Clin Exp Immunol 1997; **108**:167–174

Binding and inhibition of myeloperoxidase (MPO): a major function of ceruloplasmin?

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(Accepted for publication 31 December 1996)

SUMMARY

Interactions between plasma proteins and MPO were studied. The protein fraction of normal plasma and serum was shown to exhibit an inhibitory effect on the peroxidase activity of MPO. Most of the inhibitory effect could be retained on an MPO-coupled affinity chromatography column. In particular, a protein with apparent mol. wt of 130 kD showed affinity for MPO. The protein was identified as ceruloplasmin by N-terminal amino acid sequencing and immunochemistry. During separation procedures the peroxidase inhibitory effect was limited to ceruloplasmin-containing fractions of plasma. Purified ceruloplasmin inhibited the peroxidase activity of MPO in a concentration-dependent manner, and exhibited selective binding to MPO-coated microtitre plates. This binding could be inhibited by MPO dissolved in buffer. Correspondingly the binding of MPO to ceruloplasmin-coated plates could be blocked by ceruloplasmin in solution, showing a physical interaction to occur between the two proteins under physiological conditions. We also found affinity to exist between MPO and C3 (and its C3d-containing fragments). However, C3 and C3 fragments did not inhibit the peroxidase reaction *in vitro*. We propose that ceruloplasmin takes part in the clearance and inactivation of MPO, *in vivo*. We also speculate that impaired inactivation of MPO may have a pathophysiological role in inflammatory diseases characterized by autoantibodies to MPO, such as rapidly progressive glomerulonephritis with P-ANCA (perinuclear anti-neutrophil cytoplasmic antibodies).

Keywords myeloperoxidase ceruloplasmin complement C3d glomerulonephritis ANCA

INTRODUCTION

MPO is a major constituent of neutrophil leucocytes and has an important function in the killing of invading microorganisms [1]. The molecular weight of MPO is ≈140 kD, and it consists of two identical protomers, each containing a heavy chain and a light chain. MPO catalyses reactions between H_2O_2 and halides, a main product being the highly toxic oxidant HClO. Extracellulary, MPO-derived metabolites induce tissue destruction by oxidation and halidation. One important effector route is the enhancement of the proteolytic effect of serine proteinases, such as proteinase 3 (PR3) and neutrophil elastase, by oxidative inactivation of their main inhibitor α_1-antitrypsin (α_1-AT) [2]. In rapidly progressive glomerulonephritis (RPGN), MPO can be detected extracellulary in the glomeruli, and may take part in the pathogenesis [3]. Furthermore, autoantibodies to MPO are common in RPGN, and in related vasculitic disorders, and they can be detected by ELISA or as perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) by indirect immunofluorescence [4]. MPO-ANCA is an important tool in the diagnosis and follow up of RPGN, and may also take part in pathogenesis by activation of neutrophils [5,6].

Even though MPO is highly toxic, produced in large quantities, and released extracellulary upon neutrophil stimulation, little is known concerning its inactivation and subsequent degradation *in vivo*. MPO can be detected in serum and plasma of healthy individuals and the concentrations are increased in different diseases [3,7]. *In vitro* MPO activity is inhibited by azide and catalase, and there are reports of an inhibitory effect of normal serum, referred to as an unspecific protein effect [1]. However, a specific 150-kD inhibitor of MPO, distinct from albumin, IgG and IgA, has been observed in serum and synovial fluids [8]. Synovial fluids of patients with rheumatoid arthritis (RA) exhibited lower values of inhibition compared with serum, consistent with a consumption of the inhibitor.

Ceruloplasmin is a single-chain 122-kD plasma protein and an acute-phase reactant with an uncertain physiological function [9,10]. It is the major copper-containing protein in serum but it does not seem to act as transport protein. Ceruloplasmin is distantly related to other blue copper-containing proteins in plants, bacteria and mitochondria acting as oxidases or electron carriers in respiration [11]. Both anti-oxidant and pro-oxidant activity have been

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attributed to ceruloplasmin [10]. As an oxidase ceruloplasmin is rather unspecific, the best known substrate being Fe^{2+}. Ceruloplasmin is believed to play a role in iron metabolism and to protect the body from catalytically active Fe^{2+}. Recently this was supported by the description of families with homozygote ceruloplasmin deficiency. Affected family members exhibited dementia and iron overload in the brain and liver, but no alterations in copper metabolism [12,13].

The aim of this study has been to elucidate the interactions between MPO and serum proteins in order to identify proteins that bind MPO and to identify possible inhibitors. We found ceruloplasmin and fractions of C3 to bind to MPO, and that these proteins have different effects on peroxidase activity *in vitro*.

MATERIALS AND METHODS

MPO and MPO activity assay

MPO was a gift from Dr Inge Olsson (Lund, Sweden) [14]. Peroxidase activity was measured using K-blue (Neogen Corp., Lexington, KY), containing tetramethylbenzidine and hydrogen peroxide. In assays for inhibition of peroxidase activity, 0·15 μg MPO in 50 μl PBS was incubated for 60 min with an equal volume of the test sample, 50 μl of the substrate solution were then added and absorbance at 660 nm was measured after 3 min. Results are presented as means of duplicates (differences in absorbance values between duplicates were not allowed to exceed 10%).

Protein determinations

If not stated otherwise, all protein determinations were made using BCA Protein Assay Reagent (Pierce, Rockford, IL).

