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Interaction of Ceruloplasmin, Lactoferrin, and Myeloperoxidase

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Abstract—When lactoferrin (LF) and myeloperoxidase (MPO) are added to ceruloplasmin (CP), a CP–LF–MPO triple complex forms. The complex is formed under physiological conditions, but also in the course of SDS-free PAGE. Polyclonal antibodies to both LF and MPO displace the respective proteins from the CP–LF–MPO complex. Similar replacement is performed by a PACAP38 fragment (amino acids 29-38) and protamine that bind to CP. Interaction of LF and MPO with CP-Sepharose is blocked at ionic strength above 0.3 M NaCl and at pH below 4.1 (LF) and 3.9 (MPO). Two peptides (amino acids 50-109 and 929-1012) were isolated by affinity chromatography from a preparation of CP after its spontaneous proteolytic cleavage. These peptides are able to displace CP from its complexes with LF and MPO. Both human and canine MPO could form a complex when mixed with CP from seven mammalian species. Upon intravenous injection of human MPO into rats, the rat CP–human MPO complex could be detected in plasma. Patients with inflammation were examined and CP–LF, CP–MPO, and CP–LF–MPO complexes were revealed in 80 samples of blood serum and in nine exudates from purulent foci. These complexes were also found in 45 samples of serum and pleural fluid obtained from patients with pleurisies of various etiology.

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Proteins contained in the granules of neutrophilic leukocytes provide primary nonspecific protection of an organism from pathogenic microorganisms. Among such proteins that participate in reactions of innate immunity, a special place is occupied by metal proteins. One of those, i.e. lactoferrin (LF), is a member of the transferrin family, has M_r ~ 78,000, is contained in secretory neutrophilic granules, and displays various antimicrobial properties. LF possesses features of an immunomodulator and of an anti-inflammatory factor. It is able to chelate transition metal ions, interact with lipopolysaccharides, and hydrolyze protein factors of bacterial colonization. Pepsin-catalyzed hydrolysis of LF results in formation of defensin-like derivatives, the so-called lactoferricins that have a strong bactericidal effect [1, 2].

Abbreviations: CP) ceruloplasmin; LF) lactoferrin; MPO) myeloperoxidase; PACAP38) pituitary adenylate cyclase-activating peptide; PBS) phosphate buffered saline.

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Myeloperoxidase (MPO, EC 1.11.1.7), a 140 kD marker protein of azurophilic granules in neutrophils, catalyzes oxidation of various substrates by involving reactive oxygen species, such as O_2^- and H_2O_2, generated by respiratory burst of phagocytes. This results in origination of efficient antibacterial and cytotoxic agents, hypochlorite ions being among these. MPO is a dimeric chlorine-containing peroxidase possessing antimicrobial and pro-oxidant properties. In neutrophilic leukocytes, it provides 1-5% of dry weight. The putative role of MPO in antimicrobial defense and in control upon malignization is supported both by studies of purified MPO or MPO-containing cells and by data on elevated sensitivity of patients with inherited MPO deficiency to infection and occurrence of tumors. On the other hand, MPO contributes to oxidative tissue damage in the course of chronic inflammation, in the development of leukemia and neurodegenerative diseases, and increases the risk of tumors in the respiratory tract [3]. Despite the fact that no direct interaction between LF and MPO had been

shown, a synergism in the bactericidal effect of these proteins was demonstrated [1].

It is known that both LF and MPO form complexes with ceruloplasmin (CP, ferro:O_2-oxidoreductase, EC 1.16.3.1), which is a 132-kD protein of blood plasma regarded as an acute phase reactant. CP displays a large variety of enzymatic activities and behaves as a universal antioxidant due to the presence in its molecule of six tightly bound copper ions [4]. It was shown that CP inhibits the peroxidase activity of MPO secondary to the direct interaction of the proteins [5, 6]. The mechanism of inhibitory effect and the primary features of the CP–MPO complex have not been explored in detail.

We were first to characterize the CP–LF complex [7, 8] and succeeded in showing enhancement of ferroxidase activity of CP affected by LF [9]. Interaction of ferroxidase (CP) with a protein of the transferrin family (LF) is likely to be indicative of the role of the CP–LF complex in iron metabolism. Along with the interaction of purified proteins *in vitro*, we have observed CP–LF complex formation in breast milk [9, 10] and in tears (unpublished data). We suggested that the CP–LF complex formed in breast milk at early stages of lactation is later preserved in the stomach of neonates (pH 5-6) where it can accomplish its functions [7, 10].

