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Effect of lactoferrin on oxidative features of ceruloplasmin

Alexej V. Sokolov · Kira V. Ageeva · Maria O. Pulina · Elena T. Zakharova · Vadim B. Vasilyev

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Abstract In our previous report we first described a complex between lactoferrin (Lf) and ceruloplasmin (Cp) with $K_d \sim 1.8$ μM. The presence of this complex in colostrum that never contains more than 0.3 μM Cp questions the reliability of K_d value. We carefully studied Lf binding to Cp and investigated the enzymatic activity of the latter in the presence of Lf, which allowed obtaining a new value for K_d of Cp–Lf complex. Lf interacting with Cp changes its oxidizing activity with various substrates, such as Fe^{2+}, *o*-dianisidine (*o*-DA), *p*-phenylenediamine (*p*-PD) and dihydroxyphenylalanine (DOPA). The presence of at least two binding sites for Lf in Cp molecule is deduced from comparison of substrates' oxidation kinetics with and without Lf. When Lf binds to the first site affinity of Cp to Fe^{2+} and to *o*-DA increases, but it decreases towards DOPA and remains unchanged towards *p*-PD. Oxidation rate of Fe^{2+} grows, while that of *o*-DA, *p*-PD and DOPA goes down. Subsequent Lf binding to the second center has no effect on iron oxidation, hampers DOPA and *o*-DA oxidation, and reduces affinity towards *p*-PD. Scatchard plot for Lf sorbing to Cp-Sepharose allowed estimating K_d for Lf binding to high-affinity ($\sim$13.4 nM) and low-affinity ($\sim$211 nM) sites. The observed effect of Lf on ferroxidase activity of Cp is likely to have physiological implications.

Keywords Lactoferrin · Ceruloplasmin · Protein–protein interaction · Enzymatic kinetics

A. V. Sokolov (✉) · K. V. Ageeva · M. O. Pulina · E. T. Zakharova · V. B. Vasilyev
Institute for Experimental Medicine of the Russian Academy of Medical Sciences, Pavlov Street 12, 197376 St Petersburg, Russia
e-mail: biochem@nm.ru

Introduction

Lactoferrin (Lf), first described as the breast milk major protein belonging to the transferrin family, can be found also in exocrine secretions and specific granules of neutrophils. It is a multifunctional protein with properties of immunomodulator, of a growth and transcriptional factor. These features result from Lf's ability to interact with various biomolecules and to bind specifically transition metals (Brock 2002).

Although Lf plays no major role in normal iron homeostasis, it may contribute to alteration in iron metabolism in case of infection and inflammation. It seems likely that Lf is able to take up iron in an inflammation focus, where low pH precludes iron retention by transferrin. The iron-binding capacity of Lf may promote other physiological functions. For instance, antimicrobial activity of Lf initially was thought to be provided only by its capacity to sequester iron ions from the medium, thus preventing their utilization by microorganisms (Brock 1980; Arnold et al. 1980). To get incorporated into

transferrins (Lf in that number) and ferritin Fe^{2+} ions have to be oxidized to Fe^{3+}. Oxidation of ferrous iron is realized by ceruloplasmin (Cp, ferro: O_2-oxidoreductase, EC 1.16.3.1) (Vassiliev et al. 2005). Therefore, the interaction between Cp and Lf that we found previously does not seem to be accidental (Zakharova et al. 2000; Pulina et al. 2002). The Cp–Lf complex has electrostatic nature as its dissociation is achieved by decreasing pH or increasing salt concentration and by adding polyanionic (DNA, lipopolysaccharides, heparin) or polycationic (protamine) molecules (Zakharova et al. 2000; Pulina et al. 2002; Sokolov et al. 2005b).

