shielding constants, particularly for systems containing hetero nuclei.

The present work, however, shows clearly that there does indeed exist a distinct difference between the conformations of the oxidized and reduced pyridine dinucleotides.

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Fluorescence Properties of Hemocyanin from *Levantina hierosolima**

Nurith Shaklai and Ezra Daniel

ABSTRACT: The emissive properties of reduced and oxygenated hemocyanin from *Levantina hierosolima* at neutral and alkaline pH were studied. A large enhancement in fluorescence intensity is observed on complete reduction, fourfold at neutral pH. In partially reduced protein the enhancement is proportional to the fraction reduced. The quenching upon oxygen binding is traced to radiationless energy transfer from the tryptophanyl residues to the Cu···O groups. Application of Förster's theory leads to a value of 25 Å for the distance between the donor and the acceptor in the "equivalent oscillators system," defined as a single donor–acceptor pair with parallel orientation which would display the same emissive properties as the actual protein molecule. From a comparison of the data obtained at neutral and alkaline pH, it is concluded that the internal arrangement of the donor–acceptor system is not affected by the extensive dissociation which the molecule undergoes in the transition between the two pH values considered. The findings indicate that the fluorimetric method provides a sensitive tool for the study of oxygen binding equilibria in hemocyanin.

Hemocyanin is the oxygen binding protein in molluscs and arthropods. Many studies have been carried out on the protein to determine the size, shape, and subunit structure of the molecule. In contrast, the binding of oxygen has received less attention. In the course of a study designed to examine the effect of changes in the quaternary structure of hemocyanin on its oxygen binding function, the need for a sensitive experimental method to measure the binding was quickly felt. Fluorescence measurements have been used with success in binding studies in some systems (Anderson and Weber, 1965; Daniel and Weber, 1966). In this paper the emissive properties of hemocyanin from *Levantina hierosolima* were studied. The results show that the binding of oxygen brings about a conspicuous change in fluorescence efficiency which may be used to advantage in the study of problems of the kind that motivated this work.

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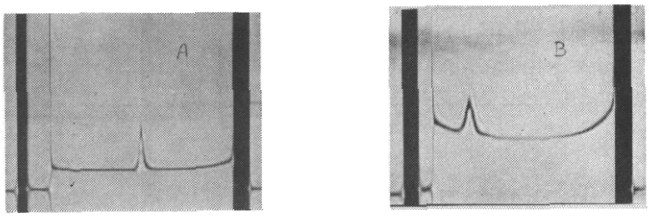

FIGURE 1: Sedimentation diagrams of *Levantina hierosolima* hemocyanin: (A) pH 6.6, 44,000 rpm, 5 min after reaching speed; (B) pH 12.0, 56,000 rpm, 24 min after reaching speed.

Experimental Section

Materials. Oxygenated hemocyanin[1] (Hcy-O) was prepared from the mollusc *L. hierosolima*. The shell covering the location of the heart was imbibed with concentrated HCl (diluted 1:4) until it almost completely dissolved. The residual thin layer was peeled off with a pincette and a puncture in the pericard was made. The blood was collected in a cooled test tube. Blood from several molluscs was pooled and centrifuged for 15 min in a Sorvall centrifuge (3000*g*) to remove particulate matter. The supernatant was centrifuged at 200,000*g* in a Spinco Model L preparative centrifuge for 10 hr at 4°. The supernatant was discarded and the precipitate was dissolved in 0.1 M phosphate buffer (pH 6.6). The solution was passed through a Sepharose B-4 column equilibrated with the same buffer and the eluent hemocyanin solution—absorbance $\sim$ 6 at 280 mμ—was kept in the cold room at 4° until used. Reduced hemocyanin (Hcy) was prepared as follows: A 50-ml glass bulb was connected to a 1 $\times$ 1 cm^2, four-sided quartz cuvet. About 3 ml of Hcy-O solution was introduced and the system was deaerated by connecting to a vacuum system. To prevent excessive evaporation of the solution, the deaeration was effected through repeated cycles of short evacuations. Complete deoxygenation was indicated by the complete disappearance of the 345-mμ peak. Apohemocyanin (apo-Hcy) was prepared from Hcy-O by cyanide treatment according to the method of Ghiretti as modified by Cohen and Van Holde (1964). Again the disappearance of the 345-mμ peak was taken as an indication that the reaction was complete. All chemicals were analytical grade.

