Coordination Sphere of Blue Copper Proteins

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By unambiguous assignments of maxima in the spectra of copper proteins, the constituent ions in the complexes have been established and as a consequence it was possible to distinguish the mode of their catalytic function. A bonding scheme is also proposed to account for the intensity of the blue colour.

The unambiguity of spectroscopic assignments resulted from theoretical calculations based upon Slater's principle of atomic shielding constants supplemented by coefficients and a formula for molecules by Medved.

The life-sustaining properties and antibacterial activity of copper protein complexes, render their investigation important both for medical or pharmaceutical as well as food production purposes. The establishment of their exact function was not presented in the literature thus far. There are but hypotheses about the valences of copper ions which are contained in the various blue and nonblue protein complexes, hypotheses concerning their structure and coordination sphere, whether dimerized or nondimerized.

Their blue colour was found to be of a greater intensity than of other compounds, but no interpretation was given [9].

There is however a calculative approach, which can provide unambiguous answers to problems aforementioned. In this work a solution is presented of the above stated problems, by a spectroscopic analysis of the spectrum of bovine ceruloplasmin combined with theoretical calculations of the spectroscopic maxima.

THEORETICAL

The principle of calculating the maxima of absorption is based upon Slater's theory [1] of atomic shielding constants of nuclear charge. However, the application of such theory upon molecules, necessitated a supplementation of the atomic coefficients with the coefficients for molecules, *i.e.* including electrons which are forming the covalent bonds (shielding constant 0.8) and electrons contained in hybridized orbitals (shielding constant 0.8) [2]. It was also necessary to set up a modified equation for calculating the wavenumber $1/\lambda$, by using quantum numbers without Slater's correction, *i.e.*

$$\frac{1}{\lambda} = R\ (Z - s)''^2\ \left\{\frac{1}{n'^2} - \frac{1}{n''^2}\right\} \qquad (1)$$

The shielded nuclear charge ($Z \mp s$) is the variable parameter which can satisfactory link the frequency rule of Bohr with the wave mechanical theory. The shielding constants s, calculated for the inner electrons, can indicate the structure of the compound, the donor atoms, the coordination number, combined with the energy level involved in the electron transfer, as will be evidenced by the instructive example of Cu^{2+}

EXPERIMENTAL

Bovine ceruloplasmin was prepared in the food-processing industrial plant PODRAVKA, Koprivnica, from fresh bovine serum, by consecutive elution procedures, at pH = 7.4, at a temperature of 4°C, by the application of different chromatographic agents SEPHAROSES from Pharmacia Fine Chemicals, Uppsala.

The intensely blue coloured solution of ceruloplasmin ($\rho = 3.43$ mg cm^{-3}) was investigated by the electron spectroscopic technique. The spectrograms in the visible and ultraviolet regions of the spectrum have been registered by means of a Zeiss spectrophotometer VSU-1, whereby a manual technique of measurement, which has the advantage of greater precision, was employed. The solutions were maintained at pH = 7.4 by the use of a K_2HPO_4 buffer.

Figs. 1—3 are showing several minima of transmission *viz.* maxima of absorption, though two of them are by no means completely developed, but represent a feature resulting from a partial change in the coordination sphere or else in the bridging mode.

All maxima have been calculated theoretically until the values were tallying with the measured wavelength. From such calculations arises the reliability of assignments in this work. When the calculations are

