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EMISSION SPECTRA FROM COPPER PROTEINS CONTAINING TYPE-3 CENTRES

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Aqueous solutions of copper-proteins containing type-3 centres (ceruloplasmin, tyrosinase, haemocyanin), excited within their absorption bands at 325–345 nm, show typical luminescence spectra. The emission bands peak at 415–445 nm and their decay time is no longer than 10 ns. A strong analogous fluorescence is obtained also by excitation of concentrated solutions of carboxylic acids and amino acids, which show again absorption bands around 330 nm. Such a fluorescence, although less intense, is also observed in copper(II) carboxylate solutions. In contrast, no fluorescence has been recorded in solutions of acetic anhydride and of polypeptides (valinomycin, gramicidin D), which do not have free carboxyl groups. We tentatively attribute this novel fluorescence in the investigated copper proteins to interactions between carboxyl groups of amino acids at, or near, the active site.

1. Introduction

Three different types of copper(II)are found in copper proteins [1,2]. Type 3 is characterized by an intense absorption band between 325 and 345 nm and the lack of an EPR spectrum. This centre consists of an antiferromagnetically coupled copper(II) pair, each copper atom being in a square-planar or quasi-square pyramidal stereochemistry [3] or, following an alternative proposal [4], in a distorted tetrahedral coordination to two S and two N ligands.

The absorption band at 325–345 nm is observed in the oxidized protein but tends to disappear in the reduced form. Really a residual absorption around 330 nm appears when the proteins are reduced or deoxygenated, and such a band was recently attributed to a histidine-to-copper charge-transfer transition [5]. As for the main most intense absorption, two hypotheses have been put forward about its origin. Both agree in correlating it to a copper-copper linkage, however, the former attributes the absorption to a simultaneous pair excitation, as previously proposed for copper(II) carboxylates [6] and in agreement with resonance Raman studies of haemocyanins [7], whereas the latter assigns the band to a charge-transfer transition [8,9], in particular a peroxide-to-Cu(II) transition for oxyhaemocyanins [3,10–12].

During some investigations we are performing on the effects of ultraviolet irradiation of copper proteins, we observed that the excitation within the absorption band at 325–345 nm produced a luminescence at 415–445 nm. Such a luminescence is added to the one previously described at 300–350 nm, which is observed after excitation in the absorption band of the protein (≈ 280 nm) and is due to the aromatic amino acid residues of the protein [13].

Here we report the results of our investigations on the luminescence in copper proteins, in some dimer copper(II) complexes and in concentrated solutions of amino acids and carboxylic acids. On the basis of the very similar luminescence observed in all these cases, we propose the presence of closely related amino acid residues in, or near, the type-3 centres.

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2. Experimental

Human ceruloplasmin, mushroom tyrosinase, *Limulus polyphemus* haemocyanin, gramicidin D and valinomycin were purchased from Sigma Chemical Co. Haemocyanins from *Carcinus maenas* and *Octopus vulgaris* were prepared and purified at the Centre for the Physiology and Biochemistry of Haemocyanins (Italian National Research Council, Padua). Aqueous solutions of copper proteins were prepared by dissolving the protein in phosphate buffer (pH 7.10) so as to have a concentration in the range 10^{-5}–10^{-6} M.

Nonaqueous solutions were prepared using fluorescence-grade solvents.

In order to ensure that the fluorescence we observed is not due to impurities, we have investigated purest grade amino acids and carboxylic acids from different origins (Carlo Erba, Hoechst, BDH): the same fluorescence was observed in all cases. Further purification, by recrystallization, produced no change in the spectra or a slight increase in the fluorescence intensity, thus excluding impurities as the cause of the fluorescence.

Copper(II) acetate, benzoate and $Cu_2(salen)_2$ (salen, *N*,*N'*-ethylenebissalicylaldimine) were prepared and purified by us.