Antibodies

Polyclonal antibodies to MPO were a gift from Dr Tor Olofsson (Lund), the anti-MPO MoAb 2B11 from our laboratory has been described previously [15]. Polyclonal antibodies to ceruloplasmin, C3d, C3c, IgG, IgM and albumin were purchased from Dako A/S (Glostrup, Denmark).

ELISA

Fractions from chromatography columns were coated to Maxisorp microtitre plates (Nunc Immunoplate, Roskilde, Denmark) in coating buffer containing 0·05 M carbonate buffer at pH 9·6. Appropriate polyclonal antibodies, diluted in ELISA buffer containing 9·5 mM sodium phosphate pH 7·5, 0·135 M NaCl, 4·2 mM KCl, bovine serum albumin (BSA) 0·2% (w/v), azide 0·02% (w/v), merthiolate 0·001% and Tween 0·05%, were incubated for 1 h at room temperature. After repeated washing, bound material was detected, using alkaline phosphatase-conjugated anti-rabbit IgG antibodies followed by substrate buffer, containing 1 mg/ml *p*-nitrophenylphosphate (Sigma Chemical Co., St Louis, MO), 1 M diethanolamine, 0·5 mM $MgCl_2$ pH 9·8, by shift in optical density (OD) at 405 nm.

The binding of purified ceruloplasmin to MPO was assayed by coating MPO, ceruloplasmin or a control antigen (ovalbumin; Sigma) to microtitre plates at a concentration of 1 μg/ml in coating buffer (see above). After washing, purified ceruloplasmin or MPO in ELISA buffer was added and bound material detected as described above. The ability of MPO or ceruloplasmin to inhibit this binding was tested by preincubation of the test samples for 2 h with MPO or ceruloplasmin, dissolved in ELISA buffer at concentrations ranging from 0·01 to 100 μg/ml.

SDS–PAGE and immunoblotting

SDS–PAGE followed by either silver staining or immunoblotting was performed essentially as described before [16–18]. Samples were mixed with 2% SDS buffer and thermal denaturation was performed by boiling for 4 min. The samples were run on 3–16% or 10–16% gels with a 3% stacking gel. For immunoblotting the material was transferred to Immobilone PVDF membrane (Millipore, Saint-Quentin, France) using a semidry technique with 20 V for 1 h [19]. Antigens were visualized with polyclonal antibodies followed by alkaline phosphatase-conjugated anti-rabbit IgG (Orion Diagnostica AB, Trosa, Sweden) and bromochloro-indolyl phosphate/nitroblue tetrazolium substrate (Sigma).

Affinity chromatography

MPO (10 mg) was coupled to a 4 ml Mini-leak gel (Kem-En-Tec IS, Copenhagen, Denmark) according to the manufacturer's instructions. Serum (0·5–2 ml) from healthy blood donors was applied at a flow rate of 20 ml/h and the gel was equilibrated with PBS pH 7·5. Bound material was eluted with buffer containing 6 M urea, 50 mM citrate pH 4·0, or with 1 M NaCl in phosphate buffer pH 7·5. A column coupled with BSA was used in control experiments.

The remaining peroxidase inhibitory effect in a 0·5-ml serum sample was measured as described above after passage through the affinity columns. For comparison a serum sample of 0·5 ml was diluted to a volume equal to that of the void volume (≈10 ml). Diluted serum and the void volumes from the affinity chromatography columns were retrieved and concentrated back to original volume using an ultrafiltration cell (Amicon, Beverly, MA) with YM10 filter.

Purification of MPO-binding eluates

Fractions from the MPO-coupled column were separated according to size using a Sepharose 12 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with PBS and a flow rate of 0·5 ml/min. Fractions from the gel filtration column were further separated by reversed phase high performance liquid chromatography (HPLC) using a 4·6 × 150 mm C4 column (Vydac, Hisperia, CA). Samples were acidified by adding 0·1% trifluoroacetic acid (TFA), applied at a flow rate of 60 ml/h, and equilibrated with 30% acetonitrile with 0·1% TFA. Bound material was eluted with various two-step linear gradients of acetonitrile from 30% to 60%. In the final experiment for separation of the 40-kD and 130-kD proteins the following gradient was used: 0 min 30%, 5 min 45%, 10 min 45%, 30 min 50%, 33 min 60% and 35 min 30%.

Amino acid sequencing

After SDS–PAGE and blotting on Immobilone membrane, appropriate bands were cut out and sent to the Biomolecular Resource Facility, Lund University for N-terminal amino acid sequencing by Edman degradation using a Model 477A Protein/Peptide Sequencer (Applied Biosystems, Foster City, CA) [20]. Database searches were performed to find the highest scoring gene sequence [21].

Purification of ceruloplasmin

Plasma (50 ml) from a healthy blood donor was diluted with an equal volume of buffer containing 50 mM NaAc pH 5·7 and applied to a 200-ml DEAE-Sephacel column (Pharmacia Biotech). The column was equilibrated with acetate buffer and then eluted with a 500-ml linear 0·05–0·2 M NaCl gradient. Ceruloplasmin-rich fractions were pooled after detection by SDS–PAGE, ELISA, 610/

280 nm absorbance measurements and assay for ceruloplasmin activity. Ceruloplasmin activity was determined by measuring the absorbance at 550 nm 30 min after addition of an equal volume of 0·05% N,N-dimethyl-*p*-phenylenediamine (Sigma) in 50 mM NaAc pH 5·7. Ceruloplasmin concentration determinations were performed at the Department of Clinical Chemistry, University Hospital of Lund.