The importance of Cp as plasma and tissue ferroxidase is evident when comparing the rate of ferrous iron tissue influx with that of its non-enzymatic oxidation (Frieden and Hsieh 1976). Apart from its ability to load iron onto transferrin, Cp is likely to have other applications in iron metabolism. This notion is supported by the fact that in the absence of transferrin both humans (Hamill et al. 1991) and mice (Bernstein 1987; Huggenvik et al. 1989) develop a severe anemia due to iron-limited erythropoiesis and severe iron overload in liver and pancreas. Meanwhile neither humans nor mice with atransferrinemia manifest neural or cognitive dysfunction as seen in patients with aceruloplasminemia. This adult-onset neurological disease apart from extensive loss of neurons and massive iron accumulation in brain and liver may be attended by retinal degeneration and *diabetes mellitus* (Yoshida et al. 1995). It was found recently that membrane bound Cp (GPI-Cp) on astrocytes is essential for iron efflux (Jeong and David 2003), which is thought to be an additional function of the protein. It was also shown that GPI-Cp co-localizes on astrocyte cell surface with ferroportin 1, the iron exporter, and these two proteins may contact physically, which allows their co-immunoprecipitation (Jeong and David 2003). Later the same authors demonstrated that multicopper oxidases (including GPI-Cp, and secretory Cp) stabilize ferroportin 1 on the cell surface thus maintaining the iron metabolism (De Domenico et al. 2007). Inhibition of Cp activity or synthesis prevented stable presentation of ferroportin on the surface of glioma cells, astrocytes and macrophages, which resulted in a decreased iron export. The importance of Cp for normal cellular iron efflux was also shown for hepatocytes and reticuloendothelial cells (Harris et al. 1999), and for the cells of duodenal epithelium (Cherukuri et al. 2005). Participation of Cp in iron metabolism is also evidenced by its interactions with ferritin (Van Eden and Aust 2000) and Lf.

Another role of Cp is a marker of inflammation acute phase, possessing the features of a universal antioxidant (Gutteridge and Stocks 1981). Cp has superoxide dismutase (Vasil'ev et al. 1988) and glutathion-dependent peroxidase activities (Park et al. 1999). It also inhibits myeloperoxidase, the pro-oxidative enzyme of leukocytes (Segelmark et al. 1997). Along with ferrous ions Cp can oxidize biogenic (epinephrine, serotonin, DOPA) and synthetic (*p*-PD, *o*-DA) amines (Wallas et al. 1964).

We found that the ferroxidase activity of Cp can be affected by Lf (Sokolov et al. 2005a). To our knowledge Lf is the only protein among transferrins that is able to stimulate Cp as ferroxidase. Selective interaction between Cp and Lf is evidenced by formation of their complex revealed in the blood plasma of patients with various pathologies (Sokolov et al. 2007) and in colostrum of healthy women (Sokolov et al. 2006). The complex is the only breast milk component with ferroxidase activity, i.e., the whole bulk of Cp in milk interacts with Lf. It is known that Cp concentration in milk is low, being 0.3 μM in colostrum and 40 nM in mature milk (Kiyosawa et al. 1995), which contradicts the K_d value ~1.8 μM obtained previously (Zakharova et al. 2000). The presence of the Cp–Lf complex in plasma where Lf content remains low even after a disease-caused increase also calls in question the K_d value.

The data we obtained in course of our studies of the Cp–Lf complex are indicative of an unambiguous physiological importance of the interaction between Lf and Cp. We investigated the structure-functional peculiarities of the Cp–Lf complex in details, in particular we studied how adding Lf changes the oxidase activity of Cp towards various substrates (Fe^{2+}, *o*-DA, *p*-PD and DOPA). Kinetic parameters (K_m, V_{max}) of the respective reactions were measured. In accord with the algorithm of refinement of the equations used to calculate the constants of biparametrical inhibition or activation of enzymes (Krupyanko 2007), we determined the type of inhibition (or activation) and calculated the constants (K_a, K_i). Along with that K_d characterizing affinity of Lf towards Cp-Sepharose was deduced from Scatchard plot.

Materials and methods

Chemicals were purchased from BioRad (USA), Fluka (Switzerland), Merck (Germany), Pharmacia (Sweden), Serva (Germany), Sigma (USA), Laboratory Medigen (Russia), and SPOFA (Poland). All solutions were prepared using deionized water and treated, when necessary, with Chelex-100 to eliminate traces of heavy metals.

Ceruloplasmin was obtained by affinity chromatography on protamine-Sepharose (Sokolov et al. 2005b). Lf was isolated from breast milk as in (Zakharova et al. 2000). Cp-Sepharose was prepared by immobilization of Cp on BrCN-activated Sepharose 4B (Zakharova et al. 2000).

Spectrophotometrical measurments were performed at 20°C using spectrophotometer SF 2000-02 ("Spectr", Russia). Protein concentration was assessed using coefficients $a_{280} = 1.61$ ml mg^{-1} cm^{-1} and $a_{610} = 0.0741$ ml mg^{-1} cm^{-1} for Cp, $a_{280} = 1.46$ ml mg^{-1} cm^{-1} for Lf (Zakharova et al. 2000).

