perience the spectra of these can contain 300–600 peaks, making the identification of low molecular weight peptides from low-resolution spectra especially difficult. A further disadvantage of low resolution data of complex mixtures is that its interpretation must depend heavily on predicted relative abundances as well as masses. We also find that computer interpretation techniques are necessary for the data from complex mixtures to be certain that all sequence possibilities are considered; even for high-resolution data unexpected coincidences can arise, such as the exact mass identity cited in mixture 4 for derivatized Lys and (Val + Ala). For a thorough analysis every peak in the spectrum must be considered for its possible structural significance; this is a relatively trivial assignment for the modern computer.

This capability of analyzing mixtures makes mass spectrometry a more attractive tool in polypeptide sequencing by reducing the number of chemical derivatization operations necessary and the required quantities of individual oligopeptides. Techniques for routine chemical derivatization of smaller samples are needed; mixture samples of $\ll 0.1$ μmol should be sufficient using chemical ionization[27] and/or improved methods for collection of high-resolution data,[23] and would represent a substantial advantage over present chemical methods. Chemical ionization also promises to make possible more complete sequence information on larger peptides. Finally, this method should make possible entirely new strategies for polypeptide sequencing,[6] with polypeptide degradations and separations tailored to give the most suitable oligopeptide mixtures.

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Nuclear Magnetic Resonance Study of the Binding of Glycine Derivatives to Hemocyanin

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Abstract: Hemocyanin is a non-heme, copper-containing oxygen-carrying protein found in many molluscs and arthropods. The existence of cupric ions in oxyhemocyanin has not been proved directly, but is inferred from results of physicochemical studies. Optical absorption spectra do not indicate any interaction between oxyhemocyanin and added ligands. We have utilized nuclear magnetic resonance line broadening techniques to investigate the interactions of glycine derivatives with the copper of oxyhemocyanin. Evidence is provided which demonstrates directly the presence of paramagnetic Cu(II) ion in oxyhemocyanin and the binding of ligands to the protein-bound Cu(II) at the active sites. The line-width measurements obtained with glycine, glycine ethyl ester, acetylglycine, and glycylglycine lead us, depending on the ligand, to conclude that the amino, carboxyl, and amide groups of the ligand are involved in the binding of cupric ions of oxyhemocyanin with formation constants ranging from 2 to 1500 M^{-1}.

Hemocyanin is a non-heme, oxygen-carrying copper protein found in many molluscs and arthropods. One oxygen molecule binds two copper atoms in hemocyanin.[2] The protein, a deep blue color when oxygenated, becomes colorless when deoxygenated. It appears that the copper in deoxyhemocyanin is Cu(I), since only cuprous compounds regenerate active hemocyanin from apohemocyanin.[3] But, the oxidation state of copper in oxyhemocyanin is uncertain.[4] Oxyhemocyanin does not display a Cu(II) electron spin resonance (esr) signal unless the protein is denatured.[5,6] The existence of paramagnetic Cu(II) in oxyhemocyanin is inferred from the absorption spectra,[7,8] chemical reactions,[9,10] and radiation chemical studies.[11] The presence of Cu(II) in oxyhemocyanin has never been proved directly.

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Reducing agents, such as thiocyanate and thiourea, interact with oxyhemocyanin causing diminution of the copper bands around 340 and 580 nm.[10] The effects of reducing agents on oxyhemocyanin are attributed to reduction of Cu(II) to Cu(I) with concomitant expulsion of oxygen. To our knowledge, there is no literature report on binding of small ligands such as simple amino acids and peptides to hemocyanin in which oxygen is not expelled. Such knowledge could lead to better understanding of copper binding at the active site of hemocyanin. Accordingly, we have investigated the binding of glycine derivatives to copper in oxyhemocyanin by nuclear magnetic resonance (nmr).

