Contribution from the Department of Chemistry and Biochemistry, Middlebury College, Middlebury, Vermont 05753

# Cobalt(II)-Substituted *Limulus polyphemus* Hemocyanin: Cobalt Equilibria, Ligand Binding, and Oxygenation Chemistry

T. J. Dutton, T. F. Baumann, and J. A. Larrabee*

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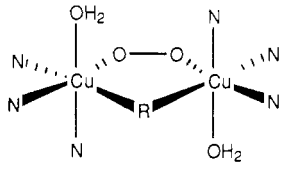
Co(II)-substituted *Limulus polyphemus* hemocyanin has been prepared and characterized. The apoprotein binds up to seven Co(II)'s per molecule; two of these are in the active site, and up to five are adventitiously bound in peripheral sites. The binding of Co(II) in the active site is cooperative with an equilibrium constant of $10^{9.34}$ $M^{-2}$. The adventitious site equilibrium constant is $10^{4.13}$ $M^{-1}$ for each of five independent binding sites. While a Co(II)-substituted hemocyanin containing only active-site cobalt was not prepared, the active-site cobalt is associated with intense visible absorption with a maximum at 566 nm ($\epsilon$ = 470 $M^{-1}$ $cm^{-1}$), and the adventitiously bound cobalts contribute insignificantly to any visible absorption. The optical spectrum of the active-site Co(II) is consistent with each Co(II) bound to three imidazole nitrogens and a fourth, exchangeable ligand, in approximate $C_{3v}$ symmetry. The active-site Co(II)'s bind a single hydroxide, chloride, or azide, bridging between the cobalts. Co(II)-substituted hemocyanin reversibly binds dioxygen, generating peroxide-to-cobalt charge-transfer transitions at 319 and 404 nm. The preliminary kinetics of oxygenation of Co(II)-substituted hemocyanin show that the rate is dependent upon [$OH^-$] to the first order. The chemistry, kinetics, and spectroscopy of the oxygenated derivative are very similar to $\mu$-peroxo, $\mu$-hydroxo dibridged Co(III) complexes. The evidence from the study of Co(II)-substituted *L. polyphemus* hemocyanin supports the hypothesis that native oxyhemocyanin has a $\mu$-hydroxo endogenous bridging ligand.

## Introduction

Hemocyanins are the oxygen transport proteins in arthropoda and mollusca.[1] The active sites of the oxyprotein contain antiferromagnetically coupled binuclear Cu(II). 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] have defined a "spectroscopically effective" oxyhemocyanin active-site structure, I. Each Cu(II)




I

is six-coordinate with three imidazole ligands from histidine residues, a water ligand, a bridging peroxide, and another bridging ligand, "R". The R bridging ligand is necessitated by the observation that methemocyanin is 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.[5] 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.[6] Finally there is an absorption peak at 425 nm in the spectra of oxy and methemocyanins, which has been associated with the endogenous bridging ligand to Cu(II) charge transfer.[1b]

In addition to the identity of the endogenous bridging ligand, other questions remain concerning the active-site structure and chemistry of hemocyanin. There is a body of evidence that suggests that the coppers within a binuclear binding site are not equivalent. In molluscan hemocyanin one copper from the active site can be selectively removed to form the half-apo derivative;[7] in both molluscan and arthropodal hemocyanins the mixed-valent half-met derivative can be prepared,[1,3] and finally, carbon monoxide binds end-on to only one copper in deoxyhemocyanin.[8] On the other hand, both coppers are simultaneously removed from arthopodal hemocyanins;[7] most of the spectral properties of hemocyanin can be interpreted on the basis of copper equivalency or near equivalency, and the X-ray crystal structure of deoxyhemocyanin shows that each Cu(I) is bound to three imidazole nitrogens in a near-equivalent but antiprismatic orientation.[6] The extent of site inequivalency or flexibility can be further probed by substitution of other metals.

Metal substitution in active sites of metalloproteins has been successfully used to study a variety of systems.[9-11] Metal substitution is often used to replace a "spectroscopically silent" metal such as Zn(II) with one conducive to magnetic and optical spectroscopic studies.[12,13] 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.[14,15] It is expected that a detailed spectroscopic

(1) (a) Solomon, E. I. *Copper Proteins*; Spiro, T. G., Ed.; Wiley: New York, 1981; pp 41-149. (b) Solomon, E. I.; Penfield, K. W.; Wilcox, D. E. *Struct. Bonding* **1983**, *53*, 1-57.
(2) (a) Himmelwright, R. S.; Eickman, N. C.; Solomon, E. I. *J. Am. Chem. Soc.* **1979**, *101*, 1576-1586. (b) Himmelwright, R. S.; Eickman, N. C.; LuBien, C. D.; Solomon, E. I. *J. Am. Chem. Soc.* **1980**, *102*, 5378-5388.
(3) Pavlosky, M. A.; Larrabee, J. A. *J. Am. Chem. Soc.* **1988**, *110*, 5349-5353.
(4) Woolery, G. L.; Winkler, M.; Solomon, E. I.; Spiro, T. G. *J. Am. Chem. Soc.* **1984**, *106*, 86-92.
(5) Wilcox, D. E.; Long, J. R.; Solomon, E. I. *J. Am. Chem. Soc.* **1984**, *106*, 2186-2194.
(6) Gaykema, W. P. J.; Volbeda, A.; Hol, W. G. J. *J. Mol. Biol.* **1985**, *187*, 255-275.
(7) Himmelwright, R. S.; Eickman, N. C.; Solomon, E. I. *Biochem. Biophys. Res. Common.* **1978**, *81*, 243-247.
(8) (a) van der Deen, H.; Hoving, H. *Biophys. Chem.* **1979**, *9*, 169-179. (b) Fager, L. Y.; Alben, J. O. *Biochemistry* **1972**, *11*, 4786-4792.
(9) Ruegg, C.; Lerch, K. *Biochemistry* **1981**, *20*, 1256-1262.
(10) Bertini, I.; Luchinat, C.; Scozzafava, A. *Struct. Bonding* **1982**, *48*, 45-92.
(11) Pantoliano, M. W.; Valentine, J. S.; Nafie, L. A. *J. Am. Chem. Soc.* **1982**, *104*, 6310-6317.
(12) Vallee, B. L.; Holmquist, B. *Methods for Determining Metal Ion Environments in Proteins*; Darnell, D. W., Wilkins, R. G., Eds.; Elsevier: New York, 1980; pp 22-74.
(13) Lever, A. B. P. *Inorganic Electronic Spectroscopy*, 2nd ed.; Elsevier: New York, 1984; pp 720-726.
(14) Gray, H. B.; Solomon, E. I. *Copper Proteins*; Spiro, T. G., Ed.; Wiley: New York, 1981; pp 1-39.
(15) Tennent, D. L.; McMillin, D. R. *J. Am. Chem. Soc.* **1979**, *101*, 2307-2311.

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study of Co(II)-substituted hemocyanins, the copper-containing oxygen transport proteins, would provide additional insight into the active-site geometry and chemistry. Two earlier reports of Co(II)-substituted *Limulus polyphemus* hemocyanin[16,17] (subsequently referred to as CoHcy) disagree in details of preparation, chemistry, and spectroscopy, confusing rather than clarifying. Reports of cobalt substitution in hemocyanin from other species[18] imply that there may be much more diversity in the way different hemocyanins bind cobalt in comparison to how they bind copper.

