Copper is not responsible for the visible fluorescence of blue oxidases

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The fluorescence of several blue copper proteins upon excitation at 330 nm has been studied. This fluorescence is not related to the redox state or even to the presence of copper, which in some cases can act as a quencher. The intensity of emission seems instead related to the amount of carbohydrate present or to tryptophan photooxidation products.

Blue oxidase *Copper emission* *Carbohydrate* *Photooxidation*

1. INTRODUCTION

Blue oxidases contain Cu^{2+} characterized by different spectroscopic features: the 'blue' paramagnetic Type 1 copper, so called because of a strong charge transfer absorption at about 600 nm (ϵ = 4–600 $m^2 \cdot mol^{-1}$), the paramagnetic Type 2 copper, with normal spectroscopic properties, and the Type 3 copper, a pair of magnetically coupled cupric ions [1,2]. Type 3 copper is considered to give rise to an absorption band at 330 nm, though some authors have suggested that Type 2 copper is also in some way involved [3]. Such uncertainty comes in part from the difficulty in elucidating the nature of the electronic transition underlaying the absorption band [4].

Recently, in analogy with findings on the copper proteins hemocyanin [5], tyrosinase [6] and thionein [7,8], two independent groups reported that tree laccase, a member of the family of blue oxidases, shows a blue fluorescence (emission maximum 430 nm) when excited at 330 nm [9,10]. This fluorescence has been attributed to the Type 3 copper. We here show that this emission is rather linked to the protein moiety and not to the metal.

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2. MATERIALS AND METHODS

Rhus vernicifera laccase and stellacyanin and *Rhus succedanea* laccase were purified from the dried acetone powder from the latex of the Japanese and Vietnamese lacquer tree, respectively. They were both supplied by Saito (Osaka). In the former case the procedure in [11] was used, in the latter one the procedure in [12] using the fraction eluted with 0.2 M phosphate from the DEAE–Sephadex column as previously described (O'Neill et al., submitted). Ascorbate oxidase was isolated from green zucchini as in [13]. Azurin was purified from *Pseudomonas fluorescens* [14].

Apo-stellacyanin and apo-azurin were prepared as in [15] and [16]. Apo-laccases and apo-ascorbate oxidase were prepared by dialysis against 0.1 M KCN in 0.1 M phosphate buffer, pH 7.2. The final pH of the solution was 9.3.

The protein concentration was determined by a biuret method [17] and by the molar extinction coefficient at 280 nm (stellacyanin: ϵ = 23500; azurin: ϵ = 10700; ascorbate oxidase: ϵ = 240000; Jap. laccase: ϵ = 93500; Viet. laccase: ϵ = 120000).

Copper content was determined by 2,2'-biquinolyl reaction [18]. Carbohydrate content was determined by the method in [19], using

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D-(+)-mannose as the standard.

Fluorescence spectra were measured with a Fica L 55 Spectrofluorimeter which gives spectra corrected for the energy of excitation and for the response of the photodetector.

Irradiation of BSA was performed in buffered solution (pH 7) with a TQ 718 high-pressure Hg Hanau UV lamp (500 W) placed at 10 cm from the sample and equipped with a quartz cooling apparatus.