The nmr measurements are particularly sensitive for the detection of interactions involving paramagnetic metal ions. Li, Scruggs, and Becker[12] have utilized the selective line broadening for detecting complexes of paramagnetic metal ions with small ligands and to elucidate their binding sites. This technique has also been used to study the binding of small molecules to proteins.[13-15] For example, Navon, Shulman, Wyluda, and Yamane[16] showed that the fluoride ion is bound directly to the manganese in the carboxypeptidase A. In this paper we present results of nmr studies on the interaction of glycine derivatives with *Busycon* (from channeled whelk) and *Limulus* (from horseshoe crab) hemocyanins.

Experimental Section

Busycon and *Limulus* hemocyanin hemolymph, obtained from the Marine Biological Laboratory, Woods Hole, Mass., was further purified as follows:[17] the hemolymph was centrifuged in the cold (4°) using a Spinco Model L ultracentrifuge at 120,000*g*, the pellet was dissolved with a small amount of water, dialyzed against water at 4° for 2 days with six changes and ultracentrifuged again, and the pellet was dissolved in D_2O. The purified protein was stored in solution at 4°. Using uv spectra as criterion, the protein stored in this manner is stable over a period of 4 weeks. In this study nmr spectra were obtained from samples stored less than a week. The copper and protein concentrations of the hemocyanin were determined from absorbances at 345 (ϵ_{345} 10^4 M^{-1} (Cu) cm^{-1}) and 280 nm (ϵ_{280} 1.571 g^{-1} cm^{-1}), respectively. The purified oxyhemocyanin solutions containing about 10^{-3} *M* copper did not display a detectable Cu(II) esr signal measured at room temperature with a Bruker B-ER 418 s esr spectrometer operating at about 9.6 GHz.

Apohemocyanin was prepared by dialyzing a 3% solution of the purified oxyhemocyanin against a 0.1 *M* phosphate buffer solution containing 0.1 *M* KCN and 0.05 *M* $MgCl_2$ at pH 7.8 for 2 days with six changes. Excess cyanide was removed by dialysis against water for 2 days with eight changes. The apohemocyanin thus prepared showed no absorption at 345 nm and no detectable copper was found by atomic absorption spectrophotometry. For nmr measurements, the apohemocyanin was further concentrated by repeated ultrafiltration using D_2O as solvent.

Proton nmr spectra were obtained at 32° and 60 MHz with Varian A-60 nmr spectrometer at Duquesne University. Oxyhemocyanin samples were freshly prepared in 99.8% D_2O at atmospheric pressure. The pD of the solution was adjusted to near 8 to minimize the protein from dissociating into subunits[18] by adding NaOD or DCl to the solutions and then calculated from pH meter readings from the equation

$$\text{pD} = \text{pH meter reading} + 0.4 \quad (1)$$

Three to five nmr spectra were obtained for each sample using a sweep width of 50 Hz. *tert*-Butyl alcohol was used as internal reference for the assignment of ligand proton peak. Side band technique was applied for the measurement of line width. The observed line width, $\Delta\nu$, was measured from the full width at half-amplitude of the $-CH_2$ proton peak. The average of three to five measurements was used to calculate the formation constant. The variation in line width was 0.05 Hz or less. The transverse relaxation time T_2 was calculated from the relation $1/T_2 = \pi\Delta\nu$.

Calculation of Formation Constants

If we assume that there are just two types of ligand molecules in an aqueous solution of oxyhemocyanin, free and bound, with mole fractions X_f and X_b, respectively, the transverse relaxation times T_{2f} and T_{2b}, then if X_b is much smaller than X_f, the observed relaxation time T_2 is given by[19,20]

$$\frac{1}{T_2} - \frac{1}{T_{2f}} = \frac{X_b}{(T_{2b} + \tau_b)} = \frac{1}{T_{2p}} \quad (2)$$

where τ_b is the lifetime of ligand nucleus in the bound environment and T_{2p} represents the contribution of the paramagnetic copper in oxyhemocyanin to the line broadening. Although X_b is small, $1/(T_{2b} + \tau_b)$ is large, due to the relatively slow tumbling of macromolecular oxyhemocyanin. Thus, a small concentration of oxyhemocyanin produces a large increase in $1/T_2$. For eq 2 to be valid, the ligand nmr frequency must be the same in the presence and absence of the macromolecule. This is indeed observed experimentally.

