Oxygenation of Cobalt(II)-Substituted *Limulus polyphemus* Hemocyanin: Kinetics, CD, and MCD Studies

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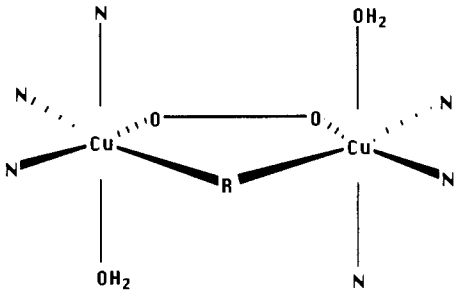
Cobalt(II)-substituted *Limulus polyphemus* (CoHcy) is characterized by circular dichroism (CD) and magnetic circular dichroism (MCD) spectroscopies. At neutral pH, the active site Co(II)'s are mostly aquoCoHcy, which gives rise to weak CD but intense low-temperature (4.2 K) MCD spectral features at 571, 552, and 526 nm. At higher pH's CoHcy is mostly hydroxoCoHcy and still has a weak visible CD spectrum, but three near-UV CD peaks at 372, 340, and 316 nm appear which are proposed to arise from ligand to metal charge transfer (LMCT). The 4.2 K MCD spectrum of hydroxoCoHcy is very rich with peaks at 304, 324, 355, 526, 556, 571, 616, and 642 nm. These spectra can be interpreted in terms of approximate C_{3v} ligand symmetry about each active-site cobalt(II) with a $(His)_3O$ ligand set. In the case of aquoCoHcy, the O ligand comes from a coordinated water, whereas, in hydroxoCoHcy, the O ligand comes from hydroxide. The hydroxoCoHcy spectrum in the d–d transition region has too many peaks to be accounted for solely from spin-allowed transitions; therefore, it is proposed that the 616- and 642-nm bands arise from spin-forbidden $^4A_2 \rightarrow {}^2A_2(G),{}^2E(G)$ transitions which gain intensity from the nearby spin-allowed $^4A_2 \rightarrow {}^4E(P)$ and $^4A_2(P)$ transitions. The aquoCoHcy and hydroxoCoHcy MCD spectral features are strikingly similar to those of the low-pH and high-pH forms of cobalt-substituted carbonic anhydrases, respectively. HydroxyCoHcy rapidly oxygenates (k = 500 M^{-1} s^{-1}) to form oxyCoHcy, which has strong CD bands at 332, 413, 518, and 618 nm. These bands are supportive of an oxyCoHcy active site, which contains μ-1,2-peroxo, μ-hydroxo dibridged Co(III) dimers. The 332- and 413-nm bands are due to $\pi_a^* \rightarrow d_\sigma^*$ and $\pi_b^* \rightarrow d_\sigma^*$ $O_2^{2-} \rightarrow$ Co(III) LMCT, while the 518- and 618-nm CD bands are d–d transitions arising from six-coordinate Co(III).

Introduction

Hemocyanins are the oxygen transport proteins in arthropoda and mollusca.[1] The active sites of the oxyprotein contain a pair of antiferromagnetically coupled Cu(II)'s, each attached to the protein through three imidazole nitrogen ligands. Preparation of a series of chemical derivatives allows the hemocyanin active site to be systematically varied.[1,2] In addition to the deoxy and oxy proteins, half-apo (one Cu(I)), half-met (one Cu(I) and one Cu(II)), met-apo (one Cu(II)) and met (two Cu(II)'s) can be prepared. The half-met and met forms do not bind dioxygen but do bind a variety of exogenous ligands such as halogens and pseudohalogens. Detailed spectroscopic studies of the native and chemically prepared derivatives using EPR, UV/vis, EXAFS, IR, and Raman spectroscopies,[1-4] as well as some recent model compound and theoretical studies,[5] have defined two possible "spectroscopically effective" oxyhemocyanin active-site structures, a μ-1,2-peroxo bridging structure with a second bridging ligand, I, and a μ-η^2:η^2-peroxo bridging structure, II. Each Cu(II) is six-coordinate with three imidazole ligands from histidine residues, a water ligand, and a bridging peroxide. It is argued that the "R" bridging ligand is necessary in I because methemocyanin is



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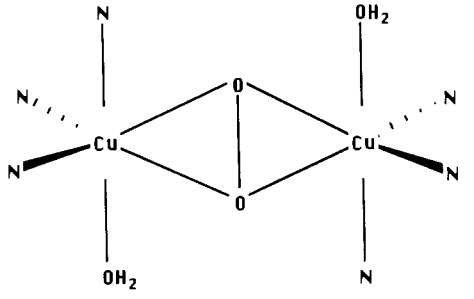

II

diamagnetic, despite the fact that both coppers are divalent, d^9. EXAFS studies have placed the Cu(II)'s at 345–366 pm, too far for a direct copper–copper bond;[4] therefore, an endogenous bridging ligand is required to allow for coupling of the unpaired electrons on each copper.[1] It has been determined from a study of the pH dependence of the EPR spectrum of a fraction of the methemocyanins that the endogenous bridging ligand has an intrinsic $pK_a > 7$ and has been suggested to be either the side group from serine, threonine, or tyrosine or simply hydroxide.[6]

