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THE EFFECT OF LASER RADIATIONS ON HUMAN CERULOPLASMIN, ABSORPTION, CIRCULAR DICHROISM AND ELECTRON PARAMAGNETIC RESONANCE STUDY

M. HERVE[X+], A. GARNIER[X+], L. TOSI[X] and M. STEINBUCH[XX]

[X]*DEPARTEMENT DE RECHERCHES PHYSIQUES, Université Pierre et Marie Curie, Laboratoire Associé au C.N.R.S., n° 71, 4 Place Jussieu, 75230 PARIS Cedex 05.*
[+]*U.E.R. DE MEDECINE ET BIOLOGIE HUMAINE, Université Paris XIII, Bobigny.*
[XX]*DEPARTEMENT DE FRACTIONNEMENT, Centre National de Transfusion Sanguine, Orsay.*

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SUMMARY : Laser radiations at wavelengths ranging from 514.5 to 360.0 nm decolorize human ceruloplasmin. Kinetic behavior of the two chromophores absorbing at 610 nm, as measured by absorption and circular dichroism data, indicate different quantum yields of the two type I copper ions, whose maximum lies approximatively at 400 nm. Furthermore, as Electron Paramagnetic Resonance measurements demonstrate, the photochemical process involves reduction of the two type I copper ions leaving type II copper unchanged.

INTRODUCTION : Ceruloplasmin (ferroxidase) (1) is a mammalian plasma metalloprotein that contains three paramagnetic cupric ions per molecule two of which - the so called type I or "blue" copper - give rise to strong absorption at 610 nm (ε = 11 000 $cm^{-1}mol^{-1}l$.) The third cupric ion which does not show detectable absorption properties is known as type II or "non blue" copper.

The native protein is sensitive to light. The action of white light - spanning a wide range of wavelengths - on ceruloplasmin has been studied in the presence of oxygen and photosensitive dyes like methylene blue and rose Bengal (2,3). The resulting effect observed was the photooxidation of histidine residues.

In the course of a resonance Raman spectroscopic study on ceruloplasmin we observed that the blue color of the protein disappears under the effect of some laser radiations (4). Surprisingly enough, the 632.8 nm exciting line of a He-Ne laser which lies inside the band enveloppe of the intense visible absorption of the protein and very near to its maximum had no effect, whereas radiation of comparable power and lower wavelength - where absorption is minimal - gave rise to rapid decolorization. Some recovery of color was also observed, the extent of which decreased with the wavelength of irradiation. Two mechanisms could be invoked to explain this behavior : 1) reduction of type I copper ions, 2) disruption of the ligand-copper(II) bond giving rise to the

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charge transfer transition at 610 nm (5,7). Hence, for the purpose of gaining further understanding of the process, we have undertaken an investigation of the effect of laser radiations of various wavelengths using absorption, circular dichroism and electron paramagnetic measurements. The present communication is a preliminary report on the results thus obtained.

EXPERIMENTAL : Human ceruloplasmin was prepared as indicated previously (8). Purity was controlled by measurements of the $\varepsilon_{610}/\varepsilon_{280}$ ratio which was higher than 0.040. All solutions were prepared with doubly distilled water.

The following radiations were used : 413.1 and 406.7 nm from a special UV Kr laser, 647.1 nm from a Kr laser, 632.8 nm from a He-Ne laser, 514.5, 488.0, 472.7, 454.5 and 360.0 nm from an Ar laser; all the four lasers are from Spectra Physics. The following technique was employed : 115 µl of ceruloplasmin solution 6.0×10^{-5}M in acetate buffer 0.05M,pH 5.5, were introduced into a special Hellma cell model of $2mm^2$ cross section and air tight closed. The cell was placed so that the laser beam went through the solution. Two successive reflexions were enough to enlarge the laser beam and ensure that the entire volume of solution was equally illuminated. At selected intervals the irradiation was stopped and spectra registered. Incident light beam power was measured by means of a Coherent radiation Model 210 power meter, and an actinometric method using potassium ferrioxalate in the same experimental conditions as ceruloplasmin samples (9). Experiments were run at 25°C, using laser radiation of 80, 100, 200 and 280 ± 10 mW.

