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Review

Structure to function relationships in ceruloplasmin: a 'moonlighting' protein

P. Bielli[a,*] **and L. Calabrese**[b]

[a] Institute of Microbiology and Genetics, Vienna Biocenter, University of Vienna, Dr. Bohrgasse 9/4, 1030 Vienna (Austria), Fax + 43 1 42779546, e-mail: pamela@gem.univie.ac.at
[b] Department of Biochemical Sciences, University of Rome 'La Sapienza', Piazzale Aldo Moro 5, 00185 Rome (Italy)

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Abstract. Specialised copper sites have been recruited during evolution to provide long-range electron transfer reactivity and oxygen binding and activation in proteins destined to cope with oxygen reactivity in different organisms. Ceruloplasmin is an ancient multicopper oxidase evolved to insure a safe handling of oxygen in some metabolic pathways of vertebrates. The presently available knowledge of its structure provides a glimpse of its plasticity, revealing a multitude of binding sites that point to an elaborate mechanism of multifunctional activity. Ceruloplasmin represents an example of a 'moonlighting' protein that overcomes the one gene-one structure-one function concept to follow the changes of the organism in its physiological and pathological conditions.

Key words. Ceruloplasmin; multicopper oxidase; moonlighting protein; iron metabolism; copper metabolism; electron transfer.

Introduction

In 1948, a blue protein from the α_2-globulin fraction of human serum, possessing oxidase activity towards aromatic diamines and catechol, was purified by Holmberg and Laurell [1]. It was denoted ceruloplasmin, literally meaning 'a blue substance from plasma'. This fascinating protein quickly became the subject of many investigations concerning its function and molecular structure and the physical properties of the copper ions bound to it. Identified as an acute-phase reactant [2] participating in inflammatory responses and seen to fluctuate greatly in several diseases and hormonal states, ceruloplasmin excited the imagination of biomedical scientists. In the ensuing years, thousand of papers were published but progress in understanding its physiological role was at best moderate: roles in copper transport, iron homeostasis, biogenic amine metabolism and defence against oxidative stress have all been plausibly proposed as physiological function(s) for ceruloplasmin [3, 4]. Parallel extensive spectroscopic investigations as well as sophisticated enzyme kinetic analyses attempted to clarify the mechanism of action and the complex nature of the multiple copper sites of this protein which had been found to be the most distinct member of the class of blue oxidases [5]. Generically reported to be a multifunctional protein, ceruloplasmin remained the most popular and the least understood among these enzymes during a half-century of intense debate. The discovery of the disease aceruloplasminaemia, an autosomal recessive disorder of iron metabolism characterised by the complete absence of ceruloplasmin [6, 7] and resolution of the three-dimensional structure of human ceruloplasmin by X-ray crystallography [8] were nearly concomitant. The clinical findings of diabetes, retinal degeneration and neurodegeneration, associated with tissue iron overload in aceruloplasminaemic patients, who eventually die after a long

* Corresponding author.

and insidious course [9], clearly indicated that ceruloplasmin plays an essential role in iron metabolism. On the structural side, the nature of ceruloplasmin as a large and complicated molecule was finally disclosed, to reveal an intriguing picture of a multiplicity of structural elements able to support multiple functions.

Although one gene-one protein-one function has been a paradigm of biochemistry, an increasing number of exceptions are being reported. The term 'moonlighting' proteins has been used to designate proteins with multiple functions, which offer the challenge of identifying when, how and why they exert their multiple roles [10]. This review will discuss the principal aspects of the structure-to-function relationships of ceruloplasmin and try to interpret the different facets of its multifunctionality in the concept of 'moonlighting' proteins.

Ceruloplasmin as a multicopper oxidase

Ceruloplasmin belongs to the family of multicopper oxidases which are among the few enzymes able to bind molecular oxygen to perform its complete reduction to water [11, 12]. The many facets of ceruloplasmin structure-to-function relationships can only be understood in the context of the chemistry and enzymology of these enzymes. Multicopper oxidases contain multiple copper-binding sites characterized by different spectroscopic properties and reactivity [13]. These enzymes are often referred to as 'blue' oxidases. This definition points to the existence in a protein of a site, the blue or type 1 (T1) site, able to bind a copper atom in a unique environment, defined by the first coordination sphere of the metal and by the protein matrix surrounding the site. An intense absorption band at 600 nm in the optical spectrum and a peculiar electron paramagnetic (EPR) spectrum are the spectroscopic signature of an oxidized blue site which confers an intense blue colour on the protein. The blue site is not unique to blue oxidases since it characterizes the so-called small blue proteins, single-domain proteins containing a single or mononuclear T1 site. The typical blue sites all have four ligands, two histidines, one cysteine and one methionine. Studies on small blue proteins have shown that much of the peculiar character of the type 1 copper is due to the fact that this copper is bound to the electron-rich cysteine thiol through a very strong, highly covalent bond, and has a tetrahedral rather than the tetragonal geometry preferred by normal copper complexes [14]. The T1 site of small blue proteins, once reduced to Cu^{1+}, is generally insensitive towards reoxidation by molecular oxygen. This important and general restriction on the reactivity of these sites is removed in the multicopper oxidases due to the presence of an oxygen-binding and -reducing site. In fact, they all contain, in addition to the blue copper centre(s), three other copper atoms sharing a common site of binding, which is therefore called the trinuclear cluster. On the basis of their spectroscopic properties, the three copper ions can be formally distinguished in one mononuclear type 2 (T2) copper site, with a normal EPR signal and nearly undetectable absorptions in the optical spectrum, and a pair of anti-ferromagnetically coupled copper ions, or type 3 (T3) copper site, with strong absorption at 330 nm in the oxidised state and no EPR signal, but they in fact functionally behave as a single multimetallic unit. This unique structure for a copper site is exclusive to blue oxidases and enables these enzymes to catalyse the four-electron reduction of dioxygen to two water molecules by one-electron oxidations of substrates [12].

During catalysis, the copper atoms of blue oxidases behave as a linear array of redox centres of a functional unit. Within this unit, the role of the T1 site is sequential uptake and transfer of single electrons from substrate to the trinuclear cluster that uses the acquired electrons to reduce oxygen to water. The catalytic mechanism of these enzymes is based on different kinds of electron transfer (ET) processes. Intramolecular ET refers to the process by which electrons travel through the protein to reach the oxygen-binding site from the T1 site. By this mechanism, they safely oxidise a variety of substrates at the expense of oxygen without releasing radical oxygen species (ROS). At variance with the majority of multicopper oxidases, which act on organic substrates, ceruloplasmin oxidises both inorganic, ferrous iron, and different organic substrates and shares this property with only a few other enzymes of unicellular organisms [15, 16].