Because both LF and MPO interact with CP, we studied the possibility of formation of a triple CP–LF–MPO complex. We also compared some features of the CP–LF and CP–MPO complexes. Also, our analysis of blood samples and of exudates from patients with purulent diseases confirmed the idea that CP can form *in vivo* complexes with LF and MPO.

MATERIALS AND METHODS

The following reagents were used in this study: BioGel P-6, colored molecular mass markers (BioRad, USA); cyanogen bromide (Fluka, Switzerland); triethylamine ($(C_2H_5)_3N$), EDTA (Merck, Germany); Sepharose 4B, Sepharose 6B, DEAE-Sephadex A-50, QAE-Sephadex A-50, CM-Sepharose, phenyl-Sepharose, Sephadex G-100 and G-150 (Pharmacia, Sweden); complete and incomplete Freund's adjuvant, NaN_3, glycerol, Coomassie R-250, molecular mass markers for gel filtration (450, 210, 160 kD), mercaptoethanol, ammonium persulfate, Tris, 2-chloroethylamine, epichlorohydrin (Serva, Germany); glycine, *o*-dianisidine, SDS, salmon protamine, phenylmethylsulfonyl fluoride, 4-chloro-1-naphthol (Sigma, USA); Toyopearl HW-55 (Toyo Soda, Japan); acrylamide, arginine, N,N′-methylene-bis-acrylamide, N,N,N′,N′-tetramethylethylene diamine (Laboratory MEDIGENE, Russia); heparin (SPOFA, Poland). Buffers: PBS (phosphate buffered saline) – 0.15 M NaCl, pH 7.4, 1.9 mM Na_2HPO_4/8.1 mM NaH_2PO_4; 0.1 M sodium acetate buffer, pH 5.5 – 0.089 M AcONa/0.011 M AcOH; 0.1 M sodium acetate buffer, pH 4.7 – 0.054 M AcONa/0.046 M AcOH.

Peptide KRYKQRVKNK corresponding to fragment 29-38 of the neuropeptide PACAP38 was obtained by solid-phase synthesis (Research Institute of Highly Pure Biopreperations, St. Petersburg) and was 99.5% pure as judged by HPLC and amino acid analysis.

Preparation of monomeric and stable CP was isolated from blood plasma by affinity chromatography on protamine-Sepharose [11].

AE-agarose for isolation of intact CP and of its complexes was obtained by treating Sepharose 6B with epichlorohydrin and 2-chloroethyl amine [12]. Serum (200 ml) was applied on the column with AE-agarose (40 ml) equilibrated with PBS and washed with the same buffer to achieve $A_{280} < 0.02$ of the effluent. Bound CP was desorbed upon elution with 0.3 M NaCl. The fraction obtained was dialyzed against PBS, and minor admixtures were eliminated by chromatography on a column with heparin-Sepharose (2 ml). Actually, all CP was eluted from the column by PBS. Minor protein admixtures were eluted from the column with 1 M NaCl and subjected to SDS-free PAGE. Protein bands possessing oxidase activity were cut from the gel and analyzed on a Bruker mass-spectrograph (Research Institute of Physico-Chemical Medicine, Ministry of Public Health Care of Russian Federation, Moscow). Peptide fingerprints of the proteins were analyzed on-line using the program MASCOT (http://www.matrixscience.com).

LF was isolated from breast milk by ion-exchange chromatography on CM-Sepharose and by gel filtration on Sephadex G-100 Superfine [7].

Human MPO was isolated from leukocytic mass that was a generous gift of Prof. V. N. Kokryakov (Department of General Pathology and Pathophysiology, Institute for Experimental Medicine, St. Petersburg). No cationic detergents were used. Leukocytic precipitate (40 g) was resuspended in 100 ml of 0.05 M sodium acetate buffer, pH 4.7, with phenylmethylsulfonyl fluoride to 2.5 mM, after which it was frozen and thawed, and subsequently subjected to three 30-sec ultrasonic treatments (44 kHz) with 60-sec intervals of cooling on ice. Thus obtained, the extract of leukocytes was centrifuged for 30 min at 15,000*g* (4°C). The supernatant was loaded on a column with heparin-Sepharose (2.5 × 7 cm), and washed with 0.05 M sodium acetate buffer, pH 4.7, until the effluent achieved $A_{280} < 0.01$. MPO and LF were eluted with 60 ml portions of linear gradient 0→3 M NaCl (in 0.05 M sodium acetate buffer, pH 4.7). Under such conditions, LF would be eluted in the same fractions as MPO. Ammonium sulfate was added to these fractions to the final concentration 2 M, and the product was loaded on a column with phenyl-Sepharose equilibrated with 2 M $(NH_4)_2SO_4$. The column was washed with equilibrating solution, and subsequently LF was eluted with 0.5 M $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 7.4. Then virtually

