$$[AH^+]_1 = \frac{(1 + K_S[HSO_3^-]_0)C_0}{(1 + K_S[HSO_3^-]_1)\{1 + (K_S + K_{S'})[HSO_3^-]_0\}} \quad \text{(VIII)}$$

Thus, the amplitude for the fast step (formation of the σ adduct S) is given by $[AH^+]_0 - [AH^+]_1$:

$$[AH^+]_0 - [AH^+]_1 = \frac{K_S([HSO_3^-]_1 - [HSO_3^-]_0)C_0}{(1 + K_S[HSO_3^-]_1)\{1 + (K_S + K_{S'})[HSO_3^-]_0\}} \quad \text{(IX)}$$

The amplitude for the slow step (formation of the σ adduct S') is given by $[AH^+]_1 - [AH^+]_f$:

$$[AH^+]_1 - [AH^+]_f = [K_{S'}([HSO_3^-]_f - [HSO_3^-]_0)C_0]/[\{1 + (K_S + K_{S'})[HSO_3^-]_0\}\{K_SC_0 + [1 + (K_S + K_{S'})[HSO_3^-]_f][1 + K_S[HSO_3^-]_1]\}] \quad \text{(X)}$$

For a typical SO_2-jump experiment C_0 is 3×10^{-5} M and $[HSO_3^-]_1 \simeq [HSO_3^-]_f = 2 \times 10^{-5}$ M. If we first assume that $K_{S'} = K_S = 1.1 \times 10^5$ M^{-1}, the ratio of the fast to the slow relaxation amplitudes is about 6. In this case, the amplitude of the slow relaxation signal is high enough and can be measured. If now $K_{S'}$ is much lower than K_S, for instance, $K_{S'} = K_S/10 = 1.1 \times 10^4$ M^{-1}, the above ratio increases to about 40. Since the amplitude of the fast signal remains constant, the slow relaxation is no longer detectable.

Chemical and Spectroscopic Comparison of the Binuclear Copper Active Site of Mollusc and Arthropod Hemocyanins

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Abstract: A series of active-site derivatives of hemocyanin have been reported which allow the binuclear copper active site to be chemically varied in a reasonably controlled manner. These include the met apo [Cu(II)-], half-met [Cu(II)Cu(I)], dimer (EPR-detectable met) [Cu(II)Cu(II)], and met (EPR-nondetectable met) [Cu(II)···Cu(II)] forms. Detailed spectroscopic study of these has demonstrated that the binuclear cupric active site of oxyhemocyanin contains both an endogenous and exogenous ligand bridge with the peroxide binding in a μ-dioxo fashion between the equatorial planes of both tetragonal coppers. Extension of these chemical and spectral studies to a series of five molluscs (*Busycon canaliculatum*, *Lunatia heros*, *Megathura crenulata*, *Octopus bimaculatus*, and *Helix pomatia*) and five arthropods (*Cancer borealis*, *Cancer irroratus*, *Cancer magister*, *Homarus americanus*, and *Limulus polyphemus*) has shown the active sites in both phyla to be quite similar; however, significant differences are observed. All the arthropod forms exhibit similar spectral features which quantitatively differ from those of the molluscs. These electronic structural differences indicate that the arthropod active site is distorted from that of the mollusc. This distortion strongly affects exogenous ligand binding at the binuclear copper site and, by extension, the peroxide regeneration of met to oxy (the arthropods have a much lower catalase activity than the molluscs). Further, the arthropods (excluding *Limulus*) are found to have an unstable active site which is irreversibly disrupted by group 2 ligands (those which break the endogenous bridge by binding the coppers with a >5 Å M-M distance). This active-site instability seems to be associated with a strain induced by the protein ligand. Finally, the *Limulus* oxy active site is found to differ from that of molluscs and other arthropods in terms of access to an axial coordination position for peroxide displacement reactions ($k_{\text{arthropods}} > k_{\text{molluscs}} \gg k_{Limulus}$).

Hemocyanin is the binuclear copper-oxygen binding protein (10_2:2Cu)[1] found in arthropods and molluscs. In the oxy form the oxygen has been shown to bind as peroxide,[2] and therefore the coppers are formally copper(II). However, this active site exhibits rather unique spectral features compared to simple inorganic cupric complexes (λ 345 nm, $\epsilon \approx 20000$ M^{-1} cm^{-1}; λ 570 nm, $\epsilon \approx 1000$ M^{-1} cm^{-1}; no EPR signal). These have recently been interpreted based on spectroscopic studies of a series of active-site derivatives[3,4] (vide infra), the 345- and 570-nm absorption features being peroxide to copper(II) charge-transfer transitions and the lack of an EPR signal being due to antiferromagnetic coupling between the coppers via an endogenous protein bridge. While the optical spectra of the oxy forms of the arthropods and molluscs are generally similar, specific differences have been stressed between the two phyla in terms of absorption band energies and, in particular, their CD spectra.[5] A second difference between the oxy forms of the two phyla which has been reported is that only the molluscs undergo ligand displacement of the peroxide, based on negative results for *Limulus polyphemus* (an arthropod).[6] However, we have recently shown that *Limulus*, which is in a different subphylum from other arthropods, was unique in this regard and, in fact, the rest of the arthropods tested underwent the most facile ligand-displacement reactions.[7] The most emphasized general difference between the arthropods (A) and molluscs (M) is related to their catalase activity.

$$2H_2O_2 \xrightarrow{Hc_M} 2H_2O + O_2 \quad (1)$$

Only the molluscs exhibit high catalase activity,[8] being regenerable

(1) A. G. Redfield, T. Coolidge, and H. Montgomery, *J. Biol. Chem.*, **76**, 197 (1928).
(2) T. B. Freedman, J. S. Loehr, and T. M. Loehr, *J. Am. Chem. Soc.*, **98**, 2809 (1976).
(3) R. S. Himmelwright, N. C. Eickman, and E. I. Solomon, *J. Am. Chem. Soc.*, **101**, 1576 (1979).
(4) N. C. Eickman, R. S. Himmelwright, and E. I. Solomon, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 2094 (1979).
(5) K. Nickerson and K. E. van Holde, *Comp. Biochem. Physiol.*, **39B**, 855 (1971).
(6) R. Witters and R. Lontie, *FEBS Lett.*, **60**, 400 (1975).
(7) A. F. Hepp, R. S. Himmelwright, N. C. Eickman, and E. I. Solomon, *Biochem. Biophys. Res. Commun.*, **89**, 1050 (1979).
(8) F. Ghiretti, *Arch. Biochem. Biophys.*, **63**, 165 (1956).

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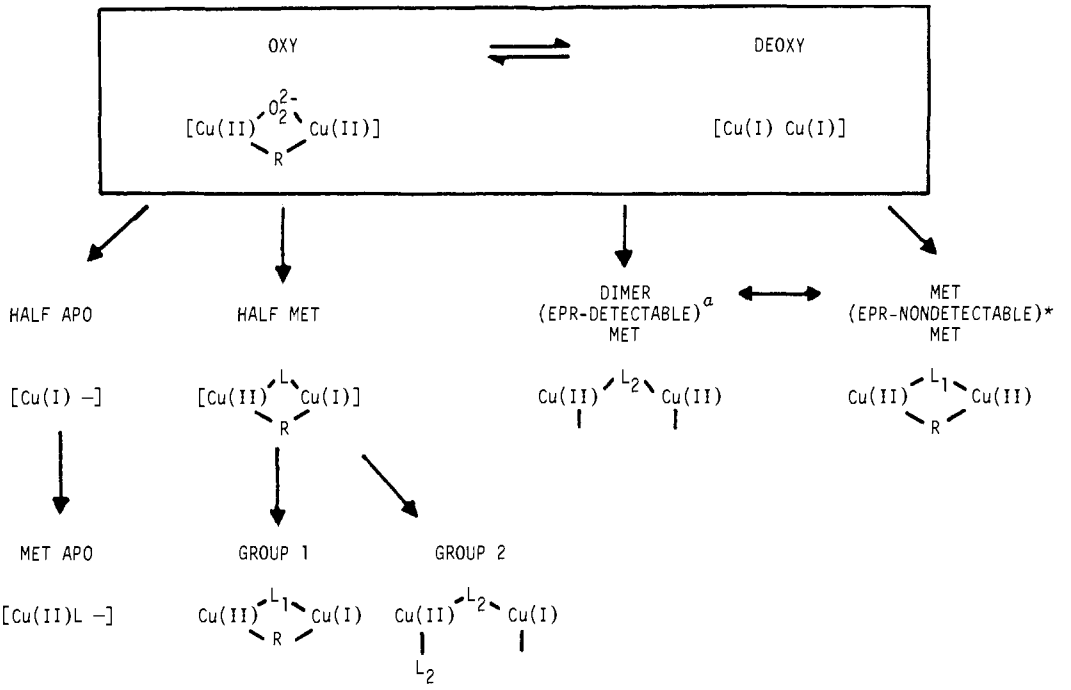
Scheme I



[a] While the nomenclature "dimer" and "met" was used in ref 4, their interconversion (ref 13) leads to the alternative nomenclature "EPR-detectable met" and "EPR-nondetectable met" suggested at the European Molecular Biology Meeting on "Comparative Study and Recent Knowledge on Quaternary Structure and Active Sites of Oxygen Carriers and Related Proteins", Tours, Aug. 20-24, 1979.

to oxyhemocyanin from forms other than deoxy.[9] Additionally, it has been shown that the metal removal by cyanide from *Busycon canaliculatum* (mollusc) differs from *Limulus polyphemus* (arthropod) in that only for the mollusc protein can one copper be removed which leads to a half-apo [Cu(I)–] derivative.[10] Finally, differences in subunit molecular weight, aggregation states of the subunits, and oxygen binding behavior (P_{50} and Bohr effects) have been studied in some detail for the different hemocyanins.[11]

In a series of recent papers, we have reported the preparation and characterization of a series of active-site derivatives of *Busycon* hemocyanin which allowed this active site to be systematically varied for the purpose of spectroscopic study.[3,4,10,12,13] These derivatives are summarized in Scheme I. Chemical and spectroscopic studies of these forms have generated the effective active site of oxyhemocyanin presented in Figure 1. The purpose of this paper is to extend these studies of *Busycon* to a series of mollusc and arthropod hemocyanins to determine their generality and the nature of correlations that exist between phyla. We will present evidence that demonstrates that the active sites of all oxyhemocyanins are quite similar. However, important differences are observed in these chemical and spectral properties which directly relate to the large difference in catalase activity and protein ligand effects on the active site and which indicate significant variation in accessibility for axial coordination at the binuclear copper active site (arthropods > molluscs ≫ *Limulus*).

