



# Looking for a partner: ceruloplasmin in protein–protein interactions

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**Abstract** Ceruloplasmin (CP) is a mammalian blood plasma ferroxidase. More than 95% of the copper found in plasma is carried by this protein, which is a member of the multicopper oxidase family. Proteins from this group are able to oxidize substrates through the transfer of four electrons to oxygen. The essential role of CP in iron metabolism in humans is particularly evident in the case of loss-of-function mutations in the *CP* gene resulting in a neurodegenerative syndrome known as aceruloplasminaemia. However, the functions of CP are not limited to the oxidation of ferrous iron to ferric iron, which allows loading of the ferric iron into transferrin and prevents the deleterious reactions of Fenton chemistry. In recent years, a number of novel CP functions have been reported, and many of these functions depend on the ability of CP to form stable complexes with a number of proteins.

**Keywords** Ceruloplasmin · Protein–protein interactions · Acute phase reactants · Neutrophilic proteins

## Introduction

Ceruloplasmin (CP), which was described for the first time almost 75 years ago (Holmberg 1944), was subsequently characterized in detail (Holmberg and Laurell 1948, 1951) and named according to its blue colour and the biological fluid (i.e., blood plasma) where it is usually present as a component of the alpha-2-globulin fraction. When the capacity of CP to oxidize  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  was discovered and the physiological role of this reaction was established (Osaki et al. 1966), the protein received a systematic name and number from the Enzyme Commission, ferro- $\text{O}_2$ -oxidoreductase (EC 1.16.3.1). An abbreviated term, ferroxidase, is commonly used. CP is one of the so-called “blue proteins” that have a common evolutionary history. These proteins include a small group of “blue oxidases” (comprising CP). Solutions of these proteins have intense blue colour due to the presence of type I copper with an intense absorption at 610 nm (see below). The blue oxidases display considerable amino acid sequence homology and have a similar polypeptide chain folding pattern, a so-called “cupredoxin” structure.

CP is found in the blood plasma of virtually all mammals; however, to date, the human protein has been studied the most. Hence, a large portion of the data presented in this manuscript refers to human CP.

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## Basic structural features of ceruloplasmin

A controversy concerning the number of polypeptide chains in CP arose from early studies that proposed dimeric (Shokeir 1973), tetrameric (Simons and Bearn 1969; Freeman and Daniel 1973; McCombs and Bowman 1976) and octameric (Poulik 1962, 1968; Kasper and Deutsch 1963; Poillon and Bearn 1966) structures of CP. The hypothesis of a single-chain structure, first proposed by Rydén (1972), was confirmed by sequencing a polypeptide composed of 1046 amino acids (Takahashi et al. 1984). *N*-glycoside bonds link oligosaccharides to Asn residues in the polypeptide chain. The predominant species (CP-I) contains four oligosaccharides shaped as two or three antennas, and the minor form (CP-II) has only three sugar-binding sites (*ibid.*). Soon after these findings, the gene encoding single-chain CP was characterized (Koschinsky et al. 1986; Royle et al. 1987).

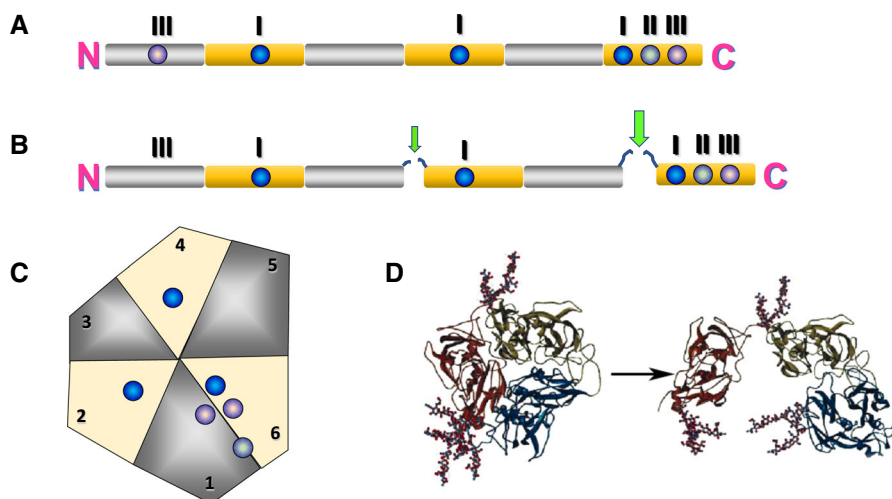
The molecular mass of fully glycosylated CP was determined in a crystallographic study as  $132 \pm 4$  kDa (Magdoff-Fairchild et al. 1969). Approximately 120 kDa of that molecular mass accounts for the protein, while approximately 12 kDa accounts for the carbohydrates.

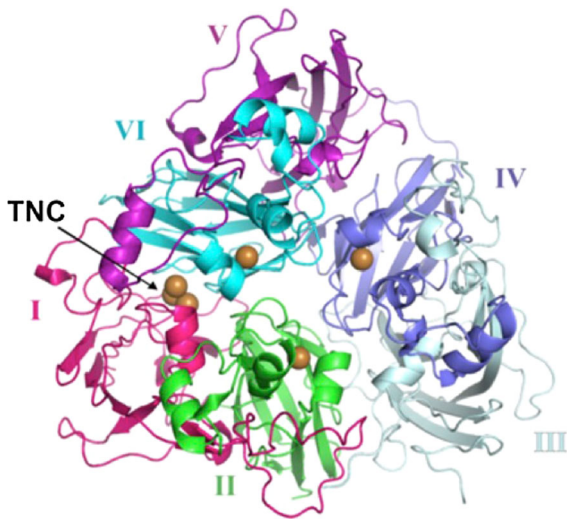
An important feature of CP is its internal homology. The polypeptide chain consists of three homologous cupredoxin domains (Ortel et al. 1984). Each of the domains contains two structurally different parts. Hence, CP comprises six domains, of which the plastocyanin-like even units differ from the odd units

that are also homologous to one another but have no similarity with domains 2, 4, and 6 (Fig. 1a).