pure MPO was eluted with 25% glycerol containing 5 mM $CaCl_2$ and 0.05 M sodium acetate buffer, pH 4.7. Additional purification of MPO was achieved by gel filtration on a column with Sephadex G-150 Superfine (5 × 100 cm) in 0.15 M NaCl, 5 mM $CaCl_2$, 0.05 M sodium acetate buffer, pH 4.7. The ratio A_{430}/A_{280} (R_z) = 0.85 was calculated for the MPO obtained, which is evidence of its high homogeneity [13]. Canine MPO was isolated from leukocytes according to a similar protocol [14].

Concentration of the homogeneous protein preparations was measured spectrophotometrically using coefficients a_{280} = 1.61 ml/mg per cm and a_{610} = 0.0741 ml/mg per cm for CP [15], a_{280} = 1.46 ml/mg per cm for LF [16], and a_{430} = 0.6 ml/mg per cm for MPO [13].

Molecular mass of proteins and of proteolytic fragments of CP was determined by SDS-PAGE [17]. CP was revealed in SDS-free PAGE [18] by specific staining with *o*-dianisidine [19]. To reveal the peroxidase activity of MPO after SDS-free PAGE, the gel was washed for 30 min in 0.4 M sodium acetate buffer, pH 5.5, and then stained in solution containing 20 mg of 4-chloro-1-naphthol in 10 ml of ethanol, 5 μl of 8 M H_2O_2, and 90 ml of 0.1 M sodium acetate buffer, pH 5.5. Purple bands appeared against the transparent background within 20 min. Colored gels were stored in the dark.

Antibodies against CP, LF, and MPO were raised by three immunizations of rabbits with respective proteins [20]. To evaluate the amounts of LF and MPO, rocket immunoelectrophoresis was run [21]. Activity of MPO in immune precipitates was revealed when agarose gels were 3 times subjected to a 20-min wash in 0.4 M sodium acetate buffer, pH 5.5, which allowed staining the precipitates with 0.08% solution of *o*-dianisidine in 0.1 M sodium acetate buffer, pH 5.5, containing 80 μM of H_2O_2. After that, the gels were incubated at 37°C for 30 min (until brown coloration developed). The background was washed clean in 5% acetic acid. CP, LF, MPO, heparin, and protamine (10, 10, 5, 20, and 4 mg per ml of wet gel, respectively) were immobilized on BrCN-activated Sepharose 4B [11].

Samples of blood serum and of exudates from patients with purulent infection were obtained at the Purulent Surgery ward of the 5th Municipal Hospital (St. Petersburg) thanks to the courtesy of Prof. Yu. A. Spesivtsev. Blood serum, pleural fluid, and exudates were sampled without adding anticoagulants and subsequent freezing. The material was centrifuged at 3000*g* (4°C) and the supernatant was analyzed.

Gel filtration of proteins was performed on a column (1 × 50 cm) packed with Toyopearl HW-55 fine. Proteins were eluted with 0.15 M NaCl in 10 mM Tris-HCl, pH 7.4, at a flow rate 0.5 ml/min. Samples having volume 100 μl contained: a) mixture of CP (1 mg) and LF (0.6 mg); b) CP (1 mg) and MPO (1.1 mg); c) CP (1 mg), LF (0.6 mg), and MPO (1.1 mg). Ferritin, catalase, MPO, CP, aldolase, and LF (1 mg per 100 μl of solution) were used as molecular mass markers. Based on the results of three measurements, a calibration graph was plotted and the molecular mass of the complexes was calculated.

To perform affinity chromatography of LF and MPO, two columns with CP-Sepharose (0.5 ml) were equilibrated with 0.1 M NaCl in 20 mM Tris-HCl, pH 7.4. Portions of the same buffer containing 0.5 mg of LF and 1 mg of MPO were loaded on both columns. Elution from the first column was performed with a stepwise gradient of 0.15, 0.20, 0.25, 0.30, 0.35, and 0.40 M NaCl in 20 mM Tris-HCl buffer, pH 7.4. Proteins from the second column were eluted with portions of 100 mM sodium acetate buffer having pH 4.7, 4.5, 4.3, 4.1, 3.9, and 3.7. Rocket immunoelectrophoresis was run to measure the content of LF and of MPO in the effluent fractions. The experiment was repeated three times.

