Phosphorescence Properties of Hemocyanin from *Levantina hierosolima*†

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ABSTRACT: The phosphorescence properties of hemocyanin from *Levantina hierosolima* were studied. From the intensity spectrum and the lifetime values at different wavelengths, the triplet emission energy was resolved into its tyrosyl and tryptophanyl components. A comparison of the emission characteristics of each amino acid in apohemocyanin, deoxygenated hemocyanin, and oxygenated hemocyanin was carried out. It was found that copper increases the tyrosyl quantum yield but does not affect the tryptophanyl emission. In contrast, oxygenation quenches the phosphorescence of both tyrosyl and tryptophanyl residues. Energy pathways in the hemocyanin molecule are discussed.

In a previous work (Shaklai and Daniel, 1970), the fluorescence properties of hemocyanin were studied. It was found that there is a mutual energy interaction between the copper–oxygen complex and the singlet state of the aromatic amino acids. A need was subsequently felt to extend our understanding of this system by studying interactions involving the amino acids in their triplet state as well.

Because of difficulties in experimentation and interpretation, work on the triplet state of proteins has been less abundant than that on the singlet state. Nevertheless, recent studies have thrown light on some aspects of the problem. The importance of tyrosine in understanding the emission of protein triplet has come to be recognized (Longworth, 1962; Augenstein and Nag-Chaudhuri, 1964; Yeargers *et al.*, 1966; Weinryb and Steiner, 1970). The existence of different kinds of tryptophans, differing in their energy levels and their ability to be selectively quenched by energy acceptors, has been realized (Purkey and Galley, 1970). Further, the effects of metals on the excited state of proteins have been emphasized (Augenstein and Nag-Chaudhuri, 1964; Shiga *et al.*, 1966).

Shiga *et al.* (1966) studied the tryptophan triplet state in hemocyanin. They found that upon oxygenation this state is quenched. It is the purpose of the present communication to report new findings on tyrosyl and tryptophanyl phosphorescence in hemocyanin. The results of this work, together with the information obtained in previous studies (Shaklai and Daniel, 1970; Shiga *et al.*, 1966), permit one to draw a more complete picture than hitherto possible of the interrelations among the excited states of the hemocyanin macromolecule and its oxygen carrier site.

Experimental Section

Materials. Oxygenated hemocyanin (Hcy-O)[1] was prepared from the snail *Levantina hierosolima* as described previously (Shaklai and Daniel, 1970). Deoxygenation of hemocyanin to obtain reduced hemocyanin (Hcy) was carried out in the quartz capillary cuvet used for the phosphorescence measurements. The capillary was connected to a vacuum system and deaeration was effected through repeated cycles of short evacuations and equilibrations with the residual atmosphere. Complete deoxygenation was inferred upon complete disappearance of the 345-nm absorption band, measured in a Cary 14 spectrophotometer. For this purpose, a special mask with a narrow slit was inserted in the path of the incident light, thereby reducing the width of the beam to suit the dimensions of the capillary. Apohemocyanin (apo-Hcy) was prepared as before (Shaklai and Daniel, 1970). Protein solutions were prepared in a 0.1 M Tris-HCl buffer (pH 8.2). Emission measurements were made in a medium containing 0.5 wt % glucose cooled to form rigid glass at 77°K.

A critical consideration in the choice of the above-mentioned experimental conditions was the desire not to cause denaturation of the protein. A good criterion to test this point in hemocyanin is the 345-nm absorption band which reflects the ability of the protein to preserve its physiological oxygen-binding activity. It was found that (1) the addition of 0.5% glucose did not change the absorption spectrum of Hcy-O and (2) a sample of hemocyanin in glucose medium retained its spectral properties upon cooling to 77°K and subsequent warming to room temperature.

Methods. Phosphorescence measurements were carried out in a Hitachi Perkin-Elmer spectrofluorophotometer, Model MPF-2A, equipped with a phosphorescence attachment. Excitation was carried out at 280 nm and emission was observed at 90° to the incident beam. Phosphorescence signals were obtained using a chopper of the Becquerell type rotating at 25 cps to cut off short-lived signals (fluorescence and stray light). Decay curves were obtained using a Tektronix storage oscilloscope. Special care was taken to include in the trace the initial intensity of the signal and its baseline. A representative decay curve is given in Figure 1.