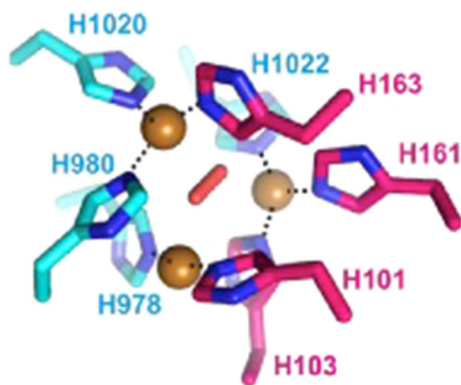
An X-ray study of human CP crystals at 3.1 Å resolution revealed six tightly bound copper ions (Zaitseva et al. 1996) (Fig. 2) classified into three types according to their spectral features (Fee 1975). The copper ion ligands are the same as those in other multicopper oxidases (laccase and ascorbate oxidase), i.e., His or a combination of His + Cys/Met residues. The three copper ions in CP belong to type I with strong absorption at 610 nm (“blue” copper). These copper ions are coordinated by two nitrogen atoms from His imidazole rings, one sulfur atom from Cys, and another sulfur atom from Met, providing a distorted tetrahedral geometry. This structure may be considered intermediate between the shape of a regular tetrahedron and the square-planar coordination of  $\text{Cu}^{2+}$  in low-molecular complexes and in non-blue copper proteins (Lu et al. 1993). The square-planar structure is typical of the binding site for type II  $\text{Cu}^{2+}$  in CP. The remaining two type III  $\text{Cu}^{2+}$  ions are located in close proximity to one another; the uncoupled electrons in their outer orbitals are compensated for, which forms a diamagnetic centre in CP. These diamagnetic ions together with type II  $\text{Cu}^{2+}$  form a trinuclear cluster (Figs. 1c, 3) that stabilizes the spatial structure of CP by buckling the holo-protein *N*- and *C*-termini (Vachette et al. 2002), which sustains the compact folding of the molecule (ca.  $214 \times 85$  Å). The distribution of the copper ions among the CP domains is shown in Fig. 1a and c. In blood plasma, the tightly bound copper ions are not easily released

**Fig. 1** Schematic representation of the six-domain structure of CP, the location of type I, II and III copper ions in its molecule, and the peptide bonds most susceptible to proteolysis





**Fig. 2** A cartoon representation of human CP domain structure and the six copper ions, with TNC as ‘trinuclear centre’. (Modified from Samygina et al. 2017)



**Fig. 3** The trinuclear centre of human CP formed by His residues, in which nitrogen atoms are coloured blue; copper ions are light-brown and dioxigen is pictured as red stick. (Modified from Samygina et al. 2017)

from CP. To leave the protein,  $\text{Cu}^{2+}$  must usually be reduced through the interaction of CP with another protein or with the cell surface. Extraction of the copper ions *in vitro* requires strong reducing agents (Vassiliev et al. 1997), and after the extraction, the overall conformation of CP becomes less compact (Fig. 1d).

Copper ions are incorporated into plasma CP during its biosynthesis in hepatocytes (Sato and Gitlin 1991; Terada et al. 1995; Hellman et al. 2002). The “copper pump” ATP7B (the Wilson disease protein) within the

trans-Golgi complex acquires Cu from the metallochaperone ATOX1 and loads it into CP (Lutsenko et al. 2007). The liver is the principal producer of CP, though some other organs synthesize the protein, e.g., the mammary glands, placenta, brain (*plexus choroideus*), and kidneys (Thomas et al. 1989; Wooten et al. 1996; Donley et al. 2002; Linder 2010). In addition, mononuclear cells and macrophages produce CP under inflammatory conditions (Bielli and Calabrese 2002; Banha et al. 2008; Qi et al. 2016). However, extrahepatic CP species are mostly membrane-linked, anchored to the cell surface by the glycosylphosphatidylinositol (GPI) moiety, and have an extra stretch of 30 amino acids at the anchored protein C-terminus that replace five C-terminal amino acids from plasma CP due to alternative splicing (Patel and David 1997; Patel et al. 2000). A peculiar form of GPI-CP with 4 extra amino acids was found in the placenta (Yang et al. 1990). Overall, GPI-linked CP is present in quite a few organs, including the liver, where it is exposed on the surface of hepatocytes (Mostad and Prohaska 2011; Marques et al. 2012). Once purified, GPI-CP has ferroxidase activity (Mostad and Prohaska 2011) and is assumed to carry all 6 tightly bound copper atoms.

A number of studies have identified binding sites for loosely bound  $\text{Cu}^{2+}$  (“labile” copper) (Zaitseva et al. 1996; Lindley et al. 1997; Mukhopadhyay et al. 1997; Bento et al. 2007). The binding sites for labile copper differ from those in which  $\text{Cu}^{2+}$  is tightly bound and can be occupied by various metals, such as Fe, Co, and Ni (Lindley et al. 1997; Samygina et al. 2008). A recent paper by Samygina et al. (2017) presented data on rat CP and revealed a novel binding site for labile copper. Amino acid sequences of human and rat CP are highly homologous; however, the newly found binding site for labile copper appears to be a specific feature of the rat protein and makes rat CP more resistant to limited proteolysis.

Human CP is quite vulnerable to proteolytic enzymes (Moshkov et al. 1979; Sang 1995). For several years, the products of proteolytic cleavage isolated from human plasma were considered CP subunits, and obtaining proof of the single-chain structure of CP required considerable efforts (Rydén, 1971; Kingston et al. 1977). The limited proteolysis of CP always follows the same (or very similar) pattern, where the first cleavage occurs within the peptide loop between domains 5 and 6 (aa 885–892) which is

exposed outward from the compact protein globule, thus serving a perfect target for trypsin-like proteases. After this first proteolytic damage, the globule may become partially unfolded, at least to an extent that allows several other peptide bonds to become exposed and cleaved, thus producing a certain number of peptide fragments that vary in size (Fig. 4). If certain precautions are taken, non-proteolysed human CP can be purified from fresh serum (Sokolov et al. 2005a, 2012). The majority of the data on CP proteolysis is based on *in vitro* experiments with highly purified non-fragmented protein. It should be noted that CP subjected to limited proteolysis does not dissociate into fragments and maintains a relatively compact structure while retaining its copper. Separation of the fragments *in vitro* requires disulfide bond reduction and treatment with denaturing agents (Prozorovski et al. 1982). Until recently, no specific protease had been identified as the CP cleavage effector; hence, the cleavage was assumed to occur due to a coagulation factor, e.g., plasmin, which attacks CP during the purification process (Moshkov et al. 1979). The most convincing data so far were obtained in 2015, indicating that thrombin is the protease that causes the limited proteolysis of CP both *in vitro* during purification and *in vivo*, at least at inflammatory foci (Sokolov et al. 2015a). Limited proteolysis was shown to abrogate the inhibitory effects of CP on various proinflammatory enzymes including myeloperoxidase (Panasenko et al. 2008;

