Spectroscopy of (Carbon monoxy)hemocyanins. Phosphorescence of the Binuclear Carbonylated Copper Centers†

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ABSTRACT: The luminescence of CO-hemocyanin has been studied in several hemocyanins from both arthropods and molluscs. All of them show an emission in the region 550–560 nm with quantum yield = 0.2–0.4, though individual differences are apparent. Solvent composition (as well as the presence of calcium and lanthanides) has no effect on the emission. Lifetimes of this luminescence are in the range 60–140 μs, indicating that the emitting state may be a triplet. The presence of both copper atoms (Cu^+) per CO binding site is required for the luminescent state since half-met and half-apo derivatives do not show such an emission. Though not clearly related to any of the functional properties, systematic differences in luminescence parameters are apparent between arthropodal and molluscan hemocyanins. *Limulus* hemocyanin appears to be very different from both types of hemocyanin.

Hemocyanins, the O_2 carriers of a number of invertebrates, reversibly bind one O_2 or one CO molecule to a binuclear cuprous center (Root, 1934; Vanneste & Mason, 1966; van Holde & van Bruggen, 1971). The binding of O_2 is associated to the appearance of new absorption bands (at 340–350 and 570 nm) which allow a quantitative determination of the ligand binding curves. The binding of CO gives rise to much less intense transitions at 300–340 and 440 nm (Bonaventura et al., 1974; Kuiper et al., 1975, 1980a) which, nevertheless, have allowed the determination of the binding parameters for carbon monoxide.

We have recently reported that carboxyhemocyanin is strongly luminescent with an emission maximum around 550 nm upon excitation at 280–320 nm (Kuiper et al., 1980a). The fairly high quantum yield prompted us to investigate more extensively the spectroscopic properties of CO-hemocyanins from different species under a number of experimental conditions.

Experimental Procedures

α and β components from *Helix pomatia* hemocyanin were isolated according to Heirwegh et al. (1961) as modified by Konings et al. (1969) and by Siezen & van Driel (1973). *Panulirus interruptus* hemocyanin was prepared and stored as already described (Kuiper et al., 1975). *Octopus vulgaris* and *Carcinus maenas* hemocyanins were provided by Dr. B. Salvato, *Busycon canalicolatum* by Dr. R. Lontie, and *Limulus polyphemus* by Dr. J. Bonaventura.

All the proteins used for fluorometry were dialyzed against 0.1 M Tris buffer, pH 8.0, containing 10 mM $CaCl_2$ in order to avoid dissociation into subunits. Protein samples used to check calcium or lanthanide effects were first dialyzed against 0.1 M Tris buffer, pH 7.2, containing 3 mM EDTA and subsequently against 0.1 M Bis-Tris buffer, pH 6.5. The protein concentration is always expressed as oxygen binding sites per liter.

Fluorometric measurements were carried out with a Thunberg-type fluorometric cuvette, by flusing the solution with pure CO (Caracciolo, Rome, Italy). The extent of oxygen removal was checked spectrophotometrically. Half-apo- and half-methemocyanin samples were prepared according to Himmelwright et al. (1978) with minor modifications.

Fluorescence spectra were recorded with a FICA 55 L corrected spectrofluorometer operated at 25 °C. Phosphorescence and low temperature luminescence were performed with an Aminco-Bowman spectrofluorometer at 77 K. Relative quantum yields were determined by comparison of the area under emission peaks of each species with a standard solution of rhodamine 6B excited at the same wavelength after correction for the optical density at the excitation wavelength.

Lifetime measurements were performed at room temperature and at pH 8 if not otherwise specified. The excitation source was a pulsed nitrogen laser (λ_{em} = 337 nm, Lambda Physik, Göttingen) with a pulse width of ≈3 ns and a peak power of 1 MW.

The laser beam was focused on the sample cell by a 15-cm focal quartz lens. The emission was detected at a right angle. A 5-cm cell filled with Cl_2 at 2 atm and a Balzers broad band interference filter (K_3 or K_4) prevented laser light from reaching the photomultiplier. A Bausch & Lomb high intensity monochromator provided the selection of emission wavelength. Light detection was by means of an RCA IP28 photomultiplier tube operated at 850 V.

