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Proximity Relationships of Tryptophanyl Residues and Oxygen Binding Site in *Levantina hierosolima* Hemocyanin. A Fluorimetric Study†

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ABSTRACT: The effect of iodide on the fluorescence of *Levantina hierosolima* hemocyanin was studied. About half of the tryptophanyl fluorescence yield of the apoprotein was found to be accessible to quenching by iodide. In contrast, no quenching of the fluorescence of oxyhemocyanin by iodide could be detected. It was concluded that the residual fluorescence of oxyhemocyanin emanates exclusively from buried tryptophans. A comparison of the polarization of the fluorescence of deoxy- and oxyhemocyanin, as well as of apohemocyanin in the absence and presence of iodide, does not provide evidence for significant tryptophan-tryptophan energy transfer. The fluorescence decay of apohemocyanin could be fitted to a biexponential decay function with lifetimes of 4.6 and 1.2 ns, with amplitudes 0.30 and 0.70, respectively. The fluorescence decay of deoxyhemocyanin was virtually identical to that of the apoprotein, showing that the introduction of copper did not affect the emissive properties of the protein. Analysis of the decay curve of oxyhemocyanin showed two lifetimes, 3.8 and 0.71 ns with amplitudes of 0.17 and 0.83, respectively. Comparison of the quantum yields calculated from the decay kinetics with the ones measured directly showed that 0.55 of the tryptophanyl emission in deoxyhemocyanin was totally quenched by the introduction of oxygen. Our findings indicate that the copper-binding site in hemocyanin is located by the solvent-accessible tryptophans, near the exterior of the molecule.

Hemocyanin is a multisubunit protein that functions as an oxygen carrier in molluscs and arthropods (Van Holde and Van Bruggen, 1971). Each oxygen-binding site involves two copper ions and in the molluscs is associated with a unit of 50 000 daltons containing seven tryptophanyl residues (Ghiretti-Magaldi et al., 1966). A previous study of the luminescence properties of hemocyanin from the snail *Levantina hierosolima* showed that oxygenation brings about a marked quenching of the fluorescence of the protein tryptophans (Shaklai and Daniel, 1970). The observed similarity in the optical rotatory properties of oxy- and apohemocyanin (Cohen and Van Holde, 1964) ruled out an explanation of the quenching in terms of a conformational change in the protein as a result of oxygenation. The quenching was attributed to long-range nonradiative energy transfer from the tryptophans acting as donors to the Cu···O complex acting as acceptor (Shaklai and Daniel, 1970).

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It was further shown that the action of a Cu···O acceptor in the multisubunit molecule affects only tryptophans from the 50 000 dalton unit where it is situated and did not extend to tryptophans from neighboring domains.

Much information has accumulated over the years regarding the oxygen-binding properties of hemocyanin. However, a real understanding of the binding function suffers from the lack of structural information about the molecule in general and the binding site in particular. In the present work, the emissive properties of the various tryptophans in *L. hierosolima* hemocyanin were studied using fluorescence steady-state and decay techniques. The donor-acceptor relationship between the tryptophanyl residues and Cu···O was used to probe the surroundings of the oxygen-binding site. The significance of the findings is discussed in the light of the detailed information available on the environment of the oxygen-binding site in hemoglobin.

Experimental Section

Materials. Oxyhemocyanin (Hcy-O)[1] deoxyhemocyanin (Hcy), and apohemocyanin (apo-Hcy) were prepared from the snail *Levantina hierosolima* as described previously (Shaklai and Daniel, 1970). KI was an analytical reagent purchased from Merck. All solutions were prepared using double-distilled water.

