THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 9, pp. 6465–6477, March 1, 2013

Ceruloplasmin Is an Endogenous Inhibitor of Myeloperoxidase*

Received for publication, September 12, 2012, and in revised form, January 9, 2013 Published, JBC Papers in Press, January 10, 2013, DOI 10.1074/jbc.M112.418970

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Background: Myeloperoxidase promotes oxidative stress during inflammation by producing hypochlorous acid.
Results: Ceruloplasmin was a potent inhibitor of myeloperoxidase and slowed its activity in plasma from wild type mice compared with ceruloplasmin knock-out animals.
Conclusion: Ceruloplasmin is a physiologically relevant inhibitor of myeloperoxidase.
Significance: Ceruloplasmin will provide a protective shield against oxidant production by myeloperoxidase during inflammation.

Myeloperoxidase is a neutrophil enzyme that promotes oxidative stress in numerous inflammatory pathologies. It uses hydrogen peroxide to catalyze the production of strong oxidants including chlorine bleach and free radicals. A physiological defense against the inappropriate action of this enzyme has yet to be identified. We found that myeloperoxidase oxidized 75% of the ascorbate in plasma from ceruloplasmin knock-out mice, but there was no significant loss in plasma from wild type animals. When myeloperoxidase was added to human plasma it became bound to other proteins and was reversibly inhibited. Ceruloplasmin was the predominant protein associated with myeloperoxidase. When the purified proteins were mixed, they became strongly but reversibly associated. Ceruloplasmin was a potent inhibitor of purified myeloperoxidase, inhibiting production of hypochlorous acid by 50% at 25 nM. Ceruloplasmin rapidly reduced Compound I, the Fe^{V} redox intermediate of myeloperoxidase, to Compound II, which has Fe^{IV} in its heme prosthetic groups. It also prevented the fast reduction of Compound II by tyrosine. In the presence of chloride and hydrogen peroxide, ceruloplasmin converted myeloperoxidase to Compound II and slowed its conversion back to the ferric enzyme. Collectively, our results indicate that ceruloplasmin inhibits myeloperoxidase by reducing Compound I and then trapping the enzyme as inactive Compound II. We propose that ceruloplasmin should provide a protective shield against inadvertent oxidant production by myeloperoxidase during inflammation.

Myeloperoxidase is a promiscuous neutrophil enzyme that generates hypohalous acids and free radicals (1, 2). It is packaged in the azurophilic granules of neutrophils and released into phagosomes when they ingest and kill bacteria (3). The oxidants it generates provide a front-line defense against phagocytosed pathogens (4). Myeloperoxidase is also released into the extracellular space as a result of incomplete phagosome closure and during chronic inflammation when neutrophils are activated and degranulate in the absence of infection. In these situations, its oxidants have considerable potential to damage host tissue and are linked to numerous inflammatory diseases. Elevated levels of myeloperoxidase have been measured in all stages of heart disease (5) and track with major adverse cardiac events (6, 7). There are implications for the negative involvement of myeloperoxidase in cystic fibrosis, chronic obstructive pulmonary disease, rheumatoid arthritis, and Alzheimer disease (2, 8). Hypochlorous acid, the enzyme's major product, is a strong oxidant that reacts with proteins, lipids, and DNA (2). It oxidizes thiols in cells to promote apoptosis and necrosis at high and low doses, respectively (9, 10). Its chlorination footprint, 3-chlorotyosine, has been detected in proteins from inflammatory foci (8, 11, 12). The tumor suppressor protein p53 is exquisitely sensitive to hypochlorous acid and chloramines (13). Protein carbamylation, via myeloperoxidase-derived hypothiocyanous acid, is a mechanism that has been proposed to link inflammation, smoking, uremia, and coronary artery disease pathogenesis (14). When myeloperoxidase reacts with hydrogen peroxide it forms a redox intermediate called Compound I, which contains Fe^{V} in its heme prosthetic groups. Compound I is reduced via one electron to form Compound II, which is an Fe^{IV} intermediate. Myeloperoxidase generates damaging radicals when these redox intermediates oxidize tyrosine, urate, and myriad xenobiotics (2, 15, 16). The radicals promote chain reactions or couple with superoxide to form reactive hydroperoxides (17, 18).

Given the substantial potential of myeloperoxidase to exacerbate the oxidative stress associated with inflammation, it raises the question as to whether an endogenous inhibitor exists to limit its activity in the extracellular environment of neutrophils. As an analogy, α_1-antitrypsin provides an important shield against the proteolysis elicited by neutrophil elastase, which is discharged along with myeloperoxidase when these inflammatory cells are stimulated (19).

* This work was supported by the Health Research Council of New Zealand.
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The plasma protein ceruloplasmin is a possible candidate for a physiological inhibitor of myeloperoxidase. It is an abundant acute phase protein that has been demonstrated to interact with myeloperoxidase in plasma (20, 21). Ceruloplasmin binds to myeloperoxidase with a binding constant of 7.5×10^6 M^{-1} (22). It has been proposed that there is an electrostatic interaction between the two proteins due to the cationic nature of myeloperoxidase (pI ~10) and anionic charges on ceruloplasmin (pI ~4) (20).

Ceruloplasmin was first demonstrated to prevent hypochlorous acid-dependent inactivation of α_1-anti-protease by myeloperoxidase (23). In subsequent studies, it was found to be a modest inhibitor of the halogenation activity of myeloperoxidase (24, 25). Ceruloplasmin was also shown to inhibit the peroxidase activity of myeloperoxidase (20, 22), but there were inconsistencies in the extent of inhibition, with values for inhibition ranging from 40 to 70%. The degree of inhibition also increased with increasing size of the peroxidase substrate (25). This suggested that binding of ceruloplasmin to myeloperoxidase hinders large substrates from accessing the active site of the enzyme. As a corollary, this result implies that ceruloplasmin is unlikely to be an effective inhibitor of the oxidation of either chloride or thiocyanate, which are small anions.

As a result of these variable findings, ceruloplasmin has largely been ignored as a potentially important inhibitor of myeloperoxidase. Currently, there is no evidence to support ceruloplasmin functioning as an inhibitor of myeloperoxidase *in vivo*, and the mechanism of inhibition has yet to be conclusively determined. In the present study, we sought evidence for effective inhibition of myeloperoxidase by ceruloplasmin in blood plasma and aimed to clarify the mechanism by which this occurs. We show that myeloperoxidase is more active in promoting the oxidation of ascorbate in the plasma of ceruloplasmin knock-out mice than in wild type animals, suggesting that ceruloplasmin will attenuate the production of hypochlorous acid *in vivo*. Furthermore, using appropriate assays, we show that ceruloplasmin is a potent inhibitor of hypochlorous acid production.

EXPERIMENTAL PROCEDURES

Materials—Purified myeloperoxidase was purchased from Planta (Austria). Its concentration was calculated from its heme absorbance (ϵ_{430} = 89,000 M^{-1} cm^{-1}/heme). All concentrations are given per heme, so that the protein concentration of myeloperoxidase was always half this value. Hypochlorous acid (Sarah Lee, Auckland, New Zealand) concentrations were determined from its absorbance at 292 nm (pH 12, ϵ_{292} = 350 M^{-1} cm^{-1}) (26). Antibodies to myeloperoxidase and ceruloplasmin were from Abcam (Cambridge, UK), and goat anti-rabbit immunoglobulin biotin conjugate secondary antibodies were from Dako (Campbellfield, Australia). All other chemicals were purchased from either Sigma or BDH (Poole, UK).

Ceruloplasmin Knock-out Mice—Wild type (Cp$^{+/+}$)[3] and homozygous ceruloplasmin knock-out (Cp$^{-/-}$) mice (males and females) were originally a kind gift from Dr. Z. L. Harris and bred at the University of Pittsburgh. The mice are on a *C57/BL6* background and back-crossed more than 12 generations. They were allowed food and water *ad libitum* and were kept on a 12-h/12-h light/dark cycle. All animal procedures were approved by the Institutional Care and Use Committee of the University of Pittsburgh. Blood was taken from the mice into heparin-containing tubes (100 units/ml units), as described previously (27). Plasma ceruloplasmin ferroxidase activity was measured in the knockouts and found to be <5% of the wild types.

Measurement of Ascorbate Oxidation in Plasma from Ceruloplasmin KO Mice by HPLC—Plasma from Cp$^{+/+}$ and Cp$^{-/-}$ mice (50 μl) was incubated with myeloperoxidase (25 nM) for 15 min prior to the addition of glucose oxidase (generating ~5 μM/min hydrogen peroxide as measured by the Fox assay (28)). Ascorbate levels in the plasma were checked, and because all ascorbate had been lost during storage, 50 μM was added to all samples with the myeloperoxidase. Reactions were stopped after 5 min by the addition of an equal volume of perchloric acid (0.54 M) to precipitate proteins. Precipitated proteins were pelleted, and the supernatants were assayed for ascorbate by reverse-phase HPLC on a Synergi 4-μm column (Phenomenex) with electrochemical detection (29).