Absorption spectra were recorded with a Cary 14 spectrometer, circular dichroism spectra with a Roussel-Jouan dichrograph model CD 185, and electron paramagnetic spectra at 77K and 9.2GHz with a Bunker type ER 420 spectrometer.

RESULTS AND DISCUSSION : Figure 1, curve 1, shows the absorption spectrum of ceruloplasmin between 800 and 300 nm. The striking feature of this spectrum is the strong absorption at 610 nm assigned to a charge transfer transition from a thiol (5,6) or thioether (7) group to the cupric ion. As the circular dichroism pattern of curve 2 indicates this is a region particularly rich in electronic transitions. In figure 2 are reported the values of the absorbancies at 610 nm versus time of irradiation at different wevelengths. The curves are characterized by an initial rapid decolorization followed by a somewhat slower process, the extent of bleaching being stronger as the wavelength of the laser beam decreases. Using the absorbancies at 610 nm at any time of irradiation to calculate type I Cu(II) concentrations and knowing the incident light intensity one can calculate primary quantum yields as defined by $\phi = \frac{dA/dt}{I_{abs.}}$ (9,10), where dA/dt is the number of molecules undergoing change per cm^3.sec and $I_{abs.}$ is the number of photons absorbed by the reactant per cm^3. sec. The values of ϕ thus obtained are not constant but depend on the time of

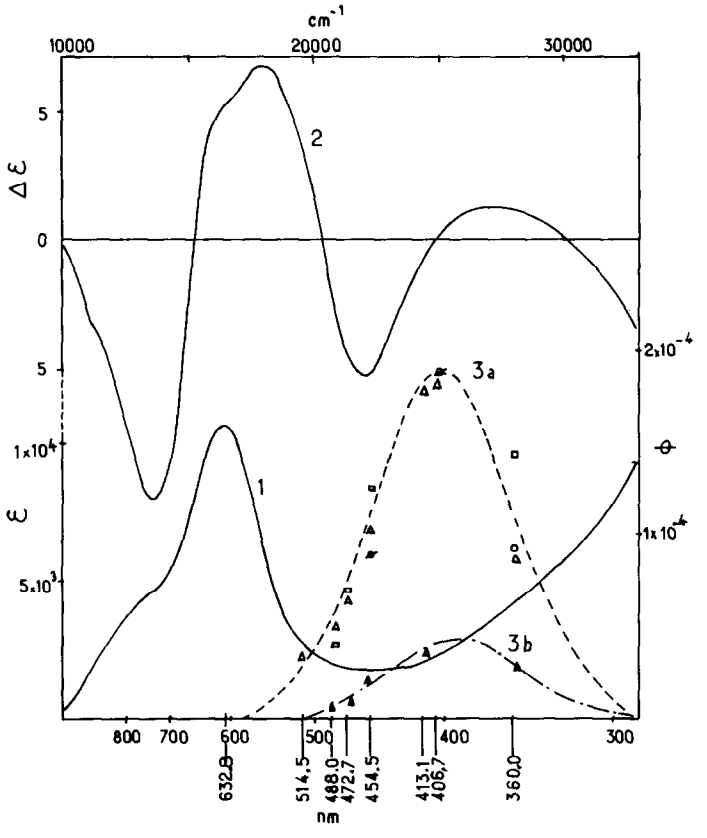

Figure 1. Absorption (curve 1) and circular dichroism (curve 2) spectra of human ceruloplasmin solution 6×10^{-5}M in acetate buffer 0.05M pH 5.5. Curve 3a and 3b give primary quantum yields of type Ia and Ib copper respectively (see text). Laser power : o 280 mW; Δ 200 mW; ø 100 mW; □ 80 mW.

