Fluorescence Studies of Oxygen Binding in *Limulus* Hemocyanin

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Abstract □ *Limulus* hemocyanin, a weakly fluorescent copper protein when oxygenated, is highly fluorescent in the deoxygenated forms. A large enhancement of the fluorescence yield, 8.8-fold at neutral pH, was observed on complete deoxygenation, and a 9.4-fold increase was observed on removal of copper from oxyhemocyanin. The weak fluorescence of oxyhemocyanin is attributed to radiationless energy transfer from the tryptophan residues to the Cu···O group. The average distance between these two moieties was 21.4 Å. In oxyhemocyanin, the active site containing copper and oxygen is strongly hydrophobic, and a change in oxygen binding may profoundly alter the hydrophobic structure of the protein. This alteration is indicated by the binding of tetracycline to the protein where the distance between tryptophans and the tetracycline binding site is significantly increased when the protein is deoxygenated or when copper is removed. From the fluorescence results, it was found that the Cu···O group in *Limulus*, an arthropod hemocyanin, is nearer and interacts more strongly with the tryptophan residues than these groups in the mollusk (*Leventina hierosolima* and *Murex trunculus*) hemocyanins. Deoxygenation of oxyhemocyanin by sodium sulfite was catalyzed by trace amounts of certain metal ions. The reaction rate was increased significantly by Co(II), Cu(II), and Mn(II), in decreasing order, but not by Zn(II).

Keyphrases □ *Limulus* hemocyanin—oxygen binding, energy transfer, fluorometric study □ Hemocyanin, *Limulus*—oxygen binding, energy transfer, fluorometric study □ Oxygen binding—*Limulus* hemocyanin, fluorometric study □ Energy transfer—*Limulus* hemocyanin, fluorometric study □ Fluorometry—study of *Limulus* hemocyanin oxygen binding and energy transfer

Hemocyanin is a nonheme, oxygen-carrying copper protein found in many mollusks and arthropods. The protein, a deep-blue color when oxygenated, becomes colorless when deoxygenated. One oxygen molecule binds to two copper atoms in the protein (1). All hemocyanins reversibly bind oxygen and, in most cases, appear to play a physiological role analogous to that of hemoglobin in higher animals, *i.e.*, the transport of oxygen to tissues.

BACKGROUND

While the oxygen binding phenomenon of hemocyanin has been known for some time, the binding mechanism and the nature of the binding site are still not clear. Spectrophotometric studies showed that the absorption spectrum of a deoxyhemocyanin is, in general, a typical protein spectrum. Upon binding oxygen, hemocyanin exhibits a profound change in its absorption spectrum. In addition to the absorption at 280 nm, strong bands in the 340–350- and 400–800-nm regions have been observed. Oxygen binding also results in profound changes in the fluorescence spectrum of the protein.

Shaklai and Daniel (2), in studying the fluorescence of mollusk *Levantina hierosolima* hemocyanin, reported that when the protein was deoxygenated or when copper was removed, the fluorescence yield at pH 6.6 increased fourfold in each case. The fluorescence change was traced to radiationless energy transfer from the tryptophan residues to the Cu···O groups with the average distance between the two units reported to be about 25 Å. Bannister and coworkers (3, 4) reported that for *Murex trunculus*, another mollusk hemocyanin, the fluorescence was attributable to tryptophan and that the fluorescence was enhanced 513% when copper was removed from the protein.

Fluorescence spectroscopy is a useful tool for energy transfer studies. In a protein, the fluorescent tryptophan residue may transfer its electronic energy to a bound ligand if, among other conditions, the two units are closely located on the macromolecule. The energy transfer data permit calculation of the distance between the energy donor and acceptor and, thus, provide useful information about the nature of the binding site. In this investigation, the energy transfer studies on *Limulus* hemocyanin, an arthropod protein that has been extensively investigated for rapid endotoxin assay (5), were carried out using the fluorescence technique. The reasons for this investigation were:

1. There are significant differences between hemocyanins from mollusks and arthropods, including differences in molecular architecture as revealed by electron microscopy, circular dichroism, and subunit dissociation properties (6). Fluorescence studies have been reported only on mollusk hemocyanin.
2. The equations used previously (2) for the energy transfer calculations need to be modified.