Results

The phosphorescence spectrum of apo-Hcy is given in Figure 2. A comparison to Figure 3 shows that the protein emission bears the structured prints characteristic of tryptophan phosphorescence. Further, the fact that the spectrum extends to wavelengths lower than 390 nm indicates the involvement of tyrosine phosphorescence as well. In order to determine the contribution of the tyrosyl and tryptohanyl residues to the total phosphorescence, an analysis of the lifetime as a function of wavelength—lifetime spectrum—

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

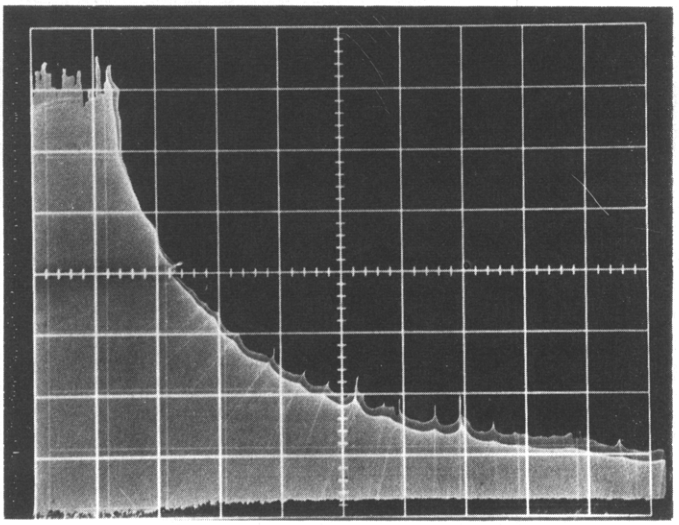

FIGURE 1: Decay curve of phosphorescence signal from *L. hierosolima* apohemocyanin in 0.5% glucose (pH 8.2) at 77°K. Excitation was effected at 280 nm, emission was observed at 410 nm.

was carried out. At each wavelength, the intensity decay curve was analyzed and the number of exponentials and lifetime values was determined. Representative plots are given in Figure 4. It is seen that at 380 nm, there is only one lifetime, $\tau = 1.3$ sec, typical of tyrosine. In contrast, at 410 nm the logarithmic plot is not linear and the decay curve may be represented as the sum of two exponentials, one with $\tau = 4.8$ sec and another with $\tau = 1.3$ sec. At 480 nm, the decay curve (not shown) has essentially one lifetime, $\tau = 4.7$ sec, characteristic of tryptophan. The lifetime spectrum analysis of the data is summarized in Table I. The table shows that the emission between 350 and 390 nm is entirely due to tyrosine and therefore emission at these wavelengths represents the 350- to 390-nm portion of the "protein-tyrosyl spectrum." A comparison of the protein emission at 350–390 nm (Figure 2) to the tyrosine spectrum at the corresponding wavelengths (Figure 3) shows that the two are identical. The fact that the protein tyrosyl spectrum is identical with that of the amino acid at 350–390 nm makes it plausible to

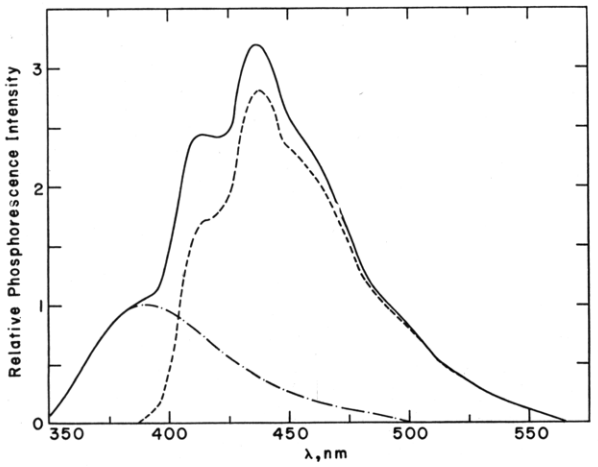

FIGURE 2: Phosphorescence emission of *L. hierosolima* apohemocyanin in 0.5% glucose (pH 8.2) at 77°K. (—) Observed protein spectrum; (–·–·) resolved tyrosyl emission; (----) resolved tryptophanyl emission.