Enzymatic activity of Cp

To assess Lf effect on Cp enzymatic activity Hanes–Woolf graphs were plotted describing oxidation of different substrates measured spectroscopically. Reaction rate was measured at 20°C as a change of absorption with time at different substrate concentrations. Lf was added in amounts providing a required molar ratio to Cp (1, 2 or 4 Lf per 1 Cp). Three parallel measurements were done for every concentration of substrates and Lf. The respective K_m and V_{max} were determined from the graphs in Hanes–Woolf coordinates using least squares fitting. Coefficients of determination for approximating straight lines in Hanes–Woolf graphs were all above 0.98. Lf displayed no oxidase activity towards any substrate of Cp.

Kinetics of ferroxidase reaction was studied by measuring the rate of Fe^{3+} formation. This novel method is based on spectrophotometry of a complex ($\lambda_{max} = 270$–320 nm) that Fe^{3+} forms with dimethylformamide. Reaction mixture contained 0.1 M sodium-acetate buffer, pH 5.5, with dimethylformamide added to achieve 1%, ferrous ammonium sulfate in 30 μM thiourea to provide its concentration 80, 100, 125, 150, 175 and 200 μM, and 0.79 μM Cp. The reaction rate was measured as ΔA_{310}/min. To prove the validity of the new method control experiments were run in which the iron oxidation rate was measured using the test with ferrozine (Van Eden and Aust 2000). The results obtained by the two methods coincided.

o-Dianisidine oxidation by Cp was studied in solution containing 0.36 μM Cp in 0.1 M sodium-acetate buffer, pH 5.5, to which portions of 2 mM *o*-DA dihydrochloride stock solution in 1% Triton X-100 were added to provide the resulting concentrations 0.1, 0.2, 0.3 and 0.4 mM. The reaction rate was measured as ΔA_{450}/min.

Oxidation of DOPA by Cp was followed by absorption at 320 nm (ΔA_{320}/min). Reaction mixture contained 0.1 M sodium-acetate buffer, pH 5.5, 1.58 μM Cp, and 0.1, 0.25, 0.5 or 1.0 mM DOPA.

Catalyzed by Cp *p*-PD oxidation rate was assayed as ΔA_{530}/min. Reaction mixture contained 0.1 M sodium-acetate buffer, pH 5.5, 1.58 μM Cp, *p*-PD as 0.25, 0.5, 0.75, 1 and 1.5 mM solution.

Dissociation constant (K_d) of Cp–Lf complex

To measure K_d of the complex we used suspension of Cp-Sepharose in 10 mM sodium-phosphate buffer with 0.15 M NaCl, pH 7.4 (total volume-10 ml, resin volume-3 ml). Activated Sepharose with blocked active groups was used for control. The same amounts of Lf (0.2, 0.4, …, 2.2 μM) were added both to the measuring and control suspensions. Test-tubes were shaken for 10 min at 20°C to achieve equilibrium. Then resin was precipitated by centrifugation (100*g*, 5 min) and Lf concentrations in supernatant portions from both test-tubes were assessed by rocket immunoelectrophoresis with polyclonal antibodies (Laurell 1967). Antibodies had been obtained by immunizing rabbits with Lf (Sokolov et al. 2007). Concentration of Lf not bound to Cp-Sepharose was denoted as [*F*]. Difference between Lf concentrations in the two test-tubes gave [*B*], i.e., the amount of Lf specifically bound to Cp, after which [*B*]/[*F*] was calculated and Scatchard graph was plotted. The experiment was repeated three times. K_d values were determined as tangent of an angle formed by abscissa and the approximating line plotted by the least squares fitting. These K_ds were presented as mean values with standard deviation. Next, to learn how varying concentrations of Lf-binding centers on Cp-Sepharose alter the Scatchard

