Labeling of Peroxide-Induced Oxidative Stress Hotspots by Hemin-Catalyzed Tyrosine Click

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Tyrosine radical generation is one of the major factors for hemin/peroxide-induced oxidative stress. A method for trapping tyrosyl radical directly was developed using N-methyl luminol derivative, a tyrosine labeling reagent. N-Methyl luminol derivative selectively forms a covalent bond with a tyrosine residue under the single-electron oxidation condition. This reaction labels oxidative stress hotspots not only at the protein level but also at the level of tyrosine residues undergoing oxidation. Human serum albumin complexed with hemin was labeled at Tyr138, the tyrosine residue closest to the hemin binding site and most strongly subjected to oxidative stress caused by hemin/H$_2$O$_2$. Oxidatively damaged proteins were visualized in protein mixtures.

Key words oxidative stress; hemin; protein labeling; albumin; peroxide

Introduction

Reactive oxygen species (ROS) are essential for normal physiological function and signaling, whereas elevated ROS levels are associated with disease. The production and elimination of ROS are regulated by enzymes and low-molecular-weight redox-active molecules, and the disruption of ROS metabolic homeostasis is involved in various diseases through the functional impairment of cells and cell death. In some cardiac diseases, cells are impaired, necrosis is induced, and extracellular hydrogen peroxide ($H_2O_2$) is released, with estimated local $H_2O_2$ concentrations reaching as high as 1 mM.1 Superoxide dismutase (SOD) and catalase are examples of enzymes that catalyze $H_2O_2$ metabolism in living cells. SOD catalyzes the disproportionation of superoxide ($O_2^−$) into $O_2$ and $H_2O_2$. Cu/Zn-SOD (SOD1) is constantly expressed in mammalian tissues and has the highest turnover rate of all enzymes.3 On the other hand, hemin is a key molecule that amplifies oxidative stress caused by ROS.4,5 Hemin was reported to react with peroxide to form ferryl intermediates, compound I ($Fe^{IV}=O+[PPOX]^{−}$) and compound II ($Fe^{IV}=O+[PPOX]$) as well as compound III ($Fe^{IV}OO+[PPIX]$).6–10 Figure 1 shows the catalytic cycle of hemin that produces compound I as a typical mechanism. Activated hemin oxidizes the tyrosine residue to produce tyrosyl radical species. The highly reactive tyrosyl radical species react immediately, causing cross-linking reactions with surrounding molecules. Tyrosyl radical species are known to produce various structures, including dityrosine, 3,3-dityrosine, hydrotyrosine (L-DOPA), nitration (3-nitrotyrosine), and halotyrosine structures. Antibodies to each of the products have been used in research related to oxidative stress.11–19 However, due to the complexity of the products, it is difficult to quantitatively compare the generated amounts of tyrosyl radical species. In this study, we developed a method for labeling oxidative stress hotspots in protein by utilizing the tyrosine click reaction that is activated by the conditions for tyrosyl radical formation.

We previously developed the tyrosine click reaction, which involves covalent bond formation between tyrosine labeling reagent and tyrosine residue under the conditions for single electron oxidation, such as hemin/H$_2$O$_2$ condition.20–22 The tyrosine labeling reagent N-methyl luminol derivative radicalizes at the same oxidation potential as the tyrosine residue and oxidatively forms a covalent bond with the tyrosine residue (Fig. 1). Therefore, using N-methyl luminol derivative, it is possible to label the activation site of the oxidative radical reaction, “oxidative stress hotspot.” Due to the high reactive property of the radical species, the tyrosine click can only proceed in nanometer-scale proximity to hemin and can selectively label oxidative stress hotspots. In this study, we developed a method to identify the oxidative stress hotspots in albumin under the hemin activation condition, and applied it to the identification...
of proteins undergoing oxidative stress in protein mixtures.