Emission and excitation spectra were recorded at room temperature on a Perkin-Elmer 650-10S or MPF4 spectrophotometer; the absorption spectra on a Perkin-Elmer Mod.552. A pulsed nitrogen laser ($\lambda = 337.1$ nm) was used as exciting source in order to estimate, from oscilloscope traces, the lifetime for the emission.

3. Results and discussion

All the proteins investigated show a broad emission at 415 nm (ceruloplasmin, haemocyanins) or 445 nm (tyrosinase) (fig. 1), of which the decay time is equal to or less than 10 ns, which is the overall response time of our detection system. The emission band at 415–445 nm is exclusively observed exciting within the absorption band peaking at 325–345 nm, while only the tail of the emission at 330 nm, due to the aromatic amino acid residues of the protein, is present when one excitation is performed at 280 nm. However, as the two absorption bands partially overlap, it is possible to excite simultaneously and efficiently the two emission bands at 314 nm (fig. 2).

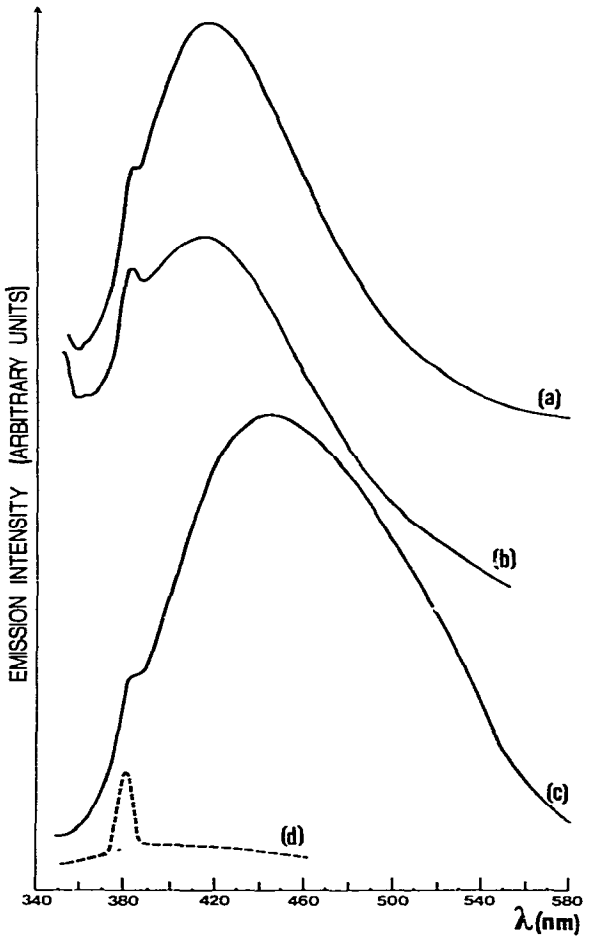

Fig. 1. Emission spectra ($\lambda_{exc} = 337$ nm) of (a) human ceruloplasmin ($\approx 10^{-5}$ M), (b) *Limulus polyphemus* haemocyanin (4 mg/ml), (c) mushroom tyrosinase (0.5 mg/ml). The above aqueous solutions were prepared in phosphate buffer, pH 7.10 (d). All the spectra were recorded under the same conditions at room temperature.

The excitation spectra have a peak at approx. 340 nm (fig. 3a) and reproduce well the absorption spectra of the proteins, thus giving further evi-

