CERULOPLASMIN-ANION INTERACTION
A RESONANCE RAMAN SPECTROSCOPIC STUDY +

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SUMMARY : Resonance Raman spectra of ceruloplasmin using the 632.8 nm He-Ne radiation show bands at 415, 402 (shoulder), 382, 360 and 340 cm^{-1} which can be assigned to the metal-ligand stretching modes of the two "blue" Cu(II) ions present in the enzyme. After addition of N_3^- and SCN^- to ceruloplasmin the Cu(II)-ligand bonds of one of the two "blue" copper sites are disrupted and three bands in the RR spectra disappear, namely, at 415, 382 and 340 cm^{-1}. RR data enable the assignment of the metal-ligand stretching vibrations of each "blue" cupric ion, give evidence of subtle conformational changes around the non disrupted one, and confirm previous results indicating the non equivalency of the two "blue" copper sites.

INTRODUCTION : Ceruloplasmin is a copper protein which probably contains seven metal ions per molecule, two of which, called "blue" or type I cupric ions, are responsible for the unusually intense absorption of its visible spectrum (ε = 11000 at 610 nm) (1). Although little is known about the specific ligands bound to "blue" Cu(II) ions, the high values of the molar extinction coefficient at 610 nm has been interpreted as arising from a charge transfer transition from an RS^- group, probably cystein to a cupric ion (2). Furthermore, on grounds of EPR (3) and anion protein interaction CD

+ *Abbreviations used throughout this paper : CD : circular dichroism, EPR : electron paramagnetic resonance, RR : resonance Raman.*

studies (4), it has been suggested that the two "blue" copper sites of ceruloplasmin are not equivalent.

Resonance Raman scattering has been extensively used as a very sensitive technique for structural studies of heme proteins (5-6-7). Moreover, it has been applied as a very sensitive analytical probe to distinguish between position isomers (8). Siiman et al. have recently reported RR spectra of several "blue" copper proteins using the 647.1 and 568.2 nm Kr^+ laser as exciting lines (9). Here we present the results of a RR spectral study of anion bound ceruloplasmin using the 632.8 nm (He-Ne) exciting line which confirm previous results indicating the non equivalency of the two type I cupric sites in native ceruloplasmin; enable the assignment of the stretching modes of each site, and give information on subtle conformational changes around the copper ion that are not detected by other methods.

EXPERIMENTAL :Human ceruloplasmin was prepared as indicated previously (10). Purity was controlled by measurements of the ratio $\varepsilon_{610}/\varepsilon_{280}$ which was higher than 0.040. Sodium or potassium thiocyanate, sodium azide, sodium chloride, sodium perchlorate, potassium cyanide, potassium fluoride and potassium sulfate were commercial products of the best reagent grade available and were used without further purification. Salts of the anions were dissolved in acetate buffer 0.05 N, pH 5.5 and the pH readjusted to 5.5 if necessary before addition of enzyme solutions. All solutions were prepared with bidistilled water, ceruloplasmin concentration was 6.8×10^{-5}M, acetate buffer 0.05 N pH 5.5. RR spectra were recorded on a Raman Coderg triple monochromator, model T800 using the 632.8 nm (He-Ne) and the 514.5 nm (Ar) exciting lines. The spectra were recorded at room temperature thirty minutes after mixing of solutions using 90° scattering. The intensities were measured as peak heights relative to the 930 cm^{-1} buffer band whenever possible or the ν_1 Raman line of $SO_4^=$ used as internal standard.

RESULTS AND DISCUSSION : Although the absorption spectrum of ceruloplasmin in the visible and near UV region shows an intense absorption centered at 610 nm, the molecule still absorbe considerably at 400 nm. Thus, we tried the three radiations at our disposal : 632.8, 514.5, and 488 nm for which the values of ε are 10,000, 3,700, and 3,400 respectively. However, taking into account that ceruloplasmin is very sensitive to radiation (11-12) previous to recording of RR spectra we investigated the effects of these

three exciting lines. The result of this previous investigation will be published elsewhere. For the present study it is sufficient to remind that after 4 hours exposure to the 632.8 radiation (54 mw), the enzyme keeps its full activity. Marked alterations are observed using the 514.5 and 488 nm lines, being stronger and irreversible for the latter. Ceruloplasmin activity is reduced to 80% after one hour exposure to the 514.5 line at 54 nw. Hence, we recorded RR spectra using the 632.8 and 514.5 nm as exciting radiations.