Suzuki et al.[16] report that *L. polyphemus* CoHcy contains exactly 2 Co atoms per active site having distorted tetrahedral ligand environments and an intense absorbance between 500 and 600 nm with a molar absorptivity per cobalt of approximately 200 $M^{-1}$ $cm^{-1}$; they do not mention additional, adventitiously bound cobalt. Lorosch and Haase[17] report that *L. polyphemus* CoHcy binds up to 2 Co(II)'s per active site and an additional 4.5 Co(II)'s in adventitious binding sites; each active site Co(II) is reported to have a molar absorptivity of 400 $M^{-1}$ $cm^{-1}$ at 548 nm, consistent with distorted tetrahedral geometry. Both papers report that exogenous ligands such as azide and chloride do not bind to *L. polyphemus* CoHcy, despite the reported ligand binding chemistry of CoHcy from other species.[18] Both papers report that CoHcy reacts with 10-fold excess amounts of cyanide, which oxidizes the Co(II) to Co(III). Lorosch and Haase report that a band at 380 nm accompanies reaction with cyanide, analogous to the reaction of cyanide with Co(II) tyrosinase.[9] Both papers report on aerobic aging effect on the CoHcy, which results in increased absorbance in the near-UV region, and both agree that this is not due to dioxygen binding. Lorosch and Haase claim that the aging is due to protein aggregation, which causes increased light scattering (proportional to $1/\lambda^4$); Suzuki et al. claim that the Co(II)'s are not binding oxygen but change coordination number from 4 to 5 or 6 in aged CoHcy.

We wanted to use Co(II)-substituted hemocyanins to expand upon our infrared spectroscopic studies of exogenous ligands bound to the coppers in the active sites of native hemocyanins;[3] however, we thought it necessary to first understand the differences among the previous reports[16-18] and to learn to consistently prepare well-characterized Co(II)-substituted hemocyanins. We wish to report our results on the reaction of Co(II) with apo *L. polyphemus* hemocyanin. These results show that there are at least two types of Co(II) binding sites: the active site and adventitious sites. Our studies on the effect of Co(II) concentration, other divalent cations, and pH resolve many of the discrepancies between the previous reports. Our evidence shows that Co(II)-substituted *L. polyphemus* hemocyanin binds exogenous ligands such as $Cl^-$ and $N_3^-$, exists with binuclear Co(II) in the active site with a bridging hydroxide ligand, and will *reversibly bind dioxygen.*

## 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 20000 × g to remove cells and debris. The supernatant was dialyzed versus 0.05 M Tris/$SO_4^{2-}$ buffer, pH 8.0. Sulfate was chosen as the buffer anion because chloride was suspected to bind to the cobalt in CoHcy's[18] (vide infra). The hemocyanin was then passed through a column packed with sterile Bio-Gel P-6DG (Bio-Rad, Richmond, CA) that was equilibrated with Tris/$SO_4^{2-}$ buffer; this step removed most of the bacteria present in the hemolymph and increases storage stability.[19]

Stripped hemocyanin, in which all the divalent cations are removed, was prepared by dialysis against 0.02 M EDTA in 0.05 M Tris/$SO_4^{2-}$ buffer,[17] 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 (vide infra).

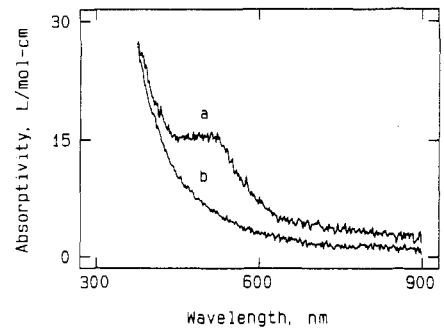



**Figure 1.** UV/vis absorption spectra of *L. polyphemus* deoxyhemocyanin: (a) Co(II) treated; (b) native.

Apohemocyanin was prepared by dialysis of oxyhemocyanin against 0.1 M KCN in pH 8.0 Tris/$SO_4^{2-}$ buffer until >98% of the copper was removed;[20] the apohemocyanin was then exhaustively dialyzed against pure buffer. The apohemocyanin was then stripped as described above.

CoHcy was prepared by anaerobic dialysis (continuous Ar sparging) of 10 mL of 0.4–0.7 mM apohemocyanin against 1 L of Tris/$SO_4^{2-}$ buffer containing between 0.01 and 0.5 mM Co(II), added as solid $CoSO_4 \cdot 7H_2O$. For the relative rate of Co(II) introduction experiments, the 10 mL of protein solution was split into five 2-mL dialysis bags, which were removed throughout the experiment. All Co(II) introduction experiments were conducted by using SPECTRA/POR dialysis tubing of 6.4-mm diameter with a molecular weight cutoff of 6000–8000 (Spectrum Medical Industries, Inc., Los Angeles, CA). CoHcy preparations were not dialyzed or treated in any way to remove excess Co(II). The dialysate from each preparation was analyzed for total cobalt, and the total cobalt of the protein solution was corrected with this value to obtain total protein-bound cobalt.

Reconstitution of hemocyanin with copper was accomplished by anaerobic incubation with a 2-fold molar excess of $[Cu(CH_3CN)_4]ClO_4$ in 0.1 M Tris/HCl buffer, pH 7.0.[20]

The effect of $Cl^-$ and $N_3^-$ on the spectrum of CoHcy was studied by preparation of the cobalt derivative with varying concentrations of the corresponding anion. This procedure was necessary, as opposed to direct addition of small volumes of concentrated anion solution, because the CoHcy was easily precipitated with rapid ionic strength changes. CoHcy became more sensitive to precipitation when it was close to cobalt saturation (7 Co's/molecule of protein).

Protein concentration was determined by absorbance at 280 nm ($\epsilon$ = 82400 $M^{-1}$ $cm^{-1}$);[21] concentration of copper in the active site of oxyhemocyanin was determined by absorbance at 342 nm ($\epsilon$ = 20000 $M^{-1}$ $cm^{-1}$).[16] Total copper and cobalt were determined by atomic absorption spectrometry (AA) with a Perkin-Elmer Model 460 AA spectrophotometer. Absorption spectra were recorded on a Cary 17 UV/vis/near-IR spectrometer that was interfaced to a computer using OLIS version 9.01 UV/vis/near-IR software (On-Line Instrument Systems, Jefferson, GA). All sample preparation and analysis was conducted at 4 °C in either an argon or nitrogen atmosphere, unless otherwise stated.

