Fluorescence and Circular Dichroism Studies with Hemocyanin from Taiwan Snails

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ABSTRACT

Several derivatives of hemocyanin from Taiwan snails (*Achatina fulica*) have been prepared. The reconstituted protein (R-HcO_2) has lower Cu content, lower circular dichroism intensity, and higher fluorescence intensity than native oxyhemocyanin (HcO_2). The Co(II) derivative (CoHc) does not take up molecular oxygen and only 50% of the total sites for Cu in native hemocyanin is taken up by Co. The half-apo derivative (half-apo-Hc) contains a single Cu per active site. Divalent cations quench the tryptophan fluorescence in the hemocyanin species and also quench the fluorescence from Tb^{3+} bound to the protein. The collisional quenching constants decrease in the order $Co^{2+} > Mn^{2+} > Ca^{2+}$. The static component is negligible. For carboxy hemocyanin (HcCO), fluorescence originates from a Cu(I) CO complex and was used to study reaction of Hc CO with CN^-.

INTRODUCTION

Hemocyanins, the copper-containing proteins that carry oxygen, are found in the hemolymph of many arthropods and molluscs. They bind oxygen in the ratio of one oxygen molecule for every two copper atoms. The protein, deep blue colored when oxygenated, becomes colorless when deoxygenated by passing N_2, and the coppers in

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Journal of Inorganic Biochemistry **29**, 231-239 (1987)

0162-0134/87/$3.50

deoxy hemocyanin are Cu(I) [1]. This paper describes studies on metal ion and cyanide interactions with several derivatives of *Achatina fulica* hemocyanin from Taiwan snails, from fluorescence and circular dichroism measurements. The following derivatives of hemocyanin were prepared: reconstituted (R-HcO_2), cobalt (CoHc), half-apo(half-apoHc), carboxy (HcCO), apo(apoHc), and met (metHc).

The interactions between CoHc, R-HcO_2, native oxyhemocyanin (HcO_2), or half-apo-Hc and divalent cations were studied by fluorescence measurements (excitation, 280 nm; emission, 340 nm). Fluorescence experiments (excitation, 295 nm; emission, 543 nm due to protein-bound Tb^{3+}) were carried out to determine whether the emission intensity at 543 nm may be used to monitor the interaction of added $TbCl_3$ with hemocyanin and the effect of added divalent cations on the interactions. For HcCO, the excitation wavelength was 295 nm and the observation wavelengths were 330 and 540 nm. We found that the fluorescence emission intensity at 540 nm is an excellent probe for studying the interaction of HcCO. CoHc, HcO_2, and R-HcO_2 were also studied by carrying out circular dichroism experiments.

MATERIALS AND METHODS

Preparations

Hemocyanin was prepared from the hemolymph of Taiwan snails and purified as previously described [2]. Protein concentration was determined spectrophotometrically by using ϵ_{280} (1%, 1 cm) 15.71. The copper content was determined by neutron activation analysis (using the Tsing Hua University reactor) to be 0.24%, and the minimal molecular weight of the protein that contains one dicopper active site was taken to be 53,000. ApoHc was prepared by dialysis of HcO_2 in 50 mM Tris-HCl buffer (pH 8.0) against the same buffer that contained 10 mM KCN at 4°C for 3 days. It was then dialyzed against the buffer alone for 2 days with changes every 4 hr. With this procedure, 97% of the total copper was removed from the active site, and the apoHc showed no absorption at 345 nm. Reconstituted Hc, R-HcO_2, was prepared by treating the apoHc solution at pH 5.7 for 24 hr at 4°C in N_2 atmosphere with an amount of $Cu(CH_3CN)_4ClO_4$ corresponding to twice the copper content of native Hc [3, 4]. R-HcO_2 was dialyzed against 0.025 *M* EDTA in 0.1 *M* acetate buffer, pH 5.7, N_2 atmosphere for 24 hr and afterwards against the acetate buffer alone for 1 day. A copper content of 0.21% was found, corresponding to a reconstitution factor of 87.5%. MetHc was prepared by treating HcO_2 with 25 mM azide in 0.1 *M* sodium acetate buffer, pH 5.0 at 37°C [5]. The excess azide was removed by extensive dialysis against 0.1 *M* acetate buffer, pH 5.7.

