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Effect of Lactoferrin on the Ferroxidase Activity of Ceruloplasmin

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Abstract—The effects of various forms of lactoferrin (Lf) interacting with ceruloplasmin (Cp, ferro-O_2-oxidoreductase, EC 1.16.3.1) on oxidase activity of the latter were studied. Comparing the incorporation of Fe^{3+} oxidized by Cp into Lf and serum transferrin (Tf) showed that at pH 5.5 apo-Lf binds the oxidized iron seven times and at pH 7.4 four times faster than apo-Tf under the same conditions. Apo-Lf increased the oxidation rate of Fe^{2+} by Cp 1.25 times when Cp/Lf ratio was 1 : 1. Lf saturated with Fe^{3+} or Cu^{2+} increased the oxidation rate of iron 1.6 and 2 times when Cp to holo-Lf ratios were 1 : 1 and 1 : 2, respectively. Upon adding to Cp the excess amounts of apo-Lf (Cp/apo-Lf < 1 : 1) or of holo-Lf (Cp/holo-Lf < 1 : 2) the oxidation rate of iron no longer changed. Complex Cp–Lf demonstrating ferroxidase activity was discovered in breast milk.

Key words: ceruloplasmin, ferroxidase, lactoferrin, transferrin

Ceruloplasmin (Cp, ferro-O_2-oxidoreductase, EC 1.16.3.1) is the major copper containing protein of plasma with M_r ~ 132,000. Owing to strong binding of six copper atoms per molecule, Cp exhibits activity towards several substrates. Since the time when this protein was discovered in 1948 [1], the Cp-catalyzed diamine oxidase reaction has been described and extensively used for measurements of Cp content in serum [2]. The ferroxidase activity of Cp was first reported in 1966 [3]. When catalyzing the oxidation of Fe^{2+} to Fe^{3+}, Cp accomplishes a four-electron transfer to O_2 with production of water and thus prevents non-enzymatic reaction resulting in formation of $O_2^{\cdot-}$ (superoxide anion) [3]. Due to this reaction in plasma the Fe^{2+}/O_2 ratio of 4 : 1 is provided.

Oxidized in the ferroxidase reaction, iron can be incorporated into serum transferrin (Tf) and transported throughout the organism as a part of the $(Fe^{3+})_2$–Tf complex [3]. In the presence of reduced glutathione, Cp exhibits peroxidase activity [4]. Along with ferroxidase activity, Cp catalyzes copper oxidation [5].

The broad spectrum of enzymatic activities makes Cp a universal antioxidant. Oxidation of Fe^{2+} prevents $OH^{\cdot}$ formation in the Fenton reaction [6]. Hereditary deficiency of the Cp gene (patients with aceruloplasminemia and Cp gene knock-out mice) results in iron metabolism disorders and oxidative stress associated with iron accumulation [7, 8].

Interactions with various proteins have different effects on the enzymatic activity of Cp. Five-fold increase in Cp diamine oxidase activity was reported upon its interaction with protein C [9]. When Cp contacted with ferritin the rate of Cp-catalyzed iron oxidation increased [10]. In our study interaction of Cp with protamine increased the ferroxidase activity of the former 1.5-fold [11]. However, binding to myeloperoxidase affected neither ferroxidase nor peroxidase activities of Cp [12].

Previously we described the complex of Cp with lactoferrin (Lf) *in vitro* and *in vivo* [13, 14]. Lf is a glycoprotein with M_r ~ 78,000 belonging to the transferrin family. Lf has been revealed in exocrine secretions: milk, saliva, tears, seminal fluid, and also in secretory granules of neutrophilic leucocytes. Lf suppresses microbial growth due to its ability to bind with high affinity Fe^{3+}, Cu^{2+}, and other transition metal ions. Among the antimicrobial effects of Lf are also binding to DNA and lipopolysaccharides, proteolysis of the protein factors of bacterial colonization, and formation of defensin derivatives [15].

Transferrins are composed of two domains that are able to bind Fe^{3+} and a synergistic anion (usually CO_3^{2-}). The latter stabilizes the red-colored Fe^{3+}–transferrin (λ_{max} = 450-470 nm). The spectral properties of transferrins can be used to measure ferroxidase activity of Cp

Abbreviations: Cp) ceruloplasmin; Lf) lactoferrin; Tf) transferrin.