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The X-ray crystal structure of *Panulirus interruptus* deoxyhemocyanin at 320-pm resolution shows no evidence of a large bridging ligand, tending to favor the hydroxide.[7] An absorption peak at 425 nm in the spectra of oxy- and methemocyanins has been associated with the endogenous bridging ligand to Cu(II) charge transfer.[1b]

It can be argued that since methemocyanin is a chemically modified form of the protein that any bridging ligands present may not relate to the ligand set in oxyhemocyanin. No bridging ligand, bulky or small, would be expected in the X-ray crystal structure if II is the correct structure. The striking spectroscopic similarity of the μ-η^2:η^2-peroxo bridging structure model compound to oxyhemocyanin[5b] and the recent theoretical studies[5c,d] favor structure II as the most likely oxygen binding mode in oxyhemocyanins, but the situation is still not settled.

Metal substitution in active sites of metalloproteins has been successfully used to study a variety of systems.[8-10] Metal substitution is often used to replace a "spectroscopically silent" metal such as Zn(II) with one conducive to magnetic and optical spectroscopic studies.[11,12a] Metal substitution can also be used to systematically vary the active site to increase the dimensionality of a spectroscopic study; the substitution of Cu(II) with Co(II) or Ni(II) in the blue copper proteins illustrated the usefulness of this approach.[13,14] Recently, we reported a Co(II)-substituted *Limulus polyphemus* (horseshoe crab) hemocyanin derivative that would bind dioxygen.[15] This Co(II)-substituted hemocyanin (subsequently referred to as CoHcy) was essentially the same as previously reported by Suzuki et al.[16a] and Lorosch and Haase;[16b] however, CoHcy prepared from *L. polyphemus* is significantly different from CoHcy prepared from *Carcinus maenas* hemocyanin reported by Salvato et al.[17] In *L. polyphemus* CoHcy, chemical and spectroscopic evidence suggest that hydroxide bridges the Co(II)'s in the active site at high pH (hydroxoCoHcy) and facilitates the oxygenation to form a μ-1,2-peroxo, μ-hydroxo dibridged Co(III) site. Chloride, other halides, and pseudohalides can displace hydroxide at pH's between 7 and 9, inhibiting oxygenation of hydroxoCoHcy. The spectroscopic properties CoHcy and kinetic behavior of hydroxoCoHcy toward oxygenation were similar to a vast number of simple Co(II) complexes which form the μ-1,2-peroxo, μ-hydroxo dibridged Co(III) structure, analogous to I.[18]

While our earlier results on the oxygenation of *L. polyphemus* CoHcy are circumstantially supportive of structure I, they are open to the same type of criticism as the methemocyanin results. CoHcy is a chemically modified form of hemocyanin that may have little to do with native oxyhemocyanin. On the other hand, structure II was never seriously considered as a possibility for oxyhemocyanin until a copper model compound was synthesized by Kitajima et al.[5] CoHcy can also be considered as a model compound of hemocyanin in which the natural ligand is retained and the metal is changed. This is an interesting contrast to the classic model compound in which the same metal is retained and the ligand is changed. As long as the limitations are kept in mind when conclusions are drawn, both approaches are valid and useful to achieve a greater understanding of this interesting and complex protein.

Since CoHcy is a potentially useful model of hemocyanin, and oxyCoHcy is a potentially useful model of oxyhemocyanin, we wanted to characterize the oxygenation of hydroxoCoHcy by kinetics and circular dichroism (CD) and magnetic circular dichroism (MCD) spectroscopies. The active site of CoHcy is a mixture of an aquoCoHcy species and a hydroxoCoHcy species, which both have four-coordinate, distorted tetrahedral ligand geometries. HydroxoCoHcy reacts with oxygen to form oxyCoHcy, which has an active site best described as a μ-1,2-peroxo, μ-hydroxo Co(III) dimer. CD and MCD are ideally suited to probe the CoHcy system. The distorted tetrahedral Co(II) in aquoCoHcy and hydroxoCoHcy is expected to have an intense MCD spectrum and a weak CD spectrum, while the six-coordinate Co(III) in oxyCoHcy is expected to have a weak MCD spectrum and an intense CD spectrum.[12b,c]

Experimental Section

Hemocyanin was prepared from the hemolymph of *Limulus polyphemus* (horseshoe crab) obtained from Marine Biological Laboratories, Woods Hole, MA. The hemolymph was centrifuged for 20 min at 20 000 × g to remove cells and debris. The supernatant was dialyzed against 0.05 M Tris/SO_4^{2-} buffer, pH 8.0. Sulfate was chosen as the buffer anion because chloride binds to the cobalt in CoHcy. The hemocyanin was then passed through a column packed with sterile Bio-Gel P-6DG resin (Bio-Rad, Richmond, CA) which was equilibrated with Tris/SO_4^{2-} buffer; this step removes most of the bacteria present in the hemolymph and increases storage stability.[15]

Stripped hemocyanin, in which all the divalent cations were removed, was prepared by dialysis against 0.02 M EDTA in 0.05 M Tris/SO_4^{2-} buffer, followed by dialysis against pure buffer. All protein samples were stripped prior to introduction of Co(II) since divalent cations affect the rate and equilibria of CoHcy formation.[15]

Apohemocyanin was prepared by dialysis of oxyhemocyanin against 0.1 M NaCN in pH 8.0 Tris/SO_4^{2-} buffer until greater than 98% of the copper was removed.[18] The apohemocyanin was then exhaustively dialyzed against pure buffer and stripped as described above.