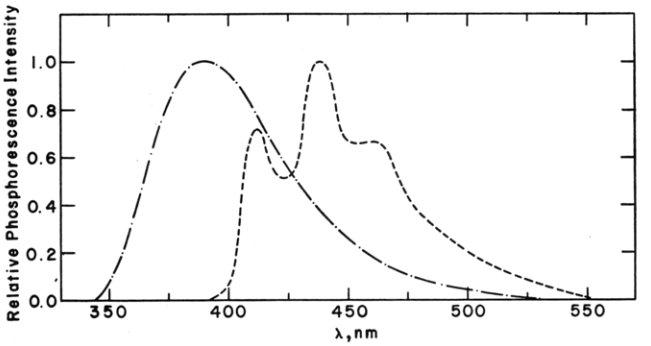

FIGURE 3: Phosphorescence spectra of tyrosine (–·–·) and tryptophan (----) in 0.5% glucose (pH 8.2) at 77°K.

assume that the identity continues to hold in the whole wavelength range of the spectrum. Subtraction of the protein tyrosyl contribution yields a residual "protein-tryptophanyl spectrum." A comparison of the latter to the spectrum of tryptophan (Figure 3) shows the similarity of the two. That the resolution of the total protein spectrum into its two components is essentially correct is shown by comparing the tyrosyl contribution to the total intensity as determined from spectral and from lifetime data (Table II).

The phosphorescence spectra of Hcy and Hcy-O (Figures 5 and 6) were analyzed in the same way as apo-Hcy. Again the ultraviolet part of the spectrum may be entirely attributed to tyrosine. However in Hcy-O the emission of the tyrosyl part of the spectrum is composed of two signals which differ in their lifetimes (Table I). Analysis of the lifetime spectrum of Hcy-O at 360–390 nm showed that the short-lived signal represented 30% of the total tyrosyl intensity.

In contrast to apo-Hcy, the protein tyrosyl emissions in Hcy and Hcy-O are shifted to the red. Figures 5 and 6 show that, provided appropriate shifts are made, the protein tyrosyl

TABLE I: Lifetime Spectrum of *Levantina hierosolima* Hemocyanin at pH 8.2 and 77°K.

Emission Wavelength, λ (nm)	Apo-Hcy, τ (sec)	Hcy, τ (sec)	Hcy-O, τ (sec)
350	1.3		
360	1.3	1.4	0.4, 1.6
370	1.4	1.3	0.4, 1.2
380	1.3	1.3	0.4, 1.2
390	1.4	1.2	0.4, (1.9)
400	(1.8)[a]	1.3	0.5, 1.1
410	1.3, 4.8	1.2, 5.5	0.4, 1.7, 4.8
420	1.3, 4.1	1.4, 5.1	0.4, 1.4, 5.0
430	4.7	4.7	(0.7), 4.9
440	4.7	5.4	(0.7), 5.3
450	4.7	4.6	(0.7), 4.5
460	5.0	4.6	(0.7), 5.1
470	5.0	4.7	(0.5), 5.5
480	4.7	4.5	(0.6), 4.9

[a] The numbers in parentheses stand for average values in cases where further resolution was impossible.

TABLE II: Comparison of the Tyrosyl Contribution to the Total Phosphorescence Calculated from Resolved Spectra with the Value Determined from Analysis of the Signal Decay Curve.

	Tyrosyl Contribution to Total Intensity					
	Apo-Hcy		Hcy		Hcy-O	
Emission Wavelength, λ (nm)	From Spectra	From Anal. of Decay Curve	From Spectra	From Anal. of Decay Curve	From Spectra	From Anal. of Decay Curve
410	0.34	0.32	0.57	0.55	0.70	0.68
420	0.26	0.22	0.46	0.40	0.69	0.64
430	0.17	0.14	0.31	0.35	0.55	0.52
440	<0.10	<0.10	0.24	0.25	0.41	0.50
450	<0.10	<0.10	0.21	0.21	0.27	0.28
460	<0.10	<0.10	0.15	0.13	0.31	0.27
470	<0.10	<0.10	0.14	0.10	0.24	0.28
480	<0.10	<0.10	0.13	0.10	0.22	0.30

emissions coincide with that of tyrosine. Table II shows that the contribution of tyrosyl phosphorescence from spectra and from lifetimes are in good agreement. In the case of Hcy-O, the residual tryptophanyl spectrum is similar to that of the amino acid. In contrast, the residual tryptophanyl spectrum in Hcy is different from that of tryptophan. In this case, part of the tryptophanyl emission is shifted to the red. Table III summarizes the emission characteristics of the tyrosyl and tryptophanyl residues in hemocyanin in comparison to those of the free amino acids.

