Catalase Catalyzes of Peroxynitrite-mediated Phenolic Nitration

Yasuhiro Kono,1 Tomoaki Yamasaki, Akane Ueda, and Hitoshi Shibata

Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690, Japan

Received July 29, 1997

Catalase catalyzed the peroxynitrite-mediated nitration of 4-hydroxyphenylacetic acid. The curve for the pH dependence of nitration was similar to that for the reaction between peroxynitrite and phenol. Cyanide, azide, and 3-amino-1,2,4-triazole inhibited the nitration in a dose-dependent way. When catalase was mixed with peroxynitrite, Compound I was detected as an intermediate. Because azide was an electron donor for the peroxidatic action of catalase, and because 3-amino-1,2,4-triazole inhibited catalase activity by binding with Compound I, peroxynitrite-mediated phenolic nitration was probably accompanied by Compound I formation. Both catalase and superoxide dismutase protected Escherichia coli from peroxynitrite toxicity.

Key words: bacterial killing; catalase; peroxynitrite; phenolic nitration; superoxide dismutase

Nitric oxide (NO•) reacts with superoxide (O2•−) to give peroxo-nitrite (ONOO•−) at the rate constant of 6.7 × 109 M−1 s−1.1

O2•− + NO• → ONOO•−. (1)

Peroxynitrous acid (ONOOH) is formed by the reaction between H2O2 and HNO2 at a mildly acidic pH.2−3 ONOO•− and/or ONOOH induces lipid peroxidation, oxidizes Met and Cys residues in proteins, causes the nitration of Tyr residues in proteins, depletes antioxidants, and damages DNA.2−3 Peroxynitrite also is cytotoxic by promoting elastase activity by inactivating its inhibitor α1-antiproteinase,4,5 so the generation of peroxynitrite in vivo can worsen both oxidative and proteolytic damages.3,4,5

Nitrination of Tyr interferes with signal transduction involving phosphorylation and de-phosphorylation, and nitrated protein is the target for degradation.6−13 As the nitration of phenolic moieties by peroxynitrite occurs spontaneously but can be catalyzed by transition metals,10 superoxide dismutase (SOD)12,13 peroxidases,14,15 and proteins containing copper,16 these results prompted us to investigate the reaction between peroxynitrite and catalase. The participation of Compound I of catalase in the phenolic nitration, and the protection by catalase and SOD against bactericidal action of peroxynitrite are reported here.

Materials and Methods

Materials. Bovine liver catalase, and Cu/Zn-SOD, horseradish peroxidase (HRP), bovine erythrocyte hemoglobin, and bovine skeletal myoglobin were purchased from Sigma. Horse heart cytochrome c and 3-amino-1,2,4-triazole (3-AT) were obtained from Wako Pure Chemical Industries. 4-Hydroxyphenylacetic acid (4-HPA) was purchased from Nakalai Tesque. Peroxynitrite was prepared by the autoxidation of hydroxylamine at alkaline pH:2,11 10 mM NH2OH was autoxidized in 1 mM EDTA and 0.5 mM NaOH for 3 h with continuous bubbling under air, or was synthesized in a quenched-flow reactor from sodium nitrite and H2O2:16,17 a 10-ml solution of 0.6 mM NaNO2 was rapidly mixed with 10 ml of 0.6 mM HCl containing 0.7 M H2O2 and then the mixture was quenched in 20 ml of 1.5 M NaOH while being stirred rapidly. MnO2 was used to remove any residual H2O2 after the quenching. The solution was freeze-fractionated. The concentration of peroxynitrite was assayed spectrophotometrically with ε = 1,670 M−1 cm−1.11,17,18 All other chemicals were of reagent grade.