Luminescence decay signals, generally at 550 nm, were acquired and reduced by using an R7912 Tektronix transient digitizer interfaced to a Z-2D Cromemco microcomputer. The analysis of data was performed by a nonlinear iterative least-squares fitting procedure (Bevington, 1969).

Results

Room Temperature Emission of CO-hemocyanin. We have investigated the luminescence properties of hemocyanins from several arthropods and molluscs and found that in all cases the carbonylated derivative exhibits an emission band at ~550 nm. Individual variations in the quantum yield and in the magnitude of the CO-linked quenching of intrinsic fluorescence are reported in Table I. The excitation spectrum given in Figure 1 shows a main peak coincident with the protein absorption, a clear-cut shoulder at 320–340 nm, and a much smaller peak around 440 nm. The latter bands are related to carbon monoxide binding (Kuiper et al., 1980a). Due to the very low extinction coefficient, it proved impossible to determine the quantum yield of emission upon excitation at 440

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Table I: Luminescence Parameters of CO-hemocyanins[a]

sample	emission (nm) protein	emission (nm) CO–Cu complex	quenching of protein fluorescence (%)	quantum yield[d]	lifetime[e] (μs)
α-Helix pomatia	335	550	0.40	0.33	71.4 ± 1.8
β-Helix pomatia	335	550	0.34	0.28	83.4 ± 2.9[f]
Octopus vulgaris	335	550	0.40	0.33	77.1 ± 1
Busycon canaliculatum	335	550	0.30		
Limulus polyphemus	340	560	0.45	0.39	113.8 ± 2.8
Panulirus interruptus	335	560	*b*	0.22	65.3 ± 5
Carcinus maenas	335	560	*c*	0.18	63.9 ± 2.8

[a] Determinations made at pH 8 in Tris buffer/$CaCl_2$ unless otherwise specified. [b] Partially saturated at 1 atm of CO ($\bar{Y} = 0.60$). [c] Partially saturated at 1 atm of CO ($\bar{Y} = 0.70$). [d] Excitation wavelength 320 nm. [e] Emission wavelength 550 nm. [f] Invariant in the pH range 7–9 and in the wavelength range 480–580 nm.

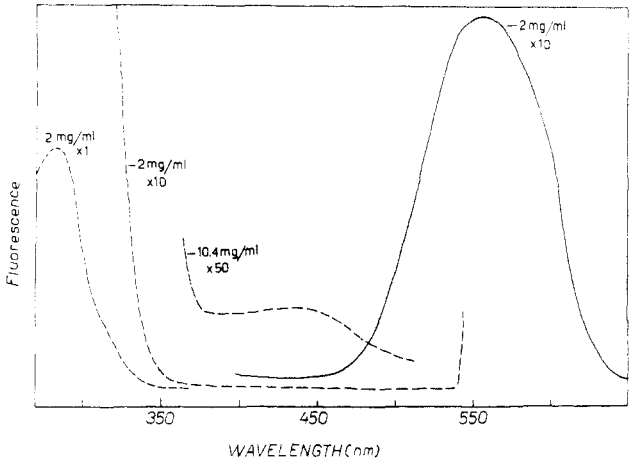

FIGURE 1: Luminescence of CO-hemocyanin at room temperature. β-Hemocyanin from *Helix pomatia* at the concentrations indicated, in 0.1 M Tris buffer, pH 8, containing 10 mM $CaCl_2$, was equilibrated with 1 atm of CO. The emission spectrum (solid line) was obtained by excitation at 320 nm. The excitation spectra (dashed lines) were obtained by setting the emission monochromator at 550 nm. Note the different amplifications in the three cases. All the experiments were carried out at 25 °C.