Current knowledge about multicopper oxidases has been derived by a combination of different experimental approaches. Kinetic studies have been widely employed to clarify the details of the electron transfer processes. Following the electron path inside a protein requires, however, very difficult theoretical and experimental approaches. Pulse radiolysis techniques have proved to be very useful, and have been extensively applied to study electron transfer processes operated by small blue proteins and multicopper oxidases, in the latter case leading to the discovery that oxygen binding to the trinuclear cluster increases the driving force of the intramolecular ET between the T1 site and the trinuclear cluster in ascorbate oxidase [17]. In addition to these techniques, spectroscopy has played a major role for understanding the nature of copper centres; as a matter of fact, the close proximity of the T2 and T3 sites was initially inferred from spectroscopic studies. It was first postulated in laccase, on the basis of studies of its anion-binding properties [18], then proposed for ceruloplasmin on the basis of its redox properties [19, 20] and finally definitely established by resolution of the crystallographic structures of ascorbate oxidase [21], human ceruloplasmin [8] and laccase [22]. In this context, one should note that cerulo-

plasmin has been, and still is, the most disputed among blue oxidases, with respect to the state and functional role of its copper sites. There are many reasons for this, the first of which is that conformational changes in ceruloplasmin lead to irreversible modification of its spectroscopic properties, especially those detected by EPR. Among the copper sites, the type 2 copper in particular is the elective spectroscopic monitor for the transition of the protein towards altered conformational states. Many investigations have recorded the particularly labile conformation of this site in ceruloplasmin [23, 24]. Various, non-human, ceruloplasmins, when isolated in a native conformation with a very rapid one-step procedure [25], apparently lack an EPR-detectable T2 copper in the resting state, and this signal manifests only after prolonged storage or manipulation of samples [19, 20, 26]. The possibility that this site establishes peculiar interactions with the T3 site leading to broadening of its EPR signal beyond the limit of detection was therefore suggested. Another possibility is that it has the tendency to stay reduced in the resting protein, another peculiarity of ceruloplasmin that also characterises its blue site(s) [27].

In human ceruloplasmin, a more or less intense signal is generally observed in the EPR signal of the purified protein, depending on the age of the donor. The direct analysis of ceruloplasmin in the plasma shows that, during ageing, the protein moiety of ceruloplasmin is subjected to oxidative modifications that induce conformational changes around its copper sites resulting in a dramatic change of its EPR spectrum in individuals over 65 years of age [28]. Another peculiarity of human ceruloplasmin is that the redox state of its copper sites is strongly affected by chloride [27], the most abundant anion in plasma. This anion can bind to the trinuclear cluster, probably to the T2 copper, and raise its redox potential [29] enhancing both the spectroscopic and, more importantly, the catalytic properties of the T1 sites(s) [30]. Given a measured K_d of ~4 mM for the purified protein with respect to a chloride concentration in plasma of over 0.1 M, one can easily infer that circulating ceruloplasmin is under the control of this anion.

These properties are exclusively observed in ceruloplasmin, and when combined to its also different structural parameters, reveal how this protein is intrinsically different with respect to other multicopper oxidases, although sharing extensive sequence similarities and a common structural organization of the polypeptide chain [31]. Multicopper oxidases are comprised of multiple modules each of which has the fold typical of the single module of small blue proteins, an eight-stranded β barrel anchoring the blue site, often referred to as 'the cupredoxin fold' [32]. This is consistent with the concept of a common origin of these proteins from a primordial cupredoxin [33]. However, evolutionary studies reveal that, phylogenetically, ceruloplasmin is much more closely related to coagulation factors (CF) V and VIII. Determination of the primary structure of the human protein, 1046 amino acids, combined with the fragmentation pattern observed in limited proteolysis, indicates, in fact, that ceruloplasmin is composed of three consecutive homology units further subdivided into sub-domains [34]. The high degree of internal homology has led to the proposal that ceruloplasmin arose by tandem triplication of a primordial gene that coded for a primitive two-domain oxidase [35], the common ancestor of multicopper oxidases. In contrast, the other blue oxidases evolved further by adding just one genetic element to the two-domain structure of the ancestor. The spatial organisation of the ceruloplasmin molecule in six domains, instead of the three domains of the blue oxidases, added to the presence of multiple blue sites, and their peculiar distribution among the six domains as anticipated by sequence alignment [31] and confirmed by X-ray studies [8], is totally consistent with this hypothesis. Thus, in the framework of a common evolutionary pathway for multicopper oxidases from a primordial cupredoxin to, and then from, a primordial oxidase, ceruloplasmin branched off very early towards its own pattern, CF V and VIII diverging soon after it obtained its six-domain form and evolved towards the structure of contemporary ceruloplasmin [35].

As a copper protein endowed with ferroxidase activity, ceruloplasmin is a link between copper and iron metabolism. In this respect, ceruloplasmin is likely to represent the current end point in the parallel development of copper- and iron-based systems of aerobic cells exposed to the harmful reactivity of these metals, which they have exploited to survive through natural selection.

Structure-to-function relationships in human ceruloplasmin

The spatial organisation of the ceruloplasmin molecule derived from the X-ray structure of human serum ceruloplasmin solved at 0.3 nm [8] is shown in figure 1. Compared to the three-domain structure typical of the other multicopper oxidases, as exemplified by ascorbate oxidase [36], the structure of ceruloplasmin is far more complex. The molecule is composed of six compact domains, with large loop insertions, and it contains six tightly bound copper atoms. The three copper ions of the trinuclear cluster lie at the interface between the first and last domains, 1 and 6 respectively, possessing ligands from each domain, an arrangement also seen in the structures of ascorbate oxidase and laccase [22] and also found in the FET3 structural model [37]. The remaining three copper atoms are mononuclear centres held by intra-domain sites; those located on domains 4 and 6 have a typical T1 copper environment with a set of four ligands, two histidines, one methionine and one cysteine, while the one