Assay of nitration. Phenolic nitration was started by the addition of 2 mM peroxynitrite to a reaction mixture that contained 50 mM potassium phosphate, pH 7.4, 0.1 mM diethylenetriaminepentaaetic acid (DPA), 0.5 mM 4-HPA, and 0.2 mg/ml catalase. Changes in the absorbance at 450 nm were monitored and the formation of 3-nitro-HPA (NO2-HPA) was calculated with ε = 2,440 M−1 cm−1 at pH 7.4.11 When the effects of pH on the yield of NO2-HPA were being tested, the reaction was started by the addition of 2 mM peroxynitrite to a reaction mixture that contained 50 mM potassium phosphate at pH 4.2 to 9.0, 0.1 mM DPA, 0.5 mM 4-HPA, and 0.2 mg/ml catalase. After the reaction at 37°C for 30 min, the pH of the reaction mixture was adjusted to 10.0–10.6 with 1 M NaOH. The formation of NO2-HPA was calculated with ε = 4,400 M−1 cm−1.18

Microorganism and assessment of bacterial killing.19 Escherichia coli NIHJ JC-2 (National Institute of Health, Tokyo, Japan) was used as the test organism. Lyophilized cultures were subcultured onto multiple nutrient agar slants composed of (in grams per liter) beef extract, 5; polypeptone, 10; NaCl, 5; and agar, 15. The slants were stored at −70°C until being used. Cultures were grown at 37°C in M9 minimal medium (42 mM Na2HPO4, 22 mM KH2PO4, 12.5 mM NaCl, 18.5 mM NH4Cl, 0.1 mM CaCl2, and 2 mM MgSO4, pH 7.4) supplemented with glucose (2 g/l). The cells in the late log

1 To whom correspondence should be addressed (Tel. 0852-32-6580; Fax. 0852-32-6598; e-mail, ykono@life.shimane-u.ac.jp).
phase were washed twice with physiological saline, and then incubated at 37°C for 30 min in a medium (1 ml), containing various compounds; see figure legends. Peroxynitrite and heat-sensitive enzymes were sterilized by filtering through a filter (0.45 μm). Viable cells were diluted with sterile 0.9% NaCl, plated onto nutrient agar, and incubated at 37°C for 18 h before the colonies were counted.

Results

Peroxynitrite-mediated nitration of 4-HPA catalyzed by catalase

In the assay of nitration of 4-HPA, absorbance at 430 nm, where NO₂-HPA absorbs, increased when catalase was added to a mixture containing peroxynitrite (Fig. 1A), and when peroxynitrite was added to a solution containing catalase (Fig. 1B). After these reactions were completed, added dithionite, which reduces nitro groups to colorless amines, caused a decrease in the increased absorbance (data not shown). However, with heat-denatured catalase, absorbance did not increased (Fig. 1C). Absorbance was maximum at about 5 min, but when more peroxynitrite was added, absorbance increased again (Fig. 1D). When catalase was added about 8 min after a first addition had been made, absorbance increased only slightly (Fig. 1E). These results indicated that the nitration of 4-HPA to NO₂-HPA needed both catalase and peroxynitrite. The hemoproteins, hemoglobin, myoglobin, and cytochrome c did not catalyze the nitration, but HRP did. The rates of nitration of 4-HPA by HRP and catalase were 37.0 and 12.4 nmol/mg protein-min, respectively.

Effects of pH on the nitration of 4-HPA

The pH optimum for catalase-catalyzed nitration of 4-HPA was about pH 7 (Fig. 2). The pH profile, increased with increasing pH and decreased at pH higher than 7, was similar to that for the reaction between peroxynitrite and phenol. Only 6.9% of added peroxynitrite was recovered as NO₂-HPA with 0.2 mg/ml catalase at pH 7, but 9.0% is reported to be recovered with 10 μM SOD at pH 7.4.

Effects of concentrations of peroxynitrite, 4-HPA, and catalase on the nitration of 4-HPA

The nitration rate increased in direct proportion to the initial concentration of peroxynitrite (Fig. 3A). However, the nitration rates plotted against the initial concentrations of 4-HPA (Fig. 3B) or of catalase (Fig. 3C) were in form of a saturation curve with 50% of maximum at 0.8 mM 4-HPA and about 1.1 μM catalase, respectively.

