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PREPARATION AND CHARACTERIZATION OF MET APO HEMOCYANIN: A SINGLE COPPER(II) ACTIVE SITE

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SUMMARY: A new derivative of Busycon canaliculatum hemocyanin has been prepared for which one copper has been removed from the binuclear active site of the holoprotein and the remaining copper has been oxidized with a variety of small molecule oxidizing agents. This met apo derivative [( )...Cu(II)] binds a number of ligands; EPR spectra of several forms are reported and compared to those obtained for a singly oxidized (half met-L) derivative [Cu(I)...Cu(II)L]. The site of the oxidized copper for both forms is found to be quite similar in structure but shows large differences in ligand binding ability.

INTRODUCTION: The unique electronic properties of the binuclear copper unit in hemocyanin[1-3] (the lack of an EPR signal and intense absorption bands in the near uv and visible regions) have made it difficult to determine the electronic and geometric structure of the active site. Recently, through detailed spectroscopic studies[4] of the effects of ligand perturbation on the half met (singly oxidized[5]) derivative of Busycon canaliculatum hemocyanin [Cu(I)...Cu(II)L, where L = N_3^-, $CH_3CO_2^-$, CN^-, etc.], we have shown that the tightly bound but exchangeable ligand L coordinates in the plane of an approximately tetragonal copper(II) site with a $d_{x^2-y^2}$ ground state. This communication reports the preparation and initial EPR studies of a new hemocyanin derivative, met apo-L, which contains a single copper(II) in the active site and reversibly binds a variety of ligands [( )...Cu(II)L].

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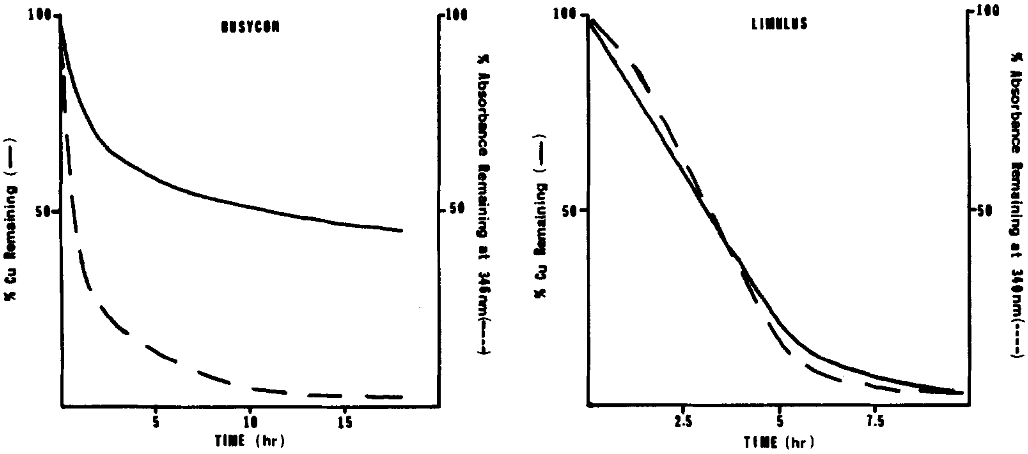
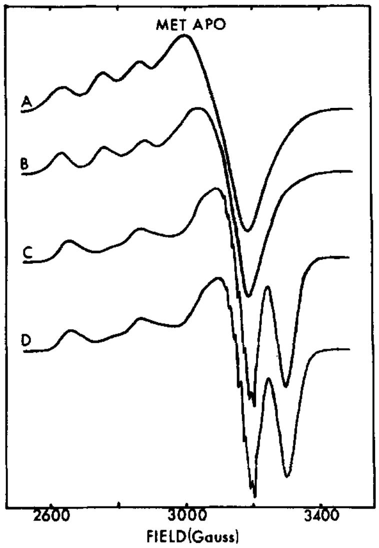


Figure 1. Copper removal from Busycon canaliculatum and Limulus polyphemus hemocyanin.

MATERIALS AND METHODS: Hemocyanin was purified from the hemolymph of the marine snail, Busycon canaliculatum, and the horseshoe crab, Limulus polyphemus, by ultracentrifugation. The pellets were redissolved in 0.1 M tris, 0.1 M $CaCl_2$, pH 8.5.[6] The half apo protein was prepared by careful dialysis of the hemocyanin against a buffered 0.05 M cyanide solution while monitoring copper content and optical absorption at 345 nm. The met apo derivative is obtained through oxidation of the copper by incubation in a tenfold excess of $NaNO_2$ at pH 6.3, 0.1 M phosphate buffer. Oxidation of the copper has also been achieved by anaerobic treatment at pH 8.0, 0.1 M tris with NaO_2 dissolved in DMF, by autooxidation at 37°C, or by addition of peroxide at pH 8.0, 0.1 M tris. Regeneration[7] of the half apo form to oxyhemocyanin is accomplished by anaerobic treatment with $Cu(CH_3CN)_4ClO_4$. Copper content was determined by atomic absorption using a Perkin Elmer 360 spectrometer equipped with a graphite furnace. EPR spectra of frozen solutions at -100°C were obtained using a Varian E-9 spectrometer operating at 9.1 GHz.