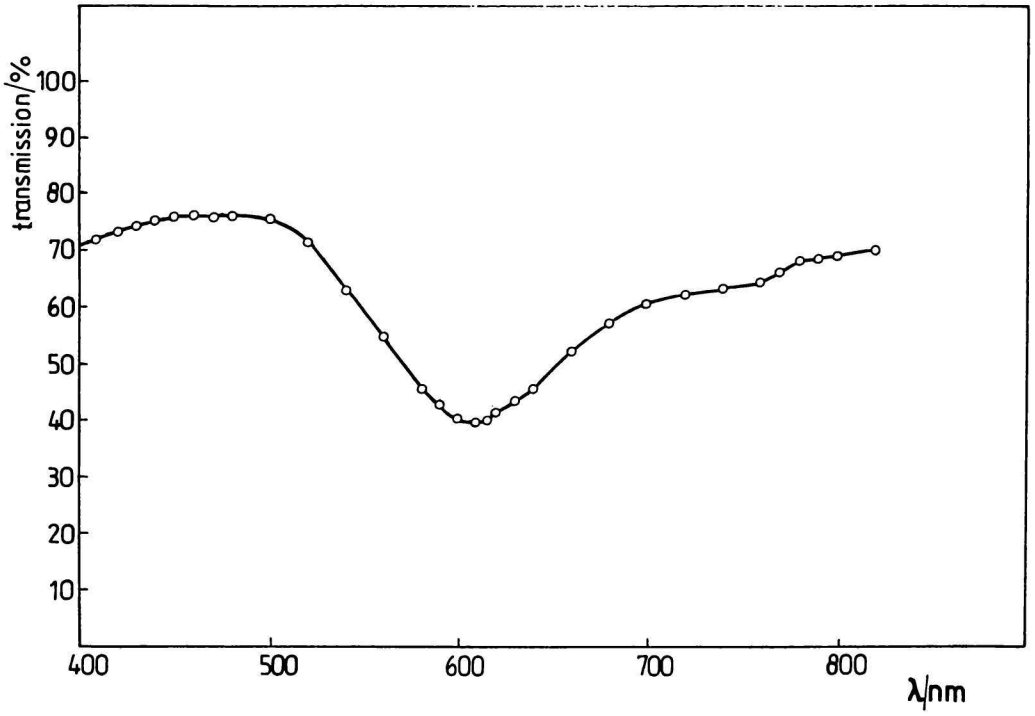


Fig. 1. Spectrum of ceruloplasmin in the visible region, fresh concentrated solution.

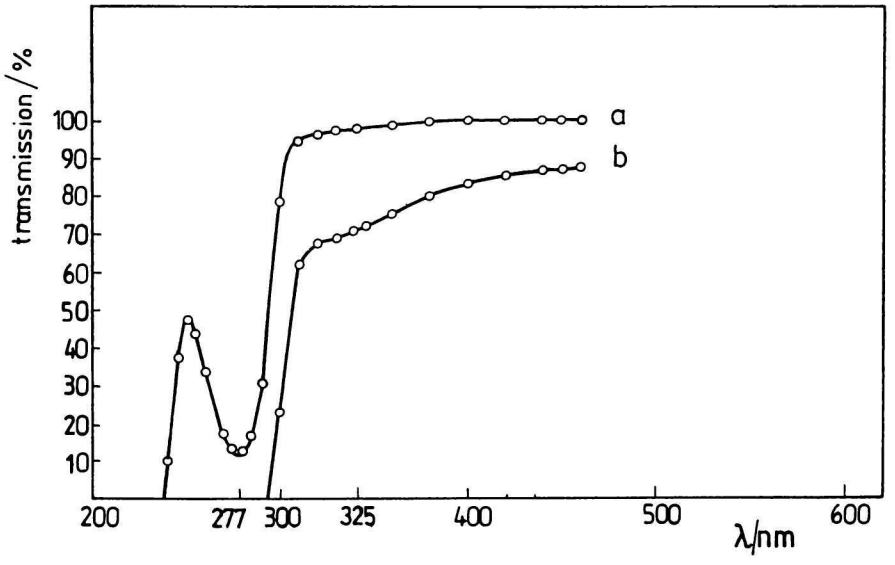


Fig. 2. Spectrum of fresh ceruloplasmin in the ultraviolet region. *a*) $\varphi_r = 2{:}10$ dilution; *b*) $\rho = 3.43$ mg cm^{-3}.

carried out for simple molecules, the measured values are accordant within a nanometer, whereas for a complex system such as blue copper proteins, the values might differ for several nanometers, due to variations in the chain structure of the ligand. Experiments described in the literature provide evidence that the C-substitution with alkyl groups might cause reading shift in complex compounds [3].

