Biochemical Pharmacology, Vol. 60, pp. 1735–1741, 2000.



ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00399-3

COMMENTARY

# The Physiopathological Significance of Ceruloplasmin

## A POSSIBLE THERAPEUTIC APPROACH

*Giovanni Floris,* Rosaria Medda,* Alessandra Padiglia* and Giovanni Musci†‡*

*Department of Sciences Applied to Biosystems, University of Cagliari, Cagliari, Italy; and
†Department of Organic and Biological Chemistry, University of Messina, Messina, Italy

**ABSTRACT.** This article reviews and comments on the physiological roles of ceruloplasmin (Cp). We show that, in addition to its ascertained involvement in iron homeostasis, the protein, by virtue of its unique structure among multicopper oxidases, is likely involved in other processes of both an enzymatic and a nonenzymatic nature. In particular, based on the analysis of the kinetic parameters, on the one hand, and of the side-products of the oxidation, on the other, we propose that the long-recognized ability of Cp to interact with and oxidize non-iron substrates may be of physiological relevance. The striking example of 6-hydroxydopamine oxidation is presented, where we show that the catalytic action is carried out readily under physiological conditions, without release of potentially toxic oxygen intermediates. BIOCHEM PHARMACOL **60**;12:1735–1741, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** ceruloplasmin; iron; biogenic amines; neurodiseases

The interest in Cp,† a vertebrate plasma protein first isolated over half a century ago [1], has risen sharply in the last decade, due to novel structural and functional findings. Cp belongs to the family of multicopper oxidases, which also comprises plant and fungal laccases, plant ascorbate oxidase, and yeast Fet3 [2, 3]. These enzymes are characterized structurally by the presence of different copper sites, generally classified into three types on the basis of their spectroscopic features [4]: type 1 copper lies in a distorted tetrahedral site, with two histidines and a cysteine as conserved ligands. The redox potential of the site depends on the distance of the fourth axial ligand—usually a methionine [5]—and/or on its solvent accessibility and the orientation of protein dipoles around the copper site [6]. Type 1 copper is also called the "blue" copper, since it confers on the protein the typical blue color, by virtue of a strong ($\epsilon \approx 5000\ M^{-1}\ cm^{-1}$) electronic absorption at around 600 nm due to charge transfer between the cysteine sulfur and the metal. The paramagnetism of type 1 copper gives rise to an unusual EPR spectrum, characterized by the small value of the copper hyperfine coupling constant in the parallel region ($A_{//} \leq 95 \times 10^{-4}\ cm^{-1}$). Type 2 copper is tetragonally coordinated by four imidazolic nitrogens, has no appreciable absorption in the optical spectrum, and has an EPR lineshape with "normal" magnetic parameters ($A_{//} \geq 140 \times 10^{-4}\ cm^{-1}$). It lies in close proximity to type 3 copper, which is constituted by a pair of antiferromagnetically coupled copper ions, spectroscopically characterized by an electronic absorption at 330 nm and a complete EPR silence. Type 2 and type 3 copper constitute, in fact, a unique structure, called the trinuclear cluster [7–9]. From a functional point of view, the different copper sites are designed to drive electrons from a reducing substrate to oxygen, the final acceptor, in a controlled way, i.e. without release of potentially toxic intermediates ($O_2^-$, $H_2O_2$). This task is accomplished through acceptance of electrons, one at a time, at the type 1 copper site(s), and subsequent intramolecular electron transfer to the trinuclear cluster, which then provides for reduction of oxygen and release of water. As far as stoichiometry is concerned, it is clear from the picture above that four copper ions—a blue site and a trinuclear cluster—would constitute the minimal functional unit of multicopper oxidases. Indeed, laccases, ascorbate oxidase, and Fet3 all show a four-copper stoichiometry (ascorbate has 8 copper ions, but in a dimeric structure). On the other hand, Cp is unique in that its total copper stoichiometry (5–6 atoms/molecule [10]) requires at least one of the copper types to be overrepresented in the molecule. Measurements of the extinction coefficient at around 600 nm, of the EPR spectrum, and of the redox potentials have led in the past to the conclusion that two nonequivalent type 1 sites are present [11–13], although this would be consistent only with a total stoichiometry of 5 copper ions (two type 1 plus a trinuclear cluster). A few years ago, however, spectroscopic evidence was presented indicating that human Cp contains three blue ions, which are in part reduced in the resting enzyme [14]. Subsequent resolution of the crystallographic structure has confirmed that the human protein contains three type 1 copper sites, symmetrically arranged in three domains arising from a

* Corresponding author: Dr. Giovanni Musci, Department of Organic and Biological Chemistry, University of Messina, Salita Sperone 31, 98166 S. Agata Messina, Italy. Tel. (39) 090-6765170; FAX (39) 090-393895; E-mail: musci@isengard.unime.it

† *Abbreviations*: Cp, ceruloplasmin; and LDL, low-density lipoprotein(s).

triplication of the ancestral gene of mononuclear blue proteins [15]. It remains to be established whether those ceruloplasmins that apparently show a lower total stoichiometry (i.e. chicken and turtle Cp [10]) are so because of the presence of only two type 1 copper sites.