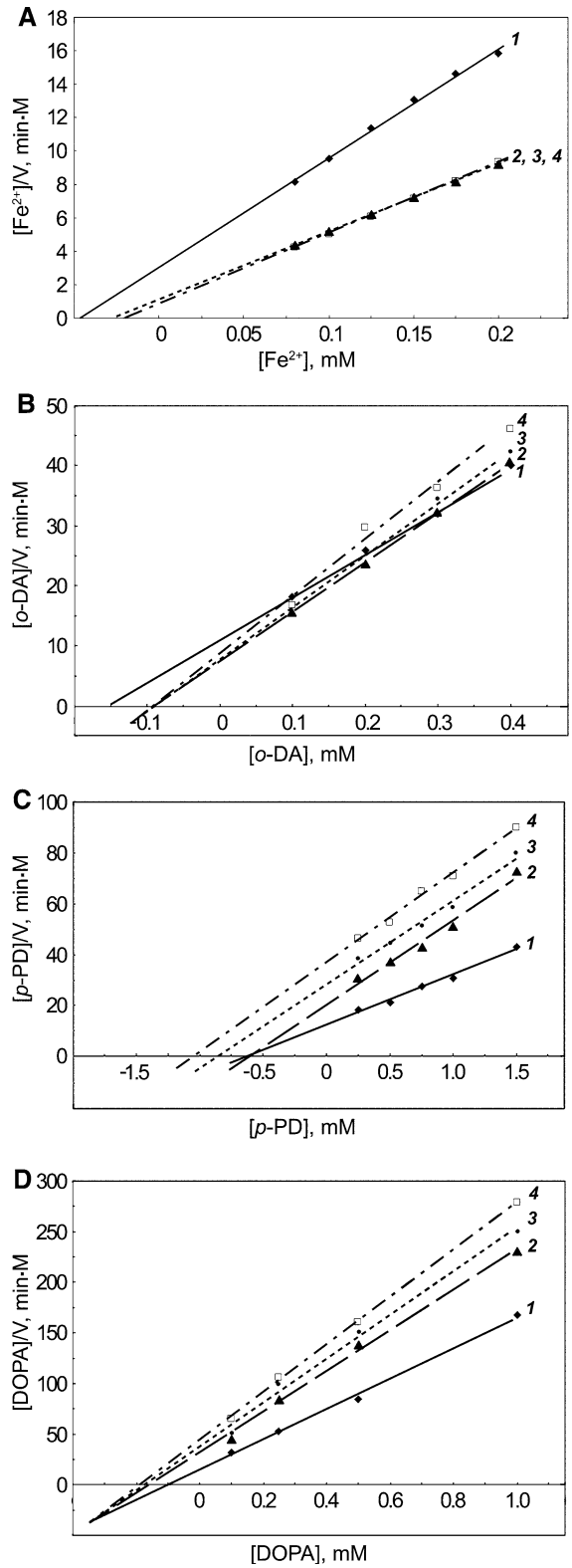

Fig. 1 Hanes–Woolf graphs for oxidation of different substrates catalyzed by Cp without Lf (*1*) and in presence of equal to Cp (*2*), twofold (*3*) and fourfold (*4*) amount of Lf. In all cases 0.1 M sodium-acetate buffer, pH 5.5 and 20°C were used. **A** Oxidation of Fe^{2+} (0.79 μM Cp); **B** Oxidation of *o*-DA (0.36 μM Cp); **C** Oxidation of *p*-PD (1.58 μM Cp) and **D** Oxidation of DOPA (1.58 μM Cp) (see "Materials and methods" for details). Kinetic parameters' values presented as M ± SD are in Table 1

plot we used different amounts of Cp-Sepharose (2 and 4 ml) and assessed the changes.

Statistical analysis

Three parallel measurements were done for every concentration of substrates and Lf in course of enzymatic studies. Similarly the experiment with immobilized Cp was repeated three times. Data were presented as mean values with standard deviation. Statistical analysis was performed using the Student's *t* test for unpaired data. *P* values <0.05 were considered significant.