CoHcy was prepared by anaerobic dialysis (continuous Ar sparging) of 5–10 mL of 0.4–0.7 mM apohemocyanin against 1 L of Tris/SO_4^{2-} buffer containing between 0.05 and 0.1 mM Co(II), added as solid $CoSO_4 \cdot 7H_2O$ or as a small volume of concentrated Co(II) solution. Dialysis (SPECTRA/POR dialysis tubing, 15.9-mm diameter, 12 000–14 000 MW cut-off, Spectrum Medical Industries, Inc., Los Angeles, CA) was continued until the concentration of Co(II) in the active site stopped increasing (3–5 days). No attempt was made to remove excess Co(II) from the CoHcy preparations. All sample preparation was conducted at 4 °C in either an argon or nitrogen atmosphere, unless otherwise stated.

The kinetics of CoHcy oxygenation were studied at pH's between 8 and 9.5 using an Applied Photophysics (Leatherhead, UK) RX1000 rapid mixing accessory (stopped-flow) and an OLIS (On-Line Instrument Systems, Jefferson, GA) modified Cary 118 spectrophotometer. The absorbance increase was measured at 320 nm using a 1-cm path length cell. The entire stopped-flow apparatus was placed in a glovebag which was filled with Ar. CoHcy (0.3–0.5 mM) was placed in one syringe, and buffer with dissolved O_2 (0.02–0.05 mM) was placed in the second syringe. Equal volume amounts of CoHcy and buffer were mixed at 20 °C, and the absorbance increase at 320 nm was monitored. The data were fit to a single exponential rise using the OLIS Levenberg–Marquardt nonlinear fitting algorithm.

CD and MCD spectra were obtained on a JASCO J600 spectropolarimeter equipped with an Alpha Scientific (Hayward, CA) 1.4-T electromagnet and an Oxford Instruments SM-4 magnet/cryostat. CoHcy samples were mixed with glycerol (50/50, v/v) to form a glass for

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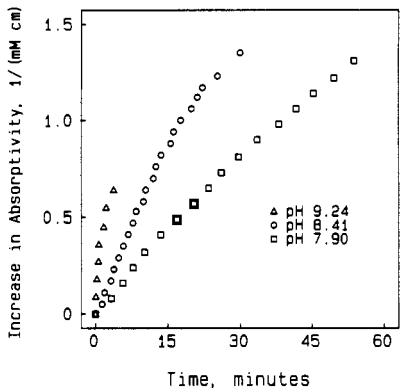


Figure 1. Increase in absorptivity at 320 nm (due to oxyCoHcy) versus time for 0.15 mM CoHcy and 0.015 mM O_2 at pH 7.90, 8.41, and 9.24. CoHcy at 0.30 mM was rapidly mixed with buffer containing 0.03 mM O_2 using a stopped-flow apparatus. Only every tenth datum point is shown for clarity.

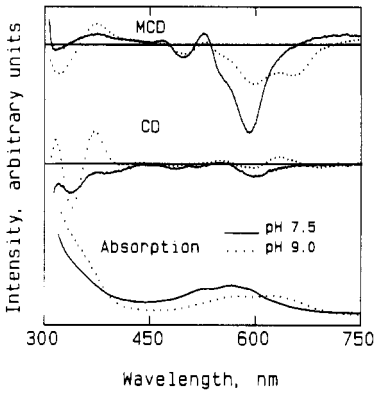


Figure 2. Room-temperature absorption, CD, and MCD spectra of CoHcy at pH 7.5 and pH 9.0. The spectra at pH 7.5 are representative of aquoCoHcy, and the spectra at pH 9.0 are due mostly to hydroxoCoHcy.

low-temperature spectra. The glycerol solutions were filtered through a 0.45-μm nylon filter (Rainin, Woburn, MA) before freezing. The MCD spectra of the sample in pure buffer and in 50/50 buffer/glycerol are the same at room temperature. This showed that glycerol does not affect the MCD spectral properties of the active site. The CD peak intensities are reported as $\Delta\epsilon$ with units of M^{-1} cm^{-1} and were calibrated against an aqueous solution of nickel tartrate with $\Delta\epsilon_{720} = -0.030\ 69$ M^{-1} cm^{-1}.[19] The MCD peak intensities are also reported in $\Delta\epsilon$ normalized to magnetic field in tesla, T, calibrated against aqueous cobalt(II) sulfate with $\Delta\epsilon_{510} = -0.018\ 50$ M^{-1} cm^{-1} T^{-1} at 298 K.[20]