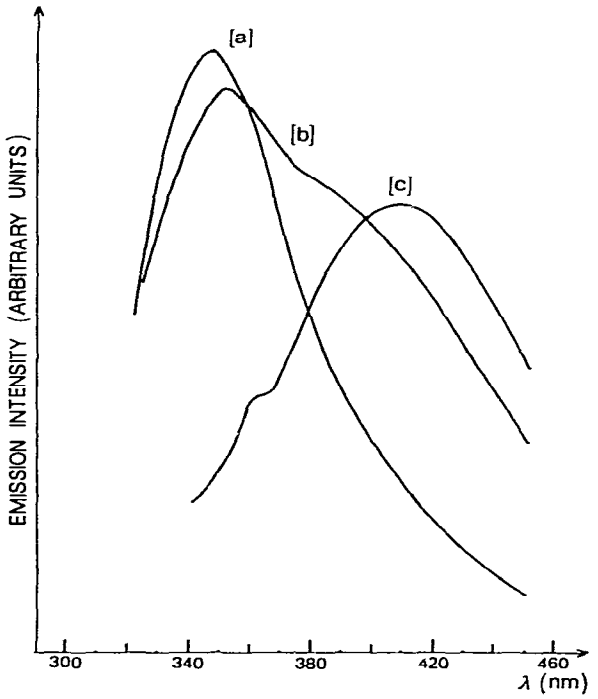

Fig. 2. Emission spectra of human ceruloplasmin ($\approx 10^{-5}$ M) in phosphate buffer, pH 7.10, under different exciting wavelengths: (a) $\lambda_{exc} = 310$ nm, (b) $\lambda_{exc} = 314$ nm, (c) $\lambda_{exc} = 320$ nm.

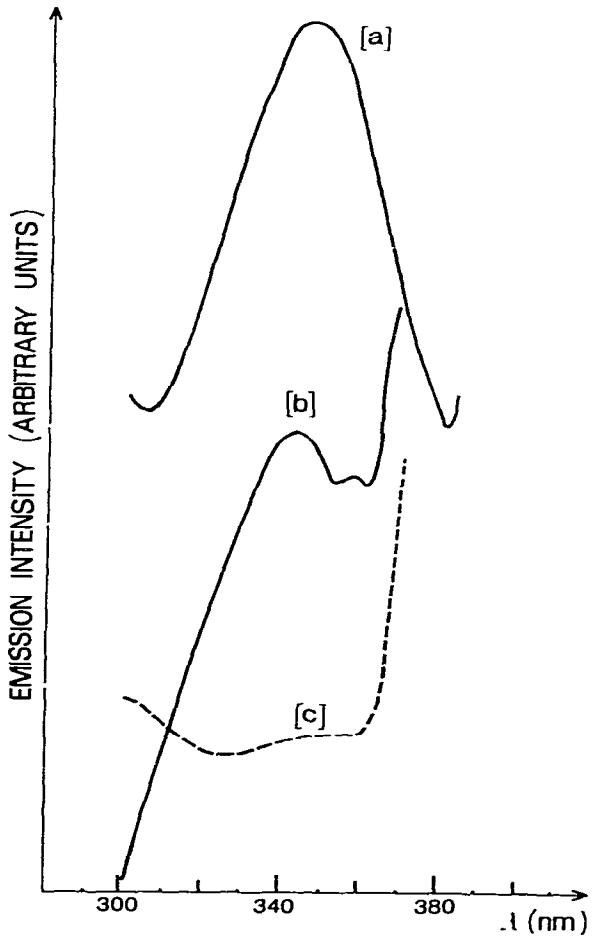

Fig. 3. Excitation spectra (uncorrected) of (a) mushroom tyrosinase in phosphate buffer, pH 7.10 ($\lambda_{em} = 445$ nm). (b) copper(II) benzoate in 1-butanol ($\lambda_{em} = 415$ nm). (c) 1-butanol ($\lambda_{em} = 415$ nm).

dence against fluorescent impurities.

Since the emission is closely related to the absorption characteristic of the type-3 centres, it could be inferred that the Cu-Cu linkage is directly involved in the fluorescence process. Actually, we have observed a quite similar fluorescence in 1-butanol solutions (10^{-3}–10^{-4} M) of copper(II) carboxylates (acetate, benzoate (fig. 3b)), where dimeric copper(II) centres are surely present. The presence of water, which breaks copper-copper bonds [6], strongly reduces the fluorescence. By comparison with the emission spectra of anthracene (10^{-7} M in 1-butanol) we have evaluated a quantum yield ϕ of the order of 10^{-3} for copper(II) acetate solutions. Similar or higher quantum yields were found in the different copper proteins.