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using Tf or ovotransferrin [3, 16]. The binding constant of Fe^{3+} to Lf is 300 times higher than that to Tf. Lf loses half of its bound Fe^{3+} at pH 3.0, while Tf does so at pH 5.7 [17].

In this study, we have investigated the effect of Lf on Cp ferroxidase activity.

MATERIALS AND METHODS

Chemicals. We used the following chemicals: Chelex 100 (BioRad, USA); $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $K_4Fe(CN)_6$, KSCN, $FeCl_3$, $CuCl_2$, EDTA (Merck, Germany); Sepharose 4B, DEAE-Sephadex A-50, QAE-Sephadex A-50, CM-Sepharose (Pharmacia, Sweden); acrylamide, arginine, methylenebisacrylamide, tetramethylethylenediamine (Medigen, Russia); sodium azide (NaN_3), glycerol, Coomassie R-250, ammonium persulfate, Tris (Serva, Germany); glycine, salmon protamine, phenylmethylsulfonyl fluoride (PMSF) (Sigma, USA); heparin (SPOFA, Poland). PBS (phosphate buffer saline) contained 0.15 M NaCl, pH 7.4, 1.9 mM Na_2HPO_4/8.1 mM NaH_2PO_4. Sodium-acetate buffer (0.1 M) contained 0.089 M AcONa/0.011 M AcOH for pH 5.5, 0.0948 M AcONa/0.0052 M AcOH for pH 6.0, and 0.09977 M AcONa/0.000023 M AcOH for pH 7.4.

Lf was purified from breast milk [13]. Milk samples were centrifuged at 15,000*g* for 30 min to remove lipids. Tf was isolated from serum [18].

Isolation of stable Cp required immobilization of arginine, heparin, and protamine on BrCN-activated Sepharose 4B (3 mg per ml of resin) [13]. Cp was isolated using affinity chromatography on protamine-Sepharose [11]. To 2000 ml of plasma containing 0.9% sodium citrate, EDTA (10 mM) and PMSF (0.1 mM) were added. Plasma was centrifuged for 30 min at 450*g* (4°C). Supernatant was diluted by half with CH_3COOH-CH_3COONa buffer (final concentration, 0.05 M), pH 5.5, and 10 g of DEAE-Sephadex A-50 was added. The mixture was subjected to gentle shaking for 2 h at 4°C that resulted in binding of Cp to the resin. After that, green DEAE-Sephadex A-50 was packed in a column (5 × 15 cm) that was eluted with a linear gradient from 0.05 M to 0.5 M CH_3COOH-CH_3COONa, pH 5.5 (2 × 1000 ml). The fractions containing Cp were pooled, diluted by half with 0.01 M Na_2HPO_4-NaH_2PO_4, pH 7.4, and applied to a column with QAE-Sephadex A-50 (5 × 10 cm). The latter was washed with 500 ml PBS, and Cp was eluted with 0.4 M NaCl in 0.01 M sodium phosphate buffer (pH 7.4).

Next, Cp was subjected to ethanol precipitation. The mixture C_2H_5OH–$CHCl_3$ (9 : 1 v/v) was added to Cp solution at a ratio 1 : 1 (v/v) on ice. The opaque suspension was centrifuged for 30 min at 1000*g* (4°C). The supernatant was supplemented with an equal volume of C_2H_5OH–$CHCl_3$ mixture (9 : 1 v/v) and centrifuged for 30 min at 1000*g* (4°C). Precipitated Cp was dissolved in 10 ml PBS, and insoluble admixtures were removed using centrifugation for 30 min at 1000*g* (4°C).

The supernatant was filtered through a column with arginine-Sepharose (3 × 10 cm). Eluate was applied on a PBS-equilibrated column with protamine-Sepharose (2 × 10 cm). The column was washed with 200 ml of 0.075 M NaCl (pH 7.4) and then eluted with 0.33 M NaCl (pH 7.4) at high rate (1 ml/min per cm^2 cross section of the column). The eluate containing Cp was dialyzed against PBS and filtered through a column with heparin-Sepharose (2 × 4 cm). Pure monomeric Cp had ratio $A_{610}/A_{280} = 0.052$ and was stable during storage. Its yield was estimated to be 42%.