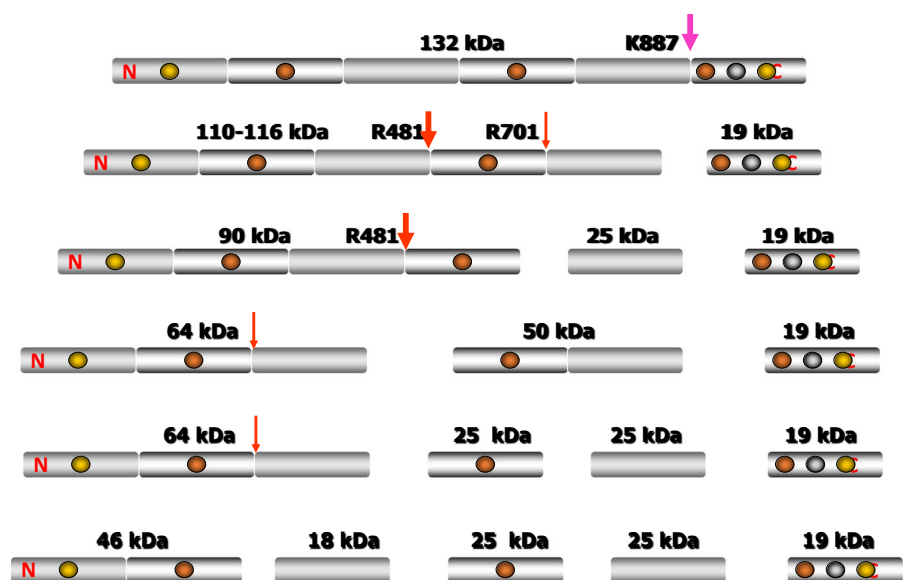
Sokolov et al. 2008; Samygina et al. 2013), eosinophil peroxidase (Sokolov et al. 2015b), and 5-lipoxygenase (Sokolov et al. 2010).

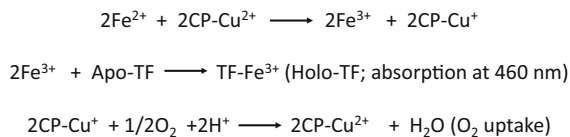
### Functions of ceruloplasmin

Bielli and Calabrese (2002) had every reason to term CP a “moonlighting protein” because of its multifunctionality. Indeed, presently, this protein has a number of known functions, and some of the functions have been revealed in the last two decades. The physiological role of CP is not limited to Fe<sup>2+</sup> oxidation (Fig. 5), even though a deficiency in the CP gene in humans (aceruloplasminaemia) provokes oxidative stress from the accumulation of ferrous iron in tissues (Vassiliev et al. 2005). The details of CP participation in iron metabolism are discussed below.

CP has activities of ferroxidase (Osaki 1966), cuproxidase, which catalyses Cu<sup>+</sup> oxidation (Stoj and Kosman 2003), superoxide dismutase (Vasilyev et al. 1988), glutathione-linked peroxidase (Kim and Park 1998) and NO-oxidase (Shiva et al. 2006); accordingly, CP actively precludes the formation and persistence of free radicals. These properties make CP an effective antioxidant that prevents oxidative damage to proteins and lipids (Samokyszyn et al. 1989). Plasma concentrations of CP in inflammation can increase from 3 to 10 μM, suggesting a role for CP in the regulation of inflammatory reactions. The

**Fig. 4** The scheme of human CP proteolytic cleavage. Arrows mark the sites of a protease attack





**Fig. 5** The ferroxidase reaction catalyzed by CP according to Osaki (1966)

proinflammatory activities of certain enzymes are suppressed when they form complexes with CP. These enzymes include myeloperoxidase (Sokolov et al. 2008; Samygina et al. 2013), members of the serprocidin family (elastase, cathepsin G, and proteinase 3) (Sokolov et al. 2007b), matrix metalloproteinases 2 and 12 (Sokolov et al. 2009a), 5-lipoxygenase (Sokolov et al. 2010), and eosinophil peroxidase (Sokolov et al. 2015b). Importantly, the inhibition of the mediators of inflammation listed above is not directly linked to CP oxidase activity. As specified by Chapman et al. (2013), ceruloplasmin has both antioxidant and prooxidant properties. Even the ferroxidase activity of CP that has been regarded as an antioxidant property of CP for a long time, was shown to evoke its prooxidant effects, at least in some cases, such as in patients with localized aggressive periodontitis. Neutrophils from patients synthesize CP, which oxidizes  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ , and the ferric ions activate NADH oxidase, increasing the production of reactive oxygen species (Iwata et al. 2009).

It is generally assumed that the functions of CP strongly depend on the presence of copper ions in the CP molecule (Linder 2016). This assumption appears to be correct not only in the case of direct participation of the copper ions in CP functions, such as substrate oxidation, but also in the case of indirect impacts, e.g., the absence of some tightly bound  $\text{Cu}^{2+}$  or occupancy of the corresponding sites in CP by labile copper. These variations in CP structure modify the spectrum of its functions.

Of the six tightly bound copper atoms, one “blue” and three “non-blue”  $\text{Cu}^{2+}$  ions form a catalytic centre typical of blue oxidases (Rydén 1982; Zaitseva et al. 1996). This centre accomplishes a four-electron transfer to oxygen (Farver et al. 1999), with two water molecules as the end products. The precise role of the two remaining type I ions in the CP molecule should be explored.