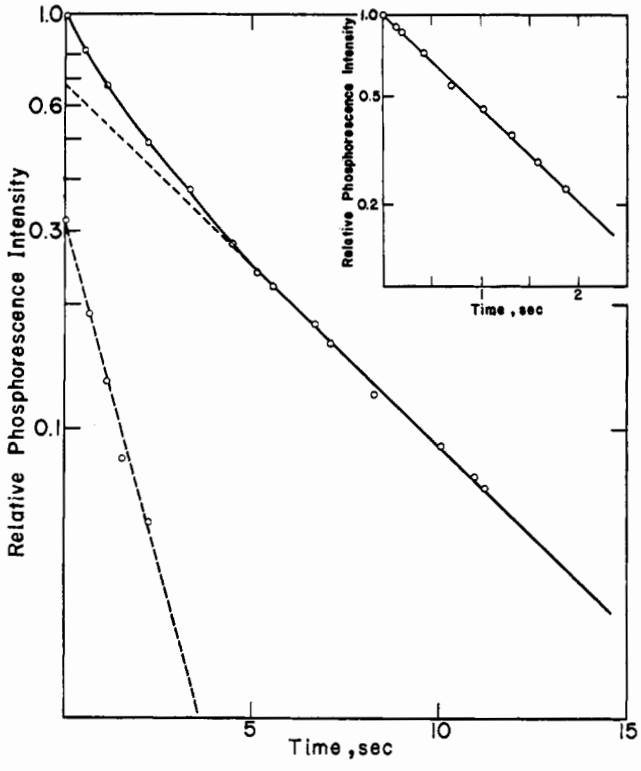

FIGURE 4: Kinetics of the phosphorescence signal for *L. hierosolima* apohemocyanin in 0.5% glucose (pH 8.2) at 77°K. Excitation was effected at 280 nm, emission was observed at 380 nm (inset) and 410 nm. At 410 nm, the observed signal (—) was resolved into a sum of two exponentials (- - - - -) and the lifetime for each determined from the slope of the corresponding straight line.

Discussion

Previous work (Shiga *et al.*, 1966) on the triplet state of hemocyanin was based on the premise that the tryptophan residues were the sole carriers of triplet emission. The results obtained in this study show that tyrosine as well as tryptophan contribute to the observed triplet state emission. This finding is in accord with studies of the last years, which show that tyrosine phosphorescence is observed in many tryptophan-containing proteins (Longworth, 1962; Augenstein and Nag-Chaudhuri, 1964; Yeargers *et al.*, 1966).

In order to eliminate a possible influence of the copper atom or the Cu···O group on the triplet state of the protein, the behavior of apo-Hcy will be considered first. The protein tyrosyl spectrum is identical with that of tyrosine and the residual protein tryptophan emission shows the three characteristic vibrational bands as in the free amino acid. The ratio of the energy contribution of the tyrosyl and tryptophanyl residues is given in Table III. Although this ratio may not be taken as a measure of the relative abundance of the two amino acids in the protein, it is nevertheless useful in comparing the emissive properties of the protein in its various states.

Introduction of copper into the protein brings about a

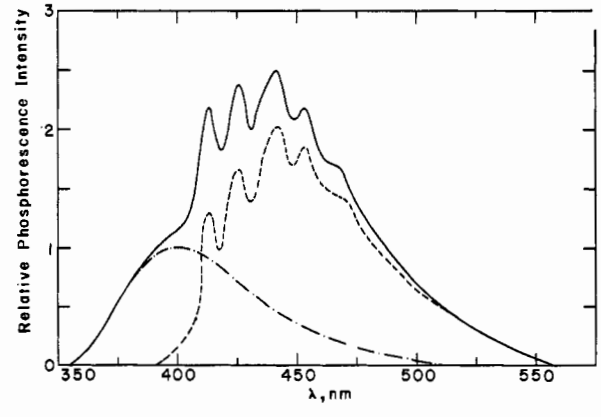

FIGURE 5: Phosphorescence emission of *L. hierosolima* deoxyhemocyanin in 0.5% glucose (pH 8.2) at 77°K. (—) Observed protein spectrum; (-·-·-) resolved tyrosyl emission; (------) resolved tryptophanyl emission.

