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Hydrogen peroxide induced oxidation of peroxisomal malate synthase and catalase

Pria Anand [a,c], Yoon Kwak [a], Rahul Simha [b], Robert P. Donaldson [a,*]

[a] Department of Biological Sciences, The George Washington University, Washington, DC 20052, USA
[b] Department of Computer Science, The George Washington University, Washington, DC 20052, USA
[c] Yale College, Yale University, New Haven, CT 06520, USA

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ABSTRACT

Peroxisomes contain oxidases that produce H_2O_2, which can result in protein oxidation. To test the vulnerability of peroxisomal proteins to oxidation *in vivo* the organelles were isolated from castor bean endosperm incubated with H_2O_2. When peroxisomes were exposed to H_2O_2 *in vivo*, the peroxisomal proteins exhibited an increase in carbonylation as detected in avidin blots of biotin hydrazide derivatized samples. Biotin-tagged peptides from trypsin digests of the proteins were analyzed by mass spectroscopy and compared to the masses of peptides from the same protein that had not been biotin-tagged and from proteins not exposed to excess H_2O_2. H_2O_2 exposure was found to increase the activity of catalase (CAT), and to increase the number of oxidized peptides found in CAT and malate synthase (MS). CAT had 10 peptides that were affected by *in vivo* exposure to H_2O_2 and MS had 8. These sites of oxidation have definable locations within the proteins' structures.

Introduction

Peroxisomes are a major site of reactive oxygen species (ROS)[1] production in eucaryotic cells, including animals, yeasts and plants [1]. In animal tissues such as liver, D-amino acid, acyl-CoA and urate oxidase activities produce H_2O_2 [2,3]. Certain yeasts will use methanol as a carbon source, the metabolism being initiated by a peroxisomal methanol oxidase, which forms striking crystals within the peroxisomes that dominate the cells [4]. In photosynthetic plant tissues, leaves, peroxisomes contain an H_2O_2-generating glycolate oxidase that is part of the photorespiratory carbon cycle, which begins in the chloroplast when O_2 replaces CO_2 in the RuBisCo reaction [5]. In those germinating seeds where triglycerides are mobilized as a source of energy, fatty acids are oxidized to acetyl-CoA in peroxisomes. This peroxisomal β-oxidation pathway, which generates H_2O_2, was first described in germinating castor bean [6] and was later found in animal tissues [7]. The peroxisomes in germinating oil seeds, such as castor bean, have been referred to as glyoxysomes because they also contain glyoxylate cycle enzymes, such as malate synthase (MS) and isocitrate lyase (ICL), which allow for the conversion of acetyl-CoA to sugars [8].

Because peroxisomes house ROS-generating processes, they also contain catalase (CAT) and other ROS-scavenging activities. Plant peroxisomes also have associated with their membranes an ascorbate peroxidase and a monodehydroascorbate reductase, which can scavenge H_2O_2 more effectively at low concentrations than can CAT, thus limiting the escape of H_2O_2 and protecting cytosolic proteins and lipids from oxidative damage [9,10]. Peroxisomal activities can also generate superoxide, which is converted to H_2O_2 by a peroxisomal superoxide dismutase. Other antioxidant activities housed within peroxisomes include peroxiredoxins and glutathione peroxidase [1,11].

Under certain conditions, which can be considered oxygen stress, the production of ROS can overcome the antioxidant systems and cause oxidative damage. A variety of circumstances can provoke oxygen stress in plants, for example, exposure to Cd [12] or high NaCl [13]. Oxygen stress is characterized by increases in H_2O_2 and decreases in antioxidant activities, CAT and dehydroascorbate reductase [11], and is correlated with increases in protein carbonylation [14]. Experimentally, oxygen stress can be provoked by exposing tissues or cells to excess H_2O_2, treating with aminotriazole to inhibit CAT, knocking out the expression of antioxidant activities or simply aging [15]. H_2O_2 can escape from peroxisomes, especially when the sources are particular oxidases,

* Corresponding author. Address: Department of Biological Sciences, Lisner Hall 340, 2023 G Street NW, Washington, DC 20052, USA. Fax: +1 202 994 6100.
E-mail address: robdon@gwu.edu (R.P. Donaldson).

[1] *Abbreviations used:* CAT, catalase; MS, malate synthase; ICL, isocitrate lyase; MCO, metal catalyzed oxidation; PMCP, peptide mass comparison program; ROS, reactive oxygen species; MALDI-TOF, matrix-assisted laser desorption-time-of-flight.

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such as urate oxidase [2,3]. Thus, H_2O_2 produced in peroxisomes is not completely consumed by CAT and other antioxidant activities within peroxisomes.

Oxidative stress often results in the oxidation of particular proteins in the affected tissues. For example, in senescing pea leaves, an increase in protein carbonylation is correlated with an increase in H_2O_2 and decreases in the activities of CAT and dehydroascorbate reductase [14]. Plant mitochondrial proteins are oxidized when exposed to H_2O_2 and Cu^{++} *in vitro*. The oxidized proteins can be tagged with DNP, immunoprecipitated and identified by LC/MS, and include malate dehydrogenase, superoxide dismutase, glycine decarboxylase, aconitase and a peroxisomal 2-hydroxyacid oxidase [16,17]. Oxidized proteins accumulate in Arabidopsis plants during vegetative growth and then precipitously disappear during flower development [18]. Also, there are indications that protein oxidation in human nervous tissue is correlated with degenerative disorders, such as Alzheimer's and Parkinson's diseases [19].