The RR spectra of ceruloplasmin upon excitation with the He-Ne radiation is shown in fig.1, curve 1. This spectrum reproduces that of Siiman et al. (9) obtained with the 647.1 (ε = 8,500) and 562.8 (ε = 7,500) Kr lines. There are four major Raman bands at 340, 360, 382 and 415 cm^{-1}, a shoulder at $\sim$ 400 cm^{-1} and a weak band at 270 cm^{-1}. Other weak bands appear at 750, 825, 1220 and 1235 cm^{-1}. Depolarization ratios in the region below 450 are of the order of 0.3-0.6. Excitation with the 614.5 Ar line yields very weak bands in the 340-415 cm^{-1} region, at 1450 and 1650 cm^{-1}. Owing to the low quality of this spectrum as well as to the sensitiveness of the enzyme to the 514.5 nm radiation, all the study on anion ceruloplasmin interaction has been done using the He-Ne laser.

As it is known, anions like ClO_4^-, $SO_4^=$, F^-, Cl^-, CN^-, N_3^- and SCN^- inhibit the enzymic activity of ceruloplasmin (13). Excepting CN^- which acts as a complexing agent for the three types of copper ions, and $SO_4^=$ and ClO_4^- which do not coordinate to them, EPR measurements have shown that all the other anions bind to "non blue" Cu(II) (14). However, as absorption and CD data indicate the binding of N_3^- and SCN^- to "non blue" copper gives rise to changes in "blue" copper as well, presumably by inducing conformational changes leading to disruption of Cu-S (cyst) bonds (4). As a matter of fact, only one of the two "blue" copper sites is affected since the absorbance at 610 nm decreases to half the value of native ceruloplasmin when N_3^- and SCN^- concentrations are high enough to

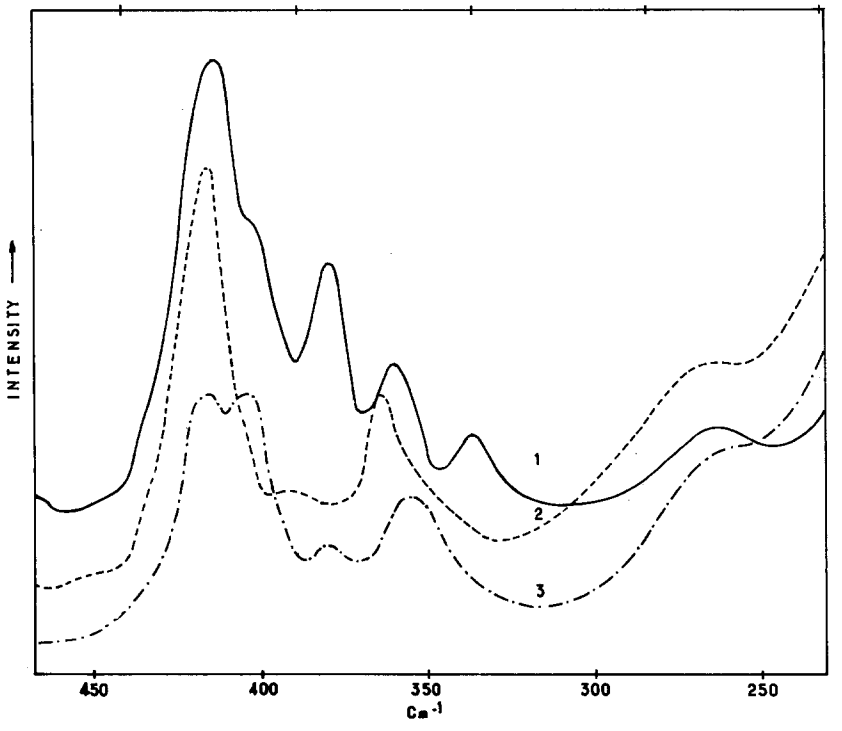

Fig.1. Resonance Raman Spectra of native and anion bound ceruloplasmin using 632.8 nm excitation, 40 mw; slit width : 5 cm^{-1}, time constant : 2.5 sec, scanning time 25 cm^{-1}/min.
1) ceruloplasmin 6.8×10^{-5} M, acetate buffer 0.05 N, pH 5.5 ;
2) id as 1, N_3^- 0.1 N ; 3) id as 1, SCN^- 0.2 N.

yield no further spectral changes (4). The other anions do not induce changes in type I Cu(II), save CN^- which removes copper from the protein giving rise to bleaching of ceruloplasmin solutions.