Experimental Section

Hemolymph was obtained from *Busycon canaliculatum*, *Lunatia heros*, *Helix pomatia*, and *Megathura crenulata* by foot puncture; from *Limulus polyphemus* and *Homarus americanus* by heart puncture; and from *Cancer borealis*, *Cancer irroratus*, and *Cancer magister* by removal of several legs. The *Octopus bimaculatus* hemolymph was purchased from Pacific Bio-Marine Laboratories, Venice, CA. The hemocyanin was isolated and purified by ultracentrifugation and extensive dialysis at 4 °C in pH 6.3 phosphate or pH 8.2 Tris buffer.

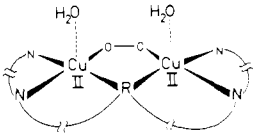


Figure 1. Structural representation of the oxyhemocyanin active site.

The half-apo and met apo forms of mollusc hemocyanins were prepared as previously described.[10] Ligand substitution reactions of met apo were carried out by dialyzing aliquots of protein in buffer solutions containing the appropriate excess of added ligand.

Half-met hemocyanin[3] was obtained by treatment of oxyhemocyanin with excess $NaNO_2$ and excess ascorbic acid as described under Results. Ligand substitution reactions were performed either by direct addition of buffered ligand solutions or dialysis in buffer solutions containing the appropriate excess of ligand. Carbon monoxide reactions were carried out in a high-pressure reaction vessel by equilibration of ~2.0 mL of protein solution with 30 psig of CO for 30 min. Spectra were recorded after removal of CO and exposure to the atmosphere.

Dimer (EPR-detectable met) hemocyanin was prepared by treatment of deoxy with NO[14a] and trace amounts of O_2[14b] or by the addition of a 100-fold excess of $NaNO_2$ to met[13] at pH <7. The dimer forms were reacted with a 50- to 100-fold excess of N_3^- (direct addition) at room temperature to produce the dimer–N_3^- forms.[13]

Met hemocyanin (EPR-nondetectable met) of the molluscs was prepared by incubation of oxy at 37 °C with a 100-fold excess of N_3^- or F^- for 48 h[6] in pH 6.3 phosphate buffer. The arthropod met forms were obtained by oxidation of deoxy with a fivefold excess of H_2O_2.[9] Direct addition of ligand solutions was used to effect ligand substitution in the met forms.

Diethylenetriamine (dien) and 1,4,8,11-tetraazacyclotetradecane (2,3,2-tet) were purchased from Strem Chemicals, Newburyport, Mass. The copper complexes were prepared by the addition of stoichiometric $CuSO_4$ to a pH 7.2 Tris (SO_4^{2-}) buffered aqueous solution of each ligand, followed by titration with NaN_3.

Ligand displacement reactions[7] of peroxide by N_3^- and SCN^- from

(9) G. Felsenfeld and M. P. Printz, *J. Am. Chem. Soc.*, **81**, 6259 (1959).
(10) R. S. Himmelwright, N. C. Eickman, and E. I. Solomon, *Biochem. Biophys. Res. Commun.*, **81**, 243 (1978).
(11) K. E. van Holde and E. F. J. van Bruggen in "Subunits in Biological Systems", Part A, S. N. Timasheff and G. D. Fasman, Eds., Marcel Dekker, New York, 1971, pp 1-53.
(12) R. S. Himmelwright, N. C. Eickman, and E. I. Solomon, *Biochem. Biophys. Res. Commun.*, **84**, 300 (1978).
(13) R. S. Himmelwright, N. C. Eickman, and E. I. Solomon, *Biochem. Biophys. Res. Commun.*, **86**, 628 (1979).
(14) (a) A. J. M. Schoot Uiterkamp, *FEBS Lett.*, **20**, 93 (1972). (b) H. van der Deen and H. Hoving, *Biochemistry*, **16**, 3519 (1977).

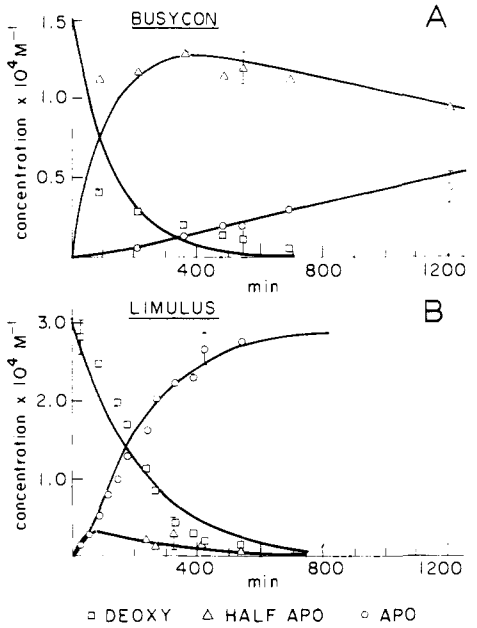


Figure 2. Predicted (solid lines) and actual (symbols) time dependence of the concentration of (A) *Busycon* and (B) *Limulus* deoxy [Cu(I)Cu(I)], half-apo [Cu(I)–], and apo [– –] forms observed during removal of the copper with cyanide.

oxyhemocyanin were performed at 4 °C in pH 6.3 phosphate buffer. Large enough volumes (10–15 mL) of oxyhemocyanin were used so that aliquots could be removed to record spectra at various reaction times.

EPR spectra were recorded on a Varian E-9 spectrometer using an Air Products, Helitran liquid helium flow system. Variable temperature optical spectra were obtained by using a Cary 17 spectrometer and a Spectrum II cryocooler. Sample forms and conditions have been previously described.[3,4]

Results

A comparison of the chemical and spectral properties of the met apo, half-met, met (EPR-nondetectable met), dimer (EPR-detectable met) and oxy forms of five mollusc (*Busycon canaliculatum*, *Lunatia heros*, *Megathura crenulata*, *Octopus bimaculatus*, and *Helix pomatia*) and five arthropod (*Cancer borealis*, *Cancer irroratus*, *Cancer magister*, *Homarus americanus*, and *Limulus polyphemus*) hemocyanins has been performed. A general result of these comparisons is that all the molluscs exhibited very similar properties; thus, the results presented below for *Busycon canaliculatum* are representative of all molluscs studied. For the arthropods, the results for *Cancer borealis* are representative of those for *Cancer irroratus*, *Cancer magister*, and *Homarus americanus*. However, *Limulus polyphemus*, which is in a different subphylum (Chelicerata) from the other arthropods (Mandibulata), exhibited different chemical properties; therefore, the results, when significantly different on this hemocyanin, will be presented independently of the other arthropods.

Met Apo Hemocyanin. One general difference which is observed in a comparison of mollusc and arthropod hemocyanins is based on metal binding to the protein ligand. Only for the mollusc hemocyanins can one metal be preferentially removed per binuclear copper site, producing the half-apo derivative (Scheme I). Figure 2A presents the metal-removal curves for *Busycon* which are representative of the mollusc hemocyanins. Here we plot the relative concentrations of deoxy, half-apo, and apo forms present after increasing dialysis times against cyanide (250-fold excess in pH 8.2 Tris buffer, 0.1 M $CaCl_2$). These were obtained from the protein concentration, the total copper content (by atomic absorption), and the amount of deoxy (by the oxygen-binding ability remaining from the decrease in intensity at 345 nm of the optical spectrum). The solid lines were obtained from a pseudo-first-order kinetic analysis (eq 2), where $k_1 = 0.48\ h^{-1}$ and k_2

$$\text{deoxy[Cu(I)Cu(I)]} \xrightarrow{k_1} \text{half-apo[Cu(I)–]} \xrightarrow{k_2} \text{apo[– –]} \quad (2)$$

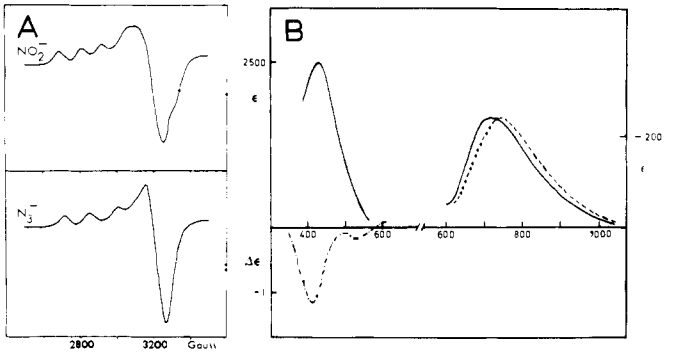


Figure 3. *Busycon* met apo spectra: (A) EPR spectra (77 K, pH 6.3 phosphate buffer, 30 × NO_2^-, 100 × azide); (B) absorption (–) and circular dichroism (–•–) spectra of met apo-N_3^-. The optical absorption spectra of met apo-aquo (- - -) has also been included. (Spectra in the 400–600 nm region recorded at room temperature and in the 600–1000 nm region at ~15 K.)

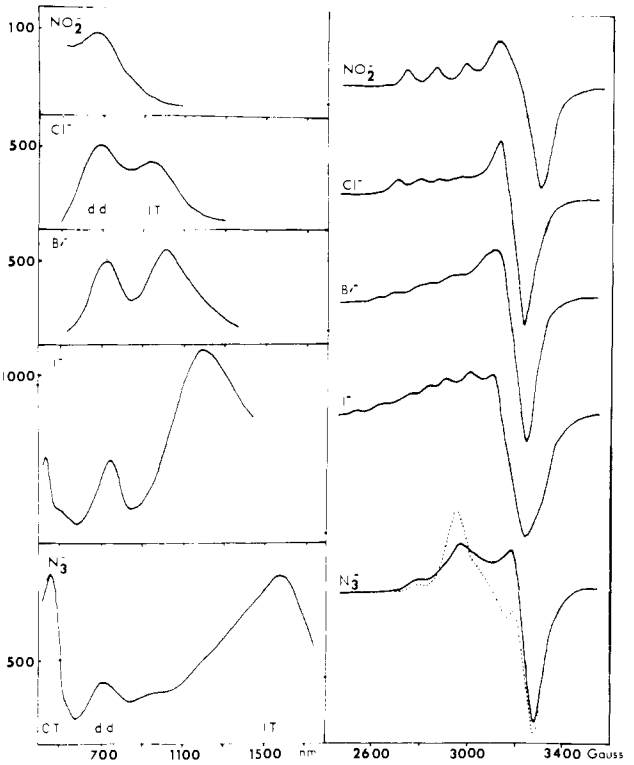


Figure 4. Absorption spectra (~15 K, pH 6.3 phosphate buffer in 1:1 sucrose glass) and EPR spectra (77 K) of the half-met-L series of *Busycon canaliculatum* hemocyanin derivatives. L is indicated for each spectrum. The EPR spectrum (7 K) of the N_3^- form is indicated by a dotted line.