## Results

**Preparation of CoHcy.** The rate of Co(II) introduction was sensitive to concentration of other divalent cations, pH, ratio of dialysate volume to apoprotein volume, and the diameter and type of dialysis tubing used. For studies of relative rates of cobalt introduction, the physical and chemical experimental set-up had to be carefully reproduced. Attempts at titrating solutions of apohemocyanin with concentrated Co(II) solutions, as was done for the preparation of Co(II)-substituted tyrosinase,[9] resulted in precipitation of protein. Furthermore, with our dialysis conditions, concentrations of Co(II) greater than 0.2 mM resulted in protein precipitation before equilibrium was reached. This protein precipitation was previously reported and could be reversed by dialysis against chelating agents such as EDTA or treatment with ion-exchange resins such as Bio-Rad Chelex 100.[16] The effect of chelating agents on CoHcy was not studied in detail, but it was noted that such treatment was found to remove some of the adventitious as well as active-site cobalt.[17]

(16) Suzuki, S.; Kino, J.; Nakahara, A. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 212–217.
(17) Lorosch, J.; Haase, W. *Biochemistry* **1986**, *25*, 5850–5857.
(18) (a) Salvato, B.; Beltramini, M.; Piazzesi, A.; Alviggi, M.; Ricchelli, F.; Magliozzo, R. S.; Peisach, J. *Inorg. Chim. Acta* **1986**, *125*, 55–62. (b) Suzuki, S.; Kino, J.; Kimura, M.; Mori, W.; Nakahara, A. *Inorg. Chim. Acta* **1982**, *66*, 41–47.
(19) DePhillips, H. A., Trinity College, Hartford, CT. Personal communication.
(20) Konings, W. N.; van Driel, R.; van Bruggen, E. F. J.; Gruber, M. *Biochim. Biophys. Acta* **1969**, *194*, 55–66.
(21) Nickerson, K. W.; van Holde, K. E. *Comp. Biochem. Physiol., B: Comp. Biochem.* **1971**, *39B*, 855–872.

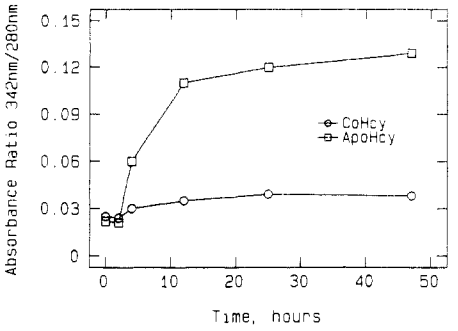



**Figure 2.** Rate of reconstitution of *L. polyphemus* hemocyanin with $[Cu(CH_3CN)_4]ClO_4$, apohemocyanin versus CoHcy, monitored by absorbance at 342 nm.

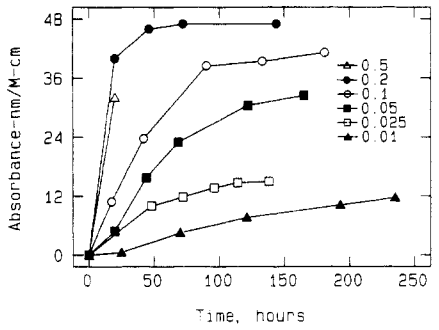



**Figure 3.** Rate of cobalt uptake by *L. polyphemus* apohemocyanin at pH 8.0, [Co(II)] from 0.01 to 0.5 mM in the dialysate, monitored by integrated visible absorbance. Note that the 0.5 mM Co(II) experiment resulted in protein precipitation and was stopped early.

Treatment of apohemocyanin with Co(II) between pH 7 and 8 produced CoHcy that had a multipeak absorption in the visible region with a maximum at 566 nm, which was consistent with all the previous reports on both *L. polyphemus* and other species of CoHcy.[16-18] These absorptions had been assigned to d–d transitions from Co(II) in a distorted tetrahedral ligand field. Comparison of absorbance at 566 nm with total cobalt showed that there was not a linear relationship, indicating at least two different types of binding site; therefore, it had to be proved that the visible absorption was due to active-site Co(II) and not adventitiously bound cobalt.

Treatment of deoxyhemocyanin with Co(II) resulted in a material containing cobalt as measured by AA but having only a very weak visible absorption (Figure 1) centered at 520 nm ($\epsilon$ = 16 $M^{-1}$ $cm^{-1}$/cobalt). Identical treatment of apohemocyanin resulted in CoHcy having more total cobalt as measured by AA but always less than two additional cobalts per molecule of protein. The difference in total cobalt of deoxyhemocyanin with added Co(II) and CoHcy is attributable to the intense visible absorption centered at 566 nm and yields a molar absorptivity of 470 $M^{-1}$ $cm^{-1}$/active site.

Apohemocyanin can be reconstituted with Cu(I) compounds such as $[Cu(CH_3CN)_4]ClO_4$;[20] the rate of reconstitution is monitored by absorption at 342 nm in the oxygenated protein. The rate of copper reconstitution into apohemocyanin versus CoHcy is compared in Figure 2; the presence of Co(II) inhibits the return of copper to the active site.

CoHcy was prepared at levels of Co(II) in the dialysate ranging from 0.01 to 0.5 mM at pH 8.0. The dialysis was allowed to continue until no further increase in total cobalt or in absorbance at 566 nm was observed. The 0.5 mM Co(II) experiment was stopped before equilibrium since protein precipitation occurred. The results are shown in Figures 3 and 4. Since there was a small increase in absorbance at 566 nm due to light scattering as the total cobalt concentration increased (due to protein aggregation), integrated absorbance was used with a valley-to-valley base line drawn from 450 to 713 nm. Extrapolation of the integrated visible absorbance at equilibrium to infinite Co(II) concentration yields an integrated molar absorptivity of 47.4 absorbance nm/(mM cm).

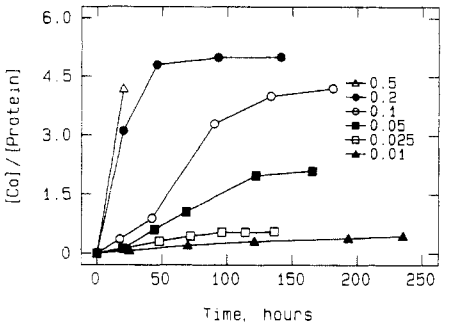



**Figure 4.** Rate of cobalt uptake by *L. polyphemus* apohemocyanin at pH 8.0, [Co(II)] from 0.01 to 0.5 mM in the dialysate, monitored by AA and corrected for active-site Co(II). Note that 0.5 mM Co(II) resulted in protein precipitation and was stopped early.

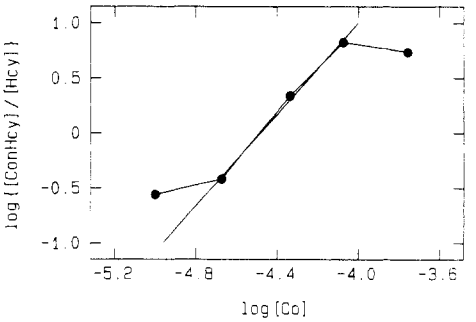



**Figure 5.** Hill plot of the data from Figure 3. The linear portion of the curve has a slope of 2.08 and an intercept of 9.34.

The area absorptivity and molar absorptivity of 470 $M^{-1}$ $cm^{-1}$ correlate very well for CoHcy prepared at low Co(II) concentrations.

