Biochemical and Biophysical Research Communications 453 (2014) 722–727

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ceruloplasmin has two nearly identical sites that bind myeloperoxidase

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ARTICLE INFO

Article history:
Received 22 September 2014
Available online 6 October 2014

Keywords:
Ceruloplasmin
Myeloperoxidase
Interaction
Inhibition

ABSTRACT

Ceruloplasmin (Cp) is a copper-containing ferroxidase with potent antioxidant activity. Cp is expressed by hepatocytes and activated macrophages and has been known as physiologic inhibitor of myeloperoxidase (MPO). Enzymatic activity of MPO produces anti-microbial agents and strong prooxidants such as hypochlorous acid and has a potential to damage host tissue at the sites of inflammation and infection. Thus Cp–MPO interaction and inhibition of MPO has previously been suggested as an important control mechanism of excessive MPO activity. Our aim in this study was to identify minimal Cp domain or peptide that interacts with MPO. We first confirmed Cp–MPO interaction by ELISA and surface plasmon resonance (SPR). SPR analysis of the interaction yielded 30 nM affinity between Cp and MPO. We then designed and synthesized 87 overlapping peptides spanning the entire amino acid sequence of Cp. Each of the peptides was tested whether it binds to MPO by direct binding ELISA. Two of the 87 peptides, P18 and P76 strongly interacted with MPO. Amino acid sequence analysis of identified peptides revealed high sequence and structural homology between them. Further structural analysis of Cp's crystal structure by PyMOL software unfolded that both peptides represent surface-exposed sites of Cp and face nearly the same direction. To confirm our finding we raised anti-P18 antisera in rabbit and demonstrated that this antisera disrupts Cp–MPO binding and rescues MPO activity. Collectively, our results confirm Cp–MPO interaction and identify two nearly identical sites on Cp that specifically bind MPO. We propose that inhibition of MPO by Cp requires two nearly identical sites on Cp to bind homodimeric MPO simultaneously and at an angle of at least 120 degrees, which, in turn, exerts tension on MPO and results in conformational change.

1. Introduction

Ceruloplasmin (Cp) is an acute-phase plasma protein made mainly by hepatocytes and secreted into the bloodstream [1,2]. The normal plasma level of Cp doubles in response to inflammation, trauma, or infection [3]. The expression of Cp can also be induced in monocytes and macrophages by interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α) [4]. The ferroxidase activity of Cp converts Fe^{2+} to Fe^{3+} and plays an important role in iron metabolism and transport, and in erythropoiesis [5–7]. Cp inhibits ferrous ion-mediated production of reactive oxygen species and, therefore, is widely accepted as an important antioxidant. It also exhibits ferroxidase-dependent bactericidal activity [8]. Cp is 132 kD single polypeptide that contains about seven copper atoms in its structure and folds into three homologous domains [9].

Premature termination of Cp mRNA translation results in aceruloplasminemia, a rare disorder with complete absence of plasma Cp and pathologic iron accumulation in brain, liver, spleen, and other tissues [10,11]. In a murine model generated by Cp gene disruption, $Cp^{-/-}$ mice are normal at birth, but reveal progressive liver and spleen iron accumulation accompanied by mild anemia [12]. $Cp^{-/-}$ mice also exhibit excessive inflammatory response in dextran sodium sulfate induced colitis where Cp made by infiltrating macrophages plays an important anti-oxidant and anti-inflammatory role [13].

Cp has been shown to bind and inhibit myeloperoxidase (MPO) [14]. Abundantly present in azurophilic granules of neutrophils, MPO is a heme-containing enzyme that catalyzes the reaction between chloride and hydrogen peroxide to generate hypohalous acids and free radicals. Upon activation of neutrophils MPO is released into phagosomes and extracellular space. The highly reactive products of its enzymatic activity such as hypochlorous acid are potent oxidants and anti-microbial reagents that have the potential to damage host tissue as well [15]. Hypochlorous acid oxidizes thiols in cells and has been linked to the induction of

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http://dx.doi.org/10.1016/j.bbrc.2014.09.134
0006-291X/

apoptosis and necrosis [16,17]. The inflammatory sites contain 3-chlorotyosine, the chlorination adduct of MPO [18,19]. It has been suggested that destructive activity of MPO at the sites of inflammation is inhibited by Cp as a mechanism to limit excessive oxidative damage. Interestingly, the binding of MPO to Cp does not inhibit important antioxidant activities of Cp, oxidation of ferrous ions and removal of peroxides [14].