Methods. Sedimentation was carried out in a Spinco Model E analytical ultracentrifuge equipped with schlieren and absorption scanner optics. Absorption spectra were measured in a Cary 14 spectrophotometer. Fluorescence spectra were measured in a Hitachi Perkin-Elmer spectrofluorophotometer, Model HPF-2A. Emission was observed at 90° to the incident light. Compensation for fluctuation of the light source was effected by ratio recording, where the emitted light is constantly compared with a sample of the incident beam. The performance of the spectrofluorophotometer was tested by recording the emission spectra of tyrosine, tryptophan, and bovine serum albumin in water and comparing the observed emission maxima and band widths with the corresponding corrected values reported in the literature. It was found that the error in the location of the maxima is $\pm$2 mμ and the error in the half-band width is $\pm$1 mμ.

[1] Abbreviations used are: Hcy-O, oxygenated hemocynin; Hcy, reduecd hemocyanin; apo-Hcy, apohemocyanin.

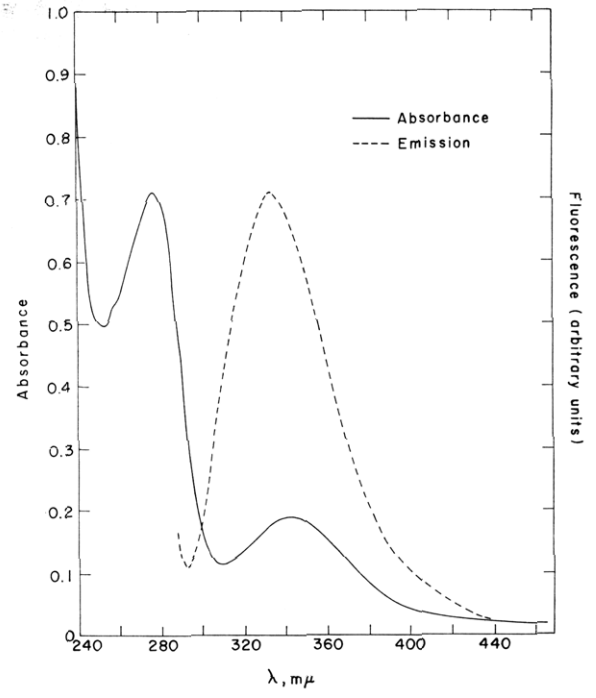

FIGURE 2: Absorption and emission spectra of *Levantina hierosolima* oxyhemocyanin, Hcy-O, pH 6.6.

Results

Sedimentation. Figure 1A shows a typical schlieren photograph of Hcy-O at pH 6.6. The material sediments giving a single boundary which remains sharp down through the cell. Absorption scans at 280 mμ showed that no absorbing material remained behind the sedimenting species. This may be taken as an indication of the purity of the material. At pH 6.6, $s_{20,w} \simeq 100$ S. At pH 12.0 the material sediments in a single peak (Figure 1B), $s_{20,w} \simeq 7$ S, indicating that considerable dissociation has taken place.

Absorption. The absorption spectrum of Hcy-O at pH 6.6 exhibits the typical hemocyanin maxima, the protein peak at 278 mμ and the Cu$\cdots$O bands—the so-called "copper bands"—at 345 and 570 mμ (Figure 2 shows a portion of the spectrum). Due to their large size, hemocyanin molecules show appreciable scattering in solution (Heirweigh *et al.*, 1961); therefore, the proportionality of the observed absorbance to protein concentration was checked. At 278 mμ the observed plot deviated from linearity. However, since the scattering follows a fourth power relation to the wavelength of the light, the deviation from linearity was expected to be smaller at a higher wavelength. Indeed, at the concentrations we used in this work, the absorbance at 345 mμ was found linear with the concentration (Figure 3). At pH 6.6, an $E^{1\%}_{1\,cm}$ value of 4.00 $\pm$ 0.20 was determined at 345 mμ from an absorbance and nitrogen determination (Kjeldahl) on a fully oxygenated hemocyanin sample, taking a nitrogen content of 15.5% (Ghiretti-Magaldi *et al.*, 1966). That full oxygenation is attained by equilibration with air is indicated by the finding that no change in absorption (and fluorescence) properties occurs upon equilibration with pure oxygen. At pH 12.0, both the protein and the "copper absorption bands" move to longer wavelengths: protein band, 278 $\rightarrow$ 283 mμ; "copper