CALCULATIONS

The electronic configuration of Cu(O) as a RYDBERG atom is corresponding to an element of the first main group

Cu(O)	↑↓		↑↓	↑↓	↑↓		↑↓	↑↓	↑↓	↑↓	↑↓		↓							
	3*s*		3*p*				3*d*						4*s*		4*p*					

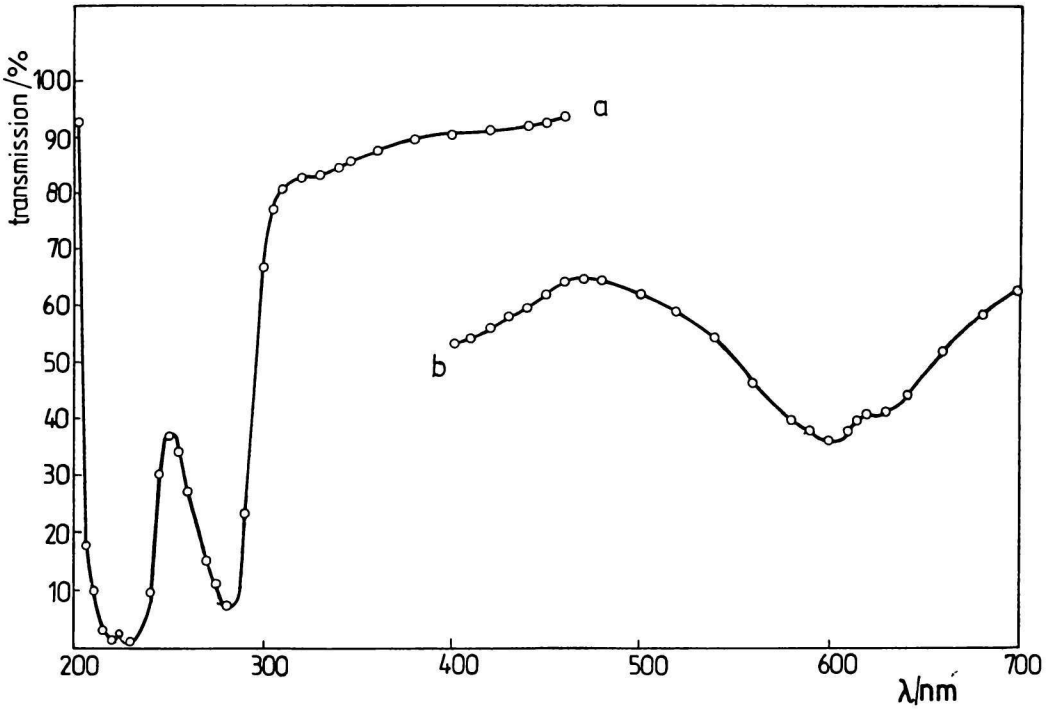


Fig. 3. Spectrum of ceruloplasmin registered after 14 days of storage at 4 °C. *a*) $\varphi_r = 2:10$; *b*) concentrated eluted solution.