The reason for the blue copper redundancy in Cp is unclear. The hypothesis that one of the blue sites is, at least in the human enzyme, permanently reduced and thus functionally irrelevant [16] lends support to the idea that the multiplicity of type 1 ions reflects an evolutionary vestige, with no important implications in the oxidase mechanism of Cp. Apparently consistent with this view is the recent report that a fraction of the blue ions can be removed reversibly from Cp without completely sacrificing the enzymatic ability of the protein [17]. However, the authors also found that any of the three blue ions can be lost, thus implying that they are all equivalent in terms of their contribution to the catalytic cycle. An alternative explanation was offered, i.e. that Cp is a multifunctional protein, possibly able to carry out, at the same time, the enzymatic role as an oxidase and the copper transport role (see below).

As Frieden has pointed out repeatedly [18–20], multifunctionality is the key concept for the comprehension of the biological function(s) of Cp. In the following, we will review and comment on the roles so far proposed for Cp. After a brief excursus on regulatory functions, we will focus on the enzymatic roles of the protein, and an explanation will be offered to link the structure of Cp to its multifaceted capabilities.

## REGULATORY ROLES

Over 95% of plasma copper is bound to Cp, and this fact generally has been taken as evidence for a role of Cp in copper transport. Experimental evidence supporting this view includes the observation that the copper atoms of Cp are a prerequisite for copper utilization in the biosynthesis of cytochrome *c* oxidase [21, 22], and that Cp can transfer copper to metal-free superoxide dismutase in cultured aortae [23] and in the erythroleukaemic cell line K562 [24, 25]. Harris and his group, in particular, disclosed several features of the mechanism of copper transfer from Cp to cells. Using K562 as a cellular model, they were able to demonstrate that copper, but not the protein moiety of Cp, was taken up by the cell [26], and that ascorbate enhanced copper transport, with possible relevance to vitamin C deficiency [27]. Their mechanism is fully consistent with the widespread notion that specific receptors for Cp are present on the membranes of numerous cell types, including aortic and heart tissues [28], liver endothelium [29, 30], erythrocytes [31], white blood cells [32], Kupffer cells [33], and human placental vesicles [34].

Patients with the inherited disorder known as Wilson's disease have moderate to undetectable levels of circulating Cp [35]. They show copper accumulation in the liver and brain, and reduced activity of cytochrome *c* oxidase [36], both observations being consistent with a role of Cp in copper transport. Moreover, the pathological symptoms can be relieved by treatment with copper-chelating agents such as penicillamine and trientine [37, 38], which can act as Cp substitutes in copper mobilization.

It should be noted, however, that Cp is not likely to be the sole carrier of copper to tissues [39, 40]. As a matter of fact, aceruloplasminemic patients who completely lack a functional Cp gene (see below) do not have appreciably impaired copper metabolism [41], suggesting that, in the absence of Cp, other systems take over in copper transport to tissues.

The ability of Cp to donate copper to cells should not be viewed only as a transport function, but may be a true regulatory role for the multicopper protein. Recently, it has been shown that ceruloplasmin is able to modulate the function of endothelial nitric oxide synthase, both in cultured aortae [42] and in cultured endothelial cells [43]. The authors demonstrated that the interaction of Cp with a specific receptor prompted the selective donation of copper to the endothelial cell and, in turn, to inhibition by the metal of the enzymatic production of nitric oxide (NO) by endothelial nitric oxide synthase. Since this enzyme is involved in the maintenance of vessel tone [44], the role of Cp would be that of controlling the NO-dependent relaxation of the vasculature.

A different role for Cp as a growth factor has been outlined by Crane's group, who showed that thymidine incorporation is enhanced in fibroblasts exposed to low amounts of exogenous Cp [45]. They suggested that Cp might act as a terminal oxidase for ferrous ion to stimulate transmembrane electron transport, which, in turn, would increase cell growth and thymidine incorporation. Indeed, they were able to show that NADH oxidation by plasma membranes is stimulated by Cp [46], and that Fe(II) chelators abolish the effects of Cp [47]. It should be noted that, although the role of Cp as a growth factor can be considered a regulatory function of the protein, it is mediated by the enzymatic ability of Cp to convert Fe(II) to Fe(III) (see below).

A further activity of Cp worth mentioning is at the border between a regulatory and an enzymatic function. Due to its ability to react with and scavenge oxygen species such as superoxide and hydrogen peroxide, Cp always has been considered as a type of plasma antioxidant [48, 49]. Recently, however, it has been reported that highly purified, undegraded human Cp enhances rather than suppresses copper ion-mediated oxidation of LDL and that the activity depends on the presence of a single, chelatable copper atom [50]. In contrast, proteolytically degraded Cp inhibited cupric ion oxidation of LDL. These results suggested that Cp may be in part responsible for oxidation of LDL in blood or in the arterial wall and thus may have a physiological role that is quite distinct from what is commonly believed. By using site-directed mutagenesis, the prooxidant site was localized precisely [51]. Quite interestingly, the prooxidant action of Cp was shown to be

superoxide-dependent, in that superoxide reduction of Cp copper is a critical reaction in cellular LDL oxidation [52].

