COMMUNICATION

Studies of the ceruloplasmin–lactoferrin complex

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Abstract: We have previously shown that iron-containing human lactoferrin (LF) purified from breast milk is able to form both in vitro and in vivo a complex with ceruloplasmin (CP), the copper-containing protein of human plasma. Here we present evidence that the CP–LF complex is dissociated by high concentrations of NaCl, $CaCl_2$, or EDTA, or by decreasing the pH to 4.7. In addition, DNA, bacterial lipopolysaccharide, and heparin can displace CP from its complex with LF. Antibodies to either of the two proteins also cause dissociation of the complex.

Key words: lactoferrin, ceruloplasmin, ferroxidase.

Résumé : Nous avons déjà montré que la lactoferrine (LF), une protéine contenant du fer, purifiée à partir de lait humain, forme un complexe in vitro et in vivo avec la céruloplasmine (CP), une protéine plasmatique humaine contenant du cuivre. Dans cet article, nous montrons que le complexe CP–LF se dissocie à forte concentration de NaCl, de $CaCl_2$, ou d'EDTA, ou lorsque le pH est abaissé à 4,7. De plus, l'ADN, le lipopolysaccharide bactérien et l'héparine peuvent dissocier la CP de la LF. Des anticorps dirigés contre l'une ou l'autre de ces protéines entraînent également la dissociation du complexe.

Mots clés : lactoferrine, céruloplasmine, ferroxydase.

[Traduit par la Rédaction]

Introduction

In our recent paper (Zakharova et al. 2000), we showed that two human proteins found in various body fluids, ceruloplasmin (CP, ferro-O_2-oxidoreductase, EC 1.16.3.1) and lactoferrin (LF), selectively interact to form a complex with a $K_d = 1.8 \times 10^{-6}$ M. We demonstrated that the CP–LF complex is formed both in vitro and in vivo. CP, a copper-containing protein of vertebrate plasma, is believed to play an important role in oxidizing iron (Osaki et al. 1966), which enables the latter to get incorporated into the molecules of transferrins. Lactoferrin (or milk transferrin) is a component of secretory granules of neutrofil leukocytes and is excreted in the foci of inflammation, where CP is also present. The CP–LF complex described in our previous paper might be involved in the host defense of a mammalian organism. The antimicrobial effect of both proteins has been documented (Bullen et al. 1972; Klebanoff 1992). Because of this possible physiological importance, we investigated some features of the CP–LF complex, and the results of the study are presented below.

Materials and methods

All solutions were prepared using deionized water and treated, when necessary, with Chelex-100 to eliminate traces of heavy metals. Chemicals were purchased from Serva (Heidelberg, Germany), Merck (Frankfurt, Germany), Pharmacia (Uppsala, Sweden), Stratagen (Moscow), and Sigma Chemical Co. (Moscow).

Obtaining protein preparations

Human CP was purified as described by Prozorovski et al.

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Abbreviations: CP, ceruloplasmin (ferro-O_2-oxidoreductase; EC 1.16.3.1); LF, lactoferrin; PAG, polyacrylamide gel; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; LPS, bacterial lipopolysaccharide.

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(1982). Apo-CP was obtained by cyanide treatment of holo-CP according to Vassiliev et al. (1997). Apo-LF was purified from breast milk according to Zakharova et al. (2000). Saturation of apo-LF with iron was achieved as previously described (Zakharova et al. 2000). Coppercontaining LF was obtained as described for the iron-containing form (Zakharova et al. 2000), with the exception that $CuCl_2$ was used instead of iron trichloride. To check the incorporation of copper into LF, the molar absorption coefficient of 0.616 $M^{-1}\cdot cm^{-1}$ for copper-saturated LF at 435 nm was used (Ainscough et al. 1983).

Interaction of LF with partially proteolyzed CP was studied using highly purified CP stored at 4°C for more than 3 years, which results in spontaneous fragmentation of the protein (Prozorovski et al. 1982).

Neutrophil extract was obtained as described by Roy et al. (1997).