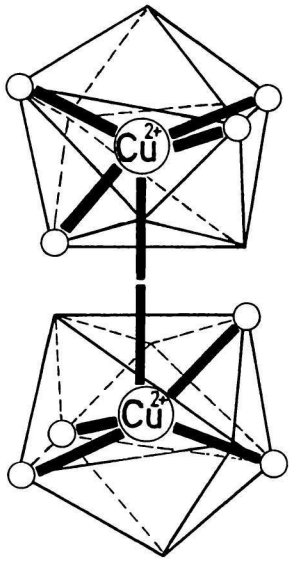


Fig. 4. Dodecahedral, dimeric structure of the blue copper protein complex.

and the divalent copper ion has a d^9 configuration

$$\underset{}{Cu^{2+}} \quad \underset{3s}{\uparrow\downarrow} \quad \underset{3p}{\uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow} \quad \underset{3d}{\uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow\ \downarrow} \quad \underset{4s}{\_} \quad \underset{4p}{\_\ \_\ \_}$$

The most accordant value with a maximum in the region complementary to the blue colour was obtained by coordination of four ligands in a dodecahedral geometry (Fig. 4).

$$Cu(L)_4^{2+} \quad \uparrow\downarrow \quad \uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow \quad \underbrace{\_ \quad \uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow}_{d^4sp^3} \quad \downarrow \quad \downarrow\ \downarrow\ \downarrow$$

The wavelength of the maximum does comply with an electron transition from sulfur upon copper(II)

S $(3p) \rightarrow Cu^{2+}$ $(4d)$ Selection rule $\Delta l = \pm 1$

$$(Z - s) = 29 - 2 - 8 - 9 \times 0.85 - 12 \times 0.8 = 1.75$$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times (1.75)^2 \times \left\{\frac{1}{3^2} - \frac{1}{4^2}\right\}$$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times 3.06 \times 0.0485 = 0.163 \times 10^7\ \mathrm{m}^{-1}$$

$\lambda = 614$ nm (measured 610 nm)

The calculation reveals that the electron is promoted from the third into the fourth energy level, indicating thereby the feasibility of a charge transfer from sulfur upon copper, whereas the transition is not possible from nitrogen upon Cu(II). However, the intensity of the colour, as well as a slightly doubled form of the maximum, are indicating that another transition does occur also at the same wavelength or within a nonresolvable difference of the wavelength.

The calculations indicate that a coinciding maximum is formed by a charge transfer between two copper ions, in a dimerized molecule containing 2 Cu(II). In such a dimerized molecule the subsequent electron transition does cause a blue colour. The electron is

promoted from the 3rd energy level of the Cu(II) into the 4th energy level of the other Cu(II) ion

$3d' \rightarrow 4d''$ Selection rule $\Delta l = \pm 0, \pm 1$

5s

$3\sigma^*$ $4d''$

$\downarrow\uparrow\downarrow\uparrow\downarrow\uparrow\downarrow\downarrow\downarrow\downarrow$ $\underline{\downarrow\downarrow\downarrow\downarrow\uparrow\downarrow\uparrow\downarrow\uparrow\downarrow\downarrow}$

d^4sp^3

$\uparrow\downarrow$

3σ

$(Z - s) = 29 - 2 - 8 - 9 \times 0.85 - 12 \times 0.8 = 1.75$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times (1.75)^2 \times \left\{\frac{1}{3^2} - \frac{1}{4^2}\right\}$$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times 3.06 \times 0.0485 = 0.163 \times 10^7\ \mathrm{m}^{-1}$$

$\lambda = 614$ nm (measured 610 nm)

The promoted electron absorbs a second photon in the visible region and rises to the 5th energy level

$4d'' \rightarrow 5s'$

$(Z - s) = 29 - 2 - 8 - 9 \times 0.85 - 7 \times 0.8 - 4 \times 0.8 = 2.55$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times (2.55)^2 \times \left\{\frac{1}{4^2} - \frac{1}{5^2}\right\}$$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times 6.50 \times 0.0225 = 0.161 \times 10^7\ \mathrm{m}^{-1}$$

$\lambda = 620$ nm

Accordingly the explanation of the intensity of the blue colour of copper proteins is in a sequence of transitions caused by photons of similar energy.