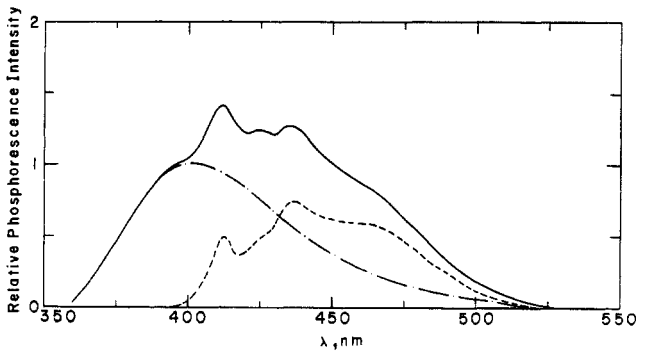

FIGURE 6: Phosphorescence emission of *L. hierosolima* oxyhemocyanin in 0.5% glucose (pH 8.2) at 77°K. (—) Observed protein spectrum; (-·-·) resolved tyrosyl emission; (------) resolved tryptophanyl emission.

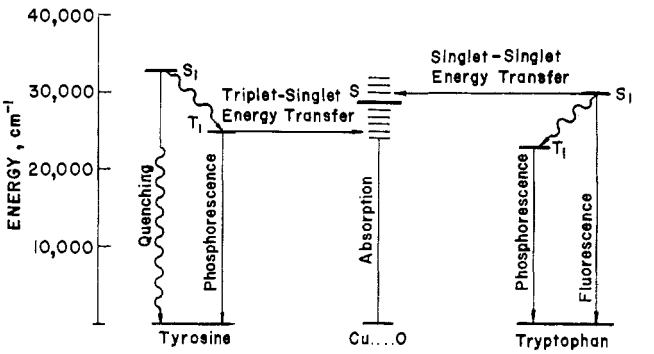

FIGURE 7: Energy level diagram for the tyrosyl and tryptophanyl amino acid residues and the copper–oxygen complex in hemocyanin. Energy pathways of the excitation energy are indicated.

few changes in the phosphorescence behavior. Compared to apo-Hcy, the tyrosyl component in Hcy is shifted by 12 nm. The tryptophan protein emission is also shifted to the red; however only part of the tryptophan undergoes the shift, while the rest shows normal emission characteristics. The splitting of the tryptophan residual spectrum under the action of copper shows that the metal affects only part of the tryptophan residues.

The tyrosyl to tryptophan phosphorescence ratio increases upon introduction of the copper (Table III). This may be the result of either an enhancement in tyrosyl intensity or a quenching of tryptophanyl energy. The fact that the lifetime of tryptophan in Hcy equals that in apo-Hcy indicates that no quenching of tryptophan from the triplet state occurs. In a previous work, Shaklai and Daniel (1970) showed that the fluorescence quantum yield of tryptophan is the same in Hcy and apo-Hcy. This may be taken as an indication that no quenching of the singlet takes place under the action of copper. The fact that no quenching occurs in either singlet or triplet states suggests that the tryptophan phosphorescence energy remains the same. Therefore the change in the tyrosine to tryptophan phosphorescence yield has to be interpreted as an increase in tyrosine triplet energy. The increase in tyrosine phosphorescence energy can in turn be a result of either a change in the lifetime of the triplet state or a change in its population. The fact that no change in tyrosine lifetime occurs in the transition apo-Hcy → Hcy (Table I) rules out the first possibility. An increase in the population of the tyrosine triplet state under the action of copper is inferred and attributed to an increase in the rate constant for the intersystem crossing. The ability of a heavy atom to perturb intersystem crossings has been discussed by Kasha (1960) and was suggested for protein systems by Augenstein and Nag-Chaudhuri (1964).