## ENZYMATIC ROLES

Divalent iron, Fe(II), is oxidized by Cp efficiently, both in terms of $K_m$ and $V_{max}$ [19], and for this reason the ferroxidase activity historically has been considered the principal role of the protein *in vivo* [53–56]. Investigations carried out in the last few years (reviewed in Ref. 57) have lent support to the idea that ferroxidation by Cp is a physiologically crucial event. Individuals with mutations in the Cp gene leading to the expression of a truncated protein suffer from the rare genetic disorder aceruloplasminemia, characterized by iron deposition in several tissues, including the brain, liver, and pancreas, with pathological consequences ranging from dementia to diabetes mellitus [41, 58, 59]. Nevertheless, in contrast to expectations, Cp recently has been proposed to enhance iron uptake by iron-deficient HepG2 cells [60]. Interestingly, the authors show that Cp must be enzymatically active to perform this task, thus establishing the point that Cp controls iron traffic through its catalytic activity. Although difficult to reconcile with the reported pathological consequences of aceruloplasminemia, this finding would be in line with the role of Fet3, the most recently discovered among multicopper oxidases, which is part of the Fet3-Ftr1 multiprotein complex involved in high-affinity iron uptake by yeasts [61–63], and, in fact, Attieh *et al.* subsequently have proposed that the mechanism of Cp-assisted iron uptake requires a trivalent cation-specific transporter [64], functionally equivalent to yeast Ftr1. Two reports that appeared in 1999, however, have suggested that Cp may control iron efflux, rather than iron uptake. According to Richardson [65], iron uptake by HepG2 cells indeed takes place only when physiologically irrelevant conditions (in terms of iron donor, temperature, and Cp concentration) are used, and that instead, under properly controlled conditions, Cp induces iron release. Gitlin and coworkers [66], on the other hand, have shown that transgenic mice with a mutated Cp gene, taken as a model for aceruloplasminemia, have normal kinetics for iron uptake, yet accumulate the metal in tissue parenchyma. Whatever the case, the reported data constitute convincing evidence that Cp is involved in iron homeostasis, possibly through its ferroxidase activity, which therefore should be considered as one of the main functions of the protein.

It is worth noting that Cp is mainly a circulating protein, whereas Fet3 is tightly embedded in the yeast membrane. This observation makes it difficult to understand how the two proteins can play similar roles, i.e. regulate the iron traffic across the membrane. However, the recent findings of membrane-associated forms of Cp in mammalian astrocytes [67] and Sertoli cells [68] are consistent with the involvement of the protein in membrane processes, and can explain the observed presence of Cp receptors on a number of cell types.

An important point regarding the ferroxidase activity of Cp is that Fe(II) readily oxidizes, at physiological pH, even in the absence of a protein catalyst. This is why the enzymatic contribution of the blue protein is often assessed at acidic pH values in *in vitro* measurements [69], to disfavor iron autoxidation. However, the spontaneous conversion of Fe(II) to Fe(III) is a potentially dangerous event, in that it triggers the formation of superoxide anion and, in turn, of hydrogen peroxide and hydroxyl radicals through Fenton chemistry. The prooxidant action of Fe(II) is so strong that the aerobic mixture of iron and ascorbate, the latter being able to bring the metal back to the reduced state, is the standard inducer of oxidative stress in *in vitro* experiments [70, 71]. Thus, the ferroxidase action of Cp would be aimed, on one hand, at preventing iron-induced oxidative stress; on the other, since Fe(II) is normally absent as a free ion *in vivo*, as it is shielded from autoxidation by proper chelating systems [72], it can be postulated that Cp may be able to oxidize specifically coordinated derivatives of the metal. Within this schematic picture, the conclusion is that Cp would serve more as a modulator, or controller, of iron oxidation, rather than as an accelerator of the reaction.

The question now is: does Cp exclusively use Fe(II) as an electron source for reducing oxygen to water *in vivo*? At variance with all other multicopper oxidases, for which the canonical substrate is well established [polyphenols for laccases, ascorbate for ascorbic oxidase, Fe(II) for Fet3], Cp has in fact the peculiar ability to utilize, at least *in vitro*, a number of structurally unrelated molecules as electron donors, including, besides Fe(II), aromatic amines and catechols [19], and even nitric oxide [73]. However, although the oxidase activity of plasma is often expressed in terms of Cp-assisted oxidation of aromatic amines [74], oxidation of non-iron substrates has long been considered physiologically irrelevant, mostly because of poor kinetic parameters [19]. One problem is that the pH optimum of Cp is between 6 and 6.5 [75], and for this reason the enzymatic parameters of the protein have always been measured at acidic pH. *In vivo*, however, the pH is usually slightly alkaline, at least in the blood where most Cp can be found, and the assessment of a "true" substrate for Cp should be done at this pH. A few years ago, Musci and colleagues [76] made the novel observation that, in the presence of chloride, the oxidase activity of Cp is enhanced significantly at neutral pH. The study disclosed how misleading the choice of wrong experimental conditions can be, since chloride always had been considered an inhibitor of Cp, and it is indeed so when the activities are run at acidic pH. In a very recent follow-up [77], the phenomenon was better substantiated, with surprising conclusions: (i) the enhancement of the activity at neutral pH can be elicited also by stoichiometric concentrations of another anion, namely azide; (ii) the effect of both chloride and azide is kinetically complex, with both $k_{cat}$ and $K_m$ affected by anion binding, and up to 100-fold activation is observed when expressed in terms of the $k_{cat}/K_m$ ratio; and (iii) the effect of anions is specific for the oxidase activity toward

non-iron substrates, whereas oxidation of Fe(II) is unaffected. This latter point is of utmost importance, in our opinion. As a matter of fact, anions seem to optimize the oxidase activity of Cp versus non-iron electron donors, pushing the protein to utilize them as efficiently as it does with Fe(II). The main criticism of the physiological relevance of non-iron substrates, i.e. their poor kinetic parameters, thus vanishes. Of course, the existence of modulators of Cp activity different from anions should not be ruled out.