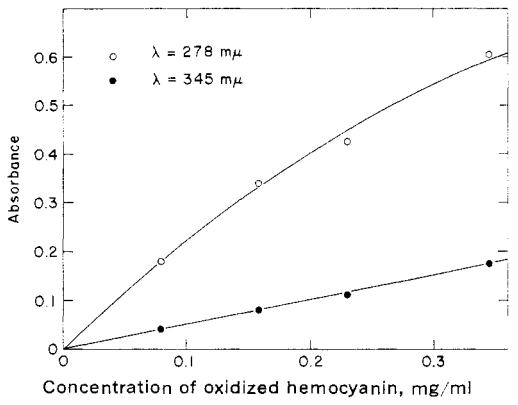

FIGURE 3: Dependence of absorption at 278 and 345 mμ upon the protein concentration in *Levantina hierosolima* oxyhemocyanin

band," 345 → 348 mμ. At the alkaline pH and at 348 mμ, $E^{1\%}_{1\,cm}$ is 3.40 ± 0.20.

Fluorescence. The fluorescence spectrum of Hcy-O at pH 6.6, excited at 280 mμ, is given in Figure 2. Table I summarizes the wavelength maxima, band widths, and fluorescence yields of the emission bands at pH 6.6 of Hcy-O, Hcy, and apo-Hcy. The fluorescence characteristics of hemocyanin at pH 12.0 are shown in Table II.

Extent of O_2 Binding from Fluorescence Intensity Measurements. The absorbance at 345 mμ is a measure of concentration of Hcy-O. Therefore the correlation between the fluorescence and the absorbance at 345 mμ may be used to obtain the relation between oxygen binding and fluorescence intensity. In order to obtain the latter relation at pH 6.6, experiments were carried out in the following way. An Hcy-O solution was completely reduced through evacuation (see Experimental Section). Reoxygenation of the reduced Hcy was effected in steps by introducing small quantities of air into the system. After each step, the absorbance at 345 mμ and the fluorescence were measured. It is seen from Figure 2 that the band at 278 mμ contributes a residual absorbance at 345 mμ. The observed absorbance at 345 mμ was therefore corrected for the residual absorbance, the exact value of the contribution being estimated by comparison with the apo-Hcy spectrum.

TABLE I: Fluorescence Characteristics of *Levantina hierosolima* Hemocyanin at pH 6.6.

	Wavelength at Maximum Emission (mμ)	Half-Band Width (mμ)	Yield[a] (%)
Hcy-O	331	24	2.1
Hcy	335	22	8.4
apo-Hcy	335	22	8.3

[a] Estimated by comparison of area under the emission peak with that of a bovine serum albumin solution of equal absorbance at 280 mμ, and taking 15.2% for the quantum yield of the latter (Teale, 1960).

TABLE II: Fluorescence Characteristics of *Levantina hierosolima* Hemocyanin at pH 12.0.

	Wavelength at Maximum Emission (mμ)	Half-Band Width (mμ)	Yield[a] (%)
Hcy-O	342	26	2.1
Hcy	342	24	4.0

[a] Estimated by comparison of area under the emission peak with that of a bovine serum albumin solution of equal absorbance at 280 mμ, and taking 15.2% for the quantum yield of the latter (Teale, 1960).

In a mixture of Hcy-O and Hcy, the fraction oxygenated is therefore given by $A_{345}/A_{0,345}$, where A_{345} and $A_{0,345}$ are the corrected absorbances in the partly reduced and fully oxygenated hemocyanin. If the corresponding fluorescence intensities are F and F_0, the fluorescence enhancement is given by F/F_0. In Figure 4 the fluorescence enhancement is plotted as a function of the fraction reduced. The plot is linear.