Although the Cp–MPO interaction was first described in late 1990s [14,20], there still is no structural study aimed at elucidating Cp–MPO interaction. In present study we determined minimal Cp peptides that binds MPO using a series of overlapping peptides spanning Cp's entire amino acid sequence. Furthermore, we generated rabbit antisera specific to the minimal interacting peptide and showed that it interrupts Cp–MPO binding and rescues MPO activity.

2. Materials and methods

2.1. Cp–MPO binding ELISA

Purified human Cp (Vital Products) was coated on Nunc MaxiSorp® flat-bottom 96 well plates (Nalgene). 100 nM solution of purified human MPO (Lee Biosolutions), either alone or preincubated with excess amount of free Cp or ascorbate oxidase (Sigma), was added into wells and incubated for 1 h at room temperature. Bound MPO was detected by incubating plate with rabbit anti-human MPO antibody (DAKO), anti-rabbit alkaline phosphatase-conjugated secondary antibody (Abcam), and alkaline phosphatase substrate solution (Sigma), respectively. The absorbance was read on microplate reader at 405 nm.

2.2. MPO activity assay

MPO, either alone or preincubated with 1 to 100-fold excess of Cp, was incubated in Tetramethylbenzidine liquid substrate (Sigma) in wells of 96-well plate for 10 min at room temperature. The reaction was stopped by adding hydrochloric acid and absorbance was measured at 450 nm.

2.3. Surface plasmon resonance

MPO was immobilized on CM5 sensor chip via $-NH_2$ and exposed to buffer with various concentrations of Cp using Biacore3000 instrument. Flow rate for the analyte was 30 μl/min. Binding was analyzed by Biaevaluation software.

2.4. Peptide screening

Each of the 87 peptides spanning the entire amino acid sequence of human Cp was designed at 24 residues long and to overlap by 12 residues with preceding and 12 residues with following peptide. The peptides were synthesized (Mimotopes) and coated on 96 well plates (Nalgene). A total of 87 plates were designed and used for each individual peptide by coating one half of the 96-well plate with peptide and the other half with Cp as a control. MPO, either alone or pre-incubated Cp or AO, was added to the wells. Bound MPO was detected by incubating the plates