Methods. Steady-state fluorescence measurements were carried out using an Hitachi Perkin-Elmer, Model MPF-2A, spectrophotofluorometer. Fluorescence quenching experiments in the presence of iodide are complicated by the absorption of exciting light at 280 nm by I^- and the absorption of emitted light by I_3^- formed by the action of dissolved oxygen. For these reasons, an excitation wavelength of 288 nm was used in all iodide quenching experiments, and the production of I_3^- was minimized by use of freshly prepared KI solutions which were exposed to the exciting light only for the short time required to take the reading of the fluorescence intensity. The relative tryptophanyl yield of hemocyanin was determined by comparing the area under the emission band, excited at 295 nm, of a protein solution and a solution of *N*-acetyl-L-tryptophanamide (NATA) having the same absorbance at the excitation wavelength. Polarization of fluorescence was measured using the polarization accessory supplied by the manufacturer. The polarization, P, was calculated from the relation (Chen and Bowman, 1965)

$$P = \frac{I_V^V - I_H^V(I_V^H/I_H^H)}{I_V^V - I_H^V(I_V^H/I_H^H)}$$

where the superscript of the fluorescence intensity, I, refers to horizontally (H) or vertically (V) polarized excitation and the subscript refers to corresponding values for emission. All fluorescence measurements were made at 20°C.

Fluorescence decay measurements were done with an instrument of the type described by Hundley et al. (1967), modified to overcome drift problems associated with the repeated averaging procedures used in fluorescence decay measurements (Hazan et al., 1974). The excitation wavelength was selected using a Jarell-Ash double monochromator (Model 82-410), the wavelength bandwidth being 6–10 nm. The fluorescence decay was observed at a right angle through a Schott KV330 cutoff filter transmitting light of wavelengths above 330 nm. The time profile of the excitation light pulse was collected using a scattering suspension. The decay curve and the excitation light profile were stored in the two halves of a multichannel analyzer.

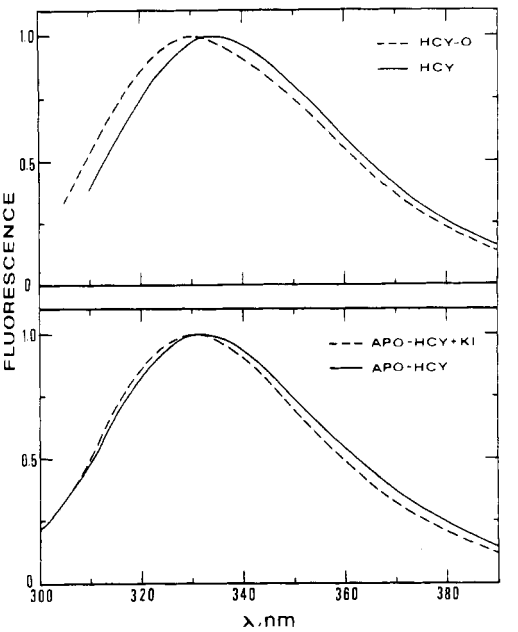

FIGURE 1: Fluorescence spectra of oxy- (- - -) and deoxyhemocyanin (—) (upper) and apohemocyanin in the presence (- - -) and absence (—) of 0.6 M KI (lower). Solutions containing about 1 mg/mL protein in 0.1 M Tris-HCl (pH 6.0) were used. Excitation was carried out at 288 nm, and bandwidths of 2.5 and 4 nm were used for excitation and emission, respectively. Instrumental amplification was used to bring the spectra in the presence and absence of quencher to equal height at their respective peaks.

The fluorescence decay curves were analyzed using the method of nonlinear least squares (Grinvald and Steinberg, 1974). The fluorescence decay mechanism was assumed to be a monoexponential or a multiexponential function of the type $I(t) = \Sigma_k \alpha_k \exp(-t/\tau_k)$, where $I(t)$ is the decay function and α_k and τ_k are the amplitude and lifetime of the kth component. $I(t)$ was convoluted with the time profile of the excitation pulse, $G(t)$, to give the calculated decay curve $F_c(t)$. The latter was compared with the experimental decay curve $F(t)$. The decay parameters—amplitudes and lifetimes—were used as free parameters to be changed so as to minimize the value of the root mean square of the deviations between the calculated and experimental curves, RMS, defined as:

$$\mathrm{RMS} = \left\{\frac{1}{n}\sum_{j=1}^{n}[F_c(t_j) - F(t_j)]^2\right\}^{1/2}$$

The summation is done over all n data points (for a typical experiment, $n = 500$).