Affinity chromatography on LF- and MPO-Sepharose allowed isolating from a long-stored preparation of CP that had undergone spontaneous proteolytic degradation peptides that displayed strong binding towards LF and MPO. A sample of CP (2 ml; 100 mg/ml in PBS containing 25% sucrose) was centrifuged for 30 min at 15,000*g* (4°C), then diluted to 1/10 with PBS and applied on a column with LF-Sepharose (1 × 3 cm). The resin was washed with PBS to achieve $A_{280} < 0.002$, after which it was eluted with 0.3 M NaCl in 10 mM sodium phosphate buffer, pH 7.4. The eluate was diluted 10 times with PBS and applied on a column with MPO-Sepharose (1 × 3 cm). Monitoring the changes of A_{220} in the eluate allowed judging whether all the peptides that were binding to LF-Sepharose could also interact with MPO. As in the case with LF-Sepharose, the resin was washed and peptides were eluted. The fraction obtained was lyophilized with subsequent dissolution in a minimum volume of water and desalted by gel filtration on a column with BioGel P-6 (1 × 30 cm) equilibrated with PBS. Molecular masses of peptides eluted in the void volume were estimated by SDS-PAGE, the error of three measurements being 0.5 kD.

Peptides were identified using mass-spectrometry of their tryptic fragments. To prepare the samples, the peptides were resolved in SDS-PAGE, after which pieces of gel containing protein bands were cut out. A Bruker mass-spectrograph (Research Institute of Physico-Chemical Medicine, Ministry of Public Health Care of Russian Federation, Moscow) was used. Peptide fingerprints of the proteins were analyzed on-line using the MASCOT program (http://www.matrixscience.com). The picture of the three-dimensional structure of CP was obtained using the RasMol program, version 2.7.1, using the coordinates obtained in a crystallographic study of CP [22].

To model the forming of the CP–MPO complex *in vivo*, three inbred male rats with body mass ~150 g were used. A 2-mg sample of purified human MPO in 100 μl of PBS was injected into the caudal vein of an animal. Blood was sampled 1, 5, 15, 30, and 45 min after injection. Serum was analyzed by rocket immunoelectrophoresis (10-μl sample) and by SDS-free PAGE (10-μl sample).

RESULTS AND DISCUSSION

Forming the complexes of CP with LF and MPO. Using SDS-free PAGE, we investigated the possibility of forming the CP–LF–MPO triple complex. Adding to CP the purified preparations of LF, of MPO, or of their mixture resulted in reduction in the electrophoretic mobility of CP, indicating the forming of its complexes with the proteins under study (Fig. 1a). In a previous study of the interaction of CP with LF, we demonstrated that upon forming their complex both proteins acquire an altered electrophoretic mobility [7].

In control experiments, purified MPO did not enter in SDS-free PAGE, which is similar to the behavior of purified LF described earlier [7]. However, MPO entered the gel upon adding various amounts of CP, which resulted in forming of CP–MPO and CP–2 MPO complexes revealed by staining the gel for peroxidase activity (Fig. 1b). One can see the band corresponding to the CP–MPO complex (at the border between the loading gel and the resolving gel) as well as the band consistent with the CP–2 MPO complex (on the verge of the concentrating gel). The presence of CP and MPO in these bands is shown by their staining with chromogenic substrates specific for both enzymes (Figs. 1a and 1b; lanes *3* and *4*). We have observed previously the forming of complexes with various stoichiometries when studying the interaction of CP with LF, e.g. 1 CP : 2 LF and 1 CP : 4 LF [7]. The triple complex CP–LF–MPO (Figs. 1a and 1b, lane *5*) was formed upon mixing equimolar amounts of the proteins (regardless of the order of mixing). When CP, LF, and MPO were mixed, the triple complex was formed predominantly as judged by the results of SDS-free PAGE, while the double complexes CP–LF and CP–MPO were less abundant (Figs. 1a and 1b, lane *5*).