3. RESULTS AND DISCUSSION

Laccase samples show a fluorescence at 430 nm when excited at 330 nm as already reported (fig.1). The same figure shows that also stellacyanin, a single copper-containing blue protein, is fluorescent with the same excitation and emission parameters, though it lacks Type 3 Cu^{2+}. On the contrary, ascorbate oxidase which has Type 3 Cu and a higher 330 nm absorption band is much less fluorescent. The reduction of these copper proteins with stoichiometric amounts of ascorbate in the absence of air increases their visible fluorescence. The total removal of copper is even more effective in increasing this emission. This finding rules out any role for copper, but a quenching ability, in the 330 nm fluorescence of blue oxidases. The fluorescence observed must therefore be originating from the protein moiety. It is interesting to point out that there appears to be a correlation between the carbohydrate content of the copper proteins investigated and the fluorescence observed (table 1). Azurin, a single Cu-containing blue protein devoid of carbohydrate, is the least fluorescent. Besides the involvement of carbohydrates an ageing process also appears to be involved. Samples of azurin aged by storing at −25°C for months to years exhibited a visible fluorescence of less intensity. The effect of ageing on the intensity of the fluorescence spectra was studied using BSA, a protein devoid of carbohydrates. An artificial ageing process was set up by irradiation with UV light. After irradiation for 2 h, the visible fluorescence of BSA was found to be almost doubled (fig.2). The intrinsic fluorescence (ex, 280 nm; em, 335 nm) dropped by a factor of 4. This is due to tryptophan photooxidation [20].

Recently one of the two groups who first described the extrinsic emission from blue oxidases

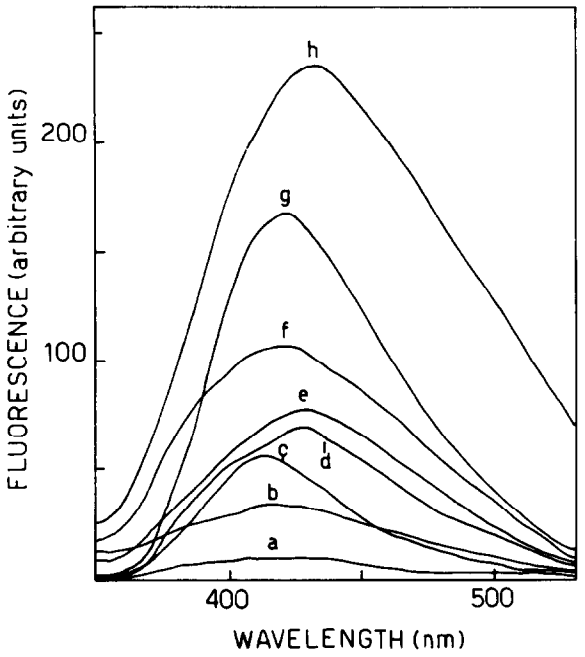

Fig.1. Fluorescence of blue proteins excited at 330 nm. All the samples had an optical absorption at 330 nm = 0.3 cm^{-1} and were dissolved in water. (a) Azurin (2.6×10^{-4} M); (b) ascorbate oxidase (2.7×10^{-5} M); (c) stellacyanin (3×10^{-4} M); (d) Vietnamese laccase (7.5×10^{-5} M); (e) Japanese laccase (9×10^{-5} M); (f) ascorbate oxidase (copper-free) (4.5×10^{-5} M); (g) stellacyanin (copper free) (2.8×10^{-4} M); (h) Vietnamese laccase (copper free) (1.2×10^{-4} M).

Table 1

Carbohydrate content and relative fluorescence of various proteins

Proteins	Carbohydrate content % (w/w)	Fluorescence[a] (arbitrary units)
Azurin	1	10
Ascorbate oxidase	8	34
Stellacyanin	20	56
Viet. laccase	26	69
Jap. laccase	27	76
Bovine serum albumin	0.02	9.5

[a] Excitation: 330 nm; emission: 430 nm. Each value is the mean of 3 different samples, which gave slightly different results