Hill plot analysis[22] of the data in Figure 3 based on the following scheme of reactions

$$n\text{Co(II)} + \text{Hcy} = \text{Co}_n\text{Hcy}$$

$$K = \frac{[\text{Co}_n\text{Hcy}]}{[\text{Co(II)}]^n[\text{Hcy}]}$$

$$\log \frac{[\text{Co}_n\text{Hcy}]}{[\text{Hcy}]} = n \log [\text{Co(II)}] + \log K$$

where [Hcy] is apohemocyanin concentration, is shown in Figure 5. A clear sigmoidal shaped curve is obtained, and the central, linear portion has a slope $n$ = 2.08 and an intercept of 9.34, corresponding to $K = 10^{9.34}$ $M^{-2}$. This indicates that Co(II) binds cooperatively in the active site with a stoichiometry of two cobalts per active site. Since the binding of Co(II) is cooperative, CoHcy that is less than saturated in cobalt consists of active sites with two cobalts or no cobalts.

The total cobalt data in Figure 4, corrected for the amount of cobalt in the active site, gives an estimate of the amount of cobalt binding to the adventitious sites. Scatchard plot analysis[22] of this data gives a best fit for five independent cobalt binding sites, each with an equilibrium constant of $10^{4.13}$ $M^{-1}$. The fit was not as good as for the active-site (absorbance) data since the range of Co(II) concentrations above 0.2 mM could not be investigated (protein precipitation). Assuming two Co(II)'s per active site with $K = 10^{9.34}$ $M^{-2}$ and five adventitious Co(II) binding sites, each with $K = 10^{4.13}$ $M^{-1}$, the ratio of active-site cobalt to adventitiously bound cobalt can be calculated as a function of Co(II) concentration in the dialysate. The trend in the calculated values compares well with the trend in the observed values, as shown in Table I. To maximize the ratio of active-site cobalt to adventitiously bound cobalt at pH 8.0, a dialysate containing between 0.025 and 0.05 mM Co(II) is ideal.

(22) Marshall, A. G. *Biophysical Chemistry: Principles, Techniques, and Applications*; Wiley: New York, 1978; pp 70–84.

**Table I.** Ratio of Active-Site Co(II) to Adventitious Co(II) in *L. polyphemus* Hemocyanin

| equil [Co(II)], mM | active-site Co(II)/adventitious Co(II) | |
|---|---|---|
| | obsd | calcd[a] |
| 0.010 | 0.37 | 1.1 |
| 0.025 | 0.68 | 1.2 |
| 0.050 | 0.75 | 0.48 |
| 0.10 | 0.64 | 0.38 |
| 0.20 | 0.54 | 0.32 |

[a] Calculated by assuming $K = 10^{9.34}$ $M^{-2}$ and $n = 2$ for active-site Co(II) and $K = 10^{4.13}$ $M^{-1}$ and five independent binding sites for adventitious Co(II).

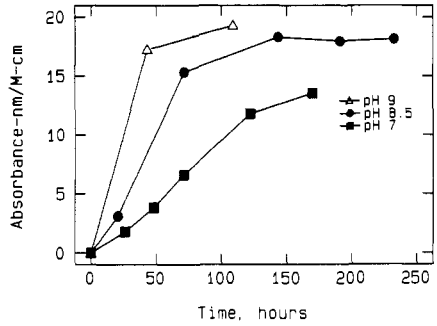



**Figure 6.** Relative rate of cobalt uptake by *L. polyphemus* apohemocyanin as a function of pH as measured by integrated visible absorbance. [Co(II)] was 0.025 mM in the dialysate.

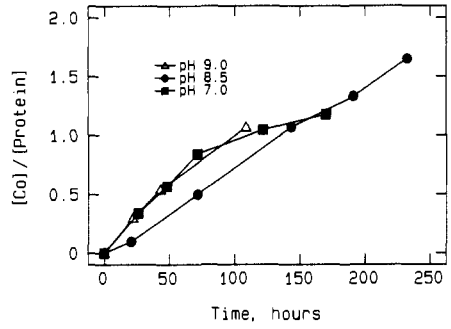



**Figure 7.** Relative rate of cobalt uptake by *L. polyphemus* apohemocyanin as a function of pH, measured by AA and corrected for Co(II) in the active site. [Co(II)] was 0.025 mM in the dialysate.

Figure 6 shows the relative rate of Co(II) uptake by apohemocyanin, measured by integrated absorbance, as a function of pH. Figure 7 shows total cobalt, corrected for active-site cobalt, for the same samples. There is a marked pH dependence on the uptake of active-site Co(II), increasing with pH. There is no significant pH dependence on the uptake of adventitious Co(II) between pH 7 and pH 9. The effective $pK_a$'s of the adventitious Co(II) ligands must be less than 7, and the effective $pK_a$'s of the active-site Co(II) ligands must be less than 9.

The rate of uptake of Co(II) by apohemocyanin was slowed by the presence of either Ca(II) or Mg(II). As an example, 0.025 M Mg(II) in a dialysate containing 0.05 mM Co(II) reduced the amount of total cobalt uptake by the protein by over half. Divalent cations affected the adventitious binding of cobalt as well as the active-site binding, although the adventitious binding was more sensitive to the presence of other divalent cations. It is well-known that divalent cations such as Mg(II) and Ca(II) bind to hemocyanins and cause protein aggregation;[23] it is likely that at least some of the adventitious binding sites for Co(II), Mg(II), and Ca(II) are the same. It is not surprising that Mg(II) or Ca(II) competes with Co(II) for active-site binding sites, since it is known that complete removal of copper by cyanide complexation from molluscan hemocyanins requires these cations.[21]

(23) Witters, R.; Lontie, R. *Physiology and Biochemistry of Hemocyanins*; Ghiretti, F., Ed.; Academic: New York, 1968; pp 61-73.

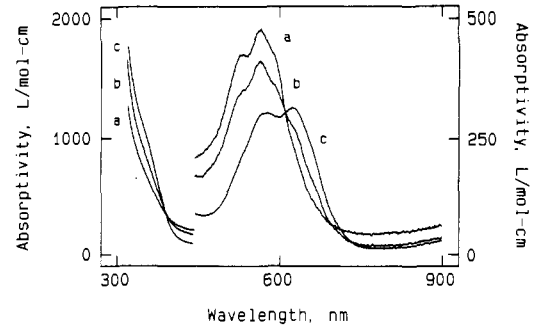



**Figure 8.** UV/vis absorption spectra of CoHcy: (a) pH 7.26; (b) pH 8.66; (c) pH 10.0.

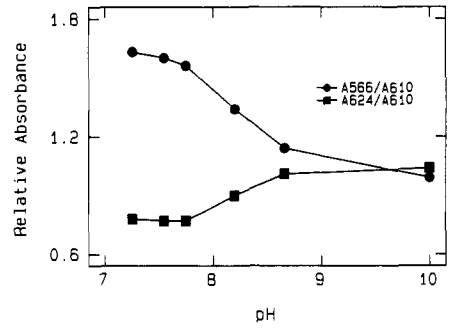



**Figure 9.** Absorbance ratios in the absorption spectrum of CoHcy versus pH. At 610 nm is an isosbestic point, 566 nm is the peak maximum for CoHcy/$H_2O$, and 624 nm is the peak maximum for CoHcy/$OH^-$.