Results

Effect of Oxygen on the Fluorescence Parameters. Figure 1 shows the fluorescence spectra of Hcy and Hcy-O. In addition to the marked quenching of the fluorescence yield noted previously (Shaklai and Daniel, 1970), oxygenation is seen to be accompanied by a blue shift in the maximum of the emission.

Polarization of the fluorescence of Hcy and Hcy-O was measured upon excitation at 280 or 295 nm and monitoring at 335 nm. The binding of oxygen was found to influence the polarization of the protein fluorescence at both excitation wavelengths (Table I).

Effect of Iodide on the Fluorescence Parameters. Figure 1 also shows the fluorescence spectra of apo-Hcy in the pres-

[1] Abbreviations used are: Hcy-O, oxygenated hemocyanin; Hcy, deoxygenated hemocyanin; apo-Hcy, apohemocyanin; NATA, *N*-acetyl-L-tryptophanamide.

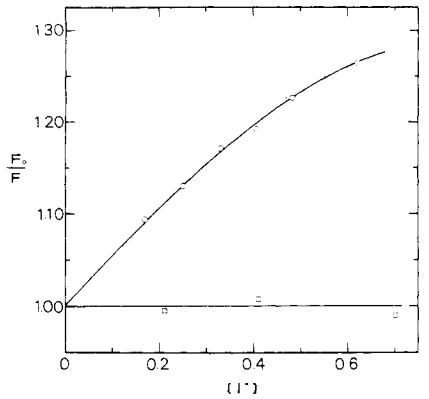

FIGURE 2: Stern-Volmer plot for the quenching by iodide of apohemocyanin (○-○) and oxyhemocyanin (□-□) fluorescence. Solutions containing about 1 mg/mL protein in 0.1 M Tris-HCl (pH 6.0) were used. Excitation was at 295 nm; monitoring was at 335 nm.

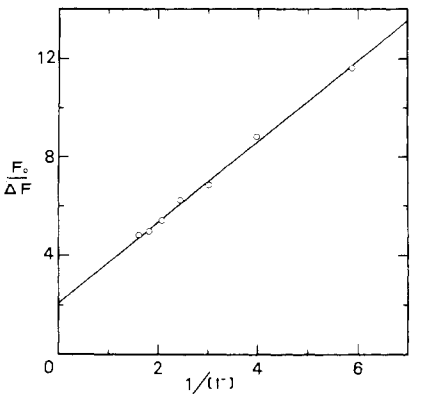

FIGURE 3: Modified Stern-Volmer plot of the data shown in Figure 3 for apohemocyanin and iodide.

TABLE I: Polarization of the Fluorescence of *L. hierosolima* Hemocyanin in the Absence and Presence of Quenchers.[a]

	P excitation at 280 nm[b]	P excitation at 295 nm[b]
Hcy	0.085	0.157
Hcy-O	0.110	0.205
apo-Hcy	0.087	0.155
apo-Hcy + 0.6 M KI	0.107	0.196

[a] In 0.1 M Tris-HCl (pH 6.0), 20 °C. [b] Emission monitored at 335 nm.

ence and absence of iodide. Iodide is seen to cause a shift in the emission maximum toward the blue. In addition, the presence of iodide brings about a quenching of the fluorescence intensity. The quenching was examined by reference to a simple Stern-Volmer equation

$$F_0/F = 1 + K_Q[I^-] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of iodide, and K_Q is a constant. A plot of F_0/F vs. $[I^-]$ (Figure 2) was found to be nonlinear, showing that the quenching cannot be described by eq 1. The curvature in Figure 2 may be explained by assuming the presence of two populations of tryptophans, one of which is accessible and the other not, to quenching by iodide. Quenching data in such a case may be described by a modified Stern-Volmer relation proposed by Lehrer (1971)

$$F_0/\Delta F = (1/[I^-]f_aK_q) + (1/f_a) \quad (2)$$

where $\Delta F = F_0 - F$ is the change in fluorescence intensity corresponding to a concentration of iodide $[I^-]$, and f_a is the fraction of accessible tryptophans with quenching constant K_q. A replot of the data according to the modified eq 2 is shown in Figure 3. From the plot, a fraction $f_a = 0.48$ of accessible tryptophans with a quenching constant $K_q = 1.26\ M^{-1}$ is calculated.

