Kinetic Investigation of Myeloperoxidase upon Interaction with Copper, Cadmium, and Lead Ions

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ABSTRACT

Background: Myeloperoxidase (MPO), which is abundantly expressed in neutrophils, catalyzes the formation of a number of reactive oxidant species. However, evidence has emerged that MPO-derived oxidants contribute to tissue damage and initiation and propagation of inflammatory diseases, particularly, cardiovascular diseases. Therefore, studying the regulatory mechanisms of the enzyme activity is of great importance. For clarifying some possible mechanism of the enzyme activity, kinetic investigations of MPO in the presence of Copper (Cu), Cadmium (Cd), and Lead (Pb) ions were carried out in vitro. Methods: MPO was partially purified from human white blood cells using ion-exchange and gel-filtration chromatography techniques. Its activity was measured spectrophotometrically by using tetramethyl benzidine (TMB) as substrate. Results: Purified enzyme had a specific activity of 21.7 U/mg protein with a purity index of about 0.71. Cu inhibited MPO activity progressively up to a concentration of 60 mM at which about 80% of inhibition achieved. The inhibition was non-competitive with respect to TMB. An inhibitory constant (Ki) of about 19 mM was calculated from the slope of repot. Cd and Pb did not show any significant inhibitory effect on the enzyme activity. Conclusion: The results of the present study may indicate that there are some places on the enzyme and enzyme-substrate complex for Cu ions. Binding of Cu ions to these places result in conformational changes of the enzyme and thus, enzyme inhibition. This inhibitory effect of Cu on the enzyme activity might be considered as a regulatory mechanism on MPO activity.

Keywords: Myeloperoxidase, Copper (Cu), Enzyme inhibition, Cadmium (Cd), Lead (Pb)

INTRODUCTION

Myeloperoxidase (MPO) is the most abundant protein of the azurophilic granules of neutrophils [1] that is found in macrophages [2], microglia [3], Kupffer cells [4], and neurons [5]. MPO is capable of catalyzing the oxidation of chloride and bromide ions by H₂O₂ generating hypochlorous acid (HOCl) and hypobromous acid (HOBr), respectively [6, 7]. At physiological conditions, HOCl, which is known as a potent oxidant, is the predominant product of the enzyme [8]. The resultant hypohalogenite (HOCl and HOBr) react readily with a wide variety of bimolecular such as proteins [9], lipids [10], DNA [11], and lipoproteins [12, 13]. In addition, increased systemic MPO activity has been associated with the production of various tissue injury and diseases, including atherosclerosis [14], vasculitis [15], stroke [16], cancer [17], Parkinson’s disease [18], Alzheimer’s disease [5] and multiple sclerosis [19]. Therefore, it is plausible to suggest that increased activity of MPO may cause the induction of the disease, and thus the regulation of the enzyme activity is useful in prevention of the disease. It is reported that the activity of MPO is affected by many biologically active agents [20]. Among heavy metals, copper (Cu) is of particular concern as an essential trace element and Cadmium (Cd) and lead (Pb) are two environmentally persistent toxic element which are non-essential in human. Cu ions act as cofactors in many enzymes, such as Cu/Zn superoxide dismutase, cytochrome C oxidase, amino acid oxidase, and laccase [21]. At the cellular level, Cu also plays an essential role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation and iron metabolism [21]. In addition, some enzymes are inhibited by Cu ions. For example, faixova and faix [22] have shown that glutamate dehydrogenase and...
urease activity are inhibited by Cu ions. Chen et al.
[23] also showed that digestive enzymes activities in
the guts of various marine invertebrates are inhibited in
the presence of Cu. Although many physiological
functions of Cu have already been clarified, scientists
are still uncovering the new information regarding the
functions of Cu in the human body.

Cd and Pb, two persistent and common environ-
mental contaminants, affect cell metabolism and
catalytic activity of several enzymes through binding to
thiol groups on protein molecules [24, 25]. Pandya et al.
[25] have studied the effect of Pb and Cd on the
activity of steroid metabolizing enzymes, 17-β
hydroxyl steroid oxidoreductase and uridine
diphosphate glucuronyltransferase, and showed the
inhibitory effects of the metals ions on the enzyme
activities. However, few studies have been carried out
on the effect of heavy metals on MPO activity.

Due to important role of MPO in inducing various
diseases, regulatory mechanisms of the enzyme activity
have attracted much attention. In this regard and for
better understanding of the possible mechanisms by
which these metals affect on the enzyme activity,
changes in the kinetic parameters of MPO upon
interaction with Cu, Cd, and Pb ions was studied.

