HYDROGEN PEROXIDE INDUCED OXIDATION OF PEROXISOMAL MALATE SYNTHASE AND CATALASE.

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Abbreviations

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.

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 derivitized 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.

Key words: biotin hydrazide, carbonylation, catalase, hydrogen peroxide, glyoxysome, MALDI-TOF MS, malate synthase, mass spectroscopy, peroxisome, protein oxidation, reactive oxygen species.

Introduction

Peroxisomes are a major site of reactive oxygen species (ROS) 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, 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, immuoprecipitated and identified by LC/MS, and include malate dehydrogenase, superoxide dismutase, glycine decarboxlyase, 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 30ºC 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 hrs at 30ºC 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^{o}C 0.1 M $KHPO_4$, pH 6.9, sealed in a vial and heated 95^{o}C for 30 min. The tissue was homogenized with a mortar and pestle, filtered through nylon net, centrifuged 15 min at 10,000 xg 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 mins 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 cm x 16 cm x 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 one hour 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 BAA04697, 55 kD) and MS (NCBI accession P17815, 64 kD) 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 56ºC for 45 mins. The gel pieces were then alkylated by adding 55 mM iodoacetamide in 100 mM NH_4HCO_3 and incubated for 45 mins 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 mins at 4ºC, then overnight at 37ºC. Peptides were extracted using sonication, dried in a speed-vac, and stored at 4ºC. 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 rasterizing scans over two duplicates of each sample were conducted. Default settings were used to generate a peak list from these data. Molecular weight data in the range of 500-5000 D were retained for four sample groups in

each trial: biotin-tagged and untagged samples from both control and H_2O_2-treated tissue. Three fully independent preparations were analyzed. A total of 74 spectra were acquired for the MS protein, and a total of 85 spectra were acquired for CAT.

Peptide Mass Comparison Program (PCMP). A novel JAVA data analysis program employing the JFree library was created and used to analyze the Kompact output, text files containing lists of peptide masses (http://www.seas.gwu.edu/~simhaweb/software/pmcp/index.html). Graphs display a comparison with simulated trypsinolysis of CAT (Catalase 1, from *Ricinus communis*, NCBI accession BAA04697) and MS (Malate synthase from *Ricinus communis*, NCBI accession P17815) generated using the ExPASy PeptideMass program. The PCMP output graphically illustrates the percentage of the total input files that contain each peptide from CAT or MS, comparing the differences between control and biotinylated samples. Peptides that were significantly more absent in biotin-treated than control samples were then considered to be oxidized.

Results

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 derivitized samples (Fig. 1). The peroxisomal proteins that were seen to be oxidized with the biotin tag were the same proteins that were previously detected with DNP tagging including MS and CAT [20]. Previously we had exposed the peroxisomal proteins to MCO *in vitro* with Cu++ and ascorbate. The extent of oxidation was time and dose dependent with *in vivo* incubation of the endosperm tissue with 10 mM and 100 mM H_2O_2 (data not shown) or *in vitro* incubation of isolated peroxisomes with the Cu^{++} and ascorbate [20]. The *in vivo* H_2O_2 exposure also resulted in an increase in CAT activity

(Fig. 1) whereas the *in vitro* MCO decreased the activity [20]. The tissue that had been incubated in H_2O_2 for any time interval was observed to contain less than 1 mM H_2O_2 based on an H_2O_2 assay of an extract obtained from tissue that had been heated to 95^{o}C for 30 min as described in the Methods [25].

Our objective here was to evaluate the peptides from peroxisomal proteins for carbonyl modification. This was done by subjecting the biotin tagged peptides to mass spectroscopy and comparing the masses of the peptides to those from the same protein which had not been biotin tagged. We would expect that not all of the peptides from a protein would be biotinylated and thus the masses of some peptides in this comparison would not be affected by the biotin tag and others would. In order to make these comparisons we created the PMCP to sort and visualize the mass data (http://www.seas.gwu.edu/~simhaweb/software/pmcp/index.html). The PMCP performs several tasks. The first task is to match peptide masses from a protein band cut from a gel to the masses of predicted peptides derived from a particular protein, having tentatively identified the protein band by MW and by immunoblotting. Thus, we enter the predicted masses of the known protein followed by the mass lists of several samplings of the protein of interest, for example the putative CAT band at ~55 kD. The program then matches all the observed masses from the samples to the known masses, retains those masses in a list and sends to another file those masses that do not match. The program generates a list of the observed, matching masses in the order of their occurrence in the primary sequence of the protein (chronological order) and also reports the frequency with which each peptide in the samplings is observed in a graphic (for example, Fig. 2A), the frequency being the fraction of samples having the peptide relative to the total number of samplings.