Results and discussions

To study in details how Lf affects the ferroxidase activity of Cp we assessed the kinetic parameters of Fe^{2+} oxidation catalyzed by Cp in the presence of different amounts of Lf and in its absence. A Hanes–Woolf plot (S, S/V) illustrates the reaction rates obtained with varying substrate concentrations (Fig. 1A). Comparing lines 1 and 2 one can see that Lf in molar amount equal to that of Cp accelerates the reaction, decreasing K_m and raising V_{max} (lines 1 and 2; for kinetic data see the Table 1). This is the so called biparametric coordinated (mixed) type activation (Krupyanko 2007). The activation constant was calculated as:

$$K_a = [\mathrm{Lf}] \Big/ \left(\left((K_m - K'_m)/K'_m \right)^2 + \left((V'_{max} - V_{max})/V_{max} \right)^2 \right)^{0.5},$$

where K_m and V_{max} stand for the absence of Lf, while K'_{max} and V'_{max} are parameters for 1Lf:1Cp ratio. Increasing Lf concentration did not change its activating effect (lines 2, 3, and 4 in Fig. 1A, virtually coincide; see also the Table 1). In our control tests Lf was added to the same reaction mixture, but Cp, and the oxidation rate was equal to

Table 1 Kinetic parameters of various substrates' oxidation by Cp in the presence of Lf

Substrate	Parameter (M ± SD)	Lf:Cp molar relation			
		0	1	2	4
Fe^{2+}	K_m, μM	46.5 ± 1.9	27.5 ± 4.5	20.3 ± 4.9	20.2 ± 5.1
	V_{max}, ΔA_{310}/h	0.918 ± 0.020	1.48 ± 0.03	1.54 ± 0.04	1.40 ± 0.05
	K_a, μM	–	0.42 ± 0.01	–	–
o-DA	K_m, μM	155 ± 5	84 ± 6	85 ± 5	91 ± 7
	V_{max}, ΔA_{450}/h	0.834 ± 0.042	0.720 ± 0.017	0.684 ± 0.015	0.630 ± 0.016
	K_i, μM	–	0.42 ± 0.02	7.9 ± 0.5	8.8 ± 0.6
p-PD	K_m, μM	612 ± 11	603 ± 15	854 ± 18	1,045 ± 22
	V_{max}, ΔA_{530}/h	3.00 ± 0.09	1.79 ± 0.06	1.81 ± 0.07	1.70 ± 0.09
	K_i, μM	–	2.3 ± 0.4	3.8 ± 0.5	6.5 ± 0.6
DOPA	K_m, μM	99 ± 4	148 ± 7	179 ± 5	186 ± 5
	V_{max}, ΔA_{320}/h	0.396 ± 0.004	0.294 ± 0.002	0.276 ± 0.002	0.252 ± 0.003
	K_i, μM	–	2.6 ± 0.3	3.4 ± 0.4	6 ± 0.6

zero. Using KSCN-method we also showed that Lf has no ferroxidase activity (Sokolov et al. 2005a).

Oxidation of *o*-DA by Cp was measured similarly (Fig. 1B). Added to Cp in equimolar amounts Lf decreased both V_{max} and K_m (lines 1, 2), which is the nonassociative (noncompetitive) inhibition described as:

$$K_i=[\mathrm{Lf}]\Big/\left(\left(\left(K_m-K'_m\right)/K'_m\right)^2+\left(\left(V'_{max}-V_{max}\right)/V_{max}\right)^2\right)^{0.5},$$

(for data see the Table 1). Increasing Lf concentration did not change K_m, whereas V_{max} continued to drop (lines 3, 4), which is the catalytic (noncompetitive) inhibition of the overall aminooxidase activity of the 1Cp:1Lf complex. We calculated K_i as $[\mathrm{Lf}]'/((V'_{max}/V''_{max})-1)$, where $[\mathrm{Lf}]'$ is the concentration of surplus Lf, V'_{max} is the maximum reaction rate for 1Lf:1Cp ratio, and V''_{max} is that in the presence of twofold or fourfold Lf excess (see the Table 1).

Catalytic (noncompetitive) inhibition by Lf of *p*-PD oxidation catalyzed by Cp was evidenced when equimolar amounts of Lf decreased V_{max} and virtually did not affect K_m (Fig. 1C, lines 1, 2; see also the Table 1), so that $K_i = [\mathrm{LF}]/((V_{max}/V'_{max}) - 1)$. Adding Lf increased K_m, while V_{max} remained constant (lines 2–4), which is the associative (competitive) inhibition of *p*-PD oxidation by the complex 1Cp:1Lf, and $K_i = [\mathrm{Lf}]'/((K''_m/K'_m) - 1)$ (data in the Table 1).

Lactoferrin added to Cp in required amounts affected DOPA oxidation as a biparametric coordinated (mixed) type inhibitor (Fig. 1D) with

$$K_i=[\mathrm{Lf}]\Big/\left(\left(\left(K'_m-K_m\right)/K_m\right)^2+\left(\left(V_{max}-V'_{max}\right)/V'_{max}\right)^2\right)^{0.5},$$

when K_m increased and V_{max} slowed down (data in Table 1).