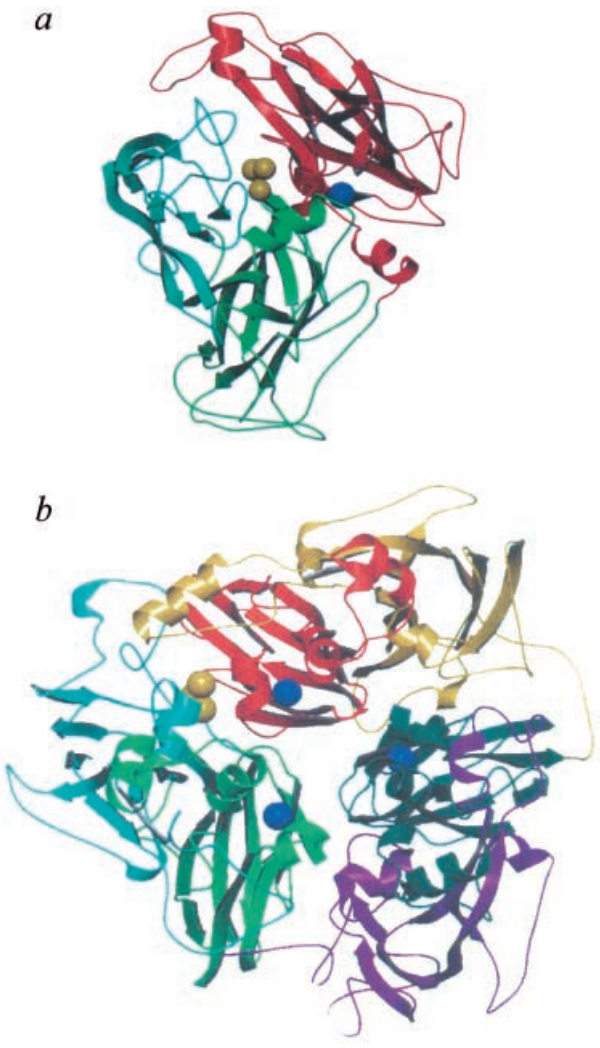

Figure 1. The overall tertiary structure of ascorbate oxidase (pdb 1AOZ) (*a*) and human ceruloplasmin (pdb 1KCW) (*b*). The polypeptide chain is shown with its structural motif colour coded to highlight the different domains, three in ascorbate oxidase and six in ceruloplasmin. The copper atoms are depicted as spheres that are shown in yellow for the trinuclear cluster and in blue for the T1 sites.

located in domain 2 has a different structure in that it lacks the methionine which is replaced in the amino acid sequence by a leucine residue, Leu329 [38]. This residue is unable to coordinate metals, the centre is therefore a tricoordinate T1 site. Another difference is that this site is not endowed with structural elements able to bind substrates that have been found close to the other two sites [39]. It is important that substrates be recognized by, and bound to, portions of the protein surface strategically located with respect to the ceruloplasmin T1 sites, which are not solvent exposed. The presence of multiple, nonequivalent, T1 sites is a long-recognized peculiarity of ceruloplasmin, picked up in numerous spectroscopic and kinetic studies. Depending on the isolation procedure, purified human ceruloplasmin contains six to seven copper atoms per molecule [25, 40, 41]. The peculiar line shape of the EPR spectrum of human ceruloplasmin has been attributed, within the uncertainty of copper stoichiometry, to two [40] or three blue sites [42] with different magnetic parameters. Functionally non-equivalent spatial and functional relationships between T1 sites and the trinuclear cluster, induced by proteolytic cleavage and ageing of ceruloplasmin in vitro and in vivo, have also been inferred, based on spectroscopic studies [19, 20, 28]. In the structures examined so far, the T1 centre and the cluster are held at a distance of 1.3 nm, prevented from coming in contact by the protein moiety; however, the cysteine ligand of the T1 site is flanked by two histidines, each bound to a copper atom of the cluster. This structural motif, His-Cys-His, was first noticed in the structure of ascorbate oxidase [21, 36], then found in the crystal structure of laccase [22], and detected also in the sequence of bacterial multicopper oxidases [16]. It is thought to provide efficient electronic coupling to the two centres undergoing the redox reaction, by offering a bifurcate pathway to electrons travelling between the T1 site and the cluster in the intramolecular ET process. This structural element is so crucial to the activity of these enzymes that, on this basis, modelling the spatial organisation of FET3 catalytic domain was possible, which is, to date, the only available three-dimensional structure of this membrane-bound blue oxidase of yeasts [37].

An intriguing feature in the structure of ceruloplasmin is that it has more copper centres than are required by its oxidase moiety where only the copper site of domain 6 is connected to the trinuclear cluster by the His-Cys pathway. The other two T1 sites are located far from this unit. As noticed by Zaitseva et al. [8] in their first report on the crystal structure of human ceruloplasmin, the six domains are organised in such a way that the odd-numbered domains point outwards from the pseudo three-fold axis whilst the even-numbered ones point towards it. Since T1 sites are confined to the latter domains, this arrangement results in a peculiar disposition of the three sites (fig. 2). Their distance is nearly 1.8 nm, a value compatible for internal ET. If these additional T1 sites are involved in the activity of ceruloplasmin, for instance increasing electron capture from substrates, they could communicate with the trinuclear cluster either via the site of domain 6 or by an independent, though improbable, mechanism. In this context, the tricoordinate site of domain 2 is most intriguing. A redox potential value of at least 1 V has been assigned to this centre [43], which is too high to allow reoxidation by the trinuclear cluster, during studies aimed at identifying the blue copper centres that stay reduced in resting ceruloplasmin samples quickly isolated from plasma by a single-step procedure. It would be a redox-inactive T1 site permanently reduced also in the resting protein and hence unable to take part in the catalytic mechanism, possibly due to its different ligating motif. To