In our experiments, the ligand concentration [L] is always in large excess over that of oxyhemocyanin, [E], so that

$$X_b \simeq [\text{EL}]/[\text{L}] \quad (3)$$

where [EL] is the oxyhemocyanin–ligand complex concentration. The formation constant takes the form

$$K = [\text{EL}]/(([\text{E}] - [\text{EL}])[\text{L}]) \quad (4)$$

Substituting in eq 2 and 3, we get

$$\frac{1}{T_{2p}} = \frac{[\text{E}]}{(K^{-1} + [\text{L}])(T_{2b} + \tau_b)} \quad (5)$$

which is the same as eq A-2 given by Navon, *et al.*[16] When eq 5 is valid, a plot of T_{2p} *vs.* [L] gives a straight line intercepting the *X*-axis at point $[\text{L}]T_{2p=0} = -K^{-1}$.

Results

Addition of glycine or glycylglycine in large excess to oxyhemocyanin at neutral pH does not change the copper bands at 345 and 580 nm. Figure 1 shows a plot of R (A_{345}/A_{280}) *vs.* pH for *Busycon* oxyhemocyanin in the presence of glycine and glycylglycine. In the pH 5–11 region R remains constant. Beyond pH >11, R decreases rapidly with increasing pH, presumably because of denaturation. While these data indicate an absence of interaction between hemocyanin and the ligands, nmr measurements show that the ligand signals are indeed broadened upon addition of oxy-

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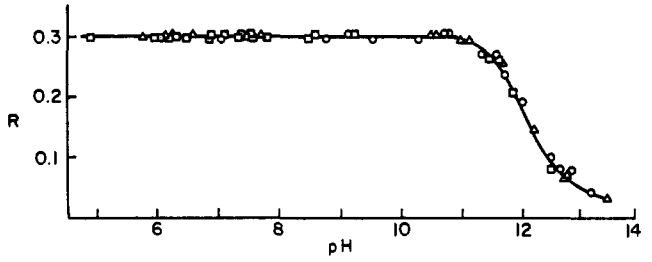

Figure 1. Plot of R (= A_{345}/A_{280}) as a function of pH. The solutions contain 1.2 mg/ml *Busycon* oxyhemocyanin (4.6×10^{-5} M Cu) in H_2O and: (□) 0.03 M glycylglycine; (○) 0.03 M glycine; (△) no additive.

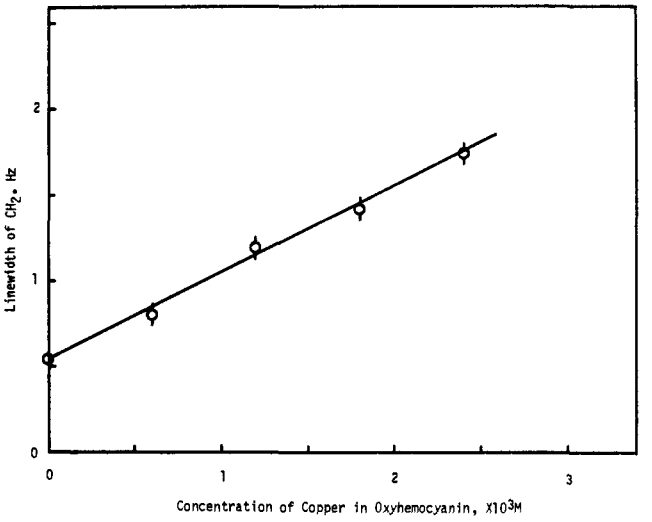

Figure 2. Line broadening of 0.3 M glycine CH_2 protons as a function of concentration of copper in *Busycon* oxyhemocyanin in D_2O, pD 7.7, 32°.

hemocyanin. At a fixed glycine concentration and pH the line width of the glycine signal is a linear function of the oxyhemocyanin concentration as illustrated in Figure 2. Moreover, the ligand signals of glycylglycine are selectively broadened. Experiments with apohemocyanin (copper-free hemocyanin) give no measurable line broadening.