irradiation, which indicates than more than one chromophore with different quantum yields participate in the overall photochemical process. This finding is by no means unexpected since, as has been demonstrated previously, there are two type I Cu(II) absorbing at 610 nm which differ in their redox potentials (11), reoxidation rates (12), electron paramagnetic signal (13), circular dichroism (14) and resonance Raman (4) patterns. In a recent circular dichroism study on ceruloplasmin-anion interaction (14) we have assigned the band at 450 nm (see figure 1, curve 2) to a transition of one of the type I Cu(II) ions - called by us Ib - which is not perturbed by anions like N_3^-, SCN^- and OCN^-. Then, we can calculate Ib concentration (C_{Ib}) at any instant by means of circular dichroism values at 450 nm. In Fig.3 are reported the values of C_{Ib} thus obtained after irradiation using the 454.5 nm laser

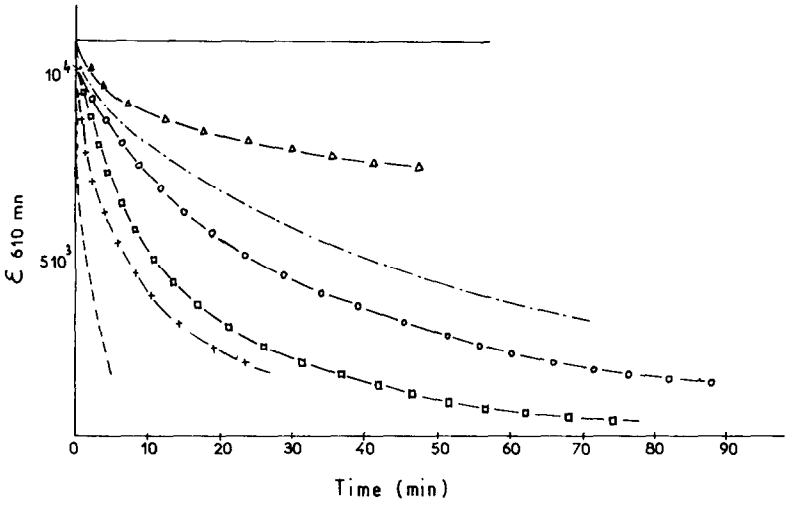

Figure 2. Molar extinction coefficients of human ceruloplasmin at 610 nm versus time of irradiation at different laser wavelengths —— 647.1 and 632.8 nm; —Δ—Δ— 514.5 nm; —·—·— 488 nm; —o—o— 472.7 nm; —□—□— 454.5 nm; —x—x— 413.1 nm and - - - 360.0 nm. Experimental conditions as in Fig. 1.
Laser power was 200mW throughout except for 632.8 (50mW).

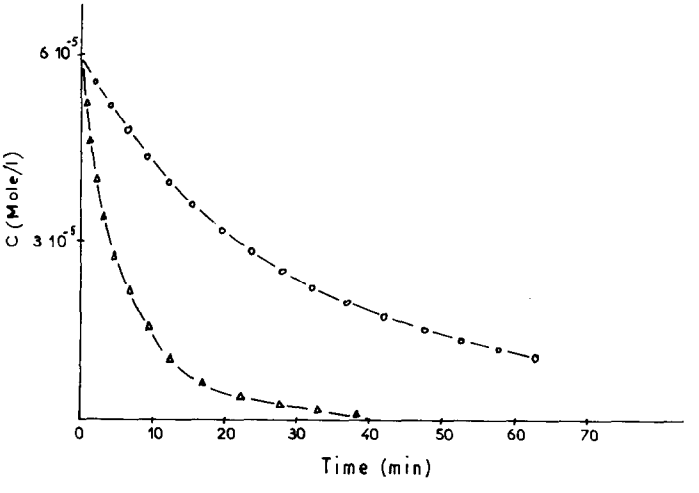

Figure 3. Type Ia (—Δ—Δ—) and type Ib (—o—o—) concentrations versus time of irradiation using the 454.5 nm line, 200 mW, calculated as indicated in text.