$= 0.024\ h^{-1}$. Thus, for the molluscs, the rate of removal of the second copper is about an order of magnitude less than the first. A very different behavior is observed for the arthropods;[10] for *Limulus*, Figure 2B and eq 2 give $k_1 = 0.27\ h^{-1}$ and $k_2 = 1.8\ h^{-1}$, while other arthropods exhibit a more complicated kinetic behavior which includes cyanide binding.[15]

The relative stability of the second copper in the molluscs allows one to obtain a protein sample containing predominantly half-apo sites (dialysis time against CN^- = 12.5 h gives ~70% half-apo sites in *Busycon*). This half-apo protein can be oxidized almost quantitatively (70–90% from EPR integration), resulting in a preparation of the single copper(II)-containing met apo [Cu(II)–] form.

This met apo [Cu(II)–] derivative undergoes labile ligand

(15) B. Salvato, M. Beltramini, F. Ricchelli, and L. Tallandini in "Invertebrate Oxygen-Binding Proteins: Structure, Active Site and Function", J. Lamy and J. Lamy, Eds., Marcel Dekker, New York, to be published.

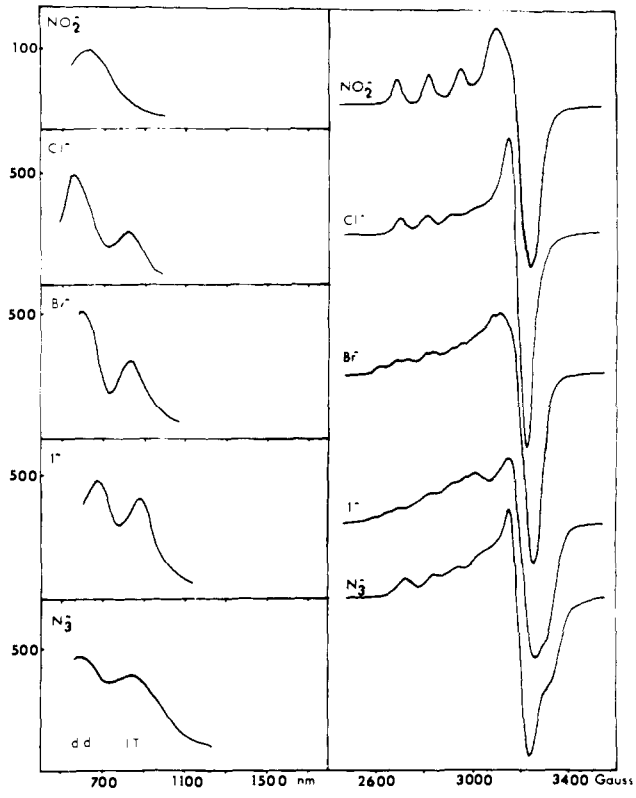


Figure 5. Absorption spectra (~15 K, pH 6.3 phosphate buffer in 1:1 sucrose glass) and EPR spectra (77 K) of the half-met-L series of *Cancer borealis* hemocyanin derivatives. L is indicated for each spectrum.

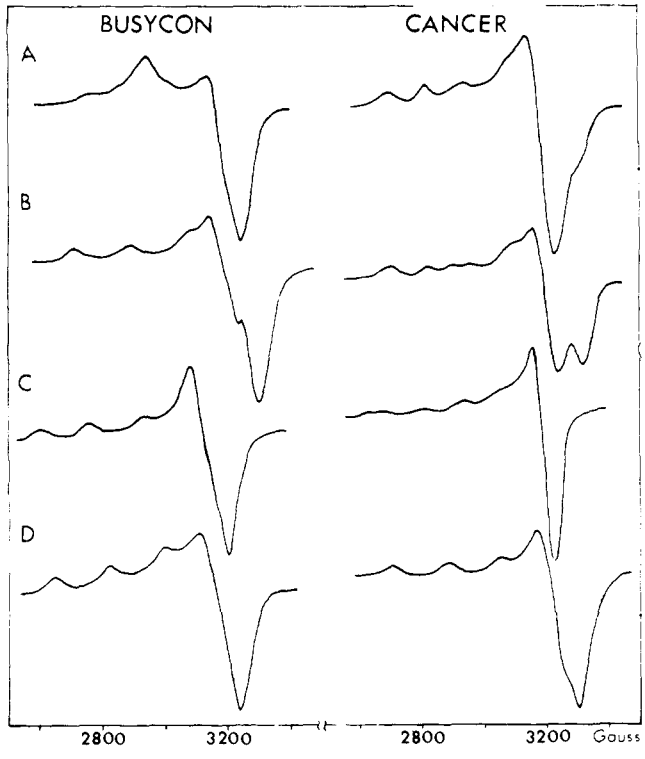


Figure 6. EPR spectra of (A) half-met-N_3^- and (B) half-met-N_3^- after treatment with carbon monoxide (77 K, pH 6.3 phosphate buffer), (C) half-met-CN^- (77 K, pH 8.0 phosphate buffer), and (D) half-met-CN^- in 100-fold excess cyanide of *Busycon canaliculatum* and *Cancer borealis* hemocyanin.

substitution chemistry[3] characteristic of one equatorial coordination position at a tetragonal copper(II) site.[16] Thus, equatorial

(16) F. Basolo and R. G. Pearson in "Mechanisms of Inorganic Reactions", 2nd ed, Wiley, New York, 1967, pp 421–423.

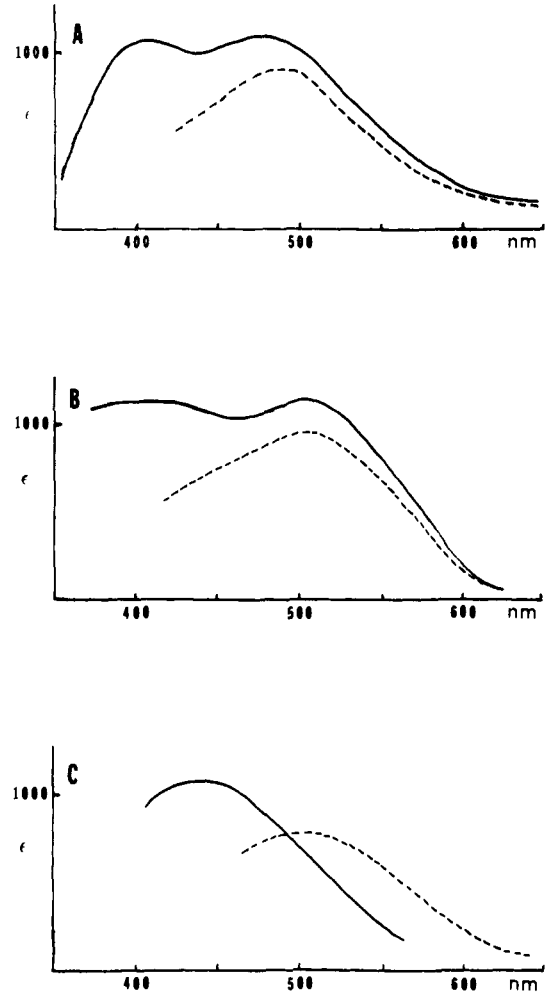


Figure 7. Absorption spectra (room temperature, pH 6.3 phosphate buffer, presented as difference spectra) of half-met-N_3^- (---) and half-met-N_3^- in 100-fold excess azide (—) of (A) *Busycon*, (B) *Limulus*, and (C) *Cancer* hemocyanin.

substitution of N_3^- on the nitrite (or any other met apo) form leads to the shifts in the EPR spectra shown in Figure 3A (NO_2^-: $g_{\|} = 2.309$, $g_{\perp} = 2.097$, $A_{\|} = 124 \times 10^{-4}$ cm^{-1}; N_3^-: $g_{\|} = 2.240$, $g_{\perp} = 2.058$, $A_{\|} = 152 \times 10^{-4}$ cm^{-1}). Coordination of N_3^- also produces the $N_3^- \rightarrow$ Cu(II) charge-transfer transitions shown in absorption and CD in Figure 3B, which allows a determination of the met apo-N_3^- binding constant of $k \approx 100$ M^{-1}. Finally, small shifts are observed ($\Delta\nu \sim 400$ cm^{-1}) in the d–d transitions of the met apo-aquo derivative upon coordination of the azide (Figure 3B).

Half-Met Hemocyanin. The half-met-NO_2^- derivative of both *Busycon* and *Limulus* hemocyanins is obtained by treatment of oxyhemocyanin in pH 6.3 phosphate buffer with a tenfold excess of ascorbic acid and nitrite. *Cancer* half-met-NO_2^- could only be obtained in pure form by initial preparation with 10-fold excess NO_2^- and 20-fold excess ascorbic acid in pH 6.3 phosphate buffer. (The excess ascorbic acid affects reduction of dimer sites produced by excess NO_2^- back to half-met-NO_2^-, vide infra; see "Dimer" under Results.) Dialysis to remove excess nitrite and ascorbic acid was then performed at pH 8.0 phosphate buffer, to prohibit further generation of NO, followed by dialysis to pH 6.3 phosphate buffer.

The series of half-met-L forms, where L = NO_2^-, $CH_3CO_2^-$, F^-, Cl^-, Br^-, I^-, aquo, N_3^-, SCN^-, and CN^-, has been generated for the mollusc and arthropod hemocyanins. The ligand substitution chemistry is similar for the half-met derivatives of all species investigated. However, in general, *Lumulus* derivatives required as much as a 1000-fold excess of ligand to effect substitution compared to the 5- to 100-fold excesses used in the other arthropod and mollusc forms. The chemistry of the half-met forms

of the arthropods (excluding *Limulus*) is further complicated by an irreversible disrutpion of the site (see later following discussion in this section) observed when treated with a >10-fold excess of N_3^- (or any excess of SCN^-). Therefore, to obtain the half-met-N_3^- form, N_3^- had to be added in less than 10-fold excess.

The EPR and optical absorption spectra of a series of half-met-L forms, where L = NO_2^-, Cl^-, Br^-, I^-, and N_3^-, of *Busycon* and *Cancer* (generally similar to *Limulus*) hemocyanin are presented in Figures 4 and 5, respectively. After dialysis of these forms for 72 h in pH 6.3 phosphate buffer, very little change is observed in the EPR spectra. This is found for all exogenous ligands and indicates that these ligands remain tightly coordinated to the half-met [Cu(II)Cu(I)] active site. The effects of carbon monoxide on the EPR spectra of the half-met-N_3^- derivatives of *Busycon* and *Cancer* hemocyanin are presented in Figure 6A,B, respectively. The half-met-N_3^- forms all undergo a reversible change upon treatment with 30 psi of carbon monoxide for 30 min. Evacuation, followed by flushing with nitrogen several times, results in recovery of the original half-met-N_3^- spectrum in each case.