MATERIALS AND METHODS

Materials. Human blood was obtained from Isfahan
Blood Transfusion Organization (Iran), tetramethyl
benzidine (TMB), H$_2$O$_2$, ammonium sulfate, Cu sulfate
(CuSO$_4$), Cd chloride (CdCl$_2$), Pb acetate
(Pb(CH$_3$COO)$_2$), cetyltrimethylammonium bromide
(CTAB), and Sephadex G150 were obtained from
Sigma Chemical Co. (USA). All other chemicals were
reagent grade.

Methods:

Buffy coat isolation. MPO was purified from normal
human leukocytes using a method described by Morita
et al. [26]. Buffy coat, the principal source of white
blood cells, was separated by centrifugation of citrated
blood at 1000 $\times$ g for 15 min. The layer between plasma
and red cells (buffy coat) was collected and kept for
analysis.

Myeloperoxidase purification. After lysis of the
buffy coat by adding 0.5% CTAB, MPO was isolated
and cell debris was removed by centrifugation at
15,000 $\times$ g at 5°C for 15 min. All subsequent
centrifugation was carried out under these conditions.
The supernatant was treated with solid ammonium
sulfate to yield a final concentration of 50% saturation.
This solution was kept at 4°C for 30 min and then
centrifuged to remove the precipitate. The resulting
supernatant was treated with solid (NH$_4$)$_2$SO$_4$ to
increase the concentration to 65% saturation, and
incubated at 4°C for 30 min prior to centrifugation.
Precipitated MPO was re-dissolved in a buffer
containing 50 mM Tris pH 7.0 and dialyzed against the
same buffer. The dialyate was applied to the first CM-
Sephadex column (4 × 50 cm) equilibrated with the
dialysis buffer to remove the surfactant from the
enzyme solution. The column was washed until the
absorption at 280 nm returned to the baseline and then
the enzyme was eluted with 0.3 M dipotassium
hydrogen phosphate solution. The enzyme fractions
were then applied to the second CM-Sephadex column
(4 × 50 cm) with a linear gradient of 0.05 M to 0.3 M
dipotassium hydrogen phosphate solution. The MPO
fractions were then subjected to gel filtration on a
column (2.5 × 100 cm) of Sephacryl S-300.

Enzyme assay. MPO activity was measured by the
method reported by Suzuki et al. [27]. Then, 0.88 mM
TMB, 1 mM of H$_2$O$_2$ and 50 µl of enzyme solution
were added to 1 ml of reaction mixture containing 50
mM acetate buffer to initiate the reaction. The
absorbance was recorded at 655 nm during 3 min using
a Perkin-Elmer 515 UV/VIS spectrophotometer.
Protein concentration was determined by the Lowry
[28] procedure with bovine serum albumin as standard.

Kinetic studies. Kinetic investigations of human
leukocyte-derived MPO was performed by adding
different concentrations of the metal ions to the assay
mixture and incubating at 25°C for 10 min. Lineweaver-Burk
plot and the corresponding replot were drawn using linear regression analysis.

Statistical analysis. Student’s $t$-test was used for the
statistical analysis. Data are expressed as mean ± SD.
P<0.05 was considered statistically significant.

RESULTS

Results of this investigation showed that MPO from
whole human blood was purified with a purity index of
about 0.71 and had a specific activity of 21.7 U/mg
protein. It was also shown that Cu inhibited human
leukocytes MPO activity progressively up to 60 mM
where 80% inhibition was achieved 10 min after
addition of Cu ions (Fig. 1). Lineweaver-Burk
reciprocal plot of MPO in the presence of two fixed
concentrations of Cu is shown in Figure 2. It can be
seen that the mode of inhibition is non-competitive,
meaning that Cu could probably bind either to the free
form of the enzyme or to the enzyme-substrate
complex, producing binary and ternary complexes
respectively. An inhibitory constant (Ki) of 19 mM

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Fig. 1. Concentration-dependent inactivation of myeloperoxidase by Cu. Copper ions were added to the assay mixture and after 10-minute incubation at room temperature, the enzyme activity was measured. Values represent mean ± SD of three independent experiments.

was calculated from the slope replot (Fig. 3). When MPO was incubated in the presence of different concentrations of Cd or Pb, no significant changes were seen between MPO activity in the absence or presence of these metals ions (Fig. 4).

DISCUSSION

The mechanisms underlying MPO oxidizing activity is a complex process thus, kinetic investigations about its behavior in the presence of various ligands may help to clarify this mechanism. One of the most common groups of ligands which are used in kinetic investigations of enzymes is heavy metals. Among these metals Cu, Pb, and Cd are concerns of many investigators due to their widespread distributions. Cu, is known to be an integral component of several enzymes, and an essential trace element in human, while Cd and Pb seem to be non-essential elements and highly toxic for general metabolism.