Polarization of the fluorescence of apo-Hcy in the absence and presence of 0.5 M KI was measured upon excitation at 280 and 295 nm, monitoring at 335 nm. It is seen that, at both excitation wavelengths, the presence of iodide causes an increase in the observed polarization of protein fluorescence (Table I).

In contrast to apo-Hcy, the fluorescence of Hcy-O is not affected by iodide. In this case, an F_0/F vs. $[I^-]$ plot (Figure 2) yields a straight line with $K_Q = 0$, showing that iodide does not cause the quenching of the residual fluorescence of oxyhemocyanin.

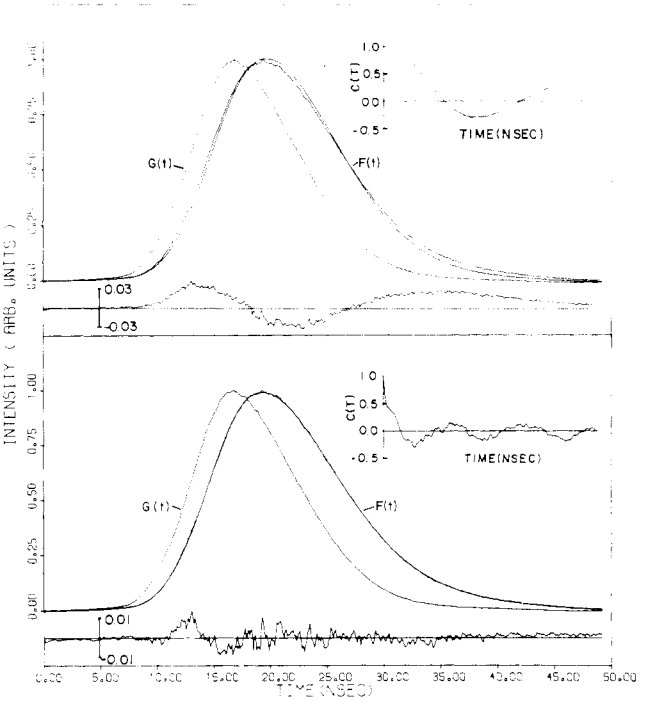

FIGURE 4: Analysis of the fluorescence decay of apohemocyanin in 0.1 M Tris-HCl (pH 8.2). The excitation wavelength was 280 nm, and the emission above 330 nm was observed. $G(t)$, excitation light profile. $F(t)$, experimental decay (noisy curve) and calculated decay curve (smooth curve). The noisy curves at the bottom of the two plots are the traces of the deviations between the experimental and the calculated curves. The inserts describe the autocorrelation function of the deviations. Upper: Analysis assuming a monoexponential decay; the decay time obtained: $\tau = 3.0$ ns; RMS = 0.017. Lower: Analysis assuming a biexponential decay; the parameters obtained: $\tau_1 = 4.6$ ns; $\tau_2 = 1.2$ ns; $\alpha_1 = 0.30$; $\alpha_2 = 0.70$; RMS = 0.0029. The fit for a single exponential decay is poor, while that for a biexponential decay is very good.