The spectrograms (Figs. 2 and 3) show impressive maxima in the ultraviolet region. The assignment of the maximum at 280 nm resulted from the calculation based upon a dodecahedral coordination sphere of Cu(III)

Cu(III) $\underline{\uparrow\downarrow}$ $\underline{\uparrow\downarrow\uparrow\downarrow\uparrow\downarrow}$ $\underline{\uparrow\downarrow\uparrow\downarrow\uparrow\downarrow\uparrow\downarrow}$ $\underline{\downarrow\quad\downarrow\downarrow\downarrow}$

d^4sp^3

The number of electrons differs in comparison with the blue protein Cu(II), but the energy levels involved in the electron transfer are identical

S $(3p) \rightarrow$ Cu(III) $4d$

$(Z - s) = 29 - 2 - 8 - 8 \times 0.85 - 12 \times 0.8 = 2.6$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times (2.6)^2 \times \left\{\frac{1}{3^2} - \frac{1}{4^2}\right\}$$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times 6.76 \times 0.485 = 0.360 \times 10^7\ \mathrm{m}^{-1}$$

$\lambda = 278$ nm

Such a transition is possible between the sulfur as a ligand and a copper(III) ion as a central ion. It is a nondimerized complex molecule, resulting from a reaction of ceruloplasmin as an enzyme oxidase.

The small maximum at 325 nm, displayed as a depression upon the transmission *vs.* wavelength plot, can be calculated by the application of similar principles.

Cu(III) $\underline{\uparrow\downarrow}$ $\underline{\uparrow\downarrow\uparrow\downarrow\uparrow\downarrow}$ $\underline{\downarrow\downarrow\uparrow\downarrow\uparrow\downarrow\uparrow\downarrow}$ $\underline{\downarrow\quad\downarrow\downarrow\downarrow}$

d^2sp^3

$3d \rightarrow 4p''$

$(Z - s) = 29 - 2 - 8 - 12 \times 0.85 - 8 \times 0.8 = 2.4$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times (2.4)^2 \times \left\{\frac{1}{3^2} - \frac{1}{4^2}\right\}$$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times 5.66 \times 0.0485 = 0.307 \times 10^7\ \mathrm{m}^{-1}$$

$\lambda = 325$ nm (measured 325 nm)

Such a small maximum is a result of differently hybridized bonds in the copper protein complex. The spectrogram in Fig. 2 indicates the relative magnitudes of such maxima paralleling the participation of the corresponding geometries in the complex.

Similar binary hybridization forms have been spectroscopically established in the visible region, *i.e.* also the maxima at 610 nm and 780 nm

Cu(II) $\underline{\uparrow\downarrow}$ $\underline{\uparrow\downarrow\uparrow\downarrow\uparrow\downarrow}$ $\underline{\downarrow\uparrow\downarrow\uparrow\downarrow\uparrow\downarrow}$ $\underline{\downarrow\quad\downarrow\downarrow\downarrow}$

d^2sp^3

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times 2.40 \times 0.0485 = 0.128 \times 10^7\ \mathrm{m}^{-1}$$

$\lambda = 781$ nm

At the calculated wavelength there can be reliably observed a small depression upon the spectrogram in Fig. 1, but such a geometry appears in the complex in a negligible proportion.

The maxima in the short ultraviolet region of the spectrum must not be omitted from the literature reports, as they belong to products of the catalytical reaction. In this work they are shown in Fig. 3. The assignments were effected by calculated values accordant with experimental wavelengths. There was ob-

served a small double minimum at 220 nm, confirmed by calculations.