Within peroxisomes, where H_2O_2 is produced in high quantities, it would be expected that the resident proteins would be subject to oxidation. We have indeed found that many of the castor bean peroxisomal proteins can become detectably oxidized when exposed to a metal catalyzed oxidation system (MCO) comprised of Cu^{++} and ascorbate *in vitro* [20]. The MCO system circumvents CAT protection. The glyoxylate cycle enzymes, MS and ICL, are especially sensitive to oxidation, and a loss of enzymatic activities is associated with protein carbonylation. CAT itself is subject to oxidation and partial loss of activity under *in vitro* MCO conditions. ICL and its product, glyoxylate, are very sensitive to H_2O_2, at least *in vitro*, and are protected from its effects by the physical association of ICL with CAT [21]. CAT appears to be physically associated with certain proteins within peroxisomes in order to protect especially sensitive proteins from oxidative inactivation by H_2O_2. A similar protective association exists between CAT and proteins involved in β-oxidation in human cells [22].

The purpose of this study was to determine the sites of oxidation in peroxisomal proteins subjected to H_2O_2 *in vivo*. Peroxisomes are especially abundant and active in castor bean endosperm during germination because all of the stored energy is in the form of triglyceride and must be metabolized through the peroxisomal (glyoxysomal) pathways to produce sucrose for the developing seedling [23]. One hundred grams of endosperm are easily obtained and this yields several milligrams of peroxisomal protein for subsequent analyses [24]. We analyzed the proteins for oxidation using Western blotting with a biotin/avidin detection system to compare oxidation in H_2O_2 incubated tissue to tissue incubated with H_2O. Mass spectrometry of the trypsin cleaved proteins was used to identify oxidized peptides within CAT and MS.

Materials and methods

Preparation of peroxisomes

Castor bean seeds (Bothwell Enterprise, Plainview, TX) were incubated in wet vermiculite at 30 °C and 80% humidity in the dark. After 4.5 days, the endosperms were separated and washed with chilled dH_2O. Endosperms were then incubated for 3 h at 30 °C with either dH_2O or 100 mM H_2O_2 with gentle shaking. Most of the H_2O_2 was immediately consumed by CAT since oxygen bubbles rapidly appeared and subsided within minutes. Thus the concentration of H_2O_2 that penetrated the tissue (~0.3 g each) was considerably less than the initial H_2O_2 concentration. The endosperms were homogenized and peroxisomes isolated through varying concentrations of sucrose using the methods and reagents described previously [21,24]. Some of the incubated endosperms were used to analyze H_2O_2 content.

Determination of enzyme activities, protein and H_2O_2 concentration

The Bradford reagent (Sigma–Aldrich) and protocols described by the manufacturer were used to measure protein concentration. The activity of CAT was measured according to the methods of Cooper and Beevers [8]. H_2O_2 content of the tissue was determined using a previously described assay that is based on phenol red and horseradish peroxidase [25]. The tissue was either incubated in 100 mM H_2O_2 or water for times ranging from 1 to 30 min, then plunged into 95 °C 0.1 M $KHPO_4$, pH 6.9, sealed in a vial and heated 95 °C for 30 min. The tissue was homogenized with a mortar and pestle, filtered through nylon net, centrifuged 15 min at 10,000*g* and the supernatant used for the H_2O_2 assay procedure.

Biotinylation and electrophoresis

Peroxisomal protein, 200 μg in PBS, was treated for an hour with biotin hydrazide (Pierce) dissolved in dimethyl sulfoxide. Biotinylation was stopped by placing the samples on ice, and sodium cyanoborohydride dissolved in PBS was added. The final concentration of biotin hydrazide in solution was 5 mM, and that of sodium cyanoborohydride was 15 mM. Samples were incubated for 40 min on ice, and then treated with an SDS sample buffer containing 2-mercaptoethanol. Proteins were separated by SDS–PAGE gel electrophoresis in 9% resolving and 5% stacking polyacrylamide gels with dimensions of 16 × 16 × 0.15 cm. Either 150 or 75 μg of protein were loaded in each well for mass spectrometry, and 20 μg of protein were loaded in each well for Western blotting.

Western blotting

Proteins were transferred to nitrocellulose for 1 h at 555 mA using a tank transfer system (Bio-Rad Laboratories). The membrane was blocked in 1% milk in TTBS, and then detected using horseradish peroxidase-conjugated avidin (1:1000, Pierce). Avidin binding was visualized using Western Lighting Chemiluminescent Reagent (Perkin Elmer) and Biomax Light Film (Kodak).

Tryptic digestion of proteins

Proteins were processed in-gel. Bands corresponding to CAT (NCBI Accession No. BAA04697, 55 kDa) and MS (NCBI Accession No. P17815, 64 kDa) were cut into 1 mm pieces and destained in 50% acetonitrile/50% 50 mM NH_4HCO_3. They were reduced by adding 10 mM DTT in 100 mM NH_4CO_3 and incubating at 56 °C for 45 min. The gel pieces were then alkylated by adding 55 mM iodoacetamide in 100 mM NH_4HCO_3 and incubated for 45 min in the dark at room temperature, and finally dehydrated in acetonitrile. Proteomics-grade trypsin (Sigma) was reconstituted in 1 mM HCl, 40 mM NH_4HCO_3 and 9% acetonitrile and added to the gel pieces. The resulting solution was incubated for 30 min at 4 °C, then overnight at 37 °C. Peptides were extracted using sonication, dried in a speed-vac, and stored at 4 °C. On the day of analysis, peptides were purified using a C18 ZipTip (Millipore) and the protocol provided by the producer.

MALDI-TOF

The samples were analyzed in an alpha-4-cyano-4-hydroxycinnamic acid matrix (Sigma–Aldrich) using an AXIMA-CFR MALDI-TOF mass spectrometer (Shimadzu Biotech) in reflectron mode. Mass spectra were acquired with Kompact Launch Pad software, version 2.3.4. (Kratos Analytical). Two to four consecutive rasteriz-