In the light of the comments above, our previous observation that Cp can accelerate the oxidation of the catecholamine 6-hydroxydopamine gains relevance [78]. The neurotoxins 2,4,5-trihydroxyphenylalanine (6-hydroxydopa) and 2,4,5-trihydroxyphenethylamine (6-hydroxydopamine) are intermediates in the formation of dopa- and of dopamine-melanins, respectively [79]. 6-Hydroxydopamine undergoes rapid autoxidation, reacting with molecular oxygen to yield a quinoidal product and several forms of reduced oxygen, including $H_2O_2$ and $O_2^{-\cdot}$ [80, 81]:

$$6\text{-}QH_2 + O_2 \rightarrow 6\text{-}QH^{\cdot} + O_2^{-\cdot} + H^+$$

$$6\text{-}QH_2 + O_2^{-\cdot} + H^+ \rightarrow 6\text{-}QH^{\cdot} + H_2O_2$$

$$6\text{-}QH^{\cdot} + O_2 \rightarrow 6\text{-}QH + O_2^{-\cdot} + H^+$$

$$6\text{-}QH^{\cdot} + O_2^{-\cdot} + H^+ \rightarrow 6\text{-}Q + H_2O_2$$

where 6-$QH_2$ is defined as 6-hydroxydopamine; 6-$QH^{\cdot}$ as the semiquinone of 6-hydroxydopamine; and 6-Q as the quinone of 6-hydroxydopamine. The reactions above closely resemble the Fenton chemistry of the reaction of Fe(II) with molecular oxygen. The relevant point is that no hydrogen peroxide is released when the oxidation of 6-hydroxydopamine is catalyzed by Cp [78], a finding consistent with the established oxidase mechanism of the multicopper oxidase. It has been postulated that the redox intermediates in oxygen reduction ($H_2O_2$ and $O_2^{-}$) and the products as well in the above reaction sequence of catechol oxidation (6-$QH^{\cdot}$ and 6-Q) might be the toxic products responsible for the degeneration of adrenergic nerve terminals [80]. A body of evidence is accumulating on the role of 6-hydroxydopamine in neuronal degeneration, possibly mediated by free radical toxicity [82]. As a matter of fact, 6-hydroxydopamine-induced lesions of the nigrostriatal system are commonly used as a model for Parkinson's disease, a disorder caused by the deterioration of dopaminergic neurons bridging the nigrostriatal system. Thus, the finding of the biochemical controller of 6-hydroxydopamine oxidation seems to be relevant. For the historical reasons reported above, our activity measurements were carried out in a non-physiological pH range (4 to 6.5). In a commentary that appeared in this journal in 1998 [83], our conclusions that Cp may have a biological role in catecholamine oxidation were criticized strongly on the basis that activity measurements had not been carried out at physiological pH due to the fast autoxidation of 6-hydroxydopamine at neutral pH. We now have performed new experiments, where the oxidation of 6-hydroxydopamine catalyzed by human Cp was measured at pH 7. The choice of the proper buffer allowed us to discriminate easily between the spontaneous and the catalyzed fraction of catechol oxidation. In particular, we found that autooxidation of 6-hydroxydopamine is considerably slower in phosphate buffer than in other buffers such as Tris-HCl. Therefore, the effect of Cp was tested in phosphate buffer. Our results are essentially identical to those previously obtained at lower pH values, in that: (i) Cp strongly accelerates the oxidation of 6-hydroxydopamine; and (ii) Cp strongly depresses the formation of hydrogen peroxide. The statement made by Sanjust and colleagues [83], that tyrosinase, but not Cp, can be considered to be an enzyme involved in catecholamine metabolism therefore should be reconsidered. It is worth recalling that, as exhaustively discussed above, the role of Cp is not only that of accelerating the oxidation of certain compounds, but also, and probably more importantly, that of preventing the formation of toxic oxygen intermediates. In this respect, the case of 6-hydroxydopamine is similar to that of Fe(II), both not needing, in principle, a catalyst to oxidize.

A role of Cp in controlling 6-hydroxydopamine levels could be of extraordinary importance. While Cp has been long recognized as a plasma protein synthesized by hepatocytes [13], evidence has been presented in the last few years indicating that other cellular types also produce it. Of particular interest is the observation that astrocytes, the glial component of the substantia nigra, can express a modified form of Cp, which remains bound on the surface of the cells through a GPI anchor [67]. Since astrocytes are involved in the production of dopamine precursors and metabolites, it would be tempting to speculate that altered levels of Cp might be the cause of neurodegenerative disorders involving catecholamine dysmetabolism. In this respect, we were not surprised to read that Cp levels are low compared with controls in the cerebrospinal fluid [84] and plasma [85] of patients with Parkinson's disease. The classical treatment for Parkinson's disease up to now has been the administration of the dopamine precursor l-dopa [86, 87], which unfortunately shows undesired long-term side-effects [88]. In the effort to obtain better therapeutic results, gene therapy is being developed, where astrocytes, synthesizing neurotrophic factors or enzymes involved in catecholamine production—in particular the rate-limiting enzyme for dopamine production, tyrosine hydroxylase—have been grafted into experimental animals [89–93]. Should astrocytic Cp synthesis be shown to be impaired in neurodegenerative disorders such as Parkinson's disease, it would be interesting to see whether gene therapy is also beneficial in this case.