The pooled fractions from the ion exchange chromatography were further purified by a two-step ammonium sulphate precipitation. Saturated ammonium sulphate solution was added to 250 ml of pooled fractions to yield a 30% saturated mixture. After 3 h of incubation the mixture was centrifuged at 20 000 ***g*** for 30 min. The pellet was discarded and a second precipitation of the supernatant was performed at 50% saturation. The mixture was incubated at 4°C overnight during constant stirring and centrifuged as above. The second supernatant was discarded and the second pellet was dissolved in 10 mM phosphate buffer pH 7·0. The dissolved pellet was dialysed three times against 1000 ml buffer at 4°C, and then the volume was adjusted to 20 ml.

The dialysed pellet was further purified by application to a 5 ml Econo-Pac CHT-II hydroxyapatite chromatography column (BioRad, Hercules, CA) at a flow rate of 0·5 ml/min. After equilibration with 10 mM phosphate buffer the column was eluted with a 60-ml linear gradient from 50 to 200 mM phosphate, followed by 500 mM phosphate buffer to remove remaining material. Again, ceruloplasmin-rich fractions were identified with ELISA, 610/280 nm absorbance measurements and assay for ceruloplasmin activity.

Pooled fractions from the hydroxyapatite column were further separated using a S-200 gel filtration column (Pharmacia) equilibrated in PBS pH 7·5. Fractions of purified ceruloplasmin were identified as described above.

RESULTS

Inhibition of MPO by serum from healthy individuals

Human serum and plasma, from healthy blood donors, had a dose-dependent inhibitory effect on the peroxidase activity of MPO (Table 1). The inhibitory capacity was only marginally affected by dialysis of serum against PBS, consistent with an effect mediated by the protein fraction of serum. The inhibitory activity of a 0·5-ml serum sample was substantially attenuated by passage through an MPO column (Fig. 1), even though < 1% of the protein content was retained on the column. Passage through a control BSA column had the same effect as dilution and concentration of the original sample. An eluate from the MPO column (after dialysis back to PBS) had no inhibitory effect on MPO, showing the inhibitory effect to be unstable under certain conditions.

Table 1. Concentration dependency of inhibition of enzymatic activity of MPO by serum, plasma and serum dialysed against PBS (cut off 12–14 kD)

	Peroxidase activity (% of positive control, mean of duplicate)		
Dilution	Serum	Plasma	Dialysed serum (cut off 12–14 kD)
1:1024	106·9	105·5	97·4
1:256	94·2	93·6	88·5
1:64	62·5	61·2	70·5
1:16	8·5	8·5	36·5
1:4	8·5	8·2	12·9

Samples were diluted in PBS and mixed with 0·15 μg MPO 60 min prior to addition of sustrate. Absorbance was read after 3 min and results are presented as mean of duplicates. Plasma and serum exhibit an equal and dose-dependent capacity to inhibit the peroxidase reaction. The inhibitory capacity is only marginally affected by dialysis, indicating its dependence on plasma proteins.

Affinity chromatography on an MPO column

The urea-buffer eluate from an MPO affinity column was analysed in order to identify serum proteins binding to MPO and possibly being responsible for the inhibitory effect described above. As shown in Fig. 2, using a 10–16% SDS–PAGE gel, two major bands were seen corresponding to proteins with apparent mol. wts of ≈40 kD and ≈130 kD. After dialysis the eluate retained its affinity for MPO and again eluted mainly as 40-kD and 130-kD polypeptides. Increasing the NaCl concentration in the application buffer from 0·15 M to 0·3 M or 0·5 M resulted in decreased binding of the 40-kD, but not of the 130-kD protein. With 1 M NaCl in phosphate buffer the 40-kD protein could, in contrast to the 130-kD protein, be eluted from the column. Thus the affinity of the 130-kD protein to bind MPO appeared to be higher compared with the 40-kD protein. The 40-kD and 130-kD proteins were not found in an eluate from a control column coupled with BSA.

Purification and sequencing of MPO binding proteins

To purify the 40- and 130-kD band, the urea buffer eluate from the MPO column was subjected to gel filtration on Sepharose followed by reversed phase HPLC with a C4 column. Modulation of the slope of the acetonitrile gradient made possible the separation of the respective proteins into distinct chromatographic peaks, yielding discrete bands on silver-stained SDS–PAGE gels. After transfer to Immobilone membrane the 40-kD and 130-kD bands were cut out and subjected to N-terminal amino acid sequencing. The 10 N-terminal amino acids of each protein are shown in Table 2. The results strongly indicated the 130-kD protein to be ceruloplasmin, the obtained sequence being identical to the 10 N-terminal amino acids of ceruloplasmin. The sequence obtained from the 40-kD protein was identical to a sequence in the middle of the C3 precursor gene. These amino acids are N-terminal in the

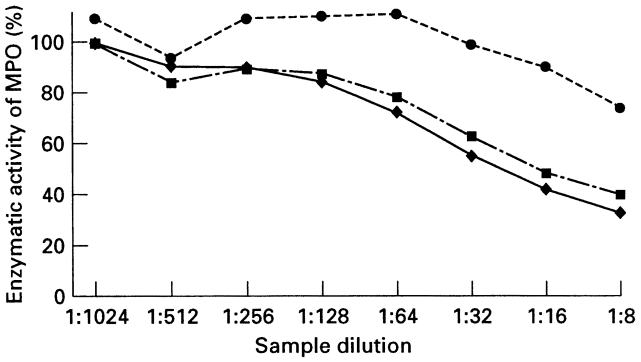

Fig. 1. The ability of serum having passed through an MPO affinity column to inhibit the peroxidase activity of MPO (●) was compared with the original serum (♦) and with serum having passed through a control bovine serum albumin (BSA) column (■). The bulk of the inhibitory capacity was retained on the MPO column but passed through the BSA column.