Isolation of Ceruloplasmin from Plasma—Protein from pooled plasma (1.4 liter from healthy volunteers) was sequentially precipitated with 30 and 55% ammonium sulfate. The second precipitate was dissolved in deionized water and then dialyzed against sodium acetate buffer (50 mM, pH 5) prior to separation on DEAE-Sephadex at 4 °C. Protein was eluted from the column via a linear gradient from pH 5 to pH 4 using 50 mM sodium acetate containing 0.4 M NaCl. Fractions with absorbance at 610 nm (due to copper in ceruloplasmin) were pooled, and protein was precipitated as described above. Precipitated protein was dissolved in sodium acetate buffer (25 mM, pH 5, containing 1 M ammonium sulfate (buffer A)) and applied to a phenyl-Sepharose column. A stepwise elution of proteins was carried out as follows; 100% A, 75% A, and 25% B (sodium acetate buffer (25 mM), pH 5), 50% A and 50% B, 25% A and 75% B, and 100% B. Fractions with absorbance at 610 nm were pooled, dialyzed against deionized water, and freeze-dried. The purified protein had a purity index of 0.042 (A_{610}/A_{280}) and was 80% pure. This is based on the purity index for pure ceruloplasmin of 0.052 (20). The protein was recognized by commercial antibodies to ceruloplasmin.

High Performance Size Exclusion Chromatography—Myeloperoxidase (30 μg, 6.8 μM), ceruloplasmin (30–150 μg, 3.9–19.5 μM), and mixtures of the two proteins were incubated in phosphate buffer (0.1 M, pH 7, containing 0.1 M NaCl) for 1 h prior to separation on an Alltech ProSphere 250 5-μm size exclusion column (7.8 × 300 mm). The mobile phase was phosphate buffer (0.1 M, pH 7, containing 0.1 M NaCl), with a flow rate of 0.5 ml/min and detection at 280, 430, and 610 nm. The column was calibrated with the following protein standards: ferritin (440 kDa), bovine serum albumin (66 kDa), and horseradish peroxidase (44 kDa), which eluted at volumes of 6.8, 8.1, and 9 ml, respectively. When myeloperoxidase was separated from plasma proteins, its activity was monitored in fractions

[3] The abbreviations used are: Cp$^{+/+}$, ceruloplasmin wild type mice; Cp$^{-/-}$, ceruloplasmin knockout mice; TMB, 3,3′,5,5′-tetramethylbenzidine; CTAC, cetyltrimethylammonium chloride; DTPA, diethylenetriaminepentaacetic acid.

using 3,3′,5,5′-tetramethylbenzidine (TMB) as a reducing substrate as described previously (26). The accumulation of blue product was detected at 650-nm oxidation over a 15-min incubation period.

Immunoprecipitation of Myeloperoxidase from Plasma—EDTA anti-coagulated plasma from a healthy donor was added to myeloperoxidase (100 nM) and incubated at room temperature with end over end mixing for three hours. Plasma alone was used as a control. Polyclonal anti-myeloperoxidase antibody was covalently bound to Protein G-Dynabeads following the manufacturer's instructions. 0.1% IPEGAL (octylphenyl-polyethylene glycol) was added to the plasma sample, followed by the beads. The sample was incubated at 4 °C with end over end rotation for 18 h. The beads were collected using a magnet and washed five times in phosphate-buffered saline (PBS; 10 mM phosphate and 140 mM Cl^-) containing 0.1% IPEGAL. Bound proteins were eluted with 0.1 M glycine, pH 3. The pH of the eluant was neutralized, and proteins were precipitated using 20% (v/v) trichloroacetic acid, 20% (v/v) acetone, 0.1% (w/v) sodium deoxycholate. Air-dried precipitates were resuspended in reducing SDS-PAGE loading buffer (2% (w/v) SDS, 10% (v/v) glycerol, 125 mM Tris-HCl buffer, pH 6.5, 1% (v/v) β-mercaptoethanol).

Simultaneous Detection of Myeloperoxidase Activity and Myeloperoxidase Protein by an ELISA—Interactions between myeloperoxidase and ceruloplasmin were investigated using a sandwich ELISA. In this assay, a standard curve was prepared with purified myeloperoxidase and used to determine the activity and protein levels in samples. Myeloperoxidase in buffer or 10-fold diluted plasma was captured by a monoclonal anti-myeloperoxidase antibody. Its enzyme activity was detected by adding hydrogen peroxide (20 μM) to Amplex Red (50 μM) in a 50 mM phosphate buffer, pH 7.4, containing 50 mM NaBr. The plate was subsequently washed to remove peroxidase substrates and products. Myeloperoxidase protein was then probed using rabbit polyclonal anti-myeloperoxidase antibody coupled to goat anti-rabbit immunoglobulin-biotin conjugate and detected with an avidin-alkaline phosphatase conjugate and *p*-nitrophenyl phosphate. To determine whether ceruloplasmin became bound to myeloperoxidase, mixtures of the two proteins were applied to the plate, and myeloperoxidase was captured as described above. The plate was washed thoroughly, and ceruloplasmin was then detected using a polyclonal antibody to ceruloplasmin as described above for myeloperoxidase.

Measurement of Hypohalous Acid Production—Formation of hypochlorous acid was measured in a discontinuous assay by capturing it with taurine and then detecting accumulated taurine chloramine (30). Myeloperoxidase (20 nM) was incubated in the presence or absence of ceruloplasmin (100 nM) and taurine (5 mM) in 10 mM phosphate buffer, pH 7.4, containing 140 mM sodium chloride (PBS) and diethylene triamine pentaacetic acid (DTPA, 100 μM) for 5–60 min. Hydrogen peroxide (50 μM) was added to start the reaction. After sufficient time to consume ~50% of the hydrogen peroxide (3–10 min), reactions were stopped by the addition of catalase (32 μg/ml), and taurine chloramine was quantified by measuring the oxidation TMB in the presence of iodide.

Production of hypochlorous acid was also measured in a continuous assay by monitoring the oxidation of ascorbate at 265.5 nm (ϵ_{265} = 15,000 M^{-1} cm^{-1}) (31). Reactions were started by the addition of a bolus of hydrogen peroxide (50 μM) to a solution containing myeloperoxidase (20 nM), cetyltrimethylammonium chloride (CTAC, 0.01%), and ascorbate (100 μM) in PBS with DTPA (100 μM) plus or minus ceruloplasmin. Initial rates were determined over the first 30 s of the reaction. In experiments in which the concentration of chloride was varied, isotonicity was kept constant by the addition of Na_2SO_4.

The halogenation of NADH was also used as a measure of the rate of hypochlorous or hypobromous acid production by myeloperoxidase (32). Myeloperoxidase was incubated with ceruloplasmin as described above, in the presence of NADH (100 μM) and either chloride (140 mM) or bromide (1 mM). Hydrogen peroxide (50 μM) was added to start hypohalous acid production, and the initial rate of NADH chlorohydrin or bromohydrin formation was determined over the first 30 s of the reaction by monitoring the increase in absorbance at 275 nm (ϵ_{275} = 11,000 M^{-1} cm^{-1}) (33).

Measurement of Peroxidase Activity—Myeloperoxidase (5 nM) was incubated with serotonin (0–1 mM) with or without ceruloplasmin (100 nM) for 1 h at room temperature in 50 mM phosphate buffer, pH 7.4, containing DTPA (100 μM) and CETAC (0.01%). Reactions were started by the addition of a bolus of hydrogen peroxide (50 μM), and the rate of serotonin dimer formation was monitored at 317 nm (34).

SDS-PAGE—Myeloperoxidase (5 μM) was incubated in PBS with or without ceruloplasmin (5 μM) for 1 h at room temperature. When used, hydrogen peroxide (50 μM) was added for 3 min prior to the addition of SDS-PAGE non-reducing loading buffer (62.5 mM Tris, pH 6.8, 20% (v/v) glycerol, 2% (v/v) SDS, 0.5% (w/v) bromphenol blue). Samples were loaded onto 7.5% T acrylamide gels. Gels were stained with Coomassie Blue G-250. In immunoprecipitation experiments, samples were resolved on 8% SDS-polyacrylamide gels under reducing conditions and silver-stained. New protein bands were excised, analyzed, and identified by mass spectrometry. All gels were run at 200 V constant for ~50 min.

Mass Spectrometry—SDS-PAGE protein bands were subjected to in-gel digestion with trypsin (35). Eluted peptides were dried and resolubilized in 5% (v/v) acetonitrile, 0.2% (v/v) formic acid. Samples were injected onto an Ultimate 3000 nanoflow ultra-HPLC system (Dionex Co.) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were separated on an in-house packed emitter tip C-18 column at 200–500 nl/min by a gradient from 5 to 80% acetonitrile, both in 0.2% (v/v) formic acid. The mass range was scanned between m/z 300 and 2000. For protein identification, MS/MS data were searched against the HumanRefSeq 2 (38,753 sequences; 18,818,966 residues) database using the Mascot search engine (see the Matrix Science Web site).

Spectral Analysis of Myeloperoxidase—An Agilent spectrophotometer was used to record spectra of myeloperoxidase (1 μM) between 350 and 700 nm at 15-s intervals after the addition of hydrogen peroxide (100 μM). Reactions were performed in PBS with methionine (1 mM) added to scavenge hypochlorous

acid. When added, ceruloplasmin or human serum albumin was present at 10 μM.