The half-met-X, where X = NO_2^-, Cl^-, Br^-, and I^-, spectra are qualitatively similar for *Busycon* and *Cancer*. The d–d transitions are observed between 550 and 720 nm, and intervalence transfer (IT) transitions are found at lower energy. The energies of the d–d and IT transitions decrease in energy over the series $Cl^- > Br^- > I^-$, and the intensity of the IT transition increases with $Cl^- < Br^- < I^-$. Accompanying this trend is an increase in the complexity of the EPR spectra, with half-met-I^- having six observable lines in the $g_{\|}$ region. It is seen that these class 2 mixed valent properties[17] correlate with the ability of the exogenous ligand to provide an effective pathway for electron delocalization between the copper(II) and copper(I).

However, significant quantitative differences do exist among these three sets of half-met-X spectra. In particular, the transitions occur at higher energy for arthropod derivatives as compared to the mollusc spectra, and the IT transitions are most intense in the mollusc half-met derivatives. (The arthropod derivatives are similar to one another, but *Limulus* does have more intense IT transitions.) Thus, the exogenous ligand provides the most effective pathway for electron delocalization between the coppers in the mollusc half-met-X forms. Similarly, in the half-met-N_3^- spectra, only the molluscs show a discontinuous shift in the absorption and EPR spectra with a decrease in temperature leading to an intense IT transition. Only very weak IT transitions are observed in the arthropod half-met-N_3^- spectra.

Exogenous ligands have been placed[3] in two groups based on coordination of a second ligand to the half-met *Busycon* active site. Both group 1 (L_1 = NO_2^-, F^-, Cl^-, Br^-, I^-, $CH_3CO_2^-$, and aquo) and group 2 (L_2 = CN^-, N_3^-, SCN^-) ligands are found to bind tightly to the half-met site of both mollusc and arthropod hemocyanins. In parallel to what has been reported in *Busycon*, the addition of a 100-fold excess of any group 1 ligand to a half-met-L_1 derivative of all molluscs and arthropods results in no spectral change, indicating that a second group 1 ligand does not bind to the site. Treatment of group 2 half-met derivatives with excess L_2 results in large changes in the EPR spectra. This is shown for half-met-CN^- in Figure 6C,D. These changes indicate the binding of an additional group 2 ligand by all mollusc and arthropod half-met forms.

However, a very significant difference in the group 2 ligand-binding behavior (for N_3^- and SCN^-) is demonstrated by the optical spectra of the half-met-N_3^- forms shown in Figure 7. All three hemocyanins exhibit a low-energy $N_3^- \rightarrow$ Cu(II) charge-transfer (CT) transition at ~500 nm associated with tightly bound bridging N_3^-. The *Busycon* and *Limulus* half-met-N_3^- forms coordinate a second N_3^- (group 2 behavior) with a binding constant similar to that observed for mononuclear copper(II) complexes and for the met apo [Cu(II)–] form. This is associated with the appearance of a second $N_3^- \rightarrow$ Cu(II) CT transition at higher energy (~400 nm) (unbridged equatorial coordination) in addition to the 500-nm band which remains in the presence of a 100-fold excess N_3^- (Figure 7A,B). For *Cancer* half-met-N_3^-, however, the 500-nm transition is irreversibly replaced by a transition at ~400 nm in the presence of a 100-fold excess of N_3^-. Loss of the 500-nm $N_3^- \rightarrow$ Cu(II) CT transition indicates that N_3^- is no longer bridging. Further, upon dialysis, all the $N_3^- \rightarrow$ Cu(II) CT intensity disappears, demonstrating that the N_3^- is no longer tightly bound at the active site and that the active site has been disrupted. This is also supported by the EPR spectral changes presented in Figure 8. Both N_3^- and SCN^-, when added in 100-fold excess to half-met-NO_2^-, result in ligand displacement of coordinated NO_2^-. However, upon dialysis to remove excess N_3^- or SCN^- for only 24 h, both half-met forms of the non-*Limulus* arthropods exhibit the same EPR spectrum (Figure 8D). This is in contrast to the mollusc and *Limulus* forms which would remain half-met-N_3^- and -SCN^- and again indicates that the site has been disrupted by excess N_3^- or SCN^- and no longer strongly binds these ligands in a bridging mode. Finally, carbon monoxide treatment of the *Cancer* half-met in the presence of 100-fold N_3^- produces very little change in the EPR spectrum, in contrast to the CO effect shown earlier (Figure 6B) for half-met-N_3^- prepared with low excess N_3^-. All of these observations indicate that the excess N_3^- (or SCN^-) has irreversibly disrupted the half-met active site.

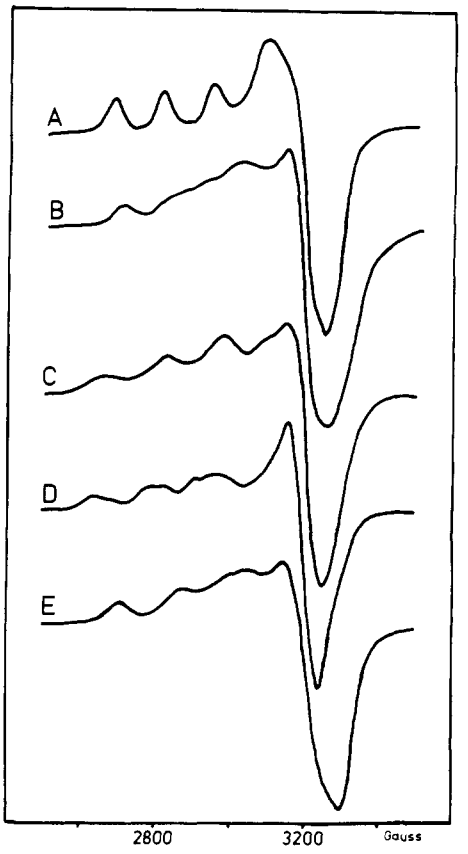


Figure 8. EPR spectra (77 K, pH 6.3 phosphate buffer) of *Cancer* hemocyanin: (A) half-met-NO_2^-, (B) half-met-NO_2^- after addition of 100-fold excess azide, (C) half met-NO_2^- after addition of 100-fold excess thiocyanate, (D) 24-h dialysis of the protein samples in B or C, and (E) as in B after treatment with carbon monoxide.

The half-met derivatives of *Busycon* and *Limulus*, but not *Cancer*, hemocyanin can be regenerated to oxyhemocyanin by reduction with $Na_2S_4O_4$. Treatment of the half-met-$CH_3CO_2^-$ or half-met-aquo forms with 10-fold excess $Na_2S_2O_4$ in pH 6.3 phosphate buffer under nitrogen results in reduction of the copper(II) to copper(I) as shown by almost complete loss of the EPR intensity for each protein. After dialysis in pH 6.3 phosphate buffer, followed by exposure to oxygen, the 345-nm absorption band in the optical spectra associated with oxyhemocyanin is recovered (>90%) for *Busycon* and *Limulus*. However, although

(17) M. B. Robin and P. Day, *Adv. Inorg. Chem. Radiochem.*, **10**, 247 (1967).

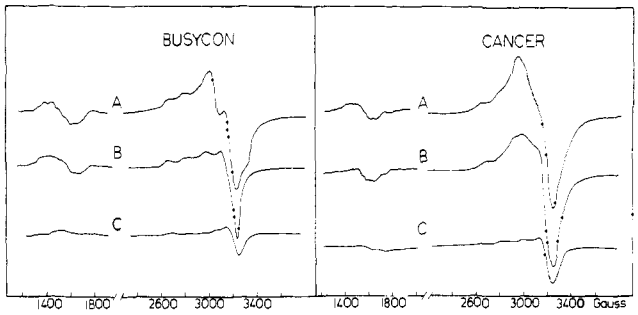


Figure 9. EPR spectra (77 K, pH 5.7 acetate buffer) of (A) NO-generated dimer, (B) NO-generated dimer after addition of 100-fold excess azide, and (C) 24-h dialysis of the protein sample in B for *Busycon canaliculatum* and *Cancer borealis* hemocyanin (10-mW power in g = 2, 100-mW power in g = 4).

no increase in EPR intensity is observed after dialysis of the *Cancer* half-met aquo that was reduced with dithionite, no increase in absorption intensity at 340 nm is observed. This indicates that the *Cancer* half-met forms cannot be regenerated to oxyhemocyanin by this method, an effect which may be related to the tendency of the arthropod, but not the *Limulus*, active site to be disrupted.

Dimer (EPR-Detectable Met) Hemocyanin. Dimer hemocyanin is obtained either by treatment of deoxyhemocyanin with nitric oxide in the presence of trace amounts of oxygen[14] or by the addition of a 100-fold excess of $NaNO_2$ at pH <7 to met hemocyanin.[13] The dimer derivative can also be generated by the addition of excess $NaNO_2$ at pH <7 in the presence of oxygen to half-met-NO_2^- hemocyanin.[13] The dimer EPR spectra of *Busycon* and *Cancer* are shown in Figure 9. The EPR spectrum of *Limulus* is very similar to that of the other dimer anthropod spectra, and these differ significantly from the dimer spectra of the molluscs. Although accurate quantitation of the NO + O_2 reaction is difficult, preparation of *Busycon* and *Limulus* dimer by the addition of excess NO_2^- to half-met-NO_2^- requires much higher concentrations (>200-fold excess) of $NaNO_2$ than do the arthropods (<50-fold excess).

The dimer derivatives of all three hemocyanins undergo a reaction with azide. Figure 9B shows the EPR spectra for each obtained after treatment of the NO dimer with 100-fold excess NaN_3. A reduction in intensity is observed along with formation of the dimer-N_3^- form. This reduction is greatest for the mollusc form (57% reduction for *Busycon*, 28% for *Limulus*, 20% for *Cancer*). The dimer-N_3^- form is found to be stable for >100 h but can be further converted to EPR-nondetectable met (as can the original dimer forms) by dialysis for ~12 h to remove excess ligand. The residual EPR spectra (Figure 9C) observed after dialysis is due to half-met necessarily present in the preparation (14% residual *B*, 19% *L*, 20% *C*). All significant intensity is lost in the $g \sim 4$ region upon dialysis of the dimer and dimer-N_3^- forms. For *Busycon*, the met obtained after dialysis can be regenerated to oxy by the addition of a fivefold excess of H_2O_2.