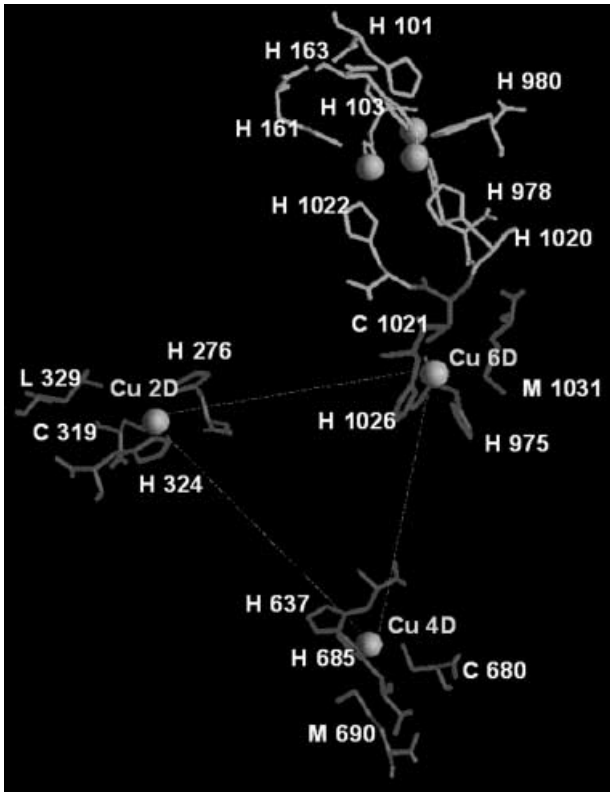

Figure 2. The configuration of the six copper sites in human ceruloplasmin. The copper atoms are depicted as spheres and are shown as surrounded by their ligands. The location of the T1 sites in domains 2, 4 and 6 is noted as 2D, 4D and 6D, respectively. The dotted-line triangle highlights the similar inter-T1 copper distances.

check this hypothesis, the replacement of Leu329 with Met by site-directed mutagenesis has been attempted, aimed at introducing into the site the ligand usually found in more typical sites [14]. The substitution resulted in a silent mutation in that it failed to modify either the spectroscopic or catalytic properties of the mutated human recombinant ceruloplasmin with respect to authentic ceruloplasmin [44]. Thus the nature of this site appears to be imposed by the protein matrix surrounding the coordination sphere of copper within a site which behaves as if it were locked in a definite frame and destined to other functions.

The concept that a blue site stays colourless and redox inactive is in contrast with the notion that these sites have been recruited during evolution as fast centres for electron transfer [45]. Bearing this kind of copper site, ceruloplasmin would be unique among all blue proteins and this underscores the importance of other approaches in trying to identify those sites involved in ET to the oxygen-reducing site of this protein, taking into account the structural data that indicate substantial differences between domains 4 and 6 with respect to binding sites for substrates [39, 46]. Pulse radiolysis studies have been performed on ceruloplasmin over many years [47], and a more recent investigation has used this approach to find possible ET pathways. The rate constants of reduction of T1 copper centres and intramolecular electron equilibration were determined, following pulsed radiolytic reduction of a disulphide bridge of the protein [48]. The evidence of a predominant role for the T1 site of domain 6 in the internal ET to the oxygen-reducing site was obtained that favours this site as the primary electron acceptor from reducing substrate(s), with a less important role of the site on domain 4. A quite different conclusion, that the internal ET between the site of domain 4 and that of domain 6 might be possible, rendering the former catalytically relevant, although not necessary for oxygen reduction, was drawn from an EPR study of the kinetics of reduction of ceruloplasmin by ferrous iron, combined with an extensive examination of all possible pathways of electron transfer [29].

A mutation study aimed at destroying the coordinating capacity of the site of domain 6, by replacing the cysteine residue of the His-Cys-His motif with serine, resulted in a heterogeneous recombinant protein, with dramatically altered molecular properties, containing, however, a small fraction of enzymatically active molecules [44]. This result, while showing that the site of domain 6 plays an important role in stabilizing the overall structure, does not however allow a definite conclusion about the role of the site of domain 4 in catalysis.

Copper incorporation during biosynthesis

Impairment of copper incorporation into ceruloplasmin during biosynthesis results in the secretion of the apoprotein which is rapidly degraded in the plasma [49, 50]. The precise mechanism of copper insertion into the nascent protein is presently unknown and is expected to be very complex, due to the multiplicity and to the different structural requirements of the six copper sites. Understanding this mechanism is important for comprehending the biosynthetic mechanism of ceruloplasmin and its derangement in various pathologic states related to disorders of copper metabolism as well as to the mechanism of its possible action as copper transferase [51].

As shown by the crystal structure, the ligands of the copper atoms of the trinuclear cluster are in both domains 1 and 6 of ceruloplasmin, holding together the N- and C-terminal portions of the protein. While there is no doubt that copper plays a stabilizing effect in such a closed configuration of the molecule, whether the metal requires a proper molecular architecture to be correctly inserted during biosynthesis or itself plays a role in organising the overall spatial arrangement of the molecule is not clear. This problem has been addressed by different experimental approaches. Calorimetric studies of various cerulo-

plasmins, including the sheep protein which is highly homologous to the human one [52], indicated a structural role for copper in the overall spatial arrangement of the protein well beyond a stabilising effect against thermal denaturation [53]. The molecule appeared to be organised in three calorimetric domains, each comprising multiple merged sub-domains, with a mismatch with respect to the three homology units of the primary structure. Copper removal caused the rearrangement of the molecule in only two calorimetric domains, likely corresponding to an open, thermally unstable form of the molecule. This property, an inactive apoceruloplasmin with a much different conformation with respect to the oxidase-active holoceruloplasmin, has been exploited to follow the intracellular process of copper incorporation into ceruloplasmin in human [54] and rat liver [55]. A conformational change from the apo- to the holo-form was detected upon copper incorporation in the secretory compartment of the cell prior to secretion, thus demonstrating that serum ceruloplasmin is synthesised in hepatocytes and secreted into the plasma as holoprotein, with the six integral copper atoms incorporated during biosynthesis [54]. Copper incorporation into nascent ceruloplasmin is a very late event in the secretory pathway and occurs immediately before secretion, or before membrane anchoring of the glycosylphosphatidylinositol (GPI)-linked form [56], recently identified in brain [57–59] and in Sertoli cells [60]. In a cell-free study of the reconstitution of purified human ceruloplasmin, deprived of the metal by chelating agents, copper was found to reinsert into the various sites of the protein in a multistep process. No intermediate species with less than stoichiometric copper content were trapped and evidence was also obtained that the protein remained in an inactive form following metal entry into the various copper-binding sites unless divalent cations, Mg^{2+} or Ca^{2+}, and ATP were present [61]. A copper-glutathione complex was used as donor of the metal to apoceruloplasmin; however, whether glutathione can serve as copper carrier between the copper-transporting ATPases responsible for copper transfer to the secretory pathway [62–66] and the newly synthesised ceruloplasmin chain is not clear.