Stopped Flow Kinetics—Single turnover kinetics were measured on an SX-20MV stopped flow spectrometer (Applied Photophysics Ltd., Leatherhead, UK), using either a photomultiplier to follow single wavelengths or a photodiode array to follow spectral changes. The temperature was maintained at 25 °C using a Haake model DC10-K10 refrigerated water circulator thermostat. All kinetic measurements were carried out at pH 7.4 using 50 mM phosphate buffer. In single mixing experiments, myeloperoxidase (1.0 μM final) with or without ceruloplasmin (1 μM final) was reacted with hydrogen peroxide (10 μM final), and the formation and decay of Compound I and Compound II were followed. Sequential mixing experiments allowed the reaction of Compound II with an external reductant to be studied. Myeloperoxidase (1.0 μM final) with or without ceruloplasmin (1 μM final) was premixed with hydrogen peroxide (10 μM final) and then reacted with tyrosine (200 μM final) after complete conversion of myeloperoxidase into Compound II (20 s for enzyme alone and 2 s in the presence of ceruloplasmin). Data were analyzed using Pro-Data Viewer software (Applied Photophysics Ltd.) and Prism (GraphPad, La Jolla, CA).

Statistics—To determine whether there were differences in the extent of ascorbate oxidation by myeloperoxidase in the plasma from wild type and $Cp^{-/-}$ mice, a one-way analysis of variance was performed (SigmaStat, Jandel Scientific, San Rafael, CA). Wilcoxon's paired signed rank test was used to determine whether there was a significant difference between the concentration of myeloperoxidase protein and its activity when the enzyme was added to human plasma.

RESULTS

Ceruloplasmin Inhibits Myeloperoxidase in Plasma from Mice—We first sought evidence that inhibition of myeloperoxidase is a physiologically relevant function of ceruloplasmin by comparing the oxidation of ascorbate in plasma from either wild type ($Cp^{+/+}$) mice or those deficient in ceruloplasmin ($Cp^{-/-}$). Plasma was supplemented with ascorbate so that its initial concentration was 50 μM. The addition of myeloperoxidase alone to plasma had no effect on ascorbate concentrations (Fig. 1). Glucose oxidase was also added to plasma so that it generated a flux of hydrogen peroxide at a rate of ~5 μM/min. This promoted a small decrease in the concentration of ascorbate, which was similar in wild type and ceruloplasmin knockout mice. When myeloperoxidase and glucose oxidase were added in combination, 75% ($p < 0.001$) of the ascorbate was oxidized in plasma from the $Cp^{-/-}$ animals, whereas there was no significant loss in the plasma from wild type mice. This result demonstrates that the presence of ceruloplasmin in plasma is associated with a substantial decrease in the ability of myeloperoxidase to promote the oxidation of ascorbate.

Proteins in Human Plasma Bind Reversibly to Myeloperoxidase and Inhibit Its Activity—To seek evidence that ceruloplasmin inhibits myeloperoxidase in human plasma by becoming associated with it, purified myeloperoxidase was added to plasma, which was then fractioned by size exclusion chromatography. Fractions were analyzed for peroxidase activity (Fig. 2*A*). In the absence of added myeloperoxidase, little peroxidase activity was detected in plasma. Upon adding the enzyme to plasma, two major peaks of activity were eluted with 7.7 and 8.7 ml of running buffer (Fig. 2*A*). The later peak eluted at the same volume observed when myeloperoxidase alone was passed through the column (Fig. 2*B*). Thus, the presence of an earlier eluting peroxidase-positive peak indicates that myeloperoxidase becomes associated with proteins in plasma. This earlier peak had the same elution volume as that for a combination of ceruloplasmin and myeloperoxidase (Fig. 2*B*). This result suggests that myeloperoxidase may bind to ceruloplasmin in plasma.

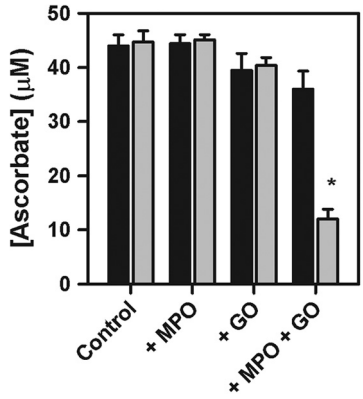

FIGURE 1. **Ceruloplasmin inhibits myeloperoxidase in murine plasma.** Plasma from $Cp^{+/+}$ (*black bars*) and $Cp^{-/-}$ (*gray bars*) mice (50 μl) was incubated with myeloperoxidase (*MPO*) (25 nM) for 15 min at 37 °C. Glucose oxidase (*GO*), generating ~5 μM/min hydrogen peroxide, was added, and samples were incubated for a further 5 min. Ascorbate levels were measured in the plasma by HPLC. Data are the means and S.E. (*error bars*) of three and four replicates for $Cp^{+/+}$ and $Cp^{-/-}$ mice, respectively. Significant differences ($p < 0.05$) were determined by one-way analysis of variance.

Next an ELISA was used to measure both the protein concentration of myeloperoxidase and its activity when the purified enzyme was added to human plasma. When 60 ng/ml 100% active myeloperoxidase was added to plasma, its additional protein concentration was fully accounted for (Fig. 2*C*). In contrast, less than half of the added enzyme activity could be detected. This result indicated that when myeloperoxidase is added to plasma, its specific activity is decreased. To understand the nature of this inhibition, we added increasing concentrations of fully active myeloperoxidase to plasma. As shown in Fig. 2*D*, this resulted in increasing detection of enzyme activity in plasma, but less than 20% of the added activity could be detected. However, when the enzyme was extracted from the plasma using cation exchange chromatography, most of its activity was recovered. In comparison, most of the added myeloperoxidase protein was detected before and after ion exchange chromatography (Fig. 2*E*). Based on these results, we conclude that proteins in plasma bind reversibly to myeloperoxidase and inhibit its activity.

To identify the proteins in plasma that myeloperoxidase becomes associated with, the enzyme was added to plasma and immunoprecipitated with a polyclonal antibody to myeloperoxidase. Co-precipitated proteins were resolved by SDS-PAGE under reducing conditions (Fig. 2*F*). The dominant protein band in the plasma sample containing myeloperoxidase had a molecular mass of ~130,000 Da and was identified by mass

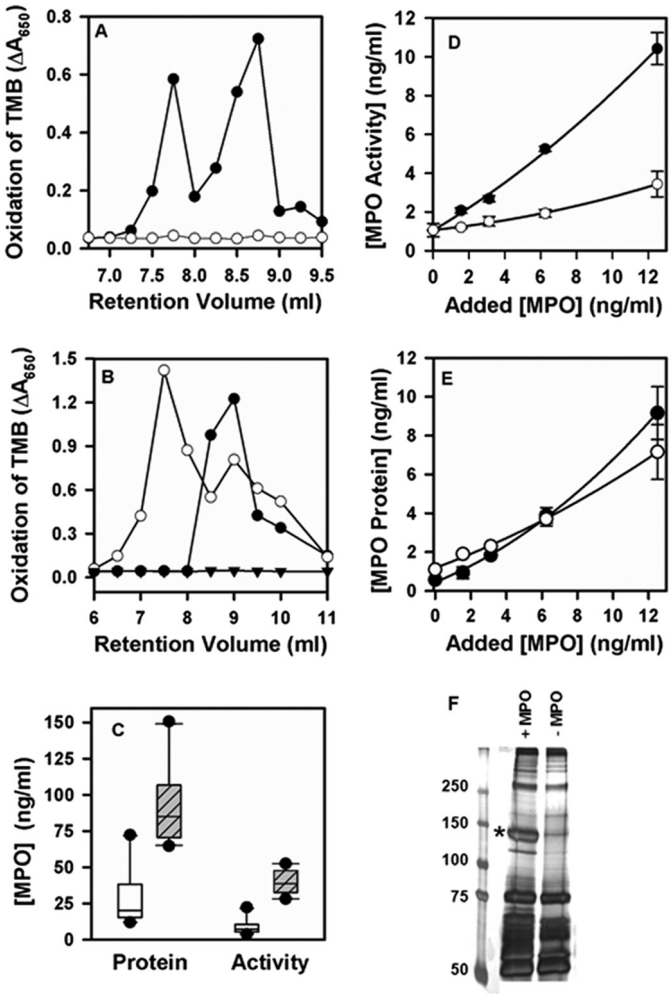

FIGURE 2. **Associations of myeloperoxidase and with proteins in human plasma.** *A*, myeloperoxidase (*MPO*; 5 μM) was incubated in heparinized plasma (11 μl) for 1 h at room temperature and then diluted 10-fold prior to separation by size exclusion chromatography. Fractions were collected and assayed for peroxidase activity using TMB as the reducing substrate and monitoring the increase in absorbance at 650 nm over 15 min. ○, plasma alone; ●, plasma and myeloperoxidase. *B*, protein fractions from the separation of myeloperoxidase (30 μg, 6.8 μM) (●), ceruloplasmin (30 μg, 3.9 μM) (▼), or a mixture of the two proteins (○) were passed through a size exclusion chromatography column and assayed for peroxidase activity. *C*, fully active myeloperoxidase (60 ng/ml) was added to plasma from 10 healthy controls. Myeloperoxidase protein concentration and activity were measured by ELISA before (*open bars*) and after adding the enzyme to the plasma (*shaded bars*). Values were determined from a standard curve prepared using purified myeloperoxidase. There was a significant difference between the protein and enzyme activity in the plasma with added myeloperoxidase ($p < 0.002$). Myeloperoxidase was added to plasma, and its activity (*D*) and protein concentration (*E*) were measured by an ELISA before (○) and after (●) extracting the enzyme by cation exchange chromatography. Data are means and S.D. (*error bars*) of triplicate measurements. *F*, plasma added to myeloperoxidase (+*MPO*) or control plasma (−*MPO*) was immunoprecipitated with a polyclonal anti-myeloperoxidase antibody, and the proteins that were pulled down were separated by SDS-PAGE under reducing conditions. The dominant band (*) in the +*MPO lane* with a molecular mass of ~130 kDa was identified as ceruloplasmin by mass spectrometry.