The functions of CP have been generally viewed as enzymatic based on its capacity to transfer electrons

from various substrates to oxygen (Linder 2016). Numerous substrates have different chemical structures and therefore bind to distinct binding sites on the CP molecule (Zaitseva et al. 1996; Zaitsev et al. 1999; Bielli and Calabrese 2002; Sokolov et al. 2009b). This feature is important considering that interactions of CP with other proteins to form complexes often cause modifications of its substrate-binding sites, which, in turn, strongly affect its enzymatic functions. Sometimes, the binding sites may become inaccessible and substrates are not oxidized; this happens to biogenic amines when CP forms a complex with lactoferrin (LF) (Sokolov et al. 2009b). Otherwise, conformational changes in CP resulting from interactions with partner proteins can improve the binding and accelerate the oxidation of  $\text{Fe}^{2+}$  or *ortho*-phenylenediamine (*ibid.*). It appears likely that the high-affinity binding site for LF includes amino acid ligands for the trinuclear cluster of copper atoms in the CP catalytic centre (Sokolov et al. 2007a, b), thus explaining the marked changes in the oxidase activity of CP. However, CP also causes apparent alterations in the structure and functions of proteins with which it forms complexes. The ability of CP to engage in selective protein–protein interactions resulting in the formation of somewhat stable complexes should be regarded as instrumental for the realization of a number of functions independent of its enzymatic properties (see below).

The catalytic activity of CP was generally outlined by the scientists who discovered CP and described its capacity to oxidize aryldiamines, diphenols, and other substrates including ascorbate, hydroxylamine, and thioglycolate (Holmberg and Laurell 1948). Earl Frieden and co-workers studied the CP-catalysed oxidation of a number of substances in detail and proposed the following three major groups of substrates (McDermott et al. 1968):

- (1)  $\text{Fe}^{2+}$  is the substrate with the highest  $V_{\text{max}}$  and the lowest  $K_{\text{m}}$ ;
- (2) a long list of bifunctional aromatic amines and phenols (including two groups of biogenic amines, i.e., epinephrine with 5-hydroxyindole derivatives and phenothiazine-derived substances);
- (3) numerous reductants capable of reducing  $\text{Fe}^{3+}$  or the partially oxidized (free radical) intermediate forms of the substrates from group 2.



These substances are considered pseudosubstrates.

In principle, any reductant can act as a CP substrate provided that it is able to donate an electron to the oxidized enzyme without blocking its own autooxidation (Frieden 1980).

A relatively large spectrum of substrates oxidized by CP was thoroughly studied by Young and Curzon (1972). The authors investigated 37 substrates, and the  $V_{\max}$  values obtained for each of the substrates differed by less than 8-fold ( $1.3\text{--}10.8 \text{ e}^- \text{ Cu}^{-1} \text{ min}^{-1}$  at pH 5.5;  $K_{\text{cat}} = 0.15 - 1.26 \text{ s}^{-1}$ ). For 20 substances in that group, the variability in their  $K_m$  values was over  $6 \times 10^3$ . The authors concluded that the rate limit of the oxidase reaction catalysed by CP does not depend on the nature of the substrate.

The ferroxidase reaction is similar in this regard; in this reaction, CP oxidizes  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ , and this reaction is usually considered separate from the oxidation of other substrates (Fee 1975). The ferroxidase activity of CP was described for the first time in the early 1960s (Curzon and O'Reilly 1960; Curzon 1961) and was thoroughly studied later in the laboratory of Earl Frieden (Osaki et al. 1966; Osaki and Walaas 1968; McKee and Frieden, 1971). Since then, this activity has been regarded as the principal feature of CP and has been described as “ferro- $\text{O}_2$ -oxidoreductase” activity.

The first work by Frieden's group (Osaki et al. 1966) indicated that CP plays an important role in iron metabolism. Indeed, the authors showed a considerable increase in iron loading into apo-transferrin accompanied by stoichiometric consumption of oxygen in the presence of pure CP. The impact of CP on iron metabolism was confirmed by the work of Miyajima et al. (1987). They were the first to describe an autosomal recessive neurological disorder in a 52-year-old woman who manifested symptoms resembling Parkinson's disease and had blepharospasm, retinal degeneration, and *diabetes mellitus*. The patient and two relatives had extremely low plasma CP levels. Computer tomography revealed high-density areas in the patient's basal ganglia and *substantia nigra*. A liver scan also suggested the accumulation of iron, and serum ferritin levels were very high. A ferrokinetic analysis with  $^{59}\text{Fe}$  showed the prolonged retention of the injected isotope in the liver and spleen. The authors suggested that iron deposition in the

neurons of the basal ganglia and in other organs was caused by the lack of CP and resulted in the observed symptoms.

The involvement of CP in iron metabolism became more evident when aceruloplasminaemia emerged as a nosology and patients were thoroughly examined. Logan et al. (1994) were the first to report an Irish family in which two brothers had no detectable CP in their serum; the CP levels were also strongly diminished in sera from twelve other relatives. Both brothers had low serum iron levels, while the liver iron content was increased. The brothers presented with dementia and *diabetes mellitus*. The authors determined the autosomal recessive transmission of the trait and revealed the genetic linkage between CP deficiency and specific polymorphic markers flanking the ceruloplasmin gene on chromosome 3q25. Treatment with pure CP significantly increased serum iron in the index patient.

Then, two papers from Nobuo Yanagisawa's group described a Japanese family with extremely low levels of plasma CP; the family manifested extrapyramidal disorders, cerebellar ataxia, and *diabetes mellitus* (Morita et al. 1995; Yoshida et al. 1995). Excessive iron deposition was found in post-mortem specimens from the brain, liver, and pancreas of the proband. The lack of CP function was most likely caused by a mutation affecting CP pre-mRNA splicing; the mutation introduced a premature termination codon that led to subsequent truncation of CP at the C-terminus.