The cobalt derivative of Hc (CoHc) was prepared by dialysis of apoHc against 50 mM Tris-HCl buffer (pH 8.0), which contained 1 mM $CoCl_2$ at 4°C for 4 days under N_2 atmosphere followed by dialysis against Co-free buffer at pH 8.0 for 2 days. The resulting crude sample of CoHc was treated with Chelex 100 resin (40% by volume, preequilibrated with the Tris-HCl buffer) to remove excess Co(II). A blue solution was obtained. The Co content in CoHc is 0.11%, which corresponds to 49.4% of the total sites for Cu in native Hc. The amount of residual Cu in CoHc is in the range 0.01%–0.02%. It varies somewhat from preparation to preparation and depends partly on the duration of dialysis with KCN and pH in forming apoHc. The residual Cu constituted less than 0.1% of the total sites for Cu in native Hc. CoHc does not bind O_2 even under O_2 atmosphere. Half-apo-Hc, colorless, was prepared by dialysis at 4°C of

deoxyhemocyanin (6 mg/ml) against N_2 saturated Tris-HCl buffer pH 8.0 containing 3.8 mM CN^-. In 10 hr, the Cu content had dropped from 0.24% to 0.13%, indicating formation of half-apo-Hc. Himmelwright et al. [6] have previously shown that one copper can be selectively removed from the binuclear active site of *Busycon*, yielding a half-apo protein, and that the copper is Cu(I).

Physical Measurement

Fluorescence spectra were measured on a Hitachi MPF-4 fluorescence spectrophotometer operated at 25°C. Circular dichroism measurements were performed on a Jasco J-20 spectropolarimeter. The concentrations of Cu and Co in the hemocyanin derivatives were determined by use of inductively coupled plasma-atomic emission spectrometer.

RESULTS AND DISCUSSION

The quenching of fluorescence upon oxygen binding to hemocyanin has been traced to nonradiative electronic energy transfer from the originally excited trytophanyl residues to the Cu···O groups [2]. For *A. fulica* hemocyanin at pH 8.0, the fluorescence intensities of native oxyhemocyanin (HcO_2), reconstituted oxyhemocyanin (R–HcO_2), and cobalt hemocyanin (CoHc) are 6.8, 16.5, and 44.1, respectively (in arbitrary units; excitation, 280 nm; emission maxima at 324, 323, and 330 nm, respectively, for HcO_2, R–HcO_2, and CoHc). Since the concentration of the CuO_2Cu groups is higher in HcO_2 than in R–HcO_2 (Cu contents of 0.24% and 0.21%, respectively), it is natural that the fluorescence intensity in HcO_2 is less than in R–HcO_2. The active site of CoHc contains a single Co atom and no oxygen, so that of these three species of the protein, CoHc shows the greatest fluorescence intensity.

Divalent cations further quench fluorescence, as shown in Figure 1 for CoHc. The quenching reaction between hemocyanin and M^{2+} can be described by the following:

$$\mathrm{Hc} + \mathrm{M}^{2+} \underset{k_{-d}}{\overset{k_d}{\rightleftarrows}} (\mathrm{Hc}\cdots\mathrm{M}^{2+}) \tag{1}$$

where $(Hc\cdots M^{2+})$ is the complex formed by diffusional encounter between Hc and M^{2+} with rate constant k_d. The experimentally observed rate constant for the quenching reaction, k_q, is equal to k_d when the efficiency of the quenching process is unity [8]. The collisional quenching process, equation (1), is usually described by the Stern–Volmer equation [9]:

$$F_0/F = 1 + K_{SV}[\mathrm{M}^{2+}] \tag{2}$$

where F_0 and F are the fluorescence intensities at an appropriate emission wavelength in the absence and presence of quencher $[M^{2+}]$, K_{SV} is the collisional quenching constant, which is equal to $k_q\tau_0$, and τ_0 is the fluorescence lifetime in the absence of quencher. By plotting F_0/F at the appropriate emission maxima vs. $[M^{2+}]$, linear plots for CoHc, R–HcO_2, and HcO_2 were obtained. Figure 1 gives plots for CoHc. The values of K_{SV} for Hc = CoHc, HcO_2, and R–HcO_2, and $M^{2+} = Ca^{2+}$, Mn^{2+}, and Co^{2+}, are listed in Table 1.

A more complete treatment of the kinetics of the quenching reaction should include consideration of a "static" quenching process [8]. In a randomly distributed solution, the quencher and the chromophore molecules occasionally may come very close to

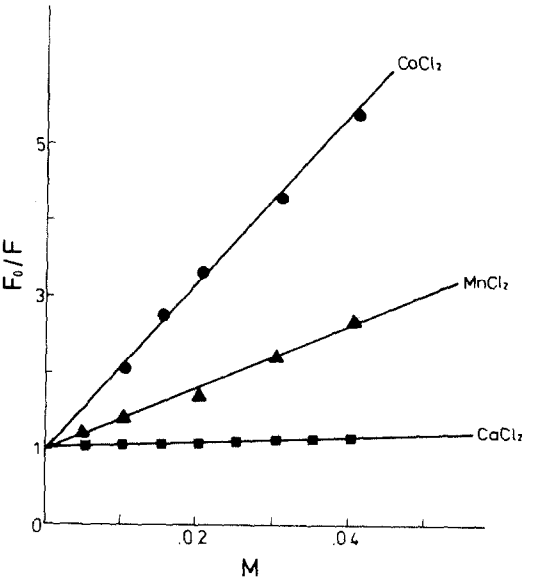


FIGURE 1. Plots of F_0/F vs. $[CaCl_2]$, $[MnCl_2]$, and $[CoCl_2]$ added to solutions containing Co Hc (2.8 mg/ml) pH 8.0.

each other when the latter become excited. In this case, the probability for the reaction may become so high that quenching occurs instantaneously (statically). The kinetics of the quenching reaction include a collisional (characterized by K_{SV}) and a static (characterized by V) component, and equation (2) becomes [8]

$$F_0/F = (1 + K_{SV}[M^{2+}])e^{V[M^{2+}]} \tag{3}$$

Since Figure 1 gives linear plots between F_0/F and $[M^{2+}]$, V must be very small and we need to consider only the collisional quenching by the cations.

Linear plots of F_0/F vs. $[M^{2+}]$ are also obtained for solutions of half-apo-Hc at pH 8.0. The values of K_{SV} are included in Table 1. For the half-apo-Hc, the emission maximum is at 330 nm (excitation, 280 nm).

On addition of $TbCl_3$ to hemocyanin solution, three emission bands, due to protein-bound Tb^{3+}, are observed at 490, 543, and 590 nm. Figure 2 gives plots of fluorescence intensity at 543 nm (excitation, 295 nm) when $TbCl_3$ is added to different hemocyanins, each at 1.2×10^{-6} M at pH 6.5, using piperazine-1,4-bis(2-ethanesulfonic acid) salt (PIPES-Na_2) as buffer. Fluorescence intensities in Tb^{3+}-hemocyanins are in the order apoHc > CoHc > R-HcO_2 > metHc > native HcO_2.