TABLE 1
Identification of ceruloplasmin

Underlined and boldface amino acids were identified in the immunoprecipitated material isolated from the major band on the gel shown in Fig. 2*F* using mass spectrometry. 541 amino acids of 1065 were identified, equating to 51% amino acid sequence coverage.

1	MKILILGIFL	FLCSTPAWAK	**EKHYYIGIIE**	**TTWDYASDHG**	**EKKLISVDTE**
51	**HSNIYLQNGP**	**DRIGRLYKKA**	**LYLQYTDETF**	**RTTIEKPVWL**	**GFLGPIIKAE**
101	**TGDKVYVHLK**	**NLASRPYTFH**	**SHGITYYKEH**	EGAIYPDNTT	DFQR**ADDKVY**
151	**PGEQYTYMLL**	**ATEEQSPGEG**	**DGNCVTRIYH**	**SHIDAPKDIA**	**SGLIGPLIIC**
201	**KKDSLDKEKE**	**K**HIDREFVVM	FSVVDENFSW	YLEDNIK**TYC**	**SEPEKVDKDN**
251	**EDFQESNRMY**	**SVNGYTFGSL**	**PGLSMCAEDR**	VKWYLFGMGN	EVDVHAAFFH
301	GQALTNKNYR	IDTINLFPAT	LFDAYMVAQN	PGEWMLSCQN	LNHLK**AGLQA**
351	**FFQVQECNKS**	**SSKDNIR**GKH	VRHYYIAAEE	IIWNYAPSGI	DIFTK**ENLTA**
401	**PGSDSAVFFE**	**QGTTRIGGSY**	**K**KLVYR**EYTD**	**ASFTNRKERG**	**PEEEHLGILG**
451	**PVIWAEVGDT**	**IRVTFHNKGA**	**YPLSIEPIGV**	**R**FNK**NNEGTY**	**YSPNYNPQSR**
501	SVPPSASHVA	PTETFTYEWT	VPK**EVGPTNA**	**DPVCLAKMYY**	**SAVDPTKDIF**
551	**TGLIGPMK**IC	K**KGSLHANGR**	QKDVDKEFYL	FPTVFDENES	LLLEDNIR**MF**
601	**TTAPDQVDKE**	**DEDFQESNKM**	**HSMNGFMYGN**	**QPGLTMCK**GD	SVVWYLFSAG
651	NEADVHGIYF	SGNTYLWRGE	RRDTANLFPQ	TSLTLHMWPD	TEGTFNVECL
701	TTDHYTGGMK	**QKYTVNQCRR**	**QSEDSTFYLG**	**ER**TYYIAAVE	VEWDYSPQRE
751	WEKELHHLQE	QNVSNAFLDK	**GEFYIGSK**YK	KVVYR**QYTDS**	**TFR**VPVER**KA**
801	**EEEHLGILGP**	**QLHADVGDKV**	**K**IIFKNMATR	PYSIHAHGVQ	TESSTVTPTL
851	PGETLTYVWK	IPER**SGAGTE**	**DSACIPWAYY**	**STVDQVKDLY**	**SGLIGPLIVC**
901	**R**RPYLKVFNP	RRKLEFALLF	LVFDENESWY	LDDNIK**TYSD**	**HPEKVNKDDE**
951	**EFIESNKMHA**	**INGR**MFGNLQ	GLTMHVGDEV	NWYLMGMGNE	IDLHTVHFHG
1001	HSFQYKHRGV	YSSDVFDIFP	GTYQTLEMFP	RTPGIWLLHC	HVTDHIHAGM
1051	ETTYTVLQNE	DTKSG			

spectrometry to be ceruloplasmin (Table 1). Minor bands that were immunoprecipitated from the plasma were identified as subunits of complement C3 as previously reported (20).

Ceruloplasmin Associates with Myeloperoxidase and Inhibits Its Halogenation Activity—Size exclusion chromatography was used to determine the likely stoichiometry of binding between myeloperoxidase and ceruloplasmin. Myeloperoxidase has a distinct absorbance at 430 nm due to its heme groups, whereas the copper in ceruloplasmin absorbs at 610 nm. When passed through the size exclusion column separately, myeloperoxidase (146 kDa) and ceruloplasmin (132 kDa) eluted with retention volumes of 8.8 and 7.9 ml, respectively. An adduct of myeloperoxidase and ceruloplasmin was seen when the proteins were incubated together at a ratio of 1:1. It eluted earlier (7.2 ml), had an approximate molecular mass of 280 kDa, and had absorbance maxima at 430 and 610 nm (Fig. 3*A*). Increasing the ratio of ceruloplasmin to myeloperoxidase to 2:1 (Fig. 3*B*) then 5:1 (Fig. 3*C*) caused a decrease in the peak for free myeloperoxidase. It also promoted the formation of another high molecular weight adduct that eluted at 6.9 ml and absorbed at both 430 and 610 nm. These results indicate that myeloperoxidase and ceruloplasmin form adducts with one another. They also suggest that two molecules of ceruloplasmin become associated with a single molecule of myeloperoxidase. Using SDS-PAGE, we found that myeloperoxidase and ceruloplasmin were mostly reversibly associated but did form a minor reducible complex (data not shown). This complex could not account either for the association of the proteins observed by gel filtration or the inhibition of peroxidase activity in plasma.

We then sought to provide evidence that association of the proteins results in inhibition of myeloperoxidase. Mixtures of the two proteins at physiologically relevant concentrations were applied to an ELISA plate coated with a monoclonal anti-myeloperoxidase antibody and then probed using an anti-ceruloplasmin polyclonal antibody. The amount of detectable ceruloplasmin on the plate increased with increasing ratios of ceruloplasmin to myeloperoxidase, although it did not reach a saturated level even when ceruloplasmin was present at a 1000-fold excess (Fig. 4*A*). Mixtures of the proteins containing increasing concentrations of myeloperoxidase and a 1000-fold excess of ceruloplasmin were also added to an ELISA plate, washed, and assayed for bound ceruloplasmin. The detection of

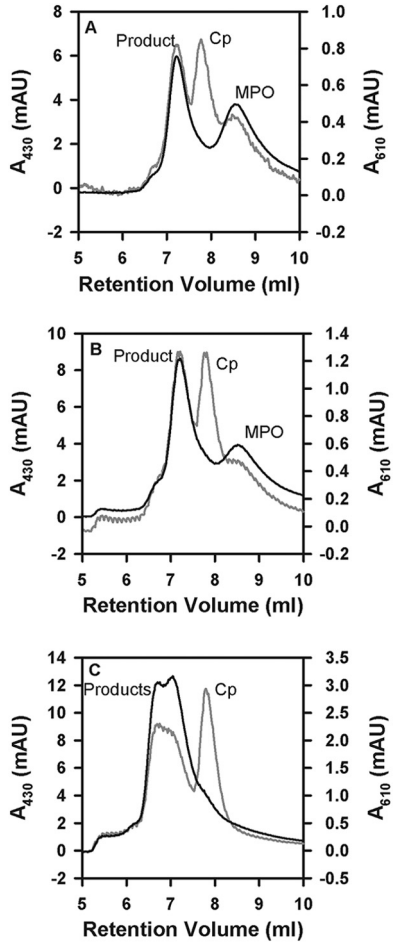

FIGURE 3. **Association of purified myeloperoxidase and ceruloplasmin.** Myeloperoxidase (30 μg, 6.8 μM) was mixed with either 30 μg (3.9 μM) (*A*), 60 μg (7.8 μM) (*B*), or 150 μg (19.5 μM) (*C*) of ceruloplasmin at room temperature in PBS for 1 h prior to separation by size exclusion chromatography. The proteins were detected at 430 nm for myeloperoxidase heme (*black*) and 610 nm for ceruloplasmin copper (*gray*).