The appearance of an additional oxidase-positive band typical of the triple complex was observed also upon adding LF and MPO to blood serum (Figs. 1a and 1b, lane *6*). This indicates that plasma proteins do not prevent CP from interacting with LF and MPO. Polyclonal antibodies against LF and MPO added to such a sample caused dissociation of the CP–LF–MPO complex and forming, respectively, of the CP–MPO (Figs. 1a and 1b, lane *7*) or CP–LF (Figs. 1a and 1b, lane *8*) complexes. It can be concluded that interaction is not impeded by any sort of rabbit immunoglobulins, as the CP–LF complex persisted in the presence of anti-MPO and *vice versa*. It seems likely that specific binding of the respective antibodies results in partial coincidence of the sites at the surface of LF or MPO needed to contact CP. We have described previously dissociation of the CP–LF complex in the presence of polyclonal antibodies to either of these two proteins [8]. Co-immunoprecipitation of CP and LF from breast milk provided by polyclonal anti-LF occurred only within a certain range of antibody concentrations [10]. When added in higher amounts, antibodies displaced CP from its complex with LF. A similar result was observed by Griffin et al. when using ligand Western-blotting [23]. These authors described the interaction of CP with MPO and an "apparent absence of interaction with LF" that is likely to be caused by partial overlap of epitopes in LF with the sites of its contact with CP. A correct protocol of such experiments should include monoclonal antibodies to a site not involved in the complex formation.

We observed *in vivo* formation of the CP–LF complex when analyzing breast milk [9, 10] and tears (unpub-

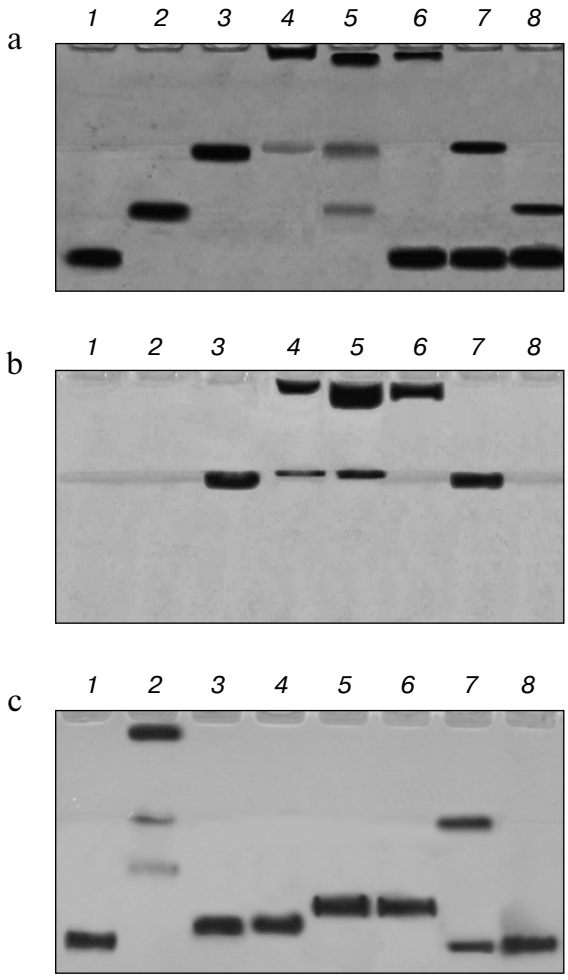

Fig. 1. Results of SDS-free PAGE of the complexes of CP with lactoferrin and/or myeloperoxidase: a) *o*-dianisidine staining; b) staining with 4-chloro-1-naphthol and H_2O_2. Lanes: *1*) CP, 0.5 μg; *2*) CP, 0.5 μg + LF, 0.6 μg; *3*) CP, 0.5 μg + MPO, 0.5 μg; *4*) CP, 0.5 μg + MPO, 1 μg; *5*) CP, 0.5 μg + LF, 0.3 μg + MPO, 0.5 μg; *6*) blood serum, 5 μl + LF, 0.1 μg + MPO, 0.2 μg; *7*) the same as (*6*) + 1 μg anti-LF; *8*) the same as (*6*) + 1 μg anti-MPO. c) *o*-Dianisidine staining. Lanes: *1*) CP, 0.5 μg; *2*) CP, 0.5 μg + LF, 0.3 μg + MPO, 0.5 μg; *3*) the same as (*2*) + PACAP38 fragment (a.a. 29-38), 0.1 μg; *4*) the same as (*1*) + PACAP38 fragment (a.a. 29-38), 0.1 μg; *5*) the same as (*2*) + salmon protamine, 0.2 μg; *6*) the same as (*1*) + salmon protamine, 0.2 μg; *7*) the same as (*2*) + heparin, 1 μg; *8*) the same as (*2*) + 7-kD and 5-kD peptides of CP, 0.1 μg.