At the same time, Jonathan Gitlin and co-workers published a detailed molecular analysis of genetic material obtained from the patient previously studied by Miyajima et al. in 1987 and her daughter (Harris et al. 1995). In both cases, DNA sequence analysis revealed a 5-bp insertion in exon 7 of the CP gene, resulting in a frame-shift mutation and a truncated open reading frame. In this work, aceruloplasminaemia was described for the first time as “an autosomal recessive disorder of iron metabolism”.

Gitlin's group performed molecular analysis on aceruloplasminaemia using DNA samples from affected humans (Klomp and Gitlin 1996; Takahashi et al. 1996; Harris et al. 1996, 1998) and also modelled the condition in mice (Harris et al. 1999). Having obtained CP(−/−) mice, the scientists observed progressive accumulation of iron in the animals by one year of age and registered a significant increase in serum ferritin and iron content in the animals' liver

and spleen. The animals had abundant stores of iron in reticuloendothelial cells and hepatocytes, and the efflux of iron from these cells was strongly reduced; the condition was efficiently corrected by administration of CP. Additionally, CP provided for the egress of iron from duodenal epitheliocytes (Cherukuri et al. 2005) and glial cells (De Domenico et al. 2007). These results underscored the role of CP in the mobilization of iron from storage cells, thus validating the primary data obtained in Earl Frieden's laboratory (Osaki and Johnson 1969; Osaki et al. 1971).

At the same time, another facet of CP's participation in iron metabolism was studied by Steven Aust and co-workers, who showed that CP is needed not only to release the deposited iron but also to secure iron storage by loading the metal into apo-ferritin (Van Eden and Aust 2000). The authors also reported a protein–protein interaction resulting in the formation of a CP:ferritin complex during iron loading into ferritin (Reilly et al. 1998).

### **Protein–protein complexes formed with CP and their role in pathological processes**

Starting from the 1990s, the interactions of CP with other proteins were described, expanding the list of possible CP functions to include participation in iron metabolism by interaction with ferritin (Van Eden and Aust 2000), ferroportin 1 (Jeong and David 2003), and lactoferrin (Zakharova et al. 2000); regulation of neural transmission and inflammation by interaction with neuropeptide PACAP38 (Tams et al. 1999) and macrophage migration inhibitory factor (Meyer Siegler et al. 2006), respectively; inhibition of the prooxidative properties of myeloperoxidase (MPO) by forming a complex with MPO (Segelmark et al. 1997); and regulation of blood clotting by interaction with protein C (Walker and Fay 1990). The assumption of the latter interaction was supported by the fact that activation of CP-like coagulation factors FV and FVIII via limited proteolysis allows these proteins to adopt conformations that favour the formation of their complexes with FIXa and FXa; these complexes participate in the subsequent activation of the coagulation cascade. Anticoagulant protein C is able to inhibit coagulation through FVa and FVIIIa proteolysis. Hence, it was suggested that CP, which has regions of amino acid homology to the protein

C-binding sites of FVa and FVIII (Church et al. 1984; Pemberton et al. 1997; Shen et al. 2008), competes with these factors for binding with protein C and thus participates in the regulation of blood clotting. Indeed, the complex formed by protein C and CP increases the ferroxidase activity of CP by fivefold and is uncoupled by the HAGMETTYTV decapeptide that mimics the sequence between amino acids 1028 and 1037 in CP. Concomitantly, the elevated ferroxidase activity of CP was abrogated. The possibility of direct involvement of CP in the regulation of blood coagulation should be thoroughly explored, even though the data on the formation of the CP:protein C complex appear quite reliable.

The first report describing CP interactions with LF was published in 2000; LF is a protein in the transferrin family detected in milk and other secretions as well as in neutrophilic leukocytes (Zakharova et al. 2000). The authors observed an unusual retardation in CP mobility when breast milk proteins were subjected to electrophoresis. This and subsequent studies detected a strong complex formed *in vitro* by CP and LF isolated either from breast milk or lacrimal fluid (Pulina et al. 2002). The latter publication suggested that CP facilitates iron incorporation into apo-LF; this was subsequently confirmed in a study of CP ferroxidase activity, which was markedly increased by LF (Sokolov et al. 2005b, 2009b). The study also showed that loading of iron into apo-LF by CP is much faster than loading of iron into apo-transferrin from blood plasma; apparently, this is an important feature of CP as a well-known participant in the acute phase of inflammation. It was suggested that LF released from neutrophils in inflammatory foci mostly as apo-protein rapidly forms a complex with CP and captures Fe<sup>3+</sup> oxidized by CP (Sokolov et al. 2005b).

The stable CP:LF complex was subsequently isolated from breast milk and its 1:1 stoichiometry was established (Sokolov et al. 2006). This finding was confirmed by a small-angle X-ray scattering study (Sabatucci et al. 2007). The authors also showed that major conformational rearrangements to either protein do not occur within the complex (*ibid.*).

At the same time, the notion that the formation of the CP:LF complex is a manifestation of an inflammatory process was supported by detection of the complex in sera from 80 patients with various inflammatory diseases and in 45 samples of serum and pleural fluid from patients with pleurisy with

various aetiologies (cancer, tuberculosis, postoperative state, etc.) (Sokolov et al. 2007a).

This finding provided a broader outlook for the role of CP in inflammation. During the acute phase of inflammation, neutrophils secrete up to 30 g of LF daily (predominantly the apo-form of LF), and approximately 10 g of LF remains in the bloodstream (Sawatzki 1987). Approximately half of the plasma iron can be bound by apo-LF, and the binding is facilitated by formation of a complex with CP, especially because the ferroxidase activity of the latter increases (Sokolov et al. 2009b). Iron becomes sequestered and is cleared from the plasma; additionally, the plasma concentration of iron can be decreased due to the restriction of circulating iron levels by hepcidin, a protein that prevents iron efflux from cells (Kemna et al. 2008). Hence, formation of the CP:LF complex may be a mechanism that allows an organism to protect itself against the neutrophil respiratory burst occurring in a focus of inflammation (Sokolov et al. 2009b). In a subsequent study, CP was proved to be a factor that opposes respiratory burst by attenuating the activation of neutrophils (Varfolomeeva et al. 2016).