For *Cancer*, a dimer-N_3^- form can also be obtained from the peroxide-generated met, independent of any NO being present (see Results, "Met"). Both have EPR spectra (Figures 9B and 10C) which are very similar, demonstrating that N_3^- has displaced any N_xO_y exogenous ligand by ligand substitution on the NO-dimer. Thus, dimer-N_3^- represents a fairly well-defined EPR-detectable binuclear cupric form of type 3 copper and is presently being subjected to detailed spectroscopic analysis.

Met Hemocyanin (EPR-Nondetectable Met). Met hemocyanin (EPR-nondetectable met), containing <10% EPR-detectable sites[13,18] (Figure 10A), is prepared for the molluscs by ligand displacement of peroxide from oxyhemocyanin with fluoride or azide[6] and for the arthropods (including *Limulus*) by 2e$^-$ oxidation of deoxy with peroxide.[9] Reaction of these EPR-nondetectable met forms with group 1 (F^-, Cl^-, Br^-, and $CH_3CO_2^-$) and group 2 (N_3^- and SCN^-) ligands has been investigated by EPR and optical spectroscopy.[7] Addition of a 100-fold excess of any of these ligands in pH 6.3 phosphate buffer to the *Limulus* met forms does not result in the appearance of EPR intensity. For the molluscs, weak variable (depending on anion, species, and pH) signals are observed in the g = 2 and g = 4 regions; however, these forms can be regenerated with peroxide to >80% oxyhemocyanin with no change in this signal.[13] Treatment of the arthropod (*Cancer* and *Homarus*) met forms with a 100-fold excess of N_3^- or SCN^- (group 2 ligands) produces an EPR spectrum that can account for up to 35% of total copper (Figure 10C,E,F). Addition of F^-, Cl^-, Br^-, and $CH_3CO_2^-$ (group 1 ligands) in molar ratios of 100 to copper, however, results in the appearance of only weak EPR signals, with Cl^- producing the largest signal which still accounts for only 6% of total copper after 36 h (Figure 10B). The reaction of *Cancer magister* met hemocyanin with N_3^- was first reported by McMahill and Mason[19] and was referred to as "springing" of the active site.

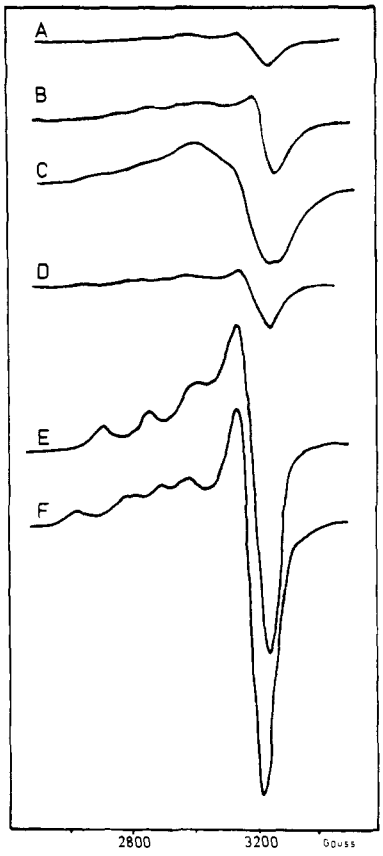


Figure 10. EPR spectra (77 K) of *Cancer* (A) methemocyanin, (B) met after addition of 100-fold excess Cl^-, (C) met 20 h after addition of 50-fold excess azide in pH 7.5 phosphate buffer, (D) 24-h dialysis of the protein sample in C in pH 7.5 phosphate buffer, (E) met 20 h after addition of 50-fold excess azide at low pH and (F) 24-h dialysis of the protein sample in E. Spectra A, B, E, and F recorded in pH 6.3 phosphate buffer.

The initial form produced on addition of N_3^- to *Cancer* met exhibits an EPR spectrum (Figure 10C) characteristic of dipolar coupled copper(II) ions which is extremely similar to that of dimer-N_3^- (Figure 9). This form can be stabilized in low excess (<10-fold) N_3^- or at pH >7. As shown in Figure 10C,D, this dimer form will convert back to EPR-nondetectable met on dialysis. In the presence of larger excesses of N_3^- or lower pH, the EPR spectrum readily changes to that of a mononuclear copper(II) ion (Figure 10E), which accounts for 30 ± 5% of total copper. On dialysis to remove excess N_3^-, the resulting EPR spectrum (Figure 10F) is found to be almost identical with that observed after removal of excess N_3^- from *Cancer* half-met-N_3^-

(18) N. Makino, H. van der Deen, P. McMahill, D. C. Gould, T. H. Moss, C. Simo, and H. S. Mason, *Biochim. Biophys. Acta*, **532**, 315 (1978).

(19) P. McMahill and H. S. Mason, *Biochem. Biophys. Res. Commun.*, **84**, 749 (1978).

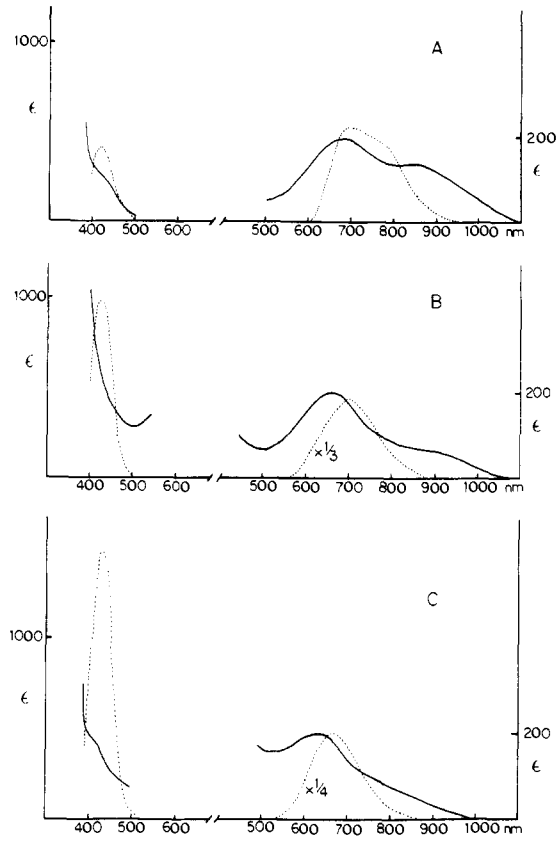


Figure 11. Absorption spectra of met-aquo hemocyanin (—) and oxyhemocyanin after subtraction of the 570-nm $O_2^{2-} \rightarrow Cu(II)$ CT transitions (···): (A) *Busycon*, (B) *Limulus*, and (C) *Cancer*.

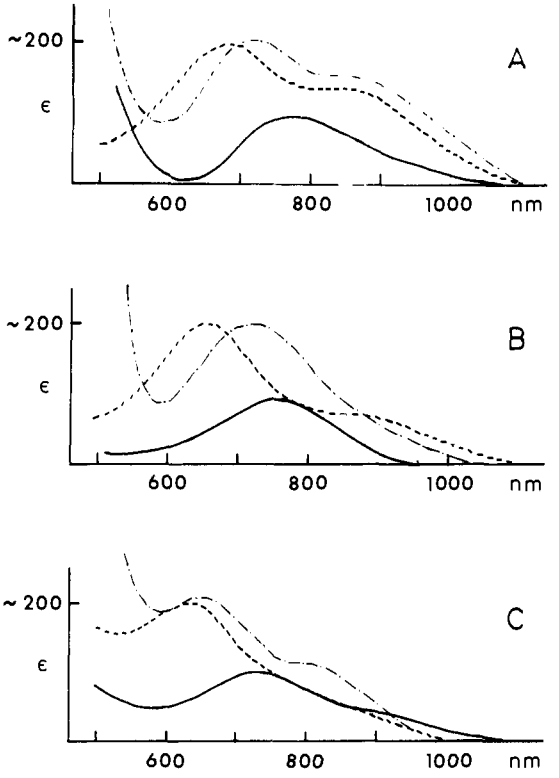


Figure 12. Absorption spectra (~15 K, pH 6.3 phosphate buffer in 1:1 sucrose glass) of met-aquo (---), met-fluoride (—) and met-azide (-•-) hemocyanin of (A) *Busycon*, (B) *Limulus*, and (C) *Cancer*.

(Figure 8D). This is again consistent with an irreversible disruption of the binuclear copper site.

The met forms of *Busycon*, *Limulus*, and *Cancer* are converted

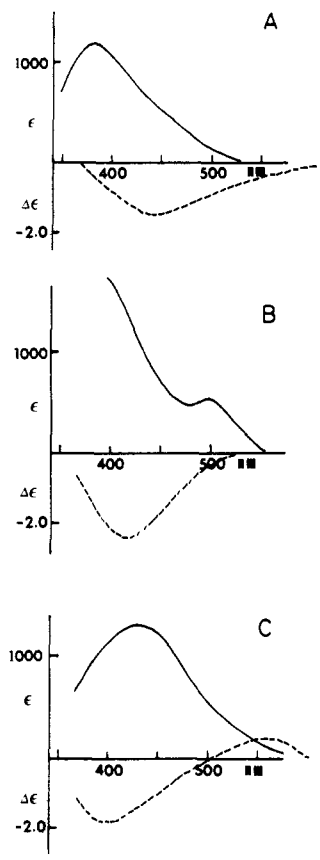


Figure 13. Absorption (—) and circular dichroism (---) spectra (room temperature, pH 6.3 phosphate buffer) of (A) *Busycon*, (B) *Limulus*, and (C) *Cancer* met-azide hemocyanin.

to the half-met-NO_2^- derivative upon anaerobic treatment with nitric oxide in pH 6.3 phosphate buffer.[13] It is important to note this reaction, since the arthropod (including *Limulus*) met derivatives cannot be regenerated to oxyhemocyanin by treatment with H_2O_2 or by any other methods which have been attempted. The half-met-NO_2^- form obtained from the *Limulus* met hemocyanin can further be regenerated to oxyhemocyanin as described in the "half-met" section (under Results) using dithionite. This indicates that, at least for *Limulus* hemocyanin, the met derivative is not an irreversibly damaged protein form.

In order to understand the lack of peroxide binding by the arthropod met forms (i.e., regeneration to oxy), the optical spectral perturbations of ligand binding to the *Busycon*, *Limulus*, and *Cancer* met forms were investigated. Addition of excess group 2 ligands to arthropod mets (excluding *Limulus*) is complicated, since a mixture of forms due to ~30% site disruption is present. However, reasonably pure forms are present for spectroscopic study of *Busycon* and *Limulus* met derivatives.