The nmr line broadening due to oxyhemocyanin is sensitive to the presence of a second ligand. Thus, addition of 10^{-3} M EDTA to 0.2 M glycine and 3.2% oxyhemocyanin ([Cu] = 1.2×10^{-3} M) at pD 8 drastically reduces the line width of glycine. The line width of the glycine CH_2 protons decreases with increasing EDTA concentration (Figure 3). Similarly, addition of sodium sulfite in the ratio of $[Na_2SO_3]/[Cu]$ = 100 reduces the line width of glycine one-half.

Addition of $CuCl_2$ to 0.3 M glycine and 3.2% apohemocyanin at pD 8.0 does not display measurable line broadening until the Cu(II) concentration exceeds 1.2×10^{-4} M. Beyond that the line width of the glycine increases sharply (Figure 4). These data indicate that each apohemocyanin molecule binds ~40 Cu(II) ions if the molecular weight of the protein is taken to be $\sim 10^7$. The actual molecular weight of *Busycon* apohemocyanin is not known.

Since copper in deoxyhemocyanin is in the Cu(I) state, no broadening of the ligand signal by deoxyhemocyanin is expected. When a solution of 0.3 M glycine and 3% oxyhemocyanin is deoxygenated by

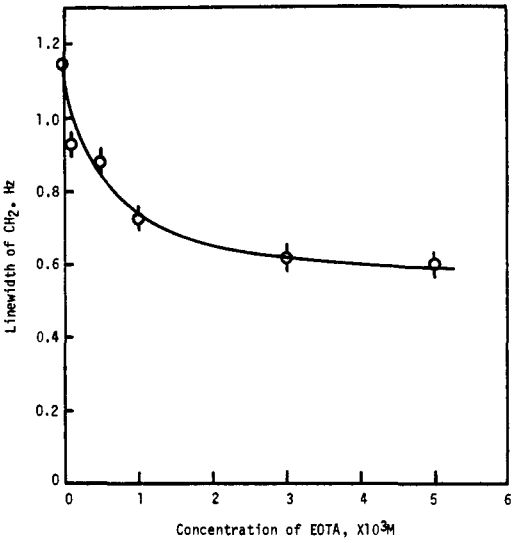

Figure 3. Line broadening of glycine CH_2 protons by *Busycon* hemocyanin as a function of EDTA concentration. The solutions contain 0.2 M glycine and 32 mg/ml *Busycon* oxyhemocyanin (1.2×10^{-3} M Cu) in D_2O, pD 8.0, 32°. In the absence of oxyhemocyanin the line width of 0.2 M glycine at pD 8.0 is 0.53 ± 0.03 Hz.

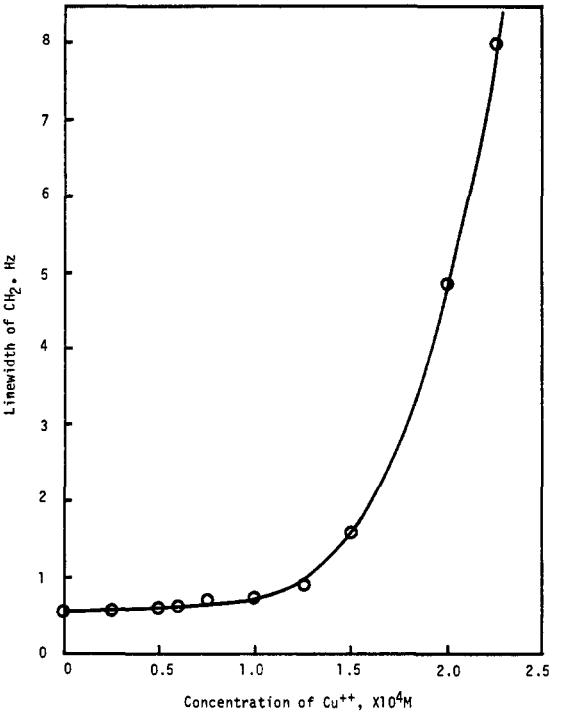

Figure 4. Line broadening of 0.3 M glycine CH_2 protons as a function of added Cu^{2+} concentration. The solutons contain 32 mg/ml *Busycon* apohemocyanin in D_2O, pD 8.0, 32°.