lished data). There is no way to find the complexes of CP with proteins of neutrophilic leukocytes in serum as their concentration is normally rather low, i.e. 265 ng/ml for LF [24] and 220 ng/ml for MPO [25]. However, we suggested that it is possible to reveal the CP–LF, CP–MPO, and CP–LF–MPO complexes upon an increase in the content of LF and MPO due to degranulation of neutrophils in the course of inflammation. We used SDS-free PAGE to screen nine samples of purulent exudates and 80 samples of serum from patients with various inflammatory diseases, and also 45 samples of serum and pleural fluid from patients with pleurisies of various etiologies (including 24 carcinomatous, seven parapneumonic, three cardiogenous, five tuberculous, three postoperative) and detected the complexes of CP with LF and MPO in all cases. The notion that additional oxidase-positive bands revealed by electrophoresis represented the complexes of CP with LF and MPO is supported by their disappearance upon adding to samples of anti-LF and anti-MPO.

Properties of the CP–MPO and CP–LF–MPO complexes. To clarify the peculiarities of the complexes forming in conditions similar to those known as physiologic (ionic strength and pH), we performed gel filtration on Toyopearl HW-55 fine. Upon mixing CP with equimolar amounts of LF, of MPO, and of LF and MPO, the proteins would elute every time as a single symmetric peak, which suggests the forming of complexes. Molecular masses of the CP–LF, CP–MPO, and CP–LF–MPO complexes were estimated as 215 ± 5, 280 ± 6, and 350 ± 5 kD, respectively. Based on the known molecular masses of CP (132 kD), MPO (140 kD), and LF (78 kD), this suggests that the proteins enter the complexes in equimolar amounts.

While under alkaline conditions of SDS-free PAGE various stoichiometries were observed for the CP–LF [7, 10] and CP–MPO complexes, gel filtration of the mixture of CP and MPO at neutral pH resulted in forming of essentially the 1 CP : 1 MPO complex, as had been shown for CP and LF. In the course of affinity chromatography of MPO and LF on CP-Sepharose, both proteins were retained by the resin. Our preliminary experiments showed that running a solution containing LF or MPO through MPO- or LF-saturated CP-Sepharose would not cause desorption of MPO (or LF), which is likely to result from the forming of the triple CP–LF–MPO complex. The proteins were simultaneously desorbed during elution with increasing concentrations of NaCl as the latter reached 0.3 M. Lowering pH of the eluent prevented the interaction with CP-Sepharose of LF at pH < 4.1 and of MPO at pH < 3.9. These results point toward the electrostatic nature of interaction of CP (p*I* 4.7) with LF (p*I* 8-9) and MPO (p*I* 9-10). Complexes of CP with LF and MPO dissociated when excessive amounts of a neuropeptide PACAP38 fragment (29-38) or of salmon protamine were added, as judged by the results of electrophoresis (Fig. 1c; lanes *1-6*). This can be explained by displacement of LF and MPO from their complex with CP provided by competitors (the PACAP38 fragment and protamine). CP can bind six or seven molecules of PACAP38 [26] or six molecules of salmon protamine [11]. Therefore, it cannot be excluded that apart from the binding sites for LF and MPO, there are binding sites for other cationic proteins on the surface of the CP molecule.

An argument supporting the high specificity of interaction of CP with MPO is the minor admixture of the latter revealed in the preparation of CP purified on AE-agarose. Electrophoresis of the effluent from heparin-Sepharose showed the presence of an oxidase-positive band at the border between the concentrating and running gels. The same mobility was acquired by CP when it formed the complex with MPO (see Fig. 1a, lane *3*). Mass spectrometry of the tryptic fragments of the material from this band revealed CP and MPO (15 and 19 peptides, respectively, were identified, the difference between the calculated and predicted molecular mass values never exceeding 0.08). It is likely that MPO, which is constantly secreted by neutrophils and the plasma concentration of which is very low, normally reaching 220 ng/ml [25], forms a strong complex with CP. This complex is retained by AE-agarose and heparin-Sepharose, the latter feature being distinctive from the previously studied CP–LF complex [7, 10] that dissociated on heparin-Sepharose. Similarly, heparin did not cause dissociation of the CP–MPO complex in SDS-free PAGE, while CP–LF would dissociate (Fig. 1c, lane *7*). This latter dissociation during affinity chromatography and SDS-free PAGE was described in our previous papers [8, 10].