The present study was carried out on human MPO in the presence of Cu, Cd, and Pb to clarify kinetic behaviors. It was shown that interaction of Cu with human MPO led to a reversible inhibition of the enzyme activity. Cu inhibited the enzyme activity in a dose-dependent manner up to 60 mM concentrations where about 80% inhibition achieved. Lineweaver-Burk plot of the kinetic data showed that the mode of inhibition in the presence of Cu is non-competitive. In the other hand, Cu binds to both the enzyme and enzyme-substrate complex, produces conformational changes close to the enzyme active site of MPO and impedes the binding of substrates to the active center of the MPO, and therefore enzyme inactivation. Different mechanisms have been reported for the influences of Cu on various enzymes activity. For example, Mengyao et al. [29] have reported that S-adenosylhomocysteine hydrolase activity is inhibited in the presence of Cu ions and the mode of inhibition was non-competitive. They showed that binding of Cu ions to this enzyme resulted in the release of NAD$^+$ cofactors, explaining the less of the enzyme activity [29]. Tormanen [30] has studied inhibition of rat liver and kidney arginase by Cu ions and has shown that the kinetics of the inhibition was non-linear allosteric.

Lisa [31] has studied directly the kinetic and equilibrium effects of Cu$^{2+}$ on the enzyme activity and showed that at equilibrium, binding of two to three Cu$^{2+}$ stoichiometrically to the enzyme resulted in enzyme inactivation. Hadzi-Taskovic sukloovic et al. [32] has shown that peroxidative and oxidative catalytic functions of class III peroxidase were inhibited by Cu. They showed that this inhibition was reversible, and accompanied by disappearance of some and appearance of new isoforms of the enzyme.

Sokolov et al. [33] have shown that ceruloplasmin, a Cu containing metalloenzyme which is synthesized in liver and carries approximately 95% of total plasma Cu, inhibits MPO activity. Kinetic studies have shown that ceruloplasmin behaves as a competitive inhibitor of MPO [33]. In the other hand, ceruloplasmin binds to the active site of the MPO and impedes the binding of substrates to the active center of MPO, and therefore enzyme inactivation. It is very interesting that ceruloplasmin and MPO in complex affect the enzymatic activity of each other [33]. Although Cu ions inhibited MPO activity, Cd and Pb could not do so. There are reports indicating that Cd and Pb ions can
bind to enzymes having functional thiol groups, and effectively inactivate them. Thus, the presence of thiol groups in MPO which are able to bind to Cd or Pb seems to be far from the active site of the enzyme and this explains why the activity of this enzyme is not very much affected. Therefore, conformational changes upon Cd and Pb binding to the enzyme are not able to change active site of the enzyme that impedes the substrate binding to the active center of enzyme.

As mentioned before, MPO is abundantly expressed in neutrophils and, to a lesser extent, in monocytes and certain type of macrophages. Elevated serum activity of MPO has been reported in many inflammatory diseases, particularly cardiovascular and neurological diseases [34]. Therefore, therapies capable of suppressing MPO activity may be useful in controlling diseases associated with elevated MPO levels. Although many metals are involved in changes in activity of many enzymes, little is understood about their effect on MPO activity. As a result, it is plausible to investigate the MPO kinetic in the presence of the metal ions to clarify the mechanisms by which these affects are brought about.

According to the results presented in our study, there may be some places both on the free enzyme molecules and enzyme-substrate complex for Cu ions. Binding of the metal ions to these places may induce conformational changes in the enzyme molecule, leading to reversible enzyme inhibition. Due to the inhibition of the enzyme in the presence of Cu ions and also inhibition of the enzyme in the presence of ceruloplasmin, a ferroxidase that contains greater than 95% of plasma Cu, it is suggested that Cu may be a biological regulator of activated neutrophils. Moreover, it may be proposed that Cu ions bound to the ceruloplasmin molecules may be the cause of the inhibitory effect of ceruloplasmin on MPO activity.

![Fig. 3. Replot of inverse slope of the Lineweaver-Burk plot vs. Cu concentration. Values were obtained from Figure 2.](http://IBJ.pasteur.ac.ir)

![Fig. 4. The effect of different cadmium (A) and lead (B) concentrations on myeloperoxidase activity. Cadmium chloride and lead acetate were added to the assay mixture and incubated at 25°C for 10 min. The enzyme activity was measured as described in Methods at intervals of 15 min for 180 min.](http://IBJ.pasteur.ac.ir)

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**REFERENCES**