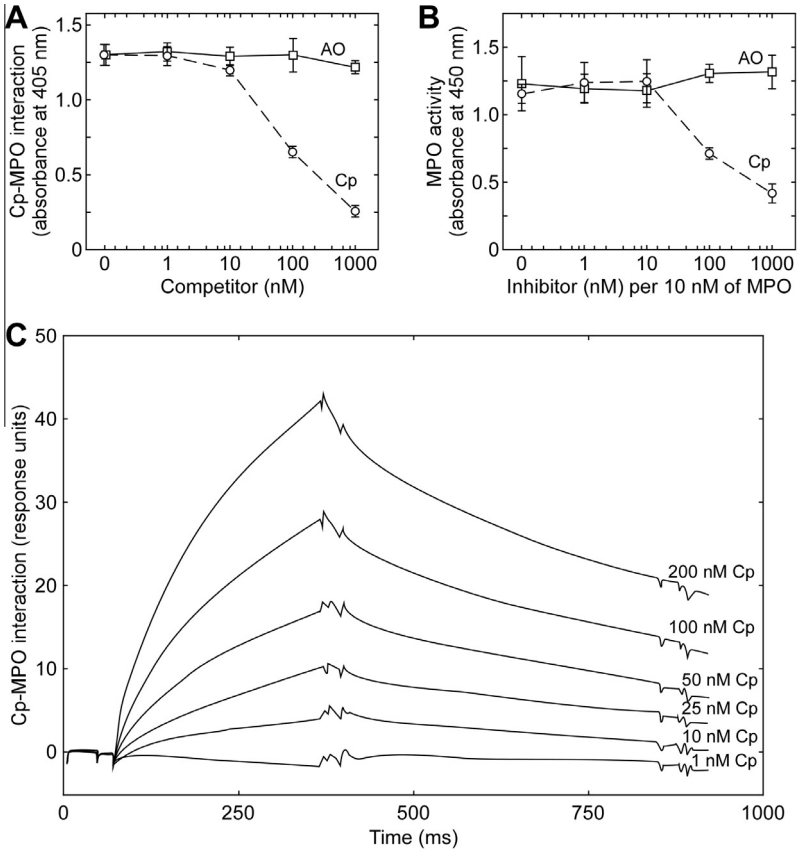

Fig. 1. Cp–MPO interaction. (A) Interaction between surface-adsorbed Cp and free MPO was inhibited by pre-incubating MPO with excess free Cp or ascorbate oxidase (AO) (mean ± SEM, $n = 4$ well per reaction). (B) MPO activity measured by incubating MPO with peroxidase substrate. Various concentrations Cp and AO were preincubated with MPO to inhibit its peroxidase activity (mean ± SEM, $n = 4$ well per reaction). (C) K_d of Cp binding to immobilized MPO was determined by surface plasmon resonance.

with rabbit anti-human MPO antibody (DAKO), anti-rabbit alkaline phosphatase-conjugated secondary antibody (Abcam), and alkaline phosphatase substrate solution (Sigma), respectively. The absorbance was read by microplate reader at 405 nm.

2.5. Anti-P18 antibody

Anti-P18 antiserum was raised by repeated immunization of rabbit by HPLC-purified 24-mer P18 peptide (AbD Serotec). The blood was obtained from the animal on day 56 after immunization and IgG fraction was affinity purified from serum using protein AG agarose columns (Pierce).

2.6. Statistical analysis

Differences in parametric data were evaluated by the Student's two-tailed t test. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Ceruloplasmin binds myeloperoxidase and inhibits its enzymatic activity

In order to confirm Cp–MPO interaction, a 96-well plate was coated with Cp. Following blocking with gelatin, MPO was added and bound MPO was detected by anti-MPO antibody. This binding was gradually inhibited by mixing and pre-incubating MPO with increasing concentration of free ceruloplasmin (Fig. 1A). As a negative control, Cp–MPO binding was not inhibited by pre-incubating MPO with increasing concentration of free ascorbate oxidase (AO), a copper-containing oxidoreductase. Parallel to previous reports [14,21], MPO activity assay showed that Cp potently inhibits MPO enzymatic activity at and higher than 1:10 Cp-to-MPO ratio, while AO did not alter its activity (Fig. 1B).

The kinetics of Cp–MPO interaction was investigated by using surface plasmon resonance. Sensor chip was prepared by immobilizing MPO on its surface and Cp ranging from 1 to 200 nM was passed over the MPO. The response in the sensogram increased gradually with increasing concentration of Cp indicating the binding between Cp and MPO (Fig. 1C). BiaCore evaluation software generated a binding affinity (K_d) of 30 nM.