Cu(III) $\underset{3s}{\uparrow\downarrow}$ $\underset{3p}{\uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow}$ $\underset{3d}{\downarrow\ \uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow}$ $\underset{4s}{\downarrow}$ $\underset{4p}{\downarrow\ \downarrow\ -}$

$(Z - s) = 29 - 2 - 8 - 16 \times 0.85 - 3 \times 0.8 = 3.0$

$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times 2.90 \times 0.0485 = 0.449 \times 10^7\ \mathrm{m}^{-1}$

$\lambda = 208$ nm

Cu(I) $\underset{}{\uparrow\downarrow}$ $\underset{}{\uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow}$ $\underset{}{\uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow\ \uparrow\downarrow}$ $\underset{}{-}$ $\underset{}{-\ -\ -}$

$(Z - s) = 29 - 2 - 8 - 18 \times 0.85 - 0.8 = 2.9$

$\frac{1}{\lambda} = 1.097 \times 10^7\ \mathrm{m}^{-1} \times (2.9)^2 \times 0.0485 = 0.449 \times 10^7\ \mathrm{m}^{-1}$

$\lambda = 222$ nm

Particularly notable is the nonhybridized form of the complex causing the absorption at $\lambda = 208$ nm. A ready interpretation was the occurrence of such a complex as a residue after the cleavage of the peroxide.

A monovalent copper is causing the maximum at 222 nm. The covalent bond indicated by the electron in the 4*s* orbital, might result either from dimerization or from a heterocovalent bond.

RESULTS AND DISCUSSION

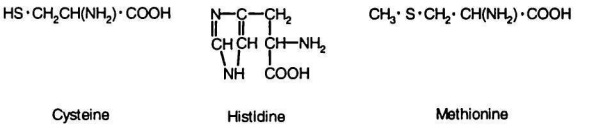
A rich harvest of conclusions has resulted from calculations presented in this work. It is discernible that a special structure was found to satisfy the condition of "best fit" with experiment, *i.e.* a dodecahedral structure. In the published articles on copper proteins [4, 5], a flattened tetrahedron or near tetrahedron appeared to be the characteristic geometry of such compounds, but the peculiarity of such a structure was not explained. However, the dodecahedral structure established by calculations, with four sites occupied by ligands and the other four by electron pairs, can account for the seemingly tetrahedral but slightly distorted structure arising within the dodecahedron (Fig. 4).

The spatial arrangement of the ligands in copper protein complexes profoundly influences their chemical and biological properties and therefore of importance is the establishment of a dodecahedral instead of a tetrahedral structure. Similarly as the porphyrin environment corresponds to an octahedral coordination sphere of iron, the copper configuration requires a dodecahedral coordination sphere. In addition, a tendency of dimerization was established resulting from symmetry requirements of the electronic configuration of Cu^{2+} This, together with the d^4sp^3 directionality of the bonds, is a prerequisite of oxygen binding.

The bridging mode of oxygen bonding was found consistent with the mode of operation. A similar structure is cited in the literature as characteristic of other enzymes, too, *e.g.* hemocyanin, as described in Lever's *Inorganic Electronic Spectroscopy* [3].

Ligands

As ligands there are mentioned in the literature S and N atoms from amino acid residues cysteine and histidine and tentatively methionine [5]

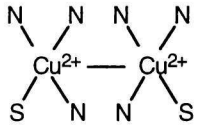


(*A*)

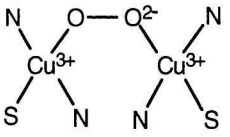
The sulfur atom, having valence electrons in the third energy level, can participate in the charge-transfer transitions, whereas for nitrogen and oxygen, having valence electrons in the second energy level, the charge transfer into the 4th energy level of copper is not possible.

Literature data reveal that the coordinative bond with copper forms 2 histidine residues and a cysteine residue, whereas the 4th ligand is still a subject of active investigation [6]. Ceruloplasmin as an enzyme oxidase is a complex protein molecule having a relative molecular mass of 132.000, comprising a polypeptide chain of 106 amino acid residues, of which the sequence is but half resolved [7].

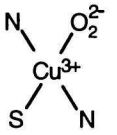
In this work the ligands are designated by donor atoms, regardless of the corresponding amino acid residues, because the calculative approach enables the establishment of the copper complex conformation without a detailed investigation of the polypeptide chain. The maxima of absorption correspond to five coordination geometries, containing copper ions of variable valences.