Lactoferrin displayed no oxidase activity towards *p*-PD despite of its homology to Cp and diamine oxidase described earlier (Metz-Boutigue et al. 1981; Houen et al. 1996). We also found no aminooxidase activity of Lf in our control experiments. However, even if such were revealed its presence would provide no explanation to the cases when oxidase activity of Cp was inhibited.

Analyzing the data obtained we found that some constants characterizing the influence of Lf on oxidation of various substrates by Cp are coinciding though effects may differ. For example, the inhibition constant (0.42 μM) for *o*-DA oxidation by the 1Lf:1Cp complex is equal to the activation constant of the ferroxidase reaction. Besides, all the inhibition constants for oxidation of *p*-PD and DOPA in the presence of Lf are virtually the same. Since an effector's binding to an enzyme (Lf to Cp) is reflected by inhibition and activation constants, the coincidence of K_i and K_a values argues in favor of a correct method of their calculation. Binding a mole of Lf is

sufficient both for activating the ferroxidase activity and for inhibiting the amines' oxidation.

Various modifications by Lf of Cp-catalyzed oxidation of substrates probably reflect the localization of substrate-binding sites in Cp molecule. Cp molecule is composed of six domains as judged by the X-ray structure (Zaitsev et al. 1999). Six copper ions that belong to three spectral types are distributed among these domains. Three type I ('blue') coppers are located in domains 2, 4 and 6. Type II ion and two type III coppers form the trinuclear center. Together with a 'blue' ion they compose the catalytic center of Cp between domains 1 and 6 (Zaitsev et al. 1999). Each substrate-binding site is in close proximity to type I Cu^{2+} ions that accept electrons from oxidized substrates. X-ray structure of Cp with attached substrates demonstrated that biogenic amines (e.g., DOPA) are bound in domain 6 (E935, H940, D1025), close to type I copper. Fe^{2+}-binding site includes the amino acid residues listed and E272. Synthetic amines (e.g., *p*-PD) are proved to be bound by amino acids H667 and W669 of domain 4, close to another blue copper (Zaitsev et al. 1999).

Our data suggest that Cp molecule has two Lf-binding sites. It seems logical that if association with Lf affects the affinity of Cp towards substrates, the binding center for Lf is in close proximity to the sites of substrates' binding. Attached to the high-affinity site Lf promotes Fe^{2+} and *o*-DA binding to Cp, impedes DOPA binding, and has no effect on the *p*-PD affinity towards Cp. Indeed, if to assume that the high-affinity center is close to the binding site for biogenic amines (DOPA), the interaction with Lf may provide a steric barrier for such a substrate. The geometry of the Fe^{2+}-binding site located nearby can get altered so that the affinity towards the substrate increases. Perhaps the high-affinity site is partly composed of the amino acid stretches 50–109 and 929–1,012 that were spotted previously as Lf-binding sequences in Cp (Sokolov et al. 2007). These stretches provide ligands for type I Cu^{2+} in domain 6 and for coppers of the trinuclear cluster that oxidize Fe^{2+} together. Binding Lf at this site may modify the active center of Cp, enhancing ferroxidase reaction, but inhibiting oxidation of *o*-DA, *p*-PD and DOPA. Since excessive Lf interacting with the 1Cp:1Lf complex diminished affinity of Cp towards *p*-PD, while V_{max} of the substrate's oxidation remained unaltered, we propose that the low-affinity Lf-binding site in Cp is probably located in domain 4. Lf binding at the low-affinity site reduces the maximum rate of DOPA and of *o*-DA oxidation, but does not alter the Cp ferroxidase activity as compared to the complex 1Cp:1Lf.

A decrease of the affinity of Cp towards *o*-DA upon formation of the complex with stoichiometry 1Cp:1Lf was unexpected. It had been presumed that *o*-DA binds to domain 4 like it happens with *p*-PD (Brown et al. 2002). However, our data point at the more likely binding to domain 6. Besides, the mechanisms of oxidation of the two substrates are different. Another unexpected observation was a decrease of the affinity of Cp towards DOPA upon adding Lf in amounts exceeding the equimolar ratio. This effect allows suggesting that domain 4 contains another binding site for DOPA unrevealed by crystallography (Zaitsev et al. 1999).