Results and Discussion

Labeling Oxidative Stress Hotspots in Albumin  Albumin is known to bind to hemin, and their complex is called methemalbumin. Such binding is reported to be involved in the “detoxification” of hemin-induced oxidative stress in severe intravascular hemolysis.\(^24\) Trapping hemin radical species by tyrosine(s) in albumin is important for the protective effect of albumin against hemin-induced damage.\(^25\) Therefore, the detection of tyrosyl radicals on methemalbumin is important for the visualization of oxidative stress hotspots in methemalbumin.

We previously reported the labeling of bovine serum albumin (BSA) using hemin and \(N\)-methyl luminol derivative. At that time, we used 1 mM hemin (100 equivalents) against 10 \(\mu\)M BSA, and found that the most exposed Tyr400 was labeled most efficiently.\(^20\) In the present study, 10 \(\mu\)M hemin (1 equivalent) was added to 10 \(\mu\)M human serum albumin (HSA), and the resulting methemalbumin was used as the substrate for the reaction. Methemalbumin was modified with azide-conjugated \(N\)-methyl luminol derivative (1), and this was followed by a Cu-free click reaction with dibenzocyclooctyne (DBCO)-conjugated cyanine dye (DBCO-Cy3) to obtain Cy3-labeled methemalbumin. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), labeled protein was digested in gel by trypsin and peptide fragments were analyzed by LC-MS (Fig. 2A). Cy3-labeled peptide fragments were quantified by measuring the absorbance at 550 nm on a LC-MS detector. The results showed that the labeling reaction proceeded relatively selectively at specific sites when 30–100 \(\mu\)M 1 was used (Fig. 2B). The detected \(m/\z\) of the peptide fragment matched that of labeled Tyr138-Arg144 containing Tyr138 by electrospray ionization-time-of-flight (ESI-TOF) MS (Fig. 2C). Although this peptide contains two tyrosine residues (Tyr138 and Tyr140) on the sequence, MS/MS analysis identified the labeling site as Tyr138 (data not shown). When concentrations higher than 300 \(\mu\)M of 1 were used, selectivity was reduced and various labeled fragments were detected. It is considered that when a low concentration of 1 was used, the region where the oxidative stress was most remarkable (Tyr138) would be selectively labeled. Even though the concentration of 1 was increased beyond 300 \(\mu\)M, because
the labeling reaction with Tyr138 was saturated, the labeling reaction at other sites proceeded, resulting in decreased selectivity (Fig. 2B).

As regards the three-dimensional (3D) structure of methemalbumin, the hemin-Fe atom is coordinated by Tyr161 to form a five-coordinated species. Tyr138 is the closest tyrosine residue to Fe atom, with the exception of Tyr161, the hemin-coordinated tyrosine26) (Fig. 3). The distance between the Fe atom of hemin and the oxygen atom of Tyr138 is 0.8 nm, which is sufficient for direct electron transfer to take place.27) Therefore, Tyr138 seems to be the most likely residue to become a tyrosyl radical, and the results in Fig. 2 are reasonable.

We also detected peptide fragments labeled with I by matrix-assisted laser desorption/ionization (MALDI)-TOF MS to identify labeling sites other than Tyr138 (Fig. 4). Peaks corresponding to labeled peptide fragments 146–160, 324–336, and 337–348 were detected. However, their exact tyrosine residues could not be identified due to the low labeling efficiencies or the low ionization efficiencies. Peptide fragment 146–160 contains Tyr148 and Tyr150, and these residues are relatively close in distance to hemin. The distances between the Fe atom of hemin and the oxygen atoms of Tyr148 and Tyr150 are 2.1 nm and 1.9 nm, respectively. Fragment 337–348 includes Tyr341, and fragment 324–336 includes Tyr332 and Tyr334. Interestingly, it is unclear why these residues were preferentially labeled over other tyrosine residues because these labeled tyrosines are located behind the hemin-binding...
site and buried in the protein structure. The labeling of these residues might involve a specific radical generation mechanism, such as intramolecular long-range electron transfer. 28) As there are few examples of electron transfer analysis for each albumin residue, this method, which can trap tyrosyl radicals and analyze them at the residue level, could be useful.