line, 200 mW. From them it is possible to compute ϕ_{Ib} which turns out to be independent of time of irradiation. Concentration of the second type I Cu(II) - called by us Ia - can now be calculated at any instant by substracting C_{Ib} from total type I Cu(II) concentration calculated by absorbancies at 610 nm, and recalling that Ia and Ib copper have the same molar extinction coefficient (5 500 M^{-1} cm^{-1}) (14) at this wavelength. C_{Ia} values obtained by this method are reported in Fig.3. From them, and knowing incident intensities, ϕ_{Ia} can be obtained which, like ϕ_{Ib}, is independent of time of irradiation. On figure 1, (curves 3a and 3b) are reported the values of ϕ_{Ia} and ϕ_{Ib} at the wavelengths studied. ϕ_{Ia} and ϕ_{Ib} have been calculated for different values of laser beam intensity (80, 100, 200 and 280 mW) and proved to be independent of them. As can be noticed the maximum primary quantum yield for the two species lies approximately at 400 nm.It may be worth recalling that this wavelength corresponds roughly to a tyrosyl → Cu(II) charge transfer transition (15,16). The reduction of the two chromophores followed apparent first order kinetics. The first order rate constants calculated from C_{Ia} and C_{Ib} data of figure 3 are $k_a = 21 \times 10^{-4}$ s^{-1} and $k_b = 5 \times 10^{-4}$ s^{-1}. Irradiation of ceruloplasmin in the absence of oxygen gives the same results as above showing that under our experimental conditions photooxidation, if present, is not significant. Moreover, circular dichroism measurements in the ultraviolet region indicate no discernible modification of the secondary structure.

Additionnal information on the nature of the mechanism involved is given by electron paramagnetic resonance measurements under the same experimental conditions using the 454.5 nm laser radiation. Fig.4 A shows a low temperature EPR spectrum of non irradiated human ceruloplasmin at about 9.27 Ghz. The spectrum agrees in essential features with those reported previously (13, 17). The line at 2685 G is due to type II copper (17); the three hyperfine peaks at 2884, 2957, and 3030 G have been assigned by GUNNARSSON and PETTERSSON (13) to one form of type I copper and the peak at 2837 G to a second form of type I copper. As can be noticed, a line which arises from this second form of type I copper is clearly apparent at 2942 G. For the first type I copper one can thus calculate $A_{//}$ =73 G and $g_{//}$ =2.213; for the second type I copper one obtains $A_{//}$ =105 G and $g_{//}$ =2.214.

EPR spectra exhibit evident changes with increasing time of irradiation. The measurements of ε_{610} and $\Delta\varepsilon_{450}$ at each time of irradiation allow the calculation of C_{Ia} and C_{Ib} which are indicated by the vertical bars in Fig.4 under curves B, C, D, E. The low-field part of the spectrum of the irradiated protein at C_{Ia}=40%, C_{Ib}=85% (curve B) and C_{Ia}=15%, C_{Ib}=70% (curve C) show a decreasing of the amplitude of the lines at 2884, 2957 and 3030 G. In particu-