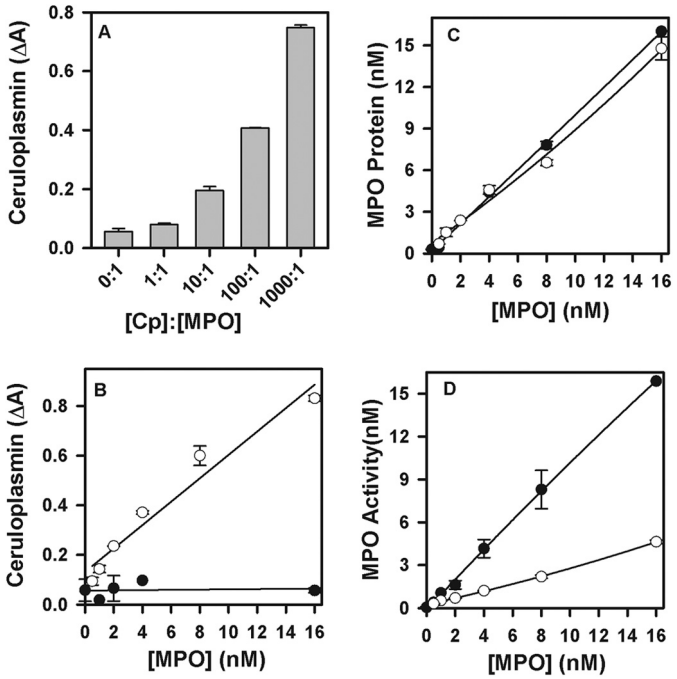

FIGURE 4. **Binding of ceruloplasmin inhibits myeloperoxidase.** *A*, myeloperoxidase (8 nM) was incubated with increasing ratios of ceruloplasmin as indicated for 1 h in PBS. Samples were applied to an ELISA plate coated with an anti-myeloperoxidase monoclonal antibody, and ceruloplasmin was detected using a polyclonal anti-ceruloplasmin antibody. *B*, detection of ceruloplasmin after incubating the indicated concentrations of myeloperoxidase either in the presence of a 1000-fold excess of ceruloplasmin (○) or in its absence (●). Myeloperoxidase protein (*C*) or activity (*D*) was detected on the ELISA plate after it had been mixed with (○) or without (●) a 1000-fold excess of ceruloplasmin. *Error bars*, S.E.

ceruloplasmin on the plate increased with increasing concentrations of myeloperoxidase (Fig. 4*B*). No ceruloplasmin was detected in the absence of myeloperoxidase, and no signal was observed in the absence of ceruloplasmin. These results suggest that, when mixed, myeloperoxidase and ceruloplasmin become associated. The association must be largely reversible because ceruloplasmin failed to saturate the plate, and it is likely that it was dissociated from myeloperoxidase during the washing steps unless it was present at very high concentrations.

We also used a polyclonal anti-myeloperoxidase antibody to detect myeloperoxidase in the ELISA experiments just described. The presence of ceruloplasmin did not affect the signal for myeloperoxidase protein over a range of concentrations (Fig. 4*C*). This result demonstrates that ceruloplasmin did not affect either the binding or the detection of myeloperoxidase on the ELISA plate. An intermediate step in the ELISA allows myeloperoxidase activity to be determined by measuring the hypobromous acid-dependent oxidation of Amplex Red (Fig. 4*D*). When ceruloplasmin was incubated with myeloperoxidase at a 1000-fold excess (*open circles*), enzyme activity was inhibited by ~70% compared with that measured for myeloperoxidase alone (*closed circles*). Thus, binding of ceruloplasmin to myeloperoxidase inhibits the halogenation activity.

Ceruloplasmin Inhibits the Enzymatic Activities of Myeloperoxidase—Next we investigated the mechanism by which ceruloplasmin inhibits the production of hypochlorous acid by myeloperoxidase. Initially we used an assay that has previously been used to test whether ceruloplasmin inhibits the chlorination activity of myeloperoxidase (24, 36). In this assay, myeloperoxidase and chloride are incubated with taurine, and the concentration of accumulated taurine chloramine formed is determined when about half of the added hydrogen peroxide has been consumed. A 5-fold excess of ceruloplasmin inhibited myeloperoxidase-dependent production of hypochlorous acid by 9–49% with a mean and S.E. of 30 ± 3% (n = 12). These results are in accord with previous studies in which ceruloplasmin was found to inhibit accumulation of taurine chloramine by 40–60% (24, 36). When urate (100 μM) was added to the reaction system to ensure that the enzyme could cycle via Compound II (37, 38), the inhibitory effect of ceruloplasmin increased to 56 ± 10% (n = 3).

To gain a better appreciation of how effective ceruloplasmin is as an inhibitor of myeloperoxidase, the initial rates of hypochlorous acid were measured in continuous assays. These included the oxidation of ascorbate (31) and the chlorination of NADH (32). Ascorbate can be oxidized either by reaction with hypochlorous acid (39) or by the peroxidation cycle involving Compound I and Compound II (40). In the presence of 140 mM

TABLE 2

Oxidation of ascorbate by myeloperoxidase

The oxidation of ascorbate (100 μM) by myeloperoxidase (MPO; 20 nM) was monitored over time in the presence and absence of ceruloplasmin (Cp; 100 nM). Reactions containing Cl^- were performed in PBS, and those without Cl^- were in 50 mM phosphate buffer, pH 7.4. Reactions were started by the addition of hydrogen peroxide (50 μM). Rates were measured over the first 30 s of the reaction. Data are the mean and S.E. of *n* experiments.

Sample (+ 100 μM ascorbate)	Rate of ascorbate oxidation
	$\mu M/s$
MPO + H_2O_2 + Cl^-	0.60 ± 0.03 (n = 23)
MPO + H_2O_2	0.08 ± 0.01 (n = 4)
H_2O_2	0.02 ± 0.01 (n = 4)
Cp + MPO + H_2O_2 + Cl^-	0.30 ± 0.02 (n = 17)
ApoCp + MPO + H_2O_2 + Cl^-	0.86 ± 0.03 (n = 5)
Cp + MPO + H_2O_2	0.10 ± 0.04 (n = 6)
Cp + H_2O_2	0.09 ± 0.04 (n = 4)
MPO + HSA + H_2O_2 + Cl^-	0.68 ± 0.13 (n = 5)

chloride, ascorbate was oxidized predominantly via its reaction with hypochlorous acid with a minor loss due to its direct oxidation by the enzyme (Table 2). Ceruloplasmin inhibited the oxidation of ascorbate, whereas the apoprotein did not (Fig. 5*A* and Table 2). Ceruloplasmin alone did not oxidize ascorbate at rates comparable with that for myeloperoxidase. Furthermore, no inhibition was seen when ceruloplasmin was replaced with human serum albumin.

Measurement of the initial rate of ascorbate oxidation was used to determine the concentration of ceruloplasmin that inhibited production of hypochlorous acid by 50% (IC_{50}). At concentrations of myeloperoxidase of 20 and 40 nM/heme group, the IC_{50} values for ceruloplasmin were 25 and 50 nM, respectively (Fig. 5*B*). Maximal inhibition occurred with ~2 mol of ceruloplasmin/heme. These results indicate that ceruloplasmin is a potent inhibitor of myeloperoxidase.

To assess how ceruloplasmin inhibits oxidation of halides by myeloperoxidase, its effect on the initial rate of ascorbate oxidation was assessed at varying concentrations of chloride or bromide. The initial rate of ascorbate oxidation increased with increasing concentrations of chloride and bromide up to a maximum (Fig. 5, *C* and *D*). Kinetic constants for the halides were obtained from such curves and are shown in Table 3. These constants were k_{cat}/K_m and k_{cat} (V_{max}/[myeloperoxidase]), which define the rate at which enzymes capture their substrates into productive complexes and release their products (41, 42). Incubation of myeloperoxidase with ceruloplasmin lowered the k_{cat} for both halides by ~50%. The ratio k_{cat}/K_m was also decreased in the presence of ceruloplasmin. This pattern of inhibition is typical of a mixed type inhibitor (42) (*i.e.* ceruloplasmin displays properties of both a competitive and uncompetitive inhibitor with respect to halides). It indicates that in the presence of ceruloplasmin the rate-determining step in turnover of myeloperoxidase must change so that it is much less dependent on the concentration of halide than in the absence of ceruloplasmin.

To determine how ceruloplasmin affected the kinetic constants for hydrogen peroxide, we used a more sensitive assay that monitors the chlorination of NADH to produce a chlorohydrin with a distinctive absorption spectrum (43). The initial rate of chlorohydrin formation increased to a maximum with increasing concentrations of hydrogen peroxide (Fig. 5*E*). Ceruloplasmin dramatically affected the initial rate of chlorohydrin formation and caused a 75% reduction in k_{cat} but had little effect on k_{cat}/K_m (Table 3). This pattern of inhibition is typical of an uncompetitive inhibitor and suggests that ceruloplasmin does not compete directly with hydrogen peroxide for reaction with myeloperoxidase.

