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Kinetic studies of the reaction of heme-thiolate enzyme chloroperoxidase with peroxynitrite

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Abstract

The kinetics of the reaction of chloroperoxidase with peroxynitrite was studied under neutral and acidic pH by stopped-flow spectrophotometry. Chloroperoxidase catalyzed peroxynitrite decay with the rate constant, k_c, increasing with decreasing pH. The values of k_c obtained at pH 5.1, 6.1 and 7.1 were equal to: $(1.96 \pm 0.03) \times 10^6$, $(1.63 \pm 0.04) \times 10^6$ and $(0.71 \pm 0.01) \times 10^6\ M^{-1}\ s^{-1}$, respectively. Chloroperoxidase was converted to compound II by peroxynitrite with pH-dependent rate constants: $(12.3 \pm 0.4) \times 10^6$ and $(3.8 \pm 0.3) \times 10^6\ M^{-1}\ s^{-1}$ at pH 5.1 and 7.1, respectively. After most of peroxynitrite had disappeared, the conversion of compound II into the ferric form of chloroperoxidase was observed. The recovery of the native enzyme was completed within 1 s and 5 s at pH 5.1 and 7.1, respectively. The possible reaction mechanisms of the catalytic decomposition of peroxynitrite by chloroperoxidase are discussed.

Keywords: Chloroperoxidase; Peroxynitrite; Stopped-flow spectrophotometry

1. Introduction

Chloroperoxidase (CPO), isolated from the fungus *Caldariomyces fumago*, is the most versatile and unusual among the heme enzymes. CPO possesses halogenating (except fluorination reactions), peroxidase, catalase and monooxygenase activities [1]. Very recently, also dehalogenase activity of CPO has been reported [2]. This enzyme is active in the broad pH range from 2.0 to 7.5 [3,4]. CPO differs from other heme peroxidases and catalases as it possesses a proximal cysteine thiolate heme iron ligand [5,6]. A similar heme-thiolate prosthetic group has been found in enzymes with very different functions such as cytochromes P450 or nitric oxide synthases [7,8]. CPO is also unique in the peroxidase family because it uses glutamate (Glu183) residue as an acid–base catalyst in the reaction with hydrogen peroxide [6,9]. In other peroxidases this role is played by histidine residue.

Peroxynitrite ($ONOO^-$/ONOOH), a highly reactive and biologically important species is formed in the diffusion-controlled reaction of superoxide anion ($O_2^{\cdot-}$) and nitric oxide ($^{\cdot}NO$). The peroxynitrite anion is relatively stable in alkaline solution, but under physiological conditions undergoes protonation (pK 6.8). Peroxynitrous acid rapidly isomerizes to nitrate, partially (about 30%) via intermediate radical products ($^{\cdot}OH$ and $^{\cdot}NO_2$), [10–14].

Under physiological conditions peroxynitrite reacts with almost all classes of biologically important molecules as a strong oxidizing and nitrating agent [[15–17] and references therein]. Several heme proteins reacts rapidly with peroxynitrite [[18] and references therein], some of them catalyze its decomposition [19–23]. It has been shown that some heme-thiolate enzymes (prostacyclin synthase, nitric oxide synthase, cytochromes: $P450_{BM-3}$, P450 2B1) are inactivated already in the presence of peroxynitrite at micromolar or even submicromolar concentration [24–27]. Destruction of the heme-thiolate catalytic site and/or nitration of tyrosine located in a close neighbourhood of the heme are the processes responsible for the inactivation of the enzymes mentioned above. Contrary to such

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behaviour, other heme-thiolate enzymes catalytically accelerate the decay of peroxynitrite at alkaline pH [19,28], while at neutral pH this effect has been reported only for CPO [27]. Heme-thiolate enzymes also efficiently catalyze nitration of phenolic compounds by peroxynitrite [19,28,29]. It has been suggested that high reactivity of heme-thiolate enzymes with peroxynitrite is connected with the presence of thiolate ligand in the active site [19,28,30].

In this work we investigated reaction kinetics of chloroperoxidase with peroxynitrite. We discussed the results obtained in this work with those reported for heme enzymes lacking a thiolate ligand in the active site.

2. Materials and methods

Chloroperoxidase from *Caldariomyces fumago* was purchased from Sigma Chemical Co. (St. Louis, MO). The concentration of CPO was determined spectrophotometrically at 400 nm using $\varepsilon_{400} = 9.1 \times 10^4\ M^{-1}\ cm^{-1}$ [31]. The purity number values, $R_z = A_{400}/A_{280}$, of the enzyme used by us were in the range 1.24–1.31.