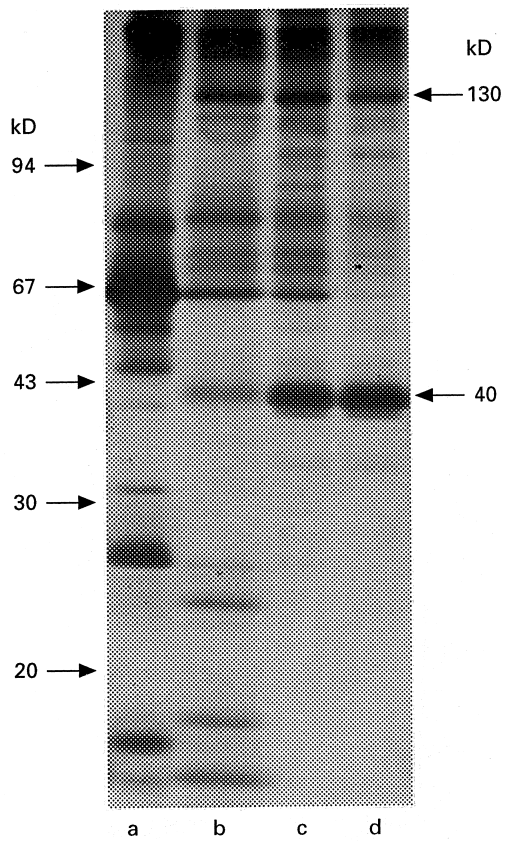

Fig. 2. MPO affinity chromatography of serum. Silver-stained 10–16% SDS–PAGE gel, ≈ 1 μg was applied to each lane. Distinct bands at 40 kD and 130 kD are seen in the eluate (lane c) but not in the void volume (lane a). These bands are also seen at re-chromatography of the eluate (after dialysis against PBS) while other bands (i.e. 67 kD) are attenuated (lane d). These bands were retrieved in the second void volume (lane b).

fragment designated g, which indicates the 40-kD protein to be the C3dg fragment with a reported mass of 41 kD [22].

Immunoblotting

The identity of the bands obtained by MPO affinity chromatography was confirmed by immunoblotting using polyclonal antisera.

Table 2. Results of N-terminal amino acid sequencing of proteins purified from the eluate of the MPO affinity column (possible alternatives in parentheses). Results are compared with the highest scoring gene sequences obtained from a database search [21]

Result of sequencing	Highest scoring gene sequence
≈40-kD fragment:	Human complement C3 precursor gene 955–964:
Glu - Gly - Val - Gln (Glu) - Lys - Glu- Asp - Ile - Pro - Pro	Glu - Gly - Val - Gln - Lys - Glu- Asp - Ile - Pro - Pro
≈130-kD fragment:	Human ceruloplasmin precursor gene 20–29:
Lys - Glu - Lys - His - Tyr Tyr - Ile (Asp) - Gly - Ile (Asp,Glu) - Ile	Lys - Glu - Lys - His - Tyr - Tyr - Ile - Gly - Ile - Ile

No MPO could be detected in the eluate of the MPO column. The C3d antiserum reacted strongly with the ≈40-kD band identified as C3dg by amino acid sequencing (Fig. 3). The C3d antiserum also reacted with a weaker band of larger mol. wt, indicating binding also of the whole C3 (or C3b) molecule to the MPO column. The band corresponding to intact C3 also reacted with anti-C3c, but anti-C3c reactivity was not seen with other bands in the eluate. The 130-kD protein, identified as ceruloplasmin by sequencing, reacted strongly with anti-ceruloplasmin antiserum, and other bands smaller in size also showed reactivity with this antiserum, suggesting these peptides to be fragments of ceruloplasmin. No immune reactivity for C3d or ceruloplasmin could be detected in the eluate from the BSA column (Fig. 3). Further polypeptides present in the void volumes and eluates from both columns were identified as IgG, IgM and albumin. These bands were attenuated when the eluate was dialysed against PBS and reapplied to the MPO column (Fig. 2), proving the unspecific nature of this binding.

Purification of ceruloplasmin

Since the eluate of the MPO affinity column did not exhibit any peroxidase inhibition, ceruloplasmin and C3 fragments were purified from serum under more gentle conditions in order to investigate their inhibitory capacity. Serum or plasma were subjected to ion exchange chromatography using DEAE-Sephacel. Presence of ceruloplasmin and C3 fragments in the fractions obtained was detected by ELISA, immunoblotting, and by measurement of ceruloplasmin enzymatic activity. This single-step procedure was effective in separating ceruloplasmin from the bulk of the major plasma proteins (Fig. 4). The DEAE chromatography also partly separated anti-C3d reactivity from anti-ceruloplasmin reactivity. The fractions were tested for peroxidase inhibitory effect. The maximum inhibitory effect corresponded to the maximum ceruloplasmin activity and to the maximum of anti-ceruloplasmin ELISA absorbance (Fig. 4). Fractions showing high anti-C3d reactivity and low ceruloplasmin reactivity did not show any inhibitory capacity, indicating the inhibitory effect to be attributed to ceruloplasmin rather than the C3 fragments.