Fig. 1. Changes with Time in Peroxynitrite-mediated Phenolic Nitration Catalyzed by Catalase.

At the times indicated by arrows, native or heat-denatured catalase (each 0.2 ngs), and peroxynitrite (final concentration, 2 mM) were added to a reaction mixture (1 ml) containing 50 mM potassium phosphate, pH 7.4, 0.1 mM DPA, and 0.5 mM 4-HPA.

Fig. 2. Effects of pH on Peroxynitrite-mediated Nitration of 4-HPA by Catalase.

The reaction mixture (1 ml) contained 50 mM potassium phosphate at the indicated pH, 0.1 mM DPC, 0.5 mM 4-HPA, and 0.2 mg catalase.
Fig. 3. Effects of Initial Concentrations of Peroxynitrite (A), 4-HPA (B), and Catalase (C) on the Peroxynitrite-mediated Nitration.

The basal reaction mixture (1 ml) contained 50 mm potassium phosphate, pH 7.4, 0.1 mm DPA, 0.5 mm 4-HPA (A and C), 2 mm peroxynitrite (B and C), and catalase 0.2 mg/ml (A and B). Peroxynitrite (A), 4-HPA (B), or catalase (C) were added at the indicated concentrations.

Effects of catalase inhibitors on the nitration of 4-HPA

In a check of the participation of the heme moiety of catalase in the phenolic nitrination, the effects of inhibitors for catalase were studied (Table I). Three kinds of inhibitors, cyanide, 3-AT, and azide inhibited catalase-mediated nitrination, in a concentration-dependent way. 3-AT which irreversibly inhibits catalase by binding its Compound I, inhibited the nitrination in a concentration-dependent way, suggesting the participation of Compound I in the nitrination. Azide also inhibited the reaction in a concentration-dependent way, suggesting that azide competes with the electron donor for Compound I and/or Compound II of catalase. From these results, it is likely that 4-HPA could be nitrated by the one-electron oxidation/reduction mechanism involving Compounds I and II.

Changes in catalase spectrum with peroxynitrite

The addition of peroxynitrite to a solution of catalase in 50 mm potassium phosphate, pH 7.4 resulted in a rapid but measurable decrease in Soret absorbance at 405 nm (Fig. 4). But absorption of Soret band did not shift. A new band near 655 nm appeared after 5 min of reaction. Figure 5 shows changes in absorbance at 405 nm after the addition of peroxynitrite, and the further addition of 4-HPA. The addition of peroxynitrite to the buffered solution of catalase caused a decrease in the absorbance. After the reaction was almost completed, further addition of 4-HPA to the mixture caused a linear increase in absorbance at 405 nm, showing that 4-HPA reverted the form of catalase (Compound I) being changed by the addition of peroxynitrite to the native form of catalase. An almost full restoration was observed when catalase was reacted with 1 mm peroxynitrite and thereafter with 0.5 mm 4-HPA (Fig. 5A), but 75% of restoration was obtained when catalase was incubated firstly with 2 mm peroxynitrite (Fig. 5B), probably because catalase was inactivated with 2 mm peroxynitrite.

Bactericidal activities of peroxynitrite after the reaction with catalase or SOD

Bactericidal effects of peroxynitrite after the reaction with catalase or SOD were compared. After the pre-reaction of peroxynitrite with SOD or catalase, the activities

<table>
<thead>
<tr>
<th>Addition</th>
<th>Nitration (nmol/min)</th>
<th>Relative value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.5</td>
<td>100</td>
</tr>
<tr>
<td>NaCN</td>
<td>1 mm</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>5 mm</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>10 mm</td>
<td>0.1</td>
</tr>
<tr>
<td>3-AT</td>
<td>10 mm</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>50 mm</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>100 mm</td>
<td>0</td>
</tr>
<tr>
<td>NaN₃</td>
<td>1 mm</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>5 mm</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>10 mm</td>
<td>0</td>
</tr>
</tbody>
</table>

Table I. Effects of Inhibitors for Catalase on the Phenolic Nitrination Mediated by Peroxynitrite.