At approximately the same time of that discovery, a novel function of CP was reported: its ability to stabilize ferroportin 1 on the cell surface (De Domenico et al. 2007). It had been known that a GPI-anchored species of CP (GPI-CP) can be present on the surface of astrocytes (Jeong and David 2003), likely due to the intracellular pathway described by Polishchuk et al. (2004). Stabilization of ferroportin 1 by CP and colocalization of these two proteins on the astrocyte membrane suggested an essential role for GPI-CP in iron egress from these cells. In fact, this feature of CP led to the suggestion that this protein is an important factor regulating iron homeostasis.

However, the regulation of iron metabolism by CP is unlikely to be limited only to this function. Formation of the stable CP:LF complex was suggested as another regulatory step in iron metabolism (Sokolov et al. 2009b). In addition to participation in the pathway regulating ferroportin-mediated iron export, the CP:LF complex may play an important role during the early stages of iron import from the intestine to the blood (*ibid.*). Incorporation of  $\text{Fe}^{2+}$  into a plasma carrier protein requires  $\text{Fe}^{2+}$  oxidation by hephaestin, a homologue of CP tethered to enterocyte membranes. The carrier role is normally

performed by plasma transferrin, which is unlikely to form a complex with CP or hephaestin (Hudson et al. 2008). Therefore, *in vivo* formation of a specific complex involving CP and LF may be an accessory link between the iron and copper metabolic pathways (Sokolov et al. 2009b).

A study in patients with inflammatory diseases revealed another stable plasma complex formed by CP and MPO (Sokolov et al. 2007a). The MPO enzyme is an acute phase reactant released from neutrophils in inflammation foci (Klebanoff 1970; Kettle and Winterbourn 1997). A triple “CP:LF:MPO” complex was discovered in a number of serum samples obtained from patients (Sokolov et al. 2007a). The authors investigated this complex under various conditions and suggested a 1:1:1 stoichiometry. In contrast, photon correlation spectroscopy results supported a pentameric arrangement 1MPO:2CP:2LF (Sokolov et al. 2009c). Subsequently, a specific complex formed by CP and eosinophil peroxidase (EPO) was described and was shown to have similar characteristics to those of the CP:MPO complex (Sokolov et al. 2015b).

The ability of CP to inhibit the production of hypochlorous acid by MPO was shown quite a while ago by Taylor and Oey (1982), and several years later, this ability was designated as another major function of CP (Segelmark et al. 1997). Later, Park et al. (2000) showed that CP is able to bind MPO without any major alterations of the principal properties of both proteins. This finding was of interest because the formation of the complex influenced the enzymatic properties of both proteins (Sokolov et al. 2008). CP strongly suppresses the peroxidase and chlorinating activities of MPO when the external peptide loop (amino acids 885–894) connecting domains 5 and 6 of CP enters the MPO haem pocket, thus creating a barrier for MPO substrates (Samygina et al. 2013). The mechanism of EPO inhibition is essentially the same. The entry of the peptide loop into the haem pocket, monitored by a shift in the Soret band in the spectra of peroxidases, provided a secure shielding of the scissile K887–V888 peptide bond within the loop, thus protecting CP from proteolysis (Sokolov et al. 2008; Samygina et al. 2013).

Formation of the CP complex with MPO and EPO is likely to change the CP conformation in the vicinity of the binding site of such substrates as *p*-phenylenediamine so that it is more rapidly oxidized by the CP



complex. It should be noted that after formation of the complex with MPO or EPO, CP retains its ferroxidase activity (Sokolov et al. 2015b). The physiological importance of these interactions implies the ability of CP to regulate inflammation by controlling the production of inflammatory products including HOCl, HOBr, and HOSCN. HOCl and HOBr are the most harmful of these products, at least with respect to their damaging effects on low-density lipoproteins, resulting in the accumulation of cholesterol in monocytes or macrophages (Sokolov et al. 2014). Moreover, the deleterious effect of MPO on the synovium in the joints of patients suffering from rheumatoid arthritis is likely to be due to MPO halogenating activity (Sokolov et al. 2015a; see below).

These data and earlier reports on the involvement of CP-MPO interactions in pathological processes (Griffin et al. 1999) stimulated a study on the associations of CP with other leukocyte proteins apart from LF, MPO, and EPO. The first report on the interactions of CP with five cationic proteins, i.e., cathepsin G, eosinophilic cationic protein, neutrophil elastase, proteinase 3, and azurocidin, was published by Sokolov et al. (2007b). The authors suggested that the interaction of antimicrobial cationic proteins with CP may reduce their antimicrobial activities and cytotoxic effects upon their secretion into the bloodstream. Similar to LF and MPO, the cationic proteins identified in the study can behave as autoantigens provoking systemic vasculitis (Malenica et al. 2004; Jennette et al. 2011). Their selective interaction with CP may be part of a mechanism regulating disease pathogenesis, and CP is likely to play the key role in this regulation. Generally speaking, CP seems to be an important part of the immune response; as a partner for a number of neutrophil proteins it behaves as an immunomodulator (Sokolov et al. 2007b; Varfolomeeva et al. 2016).

Specific attention was focused on the interactions of CP with proteases and on the limited proteolysis of the CP molecule, which affects its functions. As mentioned above, early studies proved the crucial role of proteolytic cleavage in the formation of large fragments previously regarded as CP subunits (Rydén 1971, 1972; Moshkov et al. 1979). Several laboratories detected the same or very similar sets of fragments due to “spontaneous” proteolysis during purification of CP from plasma or after storage of apparently pure protein (Rydén 1971; Kingston et al. 1977;

Prozorovski et al. 1982). The key importance of CP integrity for its interactions with other enzymes, which significantly modifies their activity, was documented much later (Sokolov et al. 2008, 2010).