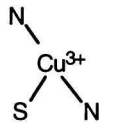
$\lambda = 614$ nm (*B*)



$\lambda = 277$ nm (*C*)



$\lambda = 277$ nm (*D*)



$\lambda = 208$ nm (*E*)

Cu^+ $\lambda = 222$ nm (*F*)

Valences of Copper Ions

The carrier of the blue colour is the divalent copper ion in the dimerized complex, with a d^4sp^3 hybridized coordination sphere, absorbing at $\lambda = 614$ nm. Such a structure confers on copper the ability of multiple absorption at different sites of the complex, of photons not differing or differing little in the wavelength.

For the formation of the copper(III), a high potential is required $E^\circ(Cu^{3+}/Cu^{2+}) > +1.8$ V, but in alkaline solutions or solutions of strong complexes, respectively [8] the redox potential is lowered, *e.g.* $E(CuO_2^-/Cu(OH)_2) > +0.8$ V, a value which is surpassed by the redox potential of peroxide by which the oxidation can be effected.

The copper(I) ion does not form complexes with organic ligands involved in blue copper proteins, and accordingly the Cu^{3+}/Cu^+ couple might be paralleling the acceptance of electrons by O_2 in the end phase of any oxidation cycle.

Table 1. Table of Potentials [8, 9]

Redox System	Potentials
$E^\circ(Cu^{2+}/Cu^\circ)$	+ 0.337 V
$E^\circ(Cu^{2+}/Cu^+)$	+ 0.153 V
$E^\circ(Cu^+/Cu^\circ)$	+ 0.521 V
$E^\circ(Cu^{3+}/Cu^{2+})$	> + 1.8 V
$E^\circ(Cu^{3+}/Cu^+)$	> + 1.9 V
$E^\circ(CuO_2^-/Cu(OH)_2)$	> + 0.8 V
$E^\circ((O_2 + 2H^+ + 2e^-)/H_2O_2)$	+ 0.68 V
$E^\circ(H_2O_2/OH^-)$	+ 0.87 V
$E^\circ(O_2/(H_2O_2 + OH^-))$	+ 0.076 V

Catalytic Activity

Ceruloplasmin is in the literature classified generally as an enzyme oxidase, *i.e.* an enzyme activating oxygen. In biochemistry [10], such compounds are classified into three groups.

1. Oxidase enzyme transferring electrons

$$O_2 + 2e^- \rightarrow O_2^{2-} + 2H^+ \rightarrow H_2O_2$$

$$O_2 + 4e^- \rightarrow 2O^{2-} + 4H^+ \rightarrow 2H_2O$$

At the terminal site of an electron transport chain, the electrons are transferred upon oxygen, which is subsequently binding protons and forming water.

2. Oxygenase enzymes, transferring O_2

$$A + O_2 \rightarrow AO_2$$
$$\downarrow$$
substrate

An oxygen molecule is bonded directly with a substrate, by the mediation of the enzyme.

3. Hydroxylase enzyme, transferring O

$$AH + DH_2 + O_2 \rightarrow AOH + D + H_2O$$
$$\downarrow$$
donor of hydrogen

How does such scheme comply with the ions established by the spectroscopic analysis in this work? Obviously the dimeric copper(II) complex is capable of bonding O_2 in a bridgelike structure. The change of valence of copper(II) is a result of the attachment of O_2 paralleled by a conformation change (a disrupting of the dimerization bond). Thereupon electrons are transferred from copper upon oxygen by a spontaneous inner redox process. Such a process might be termed catalytic, because the electron transfer is facilitated by the copper(II) configuration and the spatial d^4sp^3 hybridized conformation.