However, the lack of fluorescence in some copper(II) dimer complexes we have investigated, like $Cu_2(salen)_2$, and the fluorescence enhancement in the apohaemocyanins [14], cast some doubt upon the primary role of Cu_2 entities in the emission process. On the other hand, once the absence of fluorescent impurities is ascertained, the struct-

ural simplicity of copper(II) acetate does not offer many possibilities about the origin of the fluorescence, which has to be sought in molecular orbitals characteristic of the bridging π-electron system [8]. Indeed, very recently, it has been shown [15,16] that molecular associates containing delocalized π-electrons are characterized by an absorption in the range 300–400 nm, to which corresponds an emission in the range 400–600 nm.

To examine further the origin of this novel fluorescence, we began to investigate the spectral behaviour of different amino acids and carboxylic acids in concentrated solution, where the formation of associates is favoured.

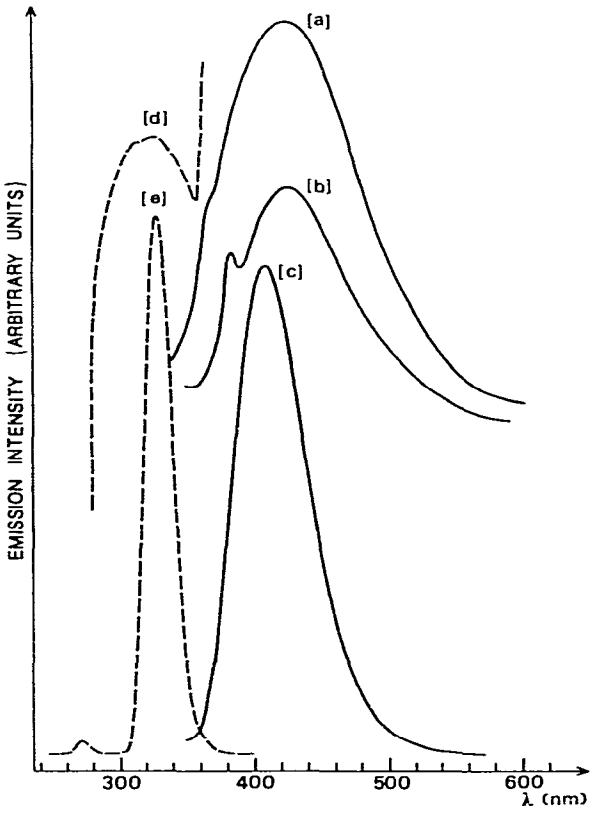

Fig. 4. Emission (———) and excitation (— — —) spectra of: (a) pure acetic acid, $\lambda_{exc} = 330$ nm; (b) histidine, 0.2 M in water, $\lambda_{exc} = 340$ nm; (c) tyrosine, 4×10^{-3} M in ethanolic solution of KOH, $\lambda_{exc} = 340$ nm; (d) acetic acid, 1.7 M in benzene, $\lambda_{em} = 410$ nm; (e) same as c, $\lambda_{em} = 410$ nm.

All the acids we have considered present a very weak and broad absorption at 330–340 nm. By irradiating within this absorption band, a strong emission ($\phi \simeq 0.1$–0.3) appears in the range 410–430 nm (table 1, fig. 4a–c) for all the compounds we have examined, except tryptophan, the emission of which is displaced towards shorter wavelengths (375 nm). A similar emission for tryptophan had already been described some years ago [17] and tentatively attributed to the formation of a tryptophan photoproduct hydroxylated at C_3. Such a hypothesis appears at least questionable, in the light of the present results, since the observed fluorescence is not limited to the tryptophan molecule alone. Analogously to the copper(II) carboxylates and to the copper proteins, the excitation spectrum has a peak at approx. 330 nm (fig. 4d and e) and the decay time of the emission is of the order of 10 ns. The addition of copper(II) ions to the above solutions quenches to a large extent the fluorescence, so it can be deduced that, also in the carboxylate complexes, copper(II) indeed decreases the fluorescence, its role being simply to keep close to the carboxyl groups even in diluted solution. The presence of sulphur atoms in the molecule (cysteine, methionine) quenches the fluorescence and decreases the intensity by about one order of magnitude. Since the dissociation constants of alanine