We had shown that binding of Lf to Cp-Sepharose does not depend on temperature (Zakharova et al. 2000). Therefore, our results concerning inhibiting/activation of enzymological reactions accomplished by Cp obtained at 20°C seem to be relevant to physiolgical conditions.

On the basis of our findings one can conclude that Lf modulates activity of Cp so that among various substrates Fe^{2+} is preferentially oxidized by the 1Cp:1Lf complex. Indeed, ferrous iron has the highest affinity towards Cp, while Lf is able to augment that affinity and to increase Fe^{2+} oxidation rate.

Experiments with immobilized Cp confirmed our suggestion about two Lf-binding sites in Cp. Scatchard plot describing Lf binding to Cp-Sepharose comprised three linear segments (Fig. 2). The first one (line a) is characterized by $K_d = 13.4 \pm 0.2$ nM, while $K_d = 211 \pm 11$ nM corresponds to the third one (line c). The ascending middle segment (line b) had invariable [*F*] value 152 ± 7nM, whereas [*B*]/[*F*] ratio varied from 4 to 8. The approximating line passes through the coordinate origin, which explains why [*F*] did not change in response to varying the amounts of resin (2–4 ml).

Comparing fragments of abscissa delimited by the approximating lines of the first and the third segments of the Scatchard plot showed that the number of the high-affinity Lf-binding sites is 6 times lower than the total number of Lf-binding sites on Cp-Sepharose. Therefore, a comprehensive pattern of Lf interaction with Cp-Sepharose can be proposed.

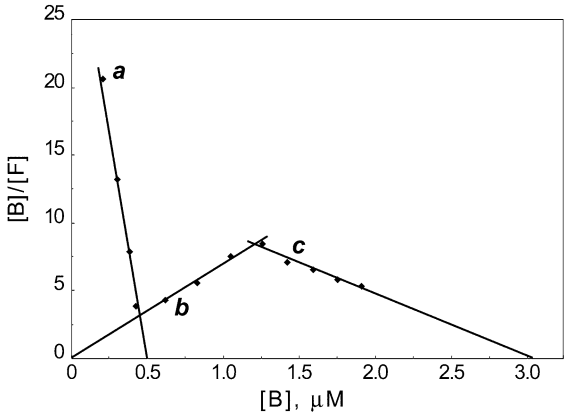

Fig. 2 Scatchard plot illustrating Lf binding to Cp-Sepharose. Lf (0.2–2.2 μM) was added to 3 ml of resin in 10 mM sodium-phosphate buffer with 0.15 M NaCl, pH 7.4 (totally 10 ml) and incubated for 10 min at 20°C, after which the resin was precipitated. Concentration of bound [*B*] and free [*F*] Lf was assessed by rocket immunoelectrophoresis (see "Materials and methods" for details). Linear segments *a* and *c* gave us K_d values for high-affinity (13.4 ± 0.2 nM) and low-affinity (211 ± 11 nM) binding. The ascending segment *b* had invariable [*F*] = 152 ± 7 nM. Data presented as M ± SD

Lactoferrin first binds at the high-affinity sites in Cp. Upon their saturation excessive Lf is not likely to bind to Cp, but rather to Lf molecules already bound (the tendency of Lf to aggregate was reported in (Babina et al. 2006)). As [*B*]/[*F*] reaches its critical value (about 8), Lf again binds to Cp and the low-affinity sites become saturated, which predetermines formation of multimolecular complexes on immobilized Cp. Previously we found the multimolecular complexes of Cp and Lf (Zakharova et al. 2000; Sabatucci et al. 2007).

K_d measured before as 1.8 μM (Zakharova et al. 2000) may reflect the low-affinity binding of Lf to Cp. K_d characterizing the high-affinity binding was discovered in this study in course of immunological measurements of Lf. Dissociation constants for Lf bound to the high- and low-affinity sites in Cp are in general agreement with activation and inhibition constants presented in the Table 1. Some discrepancies can be explained by different experimental conditions e.g., pH (that was 7.4 for K_d evaluation and 5.5 for oxidase reaction) or buffers (sodium-phosphate and sodium-acetate, respectively).

