ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 8, pp. 872-877. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 8, pp. 1072-1077.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-107, May 27, 2007.

Identification of Leukocyte Cationic Proteins That Interact with Ceruloplasmin

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Received April 16, 2007

Abstract—Proteins from leukocytes were investigated for their ability to interact with ceruloplasmin (Cp), a copper-containing glycoprotein of human plasma. Extract from leukocytes was subjected to affinity chromatography on Cp-Sepharose, after which proteins were eluted from the resin with 0.5 M NaCl in Tris-HCl, pH 7.4. SDS-PAGE of the eluate revealed protein bands with molecular weights 78, 57, 40, 30, 16, and 12 kD. Among these, Western blotting detected myeloperoxidase (57, 40, and 12 kD) and lactoferrin (78 kD). Also, the 30-kD component had a sequence ^{1}I-^{2}I/V-^{3}G-^{4}G-^{5}R/H at the N-terminus that is likely to indicate the presence of neutrophilic elastase, cathepsin G, proteinase 3, and azurocidin (CAP 37) – all from the family of serprocidins. Mass spectrometry of tryptic fragments indicated the presence of the 16-kD eosinophilic cationic protein (seven peptides), 27-kD cathepsin G (eleven peptides), 27-kD azurocidin (eight peptides), 29 kD neutrophilic elastase (seven peptides), and 27-kD proteinase 3 (six peptides). Myeloperoxidase was represented by 57-, 40-, and 12-kD fragments (thirteen, ten, and four peptides, respectively). Thus, interaction with Cp of five cationic proteins, i.e. of eosinophilic cationic protein, cathepsin G, neutrophilic elastase, proteinase 3, and azurocidin is reported for the first time.

DOI: 10.1134/S0006297907080093

Key words: ceruloplasmin, eosinophilic cationic protein, cathepsin G, neutrophilic elastase, proteinase 3, azurocidin, serprocidin, protein–protein interactions

Ceruloplasmin (Cp) is a copper-containing glycoprotein with molecular weight 132 kD. Synthesized in hepatocytes, Cp is secreted into the bloodstream; however, an alternative splicing of Cp mRNA in astrocytes and Sertoli cells results in the occurrence of an immobilized form of the protein bound to the cell membrane by a glycerophosphoinositol anchor [1]. Antioxidant features of Cp mostly result from its ability to oxidize various substrates in different manners: i) a four-electron transfer to oxygen with water being the end-product is used by Cp to oxidize Fe^{2+} [2], Cu^{+} [3], O_2^{-} [4], NO, and GSH [5]; ii) the enzyme can behave as a glutathione-dependent peroxidase [6]; iii) Cp catalyzes the oxidation of biogenic (norepinephrine, serotonin) and synthetic (*p*-phenylenediamine, *o*-dianisidine) amines [7]. In the 1990s, interactions of Cp with other proteins were described, which enlarged the overview on its probable functions, such as: regulation of clotting by interacting with protein C [8]; participation in iron metabolism by interacting with ferritin [9], ferroportin 1 [10], and lactoferrin (Lf) [11]; regulation of neural transmission and of inflammation by interacting, respectively, with neuropeptide PACAP 38 [12] and with macrophage migration inhibitory factor [13]; inhibition of pro-oxidative properties of myeloperoxidase (MPO) by forming a complex with the latter [14]. Thus, considering its various localizations, multiple enzymatic activities, and the ability to form complexes with other proteins, Cp may well be related to the group of *moonlighting proteins* [15].

In our previous studies, we observed the selective retention of Lf on a column with Cp-Sepharose when such exocrine secretions as tears and breast milk were subjected to chromatography [16]. This was explained by interaction of the cationic Lf with the negatively charged Cp (p*I* 4.7). It is worth noting that other cationic proteins present in each secretion (e.g. lysozyme) did not bind to Cp.

Chromatography of blood serum on immobilized MPO resulted in selective binding of Cp to that cationic protein [14]. Neither serum albumin nor pre-albumin

Abbreviations: CAP 37) azurocidin; Cp) ceruloplasmin; Lf) lactoferrin; MPO) myeloperoxidase; PACAP 38) pituitary adenylate cyclase activating peptide; PBS) phosphate buffered saline.

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interfered with that interaction despite the fact that their p*I* values are close to that of Cp.