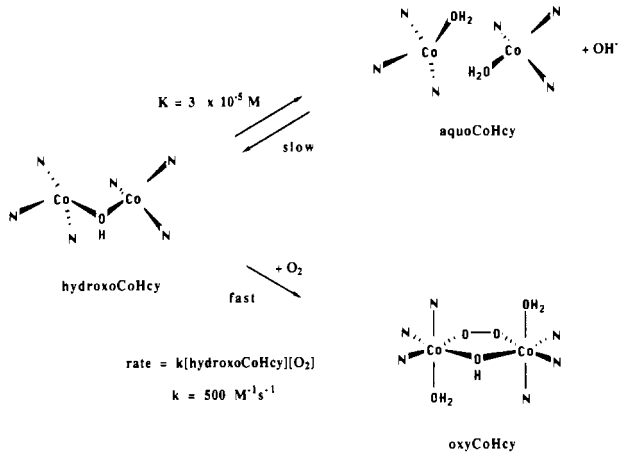
The protein concentration was determined by absorbance at 280 nm (ϵ = 82 400 M^{-1} cm^{-1}).[21] The concentration of copper in the active site of oxyhemocyanin was determined by absorbance at 342 nm (ϵ = 20 000 M^{-1} cm^{-1}).[16a] The amount of Co(II) in the active site was determined by integrated visible absorbance between 450 and 713 nm using an integrated molar absorptivity of 47.4 absorbance nm/(mM cm).[15] Total amounts of copper or cobalt were determined by atomic absorption spectrometry (AA) using a Perkin-Elmer Model 460 AA spectrophotometer. Absorption spectra were recorded on a Cary 17 UV/visible absorption spectrometer which was interfaced to a computer using OLIS version 9.01 software.

The ultraviolet CD spectrum between 190 and 240 nm is sensitive to the protein secondary structure,[22] and there is ample evidence to indicate that peripherally bound or adventitiously bound metal ions can change the secondary structure of polypeptides and proteins.[22a,b] However, the ultraviolet CD spectra apohemocyanin, oxyhemocyanin, and oxyCoHcy (0.05 M sodium borate buffer, pH 8.0) were the same; therefore, no major changes in the secondary protein structure are induced by binding of cobalt to active sites or adventitious sites.

Results and Discussion

Kinetics. When CoHcy is exposed to air, the absorbance at 320 nm rapidly increases, and the absorbance due to hydroxoCoHcy at 619 and 655 nm decreases (vide infra). The CD and MCD spectral features due to hydroxoCoHcy decrease in intensity as well. New CD bands at 332, 413, 518, and 618 nm appear in the CD spectrum of oxyCoHcy, but these peaks have no detectable MCD intensity. The rate of the oxygenation reaction can be measured by using a stopped-flow apparatus and monitoring the formation of oxyCoHcy at 320 nm. Figure 1 shows three such reactions at pH 7.90, 8.41, and 9.24 under pseudo-first-order conditions (CoHcy at 0.15 mM and O_2 at 0.015 mM).

Scheme I



The apparent rate of oxygenation is dependent on pH since hydroxoCoHcy is in equilibrium with aquoCoHcy and hydroxide. The equilibrium constant has been estimated to be 3×10^{-5} M ($\pm 2 \times 10^{-5}$ M) by a combination of equilibrium dialysis and spectrophotometric titration.[15] When the equilibrium is taken into account to adjust the kinetics result to the initial concentration of hydroxoCoHcy, the rate of oxygenation is found to be first-order in $[O_2]$ and first-order in [hydroxoCoHcy] with a rate constant of k = 500 M^{-1} s^{-1} (± 200 M^{-1} s^{-1}). These results are summarized in Scheme I. The initial equilibrium between aquoCoHcy and hydroxoCoHcy is *not* rapid, and very little aquoCoHcy is converted to hydroxoCoHcy during the brief oxygenation experiments.

The reaction of aquoCoHcy with hydroxide to form hydroxoCoHcy is very slow compared to the rate of oxygenation of hydroxoCoHcy. The rates of reaction of aquoCoHcy with azide and chloride are also slow. Charged exogenous ligands will bind to the active site,[15] but they are introduced into the active site by long equilibrium dialysis. We found that the amount of hydroxoCoHcy that forms at a given pH depends on the ionic strength, increasing with increasing ionic strength. The rate of oxygenation, however, is independent of ionic strength as one would expect since there is no net change of charge at the active site upon oxygenation.