TABLE 1. Values of K_{SV} for Hc + M^{2+} = (Hc$\cdots M^{2+}$) at pH 8.0, 25°C

	M = Ca^{2+}	Mn^{2+}	Co^{2+}
CoHc	4	44	110
HcO_2	2	10	54
R-HcO_2	2	8	50
Half-apo-Hc	2		68

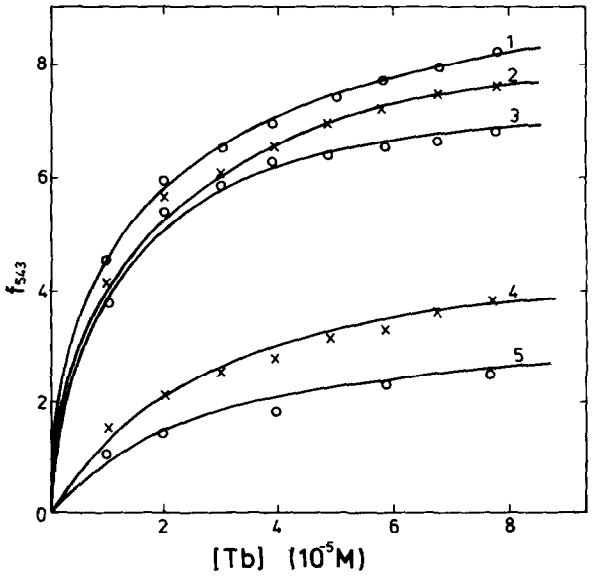


FIGURE 2. Fluorescence intensity at 543 nm (arbitrary units), pH 6.5, as function of Tb^{3+} concentration added to various hemocyanins: 1, apoHc; 2, CoHc; 3, R-HcO_2; 4, metHc; 5, HcO_2 (Hc concn. 1.2×10^{-6} *M*).

In Figure 2, correction for the Tb^{3+} fluorescence intensity in the absence of hemocyanin is entirely negligible. ApoHc has both copper sites vacant. In the preparation of CoHc, the amount of Co(II) introduced into apoHc reached only up to 50% of the total sites for Cu in the native Hc. The vacancies of 2 and 1 copper sites, respectively, in the active sites of apoHc and CoHc, explains the order of fluorescence enhancements when Tb^{3+} is added to these two protein species. In metHc, both coppers are Cu(II), which is an effective fluorescence quencher. HcO_2 contains both Cu(II) ions and, in addition, an O_2^{2-}. A previous paper [10] has indicated that oxygen is an effective quencher of Tb^{3+} bound to *Panulirus* hemocyanin. In the present research the presence of dioxygen in native HcO_2 causes this species to be the most effective fluorescence quencher, resulting in the lowest fluorescence intensity among the various hemocyanin species investigated.

Addition of $CaCl_2$, $MnCl_2$, or $CoCl_2$ to a solution of hemocyanin containing $TbCl_3$ causes a decrease in fluorescence at 490 and 543 nm. The results for HcO_2 are shown in Figure 3. These are plots of F_0/F at 543 nm vs. $[Ca^{2+}]$, $[Mn^{2+}]$, and $[Co^{2+}]$, at pH 6.5, where F_0 and F are fluorescence intensities for the HcO_2-$TbCl_3$ solution (Hc, 1.2×10^{-6} *M*; Tb^{3+}, 4×10^{-5} *M*) in the absence and presence of a divalent cation, respectively.

The interaction of a divalent cation, M^{2+}, with Tb^{3+}. Hc may be written

$$Tb^{3+}\cdot Hc + M^{2+} \underset{k_{-d}}{\overset{k_d}{\rightleftarrows}} (Tb^{3+}\cdot Hc\cdots M^{2+}) \qquad (4)$$

Since the fluorescence intensity at 543 nm is due to the protein-bound Tb^{3+}, equation (2) holds also for equation (4). Figure 3 shows linear plots of F_0/F vs. $[M^{2+}]$, with intercept equal to 1.0, for HcO_2. For other hemocyanin derivatives with Tb^{3+} concentrations varying from 2.4×10^{-5} *M* to 1.1×10^{-4} *M*, our results show that