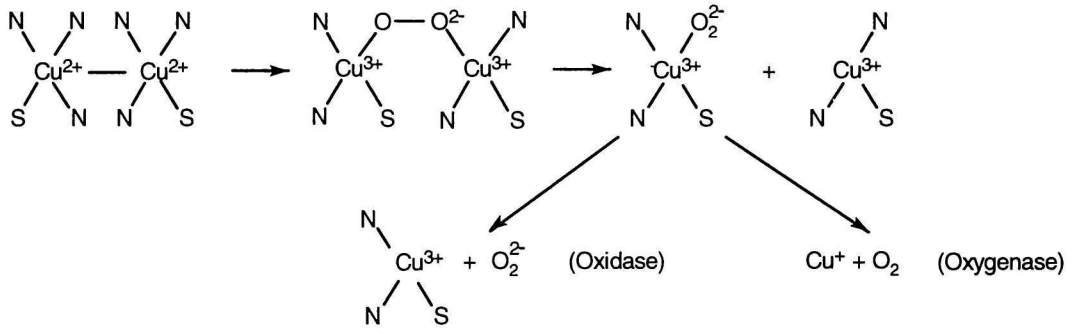
The peroxide ion might be set free from this complex in two ways, either by disjoining any of the two bonds with copper or both. Of these two possibilities, the former route has a secondary probability.

In the second stage the peroxide ion can be cleaved off, or an oxygen molecule can be released.

The resulting copper ions are a copper(III) ion bonded in a nonhybridized complex and a copper(I) ion which has been released from the complex in an oxygenated form. The presence of such an ion in blue copper proteins is mentioned in the literature [4], identified as an "EPR nondetectable" form. Its content was found to be approximately 50 % of the whole amount of the copper. By interaction with the substrate, oxygen is transferred and the copper(I) ion can recombine with the coper(III) complex regenerating the blue dimer.

Alternatively, an interaction of the peroxide with the substrate might involve a dehydrogenation, producing hydroxyl ions. It seems likely that the resulting alkalinity can enhance the precipitation of CuOH and its excretion as Cu_2O.

Perhaps the biochemists in the future shall be able



(G)

to quantify the parts of the suggested reactions in copper metabolism [11].

$$O^{2-} \xrightarrow{2H} 2\,OH^-$$

It is also instructive to elucidate the cause of Wilson's disease, *i.e.* the precipitation of elemental copper in the body of men or animals. From the complex proteins mentioned in the preceding paragraphs, copper might precipitate if substituted by another metal. The protein complex of copper(III) has a high redox potential, and the ability to oxidize even a noble metal such as mercury. The hypothesis that mercury substitution causes Wilson's disease can be based upon the volatility of Hg and the occasional possibility of the vapour to be included and transported in the blood stream, where the bond of Hg with a thiol—SH group is of a comparable stability with copper.

Among the maxima established in the spectrum of ceruloplasmin, the maximum at 450 nm mentioned in the literature [3], can be assigned to elemental copper

$$\underset{\quad 3s \quad 3p \quad\quad 3d \quad\quad 4s \quad 4p}{\text{Cu(O)}\ \underline{\uparrow\downarrow}\ \ \underline{\uparrow\downarrow}\ \underline{\uparrow\downarrow}\ \underline{\uparrow\downarrow}\ \ \underline{\uparrow\downarrow}\ \underline{\uparrow\downarrow}\ \underline{\uparrow\downarrow}\ \underline{\uparrow\downarrow}\ \underline{\uparrow\downarrow}\ \ \underline{\uparrow\downarrow}\ \ \_\ \_\ \_}$$

$$(Z - s) = 29 - 2 - 8 - 19 \times 0.85 - 0.8 = 2.05$$

$$\frac{1}{\lambda} = 1.097 \times 10^7\ \text{m}^{-1} \times (2.05)^2 \times 0.0485 = 0.224 \times 10^7\ \text{m}^{-1}$$

$$\lambda = 447\ \text{nm}$$

Accordingly the spectroscopic analysis can be used for diagnostic purposes, prior to symptoms, and even for food control purposes.

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