Protein concentrations were determined spectrophotometrically at 280 nm using the following coefficients: 1.46 $mL\cdot mg^{-1}\cdot cm^{-1}$ for Fe–LF (Masson 1970) and 1.61 $mL\cdot mg^{-1}\cdot cm^{-1}$ for human CP (De Filippis et al. 1996).

Electrophoretic experiments

PAGE under non-denaturing conditions (without SDS) was run according to Davis (1964). PAGE with SDS was carried out as described by Laemmli (1970). Immunoelectrophoresis was performed as previously described (Laurell 1967). Monospecific rabbit antibodies to either human CP or human LF were obtained as described by Zakharova et al. (1983). Oxidase activity of CP was assayed qualitatively in PAG slab after electrophoresis using *o*-dianisidine staining (Owen and Smith 1961).

Obtaining CP- and LF-Sepharose

A respective protein was immobilized on CNBr-activated Sepharose 2B according to Zakharova et al. (2000).

Results

Affinity chromatography on CP–Sepharose allowed us to obtain LF from breast milk whey, tears, and neutrophil extract. In the case of milk whey and tears the fraction that contained LF in SDS–PAGE (according to immunochemical assay; Fig. 1) was represented by a single band with an M_r of about 80 kDa. Some minor protein bands whose content did not react with anti-LF were revealed in the fraction purified from neutrophil extract. Despite the immunological evidence for its presence, LF was not purified from seminal plasma by the same method.

Since affinity chromatography on CP–Sepharose proved to be convenient in evaluation of the interaction of CP and LF (Zakharova et al. 2000), we used this method to study the stability of the CP–LF complex under various conditions and after adding various substances. Purified LF was applied on a column with CP-linked Sepharose 2B, after which pure Buffer A or the same buffer containing 50% ethylene glycol was run through the column. Under such conditions LF was retained on the resin. However, it was efficiently eluted from CP-linked Sepharose with Buffer A containing 50 mM $CaCl_2$, 300 mM NaCl, or 75 mM EDTA. Apo-LF as well as iron- or copper-saturated LF was eluted with 50 mM $CaCl_2$. When CP-linked Sepharose was sequentially treated with ascorbic acid, cyanide, and EDTA, which causes the release of copper from CP (Vassiliev et al. 1997), the resulting apo-CP–Sepharose retained the ability to bind LF.

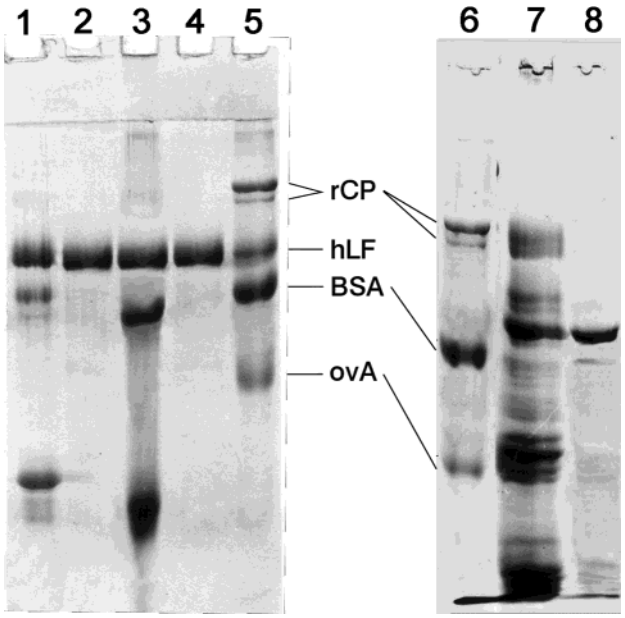


Fig. 1. SDS–PAGE of lacrimal fluid (lane 1), breast milk whey (lane 3), neutrophil extract (lane 7) and of LF-containing fractions of the same fluids (lanes 2, 4 and 8, respectively) eluted from CP–Sepharose with 0.3 M NaCl. M_r markers on lanes 5 and 6: rat CP (rCP, 130 and 110 kD), human LF (hLF, 80 kD), BSA (67 kD), and ovalbumin (OVA, 45 kD). The gel was stained with Coomassie R-250.