Visible CD Spectroscopy. The visible absorption, CD, and MCD spectra of CoHcy at pH 7.5 and at pH 9.0 are shown in Figure 2. Previously we showed that the absorption spectrum of CoHcy was sensitive to the pH and that the stoichiometry was one OH^- to two active-site Co(II)'s.[15] The visible maximum shifts slightly and new peaks at 660, 625, and 360 nm develop at higher pH's. Both the CD and the MCD spectra of CoHcy are also pH dependent. The visible absorption bands in aquoCoHcy were assigned on the basis of a $Co^{II}(his)_3O$ chromophore

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Table I. Summary of CD Results for CoHcy and oxyCoHcy

Co species	ν, 10^{-3} cm^{-1} (nm)	$\Delta\epsilon$, M^{-1} cm^{-1}	γ^a	assgnt^b
		Four-Coordinate Cobalt		
aquoCoHcy	16.7 (599)	−0.16	0.0004	$^4A_2 \rightarrow {}^4E(P)$, C_{3v}
	18.1 (552)	+0.04	0.0001	$^4A_2 \rightarrow {}^4A_2(P)$, C_{3v}
hydroxoCoHcy	15.8 (634)	+0.19	0.0006	$^4A_2 \rightarrow {}^2A_2(G), {}^2E(G)$, C_{3v}
	16.8 (595)^c	−0.04	0.0001	$^4A_2 \rightarrow {}^4E(P)$, C_{3v}
	18.2 (549)^c	+0.10	0.0003	$^4A_2 \rightarrow {}^4A_2(P)$, C_{3v}
	26.9 (372)	+0.68	0.002	LMCT
	29.4 (340)	−0.73	0.003	LMCT
	31.6 (316)	+0.47	0.002	LMCT
		Six-Coordinate Cobalt		
advent. Co(II)	20.5 (488)	−0.023	0.002	$^4T_{1g} \rightarrow {}^4T_{1g}(P)$, O_h
oxyCoHcy	16.2 (618)	+0.29	>0.02	$^1A_1 \rightarrow {}^1E_a$, D_3
	19.3 (518)	−1.23	>0.1	$^1A_1 \rightarrow {}^1A_2$, D_3
	24.2 (413)	+0.13	0.0001	$^1A_1 \rightarrow {}^1E_b$, D_3, and $\pi_a^* \rightarrow d_{\sigma}^* O_2^{2-} \rightarrow$ Co(III) LMCT
	30.1 (332)	−3.45	0.0005	and $\pi_b^* \rightarrow d_{\sigma}^* O_2^{2-} \rightarrow$ Co(III) LMCT

^{a} $\gamma = |\Delta\epsilon/\epsilon|$ ϵ's are taken from ref 15; absorption bands not observed are estimated to have ϵ's < 15. ^{b} Assignment given in point group of highest possible ligand symmetry about cobalt. ^{c} May be a mixture of aquoCoHcy and hydroxoCoHcy.

with C_{3v} symmetry. In this assignment the $^4A_2 \rightarrow {}^4T_1(P)$ visible transition of T_d Co(II) splits into a $^4A_2 \rightarrow {}^4E(P)$ and $^4A_2 \rightarrow {}^4A_2(P)$ for the approximately C_{3v} aquoCoHcy. The CD spectrum of aquoCoHcy (Table I and Figure 2) is consistent with this assignment. The $^4A_2 \rightarrow {}^4T_1(P)$ tetrahedral transition is magnetically forbidden and would be expected to have a small (<0.01) Kuhn anisotropy factor, $\gamma = |\Delta\epsilon|/\epsilon$. The Kuhn anisotropy factor is greater than 0.01 for magnetically allowed transitions.[23,24] Lowering the symmetry to C_{3v} splits the transition into $^4A_2 \rightarrow {}^4E(P)$, which is magnetically allowed in C_{3v}, and $^4A_2 \rightarrow {}^4A_2(P)$, which is magnetically forbidden in C_{3v}. The anisotropy factor for the $^4A_2 \rightarrow {}^4E(P)$ is much larger than for the $^4A_2 \rightarrow {}^4A_2(P)$ transition (Table I), which is in accordance with this assignment. The anisotropy factors are still both small because Kuhn anisotropy factors tend to depend a great deal on the "parentage" of the transition.[24] A Kuhn anisotropy factor greater than 0.01 is a reliable indicator that the transition is magnetically allowed; however, a Kuhn anisotropy factor less than 0.01 cannot be used as a reliable criterion for a magnetically forbidden transition.[24]

At high pH the spectrum of hydroxoCoHcy emerges. It is complicated by the presence of aquoCoHcy and the possibility of a small contamination with oxyCoHcy, which has an intense CD spectrum (vide infra). Nevertheless new absorption bands associated with hydroxoCoHcy at 360 nm (shoulder) and a broad band between 600 and 670 nm (at least two bands) are apparent. In the CD spectrum of hydroxoCoHcy three intense peaks at 372 (26 900 cm^{-1}), 340 (29 400 cm^{-1}) and 316 nm (31 600 cm^{-1}), associated with the 360-nm shoulder, and a weak, broad CD band at 634 nm (15 800 cm^{-1}) associated with the broad 600–670-nm absorption band are observed.