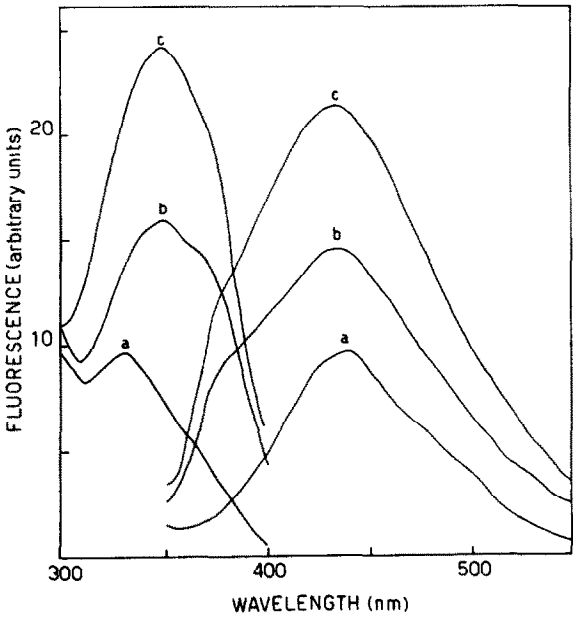

Fig.2. Fluorescence of irradiated bovine serum albumin. Experiments were performed with 1.5 mg/ml BSA in 100 mM phosphate buffer pH 7. (a) Freshly prepared solution; (b) after 120′ irradiation; (c) after 180′ irradiation.

disproved their previous assignment to Type 3 Cu^{2+} but assigned the emission to carboxylate groups of proteins [21]. This investigation shows that the faint blue fluorescence observed in many copper proteins may be of various origins. It is definitely not due to any type of copper present and particularly important could be the carbohydrate component and/or the oxidation products of tryptophan.

REFERENCES

[1] Malmström, B.G., Reinhammar, B. and Vänngård, T. (1968) Biochim. Biophys. Acta 156, 67–76.
[2] Malkin, R., Malmström, B.G. and Vänngård, T. (1969) Eur. J. Biochem. 10, 324–329.
[3] Morpurgo, L., Graziani, M.T., Finazzi-Agrò, A., Rotilio, G. and Mondovì, B. (1980) Biochem. J. 187, 361–366.
[4] Beinert, H. (1980) Coord. Chem. Rev. 33, 55–85.
[5] Finazzi-Agrò, A., Zolla, L., Flamigni, L., Kuiper, H.A. and Brunori, M. (1982) Biochemistry 21, 415–418.
[6] Kuiper, H.A., Lerch, K., Brunori, M. and Finazzi-Agrò, A. (1980) FEBS Lett. 111, 232–234.
[7] Beltramini, M. and Lerch, K. (1981) FEBS Lett. 127, 201–203.
[8] Beltramini, M. and Lerch, K. (1983) Biochemistry 22, 2043–2048.
[9] Wynn, R.M., Hemanta, K.S., Holwerda, R.A. and Knaff, D.B. (1983) FEBS Lett. 156, 23–28.
[10] Bacci, M., Baldecchi, M.G., Fabeni, P., Linari, R. and Pazzi, G.P. (1983) Biophys. Chem. 17, 125–130.
[11] Reinhammar, B. (1970) Biochim. Biophys. Acta 205, 35–47.
[12] Brändèn, R. and Deinum, J. (1978) Biochim. Biophys. Acta 524, 297–304.
[13] Avigliano, L., Gerosa, P., Rotilio, G., Finazzi-Agrò, A., Calabrese, L. and Mondovì, B. (1972) Ital. J. Biochem. 21, 248–255.
[14] Ambler, R.P. (1963) Biochem. J. 89, 341–349.
[15] Morpurgo, L., Finazzi-Agrò, A., Rotilio, G. and Mondovì, B. (1972) Biochim. Biophys. Acta 271, 292–299.
[16] Yamanaka, T., Kijimoto, S. and Okunuki, K. (1963) J. Biochem. (Tokyo) 53, 256–259.
[17] Goa, J. (1953) Scand. J. Clin. Lab. Invest. 5, 218–223.
[18] Brumpy, P.E. and Massey, V. (1967) Methods Enzymol. 10, 473–474.
[19] Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350–356.
[20] Fujimori, E. (1981) FEBS Lett. 135, 257–260.
[21] Bacci, M., Linari, R., Ricchelli, F. and Salvato, B. (1983) Proc. 1st Internat. Confer. Bioinorg. Chem., Florence, Italy, June 13–17, 1983, no.U21.