What is the link between the structure of Cp and its multifunctionality? As anticipated in the introductory paragraphs, the copper content of Cp is unique among multicopper oxidases, in that three nonequivalent type 1 sites are

present. The work recently published on partial removal of copper from Cp [17] has shown clearly that all three sites may be functionally active, although the redox potential of one of the sites has been reported to be, at least in the human protein, extremely high [16]. Thus, a rational explanation could be that the three blue ions are preferentially used as "electron gates" by different classes of substrates, and that modulators of Cp activity exert their action by inducing conformational changes of the protein leading to a "remodulation" of the redox potentials of the blue sites. According to this hypothesis, we should be able to prove, in the future, that partially depleted forms of ceruloplasmin, where one blue site has been emptied preferentially, become more selective in their substrate specificity.

## CONCLUSIONS

The scientific evidence reviewed in this article clearly suggests that Cp can play several roles *in vivo*. The versatility is at two levels:

1. Cp is multifunctional as an enzyme, being able to utilize diverse, structurally unrelated substrates, likely by virtue of its unique structure and copper content;
2. Cp is multifunctional as a protein, likely able to serve as a copper reservoir for both metal transport and cellular signaling purposes.

Whereas the involvement of Cp in iron homeostasis is now well established, it remains necessary to clarify when, where, and why Cp exerts its alternative functions.

*We acknowledge the University of Messina ("ex60%" funds) for financial support.*

## References


1. Holmberg CG and Laurell C-B, Investigations in serum copper. Isolation of the copper containing protein and a description of some of its properties. *Acta Chem Scand* **2:** 550, 1948.
2. de Silva D, Davis-Kaplan S, Fergestad J and Kaplan J, Purification and characterization of Fet3 protein, a yeast homologue of ceruloplasmin. *J Biol Chem* **272:** 14208–14213, 1997.
3. Malmstrom BG, Early and more recent history in the research of multi-copper oxidases. In: *Multi-Copper Oxidases* (Ed. Messerschmidt A), pp. 1–22. World Scientific, Singapore, 1997.
4. Malkin R and Malmstrom BG, The state and function of copper in biological systems. *Adv Enzymol Relat Areas Mol Biol* **33:** 177–244, 1970.
5. Wittung-Stafshede P, Gomez E, Ohman A, Aasa R, Villahermosa RM, Leckner J, Karlsson BG, Sanders D, Fee JA, Winkler JR, Malmstrom BG, Gray HB and Hill MG, High-potential states of blue and purple copper proteins. *Biochim Biophys Acta* **1388:** 437–443, 1998.
6. Olsson MH and Ryde U, The influence of axial ligands on the reduction potential of blue copper proteins. *J Biol Inorg Chem* **4:** 654–663, 1999.
7. Calabrese L, Carbonaro M and Musci G, Chicken ceruloplasmin. Evidence in support of a trinuclear cluster involving type 2 and 3 copper centers. *J Biol Chem* **263:** 6480–6483, 1988.
8. Messerschmidt A, Rossi A, Ladenstein R, Huber R, Bolognesi M, Gatti G, Marchesini A, Petruzzelli R and Finazzi-Agro A, X-ray crystal structure of the blue oxidase ascorbate oxidase from zucchini. Analysis of the polypeptide fold and a model of the copper sites and ligands. *J Mol Biol* **206:** 513–529, 1989.
9. Holm RH, Kennepohl P and Solomon EI, Structural and functional aspect of metal sites in biology. *Chem Rev* **96:** 2239–2314, 1996.
10. Calabrese L and Musci G, Molecular properties of ceruloplasmin from different species. In: *Multi-Copper Oxidases* (Ed. Messerschmidt A), pp. 307–354. World Scientific, Singapore, 1997.
11. Gunnarsson PO, Nylen U and Pettersson G, Effect of pH on electron-paramagnetic-resonance spectra of ceruloplasmin. *Eur J Biochem* **37:** 47–50, 1973.
12. Deinum J and Vanngard T, The stoichiometry of the paramagnetic copper and the oxidation-reduction potentials of type I copper in human ceruloplasmin. *Biochim Biophys Acta* **310:** 321–330, 1973.
13. Ryden L, Ceruloplasmin. In: *Copper Proteins and Copper Enzymes* (Ed. Lontie L), pp. 37–100. CRC Press, Boca Raton, 1984.
14. Musci G, Bonaccorsi di Patti MC and Calabrese L, The state of the copper sites in human ceruloplasmin. *Arch Biochem Biophys* **306:** 111–118, 1993.
15. Zaitseva I, Zaitsev V, Card G, Moshkov K, Bax B, Ralph A and Lindley P, The X-ray structure of human serum ceruloplasmin at 3.1 Å: Nature of the copper centres. *J Biol Inorg Chem* **1:** 15–23, 1996.
16. Machonkin TE, Zhang HH, Hedman B, Hodgson KO and Solomon EI, Spectroscopic and magnetic studies of human ceruloplasmin: Identification of a redox-inactive reduced Type 1 copper site. *Biochemistry* **37:** 9570–9578, 1998.
17. Musci G, Fraterrigo TZ, Calabrese L and McMillin DR, On the lability and functional significance of the type 1 copper pool in ceruloplasmin. *J Biol Inorg Chem* **4:** 441–446, 1999.
18. Frieden E and Hsieh HS, Ceruloplasmin: The copper transport protein with essential oxidase activity. *Adv Enzymol Relat Areas Mol Biol* **44:** 187–236, 1976.
19. Frieden E, Ceruloplasmin: The serum copper transport protein with oxidase activity. In: *Copper in the Environment, Part II* (Ed. Nriagu JO), pp. 241–284. John Wiley, New York, 1979.
20. Frieden E, Perspectives on copper biochemistry. *Clin Physiol Biochem* **4:** 11–19, 1986.
21. Hsieh HS and Frieden E, Evidence for ceruloplasmin as a copper transport protein. *Biochem Biophys Res Commun* **67:** 1326–1331, 1975.
22. Frieden E and Hsieh HS, The biological role of ceruloplasmin and its oxidase activity. *Adv Exp Med Biol* **74:** 505–529, 1976.
23. Dameron CT and Harris ED, Regulation of aortic CuZn-superoxide dismutase with copper. Caeruloplasmin and albumin re-activate and transfer copper to the enzyme in culture. *Biochem J* **248:** 669–675, 1987.
24. Percival SS and Harris ED, Regulation of Cu,Zn superoxide dismutase with copper. Caeruloplasmin maintains levels of functional enzyme activity during differentiation of K562 cells. *Biochem J* **274:** 153–158, 1991.
25. Harris ED, Copper as a cofactor and regulator of copper,zinc superoxide dismutase. *J Nutr* **122:** 636–640, 1992.
26. Percival SS and Harris ED, Copper transport from ceruloplasmin: Characterization of the cellular uptake mechanism. *Am J Physiol* **258:** C140–C146, 1990.
27. Percival SS and Harris ED, Ascorbate enhances copper