Cp was revealed after PAGE in an SDS-free gel [19] by staining with *o*-dianisidine [20]. Upon 1 h of incubation of gel slabs at 25°C in 0.01% $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ buffered with 0.4 M sodium acetate, pH 5.5, and subsequent staining with 25 mM $K_4Fe(CN)_6$, the bands with ferroxidase activity acquired blue color against the light-green background.

The holo-transferrins were obtained by dialysis of apo-transferrins against 10 molar excess of $FeCl_3$ or $CuCl_2$ and $NaHCO_3$ in 0.1 M sodium citrate buffer, pH 8.0. Solutions of metal-containing transferrins were passed through a Chelex-100 column.

To compare the rate of oxidized iron incorporation into apo-transferrins, the time dependence of absorption at 460 nm was followed. The reaction mixture contained 0.55 μM Cp, 0.5 mM $NaHCO_3$, 0.34 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and 0.13 mM apo-Lf or apo-Tf in 0.1 M sodium acetate buffer (pH 5.5 and 7.4). The same mixture without Cp was used as a blank. The time dependence of Fe^{3+} concentration was plotted using extinction coefficients for $(Fe^{3+})_2$–Lf and $(Fe^{3+})_2$–Tf. The rate of Fe^{3+} incorporation into transferrins was estimated from the slope of the linear part of the plot. The standard deviation in five experiments was less than 1.5%. All protein solutions were treated with Chelex-100, buffers were prepared in deionized water, and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ solution was used within 5 min after its preparation.

To compare the effect of different Lf forms on Cp ferroxidase activity, the amount of ferric iron was detected spectrophotometrically in reaction with isothiocyanate (KSCN) in acidic medium. The reaction mixture contained 0.1 M sodium acetate buffer, pH 6.0, 150 μg/ml Cp, and 50 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$. Lf or Tf were added in amounts providing the required molar excess. Every 2 min 1/6 volume of the reaction mixture was sampled and added to equal volumes of 0.5 M KSCN and 6 M H_2SO_4. The concentration of Fe^{3+} was estimated from absorption at 450 nm (a_{450} = 107 ml/mg per cm). Blanks were collected from mixture without Cp. The standard deviation in four experiments was less than 2%. The time dependence of Fe^{3+} concentration was plotted.

The rate of Cp ferroxidase reaction was estimated from the slope of the linear part of the plot.

Concentrations of homogeneous proteins were measured using the following coefficients: for Cp, a_{280} = 1.61 ml/mg per cm and a_{610} = 0.0741 ml/mg per cm [21, 22]; for apo-Lf, a_{280} = 1.46 ml/mg per cm; for apo-Tf, a_{280} = 1.49 ml/mg per cm. The extent of metal ion incorporation into transferrins was estimated using coefficients for $(Fe^{3+})_2$–Lf a_{460} = 0.0547 ml/mg per cm, for $(Cu^{2+})_2$–Lf a_{435} = 0.0616 ml/mg per cm, for $(Fe^{3+})_2$–Tf a_{460} = 0.057 ml/mg per cm, and for $(Cu^{2+})_2$–Tf a_{435} = 0.0525 ml/mg per cm [23].

RESULTS

Incorporation of Fe^{3+} into apo-Lf and apo-Tf upon oxidation of Fe^{2+} to Fe^{3+} catalyzed by Cp. Oxidation of Fe^{2+} in the system Fe^{2+}–Cp–apo-transferrins–HCO_3^- catalyzed by Cp resulted in formation of colored Fe^{3+}–transferrins, this process being faster at pH 7.4 than at pH 5.5. The rate of Fe^{3+} incorporation into apo-Lf was 7 times (at pH 5.5) and 4 times (at pH 7.4) higher than in the system Fe^{2+}–apo-Tf–Cp–HCO_3^- (Fig. 1).

Evidence of ferroxidase activity of complex Cp–Lf. Cp, complex Cp–Lf (1 : 2, *in vitro*), and complex Cp–Lf in breast milk on the 6th day of lactation were revealed in SDS-free PAGE both by *o*-dianisidine staining and in the reaction for ferroxidase activity. When stained with Coomassie, pure Lf and its excess in breast milk was devoid of both ferroxidase and diamine oxidase activity (Fig. 2).

