational changes upon ligand binding (Fig. 4, left).

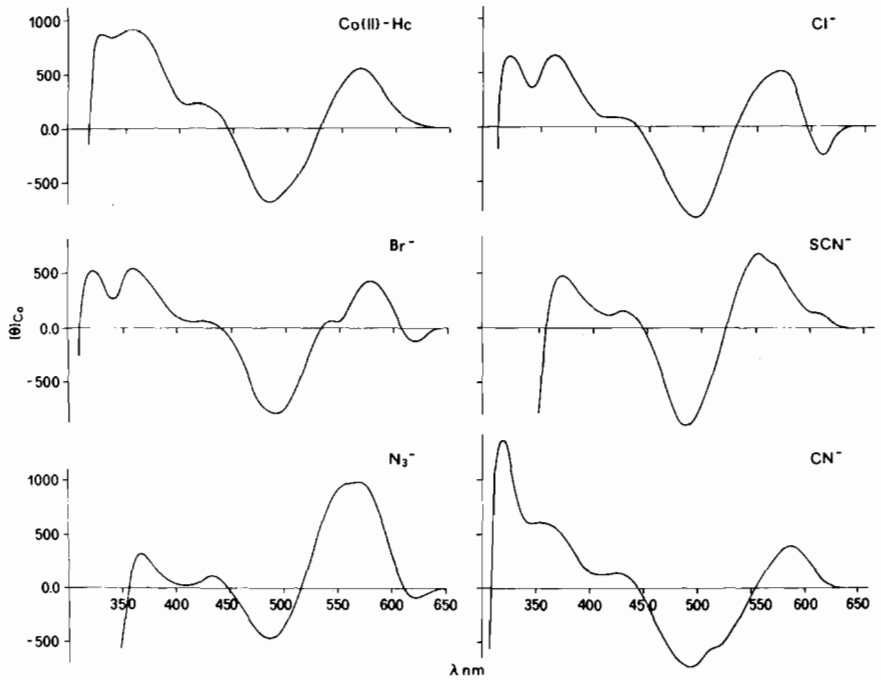

Fig. 3. Circular dichroism spectra in the visible region of Co(II)–Hc in 20 mM phosphate buffer, pH = 7.0 and in buffer containing 1 M Cl^-, 1 M Br^-, 0.2 M SCN^-, 0.2 M N_3^-, or 5 mM CN^- as labelled. The spectrum of Co(II)–Hc is unaltered by the addition of 0.2 M F^-, I^-, SO_4^{2-}, HPO_4^{2-}, ClO_4^-, $HCOO^-$, or CH_3COO^- (data not shown).

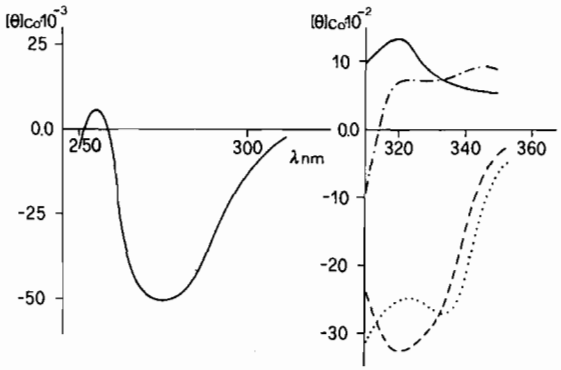

Fig. 4. Circular dichroism spectra in the UV region of Co(II)–Hc in 20 mM phosphate buffer, pH = 7.0. The spectrum from 250 to 300 nm is unaltered by the addition of 1 M Cl^-, I M Br^-, 0.2 M SCN^-, 0.2 M N_3^-. The spectra in the 310–360 nm range are shown for the protein in 20 mM sodium phosphate buffer, pH = 7.0 (– . –), and in buffer plus 5 mM CN^- (——), 0.2 M N_3^- (·····), or 0.2 M SCN^- (- - - -).

The fluorescence properties of Co(II)–Hc are summarized in Table I. The emission maximum is 330 nm (excitation at 295 nm) and the fluorescence intensity is quenched approximately 30% compared to the apoprotein. The shape and intensity of fluorescence emission of Co(II)–Hc is not modified by CN^-, Cl^-, or Br^-. Addition of N_3^- and SCN^-, however, causes quenching of tryptophan fluorescence of 30% and 50% respectively (Table I).