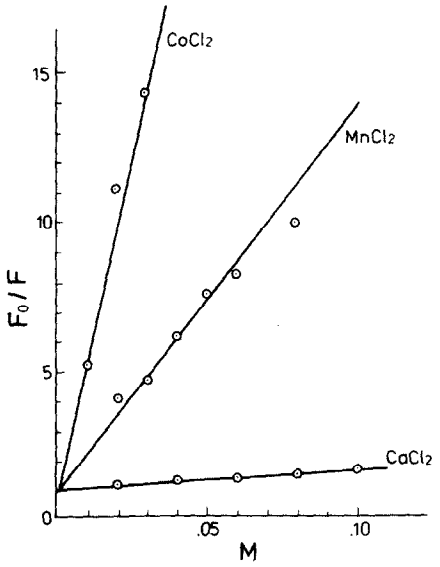


FIGURE 3. Plots of F_0/F at 543 nm vs. $[CaCl_2]$, $[MnCl_2]$, and $[CoCl_2]$ added to HcO_2–$TbCl_3$ solution (Hc, $1.2 \times 10^{-6} M$; Tb^{3+}, $4 \times 10^{-5} M$), at pH 6.5.

equation (2) is also valid for equation (4). The average values of K_{SV} are given in Table 2.

In forming complexes, both Ca^{2+} and Tb^{3+} prefer oxygen-donor ligands (in proteins, RCO_2^-, RO^-, C=O and H_2O), whereas Co^{2+} prefers nitrogen-donor ligands. Of the divalent cations listed in Tables 1 and 2, Co^{2+} gives the largest K_{SV} values for all of the Hc species.

The values of K_{SV} for Mn^{2+} are smaller than for Co^{2+} (Tables 1, 2). These are in the same order as the overall binding constants of histidine complexes: log K for Mn–histidine and Co–histidine complexes are 3.3 and 6.9, respectively [11]. Since the metal ligands in the active sites of Hc are predominantly histidine [12], the higher K_{SV} for Co^{2+} over Mn^{2+} is reasonable.

The circular dichroism and absorption spectra of CoHc (pH 8.0) are very similar to those reported for the Co derivative of squid hemocyanin [13]. The absorption of the Co(II) chromophore at 550–600 nm is very similar to the 1:1 cyanide complex of Co(II)–human carbonic anhydrase [14] and has been attributed to the transitions arising from a high-spin tetrahedral Co(II) center. The successful preparation of a half-filled Co(II) hemocyanin from the hemolymph of the squid, *Sepioteuthis lessoniana*,

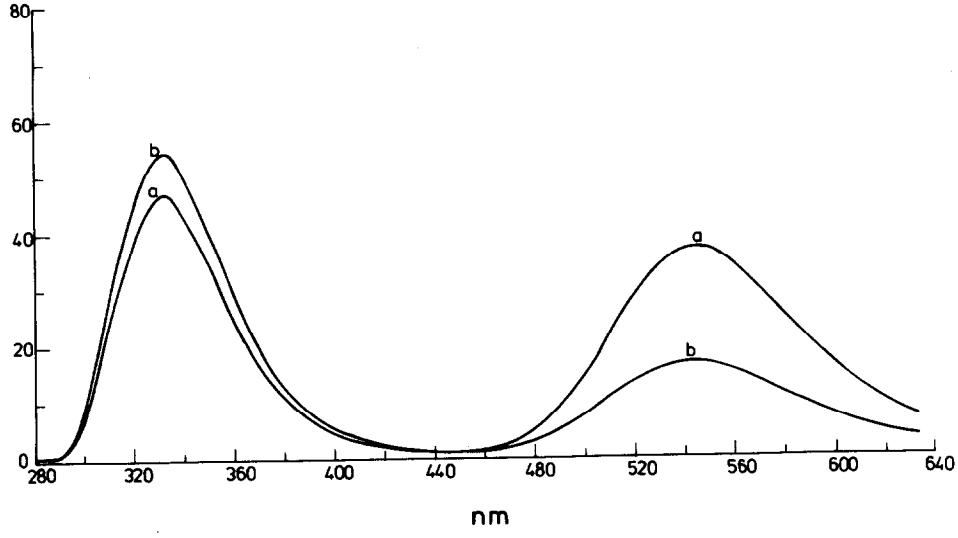
TABLE 2. Values of K_{SV} for $Tb^{3+} \cdot Hc + M^{2+} = (Tb^{3+} \cdot Hc \cdots M^{2+})$ at pH 6.5, 25°C