As would be expected, some of the predicted peptides were never observed (CAT peptides

#10, 16-18, 21-23, 26, 30, 34 and 41-42, MS peptides #14, 25, 27, 34 and 36), others were very frequently observed. In CAT 24 peptides, representing 55.5% of the residues, were recovered in the control samples and 26 peptides, or 53.6% of the residues, were recovered in the H_2O_2-treated samples. In MS 22 peptides, 54.6% of the residues, were recovered in the control sample groups and 58.6% of the residues in the H_2O_2-treated samples. When the parent protein was subjected to biotinylation of carbonyl groups, the frequency of some of the peptides was altered more than others. For example, several peptides near the N-terminus of CAT, #2, 7, 8, 9 and 12, were affected differently by the biotinylation (Fig. 2A). Peptides #2 and #6 were observed slightly less frequently in the biotinylated samples (dark gray) than in the non-biotinylated samples (light gray), #4 and #5 were more frequent with biotin tagging and #7 was not affected by the tag. The frequencies of peptides #24 and #38 were also unchanged. Only three CAT peptides were significantly decreased in frequency by the biotin tagging, #12, #25 and #36. We interpret this to mean that the biotin tag caused these peptides to shift to different masses or to not be desorbed by the laser. We did not attempt to identify or locate the new masses of the biotin tagged peptides, but simply conclude that biotinylation altered the mass of those peptides.

Prior to isolating the peroxisomes, we incubated some of the tissue samples with 100 mM H_2O_2. Peroxisomal proteins from these tissues were then subjected to an analysis of biotin-induced changes in observed frequency of peptide masses as described above. For example, CAT peptide #2 near the N-terminus was displaced from a frequency of 45% to 21% with the biotin (Fig. 2B). Several other CAT peptides were similarly affected by the biotin tagging, #2, 7, 8, 9, 12, 13, 15, 25, 28, 36, 38, 39 and 40. The greatest differences were seen in peptides #12, 15, 25, 34 and 38-40. Note that three of the peptides had also been displaced in samples from the tissue that was incubated without the H_2O_2. This indicates that the H_2O_2 treatment increased the

oxidation of some regions of the CAT protein.

The biotin tagged peptides were not reliably detectable perhaps because they were not desorbed from the matrix or because the new masses were not predictable. The mass of an oxidized, tagged peptide would depend on the particular amino acid that was modified and how that affected trypsin digestion.

The H_2O_2 treatment and biotin tagging of peptides produced similar effects in MS. The peptides that were shifted by the biotin tag in the H_2O_2 treated tissue were #5, 13, 17, 20, 25, 31, 46 and 47 (Fig. 3B), None of these peptides were affected by the biotin tagging of the MS from the tissues that had been incubated in the absence of the H_2O_2 (compare Fig. 3A and 3B).

Discussion

Figure 4 shows the locations of the biotin-displaced peptides within the sequences of CAT and MS resulting from exposure of the tissue to excess H_2O_2. This indicates that oxidation sites are located throughout the proteins. There were also regions of each protein that were apparently not prone to oxidation. Furthermore, some peptides were never detected in MALDI-TOF mass spectrometry, suggesting that some sites of oxidation may be missed. Each of the biotin-displaced peptides contains at least one residue that would be susceptible to oxidation, a histidine, lysine, arginine, threonine, proline [26] or tryptophan [17].

The CAT and MS peptides that were found to contain oxidized residues were mapped onto 3D structures of homologous proteins from bacteria (Fig. 5 and 6). Some of the peptides with oxidized residues are found around the H_2O_2 entry channel leading to the heme in CAT [27]. Some of the peptides that were oxidized following the exposure of the tissue to H_2O_2 were found in the vicinity of the Mg^{++} binding site in MS. Residues near this site may be subject to metal

catalyzed oxidation if endogenous Fe or Cu were to replace the Mg in this site. Also, since this is the active site for MS the loss of activity upon oxidation is expected [20].