Copper-transporting ATPases are not solely responsible for copper availability within the secretory pathway. Extensive research on the genes of the copper pathway has made it clear that a complex network of a family of proteins, functioning as copper chaperones, works in concert with copper transporters and copper-transporting ATPases to regulate copper traffic across the cell membrane, and then to the appropriate target within the cell [67, 68]. Only copper which has been moved by this system is effective for holoceruloplasmin synthesis, as well as for other copper-dependent enzymes [69, 70]. In patients with Wilson's disease, the dysfunction or the absence of the copper-transporting ATPase essential for copper transfer into the secretory pathway of hepatocytes abrogates copper transfer into the secretory pathway of these cells [71], resulting in diminution of the serum concentration of ceruloplasmin consequent to the rapid removal from circulation of the secreted apoprotein.

Plasma contains protease(s) that selectively attack 130-kDa ceruloplasmin at a primary proteolytic site(s) [72]. As a result, a fraction of ceruloplasmin circulates as cleaved into two major fragments of 19 and 116 kDa [28]. Following this attack, the fragments initially do not dissociate and mimic the intact molecule, although with altered redox properties of the copper sites [73]. The isolation of the protein from plasma, unless performed with proteases removed [41] or by a very rapid single-step method [25], often results in massive fragmentation of the polypeptide chain, and subsequent loss of copper. With a much less compact structure, the apoprotein is expected to be even more susceptible to proteolytic cleavage with respect to the holoceruloplasmin and to turn over rapidly in the circulation. The lability of ceruloplasmin to proteases is the major property that has hampered all studies on this protein except those related to its amino acid sequence. As a matter of fact, the amino acid sequence of human ceruloplasmin was determined on the basis of the sequence of its 'natural' fragments well before that of other similar copper enzymes of the class of multicopper oxidases [38].

Ceruloplasmin as multifunctional protein

Figure 3 shows the different facets of ceruloplasmin multifunctionality. The picture represents just some of the many reactions that ceruloplasmin has been shown to mediate in the overwhelming number of investigations aimed at clarifying its biological role. The action of ceruloplasmin as a copper transport protein is not depicted: such a role was proposed early for the newly discovered ceruloplasmin, based initially on its abundance in the blood. Numerous investigators have since then vigorously pursued this issue, their interest being elicited by numerous reports on the existence of ceruloplasmin receptors on a variety of cell types [51, 74–77]. These investigations disclosed a peculiar ability of this protein to donate its copper to recipient systems, either cells or tissues. The discovery that copper metabolism is normal in patients with aceruloplasminaemia [78] is now taken as a clear-cut demonstration that ceruloplasmin has an essential role in iron, but not copper, metabolism, leaving the matter an open question.

The ceruloplasim molecule, presented in figure 3 with its peculiar shape disclosed by X-ray crystallography, shows a three-tower-like structure at the top and a nearly flat surface at the bottom. It is surrounded by a variety of substrates, and effectors, shown at their binding sites as di-

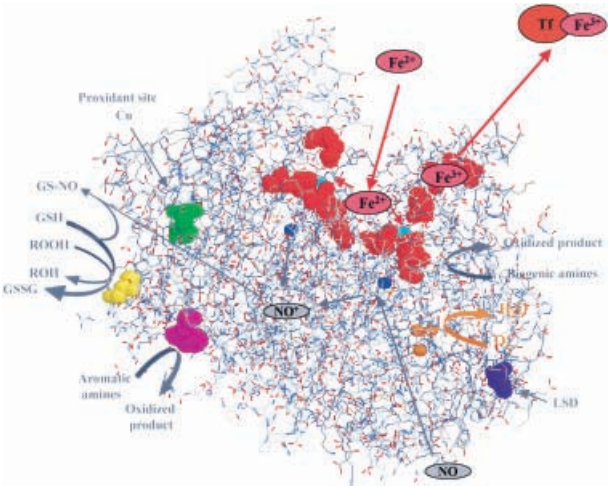

Figure 3. The multifunctional action of ceruloplasmin. The ceruloplasmin molecule is shown in side view with respect to the top view shown in figure 1. The amino acid residues implicated in the different activities are represented as a space-filled atom model and are shown in green (His426 of the prooxidant site [81]), in yellow (Cys699 of peroxidase active site [79]), in magenta (W669 of the *p*-phenylenediamine-binding site [46]), in purple (N119 near the lysergic acid diethylamide (LSD)-binding site [46]), in red-orange (residues of labile copper-binding sites of domains 4 and 6, ref. [39]) and in red (residues of the 'holding' site of domain 6 [39]). The two labile copper atoms are shown as cyan spheres between the red-orange residues of the labile sites of domains 4, on the left, and 6, on the right. These residues almost completely screen the integral copper atom of the T1 site of domain 2, which is located behind them in this view of the molecule, and therefore has not been marked with colour. The remaining five integral copper atoms are depicted as spheres, coloured in blue (T1 sites of domains 4 and 6) or orange (trinuclear cluster). The ferrous ion is shown at the top of the molecule as approaching the two islands of negatively charged residues anchoring the labile copper atoms. The ferric ion is represented as interacting with residues of the 'holding' site of domain 6 prior to the incorporation into transferrin (Tf) (GSH, glutathione; GS-NO, S-nitrosoglutathione parentheses.)

rectly identified on the native crystals of human ceruloplasmin by soaking experiments, or inferred by inspection of the crystal structure. The presence of multiple prosthetic copper sites with different reactivity and the possibility that they can be preferentially or simultaneously operative can lead to the involvement of ceruloplasmin in a variety of metabolic pathways. Beside ferrous ions, ceruloplasmin is able to oxidise an extensive group of organic compounds that includes p-phenylenediamines, amino-phenols, catechols and 5-hydroxyindoles [3]. Moreover, the presence of particularly reactive groups on the lateral chains of amino acid residues, and binding of additional copper outside the multicopper complex of the oxidase activity, can lead to a gain of function that further expands ceruloplasmin modes of action, giving, however, an intriguing picture. As depicted on the left of figure 3, independent of and apart from the multicopper complex responsible for oxidase activity, there is another catalytic site in ceruloplasmin that involves Cys699. It performs a copper-independent glutathione peroxidase activity, with both hydrogen peroxide and/or organic peroxides [79, 80]. It enables ceruloplasmin to remove reactive intermediates that, in the case of hydrogen peroxide, a membrane-permeable oxidant, could even lead to an altered intracellular redox state of vascular cells. The reaction with organic hydroperoxides would have significance in the metabolism of lipid hydroperoxides in the extracellular space.