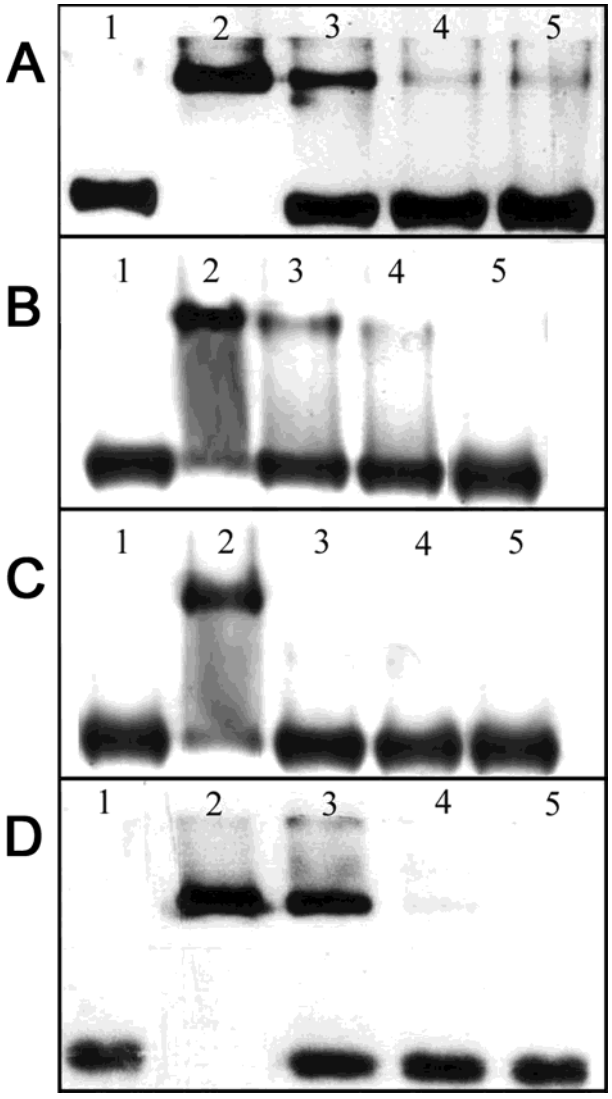
The effect of pH upon the CP–LF complex was studied when LF bound to CP–Sepharose was eluted with a stepwise pH gradient provided by various buffers. The values of pH decreased from 9.0 to 2.0. We used 0.1 M Tris–HCl as a buffer to maintain the pH between 9.0 and 7.0. To keep the pH in the range from 6.5 to 4.0 we used 0.1 M CH_3COONa as the buffer. The pH values from 3.5 to 2.0 were obtained using 0.1 M glycine–HCl. Lactoferrin was eluted from CP–Sepharose as a compact peak at a pH of 4.7. At this pH value the Sepharose-bound CP retained its blue colour, which was lost upon achieving a pH of 4.5.

Chicken DNA in Buffer A served as an efficient eluent of LF from CP–Sepharose when applied at concentrations increasing from 3 μg/mL.

Along with affinity chromatography, non-denaturing PAGE showed that upon adding DNA to the complex CP was released and acquired the electrophoretic mobility of the uncomplexed protein (Fig. 2A). Bacterial lipopolysaccharide and heparin also caused the dissociation of the complex (Figs. 2B and C). Dissociation of the CP–LF complex was also achieved by adding antibodies to either of the two proteins. An example of such an effect is shown in Fig. 2D where anti-LF provoked the release of CP from the complex.