Siiman et al. have assigned the peaks at 415-340 cm^{-1} to Cu-N and/or Cu-O stretching vibrations and the band at 270 cm^{-1} to a Cu-N (imidazole)or a Cu-S(cyst.) stretching mode (9). It seemed thus possible to test the latter assignment, using the RR spectra of anion bound ceruloplasmin. Since the RR spectrum of fig.1, curve 1 is the sum of the two metal-ligand stretching modes, the disruption of one of the two Cu-S bonds should lead to the decrease in the intensity of all the metal-ligand vibra-

tions corresponding to this chromophore. On the other band, any change in local symmetry should be attested by changes in both band position and intensity.

As was expected the spectra of the enzyme after addition of 0.1 M solutions of $C\ell O_4^=$, $SO_4^=$, F^- and $C\ell^-$ reproduces that of native ceruloplasmin, although there is a slight shift toward higher frequencies (3-5 cm^{-1}) in the RR bands of the $C\ell O_4^-$ bound protein. The decolorated CN^--ceruloplasmin, on the other hand, does not display any RR spectrum. The RR spectra of ceruloplasmin after addition of 0.1 M N_3^- and 0.2 M SCN^- are shown in fig.1, curves 2 and 3 respectively. As can be seen two bands at 340 and 380 cm^{-1} decrease dramatically in intensity while the band at 270 cm^{-1} remains unchanged. Although the visible and CD spectra of N_3^- and SCN^- bound ceruloplasmin are quite equivalent (4), the two corresponding RR spectra show striking differences particularly at 415-402 cm^{-1}. The band at 417 cm^{-1} in the azide-ceruloplasmin spectrum is slightly asymmetrical and two times as intense as that of the thiocyanate complex at 415 cm^{-1}. Besides, the shoulder at 402 cm^{-1} has almost completely disappeared in the azide-ceruloplasmin spectrum while a well resolved band is present in that of the SCN^- bound protein. There are also minor differences in the band at 360 cm^{-1} : it shifts to 364 cm^{-1} in the azide-ceruloplasmin spectrum and to 355 cm^{-1} in that of thiocyanate-ceruloplasmin. These results led us to suppose that the peak at 415 cm^{-1} and the shoulder at 402 cm^{-1} in the spectrum of the native enzyme correspond to the superposition of three bands : two at $\sim$ 415 cm^{-1} and a third one at 402 cm^{-1}. The binding of N_3^- or SCN^- to the enzyme and the consequent disruption of one Cu(II)-S bond eliminates three bands in the RR spectra of native ceruloplasmin, namely at 415, 380 and 340 cm^{-1}. When N_3^- binds to ceruloplasmin, two of the remaining peaks at 360 and 400 cm^{-1} shift to higher frequencies, the latter giving rise to an increase of intensity at 415 cm^{-1} by accidental degeneracy. When SCN^- binds to the enzyme two of the three remaining bands shift to

lower frequencies giving rise to the well resolved peaks at 415 and 402 cm^{-1}.

Thus, each "blue" copper site gives a different RR spectrum : one, that we shall call type Ia displays bands at 340, 380 and presumably at 415 cm^{-1}, the other (type Ib) at 360, 402 and 415 cm^{-1}. The disruption of one of the two Cu(II)-S bonds eliminates from the RR spectra the Cu-S stretching mode that is vibronically coupled with the charge transfer transition responsible of RR effect. Since three, or at least two, bands disappear other stretching modes are involved in vibronic coupling. This is not unexpected as metal-ligand stretching modes are usually coupled among themselves. Even though these modes are more or less mixed, one of them can be assigned mostly to a Cu(II)-S (cyst) stretching mode and the other two to a Cu(II)-N and/or Cu(II)-O stretching mode. It is tempting to assign the highest frequency bands at 415, 402 and 380 cm^{-1} to Cu(II)-N and/or Cu(II)-O stretching modes and the lower at 360 and 340 cm^{-1} to the Cu(II)-S vibration. These latter values compares with those of Fe-S(cyst) vibrations found in the RR spectra of adrenoxine (15) and rubredoxine (16-17) at 350 and 311, 360 cm^{-1} respectively.

The weak bonds at 270 cm^{-1} as well as the other ones at 750, 825, 1220 and 1235 might come from the normal Raman spectrum that superimposes to the RR effect.

It is worth noting that the RR spectrum of laccase (9), a "blue" copper protein containing four metal atoms per molecule, and one type I Cu(II) not modified by N_3^- and SCN^- binding (19), presents three bands at somewhat higher frequencies than those of type Ib Cu(II) in the spectrum of thiocyanate-ceruloplasmin. The RR spectrum of stellacyanin, another "blue" copper protein containing one type I cupric ion per molecule, shows two bands at somewhat lower frequencies than those of type Ib Cu(II) in the RR spectrum of azide-ceruloplasmin.

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