Labeling Oxidative Stress Hotspots in Fetal Bovine Serum (FBS) Next, we detected the labeled proteins when hemin and H₂O₂ were added to bovine serum. Bovine serum contains various proteins, and BSA is the main component protein (Fig. 5, Coomassie brilliant blue (CBB) stain). We performed the labeling in the absence or presence of hemin (0–300 µM). The results showed that albumin (65 kDa) was selectively labeled when hemin was added (Fig. 5, fluorescence). This could be attributed to the fact that albumin binds to hemin and protects biological components in the serum from hemin/H₂O₂-induced oxidative stress.

Labeling Using Hemin and Alkyl Peroxides We next applied this method to mouse liver lysates in order to detect oxidative stress hotspots in a more complex protein-mixing system. A demonstration experiment in which BSA was added to mouse liver lysate showed that catalase in liver completely quenched H₂O₂ and inhibited the labeling (Fig. 6B, lane 3). When H₂O₂ was added to the mixture, instantaneous bubbling of O₂ was observed, indicating that H₂O₂ is degraded by catalase before the reaction with hemin because catalase has the highest turnover rate of all enzymes, 10⁷ M⁻¹ s⁻¹. 3,4) In other words, the addition of H₂O₂ to the liver lysates did not cause any oxidative stress to the proteins in the lysate. On the other hand, lipid peroxides are also present in vivo and contribute to radical protein damage. Reaction conditions combining tert-butyl hydroperoxide (tBHP) or cumene hydroperoxide with hemin are used for lipid peroxide research. 29,30)

Therefore, the reactions with tBHP and cumene hydroperoxide were investigated. In addition, meta-chloroperoxybenzoic acid (mCPBA) with the –OOH structure was also tested (Fig. 6A). tBHP labeled albumin in a concentration-dependent manner, although the fluorescence was weaker than that when H₂O₂ was used. Labeling was also confirmed with cumene hydroperoxide, but it reached plateau at 1 mM and labeling efficiency was low. On the other hand, mCPBA did not label albumin. Whereas albumin labeling by H₂O₂ was completely inhibited in the presence of mouse liver lysate (Fig. 6B lane 1 vs. lane 3), the labeling by tBHP proceeded even in the presence of liver lysate (Fig. 6B lane 2 vs. lane 4). These results may be due to the high specificity of catalase for H₂O₂. Hemin/tBHP-induced damage may serve as a model of lipid peroxidation-induced oxidative damage.

Labeling Oxidatively Damaged Protein in Mouse Liver Lysate We detected labeled proteins using tBHP. Even in the absence of hemin, various proteins were labeled by tBHP in mouse liver lysate (Fig. 7, lane 2). Although all the labeled proteins could not be identified, the results of in-gel trypsin digestion and peptide mass fingerprinting (PMF) analysis revealed that the characteristic and somewhat abundant protein between 210 and 140 kDa was carbamoyl phosphatase synthetase (CPS, approx. 160 kDa). The overall labeling efficiency was increased with increasing amount of hemin added (lanes 3–7). The decreased intensity of the CBB-stained band and the increased intensity of the fluorescence band at the top in SDS-PAGE gel with increasing hemin concentration could be
due to oxidative degradation or denaturation/protein aggregation. In particular, the CPS band became less visible with increasing hemin concentration and instead, a high-molecular-weight band shown by the arrow in Fig. 7 appeared. This band was also identified as CPS in in-gel trypsin digestion, and was thought to be a dimerized/cross-linked CPS. There are no reports that CPS is a hemin-binding protein, however, CPS is reported to be oxidatively inactivated.31 Based on the changes in CBB band intensity in Fig. 7, it is clear that CPS is oxidized and cross-linked by hemin and tBHP. These results also indicate that the current method can selectively label proteins that are undergoing oxidative damage.