The reversibility of O_2 binding at pH 6.6 was indicated by the finding that the absorbance and fluorescence intensity of hemocyanin that had been reduced and then fully reoxygenated attained the value of the native Hcy-O. At pH 12.0 the absorbance at 345 mμ shows a tendency to decrease slowly with time, indicating that the protein might be undergoing partial irreversible denaturation at this high pH, with consequent loss of oxygen binding ability. Therefore, experiments designed to measure the fluorescence enhancement at pH 12.0 were limited to determine the maximum enhancement upon full reduction (see Table II). We estimate that not more than 10% of the protein loses its oxygen binding ability during the time needed for the experiment, *ca.* 2 hr.

Discussion

The results of this study show that apo-Hcy, Hcy, and Hcy-O exhibit fluorescence when excited at 280 mμ. Based on the amino acid content of hemocyanins from molluscs (Ghiretti-Magaldi *et al.*, 1966), the ratio of tyrosyl to tryptophanyl residues may be taken as 18:7. Both tyrosine and tryptophan absorb at 280 mμ and the free amino acids emit, respectively, at 303 and 348 mμ (Teale and Weber, 1957). At neutral pH, *L. hierosolima* hemocyanin's maximum emission is in the 331–335-mμ range. Earlier studies (Teale, 1960) indicated that in proteins containing tyrosine and tryptophan the emission is due to tryptophan only. Shifts in maximum emission of proteins with respect to the corresponding value in tryptophan were attributed to hydrophobic environment of the tryptophanyl residues (Weber, 1961a). However, more recent studies (Weber, 1961b,c) show that tyrosine fluorescence is not completely quenched and the shift in maximum emission might be due to the contribution from tyrosine. The similarity of fluorescence yield of Hcy and apo-Hcy indicates that the binding of copper by itself does not affect the emission. The remarkable

quenching of fluorescence in Hcy-O must be attributed to the Cu···O complex. As may be seen from Figure 2, there is an overlap between the Cu···O band at 345 mμ and the emission of hemocyanin. Not more than 10% of the observed quenching may be attributed to trivial reabsorption (our solutions had a maximum $A_{345} = 0.1$ for an optical path of 1 cm). The quenching of fluorescence may be traced to nonradiative electronic energy transfer from the originally excited tryptophanyl residue (donor)[2] to the Cu···O complex (acceptor). According to Förster's theory (Förster, 1947), the transfer of energy between a pair of dipole oscillators is given by the equation:

$$\frac{\nu}{\lambda} = \chi^2\left(\frac{R_c}{r}\right)^6 \tag{1}$$

where ν is the transition probability of transfer, λ is the transition probability of emission, r is the distance between donor and acceptor, and χ^2 is the angular probability of transfer which depends upon the mutual orientation of the dipoles. R_c is a characteristic distance at which transfer of the excited state and emission without transfer are equaly probable for parallel oscillators and is given by the equation[3] (Weber, 1960):

$$R_c = \left[\frac{2540}{n^2}\,\frac{\tau J_{\bar\nu}}{\left(\frac{\bar\nu_a + \bar\nu_e}{2}\right)^2}\right]^{1/6} \times 10^{-8}\ \text{cm} \tag{2}$$

Here n is the refractive index of the solvent, $\bar\nu_a$ is the wave number of maximum absorption of the acceptor, $\bar\nu_e$ is the wave number of maximum emission of the donor, τ is the lifetime of the excited state, and $J_{\bar\nu}$ is the overlap integral. Assuming Gaussian shape for the absorption and emission bands, $J_{\bar\nu}$ is given by the relation (Weber, 1960):

$$J_{\bar\nu} = E_1 E_2\,\frac{\sqrt{\pi}}{\left[\frac{1}{\sigma_a^2} + \frac{1}{\sigma_e^2}\right]^{1/2}} \exp - \left[\frac{(\bar\nu_a - \bar\nu_e)^2}{\sigma_a^2 + \sigma_e^2}\right] \tag{3}$$