Taking into account that basic amino acids prevail in the content of some proteins that were shown to interact with Cp (i.e. Lf, MPO, protamine [17], and PACAP 38), in this study we searched for its partners among the proteins of leukocytes. Cationic properties of the latter are directly connected with their protective functions.

MATERIALS AND METHODS

The following reagents were used in this study: colored molecular mass markers (BioRad, USA); cyanogen bromide (Fluka, Switzerland); triethylamine ($(C_2H_5)_3N$), EDTA (Merck, Germany); Sepharose 4B, DEAE-Sephadex A-50, QAE-Sephadex A-50 (Pharmacia, Sweden); complete and incomplete Freund's adjuvant, sodium azide (NaN_3), human serum albumin, glycerol, Coomassie R-250, mercaptoethanol, ammonium persulfate, Tris (Serva, Germany); glycine, *o*-dianisidine, horseradish peroxidase-labeled second antibodies to rabbit IgG, salmon protamine, phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), 4-chloro-1-naphthol, Folin's reagent (Sigma, USA); acrylamide, arginine, N,N′-methylene-bis-acrylamide, N,N,N′,N′-tetramethyl ethylene diamine (Laboratory MEDIGENE, Russia); heparin (SPOFA, Poland). PBS (phosphate buffered saline) (0.15 M NaCl, pH 7.4, 1.9 mM Na_2HPO_4/8.1 mM NaH_2PO_4) was also used.

Monomeric and stable Cp was isolated from blood plasma by affinity chromatography on protamine-Sepharose [17].

Lf was isolated from breast milk by affinity chromatography on CM-Sepharose followed by gel filtration on Sephadex G-100 Superfine [18].

Proteins were extracted from human leukocyte mass, which was a generous gift of Prof. V. N. Kokryakov (Department of General Pathology and Pathophysiology, Institute of Experimental Medicine, St. Petersburg). No cationic detergents were used. Leukocyte precipitate (40 g) was resuspended in 100 ml of 0.05 M sodium acetate buffer, pH 4.7, and then it was frozen and thawed and subsequently subjected to three 30-sec sonications (44 kHz) with 60-sec intervals of cooling on ice. Thus obtained, the leukocyte extract was centrifuged for 30 min at 15,000*g* (4°C). The supernatant was used for isolation of human MPO and proteins interacting with Cp.

Human MPO was isolated from the supernatant of the extract by chromatography on heparin-Sepharose and phenyl-Sepharose and gel filtration on Sephadex G-150 [19].

Immobilization on BrCN-activated Sepharose 4B of Cp, heparin, and protamine (10, 20, and 4 mg per ml wet gel, respectively) was carried out as described [17]. Antibodies against Cp, Lf, and MPO were raised by three immunizations of rabbits with the respective proteins [20]. Protein concentrations were determined according to Folin–Lowry [21] in three parallel samples, human serum albumin being used as a standard.

Affinity chromatography of the proteins from leukocyte extract on Cp-Sepharose. The supernatant of the extract (10 ml) was loaded on a column with Cp-Sepharose (4 ml), and then non-bound proteins were washed away with PBS. Washing was stopped when the effluent showed $A_{280} < 0.002$. The retained proteins were eluted from the column with 0.5 M NaCl in 10 mM Tris-HCl buffer, pH 7.4. Molecular mass of the proteins in the eluate was evaluated by SDS-polyacrylamide gel electrophoresis [22]. Western blotting identified the bands in polyacrylamide gel that contained Lf and MPO [23]. Unknown proteins were identified by mass spectrometry of their tryptic fragments. To prepare samples for mass spectrometry, the proteins were resolved by SDS-PAGE and the protein-containing bands were cut from the gel. Spectra were registered on a Bruker mass-spectrograph (Research Institute of Physico-Chemical Medicine, Moscow). Peptide fingerprints of the proteins were analyzed on-line using the program MASCOT (http://www.matrixscience.com).

N-Terminal sequencing was accomplished at the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry (Moscow), for which proteins after SDS-PAGE were transferred to Immobilon P membranes [12].

RESULTS AND DISCUSSION

We used a column with Cp-Sepharose (ca. 40 mg of immobilized Cp) to load 1500 ± 50 mg of total protein from leukocyte extract. After washing away the non-bound proteins with PBS, elution with 0.5 M NaCl in 10 mM Tris-HCl, pH 7.4, yielded 0.20 ± 0.05 mg protein in the eluate. The fraction containing non-bound proteins was again loaded on the column, after which washing and elution were repeated. This time the eluate contained no proteins.