The low-temperature met-aquo absorption spectra of *Busycon*, *Limulus*, and *Cancer* are given in Figure 11. The only spectral features in the visible and near-IR region are the overlapping d–d transitions of the two unresolvable cupric ions. The spectra are, in general, similar in energy and intensity. The peak maximum shifts to higher energy over the series *Busycon* < *Limulus* < *Cancer* ($\bar{\nu}_B$ = 14500 cm^{-1}, $\bar{\nu}_L$ = 15384, $\bar{\nu}_C$ = 15750), and the shoulder to lower energy appears to lose intensity.

Fluoride (group 1 ligand) binds to all three mets and greatly perturbs the d–d spectra (Figure 12) without site disruption. In all cases, the binding constant is on the order of 10^3 M^{-1}. Titrations of met with fluoride were done by monitoring the shift in d–d spectra, and K is in the range $600 < K < 4000$ M^{-1}. The spectra show a significant loss in intensity along with a large shift (~2000 cm^{-1}) down in energy for each of the three hemocyanins.

Azide also binds to met but, unlike fluoride, appears to interact differently with the various species. The d–d transitions of *Busycon* met shift down in energy by ~400 cm^{-1} upon binding azide, while

for *Limulus* a much larger shift of almost 1500 cm^{-1} is observed (Figure 12). The situation is complicated for *Cancer* met hemocyanin because of the site disruption. It is not obvious from the d–d spectrum (Figure 12C) that the ~70% EPR-nondetectable *Cancer* met is binding azide. The spectrum shows an increase in absorption around 800 nm and there is a slight shift in the peak maximum of ~250 cm^{-1}.

The azide to copper charge-transfer spectra of the three met azides also show large differences (Figure 13). One broad band with an extinction coefficient of ~1500 M^{-1} cm^{-1} and a peak maximum at 380 nm is found for *Busycon* met-N_3^-. The binding constant is calculated to be 500 M^{-1}. *Limulus* met-N_3^- shows a much lower binding constant. Appreciable conversion to the azide-bound form occurs only at high anion concentrations (0.1 M), indicating that K is on the order of 10 M^{-1}. The charge-transfer band for *Limulus* met-N_3^- (Figure 13B) is also quite different from that observed for *Busycon* met-N_3^-. The *Limulus* spectrum shows a band at ~500 nm with an ϵ of 500 M^{-1}. To higher energy another very intense band is growing in, but its peak maximum is not well-defined (λ <375 nm; ϵ >1500 M^{-1} cm^{-1}). The charge-transfer band in the *Cancer* met-N_3^- spectrum grows in simultaneously with the EPR signal. This suggests that at these concentrations azide is binding only to the EPR-detectable copper in the protein. The binding constant for this site is then 100 M^{-1}, and the N_3^- CT has an ϵ of ~1200 M^{-1} cm^{-1} at 420 nm. The circular dichroism (CD) spectra of the charge-transfer bands have also been included in Figure 13. For the *Cancer* azide-bound form, two bands are observed which can be closely correlated with absorption bands. Alternatively, the CD spectra of *Busycon* and *Limulus* met-N_3^- are more complicated, with three or more transitions required to fit the observed spectra.

Further information on the mode of azide binding to the met can be obtained through a comparison to model studies. From EPR and optical titrations Cu(dien) binds one azide in the equatorial plane; the azide to copper CT occurs at 345 nm with an ϵ of 2600 M^{-1} cm^{-1}. Azide binding is pH dependent; at pH 7.2, K = 40 M^{-1} and the d–d transitions of Cu(dien) shift by 130 cm^{-1} (615 to 620 nm). Axial coordination of azide was investigated using Cu(2,3,2-tet). Cu(2,3,2-tet) has four amine nitrogens forming the equatorial plane and thus the azide is forced to bind in an axial position. The binding constant is extremely low, on the order of 1 M^{-1} or less, and no intense (ϵ >300 M^{-1} cm^{-1}) charge-transfer band is observed. The d–d bands shift by 450 cm^{-1} (525 to 538 nm).

Oxyhemocyanin. The oxyhemocyanins of all species, both mollusc and arthropod, excluding *Limulus*, were found to undergo a peroxide-displacement reaction with excess N_3^- or SCN^-. The effects observed in the optical spectra of oxyhemocyanin upon addition of excess N_3^- or SCN^- at 4 °C in pH 6.3 phosphate buffer were reported in a previous publication.[7] The absorption features at 340 and 570 nm are replaced by an N_3^- or $SCN^- \rightarrow$ Cu(II) charge-transfer transition in the 400-nm region of the spectrum. Dialysis to remove excess N_3^- or SCN^- in pH 6.3 phosphate buffer for 24 h does not result in recovery of the oxy spectral features, indicating that met hemocyanin has been produced.

The pseudo-first-order rate constants (k) for the N_3^- (and SCN^- in several cases) displacement of peroxide from a series of hemocyanins are given in Table I. In general, the arthropod hemocyanins exhibit more than an order of magnitude greater reaction rate than the molluscs, with *Limulus* showing essentially no reaction. While F^- and, to a much lesser extent, Cl^-, Br^-, and I^- have been reported to also displace peroxide from mollusc hemocyanins,[6] the arthropod oxyhemocyanins showed no reaction at 4 °C with any halide anions.

Along with the loss of the oxy absorption features is a gradual appearance of an EPR spectrum for the arthropod hemocyanins. With the converison of 70–90% of oxy sites to met, the resulting EPR spectrum only can account for 30 ± 5% of total copper, and no reduction in intensity is observed upon dialysis to remove the excess N_3^- or SCN^-. These EPR spectra are identical (in quantitation, ~30% of total copper, and shape) with those obtained by direct addition of excess N_3^- (or SCN^-) to peroxide-

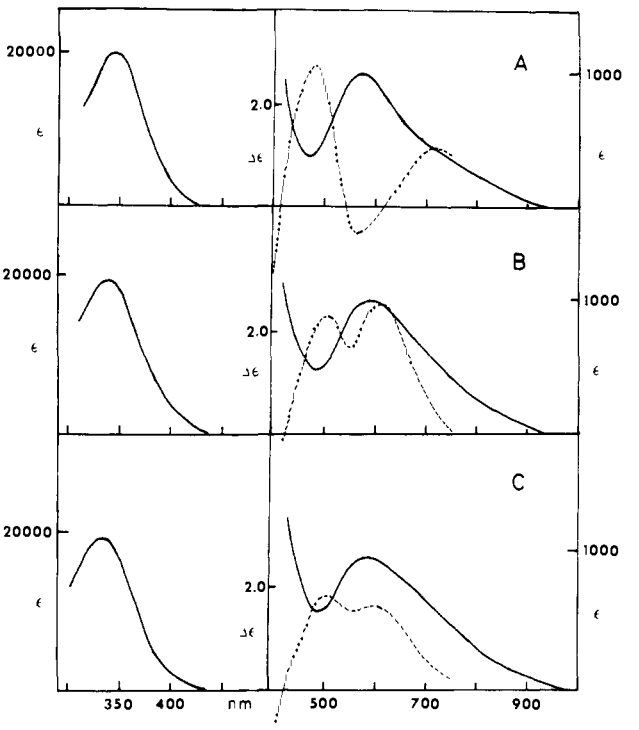


Figure 14. Absorption (—) and circular dichroism (---) spectra of (A) *Busycon*, (B) *Limulus*, and (C) *Cancer* oxyhemocyanin recorded at room temperature.

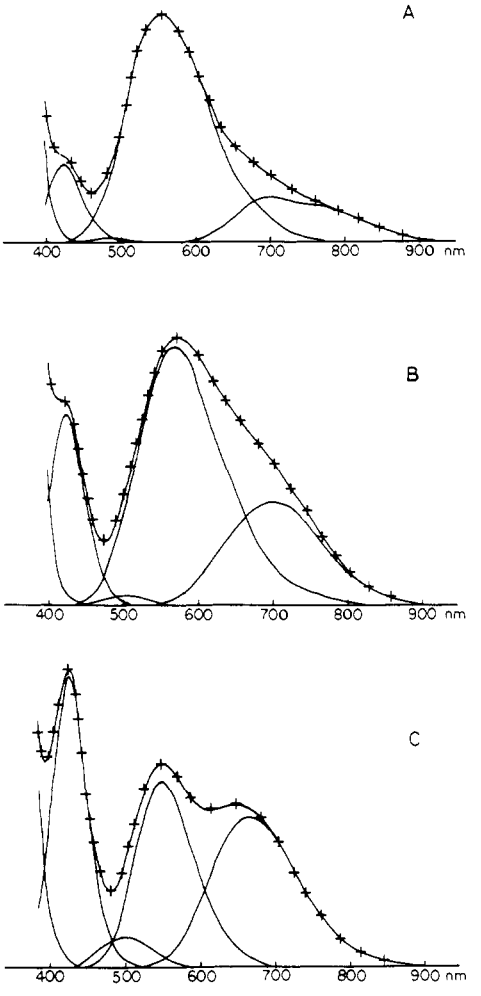


Figure 15. Gaussian resolution of (A) *Busycon*, (B) *Limulus*, and (C) *Cancer* oxyhemocyanin low-temperature (~15 K) spectra.

Table I. Pseudo-First-Order Rate Constants for Displacement of Peroxide from Oxyhemocyanin by Excess Ligand[a]

	N_3^-	SCN^-
molluscs[b]		
Busycon canaliculatum	0.002[c]	0.0004[c]
Lunatia heros	0.006[d]	
Megathura crenulata	0.004[e]	
arthropods		
Homarus americanus	>0.4[d]	
Cancer magister	0.3[d]	
Cancer irroratus	0.09[d]	
Cancer borealis	0.06[d]	0.005[f]
Limulus polyphemus	<0.0001[c]	<0.0001[c]

[a] In h^{-1}. [b] The reactions of mollusc hemocyanins are complicated by the biphasic nature of the reactions, most noticeably for the reactions with SCN^-. The loss of oxygen as O_2 initially proceeds at a much higher rate, which is then superceded by the loss of oxygen as O_2^{2-} to form met. [c] 200-fold excess. [d] 500-fold excess. [e] 600-fold excess. [f] 100-fold excess.

generated met forms (Figure 10). Similar but much less intense (<10% of total copper) EPR signals are obtained with the mollusc hemocyanins as described in the "Met" section (under Results).