transport from ceruloplasmin into human K562 cells. *J Nutr* **119:** 779–784, 1989.
28. Stevens MD, DiSilvestro RA and Harris ED, Specific receptor for ceruloplasmin in membrane fragments from aortic and heart tissues. *Biochemistry* **23:** 261–266, 1984.
29. Kataoka M and Tavassoli M, Ceruloplasmin receptors in liver cell suspensions are limited to the endothelium. *Exp Cell Res* **155:** 232–240, 1984.
30. Omoto E and Tavassoli M, Purification and partial characterization of ceruloplasmin receptors from rat liver endothelium. *Arch Biochem Biophys* **282:** 34–38, 1990.
31. Barnes G and Frieden E, Ceruloplasmin receptors of erythrocytes. *Biochem Biophys Res Commun* **125:** 157–162, 1984.
32. Kataoka M and Tavassoli M, Identification of ceruloplasmin receptors on the surface of human blood monocytes, granulocytes, and lymphocytes. *Exp Hematol* **13:** 806–810, 1985.
33. Dini L, Carbonaro M, Musci G and Calabrese L, The interaction of ceruloplasmin with Kupffer cells. *Eur J Cell Biol* **52:** 207–212, 1990.
34. Hilton M, Spenser DC, Ross P, Ramsey A and McArdle HJ, Characterisation of the copper uptake mechanism and isolation of the ceruloplasmin receptor/copper transporter in human placental vesicles. *Biochim Biophys Acta* **1245:** 153–160, 1995.
35. Scheinberg IH and Gitlin D, Deficiency of ceruloplasmin in patients with hepatolenticular degeneration (Wilsonís disease). *Science* **116:** 484–485, 1952.
36. Shokeir MH and Shreffler DC, Cytochrome oxidase deficiency in Wilson's disease: A suggested ceruloplasmin function. *Proc Natl Acad Sci USA* **62:** 867–872, 1969.
37. Brewer GJ, Practical recommendations and new therapies for Wilson's disease. *Drugs* **50**: 240–249, 1995.
38. Shimizu N, Yamaguchi Y and Aoki T, Treatment and management of Wilson's disease. *Pediatr Int* **41:** 419–422, 1999.
39. Harris ED, The transport of copper. *Prog Clin Biol Res* **380:** 163–179, 1993.
40. Linder MC, Wooten L, Cerveza P, Cotton S, Shulze R and Lomeli N, Copper transport. *Am J Clin Nutr* **67:** 965S–971S, 1998.
41. Miyajima H, Nishimura Y, Mizoguchi K, Sakamoto M, Shimizu T and Honda N, Familial apoceruloplasmin deficiency associated with blepharospasm and retinal degeneration. *Neurology* **37:** 761–767, 1987.
42. Cappelli-Bigazzi M, Ambrosio G, Musci G, Battaglia C, Bonaccorsi di Patti MC, Golino P, Ragni M, Chiariello M and Calabrese L, Ceruloplasmin impairs endothelium-dependent relaxation of rabbit aorta. *Am J Physiol* **273:** H2843–H2849, 1997.
43. Bianchini A, Musci G and Calabrese L, Inhibition of endothelial nitric-oxide synthase by ceruloplasmin. *J Biol Chem* **274:** 20265–20270, 1999.
44. Furchgott RF and Zawadzki JV, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288:** 373–376, 1980.
45. Alcain F, Low H and Crane FL, Ceruloplasmin stimulates thymidine incorporation by CCL-39 cells in the absence of serum or growth factors. *Biochem Biophys Res Commun* **180**: 790–796, 1991.
46. Alcain FJ, Villalba JM, Low H, Crane FL and Navas P, Ceruloplasmin stimulates NADH oxidation of pig liver plasma membrane. *Biochem Biophys Res Commun* **186:** 951–955, 1992.
47. Alcain FJ, Low H and Crane FL, Iron reverses impermeable chelator inhibition of DNA synthesis in CCl 39 cells. *Proc Natl Acad Sci USA* **91:** 7903–7906, 1994.
48. Dormandy TL, Caeruloplasmin: Acute-phase antioxidant. *Agents Actions Suppl* **8:** 185–197, 1981.