The reaction mixture (1 ml) contained 50 mm potassium phosphate, pH 7.4, 0.1 mm DPA, 0.2 mg catalase, 0.5 mm 4-HPA, and an inhibitor at the concentrations indicated.

Fig. 4. Changes in Catalase Spectrum by the Addition of Peroxynitrite.

Catalase (2 mg/ml) was incubated with 1 mm peroxynitrite in 50 mm potassium phosphate, pH 7.4, and 0.1 mm DPC. Curve 1 is the spectrum of native catalase before the reaction. Curve 2 is the spectrum measured at 5 min after peroxynitrite was added.
of bacterial killing by peroxynitrite were decreased as the enzyme concentrations added to the pre-reaction mixtures increased. Of all concentrations tried, the protective effect was maximum (36% and 46%) with 200 μM SOD or 70 μM catalase, respectively.

Discussion

Although Floris et al. did not observe the interaction of the heme-containing catalase with peroxynitrite, we showed here peroxynitrite-mediated nitration of HPA by catalase. There are three enzyme-substrate com-

pounds of catalase. Compounds I, II, and III, where only Compound I or both Compounds I and II participate in catalytic peroxidative reactions of catalase. The absorption peaks of Compound I are 405 and 655 nm, whereas those of Compound II are 429, 536, and 568 nm. It is possible that catalase was converted to an intermediate, Compound I, during the reaction with peroxynitrite. This was supported by the observations for characteristic spectrum of Compound I, and also by inhibitions by 3-AT, and azide. Recently, van Zyl and van der Walt reported the peroxynitrite-mediated Tyr nitration by catalase, and proposed that peroxynitrite oxidizes univalently catalase to form its ferryl state, Compound II and NO2·. Compound II can easily oxidize phenolics to phenoxyl radical which can react with NO2· to give nitrated phenolics.

We now propose an alternative mechanism. Because peroxynitrite is an one- and two-electron oxidant, it could bind and oxidize catalase to Compound I, releasing NO2−, that is a good electron donor for Compounds I and II. Compounds I and II can readily oxidize 4-HPA to form phenoxy radicals of 4-HPA (4-HPA·). Nitrite reduces bivalently Compound I and univalently Compound II to ferric catalase at the rates of 1.4 × 105 and 1.5 × 109 M−1 s−1, respectively. The bivalent oxidation of nitrite by Compound I and the univalent oxidation by Compound II produce NO3− and NO2−, respectively. NO2· and 4-HPA· then react each other to give NO3− and 4-HPA.

\begin{align*}
\text{Catalase} + \text{ONOO}^- \rightarrow \text{Compound I} + \text{NO}_2^-
\end{align*}

(2)

\begin{align*}
\text{Compound I} + \text{NO}_2^- \rightarrow \text{Catalase} + \text{NO}_3^-
\end{align*}

(3)

\begin{align*}
\text{Compound I} + 4\text{-HPA} \rightarrow \text{Compound II} + 4\text{-HPA}·
\end{align*}

(4)

\begin{align*}
\text{Compound II} + \text{NO}_2^- \rightarrow \text{Catalase} + \text{NO}_3^-
\end{align*}

(5)

\begin{align*}
\text{Compound II} + 4\text{-HPA} \rightarrow \text{Catalase} + 4\text{-HPA}·
\end{align*}

(6)

\begin{align*}
\text{NO}_2· + 4\text{-HPA} \rightarrow \text{NO}_3^-, 4\text{-HPA}.
\end{align*}

(7)

Peroxynitrite preparation could contaminate NO3− and NO2−. As reported by van Zyl and van der Walt, NO3− protects catalase from destruction of heme portion by peroxynitrite. NO3− in reaction mixture could protect catalase by acting as a substrate.