The oxyhemocyanin absorption spectrum of *Busycon* has been studied in detail and assigned[4] using a transition dipole–vector coupling model[20] in combination with comparisons to spectra of hemocyanin derivatives. Two components are expected for each $O_2^{2-} \rightarrow$ Cu(II) charge-transfer transition [π_v^* and $\pi_\sigma^* \rightarrow$ Cu ($d_{x^2-y^2}$)] for a bridging peroxide based on coupling to the two coppers. The intense band at 345 nm is assigned as one component of the peroxide $\pi_\sigma^* \rightarrow$ copper $d_{x^2-y^2}$ charge-transfer transition. The band at ~570 nm in the absorption spectrum and the positive CD band at ~480 nm are the two components of the peroxide $\pi_v^* \rightarrow$ Cu charge transfer. The weak shoulder at 425 nm is associated with a protein ligand (phenolate or oxo[4]) to copper charge transfer, and the shoulders on the low-energy side of the 570-nm band are the ligand field transitions of the cupric ions.

All of these same transitions are found in the spectra of the *Limulus* and *Cancer* oxyhemocyanins and, although the room-temperature absorption spectra (Figure 14) of all the hemocyanins are similar, variations are discerned in the visible region when the spectra are taken at low temperature (Figure 15) and in the room-temperature CD spectrum (Figure 14). These spectra can be resolved into Gaussian bands, and it is observed that one set of Gaussians fits the peroxide charge-transfer spectra of all three with shifts in energy and intensity. The ligand field transitions and the 425-nm band were obtained by subtracting out the absorption due to the more intense peroxide charge-transfer bands. The d–d transitions were simply fit to an effective band envelope. The peak positions and intensities for both room- and low-temperature spectra are given in Table II, and the 15 K spectra with Gaussian components are shown in Figure 15.

Differences are found in peak positions and intensities over these three oxyhemocyanins, which account for the observed low-temperature absorption and room-temperature CD spectral differences. The observed component of the peroxide $\pi_\sigma^* \rightarrow$ copper CT (~29000 cm^{-1} or ~340 nm) shifts to higher energy going from *Busycon* to *Limulus* to *Cancer*. The splitting between the two components of the peroxide $\pi_v^* \rightarrow$ copper CT transition decreases and the higher energy component increases in intensity in the order *Busycon* < *Limulus* < *Cancer*. The protein ligand → Cu(II) CT at 425 nm (~23 000 cm^{-1}) does not show any significant change in energy, but its intensity does increase over the series *Busycon* < *Limulus* < *Cancer*. The intensity of the d–d bands also increases over the series *Busycon* < *Limulus* < *Cancer* and the bands shift somewhat to higher energy. The peak maximum of the *Cancer* band envelope is 700 cm^{-1} higher than either *Busycon* or *Limulus* and, although the peak maxima of the latter two coincide, the *Limulus* bands are not skewed to lower energy as is the *Busycon* band shape. Thus, the double peak in the low-temperature visible absorption spectrum in the arthropod oxyhemocyanins (Figure 15) is associated with a relative increase in energy and intensity of the ligand field transitions. This increase in energy of the d–d transition is also accompanied by a shift to higher energy of a positive CD spectral feature. This overlaps the weak negative band associated with the low-energy component of the O_2^{2-} $\pi_v^* \rightarrow$ Cu CT transition and dominates this region of the CD spectrum of the arthropods (Figure 14).

Table II. Gaussian Resolution of Oxyhemocyanin Spectra

	Busycon canaliculatum	*Limulus polyphemus*	*Cancer borealis*
room temperature			
$\pi_\sigma^* \rightarrow$ Cu CT	28 850 (~20 000)[b]	29 520 (~20 000)	29 850 (~20 000)
(endog)L → Cu CT	23 000 (360)	23 000 (580)	23 000 (830)
$\pi_v^* \rightarrow$ Cu CT	20 250 (50)	20 000 (35)	19 500 (310)
	17 500 (1000)	17 000 (960)	17 250 (780)
d–d bands[a]	13 800 (230)	13 700 (300)	14 390 (520)
low temperature			
$\pi_\sigma^* \rightarrow$ Cu CT	29 350 (~20 000)	30 000 (~20 000)	30 350 (~20 000)
(endog)L → Cu CT	23 500 (400)	23 600 (890)	23 500 (1700)
$\pi_v^* \rightarrow$ Cu CT	21 000 (20)	20 250 (45)	20 000 (180)
	17 850 (1180)	17 500 (1200)	18 125 (1090)
d–d bands	14 280 (250)	14 280 (500)	15 000 (890)
$\Delta\pi_v^*$			
room temp	2 750	3 000	2 250
low temp	3 150	2 750	1 875
av π_v^*			
room temp	18 875	18 500	18 375
low temp	19 425	18 875	19 062

[a] The value given for "d–d bands" is the peak maximum of the remaining absorption in the ligand field region after the absorption due to charge-transfer bands is subtracted. The presence of six possible transitions (three d–d transitions for each copper) would make any Gaussian analysis of this region inconclusive. [b] $\nu = cm^{-1}$ (ϵ, M^{-1} cm^{-1}).

The ligand field and 425-nm absorptions of the oxy form (with the O_2^{2-} Cu CT transitions subtracted out) can now be compared to those transitions in the analogous met-aquo derivatives. The changes in energy and band shape seen in Figure 11 must be considered within the error of subtraction of the more intense π_v^* transitions; however, significant variations in intensity are observed. Essentially, no change in intensity of either the 425-nm or the ligand field region are found for *Busycon*. In contrast, for both the arthropods, large decreases (a factor of 3–4) in intensity of these transitions are observed between the oxy and met forms.

Discussion

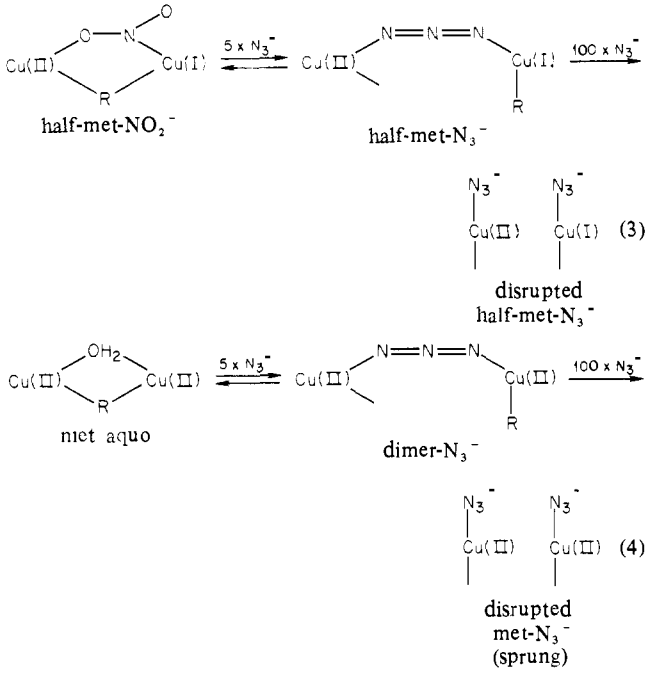
The most general observation to come from our parallel studies of five mollusc and five arthropod hemocyanins is the strong qualitative similarity in the chemistry and spectroscopy of these different species. All results are consistent with the active site pictured[4] in Figure 1. In all cases, a half-met form is accessible through similar chemical pathways. This half-met form binds ligands very tightly at the active site, reversibly coordinates carbon monoxide, and exhibits class 2 mixed valent properties which correlate with the nature of the exogenous ligand, all consistent with exogenous ligand bridging of the binuclear copper site. Further, all arthropod and mollusc hemocyanin half-met forms exhibit group 1–group 2 ligand-binding behavior. A second coordination position is present at the copper(II) only in group 2 forms (half-met-L_2, where L_2 = N_3^-, SCN^-, CN^-) where these ligands force the coppers >5 Å apart and rupture an endogenous protein bridge. Further, all exhibit an EPR-nondetectable met and dipolar-coupled dimer form which can be interconverted (addition of excess NO_2^- at low pH to met and dialysis of dimer which yields met) with little difference observed in the ligand field transitions, again consistent with breaking an endogenous protein bridge in the dimer form and eliminating the strong antiferro-

(20) R. R. Gay and E. I. Solomon, *J. Am. Chem. Soc.*, **100**, 1972 (1978).

magnetic coupling. The dimer form of all hemocyanins undergoes a reaction with N_3^- producing a new EPR-detectable binuclear cupric dimer-N_3^- derivative that also converts to met upon dialysis. Finally, the CD and absorption spectral differences of the oxy forms simply reflect increases in energy and intensity of the ligand field transitions over the series $\nu_{Busycon} < \nu_{Limulus} < \nu_{Cancer}$ and a somewhat smaller splitting of the O_2^{2-} π_v^* → Cu(II) $d_{x^2-y^2}$ charge-transfer transition for the arthropods.

There are, however, some very specific quantitative differences between the arthropods and molluscs in terms of reactivity and spectral properties. Further, we find that *Limulus* behaves differently from the other arthropods in terms of reactivity and thus must be considered separately in a comparison of these active sites.[7] This is first apparent in the ligand-displacement reactions of peroxide from the oxyhemocyanin site. Although all the oxy spectra, and therefore sites, are quite similar, *Limulus* is observed to be the *least* reactive: $k_{Limulus} \ll 10^{-4}$ h^{-1}, $k_{mollusc} \sim 10^{-3}$ h^{-1}, and $k_{arthropod} \sim 10^{-1}$ h^{-1} for N_3^- reactions. This is most reasonably explained by the lack of access to an axial coordination position on the copper(II) in *Limulus* which is required for associative type ligand substitution chemistry to occur in tetragonal cupric systems. Thus, it appears that from the average energies of the ligand field transitions[21] and from the ligand substitution reactivity of the oxy sites the coordination of the cupric sites is best considered tetragonal five or six coordinate with an axial position exchangeable (H_2O) for *Cancer* and, to a lesser extent, *Busycon* and blocked by a weakly coordinating protein ligand in the case of *Limulus*. In the active site pictured in Figure 1, the two coppers are further considered to be close to equivalent based on all present spectral data.[22]

Continuing this comparison of the reactivity over the different hemocyanin derivatives leads to a significant correlation concerning instability of the active site in the arthropods (with *Limulus* again being the exception). It is observed for the arthropods (but not *Limulus*) that a group 2 ligand will irreversibly disrupt the active site of the half-met and met forms (referred to as "springing" for the latter[19]). We find this disruption mechanism proceeds via a stable group 2 ligand-bridged intermediate (the *Cancer* half-met-N_3^- and dimer-N_3 forms) which is reversible, followed by irreversible disruption of the site with excess ligand (eq 3 and 4). An analogous propensity of the metals to come apart in the arthropods (but not *Limulus*) as compared to the molluscs can also be found in the dimer chemistry. The arthropod dimer is easily formed from half-met-NO_2^- and is not significantly converted to met upon addition of excess exogenous ligand. This is in contrast to the behavior observed for the molluscs and *Limulus*, which require large excesses of NO_2^- to generate the dimer and further show a large decrease in intensity upon the addition of azide corresponding to the production of a significant (57%) amount of the met derivative. The quantitative spectral differences, however, follow a very different correlation over these forms (vide infra), and, in particular, the electronic structures (and thus detailed geometric structure) of all arthropods including *Limulus* seem to be similar to each other but quantitatively different from that of the molluscs. These spectral correlations indicate that the arthropods, including *Limulus*, have very similar active-site structures, yet *Limulus* shows much less tendency toward disruption of the site. This instability of the active site must then be associated with more distant protein effects. These could be tertiary or quaternary in nature. At present, some data imply that this may be a cooperative effect. For example, only 25–40% of the arthropod met sites are disrupted by group 2 ligands, while all are accessible (from ligand displacement results on oxy), and, in fact, most sites irreversibly disrupt in half-met in the presence of excess N_3^-. At this point, the alternative of protein heterogeneity cannot be excluded, and systematic studies are underway to further distinguish between these possibilities.