Conclusions and Prospects  We developed a method for labeling peroxide-induced oxidative stress hotspots. Hemin is an oxidative stress-amplifying factor in vivo and reacts with peroxide to radicalize biomolecules, such as protein tyrosine residues. Albumin acts as a detoxifying factor for the hemin/peroxide-induced oxidative stress in blood by binding to hemin. Using methemalbumin, a complex of albumin and hemin, we found that Tyr138, the closest tyrosine to the hemin-binding site of HSA, was preferentially labeled. In FBS, albumin was selectively labeled, but in mouse liver lysate, the labeling did not proceed due to the detoxifying action of catalase. When tBHP, which mimics lipid peroxide, was added and the reaction mixture incubated at room temperature for 60 min, Compound 1 (final concentration 10–1000 µM) was added and the reaction mixture incubated at room temperature for 30 min. Then, H₂O₂ (final concentration 1 mM) was added and the reaction mixture was incubated at room temperature for 60 min. Labeling was quenched by adding sodium ascorbate (final concentration 2 mM), and free cysteine was capped by adding iodoacetamide (final concentration 10 mM). The reaction mixture was incubated at room temperature in the dark for 60 min. Excess amounts of small molecules were removed with Amicon Ultra (MWCO: 10 kDa). DBCO-Cy3 (final concentration 200 µM) was added and the reaction mixture was incubated at 37°C for 60 min after removal of excess amounts of small molecules with Bio-Spin 6 (Bio-Rad, U.S.A.) and the resulting solution was added to 5× SDS-PAGE sample buffer (final concentration 50 mM Tris–HCl pH 6.8, 125 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 0.025% bromophenol blue, 10% glycerol). The solution was heated at 95°C for 5 min. Then, the solution was separated by SDS-PAGE with acrylamide gel. Fluorescence images were obtained with Fusion Solo 4S (Vilber-Lourmat, France). The gel was visualized by CBB staining and images were obtained with a Molecular Imager ChemiDoc XRS (Bio-Rad).

In-gel Trypsin Digestion Bands that were separated by SDS-PAGE were excised into approximately 1 mm pieces. The gel pieces were transferred into a tube and 1 mL of water was added. The tube was incubated at 37°C for 10 min and the solution was removed (repeat three times). A solution of 50% MeCN in 100 mM NH₄HCO₃ aq. was added to the tube for gel destaining. The tube was incubated at 37°C for 10 min and the solution in the tube was removed. Next, MeCN was added to the tube for dehydration. The tube was incubated at 37°C for 10 min and the solution was removed. After that, dithiothreitol (100 mM) in 100 mM NH₄HCO₃ aq. was added to the tube for cysteine reduction. The tube was incubated at 37°C for 30 min and the solution was removed. Subsequently, iodoacetamide (250 mM) in 100 mM NH₄HCO₃ aq. was added to the tube to induce cysteine alkylation. The tube was incubated at room temperature for 30 min in the dark and the solution was

Experimental

Albumin Labeling  A solution of HSA (Sigma-Aldrich, U.S.A.) (final concentration 10 µM) in 100 mM phosphate buffer pH 7.4 was added to hemin (final concentration 10 µM) and the reaction mixture was incubated at room temperature for 30 min. Compound 1 (final concentration 10–1000 µM) was added and the reaction mixture incubated at room temperature for 30 min. Then, H₂O₂ (final concentration 1 mM) was added and the reaction mixture was incubated at room temperature for 60 min. Labeling was quenched by adding sodium ascorbate (final concentration 2 mM), and free cysteine was capped by adding iodoacetamide (final concentration 10 mM). The reaction mixture was incubated at room temperature in the dark for 60 min. Excess amounts of small molecules were removed with Amicon Ultra (MWCO: 10 kDa). DBCO-Cy3 (final concentration 200 µM) was added and the reaction mixture was incubated at 37°C for 60 min after removal of excess amounts of small molecules with Bio-Spin 6 (Bio-Rad, U.S.A.) and the resulting solution was added to 5× SDS-PAGE sample buffer (final concentration 50 mM Tris–HCl pH 6.8, 125 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 0.025% bromophenol blue, 10% glycerol). The solution was heated at 95°C for 5 min. Then, the solution was separated by SDS-PAGE with acrylamide gel. Fluorescence images were obtained with Fusion Solo 4S (Vilber-Lourmat, France). The gel was visualized by CBB staining and images were obtained with a Molecular Imager ChemiDoc XRS (Bio-Rad).