3.2. Overlapping peptide mapping of Cp reveals two peptides interacting with myeloperoxidase

Identification and characterization of protein–protein interaction sites can lead to the discovery and development of new therapeutics. In order to identify the MPO-binding site of Cp, we designed overlapping series of peptides spanning the entire amino acid sequence of human Cp. 87 peptides, each 24 amino acid long, were synthesized in such a way that each peptide's first 12-amino acid sequence is the same as the preceding peptide's last 12 amino acid sequence. An ELISA plate was designed and used for each individual peptide by coating one half of the 96-well plate with peptide and the other half with Cp as a control. MPO, either alone or pre-incubated with Cp or AO, was added to the wells and bound MPO was detected by anti-MPO antibody. Due to the variation of

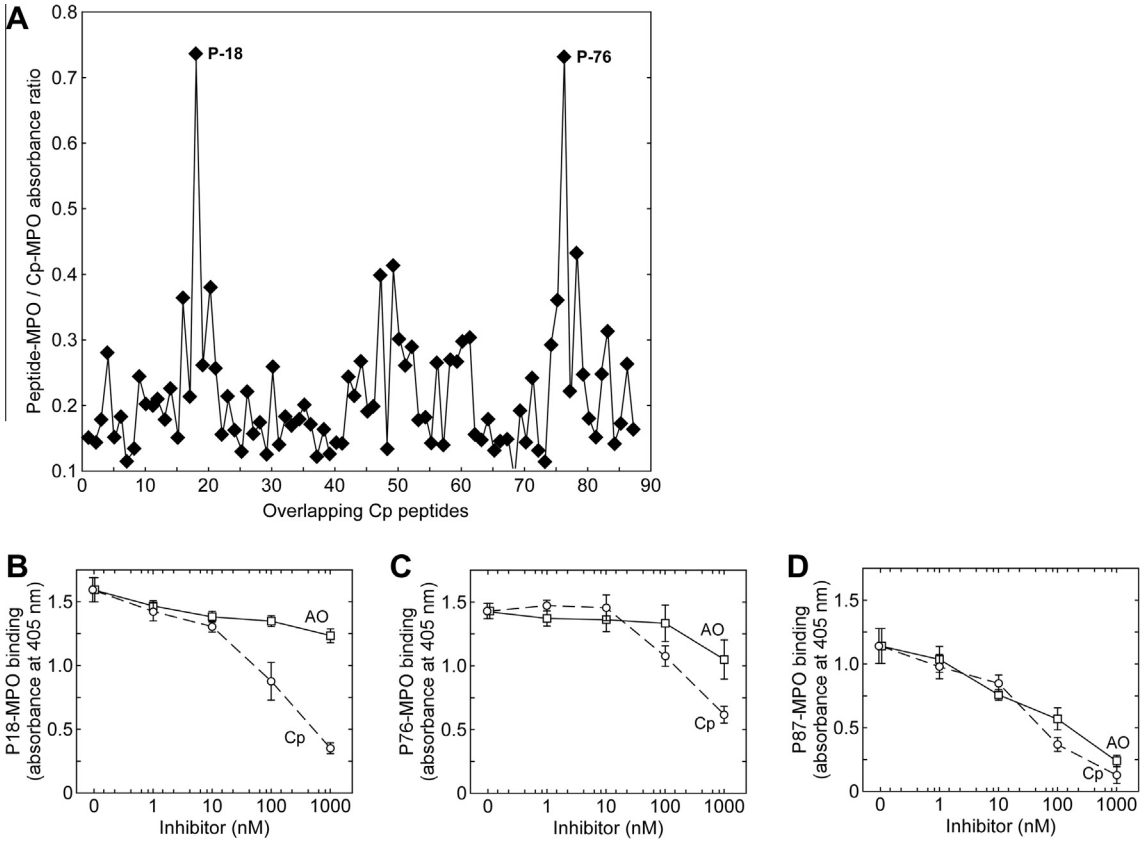

Fig. 2. Identification of MPO-binding Cp-derived peptides. (A) Peptide–MPO to Cp–MPO absorbance ratio of 87 peptides obtained by direct binding ELISA. (B) P18-MPO, (C) P76-MPO, and (D) P87-MPO binding ELISA. Interaction between surface-adsorbed peptides and free MPO was inhibited by pre-incubating MPO with excess free Cp or ascorbate oxidase (mean ± SEM, $n = 4$ well per reaction).