To provide additional insight into the oxygen binding of hemocyanin, the effect of metal ions on the deoxygenation of *Limulus* hemocyanin and the effect of oxygen binding on protein interaction with small molecules also were studied. The latter is demonstrated by the binding and energy transfer studies of tetracycline with oxy-, deoxy-, and apohemocyanins.

EXPERIMENTAL

Materials—*Limulus* hemocyanin hemolymph[1] was purified as follows (7, 8). The hemolymph was centrifuged[2] in the cold (4°) at 120,000×*g*. The pellet was dissolved with a small amount of water, dialyzed against water at 4° for 2 days, and ultracentrifuged again; the resulting pellet was dissolved in water. The purified protein was stored at 4°.

Dilutions of the protein solutions were made using 0.05 *M* tromethamine hydrochloride buffer, pH 7. The protein concentration was determined from the absorbance at 280 nm ($E^{1\%}_{1cm}$ = 15.71), using a value of 74,900 for the molecular weight carrying one oxygen binding site in hemocyanin (7, 8). The purified protein did not display a detectable Cu(II) electron spin resonance signal measured at 77° K with a spectrometer[3] operating at 9.06 GHz.

Apohemocyanin was prepared by dialyzing a 3% solution of the purified oxyhemocyanin against 0.1 *M* phosphate buffer containing 0.1 *M* KCN and 0.05 *M* $MgCl_2$ at pH 7.8 for 2 days. Excess cyanide was removed by dialyzing against water for 2 days. The apohemocyanin thus prepared showed no absorption at 340 nm.

Tetracycline hydrochloride[4] and bovine serum albumin[5] were obtained commercially. All other chemicals were reagent grade.

Instruments and Methods—Fluorescence measurements were made with a spectrophotofluorometer[6] equipped with a 150-w xenon lamp, a 1P21 photomultiplier tube, and an *x-y* spectral recorder[7]. The absorption spectra were recorded with a spectrophotometer[8] using a matched set of 1-cm cells. Unless otherwise mentioned, all experiments were carried out in pH 7 tromethamine at 25 ± 0.5°. Fluorescence quenching titrations, in which the fluorescence of the protein was recorded after each addition of a small amount of tetracycline (2 μl of a 0.01 or 0.001 *M* stock solution), were performed manually with microsyringes.

Binding Studies—The binding of tetracycline to hemocyanin was determined using the Scatchard equation:

$$\overline{V}/D_f = nK - \overline{V}K \qquad \text{(Eq. 1)}$$

where $\overline{V}$ is the number of moles of bound tetracycline (D_b) per mole of

[1] Marine Biological Laboratory, Woods Hole, Mass.
[2] Spinco model L ultracentrifuge.
[3] Model E-4, Varian Instrument Division, Palo Alto, Calif.
[4] Lot 73F503, Bristol Laboratories, Syracuse, N.Y.
[5] Lot 1662, Nutritional Biochemicals Corp.
[6] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[7] Omnigraphic, Houston Instrument, Bellaire, Tex.
[8] Cary 118, model C, Varian Instrument Division, Palo Alto, Calif.

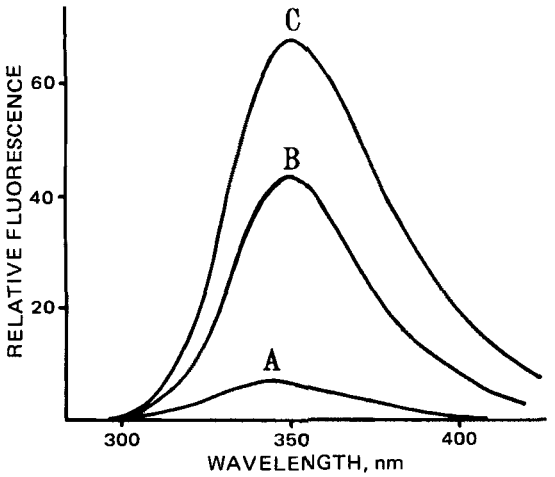

Figure 1—*Fluorescence spectra of* Limulus *hemocyanin in pH 7 tromethamine (excited at 280 nm). Key: A, 1.36 × 10⁻⁶ M oxyhemocyanin; B, 1.36 × 10⁻⁶ M oxyhemocyanin with 0.003 M sodium sulfite (the spectrum was taken after the sample was stored in the covered cell compartment for 25 min); and C, 1.36 × 10⁻⁶ M apohemocyanin.*