In-gel Trypsin Digestion Bands that were separated by SDS-PAGE were excised into approximately 1 mm pieces. The gel pieces were transferred into a tube and 1 mL of water was added. The tube was incubated at 37°C for 10 min and the solution was removed (repeat three times). A solution of 50% MeCN in 100 mM NH₄HCO₃ aq. was added to the tube for gel destaining. The tube was incubated at 37°C for 10 min and the solution in the tube was removed. Next, MeCN was added to the tube for dehydration. The tube was incubated at 37°C for 10 min and the solution was removed. After that, dithiothreitol (100 mM) in 100 mM NH₄HCO₃ aq. was added to the tube for cysteine reduction. The tube was incubated at 37°C for 30 min and the solution was removed. Subsequently, iodoacetamide (250 mM) in 100 mM NH₄HCO₃ aq. was added to the tube to induce cysteine alkylation. The tube was incubated at room temperature for 30 min in the dark and the solution was

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removed. The gels were washed with 100mMNH₂HCO₃ aq. and 50% MeCN in 100mMNH₂HCO₃ aq. Thereafter, MeCN was added to the tube for dehydration. The tube was incubated at 37°C for 10 min and the solution was removed. Finally, trypsin (Trypsin Gold, Promega) solution was added and the tube was incubated at 37°C overnight. The obtained solution was quenched by adding trifluoroacetic acid (TFA) aq. (final concentration 0.1%).

**LC-MS Analysis of Peptide Fragments**  
LC-ESI analysis of peptide fragments obtained by in-gel trypsin digestion was performed with a Bruker ESI-TOF-MS (microTOF II) and a VIOLAMO200 C18 HPLC column (2.1 mm i.d. × 250 mm). The micropump gradient method was used, as follows. Mobile phase A: 0.1% formic acid aq., mobile phase B: 100% acetonitrile. 0–5 min: 5% B, 5–40 min: 5–60% B, 40–45 min: 60–100% B, 45–51 min: 100% B, 51.1–60 min: 5% B. The labeled peptide fragments were detected by measuring the absorbance at 550 nm. For MS/MS analysis, peaks corresponding to retention times of 33.5–34.0 min were collected, and MS/MS analysis was performed with a MALDI-TOF MS (Bruker, ultraflexXtreme, Bruker, U.S.A.)

**MALDI-TOF MS Analysis of Digested Samples**  
After protein labeling with I, the digested solutions were desalted using C18 pipette tips (Agilent, U.S.A.). Each sample was mixed with 1 μL of 2,5-dihydroxybenzoic acid (DHB) solution on a MALDI-TOF plate. Peaks of the modified peptides were detected by MALDI-TOF analysis (Bruker, ultraflexXtreme). Mascot serach was performed by using BioTools (Bruker).

**Preparation of Mouse Liver Soluble Fraction**  
Animal experiments were performed in compliance with relevant laws and institutional guidelines of Tokyo Institute of Technology and with the approval of institutional committees. The liver was excised from sacrificed mouse and washed with 0.9% NaCl solution. In lysis buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 0.9% NaCl solution) with cOmplete ULTRA protease inhibitor cocktail (Roche) (approx. 30 μg/mL), the liver was cut into small pieces with scissors. The liver pieces were homogenized with a glass homogenizer (ISIS, RD440613) on ice. After centrifugation (5000 rpm, 15 min, 4°C), the supernatant was collected and used as mouse liver soluble fraction. Protein concentration was determined by the bicinchoninic acid (BCA) assay. The solution was diluted to 1 mg/mL protein with reaction buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol bis-(aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1% Triton X).

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**Conflict of Interest**  
The authors declare no conflict of interest.

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