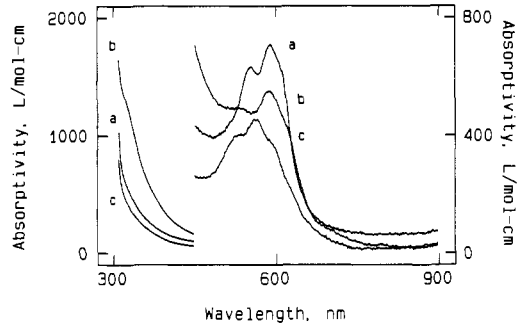



**Figure 10.** UV/vis absorption spectra of CoHcy in 0.05 M Tris/$SO_4^{2-}$ buffer, pH 7.5: (a) buffer plus 0.1 M $Cl^-$; (b) buffer plus 0.2 M $N_3^-$; (c) pure buffer.

**Spectroscopy of CoHcy.** The absorption spectrum of CoHcy was dependent on pH. There was little change between pH 7 and pH 8, but a distinct red-shift of the visible peaks and a new near-UV peak at 360 nm developed at higher pH's, as shown in Figure 8. At neutral pH, the visible maximum was found at 566 nm; at pH 10 the visible maximum was observed at 624 nm. Since the entire spectrum shifted, and there were two isosbestic points at 387 and 610 nm, both Co(II)'s in the active site had to be involved with binding hydroxide or some deprotonatable group. Hill plot analysis[22] of the spectral data from pH 7 to pH 10 gave a slope of 0.72 (rounded to closest integer, $n = 1$) with an intercept of 3.76 ($K = 10^{3.76}$ $M^{-1}$). A plot of the absorbance at 566 nm relative to 610 nm (isosbestic point) and 624 nm relative to 610 nm versus pH is shown in Figure 9. These spectrophotometric titration curves clearly show the pH at neutralization to be 8.7; therefore, the apparent $pK_a$ of the deprotonatable bridging group must be less than 8.7.

Figure 10 shows the UV/vis absorption spectra of CoHcy at pH 7.5 in Tris/$SO_4^{2-}$ buffer, buffer plus 0.1 M $Cl^-$, and buffer plus 0.1 M $N_3^-$. Hill plot analysis[21] of the chloride data from 0 to 0.5 M $Cl^-$ and the azide data from 0 to 0.2 M $N_3^-$ yields a reaction stoichiometry of one $Cl^-$ or one $N_3^-$ per two active-site Co(II)'s with equilibrium constants of $10^{1.63}$ $M^{-1}$ and $10^{2.32}$ $M^{-1}$

**Table II.** UV/Vis/Near-IR Absorption Data for CoHcy in $cm^{-1} \times 10^{-3}$ ($\epsilon$ in $M^{-1}$ $cm^{-1}$)

| CoHcy/$H_2O$ | CoHcy/$Cl^-$ | CoHcy/$N_3^-$ | CoHcy/$OH^-$ | calcd[a] | assgnt[b] |
|---|---|---|---|---|---|
| | | 31.0 (1300) | 27.7 (900) | see text | LMCT |
| 19.0 (450) | 18.1 (616) | 18.7 (410) | 17.5 (290) | 19.4 | $^4A_2 \rightarrow {}^4A_2(P)$ |
| 17.7 (470) | 16.9 (709) | 17.1 (493) | 16.0 (303) | 18.1 | $^4A_2 \rightarrow {}^4E(P)$ |
| 16.9 (390) | 16.3 (620) | 16.1 (339) | 15.2 (212) | | |
| 10.1 (weak) | 9.7 (weak) | | 10.1 (weak) | | |
| | | 9.9 (62) | | 9.9 | $^4A_2 \rightarrow {}^4E(F)$ |
| 8.6 (50) | 8.5 (74) | | 8.4 (50) | | |
| | | | | 5.7 | $^4A_2 \rightarrow {}^4A_2(F)$ |

[a] Assuming $C_{3v}$ symmetry $CoN_3L$, AOM approximation with $e_\sigma(N) = 4035$ $cm^{-1}$ and $e_\sigma(L) = 3312$ $cm^{-1}$. [b] Assignment in $C_{3v}$.

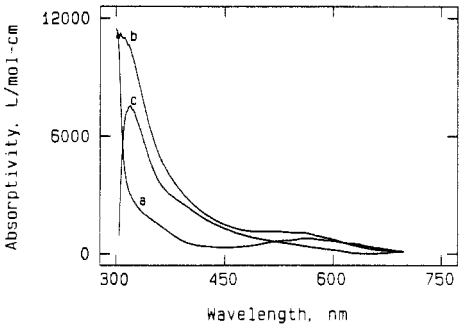



**Figure 11.** UV/vis absorption spectra of CoHcy in 0.05 M Tris/$SO_4^{2-}$ buffer, pH 8.66: (a) Ar protected; (b) exposed to air overnight; (c) difference spectrum.

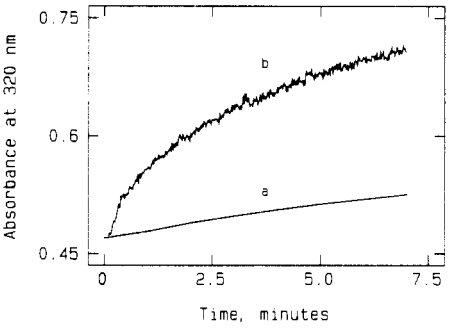



**Figure 12.** Increase in absorbance at 320 nm versus time after rapid mixing of CoHcy with $O_2$-saturated buffer: (a) pH 8.13; (b) pH 9.40. (Note that the apparent signal/noise difference is due to a much higher data collection rate for the pH 9.40 experiment versus the pH 8.13 experiment.)

for chloride and azide, respectively.

The near-IR absorption spectrum of CoHcy is also dependent on pH, [$Cl^-$], and [$N_3^-$]. The spectral data are summarized in Table II.

**Oxygenation of CoHcy.** A solution of CoHcy, pH 7.5, that was exposed to air and let stand at 4 °C over a period of several days turned from pink-violet to red-brown. At pH 9, exposure to air rapidly turned a solution of CoHcy to red-brown. At pH 10, exposure to air virtually instantaneously changed the CoHcy to red-brown. Both previous reports on *L. polyphemus* CoHcy[16,17] noted a red-brown color change with aging but discounted a possible reaction with dioxygen. It is significant that in both of the previous reports the CoHcy was prepared in Tris/$Cl^-$ buffer in the pH 7–8 range, and since chloride competes with hydroxide (or other deprotonatable group) for a bridging position between Co(II)'s, reactions of CoHcy in chloride with oxygen are less obvious and slower. In the presence of 50 mM $Cl^-$ the reaction of CoHcy with air is very slow at pH's below 8.0. At pH's above 8.5, 50 mM $Cl^-$ does not significantly inhibit the rate of oxygenation of CoHcy (vide infra).