We also determined whether ceruloplasmin affects the peroxidation activity of myeloperoxidase by measuring its effect on the oxidation of serotonin. Serotonin is the best physiological substrate for the enzyme, reacting rapidly with both Compound

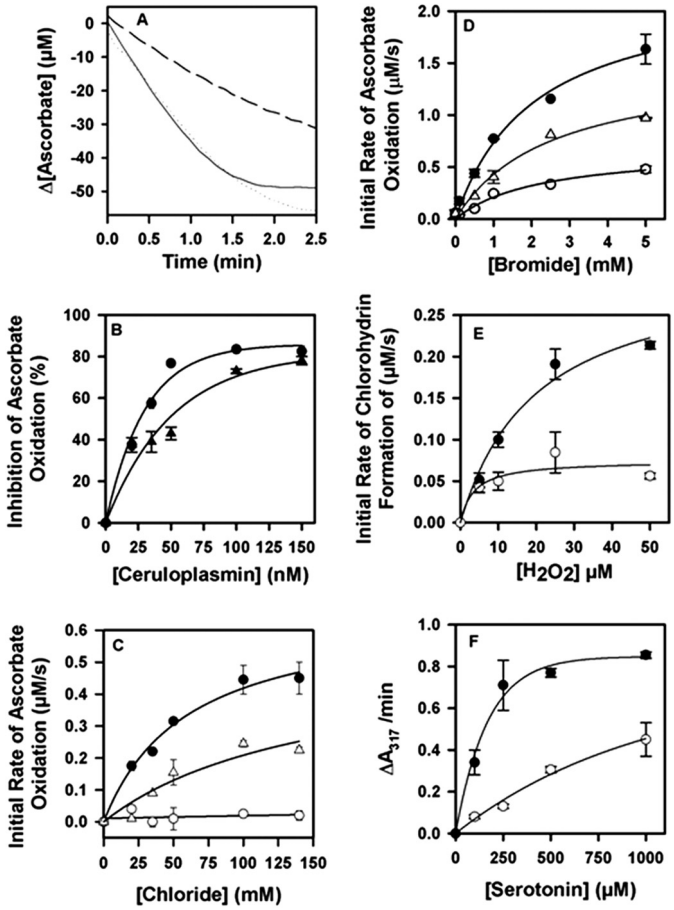

FIGURE 5. **Steady state kinetics of the inhibition of myeloperoxidase by ceruloplasmin.** *A*, the rate of hypochlorous acid production was determined by monitoring the oxidation of ascorbate at 265 nm. Myeloperoxidase alone (20 nM) (*solid line*), or myeloperoxidase preincubated with ceruloplasmin (100 nM) (*dashed line*) or human serum albumin (100 nM) (*dotted line*) was added to a solution of ascorbate (100 μM) in PBS. *B*, myeloperoxidase (20 nM (●) or 40 nM (▲)) was incubated with increasing concentrations of ceruloplasmin (25–150 nM) for 30 min prior to measurement of the initial rate of ascorbate oxidation. *C*, myeloperoxidase (20 nM) was incubated in phosphate buffer, pH 7.4, and 0–140 mM chloride, in the presence (100 nM (○) or 20 nM (△)) and absence (●) of ceruloplasmin for 1 h at room temperature prior to measurement of the initial rate of ascorbate oxidation. *D*, myeloperoxidase (20 nM) was incubated in phosphate buffer, pH 7.4, and 0–5 mM bromide, in the presence of either 40 nM (△) or 120 nM (○) ceruloplasmin, as well as in its absence (●) for 1 h at room temperature prior to measurement of the initial rate of ascorbate oxidation. *E*, myeloperoxidase (20 nM) was incubated with NADH (100 μM) in PBS in the presence (○) and absence of ceruloplasmin (100 nM) (●) for 1 h at room temperature. The initial rate of NADH chlorohydrin formation was monitored at 275 nm. *F*, myeloperoxidase (10 nM) was incubated with 0–1 mM serotonin in the presence (○) and absence of ceruloplasmin (100 nM) (●) in phosphate buffer, pH 7.4, for 1 h at room temperature. Serotonin dimer formation was monitored at 317 nm over the first 30 s of the reaction. All reactions were started by the addition of 50 μM hydrogen peroxide except as indicated in *E*. Rate data are the mean and ranges (*error bars*) of duplicate measures and are representative of two or more experiments.

TABLE 3

Kinetic parameters for the oxidation of halides and by myeloperoxidase in the presence and absence of ceruloplasmin

Values were determined by fitting rectangular hyperbola to the results presented in Fig. 5. Cp, ceruloplasmin; k_{cat}, V_{max}/[myeloperoxidase]. Data for chloride and bromide were obtained in the ascorbate assay, whereas data for hydrogen peroxide were in the NADH assay with chloride at 140 mM. The ratios of ceruloplasmin to myeloperoxidase were 1:1 for chloride and 2:1 for bromide.

Substrate	k_{cat}		K_m		k_{cat}/K_m	
	−Cp	+Cp	−Cp	+Cp	−Cp	+Cp
	s^{-1}		mM		$mM^{-1}\ s^{-1}$	
Chloride	50	27	42.1	152	1190	178
Bromide	115	75	2.1	2.5	5.5×10^4	3.0×10^4
H_2O_2	15	3.5	0.02	0.004	7.5×10^5	9.7×10^5

I ($k = 1.7 \times 10^7\ M^{-1}\ s^{-1}$) and Compound II ($k = 1.4 \times 10^6\ M^{-1}\ s^{-1}$) (44). The rate of oxidation of serotonin was determined by measuring the formation of the serotonin dimer. The rate of this reaction is determined by how fast serotonin reacts with Compound II (34). Ceruloplasmin was an excellent inhibitor of serotonin oxidation (Fig. 5*F*). It substantially decreased the k_{cat}/K_m but had a lesser effect on k_{cat}. This pattern is typical of a competitive inhibitor. It suggests that ceruloplasmin slows the rate at which serotonin can reduce Compound II.

Ceruloplasmin Reduces Compound I to Compound II—The steady state data provided insight into the mechanism of inhibition of myeloperoxidase, but additional evidence was required to establish the reactions responsible for preventing production of hypochlorous acid. We used stopped flow kinetics to determine whether ceruloplasmin affects the formation and decay of Compound I. When ferric myeloperoxidase reacts with hydrogen peroxide, it forms Compound I with a second order rate constant of $1.8 \times 10^7\ M^{-1}\ s^{-1}$ (45). Thus, when myeloperoxidase (1.0 μM) and hydrogen peroxide (10 μM) were mixed, Compound I was formed in ~40 ms. This formation was observed as a decrease in absorption at 430 nm due to the lower extinction coefficient of Compound I at this wavelength compared with the ferric enzyme (Fig. 6*A*) (45). In contrast, in the presence of one molecule of ceruloplasmin per heme group of myeloperoxidase, a decrease in absorption at 430 nm was still observed, but there was a concomitant increase at 456 nm that is associated with formation of Compound II (Fig. 6*B*).

The high rate of the initial reaction of hydrogen peroxide with ferric enzyme means that this reaction can be followed kinetically only by a series of single wavelength experiments at 430 and 456 nm using a photomultiplier. When myeloperoxidase alone reacted with hydrogen peroxide, there was an exponential decrease in absorption at 430 nm and little absorption change at 456 nm (Fig. 6*C*). This shows that only Compound I was formed during this short time period of 100 ms. Preincubation of myeloperoxidase with ceruloplasmin decreased the rate of loss of absorption at 430 nm but promoted a rapid increase in absorption at 456 nm (Fig. 6*C*). Thus, ceruloplasmin promotes fast reduction of Compound I to Compound II.

When the reaction was followed over longer periods (0–500 s), Compound I decayed into Compound II through reaction with hydrogen peroxide (46). This reaction can be followed at 456 nm, where Compound II accumulates over ~20 s, and the apparent second order rate constant we obtained was $6.9 \times 10^4\ M^{-1}\ s^{-1}$, which is well within the range of values previously reported (47). In comparison, ceruloplasmin increased this rate by a factor of 10, with maximum Compound II accumulating within 2 s. Compound II then decayed back to ferric myeloperoxidase slowly over ~400 s. Similar rates of decay were observed in the absence and presence of ceruloplasmin (data not shown).

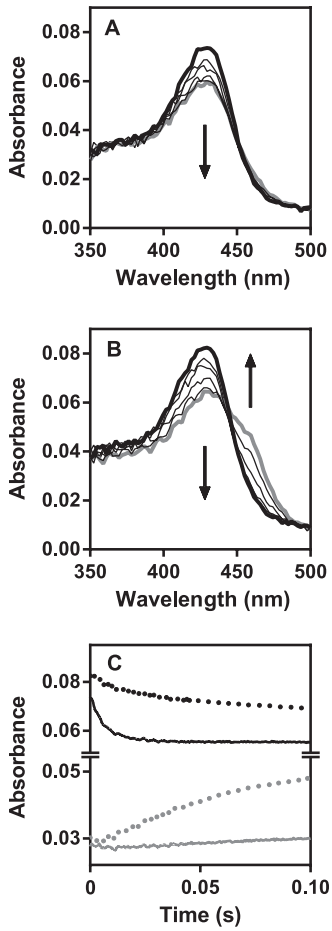

FIGURE 6. **Stopped flow spectral and kinetic observations of the initial reaction between myeloperoxidase and hydrogen peroxide in the presence and absence of ceruloplasmin.** *A*, spectral changes after myeloperoxidase (1.0 μM/heme final) was reacted with hydrogen peroxide (10 μM final). Scans were recorded at 3 ms (*thick black line*), 7 ms, 10 ms, 25 ms, 50 ms, and 100 ms (*thick gray line*). *B*, same as *A*, but myeloperoxidase was preincubated with ceruloplasmin (1 μM final). The ceruloplasmin spectrum has been removed from each time trace. *C*, single wavelength kinetic traces measured under the same conditions as in *A* (*solid lines*) and *B* (*dotted lines*) followed by the photomultiplier at 430 nm (*black lines*) and 456 nm (*gray line*). The data are representative of at least two experiments.