Fluorescence Decay. The fluorescence decay curve of apo-Hcy is shown in Figure 4, together with the results of an analysis for one and two exponential decay components. The analysis assuming a single exponential is seen to result in a very poor fit to the experimental data, while the fit using two components is very good (an analysis assuming three exponentials yielded an even better fit; however, in this case the parameters of the component with the shortest lifetime changed markedly from one experiment to another and were sensitive, to some extent, to the initial guess used to start the

TABLE II: Fluorescence Decay Parameters, Relative Quantum Yields, and Fractions of Fluorescent and Nonfluorescent Tryptophans for *L. hierosolima* Hemocyanin.[a]

	decay parameters[b,c]							
	τ_1 (ns)	τ_2 (ns)	α_1	α_2	RMS ($\times 10^4$)	q_r[d]	f_{NF}[e]	$1 - f_{NF}$
apo-Hcy	4.6	1.2	0.30	0.70	29	0.63	0.18	0.82
	(4.7)	(1.1)	(0.26)	(0.74)	(50)			
Hcy	4.6	1.2	0.29	0.71	26	0.64	0.15	0.85
Hcy-O	3.8	0.71	0.17	0.83	31	0.16	0.62	0.38

[a] In 0.1 M Tris-HCl buffer (pH 8.2). [b] Two-component analysis. [c] Excitation wavelength 280 nm (values in parentheses refer to decay curve obtained upon excitation at 300 nm). [d] Tryptophanyl quantum yield relative to NATA, measured as described under the Experimental Section. [e] Fraction of nonfluorescent tryptophanyl residues, calculated as described in the text.

analysis). Also, the parameters of the decay curves of apo-Hcy obtained upon excitation at 280 and 300 nm were the same, within experimental error. The fluorescence decay data for apo-Hcy, Hcy, and Hcy-O are summarized in Table II.

The fraction of nonfluorescent tryptophanyl residues was calculated according to Grinvald et al. (1975) from the decay parameters of the protein and its quantum yield relative to a standard whose fluorescence lifetime is known

$$f_{NF} = \left(\sum_k \alpha_k \tau_k - q_r \tau_s\right) \Big/ \sum_k \alpha_k \tau_k \qquad (3)$$

where α_k and τ_k are the decay amplitude and lifetime, respectively, of the kth decay component ($\Sigma\alpha_k = 1$), q_r is the relative quantum yield of the protein with respect to a standard whose decay time is τ_s. Quantum yields relative to NATA were determined for hemocyanin (Table II). Taking a value $\tau = 2.9$ ns for NATA in aqueous solution (Grinvald et al., 1975), values of f_{NF} for apo-Hcy, Hcy, and Hcy-O were calculated. The results are given in Table II.

Discussion

In a previous work (Shaklai and Daniel, 1970), the fluorescence properties of hemocyanin from *L. hierosolima* were studied. The fluorescence of both oxy- and deoxyhemocyanin, as well as that of the apoprotein, was found to be characteristic of tryptophan. The results of the present study indicate that about half of the tryptophanyl fluorescence in apohemocyanin—and presumably in deoxyhemocyanin—is accessible to quenching by iodide. It has been pointed out by several workers (Lehrer, 1967; Burstein, 1968; Lehrer, 1971) that tryptophan fluorophores which can be quenched by iodide represent residues exposed to solvent, whereas tryptophans which cannot be quenched by iodide represent residues buried inside the protein structure. One may thus conclude that half of the fluorescence intensity in apohemocyanin emanates from exposed and half from buried tryptophanyl residues. The tryptophan heterogeneity is also manifested by the blue shift of the emission observed upon quenching by iodide. The finding that iodide is unable to quench the fluorescence of oxyhemocyanin indicates that the residual emission after Cu···O quenching emanates exclusively from buried tryptophans. This interpretation is consistent with the blue shift of the emission observed upon oxygenation. A comparison of the degree of quenching expected upon the complete quenching of exposed tryptophans ($F/F_0 = 1 - 0.48 = 0.52$) with that actually achieved upon complete saturation of the oxygen-binding sites ($F/F_0 = 0.25$; Shaklai and Daniel, 1970) shows that in Hcy-O the fluorescence of the buried tryptophans is quenched to half of its value in the absence of oxygen.