The fraction with 0.20 ± 0.05 mg protein was analyzed by SDS-PAGE and bands corresponding to 78, 57, 40, 30, 16, and 12 kD were revealed (figure, panel (a)). Western blotting with anti-Lf and anti-MPO identified the 78-kD band as that of Lf, and the 57-, 40-, and 12-kD bands as those of MPO (figure, panels (b) and (c)). The presence in MPO preparations of a degradation product (ca. 40 kD) along with the protein protomers (57 and 12 kD) has been described [24]. It is worth noting that antibodies to Lf and MPO did not reveal these among the non-bound proteins washed away from the Cp-Sepharose.

N-Terminal sequencing of the protein(s) contained in the 30-kD band revealed the sequence ^{1}I-^{2}I/V-^{3}G-^{4}G-^{5}R/H. This suggests the presence of one or more proteins

Table 1. Peptide fingerprint of proteins interacting with Cp. Results of a search using the program MASCOT http://www.matrixscience.com (amino acids are numbered in accord with the primary structure of the full-length proteins)

N-Terminal→ C-terminal a.a. number	Expected molecular weight, daltons	Calculated molecular weight, daltons	Δ, daltons	Peptide
1	2	3	4	5
Light chain of MPO (12 kD; 60% of the sequence detected)				
1-8	918.39	918.41	0.02	VTCPEQDK
34-54	2411.13	2411.15	0.02	WLPAEYEDGFSLPYGWTPGVK
73-84	1445.66	1445.68	0.02	FPTDQLTPDQER
85-108	2817.35	2817.33	0.02	SLMFMQWGQLLDHDLDFTPEPAAR
Eosinophilic cationic protein (16 kD; 44% of the sequence detected)				
2-8	900.49	900.49	0	PPQFTR
9-23	1776.99	1776.94	0.05	AQWFAIQHISLNPPR
30-35	749.34	749.38	0.04	AINNYR
40-46	891.44	891.46	0.02	NQNTFLR
99-106	989.54	989.52	0.02	YADRPGRR
119-134	1807.99	1807.95	0.04	DSPRYPVVPVHLDTTI
123-134	1352.79	1352.73	0.06	YPVVPVHLDTTI
Cathepsin G (27 kD; 42% of the sequence detected)				
6-28	2658.26	2658.31	0.05	ESRPHSRPYMAYLQIQSPAGQSR
63-72	1196.64	1196.59	0.05	ENTQQHITAR
74-83	1281.71	1281.67	0.04	AIRHPQYNQR
77-83	941.47	941.45	0.02	HPQYNQR
84-96	1543.88	1543.84	0.04	TIQNDIMLLQLSR
101-111	1248.75	1248.71	0.04	NRNVNPVALPR
103-111	978.58	978.56	0.02	NVNPVALPR
155-162	953.49	953.46	0.03	IFGSYDPR
155-163	1109.60	1109.56	0.04	IFGSYDPRR
200-210	1174.64	1174.60	0.04	SSGVPPEVFTR
211-219	1103.65	1103.61	0.04	VSSFLPWIR
Azurocidin (27 kD; 49% of the sequence detected)				
1-16	1653.04	1653.03	0.01	RLTVLALLAGLLASSR
17-30	1368.75	1368.77	0.02	AGSSPLLDIVGGRK
35-47	1504.83	1504.78	0.05	QFPFLASIQNQGR
48-85	4022.71	4022.97	0.26	HFCGGALIHARFVMTAASCFQSQNPGVSTVVLGAYDLR
147-156	1090.58	1090.50	0.08	CQVAGWGSQR
161-166	774.44	774.45	0.01	LSRFPR
223-229	838.45	838.40	0.05	GPDFFTR
230-249	2107.08	2107.09	0.01	VALFRDWIDGVLNNPGPGPA
Neutrophilic elastase (29 kD; 33% of the sequence detected)				
36-50	1807.96	1807.97	0.01	ARPHAWPFMVSLQLR
82-92	1220.69	1220.71	0.02	VVLGAHNLSRR
97-103	846.46	846.47	0.01	QVFAVQR
145-163	2014.03	2014.04	0.01	LGNGVQCLAMGWGLLGRNR
184-191	890.46	890.46	0	SNVCTLVR
184-193	1103.65	1103.59	0.06	SNVCTLVRGR
194-220	2703.29	2703.33	0.04	QAGVCFGDSGSPLVCNGLIHGIASFVR

Table 1 (Contd.)