Table 1

Emission peaks of amino acids and carboxylic acids excited at 340 nm

Acid	Solvent	Concentration (M)	λ_{em} (nm)
Acetic	–	–	425
Acetic	Benzene	1.7	410
Benzoic	Benzene	0.2	410
DL-Alanine	Water	0.1	410
L-(+)-Glutamic	Water	0.03	420
L-(−)-Cysteine	Water	0.08	430
L-(−)-Methionine	Water	0.1	425
L-(−)-Tryptophan	Water	3×10^{-4}	375
L-(−)-Tryptophan	Ethanol	saturated	375
L-(−)-Histidine	Water	0.2	425
L-(−)-Tyrosine	Ethanol, KOH	4×10^{-3}	410

and methionine are nearly the same (alanine: $pK_1 = 2.35$, $pK_2 = 9.87$; methionine: $pK_1 = 2.28$, $pK_2 = 9.21$), a similar $COO^-/COOH$ ratio is present in the two solutions, so that quenching of the fluorescence in the sulphurated amino acids can be very likely attributed to a heavy atom effect [13]. On the other hand, on passing from the acid to the corresponding anhydride, no fluorescence is detected even in concentrated solution (i.e., acetic anhydride, 0.1 M in benzene).

In conclusion, all the experiments made on different solutions of carboxylic and amino acids and their derivatives clearly indicate the carboxyl group as being responsible for the new fluorescence.

Ab initio molecular orbital treatments performed thus far on carboxylic acids [18,19] unfortunately do not give a definite levels scheme for the low-lying states. However, on the basis of these calculations, it seems that monomeric acids cannot account for the absorption at 330 nm. On the other hand, the formation of dimers (or more complicated associates) gives rise to new levels and different spin multiplicities, which might explain both the absorption at 330 nm and the related emission.

May we confidently extend such a conclusion also to the fluorescence in copper proteins? Actually, given the similarity of the absorption, emission and excitation spectra and of the lifetime, the hypothesis of an analogous origin of the fluorescence also in the copper proteins is very attractive.

In order to ascertain that the peptidic portion of the copper proteins cannot be responsible for the fluorescence, we have investigated two polypeptides (gramicidin D, valinomycin) from which free carboxyl groups are absent. Our choice allowed us to obtain much additional information, depending on whether or not fluorescence was detected. Indeed, gramicidin D and valinomycin are linear and cyclic polypeptides, respectively, so that the eventual role of different conformations could be investigated. Furthermore, free hydroxyl and carbonyl groups are present in gramicidin D, while residues of hydroxy acids (D-α-hydroxyisovaleric and L-lactic acid) together with D- and L-valine form the 36-membered ring of valinomycin. We were not able to detect any fluorescence from solutions in methanol ($C = 10^{-4}$ M) of both polypeptides.

Moreover, the enhancement of the fluorescence in the apoproteins seems to indicate that the fluorophores are present in the type-3 centres, near or directly bonded to the pair of copper(II) ions. Since experimental data suggest two to four histidines per copper at the active site in a haemocyanins [3,7,20–22], histidine residues are very likely those responsible for the residual absorption at 330 nm and for the intense fluorescence at 415–445 nm as well.

Further experimental and theoretical studies are at present in progress to give conclusive indications about the fluorophore in the type-3 centres.

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