The complete purification of ceruloplasmin is summarized in Table 3. In the initial steps the yield of the original 0·27 g/*l* of ceruloplasmin was relatively high. The hydroxyapatite chromatography yielded two major ceruloplasmin-containing peaks, the first of which was selected and pooled for the final gel filtration procedure. After the gel filtration the ceruloplasmin was retrieved mainly in three 5-ml fractions. The middle fraction had a 610/280 nm ratio of 0·048, indicating a purity of around 94% [9]. Throughout the purification process inhibitory capacity was limited to ceruloplasmin-containing fractions.

Binding and inhibition of MPO by purified ceruloplasmin

When purified ceruloplasmin was applied to wells on MPO-coated microtitre plates, strong and dose-dependent binding could be detected by ELISA. No ceruloplasmin binding could be detected when ovalbumin was used as coating material in control experiments. Preincubation of ceruloplasmin-rich fractions with MPO in solution resulted in a dose-dependent reduction of ceruloplasmin binding to the MPO coat (Fig. 5). Similarly, when ceruloplasmin was coated onto microtitre plates, MPO binding could be blocked by free ceruloplasmin in a dose-dependent fashion. This indicates an interaction to occur between ceruloplasmin and MPO in solutions and not only when MPO is immobilized to a solid phase.

The peroxidase inhibitory activity of ceruloplasmin and a

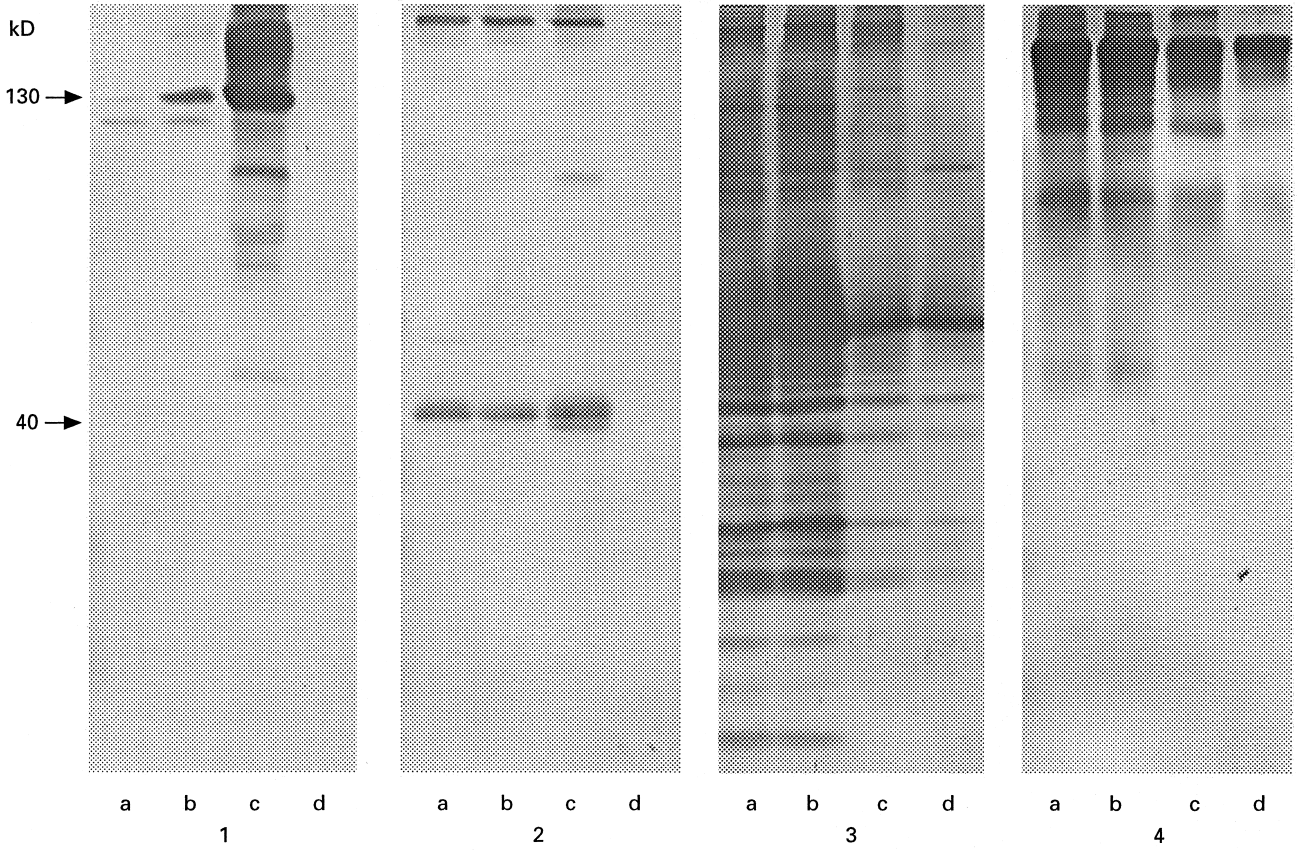

Fig. 3. Immunoblotting. Panel 1, anti-ceruloplasmin; panel 2, anti-C3d; panel 3, anti-albumin; panel 4, anti-IgG. Lanes a, b, void volumes from MPO and bovine serum albumin (BSA) columns; lanes c,d, eluates from respective column. Approximately 1 μg of protein was applied to each lane. Ceruloplasmin and C3d were found in the eluate from the MPO column but not in the eluate from the BSA column. Very little ceruloplasmin is seen in the void volume from the MPO column. Anti-C3d reactivity is seen in two bands corresponding in size to both the intact C3 molecule and to the C3dg fragment. The two bands appear both in the void and eluate of the MPO column, indicating a relatively weak interaction between the MPO column and C3dg (and C3). Minor amounts of IgG and albumin were retrieved in both eluates.

control substance (human serum albumin (HSA)) are shown in Fig. 6. Ceruloplasmin exhibited a clear dose-dependent inhibition of MPO peroxidase activity, while HSA showed no inhibitory affect at any concentration tested. Unlike plasma and serum, the inhibitory effect of purified ceruloplasmin was unstable during storage. Directly after purification, 5 μg of ceruloplasmin reduced the peroxidase activity of 0·15 μg MPO by 39%, after 1 week of storage at 4°C the inhibition had been reduced to 34% and after 3 weeks to 28%. Commercially available ceruloplasmin (Sigma) did not exhibit any peroxidase inhibition.