1	2	3	4	5
Proteinase 3 (27 kD; 40% of the sequence detected)				
1-21	2364.15	2364.16	0.01	IVGGHEAQPHSRPYMASLQMR
48-64	1899.06	1899.08	0.02	DIPQRLVNVVLGAHNVR
53-64	1289.76	1289.76	0	LVNVVLGAHNVR
133-166	3803.93	3803.94	0.01	VGAHDPPAQVLQELNVTVVTFFCRPHNICTFVPR
201-208	1041.50	1041.53	0.03	LFPDFFTR
209-217	1133.62	1133.62	0	VALYVDWIR
Fragment of heavy chain of MPO (40 kD; 21% of the sequence detected)				
164-169	778.37	778.40	0.03	LYQEAR
197-202	811.41	811.42	0.01	YLPTYR
212-221	1151.62	1151.61	0.01	IANVFTNAFR
252-258	911.47	911.47	0	VFFASWR
259-270	1279.76	1279.75	0.01	VVLEGGIDPILR
282-291	1184.62	1184.61	0.01	QNQIAVDEIR
301-312	1339.73	1339.73	0	IGLDLPALNMQR
315-325	1284.62	1284.60	0.02	DHGLPGYNAWR
400-423	2920.36	2920.44	0.08	FWWENEGVFSMQQRQALAQISLPR
414-423	1095.64	1095.64	0	QALAQISLPR
Heavy chain of MPO (57 kD; 22% of the sequence detected)				
18-24	807.40	807.42	0.02	IPPNDPR
164-169	778.38	778.40	0.02	LYQEAR
196-202	939.54	939.52	0.02	KYLPTYR
197-202	811.42	811.42	0	YLPTYR
212-221	1151.66	1151.61	0.05	IANVFTNAFR
238-246	1130.57	1130.52	0.05	YQPMEPNPR
252-258	911.49	911.47	0.02	VFFASWR
259-270	1279.79	1279.75	0.04	VVLEGGIDPILR
282-291	1184.67	1184.61	0.06	QNQIAVDEIR
301-312	1339.77	1339.73	0.04	IGLDLPALNMQR
313-325	1527.80	1527.73	0.07	SRDHGLPGYNAWR
315-325	1284.66	1284.60	0.06	DHGLPGYNAWR
414-423	1095.68	1095.64	0.04	QALAQISLPR

from the following: cathepsin G (^{1}I-I-G-G-R^5), neutrophilic elastase (^{1}I-V-G-G-R^5), proteinase 3 (^{1}I-V-G-G-H^5), and azurocidin (^{1}I-V-G-G-R^5). On account of their structural homology and antimicrobial activity as their common feature, these proteins are united in a group called serprocidins (serine protease cidins) [25].

In an attempt to identify precisely the proteins interacting with Cp, they were hydrolyzed with trypsin, and then their fragments were analyzed by mass spectrometry (Table 1). The analysis showed the presence of eosinophilic cationic protein (16 kD, seven peptides), cathepsin G (27 kD, 11 peptides), azurocidin (27 kD, eight peptides), neutrophilic elastase (29 kD, seven peptides), proteinase 3 (27 kD, six peptides), MPO (57, 40, and 12 kD; 13, 10, and 4 peptides, respectively). The interaction of Cp (p*I* 4.7) being suppressed by 0.5 M NaCl suggests that they have ionic character, and is supported by their p*I* values in the alkaline region (Table 2).