We demonstrated the possibility for CP and MPO of various species to form complexes when sera of seven mammalian species (rat, mouse, rabbit, dog, horse, dolphin, and human) were added to human MPO. Additional oxidase-positive bands are seen at the border between the concentrating and running gel (Fig. 2a), which indicates that human MPO entered the complexes with CP and modify its normal electrophoretic mobility. Similar results were obtained with canine MPO (data not shown). Contact sites in CP and MPO are likely to be evolutionarily conservative. Absence of species specificity is also typical of the complexes of CP with LF [27] and with protamine [11].

We have shown previously that human LF administered to the rat bloodstream circulated in complex with CP for about 2 h [7]. In this study, human MPO injected intravenously into rats was detected in blood samples obtained 30, but not 45 min after injection (Fig. 2b). The rat CP–human MPO complex was revealed in samples of serum also within 30 min (Fig. 2c). Its mobility in SDS-free PAGE is the same as that observed for the complex obtained upon mixing rat serum with human MPO *in vitro* (Fig. 2a, lane *1′*). Thus, elimination of MPO from blood circulation was accompanied by disappearance of the CP–MPO complex.

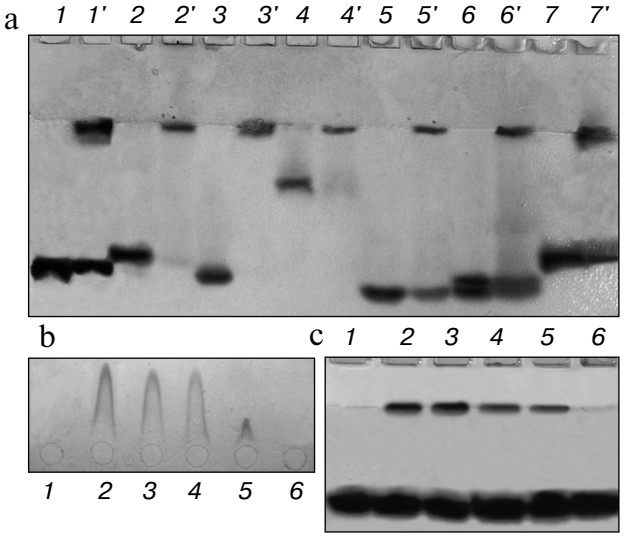

Fig. 2. Interspecific complexes of ceruloplasmin and myeloperoxidase forming *in vitro* and *in vivo*. a) Results of SDS-free PAGE of mammalian sera to which human MPO was added (N′ – 1 μg, *o*-dianisidine staining). Lanes: sera of rat, 1 μl (*1*); mouse, 5 μl (*2*); rabbit, 10 μl (*3*); dog, 20 μl (*4*); horse, 20 μl (*5*); dolphin, 5 μl (*6*); human, 3 μl (*7*). b) Rocket immunoelectrophoresis; 10 μg of anti-human MPO per ml of 1% agarose; *o*-dianisidine and H_2O_2 staining. c) Results of SDS-free PAGE; *o*-dianisidine staining. Lanes: *1*) rat serum prior to injection; *2-6*) 1, 5, 15, 30, and 45 min after human MPO was injected, respectively; 10 μl of serum were applied on each lane.

To determine the sites in the CP molecule that might be involved in interaction with LF and MPO, peptides displaying high affinity to LF and MPO were isolated from a partially hydrolyzed CP preparation. It is worth noting that peptides of CP retained by LF-Sepharose were then entirely retained by MPO-Sepharose as well. Similar results were obtained when peptides of CP were first retained by MPO-Sepharose and then by LF-Sepharose. Two peptides were isolated with molecular masses of about 7 and 5 kD. SDS-free PAGE demonstrated that these peptides provoked dissociation of the complexes formed by non-hydrolyzed CP with LF and with MPO (Fig. 1c, lane *8*), but taken separately, neither of them caused dissociation of a complex. Mass spectrometry localized these peptides in the CP molecule (see the table). Four fragments were identified in the 7-kD peptide, positioned in area of the CP chain between amino acids residues 50 and 109. Three fragments were recognized in the 5-kD peptide and ascribed to the area of the sequence between amino acids residues 929 and 1012. Molecular masses of peptides 50-109 and 929-1012 were calculated as 7.1 and 5.1 kD, respectively. The real size of the isolated peptides may be somewhat larger, since mass spectrometry of tryptic fragments does not allow determining if the identified amino acid stretches are flanked by any sequences.