DISCUSSION

The main finding of this study is that ceruloplasmin binds to MPO under physiological conditions. Ceruloplasmin was found to be a major component of the eluate of an MPO affinity chromatography column. Among the identified proteins showing affinity for MPO, ceruloplasmin exhibited the strongest binding. Binding between MPO and ceruloplasmin could also be demonstrated with other *in vitro* techniques such as immunoblotting (data not shown) and ELISA. Binding to MPO-coated microtitre plates could be inhibited by soluble MPO, and correspondingly soluble ceruloplasmin could inhibit the binding of MPO to ceruloplasmin-coated plates. This indicates that complexes between the compounds can be formed. The fact that a binding occurred at pH 7·4 and at physiological salt concentrations makes it likely that a binding occurs also *in vivo*. A direct physical interaction between MPO and ceruloplasmin has not previously been described. The nature of the binding has not been elucidated, but the fact that ceruloplasmin is anionic (pI 4·4) [23] and MPO highly cationic (pI > 10) [24] makes electrostatic interactions likely to be involved.

The second major finding in this study is that the interaction between ceruloplasmin and MPO results in inhibition of a peroxidase reaction *in vitro*. Normal human serum is known to inhibit the enzymatic function of MPO [1]. In this study we showed that > 80% of the inhibitory capacity in a sample could be retained on a MPO-coupled column, even though < 1% of the serum proteins bound to the column. The eluate from the column did not show any peroxidase inhibitory activity, most probably due to inactivation of the inhibitor by the urea-citrate elution buffer. However, when the proteins with affinity for MPO were separated under more gentle conditions the inhibitory effect co-localized with ceruloplasmin, and inhibition was never seen in any ceruloplasmin-free fractions. Purified ceruloplasmin exhibited a clear dose-dependent inhibition of peroxidase activity. Most probably ceruloplasmin is identical to the unidentified MPO inhibitor described by Yea *et al.* [8].

Purified ceruloplasmin (3 μg) exhibited approximately the same inhibitory effect on 0·15 μg of MPO as 1 μl of plasma