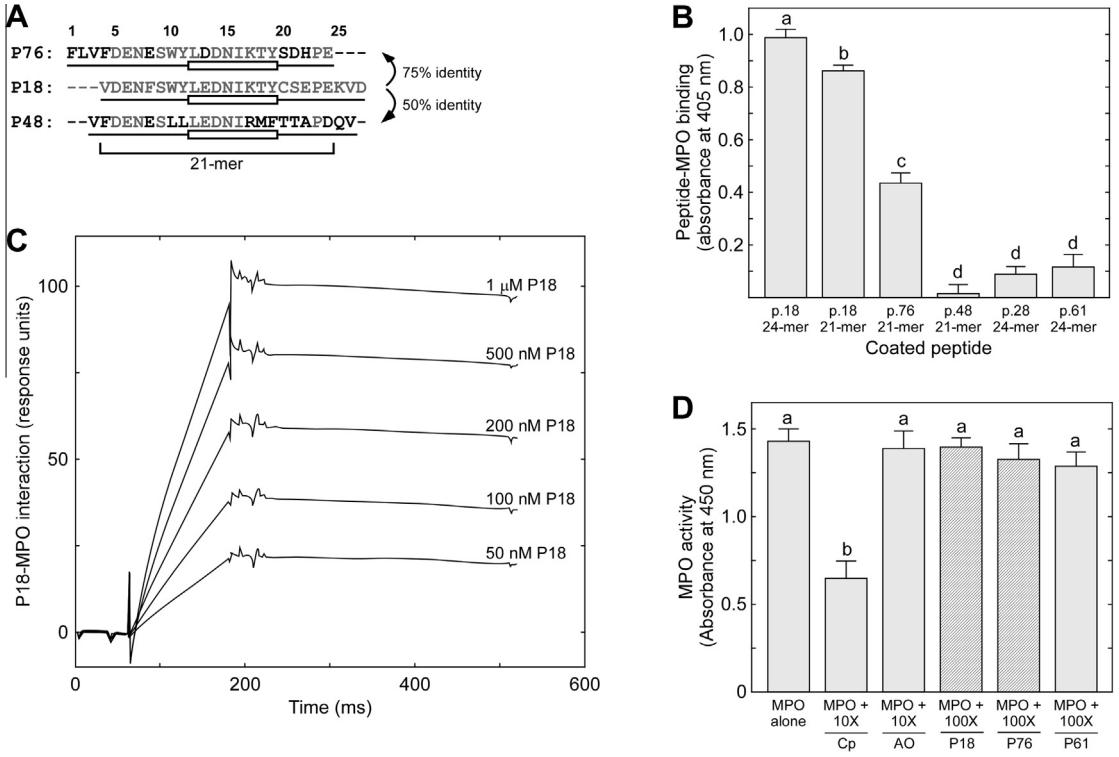

Fig. 3. P18 and P76 bind MPO but do not inhibit its activity. (A) Comparison of amino acid sequence of P18, P48, and P76. (B) Binding between MPO and HPLC-purified, surface-adsorbed peptides by direct binding ELISA (mean ± SEM, $n = 4$ well per reaction, $p < 0.05$). (C) K_d of 24-mer P18 binding to immobilized MPO was determined by surface plasmon resonance. (D) MPO activity measured by incubating MPO with peroxidase substrate. Various concentrations Cp, peptides, and AO were preincubated with MPO to inhibit its peroxidase activity (mean ± SEM, $n = 4$ well per reaction, $p < 0.05$).

detected MPO from one plate to another, the result of 87 ELISA plates was also combined by taking the ratio of peptide–MPO binding to Cp–MPO binding (Fig. 2A). The combined data revealed two major peaks at peptides number 18 (P18) and 76 (P76).

The binding ELISA result for P18 shows a strong binding of MPO to the immobilized peptide (Fig. 2B). This data also suggests that the P18-MPO binding is not nonspecific because pre-incubation of MPO with excess of free Cp disrupted the peptide–MPO interaction, while pre-incubation of MPO with AO did not. The similar binding ELISA result was obtained for P76-MPO binding (Fig. 2C). The remaining 85 peptides exhibited weak and nonspecific binding similar to P87-MPO binding ELISA result (Fig. 2D). MPO bound to P87 was equally competed out with both Cp and AO and at much lower concentrations.