Meanwhile, *in vitro* experiments showed that neither plasmin, nor neutrophil elastase regarded as enzymes likely to hydrolyze CP would cut this protein so as to produce the set of large fragments originating upon ‘spontaneous’ proteolysis. The CP polypeptide chain is cleaved into fragments that have different molecular masses from those that “spontaneously” appear. A plausible explanation for this discrepancy was proposed by Sokolov et al. (2009a) who noted that CP forms complexes with matrix metalloproteinases (MMPs) 2 and 12 during purification. These preparations contained complexes of CP with MMPs and the 19-kDa C-terminal fragment of CP; however, neither the typical large proteolytic fragments nor, especially, the products of deep proteolysis, which yields short amino acid sequences, were detected. As suggested by the authors, the complex formed by CP with MMP2 and MMP12 results in the detaching of the C-terminal fragment; however, subsequent cleavage of the molecule does not occur.

The TLKVFQPRRK loop is composed of amino acids 885–894 and links domains 5 and 6 in CP; this loop is the primary target of the proteolytic attack. It has high mobility and a poorly ordered structure that is not “resolved” even when highly purified preparations of non-degraded CP are used for X-ray studies (Samygina et al. 2008). The role of this loop in retaining the overall structure of CP should not be underestimated considering the loss of a number of CP functions after its cleavage. Indeed, if this loop is cleaved, CP loses its glutathione-dependent peroxidase activity (Kim and Park 1998), the ability to efficiently load Fe<sup>3+</sup> into ferritin (Van Eden and Aust 2000) and the safe inhibition of the prooxidant features of MPO (Panasencko et al. 2008; Sokolov et al. 2008).

Hence, limited CP proteolysis can be regarded as one of the biochemical mechanisms reducing the antioxidant defence of the body. This hypothesis is supported by the fact that increased proteolytic activity of blood plasma in haemophilic patients results in a decrease in antioxidant indices and pronounced oxidative stress (Brummel-Ziedins et al. 2009; Chen et al. 2013). In these plasma samples, CP is subjected to considerable proteolytic degradation.

The importance of limited CP proteolysis in the regulation of pathological processes was confirmed in a study of the interrelations of CP, MPO, and thrombin (Sokolov et al. 2015a). The authors studied synovial fluid samples from the affected joints of patients with rheumatoid arthritis, a systemic autoimmune disease characterized by synovial inflammation leading to progressive cartilage and bone destruction (Scott et al. 2010). It was shown that the ability of CP to inhibit MPO depends on the thrombin activity that cleaves CP (Sokolov et al. 2015a). The activity of this coagulation factor (also known as FIIa) was expected because thrombin concentrations are increased by 11-fold in the synovium of patients with rheumatoid arthritis, as has been documented previously (So et al. 2003; Gerster and Busso 2003). However, FIIa has not been regarded as a protease that cleaves CP *in vivo* since the sequence of amino acids susceptible to proteolysis in CP only partially conforms to the sequence considered optimal for thrombin cleavage of substrates (Gallwitz et al. 2012). Nevertheless, in the synovial fluid of the patients with severe rheumatoid arthritis, CP was cleaved into typical fragments produced by “spontaneous” limited proteolysis, and the same fragments were detected after *in vitro* treatment of intact CP with FIIa (Sokolov et al. 2015a). MPO activity was also increased in the synovium samples from the patients. However, intact CP (132 kDa) was detected in the joints of patients with a relatively mild form of the disease, and MPO was clearly inhibited in these samples.

These effects may be directly connected with the structure of the CP:MPO complex described above; in the complex, the CP peptide loop covers the channel leading to the catalytic site within the MPO haem pocket (see above). Hence, cleavage of interdomain loop 885–894 in CP by thrombin can abrogate the inhibition of MPO by opening the substrate entrance into the MPO active site while the MPO–CP interaction itself is not compromised (Sokolov et al. 2007a).

Limited proteolysis of CP by FIIa results in the loss of its ability to inhibit the prooxidant functions of MPO or EPO; however, this form of CP retains its ferroxidase activity (Sokolov et al. 2015b). This result can be explained by the 45–50 Å distance of the CP R481–S482 and K887–V888 scissile bonds from the ferroxidase site according to the crystal structure of CP (Bento et al. 2007). Therefore, local perturbations, possibly evoked by peptide bond cleavage, are

unlikely to be transmitted over the long range to the ferroxidase site (Sokolov et al. 2015b).

These results further underscored the crucial role of CP as an MPO inhibitor in pathology and delineated the structural basis of the interactions among CP, thrombin, and MPO. In particular, it was suggested that FIIa not only cleaves CP into large fragments, but can form a complex with CP due to its high binding affinity. Having formed a complex with CP, FIIa covers the ferroxidase catalytic site and inhibits the conversion of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  (Sokolov et al. 2015a). Therefore, thrombin can interfere with two CP antioxidant activity mechanisms, i.e., CP suppression of MPO and the decrease in toxic  $\text{Fe}^{2+}$  levels. The ability of hirudin, a specific FIIa inhibitor contained in an ointment used in rheumatoid arthritis therapy, to suppress thrombin and MPO activity by 40-fold supports the notion that MPO inhibition by CP in the synovium of the patients strongly depends on FIIa. However, essentially all thrombin and MPO in the samples from hirudin-untreated patients or placebo controls existed in their active forms (*ibid.*).

It should be noted that the functional relation between CP and FIIa had been previously suggested and confirmed to an extent in connection with their participation in inflammatory reactions. A study of experimental cerebral oedema induced by the injection of iron-containing compounds into the rat brain to mimic cerebral haemorrhage showed that small amounts of thrombin significantly attenuated brain swelling (Yang et al. 2006). In the experiments, CP was tested as a treatment against injury caused by ferrous iron. Interestingly, administration of FIIa enhanced CP gene expression. The authors suggested that thrombin-mediated production of CP increases the tolerance of the brain to oedema by inhibiting the prooxidant effects of  $\text{Fe}^{2+}$  that are abundant in haematomas after erythrocyte decomposition.

The effects of CP in inflammation foci are not limited to the formation of complexes with iron-containing proteins, such as LF, MPO, and EPO, or to the conversion of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ , which prevents the Fenton reaction (Floris et al. 2000). In severe rheumatoid arthritis, proteolysed CP loses its ability to inhibit MPO, and MPO increases the production of oxidant species that can chemically modify CP and trigger conformational changes, leading to copper release (Swain et al. 1994; Panasencko et al. 2013). The same effect is observed after CP proteolysis; in turn,

elevated levels of free copper can catalyse and amplify the production of reactive oxygen species. This observation is supported by the findings of Naughton et al. (1995), who documented the presence of higher levels of free copper in patients with rheumatoid arthritis and by the beneficial effects of the copper chelator penicillamine (Aaseth et al. 1998).