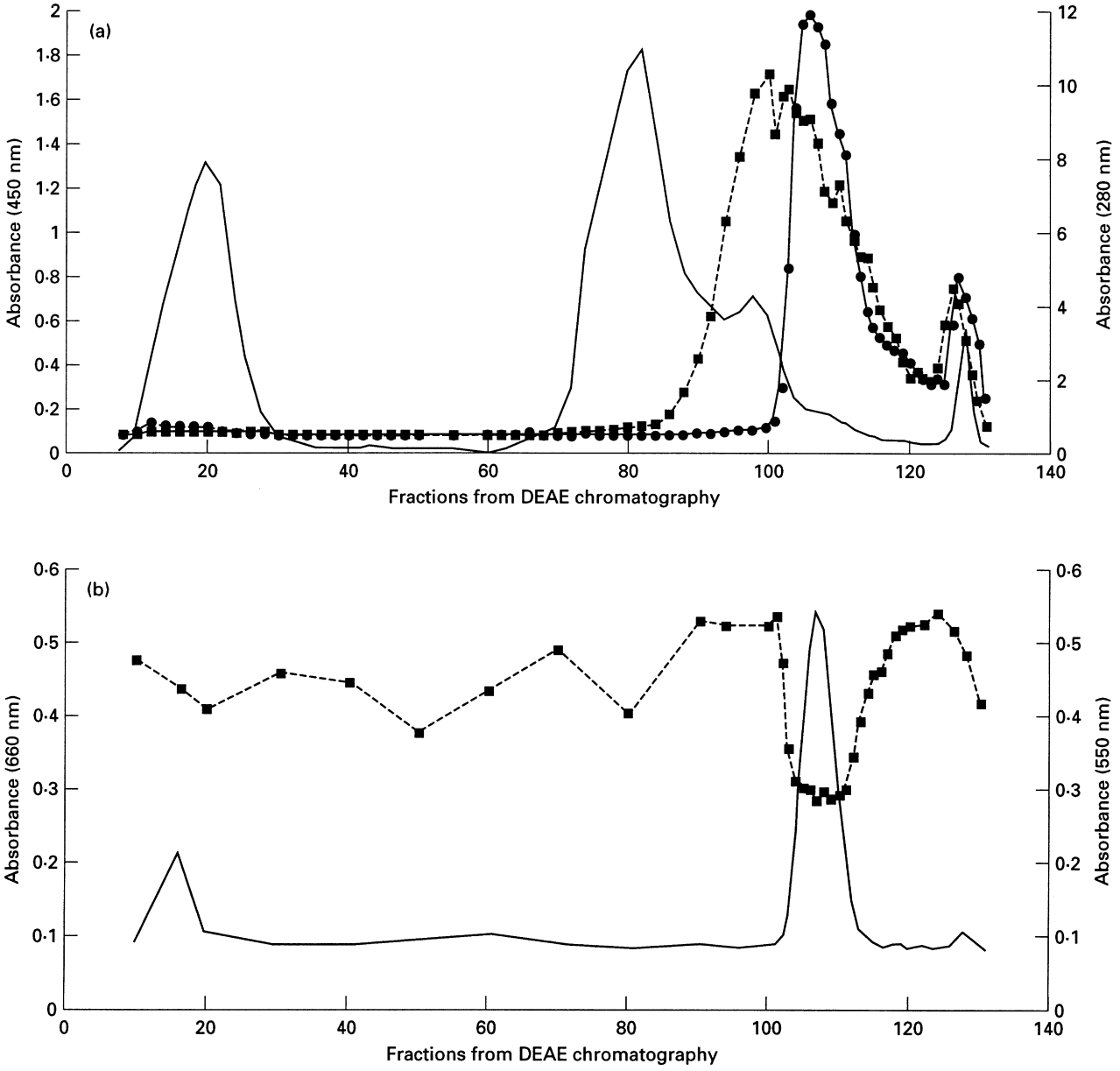

Fig. 4. Ion exchange chromatography of serum using a DEAE column. (a) Protein content of the fractions (——), and reactivities for anti-C3d (■) and anti-ceruloplasmin (●) when the fractions were used as coating material in ELISA are indicated. Ceruloplasmin was separated from the bulk of the plasma proteins, but only partially from C3. (b) Maximum inhibitory effect on the peroxidase activity of MPO (■) corresponds with the maximum intrinsic ceruloplasmin enzymatic activity (——) of the fractions and with the ELISA anti-ceruloplasmin reactivity.

containing about 60 μg of proteins. The most abundant plasma protein, HSA, did not exhibit any inhibitory effect. This shows that the inhibition of the peroxidase reaction is not an unspecific protein effect, but rather a consequence of the physical interaction between ceruloplasmin and MPO. However, purified ceruloplasmin as isolated in this study does not fully explain the inhibitory capacity of serum, since 1 μl serum contains only about 0·3 μg ceruloplasmin. There are at least two possible explanations for this discrepancy: either there is a contribution from other components of serum, or part of the inhibitory capacity is lost during the isolation procedures. The instability of the inhibitory capacity is demonstrated by the decay during storage and by the inability of commercially available ceruloplasmin to exhibit inhibition.

MPO is a powerful bactericidal protein and its enzymatic activity also has a tissue-destructive potential [2]. It is reasonable to assume that MPO released extracellulary during exocytosis or necrosis is inactivated in a regulated manner. We postulate that ceruloplasmin takes part in this process. The reactions catalysed by MPO and ceruloplasmin are highly complicated, influenced by, for example, pH, halide concentrations, reactive oxygen species, pO_2, substrate concentrations and enzyme concentrations. Furthermore, a major product of MPO, HClO, is known to inhibit MPO. Superoxide anions, besides being the major source for the MPO substrate H_2O_2, can restore the enzymatic activity of MPO, when the enzymatically less active intermediate 'compound II' is formed [1]. The superoxide anion is also a substrate for ceruloplasmin [9].

Table 3. Purification of ceruloplasmin from blood donor plasma as described in Materials and Methods

	Volume, ml	Protein conc., mg/ml	Absorbance ratio, 610/280 nm	Ceruloplasmin conc., mg/ml	Total amount of ceruloplasmin, mg	Yield, %	Purity, %
Plasma	50			0·27	13·5	100	
DEAE-chromatography	250	0·5	0·009	0·05	12·5	93	10
$(NH_4)_2SO_4$-precipitation	20	2·1	0·010	0·43*	8·6	64	20
Hydroxyapatite	6·8	0·5	0·023	0·22*	1·5	11	44
S-200 filtration fraction 48	5	0·1	0·048	0·09	0·5	4	94

*Estimated from the 610/280 nm absorbance ratio and protein concentration determination.

The complexity of the reactions makes it difficult to predict the effect of the interactions between ceruloplasmin and MPO *in vivo*, and it is beyond the scope of this study to elucidate this. Interestingly, it was reported as early as 1982 that ceruloplasmin could protect α_1-AT from oxidation, in an *in vitro* assay, using elastin degradation as output variable [25]. α_1-AT oxidation is a well known function of MPO that shifts the protease–anti-protease balance in favour of tissue destruction [2]. Thus at least *in vitro*, ceruloplasmin can protect extracellular matrix from degradation by serine proteases.

In this study we also report on an interaction between fragments of complement C3 and MPO. C3 consists of a 115-kD α-chain and a 75-kD β-chain joined by a single disulphide bridge. When activated, the small C3a fragment is cleaved off, yielding the C3b fragment, with a highly active carbonyl group. C3b is quickly either bound to surfaces or inhibited and further degraded. Major breakdown products are the C3c and C3dg fragments. The 41-kD C3dg fragment is monitored clinically in inflammatory disease as an activity parameter [22,26] and it is also the ligand for the antibody production enhancing B cell receptor CR2 (CD21) [27]. Immunoblotting of the eluate from the MPO column showed reactivity for anti-C3d with proteins corresponding in size with both intact C3 (and/or C3b) and C3dg, but for anti-C3c only with a protein corresponding in size to intact C3. Probably most fragments containing the dg moiety have affinity for MPO, raising the possibility that MPO will stick to immune complexes and microorganisms coated with complement. The affinity between MPO and C3dg was weak, but might be sufficient to influence the location of MPO at an inflammatory locus and within an organelle after phagocytosis. The C3 fragments did not inhibit the peroxidase reaction. Thus MPO would remain catalytically active if bound by C3dg to surfaces of microorganisms.