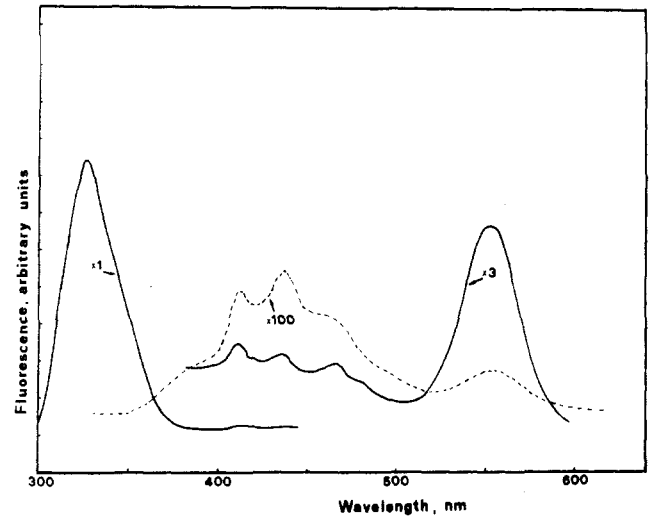

FIGURE 2: Low temperature emission of CO-hemocyanin. *Limulus* hemocyanin (≈10 mg/mL) in Tris buffer–ethylene glycol (1:1 v/v) equilibrated with 1 atm of CO. Total emission at 77 K (solid line) and emission (dotted line) obtained after introduction of a rotating shutter (≈1 ms) in the light path. Excitation wavelength 280 nm. Since the instrument output is not corrected for the phototube response, the peak position should be shifted some 20 nm toward the red.

nm. The energy and the intensity of the emission are not affected by substantial changes in the properties of the medium as obtained by dissolving hemocyanin in a 1:1 buffer–ethylene glycol mixture or by substituting H_2O with D_2O. Azide and cyanate up to a concentration of 10 mM do not affect the luminescence, while cyanide decreases the luminescence in a time-dependent way. Half-apo- and half-methemocyanin samples, in which one of the two copper atoms in a binuclear center has been either removed or oxidized to Cu(II), do not show appreciable emission in the presence of 1 atm of pure CO.

Low Temperature Emission from CO-hemocyanin. The emission of CO-hemocyanin at 77 K is characterized by three main peaks when excited at 280 nm (Figure 2). Those at shorter wavelengths represent the intrinsic fluorescence and phosphorescence of the protein moiety, while the longest wavelength peak is the CO-related emission. With respect to the spectra obtained at 298 K, minor shifts toward higher or lower energy values were observed for the intrinsic fluorescence and the CO-induced emission, respectively. The poor quality of the glass formed at 77 K prevented an exact determination of the relative quantum yields. Furthermore no information is available about the CO affinity at this temperature. The introduction of a rotating shutter in the light path, in order to discriminate between prompt and delayed emission, removes any emission below 360 nm, while the intrinsic phosphorescence centered at 420–440 nm and the emission in the yellow-green region are still observable.

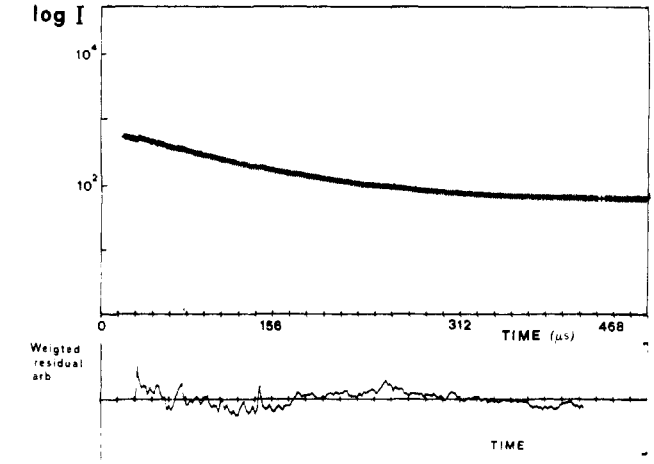

FIGURE 3: Luminescence decay of *β-Helix* CO-hemocyanin. The intensity threshold was set at $I = 10^2$ to eliminate electronic noise. For experimental details, see text.

Lifetime Measurements. The lifetime of emission for several CO-hemocyanins is reported in Table I. All of them show a single exponential decay (see Figure 3) with a τ in the range 60–140 μs. It is worth noting that for every one of the proteins the lifetime is constant throughout the emission band, indicating only one emitting state. These lifetimes are in the range expected for phosphorescence (Becker, 1969). Arthropodal hemocyanins seem to have a somewhat shorter lifetime with

respect to the molluscan ones; the lifetime of *Limulus* CO-hemocyanin is the longest among those investigated.