Ceruloplasmin Inhibits Reduction of Compound II—Next we used the stopped flow technique to determine how ceruloplasmin affects the reduction of Compound II by a classical peroxidase substrate. Due to its stability and well characterized reac-

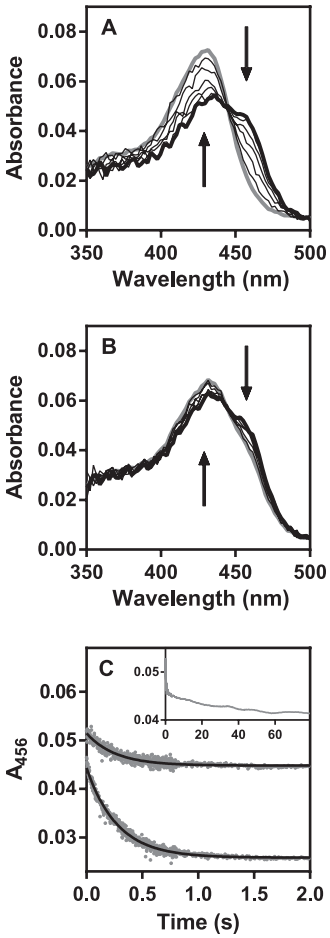

FIGURE 7. **Stopped flow spectral and kinetic observations of the reaction between Compound II and tyrosine in the presence and absence of ceruloplasmin.** *A*, spectral changes after myeloperoxidase (1.0 μM/heme final) was mixed with hydrogen peroxide (10 μM final) and incubated for 20 s to produce Compound II before reaction with tyrosine (200 μM final). Scans were then recorded at 1 ms (*thick black line*), 10 ms, 50 ms, 100 ms, 250 ms, 500 ms, and 3 s (*thick gray line*). The *arrows* indicate direction of spectral changes. *B*, same as *A* but included ceruloplasmin (1 μM final) and only incubated for 2 s before reaction with tyrosine. The ceruloplasmin spectrum has been removed from each time trace. *C*, single kinetic wavelength trace measured at 456 nm under the same conditions as *A* (*bottom*; $k_{obs} = 3.6\ s^{-1}$) and B (*top*; $k_{obs} = 3.8\ s^{-1}$). The *inset* shows the reaction of *B* over a longer time scale (0–80 s), indicating a second slower exponential decrease ($k_{obs} = 0.04\ s^{-1}$). Representative data are shown, and the observed rate constants are the average of at least two experiments.

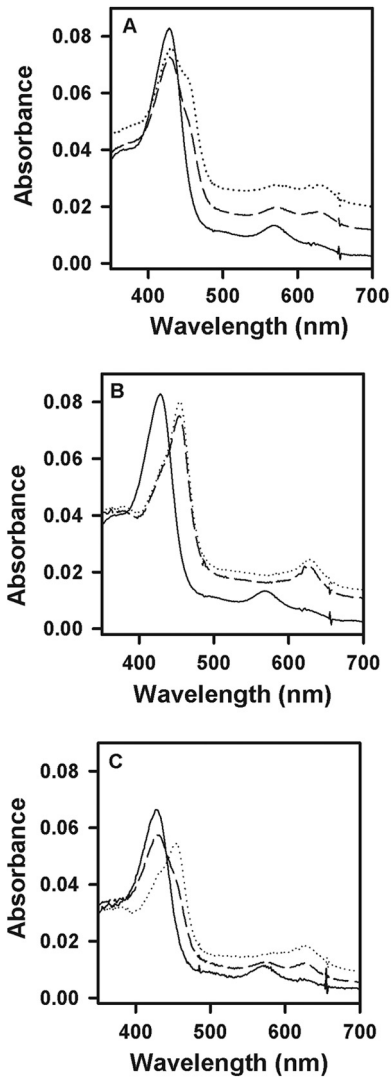

FIGURE 8. **The influence of ceruloplasmin on the formation of the redox intermediates of myeloperoxidase.** *A*, spectra of myeloperoxidase (1 μM/heme) were recorded in PBS before (*solid line*), 10 s (*dotted line*), and 120 s (*dashed line*) after the addition of hydrogen peroxide (100 μM). Methionine (1 mM) was added to scavenge hypochlorous acid. *B*, as in *A*, but myeloperoxidase was preincubated with ceruloplasmin (10 μM) for 1 h. *C*, as in *A*, but myeloperoxidase was preincubated with human serum albumin (10 μM) for 1 h. The spectrophotometer was blanked against PBS.

tions, tyrosine was used as the reducing substrate (46, 48). Myeloperoxidase in the absence or presence of one ceruloplasmin per heme group was premixed with hydrogen peroxide for 20 and 2 s, respectively, to ensure maximum formation of Compound II. The resultant mixture was then mixed with tyrosine (200 μM final), and the absorption changes were followed. In the absence of ceruloplasmin, Compound II was fully converted back to ferric myeloperoxidase by tyrosine (Fig. 7*A*). By following absorption at 456 nm (Fig. 7*C*), a first order rate constant of $3.6\ s^{-1}$ was obtained. This corresponds to an apparent second order rate constant of $1.8 \times 10^4\ M^{-1}\ s^{-1}$, similar to that previously published (46). When ceruloplasmin was preincubated with myeloperoxidase, a much smaller decrease in the amount of Compound II was observed (Fig. 7*B*). However, the absorption at 456 nm decreased with a similar first order rate of $3.8\ s^{-1}$. The loss of absorbance at 456 nm over a longer time scale of 80 s (Fig. 7*C*, *inset*) decreased with a first order rate constant of $0.04\ s^{-1}$ compared with $0.007\ s^{-1}$ for the decay of Compound II (Fig. 6*C*). The former value most likely refers to the first order dissociation of a complex between myeloperoxidase and ceruloplasmin.

Ceruloplasmin Converts Myeloperoxidase to Compound II in the Presence of Chloride—The high rate of Compound II formation in the presence of ceruloplasmin means that it was not possible to follow the reaction of Compound I with chloride when this protein is present. Therefore, to demonstrate how ceruloplasmin affects the turnover of myeloperoxidase in the presence of chloride, we monitored the Soret spectrum before and after the addition of hydrogen peroxide. In the absence of ceruloplasmin, there was partial and transient conversion of myeloperoxidase to Compound II upon the addition of hydrogen peroxide, as noticed by a shift in the Soret peak from 430 to

456 nm and the appearance of a small band at 630 nm (Fig. 8*A*). The enzyme completely reverted to the native state (Soret peak at 430 nm) after 2.5 min. Incubation of myeloperoxidase with ceruloplasmin resulted in a marked spectral shift after the addition of hydrogen peroxide, consistent with the generation of Compound II (49) (Fig. 8*B*). Compound II was stable for at least 2 min, and the ferric enzyme regenerated slowly over 8 min (not shown). In contrast, incubating myeloperoxidase with human serum albumin resulted in only a temporary generation of Compound II after adding hydrogen peroxide (Fig. 8*C*). From these results we conclude that ceruloplasmin competes with chloride to promote the accumulation of Compound II and slows its subsequent conversion back to the native enzyme.

DISCUSSION

Our finding that a deficiency of ceruloplasmin in plasma leads to enhanced oxidation of ascorbate by myeloperoxidase demonstrates that this copper-containing protein is an important endogenous inhibitor of myeloperoxidase. During inflammation, ceruloplasmin should act as an antioxidant by limiting myeloperoxidase-dependent production of reactive oxygen species. To support this conclusion, we found that ceruloplasmin is an exceptional inhibitor of myeloperoxidase, having a much lower IC_{50} than the best known inhibitors of the enzyme (50, 51). Our evidence supports a mechanism whereby the proteins become associated with each other and then ceruloplasmin reduces Compound I to Compound II as well as retards subsequent reduction of Compound II back to active myeloperoxidase.

We found that when a large concentration of myeloperoxidase was added to plasma, about one-third became associated with plasma proteins, which reversibly inhibited its activity. Our immunoprecipitation experiments support the finding of an earlier study that ceruloplasmin is the major protein in plasma that becomes associated with myeloperoxidase (20). Complement C3 also co-purified with myeloperoxidase. No other proteins were observed as specific binding partners of the enzyme. Previously, interactions between myeloperoxidase and lipoproteins *in vitro* and in plasma have been demonstrated (52–54). Compared with ceruloplasmin, these other proteins are likely to form minor associations with myeloperoxidase, as suggested recently (36).

Our experiments using size exclusion chromatography with SDS-PAGE analysis and ELISA confirm that myeloperoxidase and ceruloplasmin readily associate in a largely reversible manner. The results of size exclusion chromatography suggest that the stoichiometry of binding is 2 ceruloplasmin molecules/molecule of myeloperoxidase. Using photon correlation spectroscopy, Sokolov *et al.* (55) showed evidence for the binding of the two proteins at this ratio. Electrostatic interactions between anionic charges on ceruloplasmin and cationic residues on myeloperoxidase have been proposed as the mechanism of binding between the two proteins (20). The binding of ceruloplasmin to protein C and ferritin involves an anionic region on ceruloplasmin (amino acids 1028–1037) (56). Ceruloplasmin also binds to lactoferrin, and the cationic region ^{2}RRRR^{5} on lactoferrin is a likely binding site. A similar sequence in myeloperoxidase, ^{374}KRKGR^{378}, may be the site where ceruloplasmin attaches to the enzyme (57). However, we consistently saw inhibition of myeloperoxidase in the presence of the anionic detergent CETAC, suggesting that the interaction is unlikely to be solely electrostatic.