protein, D_f is the concentration of unbound tetracycline, n is the number of binding sites, and K is the binding constant. To calculate D_b, from which the values of $\overline{V}$ and D_f were obtained, fluorescence quenching titrations were made over a range of protein concentrations. The fluorescence intensities, expressed as percentages of the initial fluorescence of the protein, were then plotted as a function of the tetracycline–protein concentration ratio. Above a certain critical protein concentration, the titration curves were superimposable since all tetracycline molecules were bound. The concentration of bound tetracycline for a solution of lower protein concentration was then calculated by (9):

$$D_b = (D_t)(R_h)/(R_l) \qquad \text{(Eq. 2)}$$

where D_t is the total tetracycline concentration, and R_h and R_l are the tetracycline–protein ratios at the same intensity for high and low protein solutions, respectively. In this study, tetracycline binding to 1.36×10^{-6} M oxy- and apohemocyanins was determined using 3.4×10^{-5} M as the high protein concentration.

Energy Transfer Theory—The quenching of the fluorescence of deoxyhemocyanin by oxygen binding is due to the energy transfer from excited-state tryptophan residues (energy donor) to the Cu···O groups (energy acceptors). The energy transfer information allows calculation of the mean distances, R, between the donor and acceptor by (10):

$$X/(1 - X) = (R_0/R)^6 \qquad \text{(Eq. 3)}$$

where X is the fraction of the absorbed photons transferred and R_0 is the characteristic distance at which the probability of energy transfer is equal to the probability of fluorescence. The fraction of energy transfer read may be calculated from the percent quenching of the protein fluorescence.

According to Forster's theory (11):

$$R_0 = (1.69 \times 10^{-33} \tau J_v / n^2 \overline{v}_0^2)^{1/6} \text{ cm} \qquad \text{(Eq. 4)}$$

where τ is the fluorescence lifetime of the donor in the absence of transfer, n is the refractive index of the solvent, $\overline{v}_0$ is the mean of the wave numbers of the maximum absorption of the acceptor and the emission of the donor, and J_v is the overlap integral. To determine J_v and $\overline{v}_0$, Eq. 5 may be used:

$$J_v = \int_0^\infty E(v)F(v)dv \qquad \text{(Eq. 5)}$$

where $E(v)$ is the molar absorption coefficient of the acceptor and $F(v)$ is the molar emission coefficient of the donor. The molar emission function, $F(v)$, represents the photospectral distribution of the fluorescence in the unit set by the equality that $F(v)$ at maximum emission equals the molar absorption coefficient of the donor. The values of J_v and $\overline{v}_0$ can be determined graphically. A simplified approach to the determination of J_v was introduced by Weber (12) using Gaussian approximations. By letting:

$$E(v) = E_1 \exp - [(v - v_a)/\sigma_a]^2 \qquad \text{(Eq. 6)}$$

$$F(v) = E_2 \exp - [(v - v_e)/\sigma_e]^2 \qquad \text{(Eq. 7)}$$

the overlap integral was obtained as:

$$J_v = E_1 E_2 \frac{\sqrt{\pi}}{\sqrt{\frac{1}{\sigma_a^2} + \frac{1}{\sigma_e^2}}} \exp - [(v_a - v_e)^2/(\sigma_a^2 + \sigma_e^2)] \qquad \text{(Eq. 8)}$$

where E_1 and E_2 are the maximum molar absorption coefficients for the acceptor and donor, respectively; v_a is the wave number of maximum absorption of the acceptor with standard deviation σ_a; and v_e is the wave number of maximum emission of the donor with standard deviation σ_e. Equation 8 was used in the energy transfer studies of *L. hierosolima* hemocyanin (2). However, the equation is subject to modification since Eqs. 6 and 7 do not represent the Gaussian distribution. To represent the Gaussian curves, where 1 SD = ~68% of the total curve area, Eqs. 6 and 7 should be rewritten as:

$$E(v) = E_1 \exp - \tfrac{1}{2}[(v - v_a)/\sigma_a]^2 \qquad \text{(Eq. 9)}$$

$$F(v) = E_2 \exp - \tfrac{1}{2}[(v - v_e)/\sigma_e]^2 \qquad \text{(Eq. 10)}$$

By using Eqs. 9 and 10, the integration of Eq. 5 gives:

$$J_v = E_1 E_2 \frac{\sqrt{2\pi}}{\sqrt{\frac{1}{\sigma_a^2} + \frac{1}{\sigma_e^2}}} \exp - \tfrac{1}{2}[(v_a - v_e)^2/(\sigma_a^2 + \sigma_e^2)] \qquad \text{(Eq. 11)}$$

The maximum of the overlap function, $\overline{v}_0$, can be calculated from the following equation:

$$\overline{v}_0 = (\sigma_e^2 v_a + \sigma_a^2 v_e)/(\sigma_a^2 + \sigma_e^2) \qquad \text{(Eq. 12)}$$

The result calculated from Eq. 11 is significantly different from that calculated from Eq. 8, especially in a system where the absorption maximum of the acceptor and the emission of the donor are largely separated.

RESULTS AND DISCUSSION

The fluorescence spectra of 1.36×10^{-6} M oxyhemocyanin and apohemocyanin from *Limulus*, measured in pH 7 tromethamine, are shown in Fig. 1. The excitation and emission maxima for oxyhemocyanin were 280 and 345 nm, respectively. Apohemocyanin, the copper-free protein, showed an enhanced fluorescence, 10-fold in intensity as compared to oxyhemocyanin at 350 nm (curve C). Curve B shows the effect of sodium sulfite (0.003 M) on oxyhemocyanin fluorescence. The spectrum was taken after the sample was stored in the covered cell compartment for 25 min when the intensity had ceased to increase. Deoxygenation was indicated by the sixfold increase of fluorescence intensity accompanied by a shift of the emission maximum from 345 to 350 nm. The shift indicates a change of the hydrophobic environment of the tryptophan residues in the protein upon oxygen binding.

Hemocyanin denaturation in low pH solutions resulted in protein precipitation. At pH < 3, the solution became clear and colorless. The relationship of pH and fluorescence of oxyhemocyanin and of the amino acid tryptophan is shown in Fig. 2. For tryptophan, which is primarily

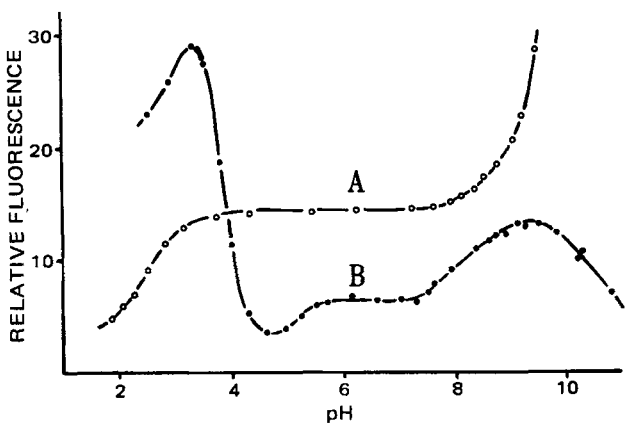

Figure 2—*The pH effect on the fluorescence of oxyhemocyanin and tryptophan in pH 7 tromethamine. Key: A, 5 × 10⁻⁶ M tryptophan; and B, 6.2 × 10⁻⁶ M oxyhemocyanin.*

Table I—Fluorescence Data of *Limulus* Hemocyanin in pH 7 Tromethamine

Type	Emission Maximum, nm	Quantum Yield, %	Fluorescence Lifetime, nsec
Oxyhemocyanin	345	1.2	0.15
Deoxyhemocyanin	350	10.5	1.26
Apohemocyanin	350	11.3	1.36

responsible for the fluorescence of the protein, a pH titration curve was obtained where the pH of the inflection points corresponded to pK_1 (2.3) and pK_2 (9.38) (13). White (14) reported similar pH titration curves for the fluorescence of tryptophan, tryptophan methyl ester, and *N*-acetyltryptophan.