where E_1 is the molar absorption coefficient at the maximum of absorption of the acceptor, E_2 is the corresponding value of the donor at the absorption band which is the mirror image of emission, and σ_a and σ_e are the standard deviations, in wave numbers, of the absorption and emission bands, respectively. In our calculations we have made use of the following numerical values: $E_1 = (2.03 \pm 0.10)10^4$ l. mole^{-1} cm^{-1} (molar absorption of Cu···O at 345 mμ, calculated from the value of $E^{1\%}_{1cm}$ and using a value of 50,800 for the molar weight carrying one oxygen binding site in hemocyanin (Ghiretti-Magaldi *et al.*, 1966)); $E_2 = (0.59 \pm 0.02)10^4$ l. mole^{-1} cm^{-1} [molar absorption of tryptophan at 280 mμ (Teale and Weber, 1957)]; $\bar\nu_a = (2.90 \pm 0.01)10^4$ cm^{-1}; $\bar\nu_e = (2.98 \pm 0.02)10^4$ cm^{-1}; $\sigma_a = \sigma_e = (0.19 \pm 0.01)10^4$ cm^{-1}; $n = 1.33$. For τ we have used a value of 1.52 ± 0.15 nsec, calculated from the equation $\tau = q\tau_n$, where τ_n, the natural lifetime of excitation in tryptophan, is taken equal to 12 nsec (Weber, 1961a) and q, the fluorescence yield of tryptophan in hemocyanin, is 12.7% (the latter value was calculated from the overall yield of fluorescence of Hcy (Table I) and the average tyrosine to tryptophan ratio in molluscs hemocyanins). It should be borne in mind that the calculation of τ rests on the assumption that the processes that reduce the quantum yield from unity are competitive with the emission. Using the values listed above we obtain, at pH 6.6, $J_{\bar\nu} = (2.62 \pm 0.55)10^{11}$ mmole^{-2} cm^3 and $R_c = 29.5 \pm 1.6$ Å.

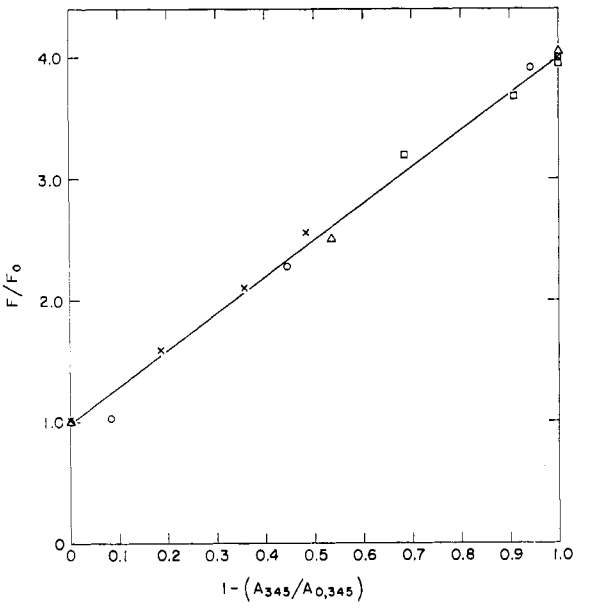

FIGURE 4: Plot of the fluorescence enhancement *vs.* the fraction of reduced protein in *Levantina hierosolima* hemocyanin. Each symbol represents values obtained in the same experiment. Total protein concentration was about 250 μg/ml.

At this point it is proper to make the following comment. Equation 1 holds for a single donor–acceptor pair. In an actual molecule of hemocyanin, we have a system of many acceptors and donors. In the absence of detailed knowledge about the exact distribution and relative orientations of the individual donors and acceptors, we define an "equivalent oscillators system," consisting of a single donor–acceptor pair, with parallel orientation, $\chi^2 = 1$, which would represent the behavior of the actual molecule as far as energy transfer is concerned (*cf.* Weber and Daniel, 1966). With this model, eq 1 may be cast in the form:

$$\frac{F}{F_0} - 1 = \left(\frac{R_c}{r_e}\right)^6 \tag{4}$$

[2] That energy transfer from tyrosyl residues to the Cu···O complex may be neglected in comparison with that from tryptophanyl residues is justified by the comparison of the corresponding characteristic distances, R_c. Substituting in eq 2 the spectral parameters of the free amino acids in water and the lifetimes of the two residues in hemocyanin (assuming for the tyrosyl $q = 0.02$ as found for bovine serum albumin (Weber, 1961c)), we obtain $R_c = 26$ and 8 Å for the tryptophanyl and tyrosyl transfer distances, respectively. The much lowered characteristic transfer distance of the tyrosyl greatly reduces the probability of its participation in energy transfer to the Cu···O complex.