Effect of different forms of Lf on the ferroxidase reaction. Oxidation of Fe^{2+} catalyzed by Cp in the presence of apo-Lf (Cp/Lf, 1 : 1) was 1.25 times faster. Holo-Lf (either iron- or copper-containing) increased the rate of Fe^{2+} oxidation 1.6 and 2 times at molar ratio Cp/Lf of 1 : 1 and 1 : 2, respectively (table). Further addition of excess apo-Lf to Cp (Cp/apo-Lf < 1 : 1) or holo-Lf (Cp/holo-Lf < 1 : 2) did not increase the oxidation rate. Apo-Tf and holo-Tf had no effect upon ferroxidase activity of Cp. Neither form of Tf or Lf possessed ferroxidase activity.

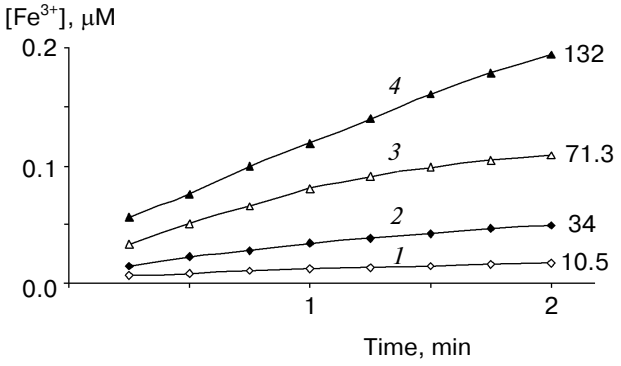

Fig. 1. Incorporation of Fe^{3+} into serum transferrin (Tf) (*1*, *2*) and lactoferrin (Lf) (*3*, *4*) in the system Fe^{2+}–apo-transferrin–Cp–HCO_3^- at pH 5.5 (*1*, *3*) and 7.4 (*2*, *4*). Numbers by the curves designate the rate of Fe^{3+} incorporation in nmol/mg Cp per min.

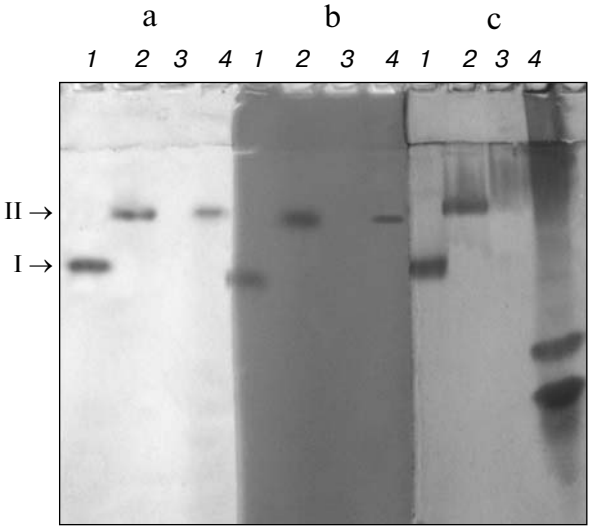

Fig. 2. Ferroxidase activity of complexes Cp–Lf. PAGE without SDS: *1*) Cp, 5 μg; *2*) Cp–Lf (5 μg + 6 μg); *3*) Lf, 6 μg; *4*) 40 μl skimmed breast milk (6th day of lactation). a) *o*-Dianisidine staining; b) staining for ferroxidase activity; c) Coomassie R-250 staining. I, electrophoretic mobility of Cp; II, mobility of the Cp–Lf complex.

DISCUSSION

In our experiments, we observed differences in the rate of incorporation of Fe^{3+} in Lf and Tf occurring on oxidation of Fe^{2+} catalyzed by Cp (Fig. 1). The higher incorporation rate of Fe^{3+} into Lf, as compared to that for Tf, can be explained by at least three different mechanisms.

First, Lf itself might oxidize Fe^{2+} on the assumption that it possessed ferroxidase activity. Such a hypothesis has been published by several authors and is based on the homology of Lf with copper-containing oxidases, e.g., diamine oxidase [24] and Cp [25]. However, staining gels after PAGE for ferroxidase activity revealed the latter only in Cp, its complex with Lf obtained *in vitro*, and that from breast milk (Fig. 2, I and II). Pure Lf and its excess migrating separately from Cp–Lf complex did not get colored. Moreover, in our control experiments we did not observe proper ferroxidase activity either in holo- or in apo-Lf.