For oxyhemocyanin, a sharp increase in fluorescence occurred at low pH values, indicating the dissociation of oxygen from hemocyanin upon denaturation in acid solution. In the pH 6.0–9.5 range, oxyhemocyanin fluorescence increased with increasing pH, indicating a reverse Bohr effect, where the oxygen binding affinity decreases with increasing pH. Most hemocyanins have a positive Bohr effect, but reverse Bohr effects have been reported for *Limulus* and several other gastropod mollusks (15, 16). The presence of a reverse Bohr effect has been proposed to have physiological significance. *Limulus* hemolymph often has low oxygen and carbon dioxide concentrations, as for example, when the animal burrows in mud or is exposed in air during egg laying. Oxygen binding and pH studies (6) showed that the biological significance of a reverse Bohr effect is to ensure a constant supply of oxygen to the tissues. When oxygen is less available, the blood pH normally drops because of an increase in the carbon dioxide concentration. The increased Bohr effect thus compensates for the lowered oxygen pressure.

Similar to the pH effect, the addition of Cu(II) ion to hemocyanin also produced a cloudy suspension. The absorption spectrum of the copper-treated hemocyanin was similar to that of aged hemocyanin, which had partly lost its oxygen binding capacity. The addition of small amounts of Co(II) and Mn(II) ions did not affect the fluorescence or absorption of oxyhemocyanin; only Cu(II) ion quenched the fluorescence of the protein. The latter effect may be explained as follows. Tryptophan has two excited single states from which fluorescence can occur and a triplet state from which phosphorescence can take place. Since the Cu(II) ion can facilitate singlet–triplet conversion (3), the addition of a small amount of Cu(II) quenches the protein fluorescence.

Oxyhemocyanin is slowly deoxygenated by sodium sulfite. Complete deoxygenation, however, can be achieved rapidly by adding traces of certain metal ions. In a solution containing 4.4×10^{-6} *M* oxyhemocyanin and 0.005 *M* sodium sulfite, the addition of 1×10^{-4} *M* Co(II), Cu(II), or Mn(II) resulted in an immediate diminution of the copper absorption band at 340 nm as well as a large enhancement of protein fluorescence. In the absence of these metal ions, the fluorescence or absorption bands did not change during the measuring time. On the other hand, the Zn(II) ion did not affect the fluorescence or absorption spectrum of the sulfite-treated protein.

Veprek-Siska and Lunka (17) discussed the role of the copper ion in

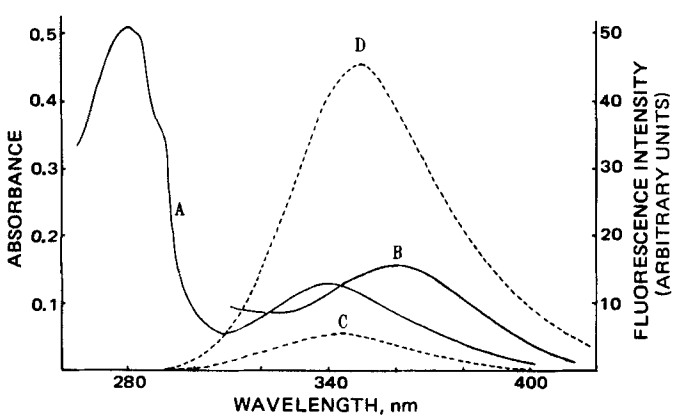

Figure 3—*Fluorescence and absorption spectra of* Limulus *hemocyanin and tetracycline. Key: A, absorption spectrum of* 4.21×10^{-6} M *oxyhemocyanin; B, absorption spectrum of* 1×10^{-5} M *tetracycline; C, fluorescence spectrum of* 4.21×10^{-6} M *oxyhemocyanin; and D, fluorescence spectrum of* 4.21×10^{-6} M *deoxyhemocyanin.*