flushing with ultrapure He gas and then carefully transferred into a nmr tube in the absence of oxygen, the glycine line width is narrowed substantially but the broadening effect is not entirely eliminated. This result is most likely due to incomplete deoxygenation because of the limitations of the deoxygenation procedure, and partial denaturation caused by vigorous bubbling of He gas.

To elucidate the mode of glycine binding to Cu(II) in oxyhemocyanin, *N*-acetylglycine and glycine ethyl ester ligands were also studied. Blocking of the

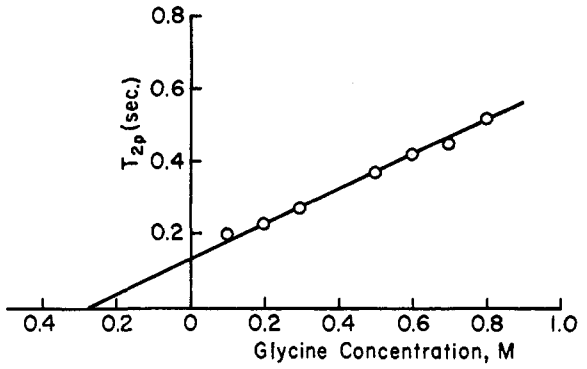

Figure 5. T_{2p} of glycine CH_2 protons as a function of glycine concentration. The solutions contain 64 mg/ml *Busycon* oxyhemocyanin (2.4×10^{-3} *M* Cu) in D_2O, pD 7.7, 32°.

amino or carboxyl group reduces the line broadening of CH_2 protons markedly. For each ligand, a plot of T_{2p} *vs.* [L] according to eq 5 always gives a straight line. A typical example with glycine as ligand is given in Figure 5. Since the experiments are carried at pD near 8, the formation constant obtained from the negative *X* intercept of eq 5 is the apparent formation constant. The true formation constant can be calculated from [21]

$$K_{ML} = K(1 + [H^+]/K_{HL}) \quad (6)$$

where K_{ML} and K are the true and apparent formation constants, respectively, $[H^+]$ is the hydrogen ion concentration at which K is obtained, and K_{HL} is the ionization constant of the ligand (amino group in this case), both corrected for the isotope effect.[22] The corrected formation constants for *Busycon* and *Limulus* with various ligands are summarized in Table I.

Table I. Formation Constants Measured by Nmr

Hemocyanin	Ligand[a]	Formation constant,[b] M^{-1}	
		K	K_{ML}
Busycon	Glycine	3.7 ± 0.5[c]	1500 ± 200
Busycon	Glycine ethyl ester	2.3 ± 0.5[c]	9 ± 2
Busycon	Acetylglycine	2 ± 0.4[d]	2 ± 0.4
Limulus	Glycine	1 ± 0.25[c]	400 ± 100

[a] The ionization constants, pK_{HL}, of the ligands (amino group) in D_2O are[22] 10.3 for glycine, 8.3 for glycine ethyl ester, and 4.2 for acetylglycine. [b] *K* values are obtained from the negative *X* intercept at the given pD. K_{ML} are obtained from *K* after correcting for pH effect according to eq 6. The error limits are estimated from variation in the negative *X* intercept of eq 5. [c] At pD 7.7. [d] At pD 6.1.

Discussion

It is well known that hemocyanin binds reversibly one oxygen molecule per two copper atoms.[2] The two copper atoms are situated so close that coupling interactions result, giving no detectable esr signals at room temperature.[23] Therefore, the lack of a copper esr signal does not necessarily rule out the presence of Cu(II) in oxyhemocyanin.