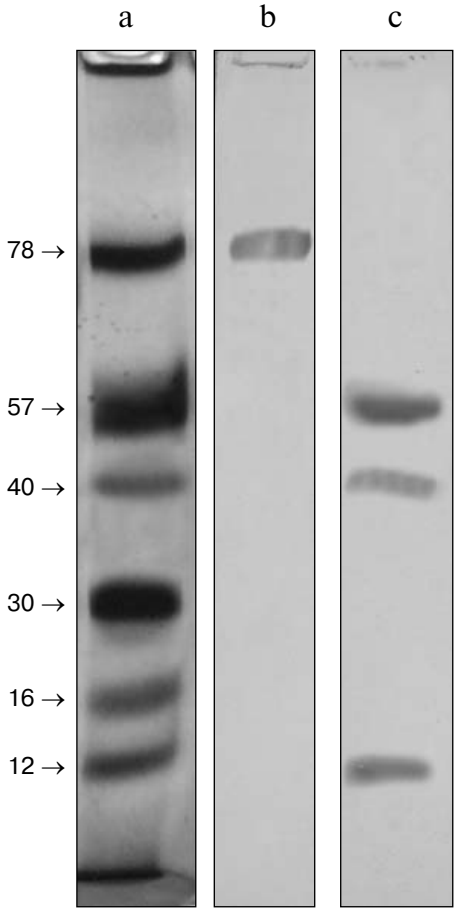
It should be noted that among the cationic proteins that interacted with Cp under the conditions of our study, we did not find a number of potentially interactive species of leukocytes, such as defensins, lysozyme, or bactericidal/permeability-increasing protein. The latter are contained in the granules of leukocytes, but the large scope of positively charged proteins from other cellular compartments must have been released upon destruction of cells in our experiments. Since these and others never bound to Cp-Sepharose, we conclude that a marked selectivity exists in the pattern of interaction of Cp with cationic proteins.

Interaction of Cp with proteins of leukocytes, namely with MPO and Lf, was discovered, respectively, in 1997 by the group of Segelmark [14] and in 2000 by our group [18]. However, in 1999 an attempt to track with antibodies Cp interacting with MPO, proteinase 3, or Lf bound to the wells of an ELISA plate resulted in detecting only MPO [26]. This discrepancy with our results is explained by the fact that anti-Cp caused dissociation of the complexes formed by Cp and Lf or proteinase 3. Such a phenomenon has been described in case of Lf in our previous study [16, 27]. One cannot exclude another possible mechanism, i.e. shielding of the sites in Lf and proteinase 3 fit for the contact with Cp when the two former proteins bind to an ELISA plate well. The same binding of MPO may involve only one of its dimers and, hence, not prevent it from interacting with Cp.

Thus, interaction with Cp of five cationic proteins, i.e. of eosinophilic cationic protein, cathepsin G, neutrophilic elastase, proteinase 3, and azurocidin, is reported here for the first time.

Table 2. Properties of the leukocyte proteins interacting with Cp (http://www.matrixscience.com)

Protein	*M*, kD	p*I*
Myeloperoxidase (MPO, EC 1.11.1.7)	150	9.2
Lactoferrin (Lf)	78	8.4-9.0
Cathepsin G (EC 3.4.21.20)	27	11.3
Neutrophilic elastase (EC 3.4.21.37)	29	9.7
Azurocidin (CAP 37)	27	9.8
Proteinase 3 (myeloblastin, EC 3.4.21.76)	27	7.8
Eosinophilic cationic protein (ribonuclease 3, EC 3.1.27.-)	16	10.7



Electrophoresis of proteins of leukocyte extract eluted with 0.5 M NaCl from Cp-Sepharose (20 μg protein per sample). a) SDS-polyacrylamide gels stained with Coomassie R-250; b, c) Western blotting with anti-Lf (1 : 10,000) (b) and MPO (1 : 10,000) (c); second antibodies labeled with horseradish peroxidase were diluted 1 : 10,000 (staining with 4-chloro-1-naphthol and H_2O_2). Arrows mark molecular weight values in kD

It can be suggested that interaction of antimicrobial cationic proteins with Cp may noticeably reduce their antimicrobial activity, and also the cytotoxic effect in the case of their secretion into the bloodstream. The fact that four members of the serprocidin family interact with Cp favors the notion of an evolutionary conservatism of such protein–protein complexes. Because the newly identified cationic proteins along with Lf and MPO studied previously are able to perform as autoantigens provoking systemic vasculitis [28], their selective interaction with Cp might point to their being a part of a regulatory mechanism in pathogenesis of the disease.

The authors are grateful to Prof. V. N. Kokryakov and Dr. M. M. Shavlovski (Institute for Experimental Medicine, Russian Academy of Medical Sciences, St. Petersburg) for presented materials, valuable advice, and fruitful discussions.

This study was supported by the Russian Foundation for Basic Research (grant No. 06-04-48602 and No. 05-04-48765).

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