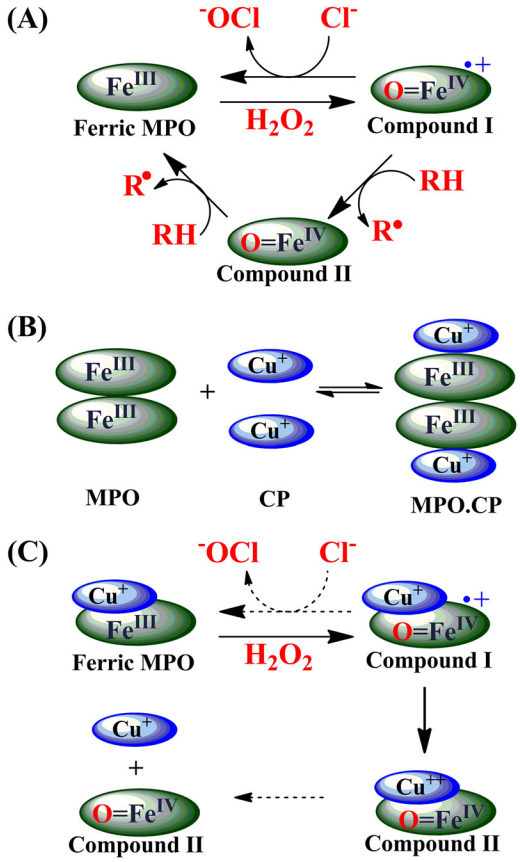

FIGURE 9. **Reactions of myeloperoxidase in the presence and absence of ceruloplasmin.** *A*, ferric myeloperoxidase (*MPO*) reacts with hydrogen peroxide to form the redox intermediate Compound I, which either oxidizes chloride by removing two electrons or removes a single electron from an organic substrate (*RH*), such as urate, ascorbate, tyrosine, or serotonin, to produce an organic radical (*R*$^{\bullet}$) and Compound II. Compound II is also reduced by these substrates to produce a second radical and regenerate ferric MPO. Only a single monomer of myeloperoxidase is illustrated. *B*, a molecule of myeloperoxidase (*MPO*) consisting of two identical dimers, each containing a heme prosthetic group, binds reversibly to two molecules of ceruloplasmin (*CP*). *C*, reaction of hydrogen peroxide with myeloperoxidase bound to ceruloplasmin enhances the one-electron reduction of Compound I to Compound II. Reduction of Compound II is slowed until it dissociates from ceruloplasmin.

The reactions myeloperoxidase undergoes to oxidize chloride and organic substrates to reactive species are shown in Fig. 9*A*. On the basis of our current evidence, the mechanism of inhibition of these reactions requires the initial reversible binding of ceruloplasmin to each monomer of myeloperoxidase (Fig. 9*B*). Steady state as well as fast kinetic data indicate that this complex still reacts rapidly with hydrogen peroxide to form Compound I. It was apparent from stopped flow experiments that ceruloplasmin then readily reduces Compound I to Compound II (Fig. 9*C*). This reaction explains how ceruloplasmin converted myeloperoxidase to Compound II in the presence of chloride. Inhibition is caused not only by reduction of the Compound I but also by the ability of ceruloplasmin to prevent the recycling of Compound II back to the active enzyme. The latter aspect of inhibition was apparent in the stopped flow experiments, whereby the association of ceruloplasmin was shown to

dramatically slow the reduction of Compound II by tyrosine. It was also apparent from the ability of ceruloplasmin to retard the steady state oxidation of serotonin. The demonstration that ceruloplasmin was more effective at inhibiting myeloperoxidase in the presence of reducing substrates, such as urate, ascorbate, and NADH, suggests that its ability to slow the turnover of Compound II is an important component of its mechanism of inhibition. Reduction of Compound I and then retardation of the turnover of Compound II fits squarely with the mixed inhibition pattern we observed when the concentration of halides was varied at differing concentrations of ceruloplasmin. The dissociation of ceruloplasmin from Compound II would then determine the rate of turnover of myeloperoxidase and consequently its chlorination activity.

The mechanism we have described has several essential attributes for an endogenous inhibitor of myeloperoxidase. First, ceruloplasmin inhibits well below its normal plasma concentration, it impedes both the production of hypohalous acids and reactive free radicals, and by binding to myeloperoxidase it should limit interactions with other susceptible proteins, including those on the endothelium. The mechanism of inhibition is similar to the action of several nonsteriodal anti-inflammatory drugs and halogenated indoles that inhibit myeloperoxidase by promoting the accumulation of Compound II (38, 58). However, these inhibitors are not as effective as ceruloplasmin because they do not impair subsequent reduction of Compound II back to the active enzyme. The ability of ceruloplasmin to essentially trap myeloperoxidase in its Compound II redox state means that the enzyme's activities will decline to a rate determined by the dissociation of the two proteins. Consequently, *in vivo* the decreased rate of hydrogen peroxide consumption by myeloperoxidase will allow endogenous scavengers of hydrogen peroxide to compete more effectively with myeloperoxidase and further limit oxidant production.

The interactions between ceruloplasmin and myeloperoxidase are intriguing. It will of interest to learn how ceruloplasmin binds to myeloperoxidase so that it can reduce Compound I and prevent substrates from reducing Compound II. This will require a detailed knowledge of electron transfer between the proteins, whether this is an enzymatic process with respect to ceruloplasmin, and the absolute kinetics of each step.

At sites of inflammation where myeloperoxidase is discharged from activated neutrophils (2, 57, 59), the likelihood of it encountering ceruloplasmin is high because ceruloplasmin is an abundant acute phase plasma protein present at concentrations of 2–4 μM (60). Indeed, complexes of myeloperoxidase and ceruloplasmin have been seen in plasma and serum from patients suffering from inflammatory conditions (36). In contrast, ceruloplasmin would not be expected to inhibit myeloperoxidase during bacterial killing by neutrophils because it would have limited access to phagosomes (3).

Ceruloplasmin is a multicopper oxidase that contains 90% of the copper in plasma. Aceruloplasminemia is a rare autosomal recessive disease that results in iron accumulation in the pancreas, liver, and brain, and patients suffer from diabetes and neurodegeneration (61). This abnormality suggests that the most important function of ceruloplasmin is its ferroxidase activity, where reactive Fe^{2+} is oxidized to Fe^{3+}, providing Fe^{3+} for transferrin and preventing Fe^{2+} from participating in the generation of hydroxyl radicals (62). Ceruloplasmin has both pro-oxidant and anti-oxidant properties and has been described as a "moonlighting protein" due to its many and varied activities (63). These also include amine oxidase (25), nitric-oxide oxidase (27), and glutathione peroxidase activities (64). Inhibition of myeloperoxidase should be added to this list but is likely to be a minor function of ceruloplasmin because a small proportion only of circulating ceruloplasmin is required to attenuate oxidant production by myeloperoxidase. In the future it will be of interest to determine whether these other activities of ceruloplasmin impinge on its ability to inhibit myeloperoxidase and thereby promote oxidative stress.

Elevated levels of ceruloplasmin have been reported in many cardiovascular disorders and correlate with cardiovascular risk (65, 66). Ceruloplasmin is capable of oxidizing LDL (67), and it has been hypothesized that oxidized LDL is involved in the progression of atherosclerosis (68). There is also much evidence that myeloperoxidase has a role in the development of heart disease (6, 69, 70), suggesting a complex redox environment where ceruloplasmin can limit myeloperoxidase activity at sites of inflammation but also contribute to oxidative damage.

In summary, we have shown that ceruloplasmin is a potent endogenous inhibitor of myeloperoxidase. However, when ceruloplasmin is overwhelmed by myeloperoxidase, as may occur in sepsis (71) or within the airways of children with cystic fibrosis (8), then it may be free to produce reactive oxygen species. Recently, increased mortality and a greater degree of protein oxidation were seen in $Cp^{-/-}$ mice in a model of inflammatory bowel disease compared with controls (72). Unrestricted activity of myeloperoxidase may have been responsible for this oxidation. This possibility warrants closer scrutiny because neutrophils are major inflammatory cells in this disease, releasing large amounts of myeloperoxidase into the intestinal mucosa (73). Alternatively, the balance between ceruloplasmin and myeloperoxidase may be tipped to a pro-oxidant state by antibodies to myeloperoxidase that occur in antineutrophil cytoplasmic autoantibody-mediated vasculitis (74). Some of these antibodies have been shown to prevent ceruloplasmin from binding to and inhibiting myeloperoxidase (22). Consequently, further investigation of the interplay between ceruloplasmin and myeloperoxidase will allow a greater understanding of how oxidative stress manifests during inflammation.

Acknowledgments—We thank Dr. Steve Brennan for help in isolating ceruloplasmin from plasma, Dr. Torsten Kleffmann for protein analysis, and Dr. Paul Pace for assistance with immunoprecipitation experiments.

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Ceruloplasmin Is an Endogenous Inhibitor of Myeloperoxidase

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J. Biol. Chem. 2013, 288:6465-6477.
doi: 10.1074/jbc.M112.418970 originally published online January 10, 2013