As mentioned previously, oxyhemocyanin selectively broadens the nmr lines of glycylglycine. The β-CH_2 signal is broadened much more than the α-CH_2 signal. The line-broadening effect is similar to that of free Cu(II) ions. Li, *et al.*,[12] have shown that the line broadening of the glycylglycine signals by Cu(II) is due to the shortening of the spin–spin relaxation time of the ligand protons by paramagnetic Cu(II) ions through Cu(II)–ligand binding. Thus, the nmr data indicate that paramagnetic Cu(II) is indeed present in oxyhemocyanin and that the ligand molecule binds directly to Cu(II) at the active sites. This postulate is further sustained by the following observations. (1) Apohemocyanin (copper-free hemocyanin) alone does not broaden the nmr signals of the ligands studied under the same experimental conditions. This eliminates the possibility that the ligand is bound to the protein moiety. (2) In the presence of EDTA, the line width of the glycine CH_2 decreases with increasing EDTA concentration (Figure 3). EDTA is known to bind Cu(II) more strongly than glycine;[24] hence, addition of EDTA would displace bound glycine and reduce its line width. However, the mode of EDTA binding to the Cu(II) of oxyhemocyanin is not understood. (3) Reducing agents such as thiourea and thiocyanate have been shown to expel oxygen from oxyhemocyanin *via* the reduction of Cu(II) to Cu(I).[25] Sodium sulfite has the same effect.[8] Addition of sodium sulfite to a solution of oxyhemocyanin and glycine reduces the line width of the glycine to about one-half. (4) Blocking of the amino or carboxyl group in glycine greatly reduces the line-broadening effect exerted by oxyhemocyanin.

To pinpoint the origin of the nmr line-broadening effect by oxyhemocyanin, we turn to consider the possible sources of Cu(II) in the protein. Three different types of Cu(II) in the protein are considered. They are: (1) extraneous Cu(II); (2) nonoxygen-carrying, *i.e.*, nonfunctional, Cu(II); and (3) oxygen-carrying, *i.e.*, functional, Cu(II). So far in the literature, only one type of copper, *i.e.*, those of oxygen-carrying, has been reported.[26]

The possibility that trace amount of extraneous Cu(II) present in the protein gives the nmr line broadening can be ruled out based on the following experimental findings: (a) treatment of the oxyhemocyanin with a chelating resin, Bio-Rad Chelex 100, at pH 8 does not remove any copper from the protein,[27] (b) Joselow and Dawson[28] have shown that there is no exchangeable copper in the protein under various experimental conditions; and (c) there is no EDTA removable copper in the protein.

The origin of the nonfunctional Cu(II) comes from denaturation of the native protein. No other type of nonfunctional Cu(II) has been detected in hemocyanin by any physicochemical methods. In our study we

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always use freshly prepared hemocyanin samples. These samples contain $\sim 10^{-3}$ *M* copper and do not show any detectable esr signal at room temperature, although we are able to detect 10^{-4} *M* Cu(II) as $CuSO_4$ in aqueous solution. Nevertheless, trace amount of nonfunctional Cu(II) produced by denaturation during the preparation of protein sample may be present. To test the effect of the nonfunctional Cu(II) on the nmr line broadening, we chose H_2O_2 inactivated *Limulus* methemocyanin, in which the copper is oxidized to Cu(II), as a model. The *Limulus* methemocyanin was prepared and purified according to the literature method.[9] We obtained a methemocyanin sample which was more than 90% inactivated. We found that the nmr line broadening of glycine CH_2 protons exerted by Cu(II) in methemocyanin is very small. Thus, a methemocyanin sample containing 1.6×10^{-3} *M* Cu(II) broadens the CH_2 line only by 1.2 Hz, whereas the same amount of free Cu(II) alone or Cu(II) added to oxyhemocyanin completely broadens the CH_2 line. No detectable line broadening is observed when the Cu(II) concentration in the methemocyanin is less than 10^{-4} *M*. One drawback of using *Limulus* methemocyanin as model for *Busycon* oxyhemocyanin is that the two species of oxyhemocyanin (native) have quite a different degree of broadening effect on the glycine protons.