49. Halliwell B and Gutteridge JM, The antioxidants of human extracellular fluids. *Arch Biochem Biophys* **280:** 1–8, 1990.
50. Ehrenwald E, Chisolm GM and Fox PL, Intact human ceruloplasmin oxidatively modifies low density lipoprotein. *J Clin Invest* **93:** 1493–1501, 1994.
51. Mukhopadhyay CK, Mazumder B, Lindley PF and Fox PL, Identification of the prooxidant site of human ceruloplasmin: A model for oxidative damage by copper bound to protein surfaces. *Proc Natl Acad Sci USA* **94:** 11546–11551, 1997.
52. Mukhopadhyay CK and Fox PL, Ceruloplasmin copper induces oxidant damage by a redox process utilizing cell-derived superoxide as reductant. *Biochemistry* **37:** 14222–14229, 1998.
53. Osaki S, Johnson DA and Frieden E, The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. *J Biol Chem* **241:** 2746–2751, 1966.
54. Osaki S and Johnson DA, Mobilization of liver iron by ferroxidase (ceruloplasmin). *J Biol Chem* **244:** 5757–5758, 1969.
55. Roeser HP, Lee GR, Nacht S and Cartwright GE, The role of ceruloplasmin in iron metabolism. *J Clin Invest* **49:** 2408–2417, 1970.
56. Evans GW, Ceruloplasmin and maternal iron mobilization. *J Nutr* **101:** 567–568, 1971.
57. Harris ZL, Morita H and Gitlin JD, The biology of human ceruloplasmin. In: *Multi-Copper Oxidases* (Ed. Messerschmidt A), pp. 285–306. World Scientific, Singapore, 1997.
58. Harris ZL, Takahashi Y, Miyajima H, Serizawa M, MacGillivray RT and Gitlin JD, Aceruloplasminemia: Molecular characterization of this disorder of iron metabolism. *Proc Natl Acad Sci USA* **92:** 2539–2543, 1995.
59. Morita H, Ikeda S, Yamamoto K, Morita S, Yoshida K, Nomoto S, Kato M and Yanagisawa N, Hereditary ceruloplasmin deficiency with hemosiderosis: A clinicopathological study of a Japanese family. *Ann Neurol* **37:** 646–656, 1995.
60. Mukhopadhyay CK, Attieh ZK and Fox PL, Role of ceruloplasmin in cellular iron uptake. *Science* **279:** 714–717, 1998.
61. Askwith C, Eide D, Van Ho A, Bernard PS, Li L, Davis-Kaplan S, Sipe DM and Kaplan J, The *FET3* gene of S. cerevisiae encodes a multicopper oxidase required for ferrous iron uptake. *Cell* **76**: 403–410, 1994.
62. De Silva DM, Askwith CC, Eide D and Kaplan J, The FET3 gene product required for high affinity iron transport in yeast is a cell surface ferroxidase. *J Biol Chem* **270:** 1098–1101, 1995.
63. Askwith C and Kaplan J, An oxidase-permease-based iron transport system in *Schizosaccharomyces pombe* and its expression in *Saccharomyces cerevisiae*. *J Biol Chem* **272:** 401–405, 1997.
64. Attieh ZK, Mukhopadhyay CK, Seshadri V, Tripoulas NA and Fox PL, Ceruloplasmin ferroxidase activity stimulates cellular iron uptake by a trivalent cation-specific transport mechanism. *J Biol Chem* **274:** 1116–1123, 1999.
65. Richardson DR, Role of ceruloplasmin and ascorbate in cellular iron release. *J Lab Clin Med* **134:** 454–465, 1999.
66. Harris ZL, Durley AP, Man TK and Gitlin JD, Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. *Proc Natl Acad Sci USA* **96:** 10812–10817, 1999.
67. Patel BN and David S, A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. *J Biol Chem* **272:** 20185–20190, 1997.
68. Fortna RR, Watson HA and Nyquist SE, Glycosyl phosphatidylinositol-anchored ceruloplasmin is expressed by rat Sertoli cells and is concentrated in detergent-insoluble membrane fractions. *Biol Reprod* **61:** 1042–1049, 1999.