The K_d value obtained for the "high-affinity" complex (13 nM) seems to be in accord with the previous notion that all Cp in breast milk interacts with Lf (Sokolov et al. 2006). Among the milk proteins known to form in vitro complexes with Lf are secretory IgA (Watanabe et al. 1984), lysozyme, casein, α-lactoalbumin and albumin (Hekman 1971). However, in our experiments their presence did not prevent formation of the Cp–Lf complex in milk. Besides, milk Lf interacted both with Cp added to breast milk and with Cp-Sepharose. These facts speak in favor of the selective interaction between the two proteins.

Despite that the Cp–Lf complex has been found in milk its role is not likely to be manifested there, but rather in the blood. Indeed, Lf is released from neutrophilic leukocytes and, according to our data, its whole bulk binds to Cp of plasma in molar ratio 1:1 (Sokolov et al. 2007). The progress of inflammation is accompanied by tissue necrosis causing an increment to ferrous iron pool. Brought about by respiratory burst the active oxygen species (namely hydrogen peroxide, hydroxyl radical, superoxide anion radical) may react with pro-oxidant ions and facilitate the oxidative injuries. Therefore, oxidation of pro-oxidant ferrous ions catalyzed by Cp should reduce the oxidative stress. It is known that cerebral hemorrhage followed by decomposition of erythrocytes and ferrous iron accumulation provokes the local expression of Cp in adjacent tissue (Yang et al. 2006). Authors showed that pretreatment with small amounts of thrombin diminished brain edema provoked by iron injection or by cerebral hemorrhage. Also thrombin administration enhanced Cp expression. It seems likely that thrombin-mediated expression of Cp increases the brain tolerance to edema by inhibiting the pro-oxidant effects of ferrous iron abundant in hematoma.

When oxidized by Cp iron ions should be secured by a chelating agent. In view of generally low pH in an inflammation focus iron-binding properties of transferrin are impaired there, and probably Lf will take up ferric ions. It is known that during the acute phase of inflammation neutrophils daily secrete up to 30 g of Lf mostly in its apo-form, of which about 10 g remains in the bloodstream (Sawatzki 1987). That Lf can bind some half of all the plasma iron. Upon interaction of Lf with Cp the ferroxidase activity of the latter is enhanced which facilitates capturing iron ions by the Cp–Lf complex. Efficient sequestration of iron by the complex and its subsequent clearance from plasma can result in decrease of plasma iron

concentration. This phenomenon may be realized along with the hepcidin-regulated restriction of iron content in circulation (Kemna et al. 2008) and thus may play a role in high occurrence of hypoferremia in case of inflammation. Thus the Cp–Lf complex that we found in the blood of patients with inflammatory diseases might be a mechanism of an organism's protection against the neutrophilic respiratory burst in an inflammation focus.

On the other hand, sequestering iron beyond reach of microorganisms, the Cp–Lf complex may contribute to the mechanisms of host defense. Antimicrobial effect of Cp based on intensive iron oxidation was demonstrated in the presence of Fe^{2+} and phosphate at pH 5.0 (Klebanoff 1992). Oxidized iron is readily bound by Lf, which is regarded as the mechanism of the protein's bactericidal activity. It seems likely that the interaction of Cp and Lf promotes cooperation of the two proteins making the overall antimicrobial defense more efficient.

It was shown recently that ferroxidase activity of Cp is required for normal iron efflux from hepatocytes and reticuloendothelial cells (Harris et al. 1999), from duodenal epitheliocytes (Cherukuri et al. 2005), and from glial cells (De Domenico et al. 2007). Moreover, the latter authors demonstrated that an additional function of multicopper oxidases is the stabilizing of ferroportin 1 on the cell surface. Cp is likely to be an element of the pathway regulating ferroportin-mediated iron export and the ability of Lf to stimulate its ferroxidase activity upon complex formation looks like a regulatory step in iron metabolism. An important metabolic point of application for the Cp–Lf complex might be an early stage of iron import from intestine into blood. Ferrous iron accepted by hephestin, a membrane-bound homologue of Cp in enterocytes, should find itself oxidized and incorporated into a carrier protein to be transferred to red bone marrow. However, it has been shown (Hudson et al. 2008) that neither hephestin nor Cp is able to form a stable complex with transferrin that has been regarded as the main Fe^{3+}-carrier in plasma. Thus in vivo formation of a specific complex involving Cp and Lf is likely to be an accessory link between iron and copper metabolism.

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