The nearby prooxidant site is an example of gain of function by ceruloplasmin, responsible for its involvement in oxidative damage. Direct prooxidant activity of ceruloplasmin can develop when a copper atom is anchored by a patch of residues exposed at the molecular surface of intact ceruloplasmin but not of the proteolytically cleaved form [81]. The most buried residue, His426, is essential to the function of this site which, once reduced by a reductant, can be directly reoxidised either by oxygen, giving rise to ROS production, or by some other oxidant species, being excluded from the functional unit of the oxidase activity. By this mechanism, ceruloplasmin can cause oxidative damage to macromolecules and may, in particular, exert a role in low-density lipoprotein (LDL) oxidation by vascular cells [82, 83]. Although the actual radical that initiates LDL oxidation has not been identified in this case, the reductant species has been proposed to be superoxide, because experimental evidence has been provided that, in metal-ion-free medium, cell-derived superoxide accounts quantitatively for the contribution of vascular cells to ceruloplasmin-dependent LDL oxidation [82]. A different mechanism underlines the enhancement of lipid peroxidation by catalytic amounts of ceruloplasmin in the presence of iron plus glutathione (GSH)-derived thiols or ascorbate, a system which is also able to promote mutagenesis [84]. In this system, the thiols produced by enzymatic cleavage of GSH by γ-glutamyl transpeptidase are strong reductants, and ceruloplasmin appears to facilitate electron transfer from thiols to iron, rather than directly producing oxygen radical species.

Nitric oxide (NO) is a new entry to the list of substrates. Ceruloplasmin has been shown to favour formation of S-nitrosoglutathione (GS-NO), when added to cultured monocytes expressing inducible NO synthase (iNOS) [85]. Nitrosothiol adducts of NO function as NO transporters and are involved in modulation of intracellular and intercellular signal transduction [86, 87]. The role of ceruloplasmin would be the oxidation of NO released by cells to nitrosonium, NO^+, a species more reactive than NO in the reaction with thiols. The mechanism of this reaction is based on the well-known property of NO to act as a ligand for copper and to engage a redox reaction with the metal once bound to it [88]. The roles of NO as a ligand or as a redox reagent in the blue sites of copper pro-

teins are difficult to disentangle from each other since, upon binding of NO, these sites bleach irrespective of their reduction. NO appears unique among substrates of ceruloplasmin since it can reach and directly bind to blue copper sites, although with low affinity [89, 90]. Further, after binding of NO, the reduction of the copper which is necessary for the formation of NO^+ is very slow. Nevertheless, during studies on the effect of ceruloplasmin on inducible NOS, we also found that it induces formation of GS-NO in the presence of RAW cells expressing iNOS [unpublished results] and a role for ceruloplasmin in the metabolism of nitrosothiols has been confirmed in clinical studies [87].

The T1 site(s)/trinuclear-cluster-dependent oxidase action of ceruloplasmin extends over a large portion of the molecule. The moiety involved in amine oxidase activity includes two distinct substrate-binding sites on domains 4 and 6, which are specific for aromatic diamines and biogenic amines, respectively [46]. A site for lysergic acid diethylamide (LSD), known to act as a modulator of the oxidase activity of ceruloplasmin towards biogenic amines, has been located on domain 1. What is intriguing in this picture is that the binding site for biogenic amines is very close to a residue of the iron-binding site of domain 6, Asp1025, while that for p-phenylenediamine is a remote site on domain 4. This compound, a classic substrate for in vitro assays [91], is an unnatural substrate, likely representing a xenobiotic in the case of a higher organism.

Interest in ceruloplasmin-catalysed oxidation of arylamines and phenols has been the object of a long and detailed investigation, stimulated by the presence of catecholamines and 5-hydroxytryptamine neurotransmitters among those substrates that could relate ceruloplasmin to neurologic diseases [92–96]. These reactions were found to be quite complex. In the most schematic reaction mechanism, the initial one-electron oxidation generates partially oxidised intermediates, which then convert to the ultimate oxidation products by either further ceruloplasmin-catalysed oxidation and/or oxygen-radical-mediated reactions [3]. The possibility that ceruloplasmin is a regulator of biogenic amine levels in the plasma and, eventually, in the brain as outlined by these early studies, and the mode of action of ceruloplasmin in these processes, is currently controversial [97, 98], due to many factors. The main problem is related to the in vitro conditions of the assays that cannot mimic in vivo conditions for the action of ceruloplasmin; poor kinetic parameters are another reason, although chloride has been shown to selectively enhance the oxidase activity of ceruloplasmin towards amine substrates [30]. The picture is complicated by the extreme reactivity of the intermediates and by the involvement of oxygen radicals in these reactions. This is particularly true for the metabolism of 6-hydroxydopamine, a neurotoxin involved in Parkinson's disease, which is oxidised in vitro by ceruloplasmin [99, 100]. A clue to justify a role for ceruloplasmin in this and other metabolic processes has been its ability to oxidise substrates at the expense of oxygen without releasing ROS that would otherwise form if substrates were to engage in direct redox reactions with oxygen. This is a recurrent hypothesis about the mode of action of ceruloplasmin that also applies to its ferroxidase activity.

The top of the ceruloplasmin molecule appears particularly designed to permit this reaction, in that it is endowed with all structural elements, depicted in red in figure 3, necessary to facilitate the diffusion and the capture of ferrous iron, its oxidation and selective retention. X-ray structural studies have shown that charge distribution on the surface of the ceruloplasmin molecule is such that negatively charged regions, necessary for attracting positively charged metal ions, are present in this region [39]. Two additional copper atoms were found in these regions close to, and connected to by a suitable electron path, the T1 sites of domains 6 and 4, but not that of domain 2, in crystals of native ceruloplasmin. Copper is anchored by the same binding motif, one His and three carboxylates from Glu and Asp residues, in both sites, referred to as 'labile' metal-binding sites, due to the different coordination with respect to the prosthetic sites. An adjoining trivalent metal-binding site has also been found, suitable for Fe^{3+} binding, and exposure of ceruloplasmin crystals induced changes indicative of loss of copper from labile sites and rearrangement of these sites, thus providing a structural explanation for the evidence of activating effects of divalent cations, contrasted to inhibitory effects of trivalent ions, obtained in kinetic studies [3, 101–103]. Displacement of copper by ferrous ions is proposed to be the initial step in the ferroxidase catalytic mechanism, followed by translocation of the resulting Fe^{3+} to the trivalent-cation-binding sites [39]. The protein to which ceruloplasmin delivers ferric ions is transferrin [104, 105], a plasma protein which transports iron in the circulation. Ferroportin 1 is an iron export protein that mediates iron release from cells and is thought to deliver iron to ceruloplasmin, at least in cells of the reticuloendothelial system [106]. With a 'holding'-site role for trivalent-cation-binding sites, ceruloplasmin can sequester ferric ions on these sites specifically to deliver them to transferrin. By this mechanism, it can either promote iron efflux, by coupling transferrin to the iron export system of mobilisable iron stores, or prevent excessive intracellular iron accumulation, by inhibiting iron uptake by the transferrin-independent iron uptake system [107] that requires ferrous iron. An impairment of this process, due to the absence or dysfunction of either ceruloplasmin or transferrin, may give rise to selective and localised areas of metal accumulation or deficiency and tissue injury. Iron is particularly prone to engage redox reactions with oxygen, resulting in a flux of ROS against many molecules and cell structures [108].