If the *in vitro* interactions between MPO, C3dg and ceruloplasmin as reported in this study parallel the situation *in vivo*, a

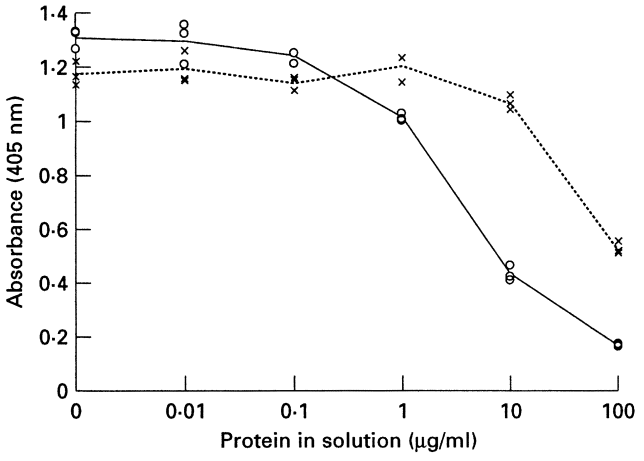

Fig. 5. Binding and inhibition of the binding between purified ceruloplasmin and MPO. Ceruloplasmin (—— and ○) at a concentration of 1 μg/ml was mixed with increasing amounts of MPO and incubated on microtitre plates coated with 1 μg/ml MPO. Bound ceruloplasmin was detected using ELISA technique. MPO (– – – and ×) at a concentration of 1 μg/ml was mixed with increasing amounts of ceruloplasmin and incubated on microtitre plates coated with 1 μg/ml ceruloplasmin. Bound MPO was detected using ELISA technique. MPO in solution inhibited the binding of ceruloplasmin to solid-phase MPO and *vice versa*. Lines are drawn between means of triplicates, individual measurement values are indicated by ○ and ×, respectively.

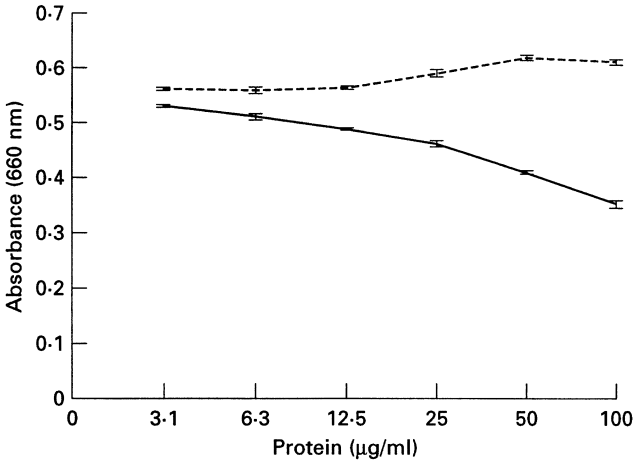

Fig. 6. Dose-dependent inhibition of an MPO-catalysed peroxidase reaction by purified ceruloplasmin (————). Human serum albumin (HSA) (– – – –) does not inhibit the reaction. The lines are drawn between mean values of duplicates, individual values are indicated by dashes. Concentrations in test solutions before addition of MPO and substrate (see Materials and Methods).

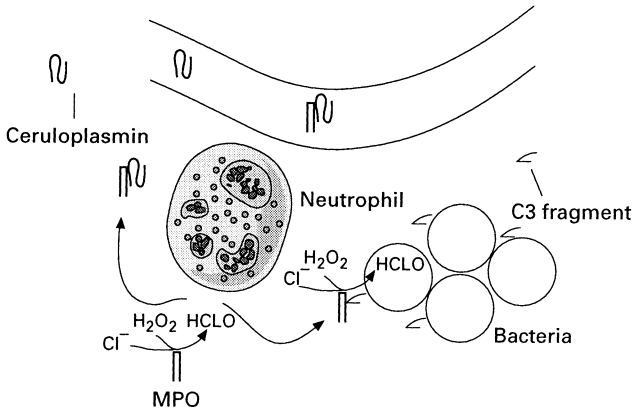

Fig. 7. Hypothesis of the extracellular regulation of the enzymatic activity of MPO. MPO released extracellularly catalyses the production of toxic oxidants in the presence of H_2O_2. Ceruloplasmin inhibits MPO by physical interaction, while MPO retains its catalytic capacity after interactions with C3 fragments. At an inflammatory locus MPO activity is directed to the surface of C3-coated microorganisms. Away from the locus, such as in the blood stream, MPO is inactivated by ceruloplasmin.

model as proposed in Fig. 7 can be constructed. In this model (i) MPO, released from invading neutrophils, retains its catalytic activity in areas of complement turnover, (ii) active MPO molecules stick to surfaces coated with C3 fragments, and (iii) MPO that has diffused away from the inflammatory locus gets readily inactivated by ceruloplasmin.

In RPGN and systemic vasculitis tissue damage stems from the concerted action of proteases and reactive oxygen species [24]. Autoantibodies are produced, directed mainly at two substances, PR3 and MPO [28,29], suggesting these enzymes play important roles in the disease process. We have previously shown that a defect in the inhibition of PR3, i.e. α_1-AT deficiency, is linked to PR3-ANCA-associated vasculitis [30]. We therefore speculate that dysregulation of MPO inactivation might be involved in MPO-ANCA-associated diseases.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (no. 16X-09487), the Swedish Kidney Foundation, the Thelma Zoega memorial foundation and the Crafoord foundation.

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