Figure 11 shows the UV/vis absorption spectra of a CoHcy sample at pH 8.2 protected under Ar, the same sample after exposure to air overnight, and the difference spectrum. The integrated absorbance in the visible region decreases with air exposure, and a peak at 319 nm ($\epsilon$ = 7400 $M^{-1}$ $cm^{-1}$) with a shoulder at 404 nm ($\epsilon$ = 2100 $M^{-1}$ $cm^{-1}$) appears in the difference spectrum. Estimates of the molar absorptivities of the peaks at 319 and 404 nm are based upon the amount of decreased integrated intensity in the visible region upon air exposure. The positions and intensities of these absorption bands are remarkably similar to those of peroxide-bridged Co(III) dimers,[24] particularly the $\mu$-$OH^-$, $\mu$-$O_2^{2-}$ Co(III) dimers. Figure 12 shows the increase in absorbance at 320 nm versus time after $O_2$-saturated buffer is rapidly mixed with Ar-saturated CoHcy at pH 8.13 and at pH 9.40. Analysis of the initial rates of these reactions shows an approximate first-order dependence on [$OH^-$] of the dioxygen uptake.[25]

The increased rate of formation of the dioxygen adduct of CoHcy with increasing pH implies that the very stable ($\mu$-peroxo)($\mu$-hydroxo)dicobalt(III) species is forming.[26] The oxygen from these complexes usually cannot be removed by reduced pressure or by purging with nitrogen or argon; rather, the oxygen is removed by treatment with acid (pH < 4), treatment with a metal complexing agent such as EDTA, or treatment with an oxygen scavenger such as dithionite. The near-UV bands at 319 and 404 nm did not go away if oxyCoHcy was exhaustively dialyzed versus Ar-saturated buffer at pH 7, nor was oxygen removed by repeated evacuations/equilibrations with Ar. When oxyCoHcy was dialyzed versus buffer containing 1 mM dithionite, under Ar, the bands at 319 and 404 nm quickly disappeared. When the dithionite was removed by dialysis against pure buffer, the CoHcy could be reoxygenated with air or oxygen to yield absorbance peaks at 319 and 404 nm.

When CoHcy is exposed to oxygen, the peaks at 319 and 404 nm do not arise from the oxygenation of adventitiously bound cobalt. Methemocyanin, which has oxidized copper in the active site, cannot bind oxygen, and does not have any intense charge-transfer bands in the visible or near-UV region, can be treated with Co(II). The Co(II)-treated methemocyanin has the same weak absorption at 520 nm, due to the adventitiously bound cobalt, observed in Co(II)-treated deoxyhemocyanin. Exposure of Co(II)-treated methemocyanin to oxygen results in no increase in absorbance at 319 or 404 nm.

## Discussion

**Preparation of CoHcy.** Our results clearly show that given the correct combination of pH, Co(II), and time, solutions of CoHcy that contain 1 mol of Co/mol of protein, 2 mol of Co/mol of protein, or any combination up to 7 mol of Co/mol of protein could be prepared. The cobalt, however, would be distributed between the active site and adventitious (peripheral) sites in any of these preparations. Recognition of two distinct types of binding sites and the fact that other divalent cations compete with Co(II) for

(24) (a) Lever, A. B. P.; Gray, H. B. *Acc. Chem. Res.* **1978**, *11*, 348–355. (b) Lever, A. B. P. *Inorganic Electronic Spectroscopy*; Elsevier: New York, 1984; pp 285–296.

(25) Baumann, T. F.; Larrabee, J. A. Unpublished data.

(26) (a) Tanford, C.; Kirk, D. C.; Chantooni, M. K. *J. Am. Chem. Soc.* **1954**, *76*, 5325–5332. (b) McCormick, B. J.; Gorin, G. *Inorg. Chem.* **1962**, *1*, 691–696. (c) Michailidis, M. S.; Martin, R. B. *J. Am. Chem. Soc.* **1969**, *91*, 4683–4689. (d) Morris, P. J.; Martin, R. B. *J. Am. Chem. Soc.* **1970**, *92*, 1543–1546. (e) Wilkins, R. G. *Adv. Chem. Ser.* **1971**, *No. 100*, 111–134. (f) Fallab, S.; Mitchell, P. R. *Advances in Inorganic and Bioinorganic Mechanisms*; Academic: New York, 1984; pp 311–377.

both types of sites account for most of the previously reported discrepancies in CoHcy preparations.[16-18] We suspect that the molar absorptivities previously reported disagree with ours because our CoHcy was prepared in Tris/$SO_4^{2-}$ buffer, whereas the others were prepared in Tris/$Cl^-$ buffer. CoHcy has one exchangeable ligand binding site for hydroxide, chloride, and azide. These anions cause shifts in the position and in the intensity of the visible absorption spectrum. Lorosch and Haase reported[17] a molar absorptivity of 800 $M^{-1}$ $cm^{-1}$ for the Co(II) in the active site of their CoHcy preparations, but since their CoHcy was prepared in 0.05 M Tris/$Cl^-$ buffer, it was probably in the chloride-bound form. Thus, our result of 709 $M^{-1}$ $cm^{-1}$ for our CoHcy/$Cl^-$ preparation (Table II) agrees reasonably well. Additionally, the previous reports did not mention the extent to which the CoHcy was protected from air after preparation; any oxygenation of the CoHcy would have affected the calculated molar absorptivities.

The absence of intense visible absorption when deoxyhemocyanin is treated with Co(II), the difference in total cobalt content between CoHcy and cobalt-treated deoxyhemocyanin, the inhibition of copper reintroduction to the active site of CoHcy, and the cooperative binding stoichiometry of two Co(II)'s all point to an active site in CoHcy that binds two Co(II)'s. Virtually all of the visible absorption intensity in CoHcy is due to active-site Co(II). These results are in complete agreement with the previous reports of *L. polyphemus* CoHcy.[16-18] The cooperativity and pH dependence of the active-site Co(II) binding can be rationalized as follows: the first Co(II) enters the active site, binds to three imidazole nitrogens, and picks up a fourth ligand of water or hydroxide; the second Co(II) can now bind with a more favorable equilibrium constant because it has three imidazole ligands plus a fourth ligand of either a bridging hydroxide or deprotonatable group.

The apparent $pK_a$ of the bridging ligand is less than 8.7 for CoHcy. This contrasts with the apparent $pK_a$ of less than 4.5 determined for the endogenous bridging ligand by EPR studies of a fraction of methemocyanin that is EPR detectable.[5] On the basis of charge-to-size-ratio arguments, Cu(II) could lower the apparent $pK_a$ of a ligand by more than 2 orders of magnitude compared to Co(II).[27] In a study of dinuclear macrocyclic complexes of Co(II) and Cu(II),[28] it was found that Cu(II) could form stable $\mu$-$OH^-$ complexes at pH's as low as 6.5, whereas the Co(II) could not form the identical $\mu$-$OH^-$ complex even at high pH's unless additional stabilizing ligands were present. It is reasonable that, for identical bridging ligands, the apparent $pK_a$ for Cu(II) is less than 4.5 while the apparent $pK_a$ for Co(II) is less than 8.7. The apparent $pK_a$ of the bridging ligand is the difference between the intrinsic $pK_a$ and log $K$ ($K$ is the equilibrium constant of the reaction between CoHcy and $OH^-$; see note 27 in ref 5). This allows the intrinsic $pK_a$ of the bridging ligand to be estimated as $\leq 12.5$, plausibly in the range of that of tyrosine ($pK_a$ = 10.7) or water ($pK_a$ = 15.7)[5] and consistent with the intrinsic $pK_a > 7$ determined by the EPR study.[5] Since X-ray diffraction studies[6] have ruled out the presence of a large amino acid side group such as a phenolate (from tyrosine) as a possible endogenous bridge, we conclude that the bridging ligand, in base, is in fact $\mu$-$OH^-$.