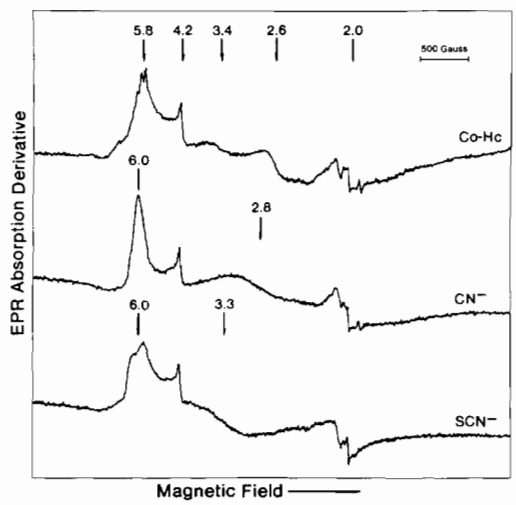

Fig. 5. EPR spectra of 1 mM Co(II)–Hc in 50 mM phosphate buffer, pH = 7.0 containing 18 mM sodium dithionite, labelled Co–Hc; CN^-, with 0.05 M KCN; SCN^-, with 0.2 M SCN^-. Samples were anaerobically reduced to decrease the signal due to residual Cu(II). EPR conditions: microwave frequency, 9.26 GHz; microwave power, 2 mW; modulation amplitude, 16 Gauss; T = 8 K.

EPR Spectroscopy of Co(II)–Hc

The low temperature (8 K) EPR spectrum of Co(II)–Hc at pH 7, both with and without added ligands, is characteristic of high spin Co(II) complexes (Fig. 5). The spectrum of Co(II)–Hc without added

ligands exhibits ^{59}Co ($I = 7/2$) nuclear hyperfine splittings at $g = 5.8$. Two values for an apparent g_{mid} feature or 'crossover line' in the absorption derivative are indicated at $g = 3.4$ and $g = 2.6$. Both these values are similar to the 'crossover lines' exhibited by Co(II) model compounds and Co(II)-substituted enzymes [25]. The high field g value is near 2.0 but is obscured by signals from contaminating species, which most likely represent residual Cu(II) bound to Hc. The data do not allow the assignment of a unique set of g values to a single rhombic or axial species expected for the high spin cobalt under these conditions.

The effect of exogenous CN^- and SCN^- on the EPR properties of Co(II)–Hc is also shown in Fig. 5. The low field feature is narrower, the hyperfine interaction is no longer resolved and only one crossover peak is evident in each case ($g_{mid} = 2.8$ for the cyanide complex and 3.3 for thiocyanate complex). The spectra of Co(II)–Hc in the presence of 1 M Cl^-, 1 M Br^-, and 0.2 M N_3^- (data not shown) are similar to the spectrum of the cyanide complex in Fig. 7, except for small variations in the position of g_{mid}.

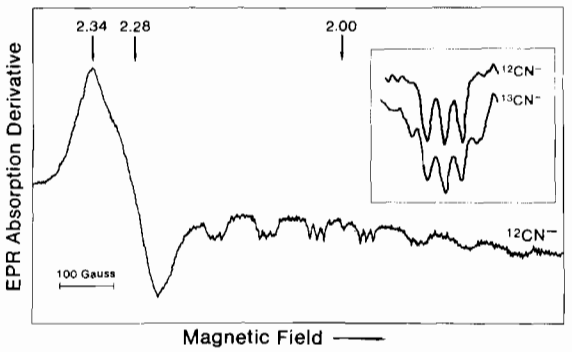

Fig. 6. EPR spectrum of low spin Co(II)–Hc in 50 mM phosphate buffer containing 50 mM KCN, pH = 9.2. The sample was reduced with sodium dithionite. EPR conditions: microwave frequency, 9.12 GHz; microwave power, 8.0 mW; modulation amplitude, 6.3 Gauss; $T = 77$ K. Inset: comparison of one cobalt nuclear hyperfine feature from the spectra of low spin Co(II)–Hc prepared with $^{12}CN^-$ or $^{13}CN^-$. The feature illustrated is centered at $g = 2.03$.