Partially proteolyzed CP (see Materials and methods) did not lose the ability to form the complex with LF, as confirmed by PAGE in non-denaturing conditions. The electro-

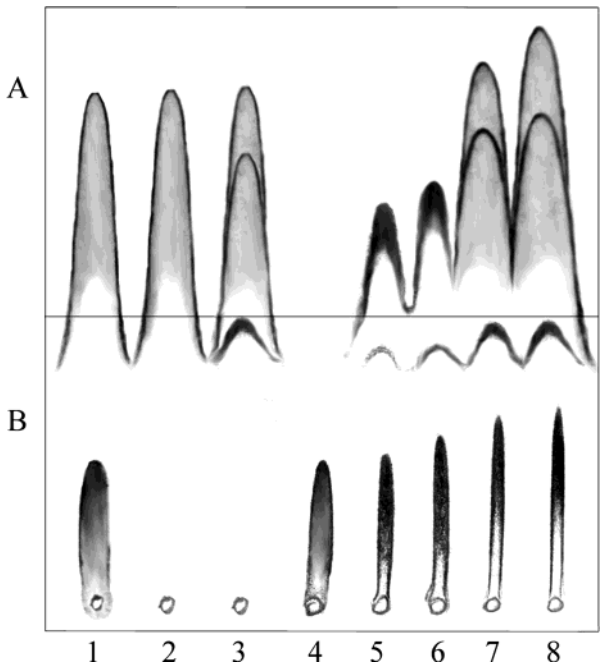
Fig. 2. Electrophoresis of CP–LF complex in the presence of (A) DNA, (B) LPS, (C) heparin and (D) rabbit anti-LF immunoglobulins. Panel A: Lane 1, 1.6 μg of purified human CP; lane 2, 1.6 μg of CP and 3 μg of LF; lanes 3, 4, and 5, the same amounts of CP and LF as on lane 2, but with 10, 20, and 30 μg of crude chicken DNA, respectively. The protocol for panels B, C, and D was the same as for panel A, except that on panel B 20, 40, and 100 μg of LPS, on panel C 1.25, 2.5, and 5 μg of heparin, and on panel D 40, 200, and 400 μg rabbit antibodies raised against LF were loaded on lanes 3, 4, and 5, respectively. The gel was stained with *o*-dianisidine for oxidase activity of CP.



phoretic mobility of the complex in this case coincided with that previously observed for LF and non-degraded CP (Zakharova et al. 2000). However, the difference in the interaction of native and proteolyzed CP with LF was detected by affinity chromatography on LF-Sepharose. Non-degraded (intact) CP did not bind to LF-Sepharose, while some part of the partially proteolyzed protein was retained by the resin and upon elution was recognized by antibodies to CP.

As mentioned above, antibodies to either of the two proteins caused dissociation of the CP–LF complex, which was

Fig. 3. Two-panel immunoelectrophoresis of CP–LF complex (1), native CP (2), partially proteolyzed CP (3), and LF (4). Wells 5–8 contained the same amount of LF and increasing amounts of partially proteolyzed CP. Agarose on panel A contained rabbit antibodies against human CP, whereas that on panel B contained antibodies against human LF. The gel was stained with Coomassie R-250.



confirmed by the results presented in Fig. 3. When the complex (well no. 1) was run through the two agarose layers, each containing antibodies either to CP (layer A) or to LF (layer B), the respective protein would precipitate liberating its counterpart. The effect of antibodies was studied on CP–LF complex, which contained partially proteolyzed CP. Unlike non-degraded CP, the proteolyzed protein formed three precipitation peaks in immunoelectrophoresis (Fig. 3). The upper peak disappeared upon adding LF to CP, as evidenced by the two-panel immunoelectrophoresis. As greater amounts of proteolyzed CP were added to LF, the precipitation peak of LF became higher. An increase of proteolyzed CP content caused the reappearance of the upper precipitation peak of that protein (see Fig. 3).

Discussion

The selectivity of the interaction between CP and LF previously demonstrated (Zakharova et al. 2000) was again confirmed by the efficient isolation of LF from several biological sources using CP–Sepharose. Lactoferrin was the only protein that was bound by immobilized CP when milk whey or lacrimal fluid was fractionated. Lactoferrin is bound to several proteins in seminal plasma (Sorrentino et al. 1999), which explains why we failed to isolate LF from that fluid. It seems likely that only free LF can be separated by CP–Sepharose from a solution.