The presence of oxygen significantly decreases the lifetime as well as the quantum yield of the CO-related emission. No change in lifetime was instead observed in the pH 7–9 interval, at least in the case of the β component from *Helix pomatia*.

Effect of Ca(II) and Lanthanides on CO Emission. We have described the binding of several lanthanides to deoxyhemocyanin and obtained evidence that some of them occupy the Ca(II) binding sites (Kuiper et al., 1979, 1981). Tb(III) shows a protein-sensitized emission while other lanthanides only induce a quenching of protein and/or bound Tb(III) emission. Since some of these lanthanides show absorption bands at energy values lower than the excitation and emission bands of CO-hemocyanin, they may be expected to act as energy sinks. However, no quenching was observed upon addition of either Tb(III) or Eu(III), but on the contrary a relative enhancement of luminescence was detected, with this effect being more prominent in *Limulus* hemocyanin. It is important to remark that the addition of Ca(II) had a similar effect.

Discussion

In previous papers we have reported that the binding of CO to the copper-containing proteins hemocyanin (Kuiper et al., 1980a) and tyrosinase (Kuiper et al., 1980b) is associated to an intense visible emission. This property seems to be uniquely related to the structure of the binuclear copper center of these proteins, since the addition of CO to laccase (Kuiper et al., 1980b) and ascorbate oxidase did not give rise to a specific luminescence.

The CO-induced luminescence proved to be a useful tool in the study of the kinetics and equilibria of CO binding to hemocyanins (Brunori et al., 1981). However, no information was available to understand the photophysical process underlying this emission. In this paper we have shown that the lifetime of the excited state, which is responsible for the emission, is in the range 60–140 μs in the hemocyanins tested. These are representative of the whole class being from arthropods (*Panulirus* and *Carcinus*), from molluscs (α and β forms from *H. pomatia* and *Octopus*), and from an animal different from both arthropods and molluscs (*Limulus*). The lifetimes are in keeping with the observed quantum yields (see Table I) and provide evidence that the CO-related emission is a phosphorescence. Two aspects of this emission deserve further comment: the efficiency of the process and the multiple bands in the excited spectrum. Concerning the former point one should note that the room temperature quantum yield of emission is 0.15–0.2 when excited at 285 nm and higher (0.3–0.4) when excited at longer wavelengths. These findings indicate that energy transfer from excited tyrosinyl and tryptophanyl residues to the emitting center is indeed fairly efficient. Moreover we believe that the quantum yield values are, if anything, underestimated due to the weakness of the CO-related absorption band at 300–320 nm compared to the contribution at the same wavelengths of protein absorption and scattering. The unusually high quantum yield of this long-lived room temperature emission of CO-hemocyanin indicates that during the excited state very few molecules undergo collisional quenching. It appears therefore that the emitting moiety is rather shielded from other solutes and even from the solvent. This is confirmed by the fact that none of the chemicals tested (i.e., azide, cyanate, ethylene glycol, etc.), which in principle may find their way to the active site, nor heavy water effects the CO emission. This latter finding seems in contrast with the proposed presence of a water molecule as a copper ligand (Himmelwright et al., 1980), though isotope effects can be deceptive. Cyanide causes a decrease in luminescence, but a in time–dependent way, typical of a slow chemical modification, perhaps related to copper removal. On the other hand, oxygen and nitric oxide are strong quenchers of this emission.

The role of the second copper in the overall process is essential, since half-met or half-apo derivatives do not show any significant emission. Furthermore, it appears that the geometry of this copper site is critical (van der Deen & Hoving, 1979). Individual differences in luminescence emission peak, quantum yield, and lifetime are observed in different hemocyanins that could recall analogous differences observed between arthropods and molluscs in the maxima of absorption of the Cu–O_2 charge-transfer bands (Himmelwright et al., 1980).