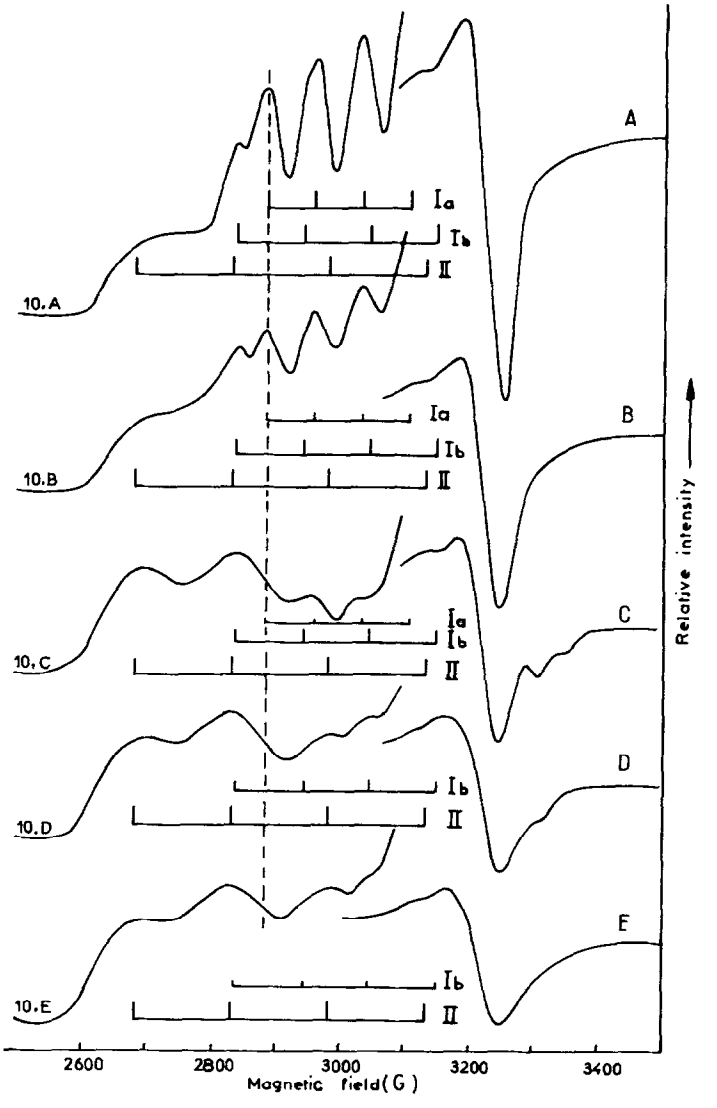

Figure 4. EPR spectra of human ceruloplasmin at 77 K and about 9.27 GHz. 6×10^{-5}M ceruloplasmin in acetate buffer 0.05M pH 5.5. registred at various time of irradiation with 454.5 nm laser beam. Curve A : C_{Ia} = 100 %, C_{Ib} = 100 %; Curve B : C_{Ia} = 40 %, C_{Ib} = 85 % ; Curve C : C_{Ia} = 15 %, C_{Ib} = 70 %; Curve D : C_{Ia} = 0, C_{Ib} = 50 %; Curve E : C_{Ia} = 0, C_{Ib} = 30 %. The height of vertical bars are proportional to each type copper concentration. Microwave frequency 9.270 GHz. Part of the spectra is shown with 10 times higher gain.

lar, the line at 2884 G does not appear in curve C. Accordingly we attributed these three lines to type Ia copper. The two lines at 2837 and 2942 G can

therefore be assigned to type Ib copper; the third hyperfine line, at 3042 G, of this copper is clearly shown in curve D since it is not any longer masqued by the 3030 G copper Ia line which has completely vanished.

As can be observed, in the five spectra of Fig.4 irradiation leads to no changes in the type II copper line at 2685 G. Spectrum E (for which C_{Ia}=0, C_{Ib}=30%) exhibits three lines at 2685, 2833 and 2983 G of comparable amplitude. Since the peak at 2685 G remains unperturbed by irradiation, and since the other two distinctly appear once type I copper signal vanishes, we assign the three peaks to type II copper. They give $A_{//}$=150 G and $g_{//}$=2.278 in good agreement with those of type II copper (13,17).

In the high-field part of the spectra the peak at 3260 G (curve A) moves to 3248 G (curve B) and its amplitude decreases (curve B to E). In curve C a new line at 3308 G appears and decreases as C_{Ib} decreases. We thus have assigned the line at 3248 G to type II copper, that at 3308 G to type Ib copper, the line of type Ia copper being localised at about 3260 G.

These EPR results rule out the second mechanism postulated in the introduction leaving the reduction of both type I copper ions as the sole mechanism responsible for decolorization.

The foregoing results provide evidence indicating different reduction rates of the two type I Cu(II) species in human ceruloplasmin when subjected to the action of light. Moreover, it follows that a laser beam of adequate wavelength should enable to study the catalytic properties of both type I copper ions separately.

Further investigation is going on in this laboratory to study the extent of recovery after irradiation as well as the effect on catalytic activity.

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