The absorption, CD, and MCD (vide infra) spectra of hydroxoCoHcy bear a striking resemblance to the high-pH spectra of cobalt-substituted carbonic anhydrase, CoCA.[25,26] Like CoCA, hydroxoCoHcy has too many bands in the d–d transition region to be accounted for entirely by spin-allowed transitions. In high-pH CoCA, the new bands at 615 and 640 nm were assigned to the spin-forbidden transitions $^4A_2 \rightarrow {}^2E(G)$ and $^4A_2 \rightarrow {}^2T_1(G)$.[26] In C_{3v} these would correspond to $^4A_2 \rightarrow {}^2E_a(G)$ and $^4A_2 \rightarrow {}^2A_2(G)$, $^4A_2 \rightarrow {}^2E_b(G)$ transitions. The unusually large intensity of these spin-forbidden transitions was rationalized in terms of strong mixing with the nearby $^4A_2 \rightarrow {}^4T_1(P)$ transition induced by a trigonal distortion from T_d symmetry on going from the low-pH CoCA to the high-pH CoCA. A number of distorted tetrahedral Co(II) complexes have intense $^4A_2 \rightarrow {}^2E(G)$ and $^4A_2 \rightarrow {}^2T_1(G)$ spin-forbidden transitions including $Co(OH)_4^{2-}$ and $Co(Cl)_4^{2-}$.[26] The carbonic anhydrase system has been extensively studied,[14,25,26] and it has been reasonably well established that the low-pH CoCA active site has a Co(II) with three imidazole nitrogen ligands and one water ligand in an approximate T_d arrangement. Raising the pH results in the deprotonation of the water ligand, yielding an active site with three imidazole nitrogens and a single hydroxide ligand. There was some controversy over whether the high-pH form of CoCA was four- or five-coordinate, but it now is almost certain that the high-pH CoCA, in the absence of exogenous ligands, is four-coordinate.[25] In the case of hydroxoCoHcy, the CD data support a four-coordinate active site. The Kuhn anisotropy factors are essentially the same for hydroxoCoHcy and aquoCoHcy (Table I). If the Co(II) in hydroxoCoHcy were five-coordinate, the $\Delta\epsilon$'s in the CD spectrum would increase and ϵ's for the absorption spectrum would decrease relative to the aquoCoHcy spectra. These changes would cause the Kuhn anisotropy factors to increase on going from aquoCoHcy to hydroxoCoHcy but they do not.

The three transitions at 372, 340, and 316 nm in the CD spectrum of hydroxoCoHcy have Kuhn anisotropy factors at least 1 order of magnitude greater than the visible CD bands for either aquoCoHcy or hydroxoCoHcy, so these may be magnetically allowed transitions. If there is to be strong mixing of the $^2T_1(G)$ and $^4T_1(P)$ levels to give intensity to the $^4A_2 \rightarrow {}^2E(G)$ and $^4A_2 \rightarrow {}^2T_1(G)$ transitions in the visible, then Dq/B must be in the 0.2–0.4 range, yielding a Dq value around 350 cm^{-1}.[26,27] The only magnetically allowed d–d transition in the neighborhood of 29 000 cm^{-1} at a Dq value of 350 cm^{-1} is the $^4A_2 \rightarrow {}^2T_2(^2F)$ spin-forbidden transition. There is no close spin-allowed transition that this can mix with to gain intensity; therefore, it is not a feasible assignment for the near-UV CD transitions. These are associated with a moderately intense absorption band and must therefore be charge-transfer bands. We previously suggested that the 360-nm absorption is σ(LMCT) from hydroxide; however, a π(LMCT) from imidazole is also feasible.[28]

A very weak CD band at 488 nm is attributable to adventitiously bound Co(II). This correlates with a weak absorption at 520 nm which is attributable to a magnetically-allowed $^4T_{1g} \rightarrow {}^4T_{1g}(P)$ transition of six-coordinate Co(II) in a moderately strong ligand field.[15] No other information about the makeup of possible ligands to the adventitiously bound Co(II) is available.

The spectrum of CoHcy at pH 7.5 changes only slightly upon exposure to air (Figure 3). This is a direct result of the fact that only the hydroxoCoHcy reacts with oxygen and that the equi-

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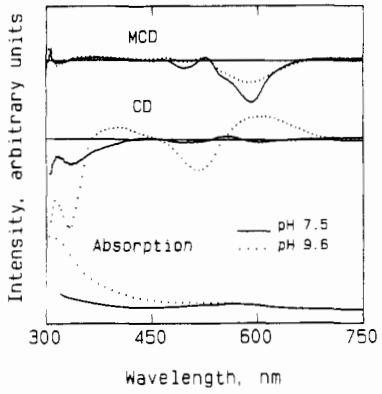

Figure 3. Room-temperature absorption, CD, and MCD spectra of CoHcy at pH 7.5 and pH 9.6 after exposure to air. The spectra at pH 7.5 are only slightly different from those at pH 7.5 in Figure 2 because of the low hydroxoCoHcy content of CoHcy at pH 7.5. The absorption and CD spectra at pH 9.6 arise mostly from oxyCoHcy, but the MCD spectrum arises from unreacted aquoCoHcy.