Second, a possible explanation is a higher affinity of Lf towards iron and a more pronounced stability of complex Fe–Lf at pH values in our experiment as compared to Fe–Tf. However, at pH 7.4 when apo-Tf no longer

Effect of different Lf forms upon ferroxidase activity of Cp

Cp–Lf form (mol : mol)	Rate of ferroxidase reaction, μmol Fe^{2+}/min per mg Cp
Cp (without Lf)	0.119 ± 0.002
Cp–apo-Lf (1 : 1)	0.149 ± 0.003
Cp–apo-Lf (1 : 2)	0.151 ± 0.003
Cp–$(Fe^{3+})_2$-Lf (1 : 1)	0.190 ± 0.004
Cp–$(Cu^{2+})_2$-Lf (1 : 1)	0.192 ± 0.004
Cp–$(Fe^{3+})_2$-Lf (1 : 2)	0.242 ± 0.004
Cp–$(Cu^{2+})_2$-Lf (1 : 2)	0.248 ± 0.004

loses bound Fe^{3+}, we observed the difference with Lf in the rate of iron incorporation. Moreover, if the second hypothesis is correct, adding to Cp of Lf in amounts exceeding the stoichiometric ratio (Cp/Lf, 1 : 2), should cause a larger increase of the reaction rate. However, in our experiments a Cp-provided increase of the iron oxidation rate was observed at Cp/Lf stoichiometric ratio (1 : 1 as obtained by gel filtration and 1 : 2 as obtained by PAGE without SDS [13]).

In our previous studies we revealed that complex Cp with Lf could be dissociated by adding heparin and DNA [14] that interact with the N-terminal arginine cluster (^{2}RRRR^5) in Lf [26]. We suggested that this cluster accounts for the interaction of Lf with Cp. Salmon protamine, a polypeptide consisting of polyarginine clusters and interacting with Cp, also increased ferroxidase activity of Cp when the Cp/protamine ratio was 1 : 2 [11].

Thus, the third and the most likely mechanism is an allosteric interaction of cationic N-terminal region of Lf with negatively charged C-terminal domain of Cp containing three of four copper ions of the active site of the enzyme.

Previously we observed no difference when comparing the affinity of Cp to apo- and holo-Lf using chromatography, electrophoresis, and immunoelectrophoresis [13, 14]. Here we present data demonstrating that the increase in Cp activity influenced by apo- and holo-Lf is connected with changes in the stoichiometric ratio of the Cp–Lf complex. It is known that apo- and holo-Lf have differences in conformation of their domains [17].

The Cp–Lf complex found in breast milk is likely to play an important role in oxidation of iron in milk and its incorporation into Lf. In the milk, 75-88% of the latter is represented by the apo-form [27]. In fact, only the band corresponding to the Cp–Lf complex of breast milk showed ferroxidase activity in $K_4[Fe(CN)_6]$-stained gels. It seems likely that complex Cp–Lf will display the ferroxidase activity also *in vivo*, e.g., as a component of breast milk. The amount of Lf in breast milk is more than 100 times higher than that of Cp. It seems likely that a higher ferroxidase activity of complex Cp–Lf should affect the results of determination of Cp content by its ferroxidase activity in breast milk. In fact, such a determination resulted in overestimation of Cp content [28] compared to that calculated from the data of rocket immunoelectrophoresis and diamine oxidase activity [29].

It can be suggested that an increase of the ferroxidase activity of Cp in the bloodstream may occur in acute phase of inflammation when neutrophils secrete up to 30 g of Lf per day and 10 g of that remain in the bloodstream. At this concentration, Lf will be able to bind more than half of the amount of iron entering the blood. Under the conditions of an inflammation focus where pH is low, Fe^{3+} can leave Tf and get incorporated into Lf [30]. Perhaps in the inflammation foci, Lf is able to bind Fe^{3+} to inhibit microbial growth. The report on frequent cases of hypoferremia in inflammation is in line with this suggestion [30]. Another report concerning bactericidal activity of Cp at pH 5.0 in the presence of Fe^{2+} and phosphate [31] demonstrates that Fe^{2+} oxidation catalyzed by Cp and phosphate plays the key role in suppression of microbial growth. Since Lf increases ferroxidase activity of Cp and possesses bactericidal properties, the Cp–Lf complex very likely takes part in host defense reactions against pathogenic microorganisms.

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