Peroxynitrite was synthesized either by the ozonation of azide [32] or by the reaction of nitrite with hydrogen peroxide under acidic conditions [33]. In the first method, to reduce the content of unreacted N_3^-, ozonolysis was carried out for additional 15 min after reaching the maximum concentration of $ONOO^-$ [34]. The concentration of azide in the peroxynitrite solutions was determined by the methemoglobin method [34]. It never exceeded 1% relative to peroxynitrite. In the second method, unreacted hydrogen peroxide was removed from peroxynitrite solution by treatment with solid manganese dioxide. In both methods nitrite remained as a contaminant. The concentration of nitrite in the peroxynitrite solutions obtained by both methods was measured by the Griess method [36] after peroxynitrite isomerization in an ice-cooled 25 mM phosphoric acid solution. Under these conditions, peroxynitrite exclusively isomerizes to nitrate. Nitrite contamination varied from 10 to 50% relative to peroxynitrite concentration.

The final concentration of peroxynitrite was equal to 25–50 mM as determined spectrophotometrically using extinction coefficient $\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$ [35]. The stock solutions of $ONOO^-$ were stored at −25 °C and used within 3–4 weeks after synthesis.

The reactions of peroxynitrite with CPO were studied at pH ranged from 5.1 to 7.1. The SX-17 MV Applied Photophysic stopped-flow spectrophotometer with 1-cm cell and with a mixing time <1 ms was used throughout the study. CPO was dissolved in 0.1 M acetate, or 0.1 M phosphate buffers. Acetate is known to bind to CPO, but with low affinity [37]. The optical spectrum of the enzyme dissolved in acetate buffer was indistinguishable from that measured in phosphate buffer. Peroxynitrite solutions were brought to the desired concentration by dilution the stock solution with 0.01 M NaOH. Equal volumes of solutions of peroxynitrite and enzyme were mixed in the cell and the reaction kinetics was followed at selected wavelengths between 265 and 450 nm. The concentration of peroxynitrite exceeded at least tenfold the enzyme concentration. The decay of peroxynitrite was monitored at 265 nm (pH 5.1) or at 302 nm (pH > 6.0).

In order to determine the amount of nitrite formed from peroxynitrite decay, peroxynitrite was allowed to decompose at the desired pH for 5 min, and total nitrite concentration in the sample was measured by the Griess method. The concentration of nitrite formed from peroxynitrite decay was calculated by subtraction from the amount of total nitrite found in the experiment at given pH, the amount of nitrite found as a contamination of peroxynitrite sample.

The activity of CPO after reaction with peroxynitrite was assayed as follows: enzyme at concentration of 1 μM was incubated at pH 5.1 or 7.1 with different amounts (30–300 μM) of peroxynitrite for 5 min. Then the samples were diluted 100-fold and the activity was measured by the guaiacol method [38]. Prior to activity measurements, CPO solution was passed through a column containing Sephadex G25.

All spectrophotometric measurements were carried out at 23 °C, at least in triplicate. Nano-pure water from MilliQ (Millipore) was used throughout.

3. Results and discussion

Peroxynitrite may inactivate enzymes by modifying their critical amino acid residues or prosthetic group [18]. We found that CPO retained full activity and unaltered absorption spectrum after incubation with peroxynitrite at pH 7.1. This result means that under above conditions peroxynitrite neither modifies the amino acid residues important for the catalytic activity of CPO nor damages the heme-thiolate active site. We, however, observed about 20% activity loss after the incubation of CPO with 50 μM of peroxynitrite (synthesized by both methods) at pH 5.1. Further increase of peroxynitrite concentration did not cause additional decrease of activity of CPO. It is worth noting that absorption spectrum of CPO at pH 5.1 after peroxynitrite treatment corresponded exactly to that of an untreated sample. It has been reported recently that 1.43 (per 11) tyrosine residues in chloroperoxidase are nitrated by peroxynitrite present in the 40-fold excess relative to the enzyme at pH 7.4 [29]. As the rate constant of the reaction between CPO and peroxynitrite increases with decreasing pH (see below), probably more tyrosine residues in CPO molecule is nitrated by peroxynitrite at acidic pH. Enhanced nitration may lead to the partial inactivation of the enzyme.

It should be noted that contaminants of peroxynitrite solution, azide as well as nitrite, bind to the heme iron. Dissociation constants of the CPO-complexes in the investigated pH range (5.1–7.1) varied from 0.6 to 43 mM for azide and from 4.2 to 415 mM for nitrite [37]. Taking into account the concentrations of N_3^- and/or NO_2^- in peroxynitrite samples one can conclude that under our experimental