Another possible regulative effect of CP in inflammation concerns its interaction with 5-lipoxygenase (5-LO), which also strongly depends on the integrity of CP and the presence of copper ions within the protein. Formation of the CP:5-LO complex reduces leukotriene synthesis in leukocytes in a dose-dependent manner (Sokolov et al. 2010). It should be noted that the concentration of CP sufficient to cause this effect is much lower than the normal CP content in plasma (50 µg/ml versus 300–400 µg/ml). An important observation of the authors included abrogation of the inhibitory effect of CP on 5-LO through the limited proteolysis of CP or copper ion loss. Copper depletion of CP converts CP into its apo-form, which acquires a peculiar conformation known as a “molten globule” (De Filippis et al. 1996). Apparently, this conformation of CP cannot interact with 5-LO. Unexpectedly, very low concentrations of CP caused the opposite effect on 5-LO, stimulating its activity (Sokolov et al. 2010). The authors suggested that low concentrations of CP are sufficient for the oxidation of NO (Shiva et al. 2006), an endogenous inhibitor of 5-LO activity (Coffey et al. 2000; Zagryazhskaya et al. 2010), but are insufficient to form significant amounts of CP:5-LO complex and to reduce leukotriene synthesis. These results provide evidence for the dual role of CP in the regulation of leukocyte cellular response in inflammation. The ability of neutrophils to synthesize CP and to provoke its degradation by proteinases secreted upon activation of these cells may represent an important feature of this regulation (Sokolov et al. 2010).

The question regarding the damaging effects of copper ions released from CP under pathological conditions was raised in the paper by Kostevich et al. (2015). The authors studied the inhibition of macrophage migration inhibitory factor (MIF) by CP that occurs when the two proteins form a specific complex. This work highlighted the utmost importance of the copper ions at the labile binding sites in CP for its interaction with MIF. This proinflammatory factor lost its tautomerase activity when CP was treated by chelators or cobalt and nickel ions, which can replace

the loosely bound copper in the labile sites (Samygina et al. 2008). However, CP-Cu<sup>2+</sup> behaved as an efficient uncompetitive inhibitor of MIF (Kostevich et al. 2015). Importantly, binding of a MIF substrate, *p*-hydroxyphenylpyruvate, was required for the formation of the CP:MIF complex (*ibid.*). Participation of copper ions loosely bound to CP in the transduction of the proinflammatory signal suggests a larger spectrum of capacities inherent in this protein, which are used to regulate various components associated with inflammation. The beneficial effects of penicillamine (Aaseth et al. 1998; Suarez-Almazor et al. 2000) and apo-lactoferrin (Guillen et al. 2000) in the treatment of rheumatoid arthritis can be attributed, at least partially, to the chelation of copper released from the CP labile binding sites (Kostevich et al. 2015).

The present discussion was focused on the “free” or “soluble” CP circulating in the plasma appearing in inflammatory foci, copper-saturated, and synthesized predominantly in the liver; however, extrahepatic synthesis of CP is also possible (Bakhautdin et al. 2013). Other forms of CP described in several studies, e.g., apo-CP (copper-free) or membrane-bound GPI-CP, are somewhat less explored.

At present, the role of GPI-CP in iron homeostasis is the only well-documented function of that species, partly due to the relatively long history of its investigations. Indeed, this species has been detected in Sertoli cells (Fortna et al. 1999), leptomenigeal cells (Mittal et al. 2003), and immune cells, i.e., lymphocytes/monocytes and macrophages (Banha et al. 2008; Marques et al. 2012). The molecular mechanism of CP-mediated control of cellular iron efflux includes its functional antagonism with hepcidin that suppresses iron egress from the cells (Kono et al. 2010). In cultured glioma cells, free and membrane-bound CP behaved as efficient antagonists of hepcidin (Kono 2013), suppressing its ability to induce ferroportin degradation in the lysosomes (Ward and Kaplan 2012). A crucial role of CP in ferroportin stabilization was observed in experiments in CP-depleted macrophages (Kono 2013) where GPI-CP is normally colocalized with ferroportin 1 in plasma membrane lipid rafts (Marques et al. 2012). Currently, the close proximity of GPI-CP and ferroportin is not considered evidence of the formation of a complex by these two proteins. However, it appears likely that ferroportin receives iron from the cytoplasm as Fe<sup>2+</sup> and that GPI-CP is needed to oxidize Fe<sup>2+</sup> to provide

Fe<sup>3+</sup> for incorporation into transferrin [for details, see (Linder 2016)]. To meet these requirements, a direct protein–protein interaction might be the best choice; this issue deserves a thorough investigation. In CP-depleted macrophages, ferroportin was rapidly degraded through a hepcidin-mediated mechanism and did not appear on the cell surface. Meanwhile, apo-CP did not have any anti-hepcidin effects (Kono et al. 2010). This effect may be regarded as an inability of ferroportin to form a complex with GPI-CP, taking into account that apo-CP is as efficient as the copper-saturated protein for the formation of complexes with other proteins such as the CP:LF complex (Pulina et al. 2002). This result requires a more thorough investigation; however, it should be noted that protein–protein interactions may exhibit considerable variability in the stability and longevity of the complexes formed with the partners. Currently, there is no evidence supporting the formation of a stable CP:ferroportin complex; however, this does not mean that the two proteins do not form a transient complex and such short-term interaction favours the regulation of very important biochemical processes.

Thus, various forms of CP function as important and sometimes crucial regulatory factors in physiological and pathological processes in organisms. The ability of CP to form complexes with other proteins has been known for a number of years; however, a noticeable increase in the functional versatility of CP complexes was only recently acknowledged (Vasilyev 2010). The present study is not a comprehensive guide for the sophisticated and ramified interactions of the “moonlighting” protein but is rather a demonstration of a few directions for further studies aimed to discover novel roles of CP multifunctionality.

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