Due to an impaired iron efflux process, individuals with aceruloplasminaemia show excessive iron deposition in selected tissues (liver, pancreas, glial cells), resulting in tissue injury. The pattern of tissue iron overloading of aceruloplasminaemics is matched by *Cp*-knockout mice [109] and differs from that observed in haemochromatosis, associated with mutations in the *HFE* or ferroportin 1 genes [110]. While both aceruloplasminaemia and haemochromatosis also result in systemic haemosiderosis and diabetes, only aceruloplasminaemics present with neural and retinal degeneration, which is consistent with the concept that ceruloplasmin expressed by glial cells, the GPI-linked form [59], promotes iron efflux from cells of the central nervous system, analogous with the role exerted by hephaestin in intestinal epithelium [111]. The severe pathology seen in brain and retina of these patients, resulting in dementia and movement disorders, associated with an increase in lipid peroxidation products in tissues [112], is consistent with ROS generation via the iron-catalysed Haber-Weiss reactions in these tissues. The ferroxidase activity of ceruloplasmin, followed by incorporation of ferric iron into transferrin, also suppresses the level of 'free' Fe^{2+} in the circulation. Thus the concerted iron-binding activity of transferrin and ceruloplasmin can be considered as a major anti-oxidant mechanism of the plasma which decreases with increasing transferrin saturation. Unbalance in this system is likely to occur in various physiopathological conditions during pregnancy, and increased oxidative stress and lipid peroxidation have in fact been detected in the pre-eclamptic state [113, 114].

Ceruloplasmin as a 'moonlighting' protein

Multifunctional proteins are now better understood as 'moonlighting' proteins which are able to change their function, switching between functions in response to changes in concentration of their ligand/substrate, their differential localisation and/or differential expression. Evolution of these proteins is thought to proceed by different mechanisms [10]. A new function can be the result of a different use of a pre-existing site, of a broadened specificity of an ancient site or of a modification of unused regions of protein surfaces, especially in the case of large molecules.

Ceruloplasmin appears to fulfil all these prerequisites. Following the hypothesis of an evolutionary pathway for ceruloplasmin from an ancestral cupredoxin [35], the three T1 sites of even-numbered domains are the only remnants of the blue site of the single domain of the ancestor initially adapted to serve the oxygen-binding site. Their characteristics and the features of their cognate binding sites for substrate, as already described, show that two of them are in the process of further adaptation to gain new functions. In this context, establishing whether the site on domain 4 is an ancient unmodified site now recruited to the metabolism of xenobiotics or an ancient site modified to cope with these compounds is quite difficult. Whatever the case, its function today is critical in view of the present risk of continuous exposure to pollutants for vertebrates.

Concerning differential expression, that ceruloplasmin gene expression and regulation are tissue-specific is now well established [115, 116]. Ceruloplasmin expressed by various tissues appears devoted to different activities, depending on the site of production. Hepatic cell synthesis of ceruloplasmin, which is responsible for the normal unevoked ceruloplasmin levels in adults, is increased by proinflammatory agonists of the acute-phase response [117, 118]. Estrogens and other hormones also increase hepatic cell synthesis of ceruloplasmin and are responsible for its high serum levels in pregnancy [119]. The lung is a prominent site of synthesis during inflammation, confined to pulmonary macrophages, and selectively during development and hyperoxia, providing an anti-oxidant defence to the tissues prior to exposure to higher concentrations of oxygen [120, 121]. In the vasculature, the synthesis of ceruloplasmin, confined to cells of monocytic origin, is also increased by proinflammatory cytokines [122].

Conditions that lead to substantial changes of acute-phase proteins, in response to an inflammatory process, include infection, trauma, surgery, tissue infarction and advanced cancer [123]. An elevation of plasma ceruloplasmin concentration commonly accompanies all these pathological states and malignancies, particularly metastatic cancers [124–128]. The elevated ceruloplasmin level in malignancy is only partly due to a generalised response to inflammation, because tumour cells themselves express ceruloplasmin [129] and the gene expression differs strongly with respect to the normal, non-cancerous, tissue. Many recent studies, based on different experimental approaches, have in fact shown that the ceruloplasmin gene is amongst the few genes expressed differentially and very early in certain types and subtypes of cancer, generally the more aggressive ones, with up-regulation levels well beyond those elicited by the inflammatory process [130–133]. The significance of dysregulation of the ceruloplasmin gene in tumours is not clear; it is generally believed that the changes found in cancers may not be completely random, because those favourable to the tumorigenesis process are usually selected for. Thus, the overexpression of ceruloplasmin in malignant cells of more aggressive tumours has to be related to some of its functional activities (see below) which are able to support tumour growth/survival.