Other attempts to identify sites of oxidation in a protein have only revealed a few peptides that were affected and identifiable in mass spectroscopy [19, 28, 29]. For example, amino acid analysis of bacterial glutamine synthetase following exposure to MCO demonstrated the loss of 2 moles of histidine per mole of protein. A change in pI indicated that other oxidations had occurred that were not revealed in the amino acid analysis [30]. Another technique, based on mass spectroscopy of fluoresceinamine derivatization of carbonyls in this protein, indicated that Arg 344 had been oxidized by MCO [31]. These two approaches made it possible to detect the oxidation of particular active site residues in the protein that accounted for loss of glutamine synthetase activity. Other proteins, such as those in peroxisomes exposed to H_2O_2, may experience particular distributions of oxidation. Our approach was based on the reasoning that oxidized, derivatized peptides would not always be detectable in mass spectroscopy since the chemical modifications could change the behavior of the peptides in unpredictable ways and the mass changes may not be predicted correctly. Also, some modified peptides may not desorp from the matrix very efficiently in the mass spectrometry. However, if a peptide is chemically modified in any way that affects its behavior in mass spectrometry, the peptide would be observed less frequently.

We developed the software, PMCP, to determine if some of the peptides from CAT or MS had been affected by the exposure to excess H_2O_2 such that some peptides were observed less frequently compared to controls in MALDI-TOF mass spectrometry. The PMCP compares peptide masses to those expected from known protein sequences and computes the percentages with which each peptide was observed. This made it possible to identify those peptides that were

significantly affected by the H_2O_2 treatment.

Previously, we had estimated that, based on spectrophotometric quantitation with 2,4-dinitrophylhydrazine, there were 2 to 3 sites of carbonylation per molecule of protein when the proteins were exposed to metal catalyzed oxidation conditions (Cu^{++} with ascorbate) *in vitro* [20]. In the current investigation, the biotin tagging indicated that 10 CAT peptides and 8 MS peptides were affected by the H_2O_2 treatment. However, none of the peptides are affected by biotin 100% of the time, suggesting that each molecule in the population has only a few oxidized sites but at varying locations within the molecule.

The methods we used in this study differ from other approaches including our own in several respects. The DNP-derivatization of the peroxisomal proteins involves the use of SDS to denature the oxidized proteins. Also, here the oxidation was brought about by exposing the peroxisomal proteins to H_2O_2 *in vivo* whereas before we had subjected the isolated peroxisomal proteins to *in vitro* MCO.

Other approaches have been employed to investigate oxidized proteins. DNP-tagged proteins can be immunoprecipitated with anti-DNP antibody and analyzed by 2D gels combined with mass spectroscopy [32]. This was applied to cultured mammalian cells and identified several ER proteins that were susceptible to oxidation when the cells were exposed to H_2O_2. DNP-tagged peptides are not amenable to mass spectroscopy since they are susceptible to decomposition in the mass spectrometer [33]. Another approach has been to use avidin affinity chromatography to recover biotin-tagged carbonylated protein followed by reverse-phase liquid chromatography and tandem mass spectroscopy [34]. Oxidized amino acids could then be identified within known peptides by the mass differences of the peptides. However, it was noted that the number of peptides detected from a protein, BSA for example, was decreased by

oxidation and biotinylation. This may occur if oxidized lysine or arginine prevents trypsin digestion, resulting in larger insoluble peptides, or because of cleavage or cross-linking of the oxidized proteins. Also, this approach depended on identifying biotinylated peptides according to their modified masses, and only one such peptide was associated with each of the several proteins examined. Thus, this approach may overlook some oxidized peptides. Our approach focuses on peptides that are displaced from the mass spectra by biotin, which allows us to identify sites of oxidation that might have been missed otherwise. Based on this, we identified several affected peptides in the two proteins that we investigated, MS and CAT. Since the recovered peptides represent less than 100% coverage, it is very likely that some sites of oxidation are missed in any of the analyses (Fig. 4). Thus, we cannot claim that this approach is comprehensive.