The high spin cobalt complex persists even upon addition of excess strong field ligands such as cyanide. The EPR spectrum of Co(II)–Hc in 10 or 50 mM CN^- at pH 7 is invariant. However, if unbuffered KCN is added to the protein under anaerobic conditions, the high spin complex is converted to a low spin form (final CN^- concentration 50 mM, final pH approximately 9). The g values for this slightly rhombic species are assigned at $g = 2.34$, 2.26 and 2.01 (Fig. 6). This spectrum is similar to those of other low spin Co(II) complexes including Co(II)–porphyrins [26] and a dicyanide complex of Co(II) carbonic anhydrase [27]. The 94 gauss splitting centered at $g = 2.00$ arises from the cobalt nuclear

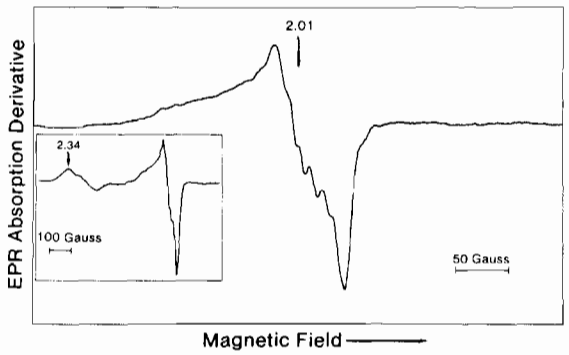

Fig. 7. EPR spectrum of Co(II)–Hc after aerobic addition of 50 mM CN^- followed by a brief incubation. This spectrum resembles that of an equimolar, aerobic mixture of Co(II) and CN^- (data not shown). Inset, spectrum of this sample recorded over a larger field sweep showing the residual signal (near $g = 2.3$) due to the low spin Co(II)–Hc cyanide complex which remains bound to the protein. EPR conditions: microwave frequency, 9.10 GHz; microwave power, 5 mW; modulation amplitude, 4 Gauss; $T = 77$ K.

hyperfine interaction while the narrow (13 gauss) 3-line superhyperfine pattern arises from the axial coordination of ^{14}N ($I = 1$) from a protein ligand.

The low spin cyanide complex was also prepared using ^{13}C-labelled cyanide and the spectrum of one ^{59}Co nuclear hyperfine feature appears in Fig. 6 (inset). This feature, compared to its counterpart from the spectrum of the complex prepared with $^{12}CN^-$, is now split into a quintet due to the combined interactions of the cobalt spin with both ^{14}N and ^{13}C ($I = 1/2$) nuclei. The additional splitting indicates that at least one cyanide ion is bound to Co(II) in this complex.

The addition of excess CN^- to Co(II)–Hc under aerobic conditions, followed by a brief incubation, produces another signal shown in Fig. 7, which is characteristic of an oxygenated Co radical species [28]. This signal is nearly indistinguishable from the broad $g = 2.0$ signal exhibited by a simple aerobic mixture of equimolar Co(II) and CN^- (data not shown) and is similar to the signal reported by Suzuki *et al.*, in their study of squid Co(II)–Hc treated with cyanide [16].

Discussion

The binding of Co(II) to apo Hc follows pseudo-first order kinetics and yields a complex with a single Co(II) per 75 000 M_r. The optical spectroscopic properties of the derivative suggest a nearly tetrahedral, four-coordinate binding site for the metal ion. This result is similar to that reported for Co(II) substitution of horseshoe crab and squid hemocyanins [15, 16].

The following observations suggest that cobalt in the derivative is bound at the Hc active site. The

fluorescence of tryptophan is quenched during Co(II) binding and the kinetics of this change parallels the development of the characteristic absorption spectrum. The fluorophore is thought to be close to one of the metal ions in the binuclear pair in the holoprotein [29]. Since the fluorescence yield of Co(II)–Hc is the same as that of deoxy Hc and a Hc derivative containing a single Cu(II) [21], it is likely that the metal-to-fluorophore distance in Co(II)–Hc is similar. Though the mechanisms for quenching may not be the same in these examples, the fluorescence change associated with Co(II) binding mimics the behavior of copper in the active site.