The nature of the emitting species is not known, but it is clearly related to CO binding. It is impossible, however, on the basis of the available information, to establish whether CO plays a direct role or simply acts through a perturbation of the ligand field of copper atoms. It should be pointed out that CO was found to bind to only one of the two coppers of each binuclear center (Alben et al., 1970; Yen Fager & Alben, 1972). Recent EXAFS experiments suggest the presence of a Cu(I)–Cu(II) pair in CO-hemocyanin (Eccles, 1979), while a Cu(II)–Cu(II) pair with a strongly bound peroxide molecule was proposed for oxyhemocyanin (Freedman et al., 1976; Larrabee et al., 1977; Himmelwright et al., 1980). In both derivatives therefore a charge delocalization involving one or two electrons from the copper center seems to take place. In the case of oxyhemocyanin this situation is associated with the presence of charge-transfer bands in the electronic absorption spectrum (Himmelwright et al., 1980). By analogy the near-UV absorption band of CO-hemocyanin may be assigned to a charge-transfer transition. The lower electron accepting ability of CO with respect to O_2 may well explain the higher energy of the charge transfer in CO-hemocyanin (315–320 vs. 340–350 nm in oxyhemocyanin). Both hemocyanin derivatives show additional weaker bands in the visible region, i.e., at 435, 500, 570, and 730 nm in oxyhemocyanin and at least one at 440 nm in CO-hemocyanin (Kuiper et al., 1980a). The position and intensity of the latter correspond to one of the bands present also in oxyhemocyanin and are attributed to a charge transfer from an endogenous ligand (phenolate or oxo) (Eickman et al., 1979). In any case we have found that absorption at this band results in the CO-related emission (Figure 1). A possible distribution of electronic energy levels in CO-hemocyanin is presented as a Jablonki's diagram in Figure 4. In this scheme two singlet excited states are represented, both referred to as the Cu–CO center; that at lower energy only tentatively is ascribed to a singlet state since its very low extinction coefficient could very well fit with a triplet state. Both excited states can populate the triplet state responsible for the emission. It should be, however, pointed out that other spin states, possible in the Cu_2–CO system, may also give similar forbidden transitions. The large efficiency of phosphorescence emission suggests the presence of a large spin–orbit coupling factor, which overcomes the spin selection rule, making the intersystem crossing rate(s) large enough to successfully compete with any other deactivation process of the excited states (Becker, 1969). Furthermore the radiative constant from the emitting state is fast enough [k_p = (2.8–4.3) $\times 10^3$ s^{-1}] to compete with nonradiative processes. In the same figure it is also indicated the energy-transfer pathway for sensitized emission through excitation of protein aromatic residues. In this case energy transfer causes a decrease of intrinsic fluorescence quantum yield (see Table I).

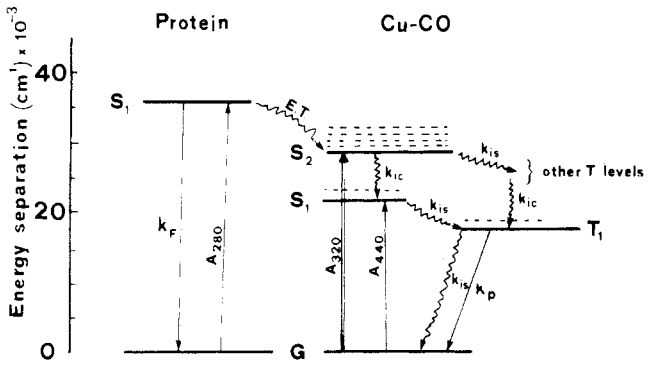

FIGURE 4: Jablonski's diagram of lowest energy CO-hemocyanin electronic excited states. The lowest singlet excited state (S) of the protein and of CO–copper centers are reported, together with the lowest triplet state (T) possibly responsible for the emission. A's are the absorption processes at the indicated wavelengths. E.T. is the nonradiative energy transfer from the protein to the CO–Cu moiety. All the k's indicate the rate constants for radiative and nonradiative processes: k_F = fluorescence; k_p = phosphorescence; k_{is} = intersystem crossing; k_{ic} = internal conversion. G indicates the ground state.

As a final remark it should be pointed out that no quenching of CO-linked phosphorescence was observed upon addition of Tb(III) or Eu(III), which suggests that cation binding sites are removed from the copper centers. Moreover the latter sites have been found largely accessible to water (Kuiper et al., 1979) while the copper centers seem to be shielded from the solvent, as already proposed (Alben et al., 1970).

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