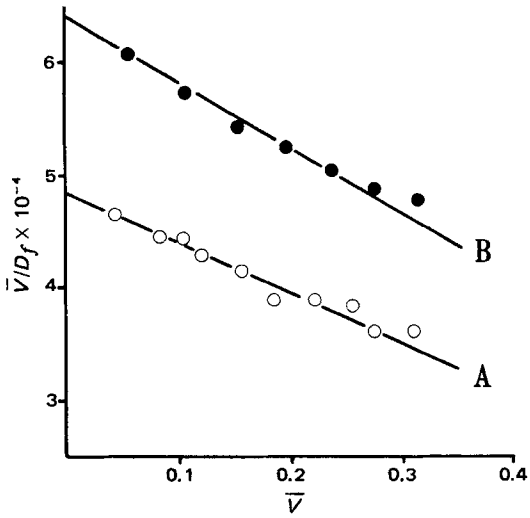

Figure 4—*Scatchard plots for the binding of tetracycline to oxyhemocyanin (A) and apohemocyanin (B).*

copper-catalyzed oxidation of sulfite and proposed the formation of a sulfito–cuprous complex $Cu(SO_3)_n^{-2n+1}$. Oxygen would react with the sulfito–cuprous complex to form intermediate products of the type $O_2Cu(SO_3)_n^{-2n+1}$. It may well be that the same mechanism holds for the copper-catalyzed reaction of sulfite with hemocyanin in the formation of deoxyhemocyanin.

Table I summarizes the fluorescence data of *Limulus* oxy-, deoxy-, and apohemocyanins (all at 4.21×10^{-6} *M*) at neutral pH. The fluorescence yield was calculated by comparing the area under the emission peak with that of a bovine serum albumin solution of equal absorbance at 280 nm and taking 15.2% for the quantum yield of bovine serum albumin (18). Complete deoxygenation of oxyhemocyanin, obtained by treating the oxygenated protein with 0.005 *M* sodium sulfite and 1×10^{-5} *M* $CuCl_2$, resulted in an 8.8-fold increase of the fluorescence yield, significantly greater than the increases reported for the mollusk *L. hierosolima* (fourfold) and *M. trunculus* (fivefold) hemocyanins (2, 3). The fluorescence lifetime data shown in Table I were calculated from the natural lifetime of tryptophan, which was taken to be 12 nsec (19), and the fluorescence yield of hemocyanins.

The fluorescence change of hemocyanin on oxygen binding is due to the energy transfer from the tryptophan residues to the Cu···O groups. The conditions necessary for energy transfer are: (*a*) the absorption spectrum of the acceptor must overlap the emission spectrum of the donor, (*b*) the relative orientations of the oscillators must permit strong interaction, and (*c*) the donor and acceptor must be within a certain distance for a given efficiency of energy transfer (20).

Figure 3 shows that the protein fluorescence band strongly overlaps the Cu···O absorption band at 340 nm. The fact that the fluorescence of hemocyanin is a function of oxygenation (2, 3) also suggests that the fluorescence change is due to the energy transfer process. When using Eqs. 4 and 11 and the following values: $E_1 = 2.996 \times 10^4$ cm²/mmole (molar absorption of the Cu···O group at 340 nm, calculated from the value of $E^{1\%}_{1\,cm} = 4.0$ and using a value of 74,900 for the molecular weight carrying one oxygen binding site), $E_2 = 0.59 \times 10^4$ cm²/mmole (molar absorption of tryptophan at 280 nm), $\upsilon_a = 29{,}410$ cm^{-1}, $\upsilon_e = 28{,}570$ cm^{-1}, $\sigma_a = 1850$ cm^{-1}, $\sigma_e = 1710$ cm^{-1}, $\bar{\upsilon}_0 = 2.9 \times 10^4$ cm^{-1}, $n = 1.33$, and $\tau = 1.26$ nsec, the energy transfer parameters were calculated as follows: $J_\upsilon = 5.26 \times 10^{11}$ cm³/mmole² and $R_0 = 30.2$ Å.

The average distance R between the tryptophan residues and the Cu···O group was calculated, based on the quantum yield data given in Table I and Eq. 3, to be 21.4 Å. The calculated R value represents a random distribution of the Cu···O group with respect to all tryptophan residues in the protein subunit. It is conceivable that the fluorescence of certain tryptophans may be 100% quenched upon oxygen binding while that of other residues is quenched to a lesser degree. Nevertheless, the results do suggest an interrelationship between the tryptophan residues and the Cu···O group.