In addition, the effect of adding Cu^{2+} to apohemocyanin on nmr line broadening was studied. Experiments with 3.2% apohemocyanin show that the presence of up to 1.2×10^{-4} *M* Cu(II) does not broaden the glycine signal (Figure 4). Presumably the Cu(II) is bound rather strongly to the protein other than in the oxygen-binding site, so that the paramagnetism of Cu(II) is not experienced by the glycine protons. Such nonspecific metal binding to copper–protein has been demonstrated recently by McKee and Frieden.[29] Appreciable line broadening is observed when excess Cu(II) is present. Thus, even if a trace amount (less than 10^{-4} *M*) of nonfunctional Cu(II) is present in oxyhemocyanin, it would not contribute significantly to the observed line broadening. The presence of 10^{-4} *M* or more nonfunctional Cu(II) in the oxyhemocyanin (about 10% of the total copper present in the protein samples used for this study) can be detected spectrophotometrically.

These results suggest that the observed nmr line broadening is mainly arisen from the functional Cu(II) in the protein. We recognize that our data do not rule out entirely the possibility that trace amount of nonfunctional Cu(II) contributes to some of the line broadening. These data lead us to conclude that paramagnetic Cu(II) is present in native oxyhemocyanin and that glycine derivatives are directly bound to Cu(II) in oxyhemocyanin.

We proceed to evaluate the strength of the Cu(II)–ligand interactions by using eq 5 to obtain the formation constant. Equation 5 is derived on the assumption that only a 1:1 complex is formed, and the ligand nmr frequency is the same in the presence and absence of the macromolecule. The validity of the assumption is borne out by the experimental data. However, the use of eq 5 in comparing various ligands which form different complexes is not strictly valid. Nevertheless, for closely related systems such as the present study, we are able to extract some useful information from the data of Table I. The formation constants of oxyhemocyanin–ligand complexes are much smaller than the second formation constant of the Cu(II)–glycinate complex, $\log K_2 = 6.9$,[30] since in oxyhemocyanin Cu(II) is bound strongly to both oxygen and the protein moiety. Felsenfeld[31] reported that the formation constant of the first copper bound to protein is $10^{16.8}$ for *Busycon* and $10^{18.7}$ for *Limulus*. The smaller formation constant of *Limulus* compared with *Busycon* listed in Table I is in accord with the general trend that the strength of the ternary complex is related inversely to the strength of the binary complex.[32] The formation constant of *Busycon* oxyhemocyanin–glycinate complex is more than 100 times larger than for the hemocyanin–glycine ester or *N*-acetylglycinate complex, indicating that glycine is bidentate while glycine ester and *N*-acetylglycine are monodentate toward hemocyanin. The same conclusion was reached by White, Manning, and Li[33] on the number of binding sites of ligands with Cu(II) by comparing the formation constants of their complexes. No attempt has been made to obtain a formation constant for glycylglycine because the α- and β-CH_2 signals overlap at pD near 8.[34]

The structure of Cu(II) binding to the protein moiety and oxygen remains unsolved. The nature and number of ligand atoms involved in binding the Cu(II) are also not known. Our data indicate that the Cu(II) at the oxygen-binding site is not saturated. The fact that glycine binds oxyhemocyanin as a bidentate ligand suggests that at least two open coordinate sites exist which are probably occupied by two water molecules in the absence of added ligand. The existence of two open coordinate sites may account for the catalatic activity of hemocyanin.[35] This postulate is in accord with the findings of Sigel[36] and Sharma and Schubert[37] that only copper complexes with two adjacent open coordinate sites possess catalytic activity. However, our data do not exclude the possibility that the binding of glycine displaces two rather loosely held protein ligands.

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