3.3. P18 and P76 are highly homologous peptides that interact with myeloperoxidase

The single polypeptide sequence of ceruloplasmin folds into three homologous domains [9]. The amino acid sequence of P18, which lies within the first domain of Cp, and P76, which lies within the third domain, are 75% identical and the remaining residues are highly-conserved. The third conserved peptide, P48, lies within the second domain and has 50% identity with P18 (Fig. 3A). First 12-amino acid sequence of P48 sequence is the same as last 12-amino acid sequence of P47, while the second half of P48 sequence is the same the first half of P49 amino acids sequence. Although P48 did not bind MPO, P47 and P49 represent a smaller biphasic peak between P18 and P76 (Fig. 2A). Moreover, according to the crystal structure of Cp, the peptides P18, P48, and P76 have similar secondary structure: alpha helix flanked with unstructured sequences on both sides.

The 87 overlapping peptides were not HPLC-purified and, thus, might result in either nonspecific binding or loss of binding due to the impurity. Therefore, in addition to the original longer version of P18 (24-mer) and two negative controls (P28 and P61), three-amino acid shorter versions (21-mers) of P18, P48, and P76, were resynthesized and HPLC-purified. The 21-aminoacid long versions of these peptides were selected to include the shortest common amino acid sequence (Fig. 3A). The HPLC-purified peptides were tested by ELISA for binding to MPO. The full length, 24 amino acid-long P18 bound more MPO than its shorter, 21-mer version. P76 also bound significant amount of MPO compared to negative controls, P28 and P61. The last conserved 21-mer, P48, bound as little MPO as the negative controls (Fig. 3B). This indicates that there are two main MPO-binding sites within the Cp amino acid sequence: P18 and P76. Because P18 and P76 have highly-conserved sequences and P18 bound more MPO than P76, we investigated P18-MPO interaction in more detail. In order to confirm the P18-MPO binding and analyze the binding kinetics, a sensor chip for surface plasmon resonance was prepared by immobilizing MPO on its surface. As buffer with 24-mer P18 ranging from 50 to 1000 nM passed over the MPO, the response in the sensogram gradually increased indicating a clear binding between P18 and MPO (Fig. 3C). BiaCore evaluation software generated a binding affinity (K_d) of about 200 nM.

Because binding of full length Cp to MPO inhibits its peroxidase activity, we investigated whether P18 inhibits MPO as well. However, peroxidase assay revealed that P18 has no effect on MPO activity, even when MPO was pre-incubated with P18 at 100× higher concentration than MPO (Fig. 3D). 10× molar concentration of Cp inhibited MPO activity and was used a positive control for MPO inhibition, while AO and P61 served as negative controls and did alter the peroxidase activity.