The results of the present study allow us to suggest that the interaction of the two proteins in the complex is likely to be ionic rather than hydrophobic, since LF can be eluted

from CP–Sepharose using high concentrations of NaCl, EDTA, or $CaCl_2$. It is possibe that low concentrations of Ca^{2+} ions or other EDTA-chelated metal ions stabilize the complex between CP and LF. The ionic nature of interaction between CP (pI = 4.5) and LF (pI = 8.6) is confirmed by the dissociation of their complex at low pH when net charges of the interacting protein molecules are altered. Dissociation of the CP–LF complex caused by polyanions such as DNA, heparin and LPS is also in agreement with this concept. However, LF has a strong affinity towards these substances (He and Furmanski 1995; Van Berkel et al. 1997), which can enable the latter to displace CP from its complex with LF. The target sequence for heparin, LPS, and DNA is the arginine cluster at the N-terminus of the LF molecule (Van Berkel et al. 1997). Since these substances displace CP from its complex with LF, we can suggest that CP is bound to the same part of LF. The negatively charged sequence in CP that is likely to be involved in binding is a C-terminal stretch of amino acid residues 1028–1037 (Juan and Aust 1998).

Substitution of iron with copper in LF does not alter its conformation (Smith et al. 1992). This might explain why apo-, Fe-, and Cu-LF showed equal ability to bind to CP under the same conditions of chromatography on CP–Sepharose. Similarly, the metalloprosthetic group in CP does not seem to play a crucial role in its ability to form the complex with LF, since the latter was efficiently bound by copper-depleted CP. Indeed, copper depletion does not result in profound conformational changes (De Filippis et al. 1996) that might alter the spatial organization of the site in CP involved in interaction with LF.

Dissociation of the CP–LF complex by chromosomal DNA can be explained by the much higher acidity of the latter as compared with CP. Hence, DNA efficiently replaces CP in the complex with positively charged LF.

Since antibodies to either CP or LF cause dissociation of the complex, it can be suggested that in both proteins the surfaces involved in the formation of the complex either coincide with the epitopes or are at close proximity to them.

The pattern of interaction of LF with partially proteolyzed CP studied by two-panel immunoelectrophoresis allows the suggestion that one of the proteolytic fragments is more tightly bound by LF. Since in immunoelectrophoresis antibodies to LF do not release the fragment from the complex with LF, it is tempting to speculate that this part of the CP molecule contains the site that interacts with the LF molecule.

Both CP and LF are acute-phase reactants and protect tissues from damage caused by free oxygen radicals in the foci of inflammation where they are usually found (Britigan 1991; Klebanoff 1992). In these foci, pH may be conditioned by the activity of neutrophils that have intracellular pH close to 5.0 (Winterbourn 1981). In our experiments, the dissociation of the CP–LF complex did not occur before pH dropped to 4.7. Hence, it seems likely that the complex maintains its integrity in the foci of inflammation. Under such conditions CP is likely to get partially proteolyzed by proteases coming out of degranulated neutrophils. However, it seems able to remain within the CP–LF complex as judged by the results of affinity chromatography on LF-Sepharose.

The existence of the CP–LF complex and its properties may relate to the mechanisms of antimicrobial protection of an organism. Both proteins are found in the milk (Lampreave et al. 1990; Puchkova et al. 1997), the bactericidal properties of the latter being dependent on LF concentration (Bullen et al. 1972). The participation of each protein in acute inflammation has been documented (Van Snick et al. 1974; Ryden 1984; Sawatski 1987). A major physiological role of CP is the oxidation of Fe^{2+} to Fe^{3+}, allowing iron incorporation into transferrin (Osaki et al. 1966; Yoshida et al. 1995). It is possible that the reciprocal affinity of LF and CP facilitates the iron uptake by apo-LF in a focus of inflammation and thus prevents the occurrence of dangerous hydroxyl radicals.

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