By using the same equations and the data reported by Shaklai and Daniel (2), the values of R_0 and R for the mollusk *L. hierosolima* hemocyanin at pH 6.6 were calculated to be 29.4 and 24.5 Å, respectively. The Cu···O group in the arthropod *Limulus* hemocyanin is closer to the

Table II—Energy Transfer Parameters in *Limulus* Hemocyanin

Donor	Acceptor	J_v, cm³/mmole²	R_0, Å	R, Å
Deoxyhemocyanin tryptophans	Cu···O group	5.26×10^{11}	30.2	21.4
Oxyhemocyanin tryptophans	Tetracycline	2.74×10^{11}	19.0	11.6
Deoxyhemocyanin tryptophans	Tetracycline	2.87×10^{11}	27.5	16.8
Apohemocyanin tryptophans	Tetracycline	2.99×10^{11}	28.1	17.2

tryptophan residues, in line with the more pronounced fluorescence change found in *Limulus* in going from the oxy to deoxy form as compared to the mollusk hemocyanins. In oxyhemocyanin, the active site containing copper and oxygen is in a hydrophobic region. The tyrosine and tryptophan residues may be located near the hemocyanin copper ions, stabilizing the hydrophobic oxygen site (2, 4, 21).

To show the effect of oxygen binding on the interaction of hemocyanin with small molecules, binding and energy transfer studies of tetracycline to *Limulus* hemocyanin were performed. The use of tetracycline is interesting, since it is capable of receiving energy from tryptophan and has been reported to bind close to the tryptophan residues of plasma protein (22). Figure 4 shows the Scatchard plots for the binding of tetracycline to oxy- and apohemocyanins. Tetracycline binds to the proteins in a ratio of one molecule per protein unit carrying one oxygen binding site. The binding affinity in apohemocyanin ($K = 5.9 \times 10^4\ M^{-1}$) is slightly higher than that in oxyhemocyanin ($K = 4.5 \times 10^4\ M^{-1}$).

The R_0 values for the binding of tetracycline to the proteins are shown in Table II. These values were calculated by Eqs. 4 and 11, using the following data: $E_1 = 1.56 \times 10^4$ cm²/mmole (molar absorption of tetracycline at 360 nm; the spectrum is shown in Fig. 3); $E_2 = 0.59 \times 10^4$ cm²/mmole; $\upsilon_a = 27{,}780$ cm^{-1}; $\upsilon_e = 28{,}990$ cm^{-1} for oxyhemocyanin and 28,570 cm^{-1} for deoxy- and apohemocyanins; $\sigma_a = 2000$ cm^{-1}; $\sigma_e = 1750$ cm^{-1} for oxyhemocyanin, 1710 cm^{-1} for deoxyhemocyanin, and 1840 cm^{-1} for apohemocyanin; $\bar{\upsilon}_0 = 28{,}500$ cm^{-1} for oxyhemocyanin, 28,240 cm^{-1} for deoxyhemocyanin, and 28,210 cm^{-1} for apohemocyanin; $n = 1.33$; and $\tau = 0.15$ nsec for oxyhemocyanin, 1.26 nsec for deoxyhemocyanin, and 1.36 nsec for apohemocyanin.

Tetracycline strongly quenched the fluorescence of hemocyanin. In a solution of $3.4 \times 10^{-5}\ M$ oxy-, deoxy-, or apohemocyanin, the fluorescence titration with tetracycline, in each case, resulted in about 95% quenching of the protein fluorescence. Based on 95% quenching, values of R were calculated using Eq. 3 (Table II). The results indicate that the energy transfer distance between hemocyanin tryptophan and bound tetracycline is the same in deoxy- and apohemocyanins but that the distance is significantly smaller in oxyhemocyanin.

Schoot-Uiterkamp *et al.* (23) reported a Cu–Cu distance of 6 Å in *N,O*-hemocyanin. Since the copper in this hemocyanin and in deoxyhemocyanin is cuprous, Freedman *et al.* (24) took 6 Å as the Cu–Cu distance in deoxyhemocyanin and compared this value with the 3.5–5-Å distance calculated for oxyhemocyanin, where both coppers are cupric and the oxygen is peroxide ion (O_2^{2-}). Considerable movement of the copper ions away from each other must have occurred when the oxyhemocyanin was deoxygenated. This finding is in line with the increase in the hemocyanin tryptophan-bound tetracycline distance when the oxyhemocyanin was deoxygenated.

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