RESULTS AND DISCUSSION: Figure 1 shows the results of copper removal from Busycon canaliculatum and Limulus polyphemus hemocyanin by dialysis against cyanide. The curves represent the percent of copper remaining and the extent of oxygen binding as a function of length of dialysis. It has previously been shown[1] that oxygen binds in the ratio 1 O_2:2 Cu in both the arthropod and mollusc hemocyanins. As seen in Figure 1A, the copper content parallels the oxygen binding ability for Limulus. In



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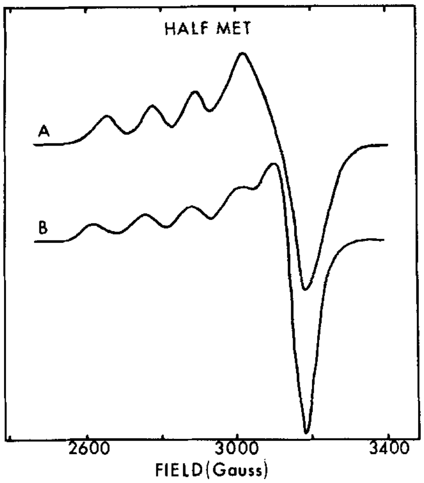

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Figure 2. EPR spectra of met apo derivatives. (A) pH = 5.7 acetate buffer, .01M NaNO$_2$; (B) pH = 5.7 acetate; (C) pH = 8.0 tris buffer, prepared by oxidation with NaO$_2$.

Figure 3. EPR spectra of half met derivatives. (A)pH = 5.7 acetate buffer, .01M NaNO$_2$; (B) pH = 5.7 acetate buffer.

Figure 1B, though, it is seen that almost complete loss of oxygen binding is obtained when 50% of copper has been removed.[8] This demonstrates that one copper is selectively removed from the binuclear active site of Busycon, yielding a half apo protein. This form shows no EPR signal, consistent with the copper being in a +1 oxidation state.

We are able to oxidize the single copper active site of this half apo derivative using a number of small molecule oxidizing agents. Incubation with excess NaNO$_2$ produces the EPR signal shown in Figure 2A. Double integration shows that approximately 50% of the sites are oxidized. Removal of the nitrite by dialysis against acetate buffer leads to a change in the EPR signal as shown in Figure 2B. The EPR spectrum given in Figure 2C shows the result of increasing the pH (0.1 M tris, pH 8.0 buffer); this

TABLE I

EPR PARAMETERS of MET APO and HALF MET DERIVATIVES

	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$ ($10^{-4}cm^{-1}$)
MET APO			
pH = 5.7 acetate, .01M $NaNO_2$	2.309	2.097	124
pH = 5.7 acetate	2.314	2.083	131
pH = 8.0 tris	2.200	2.082	220
HALF MET			
pH = 5.7 acetate, .01M $NaNO_2$	2.302	2.096	125
pH = 5.7 acetate	2.318	2.080	141

pH change is reversible. Regeneration to oxyhemocyanin is achieved by reduction and metal reincorporation. Further, we have also been able to generate the high pH met apo derivative via autooxidation at elevated temperatures, and with NaO_2, obtaining an EPR spectrum (Figure 2D) identical with that shown in Figure 2C. Finally, the half apo protein can be oxidized by treatment with hydrogen peroxide.

As previously reported[4], we have prepared the singly oxidized form of the holoprotein with a variety of ligands tightly bound at the active site [Cu(I)...Cu(II)L]. The EPR spectra of two relevant half met derivatives are presented in Figure 3 for comparison to the analogous met apo forms. The first point to make is that the EPR spectra of the parallel forms are extremely similar. (EPR parameters are given in Table I.) This shows that either the same copper is oxidized in both forms and that the Cu(I) does not play a significant role in electron delocalization and site structure formation or that the two coppers in the active site have very similar ligand environments which are not greatly affected by the presence of a Cu(I) or removal of one copper. The most significant difference between met apo-L and half met-L is in ligand binding ability. Anions bind tightly to the half met derivative and can only be displaced by competition against stronger ligands. In the met apo forms, however, all bound ligands can be removed by dialysis. Thus, the presence of the Cu(I) leads to large increases in the ligand binding constants of the site. This strongly suggests that the Cu(I) plays an active part in binding the ligand, perhaps through a Cu(I)-L-Cu(II) bridge. (The strong perturbations due to ligand variation on the EPR spectrum of the cupric ion in the half met-L active site demonstrate that the ligand is binding to the Cu(II).) Detailed spectroscopic studies of the derivatives are presently underway.

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