Hemocyanin	M = Ca^{2+}	Mn^{2+}	Co^{2+}
Apo	15	60	420
Met Hc	15	140	820
Co Hc	10	60	630
Hc O_2	8	130	480
R-HcO_2	15	80	460

has been reported [13], so that the *A. fulica* Hc behaves like squid Hc. The preparation of 1-Co(II)-substituted Hc from *Helix pomatia* and *Carcinus* [15, 16] have also been reported. On the other hand, the amount of Co(II) introduced into horseshoe apohemocyanins reached essentially 100% of the total sites for copper ions in native hemocyanins [17]. *A. fulica* CoHc was kept in a dialysis bag (pH 8.0) under O_2-atmosphere at room temperature, and the visible absorption band around 600 nm remained unchanged. This indicates that CoHc does not take up molecular oxygen at all, and may be explained by taking into account the structural restriction of the Co(II) binding which is tetrahedral [13]. The octahedral geometry would enable dioxygen binding as in the case of several Co(II)–dioxygen complexes of low molecular weights [18, 19].

The effects of CO on the luminescence properties of *Helix pomatia* α-hemocyanin and *Panulirus interruptus* hemocyanin have been discussed [10]. These proteins, when saturated with CO, show emission with maxima between 540 and 560 nm, in addition to the tryptophan resonance at 340 nm. The emission in the visible region originates from a fluorescent Cu(I)–CO complex, and this emission is interpreted as charge-transfer luminescence. With *A. fulica* hemocyanin, the excitation wavelength is 295 nm; emission for HcCO is 540 nm. A peak also occurs at 330 nm. However, the oxy, deoxy, and met hemocyanins also give fluorescence maxima at 330 nm (with varying intensities because of different extents of quenching tryptophan fluorescence), whereas only HcCO gives a fluorescence peak at 540 nm in addition to an emission maximum at 330 nm. With addition of CN^-, the intensity at 330 nm increases because Cu is removed from the protein in forming apoHc. The 540-nm peak decreases because inorganic Cu does not bind CO. The result is illustrated in Figure 4, giving the fluorescence spectra of (a) HcCO and (b) HcCO + KCN (0.0016 *M*), spectra taken 45 min after preparation of solutions. At 2.5 hr after preparation, the HcCO peak at 540 nm decreased to zero, while the 330-nm peak continued to increase. On standing overnight, the 330-nm peak was increased only slightly. During fluorescence

FIGURE 4. Fluorescence spectra of HcCO in the absence (a), and presence (b) of 0.0016 *M* KCN, pH 8.0.