[3] If no preferential orientation of oscillators is assumed, *i.e.*, random directional distribution, the numerical multiplier inside the bracket in eq 2 is to be multiplied by $^2/_3$ and becomes equal to 1690 (Weber, 1960).

TABLE III: Energy Transfer Parameters in *Levantina hierosolima* Hemocyanin.

pH	$J_{\bar{\nu}}$, $\times 10^{-11}$, mmole^{-2} cm^3	R_0, Å	r_e, Å
6.6	2.62 ± 0.55	29.5 ± 1.6	24.6 ± 1.6
12.0	2.35 ± 0.43	25.8 ± 1.2	26.2 ± 1.7

where F_0 and F are the fluorescence efficiencies with and without energy transfer, respectively, and r_e is the distance between the donor and acceptor in the equivalent system. With the value calculated for R_0 and the experimental value $F/F_0 = 4.0 \pm 0.2$ one obtains, at pH 6.6, $r_e = 24.6 \pm 1.6$ Å.

The fluorescence characteristics of Hcy and Hcy-O at pH 12.0 show that compared with the corresponding values at pH 6.6 the maxima of emission are shifted to longer wavelengths and the quantum yields are lowered. The shift in maximum emission may be attributed to a more hydrophylic environment of tryptophan and/or greater quenching of tyrosine fluorescence. Treatment of the data at pH 12.0 is carried out as before. In this case we use the following values: $E_1 = (1.73 \pm 0.10)10^4$ l. mole^{-1} cm^{-1}; $E_2 = (0.56 \pm 0.02)10^4$ l. mole^{-1} cm^{-1} (Teale and Weber, 1957; Donavan *et al.*, 1961); $\bar{\nu}_a = (2.87 \pm 0.01)10^4$ cm^{-1}; $\bar{\nu}_e = (2.92 \pm 0.02)10^4$ cm^{-1}; $\sigma_a = \sigma_e = (0.20 \pm 0.01)10^4$ cm^{-1}; $\tau = 0.74 \pm 0.07$ nsec; $F/F_0 = 1.90 \pm 0.10$. The calculated values of $J_{\bar{\nu}}$, R_0, and r_e are given in Table III.

Examination of Table III shows that the values of r_e, calculated for the neutral and alkaline pH, are within experimental error equal. The equality of r_e shows that the same "equivalent oscillators system" accounts for the emissive properties of the hemocyanin molecule at the two pH values considered. From this one may conclude that in spite of the extensive dissociation which the molecule undergoes in the transition between the two pH values (100 S → 7 S) little, if any, change occurs in the spatial arrangement of the tryptophanyl residues and the Cu···O complexes.

Our findings regarding the similarity of r_e at alkaline and neutral pH favor the explanation that the red shift in emission maximum that accompanies the transition from low to high pH is due to tyrosine quenching rather than to a change in environment at the tryptophanyl residues. Quenching of tyrosine fluorescence at high pH has been observed in tyrosyl derivatives (White, 1960; Edelhoch *et al.*, 1969). Moreover, an explanation along this line would presuppose that tyrosine emission was not totally quenched at pH 6.6 and would account for the finding that at this pH hemocyanin fluorescence occurs at shorter wavelengths compared with the emission of tryptophan.

Finally it may be pointed out that transfer of electronic energy from the aromatic residues to an acceptor group naturally present in the protein is well known in heme proteins (Weber and Teale, 1959). What is unique in the hemocyanin system is that the energy transfer is related to the oxygenation function. The linearity of the fluorescence enhancement with the fraction of reduced protein, demonstrated in this work, forms a basis on which a sensitive method for obtaining oxygen titration curves may be laid.

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