The effect of oxygen on the triplet state of hemocyanin can be estimated by comparing the properties of Hcy and Hcy-O. Table III shows that, as in Hcy, the protein tyrosyl spectrum in Hcy-O is shifted to the red. The protein residual tryptophan spectrum is however different from that of Hcy and similar to that of apo-Hcy.

A better understanding of the changes which the protein triplet state has undergone can be gathered by examination of the lifetime spectrum of Hcy-O (Table I). The tryptophan lifetime is unaffected by the introduction of oxygen. In contrast, the tyrosine emission is composed of two lifetimes, a normal one with $\tau = 1.3$ sec, and a faster one with $\tau = 0.4$ sec. This shows that in hemocyanin, there are two types of tyrosine residues, and that only one of them undergoes quenching upon the introduction of oxygen. The quenching of tyrosine may be interpreted by the occurrence of energy transfer from the triplet state of the tyrosine to the singlet state of Cu···O. Such an energy transfer is made possible by the overlap between the phosphorescence emission of the tyrosine (Figure 3) and the absorption band of the Cu···O at 345 nm. That part of the tyrosine residues does not undergo quenching by this mechanism is probably due to unfavorable orientation, which is known to be critical in energy-transfer mechanism (Forster, 1959).

The observed tyrosine:tryptophan energy ratio in Hcy-O is 1.50 compared to a value of 0.47 in Hcy. Had the tyrosine intensity remained constant, the ratio 0.47:1.50 = 0.31 would have measured the quenching of tryptophan in Hcy → Hcy-O. However, from the relative intensities of the short-lived and the normal tyrosine (0.3 and 0.7) and the respective lifetimes (0.4 and 1.3 sec), a decrease of 21% in the total tyrosine emission energy is calculated. Correcting for the decrease in tyrosine yield, the tryptophan emission energy in Hcy-O becomes 0.25 of its value in Hcy. The fact that no change in tryptophan lifetime occurs upon oxygenation indicates that the decrease in intensity is a consequence of a decrease in the population of the precursor singlet state. A fluorescence quenching of the same magnitude has indeed been demonstrated in a previous work and interpreted as energy transfer to the Cu···O group (Shaklai and Daniel,

TABLE III: Phosphorescence Characteristics of Tyrosyl and Tryptophanyl Residues in *Levantina hierosolima* Hemocyanin at pH 8.2.

	Tyr Shift[a] (nm)	Trp Shift[a] (nm)	Tyr:Trp Energy Ratio[b]
Apo-Hcy	None	None	0.35
Hcy	12	12[c]	0.47
Hcy-O	12	None	1.50

[a] Relative to the free amino acid at the same pH. [b] Calculated by comparison of the areas under the respective resolved bands of the phosphorescence spectra (Teale and Weber, 1957). [c] Only part of the tryptophanyl emission underwent this shift.

1970). The agreement between the phosphorescence ratio $P_{Hcy-O}:P_{Hcy}$ found in this work and the corresponding fluorescence ratio $F_{Hcy-O}:F_{Hcy}$ found before indicates that our interpretation of the phosphorescence findings is essentially correct.

The comparison of the phosphorescence emission spectra of Hcy and Hcy-O gives additional information on the distribution of tryptophan in the protein. Upon oxygenation, the red-shifted peaks disappear, indicating that part of the tryptophans are selectively quenched. This may be explained, as in the case of tyrosine, by favorable mutual orientation of one type of tryptophan and Cu···O oscillators. It is pertinent to recall in this context that a selective quenching of the tryptophans from the singlet state was indicated in our allied study of fluorescence, where it was shown that upon deoxygenation the tryptophan emission peak undergoes a red shift, 331 → 335 nm.

The conclusions of this study and those obtained previously in the complementary work on fluorescence make it possible to draw the path of the excitation energy in the hemocyanin system (Figure 7). The diagram shows that the special position of the Cu···O absorption band on the energy scale enables it to play the acceptor role in both singlet–singlet and triplet–singlet energy transfers. In this way, the Cu···O complex functions as a "sink" for the energy accumulated in the excited aromatic amino acids.

Acknowledgment

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