**Spectroscopy of CoHcy.** The weak visible absorption at 520 nm, due to the adventitiously bound cobalt, is entirely in accordance with Co(II) in a distorted octahedral environment of strong-field ligands. Lorosh and Haase[17] have also concluded that the "peripherally" bound Co(II)'s are in a distorted octahedral ligand field, on the basis of zero-field splitting in the EPR spectrum.

The binding stoichiometry of one chloride, hydroxide, or azide per two Co(II)'s in the concentration ranges studied indicate that these ligands are bridging Co(II)'s. Lorosch and Haase[17] reported that the active-site Co(II)'s are probably antiferromagnetically coupled with a lower limit on the exchange integral, $-2J$, of 50 $cm^{-1}$. This value is much lower than the 625-$cm^{-1}$ lower limit value established for the Cu(II)'s in oxyhemocyanin;[1] this difference may not only be due to the symmetry and metal differences but also due to the fact that the active site Co(II)'s in the Lorosch and Haase CoHcy probably were bridged by chloride (their CoHcy preparations were in 0.05 M Tris/$Cl^-$ buffer), a ligand expected to give a much weaker superexchange than hydroxide.[1b]

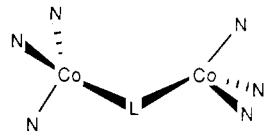
The UV/vis/near-IR absorption data of the active-site Co(II) in CoHcy/L, where L = $H_2O$, $OH^-$, $Cl^-$, or $N_3^-$ (Table II), are clearly indicative of Co(II) in a distorted tetrahedral ligand field, and the trend in the shifts is as expected from the spectrochemical series. It is fruitful to speculate on the possible distortions from $T_d$ that might best describe the Co(II) environment in the active site of CoHcy. A convenient model for estimating ligand-field transition energies for symmetries lower than $T_d$ or $O_h$ is the angular overlap model, AOM.[29] The energy level diagrams have been calculated for $T_d$, $D_{2d}$ $C_{2v}$, and $C_{3v}$ symmetries for four-coordinate Co(II),[29a] and the $\sigma$ interaction parameters, $e_\sigma$, have been tabulated for a large number of four-coordinate Co(II) complexes.[29b] From an AOM calculation, a reasonable fit to the data is obtained by assuming $CoN_3L$ units of $C_{3v}$ symmetry, $e_\sigma(N)$ = 4035 $cm^{-1}$ (imidazole) and $e_\sigma(L)$ = 3312 $cm^{-1}$ (an average of $Cl^-$, $N_3^-$, $H_2O$, and $OH^-$ values, which range from 2950 to 3635 $cm^{-1}$)[29b] (Table II). AOM calculations assuming any of the other distorted four-coordinate symmetries do not fit the observed data as well as for $C_{3v}$; nor do any of the possible five-coordinate geometries yield a reasonable fit. The lowest energy ligand-field transition, $^4A_2 \rightarrow {}^4A_2(F)$ in $C_{3v}$, was calculated to be at 5700 $cm^{-1}$ and cannot be observed because of interferences from overtone and combination vibrations from the buffer and protein. The $^4A_2 \rightarrow {}^4E(F)$ transition, calculated to be centered at 9900 $cm^{-1}$, is split in CoHcy by spin–orbit coupling, the other Co(II), and/or a lower symmetry ligand field. The $^4A_2 \rightarrow {}^4E(P)$ transition, calculated to be centered at 18 100 $cm^{-1}$, is expected to split, and since the energy of this transition is quite sensitive to $e_\sigma(L)/e_\sigma(N)$,[29a] the averaging approximation gives a poor fit. The highest energy $^4A_2 \rightarrow {}^4A_2(P)$ transition, calculated to be centered at 19 400 $cm^{-1}$, is insensitive to $e_\sigma(L)/e_\sigma(N)$,[29a] and thus, the fit is excellent, even with the average approximation.

The transitions in the $\mu$-hydroxo and $\mu$-azido CoHcy at 360 and 323 nm, respectively, are too high in energy and intensity to arise from d–d transitions of four-coordinate Co(II) in $C_{3v}$ symmetry. These must be assigned to ligand-to-metal charge-transfer transitions (LMCT). In metazidohemocyanins, a bridging azide gives rise to a $\mu$-$N_3^- \rightarrow$ Cu(II) LMCT at 380 nm.[1] On the basis of the Jorgensen optical electronegativity model[30] for calculation of LMCT energies, $\mu$-$N_3^- \rightarrow$ Co(II) should shift up from that of the copper analogue (regardless of symmetry changes) to a calculated value of 30 000 $cm^{-1}$ (333 nm), in good agreement with the observed transition at 31 000 $cm^{-1}$ (323 nm).

The LMCT peak at 425 nm that has been associated with the endogenous ligand bridge in met and oxyhemocyanins[1b] would reasonably be expected to shift up in the Co(II) analogue. Assuming the same shift up in energy (4685 $cm^{-1}$) as was observed for the $\mu$-azide LMCT of the 425-nm peak in met and oxyhemocyanins for the CoHcy analogue, a value of 28 200 $cm^{-1}$ or 355 nm is obtained, in close agreement with the 360-nm (27 700 $cm^{-1}$) LMCT observed in $\mu$-hydroxo CoHcy. Therefore, this peak can be assigned to $\mu$-$OH^- \rightarrow$ Co(II) charge transfer.

Even though the spectroscopic assignments are not rigorous, the internal consistency of the chemistry, d–d assignments, LMCT assignments, and analogy with the copper-containing hemocyanins provide a picture of the CoHcy active site as shown in structure II, where the bridging ligand, L, can be azide, chloride, or hydroxide.

(27) Huheey, J. E. *Inorganic Chemistry*, 3rd Ed.; Harper and Row: New York, 1983; pp 294–297.
(28) Motekaitis, R. J.; Martell, A. E.; LeCompte, J. P.; Lehn, J. M. *Inorg. Chem.* **1983**, *22*, 609–614.
(29) (a) Banci, L.; Bencini, A.; Benelli, C.; Gatteschi, D.; Zanchini, C. *Struct. Bonding* **1982**, *52*, 37–86. (b) Lever, A. B. P. *Inorganic Electronic Spectroscopy*, 2nd ed.; Elsevier: New York, 1984; pp 53–68, 763–765.
(30) Lever, A. B. P. *Inorganic Electronic Spectroscopy*, 2nd ed.; Elsevier: New York, 1984; pp 218–223.



II

**Oxygenation of CoHcy.** The spectroscopy and chemistry of the reaction product of CoHcy with dioxygen is quite similar to that of the ($\mu$-hydroxo)($\mu$-peroxo)dicobalt(III) species;[24,26] these similarities strongly support a $\mu$-hydroxo, $\mu$-peroxo structure for oxyCoHcy. When peroxide forms a bridge, the degeneracy of the two $\pi^*$ orbitals is removed; therefore, two $O_2^{2-} \rightarrow$ Co(III) charge-transfer transitions are expected from the dibridged species due to $\pi_a^* \rightarrow d_\sigma^*$ and $\pi_b^* \rightarrow d_\sigma^*$. These are observed at 300 and 372 nm in the L-histidine ($\mu$-$OH^-$)($\mu$-$O_2^{2-}$)$Co^{III}$ dimer.[24a] The intensities and energies of the peaks are sensitive to the angle of the peroxide relative to the plane defined by the two cobalts and hydroxide. The 319- and 404-nm bands observed in oxygenated CoHcy can be assigned to the two $O_2^{2-} \rightarrow$ Co(III) LMCT's.