69. Erel O, Automated measurement of serum ferroxidase activity. *Clin Chem* **44:** 2313–2319, 1998.
70. Cardoso SM, Pereira C and Oliveira R, Mitochondrial function is differentially affected upon oxidative stress. *Free Radic Biol Med* **26:** 3–13, 1999.
71. Moreau VH, Castilho RF, Ferreira ST and Carvalho-Alves PC, Oxidative damage to sarcoplasmic reticulum $Ca^{2+}$-ATPase AT submicromolar iron concentrations: Evidence for metal-catalyzed oxidation. *Free Radic Biol Med* **25:** 554–560, 1998.
72. Ponka P, Cellular iron metabolism. *Kidney Int* **55** (Suppl 69)**:** S2–S11, 1999.
73. Inoue K, Akaike T, Miyamoto Y, Okamoto T, Sawa T, Otagiri M, Suzuki S, Yoshimura T and Maeda H, Nitrosothiol formation catalyzed by ceruloplasmin. Implication for cytoprotective mechanism *in vivo*. *J Biol Chem* **274**: 27069–27075, 1999.
74. Jones AA, DiSilvestro RA, Coleman M and Wagner TL, Copper supplementation of adult men: Effects on blood copper enzyme activities and indicators of cardiovascular disease risk. *Metabolism* **46:** 1380–1383, 1997.
75. Bingley JB and Dick AT, The pH optimum for ceruloplasmin oxidase activity in the plasma of several species of animal. *Clin Chim Acta* **25:** 480–482, 1969.
76. Musci G, Bonaccorsi di Patti MC and Calabrese L, Modulation of the redox state of the copper sites of human ceruloplasmin by chloride. *J Protein Chem* **14:** 611–619, 1995.
77. Musci G, Bellenchi GC and Calabrese L, The multifunctional oxidase activity of ceruloplasmin as revealed by anion binding studies. *Eur J Biochem* **265:** 589–597, 1999.
78. Medda R, Calabrese L, Musci G, Padiglia A and Floris G, Effect of ceruloplasmin on 6-hydroxydopamine oxidation. *Biochem Mol Biol Int* **38:** 721–728, 1996.
79. Chapman RF, Percival A and Swan GA, Studies related to the chemistry of melanins. XII. Some spectroscopic experiments regarding intermediates in melanogenesis. *J Chem Soc [Perkin 1]* **12:** 1664–1667, 1970.
80. Heikkila R and Cohen G, Inhibition of biogenic amine uptake by hydrogen peroxide: A mechanism for toxic effects of 6-hydroxydopamine. *Science* **172:** 1257–1258, 1971.
81. Cohen G and Heikkila RE, The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. *J Biol Chem* **249:** 2447–2452, 1974.
82. Kumar R, Agarwal AK and Seth PK, Free radical-generated neurotoxicity of 6-hydroxydopamine. *J Neurochem* **64:** 1703–1707, 1995.
83. Rescigno A, Rinaldi AC and Sanjust E, Some aspects of tyrosine secondary metabolism. *Biochem Pharmacol* **56:** 1089–1096, 1998.
84. Boll MC, Sotelo J, Otero E, Alcaraz-Zubeldia M and Rios C, Reduced ferroxidase activity in the cerebrospinal fluid from patients with Parkinson's disease. *Neurosci Lett* **265:** 155–158, 1999.
85. Torsdottir G, Kristinsson J, Sveinbjornsdottir S, Snaedal J and Johannesson T, Copper, ceruloplasmin, superoxide dismutase and iron parameters in Parkinson's disease. *Pharmacol Toxicol* **85:** 239–243, 1999.
86. Emilien G, Maloteaux JM, Geurts M, Hoogenberg K and Cragg S, Dopamine receptors—physiological understanding to therapeutic intervention potential. *Pharmacol Ther* **84:** 133–156, 1999.
87. Sourkes TL, "Rational hope" in the early treatment of Parkinson's disease. *Can J Physiol Pharmacol* **77:** 375–382, 1999.
88. Murer MG, Raisman-Vozari R and Gershanik O, Levodopa in Parkinson's disease: Neurotoxicity issue laid to rest? *Drug Saf* **21:** 339–352, 1999.
89. Horellou P and Mallet J, Gene therapy for Parkinson's disease. *Mol Neurobiol* **15:** 241–256, 1997.
90. Segovia J, Vergara P and Brenner M, Astrocyte-specific expression of tyrosine hydroxylase after intracerebral gene transfer induces behavioral recovery in experimental parkinsonism. *Gene Ther* **5:** 1650–1655, 1998.
91. Fitoussi N, Sotnik-Barkai I, Tornatore C, Herzberg U and Yadid G, Dopamine turnover and metabolism in the striatum of parkinsonian rats grafted with genetically-modified human astrocytes. *Neuroscience* **85:** 405–413, 1998.
92. Krobert K, Lopez-Colberg I and Cunningham LA, Astrocytes promote or impair the survival and function of embryonic ventral mesencephalon co-grafts: Effects of astrocyte age and expression of recombinant brain-derived neurotrophic factor. *Exp Neurol* **145:** 511–523, 1997.
93. Lundberg C, Horellou P, Mallet J and Bjorklund A, Generation of DOPA-producing astrocytes by retroviral transduction of the human tyrosine hydroxylase gene: *In vitro* characterization and *in vivo* effects in the rat Parkinson model. *Exp Neurol* **139:** 39–53, 1996.