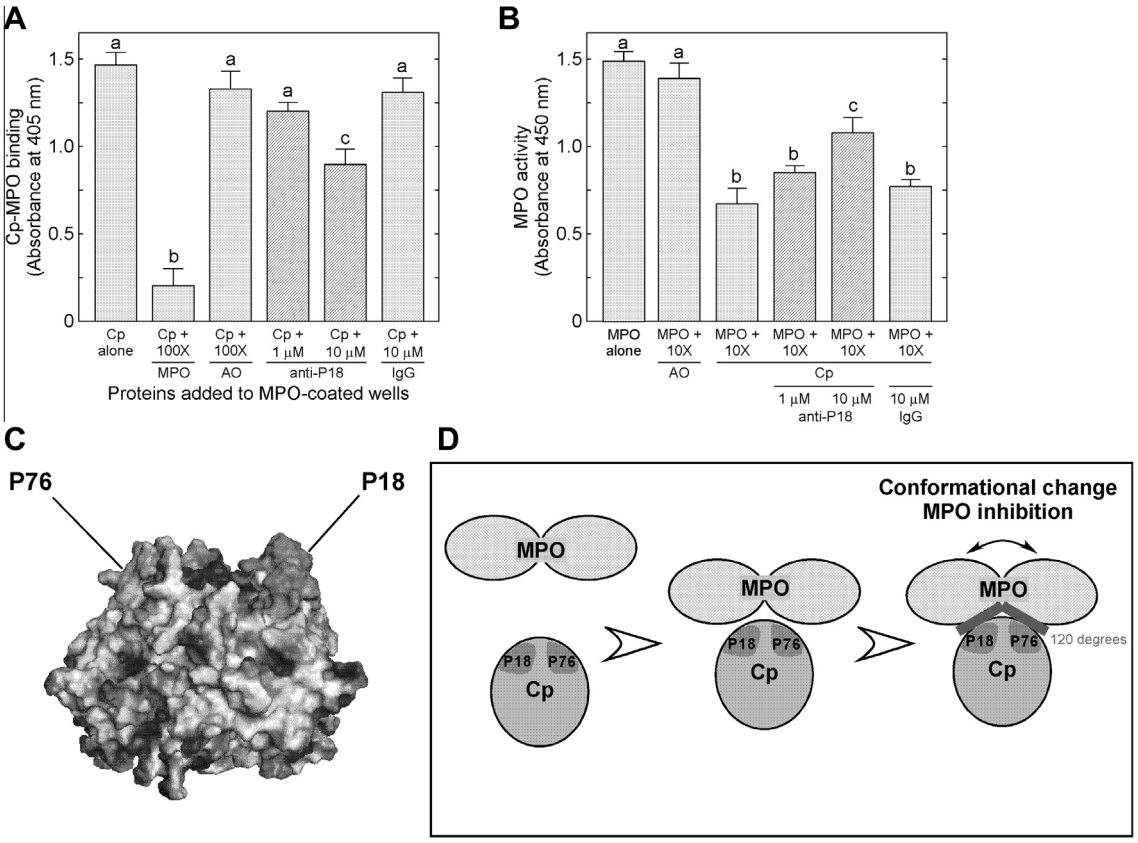

Fig. 4. (A) Interaction between surface-adsorbed Cp and free MPO was inhibited by pre-incubating MPO with anti-P18 antisera, excess free Cp, or ascorbate oxidase (AO) (mean ± SEM, $n = 4$ well per reaction, $p < 0.05$). (B) MPO activity measured by incubating MPO with peroxidase substrate. Various concentrations Cp with or without anti-P18 antisera, and AO were preincubated with MPO to inhibit its peroxidase activity (mean ± SEM, $n = 4$ well per reaction, $p < 0.05$). (C) Crystal structure of Cp with highlighted P18 and P76 sequences visualized by PyMOL. (D) A model for Cp–MPO interaction that results in MPO inhibition.

3.4. Anti-P18 antibody disrupts ceruloplasmin-MPO interaction and prevents inhibition of peroxidase activity

Although with varying degree of success, protein–protein interactions can be disrupted by antibodies. Therefore, to confirm P18 sequence as the primary site for MPO binding, we raised anti-P18 antibody in rabbit. To test whether anti-P18 antibody is capable of disrupting Cp–MPO interaction we used Cp–MPO binding ELISA. Cp was added to MPO-coated wells and the bound Cp was detected by anti Cp antibody. Cp–MPO binding was gradually disrupted by pre-incubating with increasing amount of anti-P18 antibody (Fig. 4A). As a positive control, Cp–MPO interaction was disrupted by pre-incubating Cp with excess of free MPO and, as a negative control, was unaffected by pre-incubating Cp with either pre-immune rabbit IgG or AO. In addition, MPO activity assay showed that anti-P18 antibody prevented MPO inhibition by Cp. As a negative control, Cp was also pre-incubated with pre-immune rabbit IgG (Fig. 4B).