Preliminary kinetics analysis of the oxygenation of CoHcy yielded a second-order rate constant of 11 $M^{-1}$ $s^{-1}$ at pH 8.13 and 170 $M^{-1}$ $s^{-1}$ at pH 9.4. This assumed a rate law of rate = $k$-[CoHcy][$O_2$], where $k$ is dependent on the [$OH^-$] to the first order. (These rate constants are much slower than in native hemocyanin, which has a rate constant for the oxygenation of approximately $10^7$ $M^{-1}$ $s^{-1}$.) This first-order dependence on [$OH^-$] was first observed in the formation of $\mu$-hydroxo, $\mu$-peroxo dimers of cobalt(III) glycylglycine complexes.[26a] The stabilizing effect of hydroxide in the formation of a large number of ($\mu$-peroxo)-cobalt(III) dimers has been reported.[26e,f] The rate constant for oxygenation of CoHcy is significantly lower than that observed for the oxygenation of octahedral Co(II) complexes,[26f] which are typically in the range of $10^3$-$10^4$ $M^{-1}$ $s^{-1}$. The rates do vary with steric influences,[26f] and the coordination number change that must take place upon oxygenation of CoHcy is not represented by these models. Oxygenation of tetrahedral Co(II) complexes in concentrated base has been reported but without any rate data.[26d]

The chemistry and structure of the active site in CoHcy seems to mimic that the deoxyhemocyanin: two four-coordinate distorted tetrahedral metals that upon oxygenation become six-coordinate with $\mu$-peroxo, $\mu$-hydroxo ligands. The two Co(II)'s in the active site of *L. polyphemus* CoHcy bind equivalently as determined by the equilibrium with apohemocyanin and spectroscopy of exogenous ligand binding. More detailed spectroscopic, equilibrium, and kinetics studies of CoHcy will certainly add to our understanding of the structure and function of hemocyanin active sites.

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**Registry No.** Co, 7440-48-4; OH, 14280-30-9; Cl, 16887-00-6; $N_3$, 14343-69-2; O, 7782-44-7.

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Contribution from the Department of Chemical and Biological Sciences, Oregon Graduate Institute of Science and Technology, Beaverton, Oregon 97006-1999, and Department of Chemistry, Brandeis University, Waltham, Massachusetts 02254

# Spectral Properties of *cis*- and *trans*-Metallooctaethylchlorins: Effects of Pyrroline Ring Stereochemistry and Macrocyclic Conformation

Laura A. Andersson,*[1a] Thomas M. Loehr,[1a] Matthew T. Stershic,[1b] and Alan M. Stolzenberg[1b,c]

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We recently observed that differences in the structure of *nonconjugated* substituents on the pyrroline ring of metallochlorins had a dramatic effect on the resonance Raman (RR) spectral frequencies. To explore this novel finding, we have compared the spectral properties of the cis and trans stereoisomers of planar copper(II) octaethylchlorin [Cu(OEC)] and of $S_4$-ruffled nickel(II) octaethylchlorin [Ni(OEC)], where only the configuration of the nonconjugated substituents is varied. Study of these complexes addresses both stereochemistry at the pyrroline ring and the macrocyclic conformation of hydroporphyrins as modulators of the spectral properties. (1) Electronic absorption transitions of the *cis*-M(OEC) complexes are red-shifted from those of *trans*-M(OEC), whereas the Soret/$Q_y$ intensity ratio of $S_4$-ruffled Ni(OEC) complexes is less than that for the planar Cu(OEC) complexes. (2) The infrared frequencies of $C_b$-H deformation modes of *cis*-M(OEC) are higher than those of *trans*-M(OEC). (3) The RR band at ~745 $cm^{-1}$ is lower in *frequency* for *cis*-M(OEC) than for *trans*-M(OEC), whereas this band exhibits increased *intensity* for $S_4$-ruffled Ni(OEC) vs planar Cu(OEC) complexes. (4) Extension of the core size/RR frequency correlation of metalloporphyrins to the -chlorins indicates that *cis*-M(OEC) complexes are smaller in core size than *trans*-M(OEC), whereas the Ni(OEC) complexes are smaller in core size than Cu(OEC). These data demonstrate that structural variations on the pyrroline ring of chlorins have a marked effect on the spectral properties and that macrocyclic conformation also has a significant influence. Thus, apparently localized changes in the macrocyclic structure strongly perturb the overall properties of chlorins and should be considered in spectral analyses of novel biological and model hydroporphyrins.

## Introduction

For biological hydroporphyrins[2] of known structure (e.g., chlorophylls,[3] sulfite reductases,[4] and factor F-430 of methanogenic bacteria[5,6] the stereochemistry of the substituents on the $sp^3$-hybridized carbons of the pyrroline ring is trans. The trans configuration is thermodynamically favored for hydroporphyrins with two hydrogen substituents on the pyrroline ring. Oxidative dehydrogenation of a *cis*-pyrroline ring is often facile.[7] Thus, trans stereochemistry at the pyrroline may stabilize biological macrocycles against reversion to the porphyrin.

Recently, however, a *cis*-pyrroline configuration has been suggested for the heme *d* prosthetic chlorin of the *Escherichia coli* terminal oxidase.[8-10] A cis configuration is also indicated for the chlorin catalase of *E. coli*.[11] In both of these cases, the proposed in vivo iron chlorin has no hydrogen substituents on the saturated ring, thereby eliminating the potential for oxidative

* To whom correspondence should be addressed.

(1) (a) Oregon Graduate Institute of Science and Technology. (b) Brandeis University. (c) Current address: Department of Chemistry, West Virginia University, Morgantown, WV 26506.

(2) Hydroporphyrins differ from the parent porphyrin by saturation of one or more pyrrole rings. The pyrroline (reduced) ring does not necessarily have hydrogen substituents. Biological hydroporphyrins include dihydroporphyrins (chlorins), isobacteriochlorin (iBC) tetrahydroporphyrins (two adjacent pyrroline rings), bacteriochlorin (BC) tetrahydroporphyrins (two opposite pyrroline rings), and the more highly saturated and modified corphinoid macrocycle of factor F-430.

(3) (a) *The Chlorophylls*; Vernon, L. P., Seely, G. R., Eds.; Academic: New York, 1966. (b) *The Photosynthetic Bacteria*; Clayton, R. K., Sistrom, W. R., Eds.; Plenum: New York, 1978. (c) Inhoffen, H. H.; Buchler, J. W.; Thomas, R. *Tetrahedron Lett.* **1969**, 1145. (d) Brockmann, H., Jr. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1978; Vol. II, Chapter 10. (e) Smith, K. M.; Simpson, D. J. *J. Chem. Soc., Chem. Commun.* **1986**, 1682-1684.

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