The addition of exogenous ligands to Co(II)–Hc perturbs the spectroscopic properties of the metal center with no evidence in CD spectra for significant change in the overall conformation of the molecule. The fluorescence quenching observed with N_3^- or SCN^- may be evidence for hydrogen bond formation between these large ligands bound to the Co(II) and the N1 hydrogen of a tryptophan indole located near the metal binding site. This hypothesis is supported by CD spectra in the 300–350 nm region of the N_3^- and SCN^- adducts, which exhibit reversed Cotton effects compared to spectra of the other ligand complexes. Since the H-bond would stabilize a particular geometry for the Co–azide or thiocyanate ligand complex, the CD of these could reflect a unique ligand orientation not achieved by other, smaller anions.

The appearance of nuclear hyperfine splitting near $g = 6$ in the EPR spectrum of the ligand free Co(II)–Hc is also exhibited by some 5- and 6-coordinate Co(II) complexes and a bimetallic Co(II) alkaline phosphatase [26]. This effect is often seen in the EPR spectra of octahedral Co(II) complexes and is present here even though the room temperature optical spectrum indicates a lower coordination number. It is possible that in the absence of exogenous ligands there are two populations of bound Co(II), one of which has a coordination number greater than 4. This heterogeneity may reflect a structural rearrangement that occurs upon freezing. The reconstitution of alkaline phosphatase reportedly yields two classes of Co(II) sites, one of these being octahedral and one of lower symmetry [30]. Octahedral Co(II) species, for example, could contribute to the room temperature optical absorption spectrum of Co(II)–Hc but these would have low absorptivity and could be masked by the absorption of the lower symmetry complex.

The binding of exogenous ligands, however, converts the bound Co(II) to a structure the EPR spectrum of which exhibits a single g_{mid} feature and which no longer displays the splitting at $g = 6$ usually associated with octahedral coordination. It is not yet possible to relate these EPR effects observed at low temperature in the high spin derivatives to specific structural changes, but the ligand binding effects indicate the stabilization of a single, 4- or 5-coordinate site. The spectra of ligand-bound forms are similar to those of models and Co(II)–reconstituted enzymes having a coordination number of 4 or 5, and are consistent with a tetrahedral geometry for the Co site [26, 30, 31]. Current models for the active site of hemocyanins contain three imidazoles and a bridging ligand available for metal binding and these could constitute the ligands bound to the metal ion in Co(II)–Hc. Since both optical and EPR spectra indicate that addition of one exogenous ligand does not increase the coordination number of the Co(II) center, one endogenous ligand is apparently exchangeable.

The effects of cyanide addition at pH 9 indicate that this anion binds directly to the Co(II) and the new ligand field causes a change in spin state from 3/2 to 1/2. The presence of ^{14}N and ^{13}C superhyperfine interactions in the EPR spectrum of the $^{13}CN^-$ complex indicates that cobalt is bound to both a nitrogen-containing protein ligand and cyanide. A previous study [32] of Co(II) carbonic anhydrase demonstrated the binding of 2 equatorial cyanide ions to the Co(II) center, though our data do not indicate the same phenomenon with Co(II)–Hc. The sligthly rhombic nature of the EPR of the Co(II)–Hc cyanide complex is not unprecedented, although low spin Co(II) complexes usually display axial symmetry. For example, the quinuclidine complex of Co(II) (p-OCH_3) tetraphenylporphyrin exhibits a rhombic spectrum and Co(II)–substituted heme in certain hemoglobin hybrid tetramers exhibits slightly rhombic spectra not unlike the spectrum of the CN^- complex we observe at pH 9 [25, 33].

Cyanide also labilizes the Co(II) center and under aerobic conditions at pH 9 quickly produces a species whose EPR spectrum closely resembles that of an oxygenated Co(II)–CN^- complex prepared in the absence of protein. The minor differences between the spectra of the simple complex (data not shown) and the one formed from the protein may indicate that the oxygenated Co site is protein-bound but we have no direct evidence for protein ligation. The spectrum reported by Suzuki [16] in a study of squid Co(II) Hc, which was assigned to an oxygenated Co complex having cyanide and endogenous protein ligands, may also represent a protein-free complex.

Acknowledgement

We wish to thank Dr. M. T. Foffani, Dept. of Organic Chemistry, Univ. of Padua, for making the Cary 61 Spectropolarimeter available.

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