For all the binuclear copper active-site derivatives of hemocyanins, we have found a strong correlation in spectral properties over the arthropods, including *Limulus*, which quantitatively differ from those of the molluscs. As these spectral properties probe active-site geometric structure, these differences demonstrate that the sites of the arthropods, while generally similar, are distorted from those of the molluscs in a manner which perturbs the exogenous ligand bridge. First, in all of the derivatives, the ligand field transitions are at a higher energy in the arthropods. In the half-met forms, the intervalent transfer transitions are weaker for the arthropods with no low temperature half-met-N_3^- form. This demonstrates that there is less electron delocalization between the coppers and thus a poorer bridging mode for the exogenous ligands with respect to innersphere electron transfer. In the dimer and dimer-N_3^- forms, the EPR spectra of the arthropods are similar and differ from those of the molluscs. The dimer triplet EPR spectrum in the $g = 2$ region is dominated by the zero field-splitting contribution and thus reflects Cu–Cu distance.[23] The spectral differences between the mollusc and arthropod dimer and dimer-N_3^- derivatives are also associated with a distortion which affects the exogenous ligand bridge, in this case keeping the coppers farther apart for the molluscs, which exhibit smaller zero field splittings. Further, for the oxy forms of the arthropods, the O_2^{2-} π_v^* → Cu ($d_{x^2-y^2}$) splitting is smaller with more intensity being mixed into the higher energy component of π_v^*. Associated with this is a large increase in intensity of the 425-nm absorption and of the ligand field transitions. Comparison with the met forms demonstrates that this intensity increase is due to distortion mixing with the allowed peroxide → Cu charge-transfer transitions. These oxy spectral differences indicate that the copper planes may be tilted with respect to each other, again affecting exogenous ligand (O_2^{2-}) bridging between the coppers.

Since the mechanism of catalase activity (high for the molluscs but very low for the arthropods) presented in eq 5–7

$$\text{deoxy[Cu(I)Cu(I)]} + H_2O_2 \xrightarrow{+2H^+} \text{met[Cu(II)Cu(II)]} + 2H_2O \quad (5)$$

(21) The energies of the d–d transitions of a cupric ion are predominantly dependent upon the effective geometry and ligand field strength of the four (equatorial) ligands but are also perturbed by the coordination of a fifth and/or sixth (axial) ligand. The positions of the hemocyanin peak maxima confirm the tetragonal nature of the sites, since they are similar to that observed for well-defined tetragonal five or six coordinate inorganic complexes.

(22) The coppers were proposed not to be equivalent based on a misassignment of the O_2^{2-} → Cu(II) CT transitions as d–d transitions: W. Mori, O. Yamauchi, Y. Nakao, and A. Nakahara, *Biochem. Biophys. Res. Commun.*, **66**, 725 (1975).

(23) A. J. M. Schoot Uiterkamp, H. van der Deen, H. C. J. Berendsen, and J. F. Boas, *Biochim. Biophys. Acta*, **372**, 407 (1974).

Table III. Qualitative Comparison of Spectral Features and Reactivity of Hemocyanin Derivatives

derivative/specific point of interest	mollusc	*Limulus*	arthropod
half-met			
spectral: (1) intervalent transition	intense	weak	weak
(2) delocalized low-temp half-met-N_3^-	present	not present	not present
reactivity: active site instability	not present	not present	present
dimer			
spectral: zero field splitting of triplet EPR signal	small	large	large
reactivity: (1) excess NO_2^- required for half-met → dimer production	large	large	small
(2) met production from N_3^- addition	large	medium	small
met			
spectral: (1) d–d and CT spectral changes upon adding N_3^-	normal[a]	unusual[a]	*b*
reactivity: (1) N_3^- binding constant	large	small	*b*
(2) active-site instability	not present	not present	present
oxy			
spectral: (1) O_2^{2-} π_v^* → Cu(II) splitting and intensity ratio of components	large	medium	small
(2) energy and intensity of d–d bands and 425-nm CT	low	medium	high
reactivity: rate of O_2^{2-} displacement	medium	very low	high

[a] With respect to equatorial coordination in tetragonal Cu(II) model complexes. [b] Not obtainable due to arthropod site instability.

$$\text{met[Cu(II)Cu(II)]} + H_2O_2 \rightarrow \text{oxy[Cu(II)Cu(II)]}O_2^{2-} + 2H^+ \quad (6)$$

$$\text{oxy[Cu(II)Cu(II)]}O_2^{2-} \rightleftarrows \text{deoxy[Cu(I)Cu(I)]} + O_2 \quad (7)$$

$$\text{net reaction: } 2H_2O_2 \rightarrow O_2 + 2H_2O \quad (1)$$

involves the coordination of O_2^{2-} to the met site generating oxy, structural changes which interfere with exogenous ligand bridging in the arthropod active site are likely associated with the lack of catalase activity. Our chemical and spectroscopic studies of the met derivative support the idea that a structural distortion is the dominant factor affecting peroxide binding at this active site (eq 6). For *Limulus* we have shown that an arthropod met can be regenerated to oxy by stepwise reduction to deoxy and exposure to oxygen as in eq 8. Thus, the protein ligand has not been

$$\text{met} \xrightarrow{NO} \text{half-met-}NO_2^- \xrightarrow{N_3^-} \text{half-met-}N_3^- \xrightarrow{CH_3CO_2^-} \text{half-met-}CO_2^- \xrightarrow{Na_2S_2O_4} \text{deoxy} \xrightarrow{O_2} \text{oxy} \quad (8)$$

destroyed in the met preparation (by generation of ·OH, etc.). Next, the studies on the fluoride ligand show that it binds with high affinity for all the met sites, including the arthropods, producing significant spectral changes. Fluoride is not expected to bind based on aqueous cupric chemistry, and this high binding constant suggests that the active sites of all the met (and oxy) hemocyanins stabilize a highly electronegative anion. Specific residue variation in the active-site pocket, however, cannot be excluded. Although the arthropods are generally complicated by site instability the data obtained upon binding N_3^- to the *Limulus* met site which is not unstable demonstrates that a significant structural change is present in the met form of arthropod hemocyanins which affects exogenous ligand bridging.

If we first consider met *Busycon*, addition of N_3^- causes a small decrease in the d–d transition energies and a reasonably intense ($\epsilon \sim 1000$) $N_3^- \rightarrow$ Cu(II) charge-transfer transition at 380 nm. These spectral features and the observed binding constant (k = 500 M^{-1}) are consistent with the results found for equatorial coordination of N_3^- to the Cu(dien) model complex and to the met apo site. This is also consistent with the simple replacement of the equatorial O_2^{2-} at the oxy site with N_3^-. The specifics of the charge-transfer absorption and CD spectra further suggest that the azide bridges the two coppers. This derives from the fact that the dominant CD feature at 440 nm is no longer coincident with the 380-nm $N_3^- \rightarrow$ Cu(II) CT absorption peak and that at least three transitions are required to fit the combined CD and absorption N_3^- CT region requiring N_3^- to coordinate to both coppers. For N_3^- bound to a single copper, only two transitions are possible (N_3^- $\pi_v^* \rightarrow$ Cu $d_{x^2-y^2}$ and N_3^- $\pi_\sigma^* \rightarrow$ Cu $d_{x^2-y^2}$), and this is what is observed for met apo and the disrupted *Cancer* met-N_3^-, both having N_3^- bound to a single copper. In contrast, for *Limulus* and, by extension, the other arthropods, a site distortion seems to strongly affect the binding of exogenous ligands. The N_3^- binding constant has now been greatly reduced ($k < 10$ M^{-1}) and a large distortion is required at the site for N_3^- bridging. The latter is demonstrated by the significant change in ligand field absorption energies in met-N_3^- vs. the met-aquo ($\Delta\nu$ = 1484 cm^{-1}) forms. Further, the resulting *Limulus* met-N_3^- CT spectrum is significantly different from that of *Busycon*. There are still at least three transitions supporting a bridging mode for the N_3^-; however, a weak absorption band is present at 500 nm and there is a larger separation between the major peaks in absorption and CD. These spectral changes indicate a quantitatively different bridging mode when azide is forced to coordinate to the distorted *Limulus* met site, causing a larger splitting of the charge-transfer transitions. While the nature of this distortion requires further spectroscopic study [in particular, resonance Raman studies into the $N_3^- \rightarrow$ Cu(II) charge-transfer transitions and EXAFS determination of Cu–Cu distance changes], it seems clear that this distortion is coupled to the lack of catalase activity in the arthropods.

Summary

The important features of these spectral and reactivity comparisons of hemocyanins from different phyla are listed by derivative in Table III. Thus, while emphasizing the strong qualitative similarities among the hemocyanin active sites, we summarize the differences as follows: all the arthropods (including *Limulus*) exhibit similar spectral properties quantitatively different from the molluscs and associated with a distortion of the active site which, for the met derivative, seems to relate to the lack of catalase activity. In terms of active-site instability, *Limulus* behaves more like the molluscs in that it shows little tendency toward irreversible disruption of the binuclear copper site with group 2 ligands. Thus, this active-site instability is associated with the protein ligand and can be tertiary or quaternary in nature. Finally, concerning the ligand substitution reactions of the oxy forms, there seem to be large differences in access to an axial coordination position for associative peroxide displacement over the different hemocyanins (arthropods > molluscs ≫ *Limulus*).

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