In summary, overlapping peptides of Cp revealed two major 21 amino acid sequences as the site for MPO-binding. These highly-conserved surface-exposed sequences, comprising residues 204–224 and 903–923 (Fig. 4C), lie within the first and the second homologous domains of Cp. Anti-P18 antibody disrupts the Cp–MPO interaction and protects MPO enzymatic activity.

4. Discussion

In this study we identify two highly-conserved, 21-amino acid sequences with surface exposed residues within the first and third homologous domains of Cp that strongly interacts with MPO. We also show that antibody raised to peptide 204–224 (P18) disrupts Cp–MPO interaction and rescues MPO enzymatic activity. Thus this study uncovers a novel structural aspect of Cp–MPO interaction and strengthens previously published findings on Cp–MPO interaction [14,20–22].

Our finding of two sites with highly-conserved amino acid sequences that interact with MPO correlates with the homodimeric nature of MPO as revealed by the study of its crystal structure. This may imply that two nearly-identical sites on Cp, located on first and third homologous domains of Cp, bind to two identical sites on MPO homodimer. MPO does not interact with P48 which is located on second homologous domain of Cp. Lack of P48-MPO binding can simply be explained by the low sequence homology between P18 and P48 (Fig. 3A). According to the crystal structure of Cp, all three peptides (P18, P48, and P76) on corresponding homologous domains should have "unstructured-short helix-unstructured" secondary structure motif. Based on this prediction, P18 has more charged and polar residues on unstructured parts of the peptide, whereas P48 has more hydrophobic residues on unstructured parts of the peptide. Specifically, Ala in P48 is substituted with Glu in P18 ($Glu_{P18} \rightarrow Ala_{P48}$), $Tyr_{P18} \rightarrow Leu_{P48}$ and $Trp_{P18} \rightarrow Leu_{P48}$. These key residues are surface-exposed and fully accessible in full Cp's crystal structure, which makes them accessible for interaction with MPO (Fig. 4C). The charged residues on P18, which are also conserved on P76, give the assumption that MPO-Cp interaction may be driven by hydrophilic residues on both proteins. This finding and hypothesis is also supported by the cationic nature of myeloperoxidase (pI ~ 10) and anionic charges on Cp

(pI ~ 4) [14]. However, further structural studies are needed to find the binding site on MPO and the critical residues on Cp binding site.

Our finding that P18 did not inhibit MPO activity has several implications. P18 sequence might only be responsible for binding MPO and some other Cp site might actually play role in inhibition. Thus, inhibition of MPO may actually be caused by conformational change due to the interaction with multiple sites, other than P18. The best candidate for the second site of interaction is P76. Copper, as a cofactor for Cp, may also play a key role in enzymatic inhibition of MPO. It is also possible that a conformational binding site not present on a 24 amino acid long peptide of Cp may be responsible for inhibition of MPO activity. In this case, overlapping peptide approach used in this study may not be effective at detecting conformational binding sites. On the other hand, it is unlikely that we missed any MPO-binding site on Cp other than conformational that interacts with MPO due to the overlapping design of Cp peptides.

Collectively our results confirm Cp–MPO interaction and identify two nearly identical sites on Cp that specifically bind MPO. We propose that inhibition of MPO by Cp requires two nearly identical sites on Cp to bind homodimeric MPO simultaneously and at an angle of at least 120 degrees, which, in turn, exerts tension on MPO and results in conformational change (Fig. 4D). Despite the claims that Cp–MPO interaction has very strong implications in control and resolution of highly destructive enzymatic activity of MPO during infection and inflammation, the physiologic role and *in vivo* impact of this interaction remains unknown. This study uncovers two important tools that can be used to investigate the *in vivo* role of Cp–MPO interaction: the interaction sites on Cp and antibody that disrupts it.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors thank Dr. Nicholas Tripoulas for his guidance. This work was supported in part by National Institutes of Health – United States grants R01 DK083359, P01 HL076491 and P01 HL029582 to P.L.F.

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