librium of aquoCoHcy with hydroxoCoHcy is slow. When pH 9.6 CoHcy is exposed to air, the absorption, CD, and MCD spectra change dramatically to form oxyCoHcy. The absorption spectrum shows a decrease in visible absorption but a broad intense increase in near-UV absorbance with maxima at 319 nm (ϵ = 7400 M^{-1} cm^{-1}) and 404 nm (ϵ = 2100 M^{-1} cm^{-1}). These bands can be attributed to $\pi_a^* \rightarrow d_\sigma^*$ and $\pi_b^* \rightarrow d_\sigma^*$ $O_2^{2-} \rightarrow$ Co(III) LMCT bands of a μ-1,2-peroxo, μ-hydroxo dibridged Co(III) dimer, respectively.[15,29] The CD spectrum of oxyCoHcy confirms this assignment. The highest possible local ligand symmetry around the Co(III) in oxyCoHcy is D_3 as a $Co^{III}(His)_3O_3$ chromophore (Scheme I). A magnetically-allowed d–d transition between 500 and 600 nm associated with the $^1A_{1g} \rightarrow {}^1T_{1g}$ transition in O_h is expected for this chromophore. In D_3 this would split into $^1A_1 \rightarrow {}^1E_a$ and $^1A_1 \rightarrow {}^1A_2$, both magnetically-allowed.[30] The 618-(16 200 cm^{-1}) and 518-nm (19 300 cm^{-1}) CD bands in oxyCoHcy can be assigned to the $^1A_1 \rightarrow {}^1E_a$ and $^1A_1 \rightarrow {}^1A_2$ transitions in D_3, respectively. The higher energy, magnetically-forbidden, $^1A_{1g} \rightarrow {}^1T_{2g}$ transition in O_h expected between 300 and 400 nm would also split in D_3, the lower energy transition being the magnetically-allowed $^1A_1 \rightarrow {}^1E_b$ transition. Part of the intensity at 413 nm (24 200 cm^{-1}) in the CD spectrum of oxyCoHcy can be attributed to the $^1A_1 \rightarrow {}^1E_b$ transition in D_3. The $O_2^{2-} \rightarrow$ Co(III) LMCT bands in a doubly bridged Co(III) dimer are predicted to have opposite signs in the CD spectrum and small Kuhn anisotropy factors on the basis of comparison with a L-histidine, μ-1,2-peroxo, μ-hydroxo Co(III) dimer complex.[29] The CD bands due to $O_2^{2-} \rightarrow$ Co(III) LMCT are observed at 332 (30 100 cm^{-1}) and 413 nm (24 200 cm^{-1}), with opposite signs and small Kuhn anisotropy factors. The CD results are summarized in Table I.

MCD Spectroscopy. Figures 2 and 3 show the room-temperature MCD spectra of CoHcy before and after exposure to air, respectively. The intensity of the room-temperature MCD spectrum suggests that the observed bands arise from *C*-terms.[23] Figure 4 shows the low-temperature MCD spectrum of CoHcy. The large increase in intensity (Table II) confirms that we are looking at MCD intensity gained mostly through *C*-terms, the intensity of which is inversely proportional to temperature. Intense *C*-term MCD is expected to arise from the paramagnetic ground states of all the Co(II) species, aquoCoHcy, hydroxoCoHcy, and adventitiously bound Co(II), whereas no intense MCD is expected from the diamagnetic Co(III) in oxyCoHcy.

Spin-forbidden transitions are expected to show a large MCD intensity from four-coordinate Co(II).[27] The hydroxoCoHcy peak

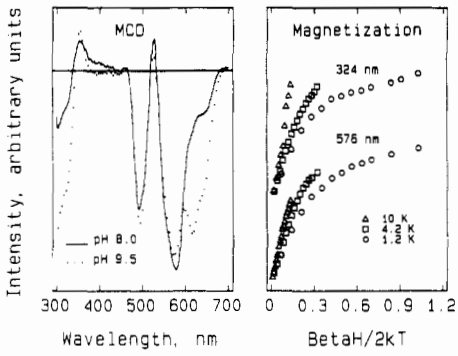

Figure 4. Left: Low-temperature (4.2 K) MCD spectra of CoHcy buffer/glycerol (50/50, v/v) at pH 8.0 and 9.5. (The pH 8.0 is estimated since the pH of Tris-buffered solutions increases with decreasing temperature. A 0.05 M pH 7.5 solution at 20 °C becomes pH 8 at 4 °C.) Right: Magnetization plots of the 324-nm peak in the pH 9.5 spectrum due to hydroxoCoHcy and the 576-nm peak in the pH 8.0 spectrum due to aquoCoHcy.

at 634 nm (15 800 cm^{-1}) in the CD spectrum that was assigned to the $^4A_2 \rightarrow {}^2A_2(G), {}^2E(G)$ spin-forbidden transitions is intensity enhanced in the MCD spectrum. At room temperature aquoCoHcy has MCD peaks at 592 (16 900 cm^{-1}), 556 (18 000 cm^{-1}, shoulder) and 526 nm (19 000 cm^{-1}). HydroxoCoHcy has room-temperature MCD peaks at 654 (15 300 cm^{-1}, broad), 599 (16 700 cm^{-1}), 556 (18 000 cm^{-1}, shoulder), 526 (19 000 cm^{-1}), 377 (26 500 cm^{-1}), and 321 nm (31 200 cm^{-1}); however, the peaks at 599, 556, and 526 nm probably contain intensity contributions from residual aquoCoHcy. At low temperature (4.2 K) the peaks sharpen and blue shift. The peak at 654 nm in hydroxoCoHcy splits into two peaks at 642 (15 600 cm^{-1}) and 616 nm (16 200 cm^{-1}), and the peak at 321 nm splits into two peaks at 324 (30 900 cm^{-1}) and 304 nm (32 900 cm^{-1}). The patterns of the MCD spectra in both aquoCoHcy and hydroxoCoHcy are indicative of four-coordinate Co(II) in a distorted T_d ligand field.[11,20a] The strong negative MCD peak at 492 nm and weak positive MCD peak at 465 nm are characteristic of six-coordinate Co(II) in a moderately strong ligand field,[20a] and these are assigned to adventitiously bound Co(II).