The central nervous system has a peculiar glial-cell-specific ceruloplasmin expression [114, 115]. More important, a transcript encoding the GPI-linked form has been

detected in a variety of tissues in both humans and rodents [56, 59]. This means that tissues can selectively express the secreted or the membrane-anchored forms of ceruloplasmin. In contrast to the liver, which mainly expresses the secreted form, the brain almost exclusively expresses the GPI-anchored form of ceruloplasmin on the surface of astrocytes [59]. The presence of a ceruloplasmin form synthesised and retained within the brain has been correlated to the needs of brain tissues which are separated from the systemic circulation [116]. Due to the blood-brain barrier, circulating ceruloplasmin cannot reach these areas. The GPI-linked isoform is generated by alternative RNA splicing that leads to replacements of the final five amino acids in secreted ceruloplasmin with an alternative 30 amino acids that constitute a hydrophobic stretch [59]. The C-terminal tail is located outside the compact core of the molecule, thus, in the mechanism of anchoring, the flat region of the ceruloplasmin molecule is likely to be involved in the interaction with negative groups on the membrane, due to its slight prevalence of positive charges. Ceruloplasmin would be exposed on astrocyte surface with its three-tower-like region bearing the iron-binding and the iron-holding sites in a precise disposition with respect to the cell membrane and/or its partners. This localisation indicates a mechanism of action for membrane-anchored ceruloplasmin very different to that of secreted ceruloplasmin and specifically adapted to the metabolic requirements of these cells. In other words, brain ceruloplasmin might engage in a different and specific function in this area compared to secreted ceruloplasmin.

In contrast, circulating ceruloplasmin, free to offer whatever surface patches to ligands (substrates, effectors and interacting partners), can serve the needs of different tissues, the ligands being responsible for the switch of function. One case is the contradictory involvement of ceruloplasmin in both the antioxidant defence mechanism and in the host defence mechanism. Localisation of secreted intact ceruloplasmin in the vessel wall combined with an availability of free copper ions and localised, vascular-cell-derived, superoxide production is an example of how the normal anti-oxidant function of ceruloplasmin can be switched to a prooxidant activity, purposely directed to oxidatively damage invasive organisms, which results, however, in oxidation of LDL, probably as a side process [83]. The copper-ion-mediated oxidation of LDL is not exerted by ceruloplasmin bearing a polypeptide chain with a single nick by a specific protease [134]. The reason for cleaved ceruloplasmin molecules in the circulation has no plausible explanations to date and can be interpreted as another sort of switch occurring in some unknown event involving this protein. According to the X-ray crystal structure, the molecule of ceruloplasmin, with three proteolytic sites located within the most exposed and flexible regions of the loops excluded from the compact core of the molecule harbouring the copper sites [8], seems especially suited to sustain proteolytic attacks in these regions. For the same reason it would also tolerate, as expected for an exposed polypeptide segment, diverse sequence variations. Consistent with this hypothesis, mutation of these residues, Arg481, Arg701, and Lys887 to alanine in the human protein by site-directed mutagenesis results in a recombinant ceruloplasmin resistant to proteolytic degradation with improved stability of the protein towards denaturation [44].

'Moonlighting' of ceruloplasmin is a peculiar case since this protein takes part in the control of some metabolic pathways as an apparently redundant factor. Many types of functional responses are complicated by the action of several 'redundant' factors to insure proper control under a variety of conditions. The metabolism of transition metals involves a variety of proteins which have evolved to bind, transport and sequester the metal [68]. An involvement of ceruloplasmin in copper metabolism is likely to be masked in normal conditions, due to redundancy of the copper transport system which can also easily cope with its absence. The X-ray structure of human ceruloplasmin shows that the molecular surface has plenty of ligating motifs able to anchor loosely bound copper atoms, some of which are displaced from the substrates of the oxidase activity [39]. Copper released from these sites by this, or other events, may confer on ceruloplasmin the property of affecting an intracellular system while performing its oxidase activity by interacting with a proper site on a cell membrane, based on its ability to induce a transmembrane flux of copper [51]. Enhanced metal transfer, due to an increased ceruloplasmin concentration and/or its interacting system, can lead to derangement of a metabolic pathway. This is the mechanism by which ceruloplasmin is able to depress nitric oxide production by endothelial cells [135] resulting in an impaired endothelium-dependent relaxation of vessels [90]. The two conditions where ceruloplasmin can exert this effect are pregnancy and inflammation, based on the dose dependence of the inhibitory effect of ceruloplasmin, which completely suppresses the activity of endothelial nitric oxide synthase at 10 μM. As for its prooxidant activity against LDL, the inhibitory effect on NO production might be a dangerous side-effect.

Angiogenesis, an event common to a variety of physiological and pathological processes [136], is another copper-dependent process that might be supported by a high level of ceruloplasmin at a proper angiogenic site. Angiogenesis is required for tumour growth, and a sufficient level of copper appears to be required for new blood vessel formation. Ceruloplasmin, at high concentration, has proven to be a good source of copper ions that are accumulated at the apical growing cone of newly forming blood vessels, in tissues where neovascularisation occurs [137]. The increase of ceruloplasmin serum levels in pa-

tients bearing different tumours is generally correlated with its capacity to stimulate neo-angiogenesis, one of the main features of malignancy. Given that some key pro-angiogenic factors are regulated by trancription factor pathways involving hypoxia-inducible factor-1 [138], the presence of hypoxia-responsive elements in the promoter region of the ceruloplasmin gene [139] might be responsible for the overexpression of this protein in malignant cells.

The involvement of ROS in cancer development and proliferation is well known and neoplastic cells are generally believed to be under persistent oxidative stress [140]. Consistent with the concept that a high level of anti-oxidants would favour tumour survival, a significant association between ceruloplasmin and glutathione peroxidase 3 genes has been found in a study of the genes overexpressed in epithelial ovarian cancer [141]. Thus, the capability to take part in the angiogenic process and in anti-oxidant defence, which are two activities of ceruloplasmin that could benefit the organism under some pathophysiological conditions, such as neovascularisation of injured tissues, in the case of tumorigenesis would facilitate, rather than counter, cancer progression. Although a precise role for ceruloplasmin in cancer development and progression has not been established, ceruloplasmin levels in malignancies repeatedly correlate with the degree of disease activity. It has also been proposed as a diagnostic and prognostic marker of cancer [126, 142]. On the other hand, it is becoming a useful therapeutic marker, based on the results of the recent approach of copper control as an anti-angiogenic anti-cancer therapy [143]. These trials are successfully exploiting the properties of the copper chelator tetrathiomolybdate, a compound that has been identified as responsible for derangement of copper metabolism by virtue of its effects on ceruloplasmin [144].

An overall conclusion should stress that ceruloplasmin is ubiquitous among vertebrates, its serum levels are low at birth and increase rapidly towards adult levels after parturition [145, 146]. It then changes during the life-span, following the different physiological and pathological conditions of the organism, behaving as a protein that senses a change in the organism and then taking part in multiple pathways as part of an overall response.

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