On mapping of the identified peptides within the tertiary structure of CP [22], one can notice that they are drawn together and belong to domains 1 and 6 of the molecule (Fig. 3). These domains assemble the catalytic center of CP that includes four copper ions ascribed to three different types based on their spectral features. The identified peptides include amino acids coordinating all four coppers of the active center, i.e. H975 (type I Cu), H101, H978 (type II Cu), H980 (type III Cu), H103 (type III Cu). Thus, the sites in CP that have high affinity to LF and MPO are localized in close proximity to the catalytic center. We have found previously that LF and protamine, upon interaction with CP, increase the ferroxidase activity of the latter [9, 11]. An allosteric effect of these proteins on the activity of CP was proposed, this notion gaining support from the present study. The absence of an effect of MPO on ferroxidase and

Peptide fingerprint of the two peptides from CP that interact with LF and MPO (search results using the MASCOT program; amino acids are numbered in accordance with the primary structure of the full-length proteins)

Start-end	Expected *M*, daltons	Calculated *M*, daltons	Δ, daltons	Peptide
Tryptic fragments identified in 7-kD peptide of CP				
50-62	1646.8269	1646.8304	–0.0035	KALYLQYTDETFR
63-79	1911.1383	1911.1233	0.0150	TTIEKPVWLGFLGPIIK
80-91	1358.7430	1358.7194	0.0236	AETGDKVYVHLK
92-109	2154.0571	2154.0646	–0.0076	NLASRPYTFHSHGITYYK
Tryptic fragments identified in 5-kD peptide of CP				
929-938	1565.7346	1565.7209	0.0137	VNKDDEEFIESNK
939-945	797.3593	797.3966	–0.0373	MHAINGR
990-1012	2668.2472	2668.2519	–0.0046	GVYSSDVFDIFPGTYQTLEMFPR

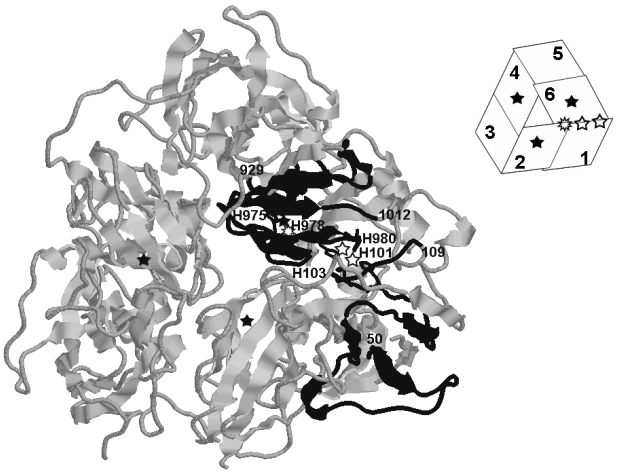

Fig. 3. Localization of amino acid 50-109 and 929-1012 stretches (in black) in the CP molecule. Type I copper ions are marked as black stars; 10-point star is for type II copper; white stars stand for type III copper. Insert at top right shows schematic subdivision of CP in six domains and localization of six copper ions [22].

diaminooxidase activities of CP [6] can be explained by relative remoteness of the MPO-binding site in CP from its catalytic center.

Interaction of LF and MPO with CP may be part of a regulatory mechanism employed for neutralizing or modulating the biological activity of the proteins of neutrophilic granules that are potentially toxic when enter the bloodstream. It cannot be excluded that forming such complexes in inflammation foci and the consequences of that interaction can entail the neutralization of the results of respiratory burst in neutrophils or the occurrence of CP derivatives possessing pro-oxidant activity. The absence of species specificity of these interactions speaks in favor of their conservative character and the need to preserve their functions during evolution.

It is possible to make a hypothesis about the function of the earlier discovered complexes. For instance, it seems logical to propose a participation of the CP–LF complex in iron metabolism. Forming the triple CP–LF–MPO complex can account for the plausible synergism of antimicrobial functions of LF and MPO. The fact that CP provides the incorporation of oxidized iron into LF also suggests the participation of the triple complex in protective reactions in the organism.

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