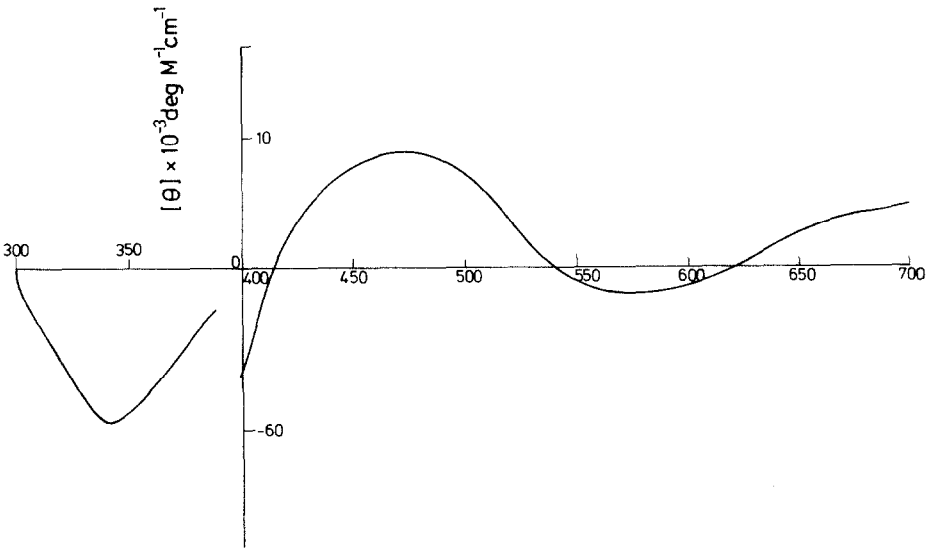


FIGURE 5. CD spectrum of native HcO_2 (2.34×10^{-4} *M*), pH 8.0.

measurements, HcCO samples were exposed to air. Although O_2 is a quencher, separate experiments have shown that the effect of dissolved O_2 in solution on protein fluorescence intensity is negligible.

The CD spectrum of native HcO_2 (2.35×10^{-4} *M*) 300–700 nm is shown in Figure 5. *A. fulica* HcO_2 exhibits two positive bands at 470 and 700 nm and one negative band at 575 nm, and one intense negative band around 340 nm. The spectra are similar to those of squid [11]. The CD spectrum of R-HcO2 (2.39×10^{-4} *M*) also exhibits maxima at 470 and 700 nm, and negative bands at 575 and 340 nm. The intensities are somewhat smaller: the values of $[\theta]$ at 340 and 470 nm for R-HcO_2 are -25.2×10^3 and 2.96×10^3 deg M^{-1} cm^{-1}, respectively, compared to corresponding values of -59.5×10^3 and 8.5×10^3 deg M^{-1} cm^{-1} for native HcO_2. For both HcO_2 and R-HcO_2, in the presence of 0.02 *M* KCN, $[\theta]$ becomes zero in the entire wavelength range. This is because apoHc is formed, as has been found for *Busycon* and *Limulus* apohemocyanins. The smaller values of $[\theta]$ for R-HcO_2 compared to HcO_2 are in line with the lower Cu content and therefore a smaller number of oxygenated active sites of the reconstituted protein.

CONCLUDING REMARKS

The very first report on hemocyanin from Taiwan snails was published in 1979 [2]. This paper describes further properties of various derivatives prepared from this species: reconstituted hemocyanin, cobalt, met, apo, and half-apo-Hc. The reconstituted oxyhemocyanin has a lower copper content, lower circular dichroism intensity, and higher fluorescence intensity than native oxyhemocyanin. Cobalt hemocyanin does not take up molecular oxygen, and only 50% of the total sites for Cu in native Hc is occupied by Co. Half-apo-Hc contains a single copper in the active site, compared to no copper in apoHc and two coppers in HcO_2. Himmelwright et al. [6] have previously shown that one copper can be selectively removed from the binuclear active site of *Busycon*, yielding a half-apo-protein, and that the copper is Cu(I). Divalent cations

quench the tryptophan fluorescence of various Hc species, including the half-apo-Hc, and quench the fluorescence due to Tb^{3+} bound to the various Hc species. The values of the collisional quenching constant decrease in the order $Co^{2+} > Mn^{2+} > Ca^{2+}$, and the static component of quenching is negligible. For HcCO, the observed emission at 540 nm (excitation, 295 nm) originates from a fluorescent Cu(I) CO complex, and was used to study reaction of CN^- with HcCO.

This work was supported by the National Science Council, ROC. The authors also thank Miss Sin Tak Chu for her very capable assistance with instrumentation.

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Received July 21, 1986; accepted November 19, 1986