The maximal nitration rates between peroxynitrite and phenol are observed near pH 6.8, in which the yield decreased rapidly above pH 7, but slowly below pH 7, and increased at pH lower than 4.5. This pH profile was similar to that for the reaction of catalase mediated nitration, in which the optimum pH was about 7. The apparent pKs of trans-peroxynitrite is about 7.9. OnOOH/ONOO− can exist as two conformers. The cis-form is more stable, less reactive with many molecules, and the only conformer found in alkaline solution. The trans-form appears to be slightly higher in energy than the cis-form, and is more reactive than cis-form, which implies that the rate of trans- to cis-isomerization must be faster than the forward reaction. The cis-conformer has to be protonated to isomerize to trans-peroxynitrosyl acid and then inonize to form trans-peroxynitrite before it reacts with SOD, and decomposes to form nitrate. Thus SOD-catalyzed nitration is
maximal at pH 7.5 and decreases to apparent pKₐ of 6.8 that is pKₐ of cis-peroxynitrous acid. A similar event could occur in the reaction with catalase. As the concentration of catalase increases, phenolic nitration could be limited by the rate of isomerization from cis- to trans-conformer of peroxynitrous acid as well as by competing pathway for peroxynitrite decomposition.

SOD catalyzes the phenolic nitration via the formation of nitronium ion (NO₂⁺) as a nitrating agent as follow.¹²

\[
\begin{align*}
\text{ONOO}^- + \text{Cu}^{2+} \cdot \text{SOD} & \rightarrow \text{ONOO-Cu}^+ \cdot \text{SOD} \quad (8) \\
\text{ONOO-Cu}^+ \cdot \text{SOD} + \text{H}^+ & \rightarrow \text{NO}_2^+ + \text{HO-Cu}^+ \cdot \text{SOD} \quad (9) \\
\text{NO}_2^+ + \text{phenolic} & \rightarrow \text{NO}_2^- + \text{phenolic} + \text{H}^+ \quad (10)
\end{align*}
\]

NO₂⁺ is a strong oxidant with \(E^\circ = 1.6\) V,¹⁹ and SOD reacted more with peroxynitrite to form nitrated phenolics than catalase as described above. SOD showed poor protection against peroxynitrite induced bacterial killing than catalase. These results suggested that the nitrating moieties formed by SOD and catalase-mediated nitratations were not identical because of different reactivity against for bacterial killing.

Peroxynitrite is attracted to the active site of SOD and can reduce active site copper. The donation of a hydrogen ion from one of the His residues involved in binding the copper, initiates the decomposition of peroxynitrite to form NO₂⁻.¹³ It is possible that NO₂⁻ was also involved in catalase-mediated nitration, because the decomposition of peroxynitrite via NO₂⁻ will be thermodynamically favored in the presence of SOD or metal ions.¹³ The fact that Compound 1 of catalase was formed during the reaction with peroxynitrite, suggested that the nitration of 4-HPA by catalase could proceed by a cycle similar to peroxidase, one-electron oxidation/reduction mechanism, thus releasing NO₂⁻, an one-electron oxidation product of NO₂⁻.

According two reaction mechanisms described above, catalase and SOD convert peroxynitrite to NO₂⁻ and NO₂⁻, respectively. These products may be less reactive against biological molecules related to the bacterial killing than peroxynitrite, whose LD₅₀ is 250 µM for E. coli.²⁹ Whereas, peroxynitrite decomposes spontaneously to give more toxic moiety, OH-like oxidant and NO₂⁻, this process is mediated by a high-energy intermediate derived from ONOOH.³⁰ Although NO₂⁻ is known to be toxic because of a potent oxidant (\(E^\circ = 0.9\) V),³⁰ NO₂⁻ cannot, but NO₂⁻ can, react with phenolics. No exact mechanism for bacterial killing is now clear, but NO₂⁻ seemed to be more reactive with biological molecules than NO₂⁻.

References