Our finding that the polarization of fluorescence of apo-Hcy in 0.6 M KI is virtually identical to that of Hcy-O (Table I) in spite of the partial quenching of buried tryptophans in the latter indicates that energy transfer does not take place among the buried tryptophans. The ability of iodide to differentiate between buried and exposed tryptophans and selectively quench the latter implies the absence of efficient energy transfer between the two classes of fluorophores. The increase in polarization of apo-Hcy fluorescence upon quenching by iodide is not, in our opinion, an indication of energy transfer among the exposed tryptophans, since the increase of polarization upon excitation at 280 nm is paralleled by a similar increase when the excitation is carried out at a longer wavelength (295 nm) not far from the red edge of the absorption spectrum where energy transfer is known to fail[2] (Weber and Shinitzky, 1970). On the whole, the polarization data do not indicate that tryptophan–tryptophan energy transfer takes place to any significant extent in *L. hierosolima* hemocyanin.

Comparison of the fluorescence decay parameters for Hcy and apo-Hcy shows that the two are identical within experimental error. This result is consistent with our previous finding that in *L. hierosolima* hemocyanin the introduction of copper does not cause a decrease in quantum yield. In contrast, the decay curves for Hcy-O differ from those for Hcy both in the decay constants and the relative amplitudes. The results indicate that upon oxygenation the mean lifetime drops by a factor of 0.57 ($\Sigma\alpha\tau = 2.19$ and 1.24 for Hcy and Hcy-O, respectively). On the other hand, as mentioned already the fluorescence yield ratio from steady-state measurements is $F/F_0 = 0.25$. The discrepancy between the two values led us to examine the possibility that oxygen binding is accompanied by a total quenching of part of the tryptophanyl fluorescence. That indeed this is the case is shown by a comparison of the nonfluorescent tryptophanyl fraction f_{NF} in Hcy-O with that in Hcy or apo-Hcy. A comparison of the fraction of tryptophans totally quenched by oxygen [$(0.62 - 0.15)/0.85 = 0.55$ (Table II)] with the iodide-quenchable fraction (0.48) shows the two to be approximately equal. Taken with our other findings, this result indicates that the tryptophan residues which undergo total quenching by oxygen must be classified as solvent-exposed tryptophans.

The quenching of hemocyanin fluorescence upon oxygen binding has been ascribed to radiationless energy transfer from

[2] A likely interpretation of the increase of polarization of apo-Hcy fluorescence upon quenching by iodide comes out of a consideration of the local freedom of rotation of tryptophan residues. The motion of solvent-exposed tryptophan residues in the protein is probably not completely frozen (Burstein et al., 1973), and rotation of the emission oscillators may be expected to occur to different degrees during the lifetime of the excited state, leading to a heterogeneous population with respect to fluorescence polarization. The observed polarization, then, is an average of the contributions of the different emittors, weighted according to their fraction in the emission. Quenching of some of the tryptophans would, according to this interpretation, lead to a change in the observed polarization.

the tryptophanyl residues to the Cu···O complex. As the efficiency of energy transfer falls off with the distance, it follows that the oxygen-binding site is situated in close proximity to the totally quenchable, identified above as solvent-accessible, tryptophans. In other words, our findings indicate that the binding site in hemocyanin is located near the exterior of the molecule. On the other hand, there is evidence from infrared spectroscopy (Fager and Alben, 1972), acid–base titration (Salvato et al., 1974), and resonance Raman spectroscopy (Freedman et al., 1976) that the copper ions in hemocyanin are situated in a hydrophobic environment. These two results, hydrophobicity and solvent accessibility, though apparently contradictory, actually represent structural features essential for reversible oxygen binding. It stands to reason that, while the binding site has to be accessible to dissolved oxygen, it has to be located in a nonpolar environment to prevent oxidation of the monovalent copper. In fact, the picture that is emerging of the binding site in hemocyanin is reminiscent of the one already established in hemoglobin, where the heme groups are located in nonpolar pockets near the protein surface (Perutz, 1969).

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