The patterns of the hydroxoCoHcy and aquoCoHcy MCD spectra closely resemble those of high-pH bovine CoCA and low-pH bovine CoCA, respectively, as indicated in Table II. This allows for confident assignment of the hydroxoCoHcy to a $Co^{II}(His)_3(OH)$ chromophore and the aquoCoHcy to a $Co^{II}(His)_3(H_2O)$ chromophore. It cannot be deduced from the peak positions in the absorption, CD, or MCD spectra whether or not the hydroxide ligand actually bridges the Co(II)'s in the active site of hydroxoCoHcy, as shown in Scheme I. However, a detailed temperature and field dependence of the MCD intensity hydroxoCoHcy peaks may provide insight into the bridging question. A plot of MCD peak intensity versus $\beta H/2kT$ at different fixed temperatures for the 576-nm peak in aquoCoHcy and the 324-nm peak in hydroxoCoHcy is shown in Figure 4. This is the expected behavior for isolated, but closely spaced Kramer's doublets.[23,31] What is interesting is that the variation for aquoCoHcy and hydroxoCoHcy are markedly different. This difference may be due to the differences in the *g* values, the zero field splitting energies, or magnetic coupling between the Co(II)'s through a bridging hydroxide in hydroxoCoHcy. The answer will have to await EPR studies and detailed temperature and field variation studies of the MCD spectra of CoHcy and model compounds.

Conclusions

The chemistry and spectroscopy of cobalt-substituted *L. polyphemus* support an active-site structure of oxyCoHcy which

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Table II. Summary of MCD Results for Active-Site Cobalt in CoHcy [ν in 10^{-3} cm^{-1} (nm, $\Delta\epsilon$ in M^{-1} cm^{-1} T^{-1})]

aquoCoHcy 298 K, pH 7.5	hydroxoCoHcy 298 K, pH 9.0	aquoCoHcy 4.2 K, pH 8.0	hydroxoCoHcy 4.2 K, pH 9.0	high-pH CoCA[a] 298 K, pH 9.0	low-pH CoCA[b] 298 K, pH 6.5
	15.3 (654, –0.5)		15.6 (642, –17)	15.6 (641, –2.0)	
			16.2 (616, –23)	16.3 (613, –1.9)	16.5 (606, –2)
16.9 (592, –1.5)	16.7 (599, –0.65)[c]	17.3 (576, –28)	17.5 (571, –26)[c]		
18.0 sh (556, –0.6)	18.0 sh (556, –0.2)[c]	18.1 sh (552, –20)	18.0 sh (556, –19)[c]	18.2 (549, –1.1)	18.2 (549, –0.8)
19.0 (526, +0.07)	19.0 (526, +0.02)[c]	19.0 (526, +4.0)	19.0 (526, +2.1)[c]	19.5 (513, +2.0)	19.0 (526, +weak)
	26.5 (377, +0.3)		28.2 (355, +5.6)	27.8 (360, –0.4)[b]	
	31.2 (321, –0.5)		30.9 (324, –16)	31.3 (319, +1)[b]	
			32.9 (304, –21)	>33.3 (<300, –)[b]	

[a] Cobalt(II)-substituted bovine carbonic anhydrase, taken from ref 26. [b] Cobalt(II)-substituted bovine carbonic anhydrase, estimated from a spectral figure in ref 26. [c] May be a mixture of aquoCoHcy and hydroxoCoHcy.

contains a μ-1,2-peroxo, μ-hydroxo dibridged Co(III) dimer. This structure is analogous to that originally proposed by Solomon as a "spectroscopically effective" active-site structure of the native oxyhemocyanin.

The CoHcy site is a mixture of aquoCoHcy and hydroxoCoHcy with the proportion controlled by the pH at the time of cobalt introduction. The hydroxoCoHcy high-pH form is the only form to oxygenate with a second-order rate constant of 500 M^{-1} s^{-1}. The absorption, CD, and MCD spectra of aquoCoHcy and hydroxoCoHcy clearly indicate a distorted four-coordinate Co(II) site. The spectra are best understood in terms of a ligand set consisting of three imidazole nitrogens and coordinated water in the case of aquoCoHcy or a coordinated hydroxide in the case of hydroxoCoHcy. The hydroxoCoHcy has additional distortion from T_d geometry as evidenced by intensity enhancement of spin-forbidden ligand field transitions. The spectral similarities of CoHcy to CoCA further support the spectral assignments and structural conclusions.

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