Peroxisomes generate H_2O_2, and the rate at which it is produced can exceed the capacity of CAT to consume it, since H_2O_2 can escape from peroxisomes [1, 2]. We observed differences in oxidized peptides from proteins that had been exposed to the H_2O_2 treatment, indicating that the H_2O_2 did have access to the peroxisomal proteins in the tissue despite the CAT activity within the organelles. The concentration of H_2O_2 attained in the peroxisomes cannot be defined since much of the H_2O_2 would have been consumed by CAT as it penetrated the tissue and the organelles. Less than 1 mM H_2O_2 was detected in the tissue after incubation with H_2O_2. Interestingly, 10 peptides from CAT itself were affected by the H_2O_2 treatment. However, CAT activity was increased in the H_2O_2 treated tissue. This is in contrast with the dose dependent loss in CAT activity that was observed when the peroxisomal proteins were subjected to MCO with Cu^{++} and ascorbate *in vitro* [20]. The novel approach we have developed to the study the oxidization of peroxisomal proteins has allowed us to conclude that the proteins, MS and CAT,

were subject to oxidation at multiple sites in the presence of H_2O_2

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Figure Legends

Fig. 1. Peroxisomal proteins are carbonylated when exposed to H_2O_2 *in vivo*. Germinating seeds were incubated with H_2O_2 or H_2O (control) prior to isolation of the peroxisomes. The peroxisomal proteins were tagged with biotin hydrazide, which was detected in Western blots with an avidin conjugate. CAT is at 55 kD and MS and isocitrate lyase (lower band) are at 64 kD CAT activity was increased in the peroxisomes isolated from the H_2O_2 treated seeds.

Fig. 2. Oxidation of peroxisomal CAT peptides by H_2O_2 exposure. Samples were obtained from H_2O_2-treated or control tissue and a portion of each was tagged with biotin. **A.** Control samples from non- H_2O_2 treated seeds. Light gray bars indicate the non-biotinylated samples, while dark grey indicate biotinylated. Only a few CAT peptides appeared to be significantly oxidized (*) in the peroxisomes collected from castor bean tissue incubated without H_2O_2. The CAT protein, either biotin-tagged or not, was digested with trypsin and the peptide masses compared using the PMCP. The number of times that a particular peptide was observed is indicated as a percent relative to the total number of times the sample was analyzed. The percentages of three CAT peptides were significantly diminished by the biotin-tag, #12, #25, and #36. (n = 20 for biotinylated samples, 21 for controls). **B.** Samples from H_2O_2 treated tissue. Thirteen peroxisomal CAT peptides were significantly oxidized (*) in seeds exposed to H_2O_2 *in vivo*. The biotin tagging significantly decreased the frequencies of peptides #2, 7, 8, 9, 12, 13, 15, 25, 28, 36, 38, 39 and 40. Three separate peroxisomal preparations from control and H_2O_2 treated tissue were analyzed, two samples from each preparation, and each sample was analyzed two to four times. (n = 19, 22).

Fig. 3 Oxidation of peroxisomal MS peptides by H_2O_2 exposure. Samples were obtained from H_2O_2-treated or control tissue and a portion of each was tagged with biotin. **A.** Control samples. None of the MS peptides were significantly affected by the biotin tag, indicating that they were not oxidized. (n = 15, 12). **B**. H_2O_2-treated tissue. Eight peptides from MS were oxidized in the seeds treated with H_2O_2. The biotin tagging affected peptides #5, 11, 15, 17, 21, 26, 39 and 40. The analysis was performed as described in Fig. 2 and the Experimental Procedures. (n = 14, 14).

Fig. 4. Locations of oxidized peptides in CAT (isozyme 1) and MS from castor bean peroxisomes (glyoxysomes). The peptides in bold were modified only in preparations where oxidation was induced with H_2O_2 treatment. This means that in seeds that were incubated with H_2O_2 prior to protein extraction, these peptides were more frequently observed in control samples than in biotinylated samples, but no change was seen in the seeds incubated without H_2O_2. Residues that would be susceptible to oxidation are shaded. The underlines indicate peptides that were observed in mass spectrometry either in the controls or in the samples from H_2O_2 treated tissue. Peptides not underlined or not bold were never observed in mass spectroscopy.

Fig. 5. Structure of *Ricinus* CAT based on an alignment with the structure of CAT from *Exiguobacterium oxidotolerans*. **A.** The entire tetrameric structure with black segments representing peptides that were found to contain oxidized residues after the tissue had be exposed to H_2O_2. **B.** Only the oxidized (black) segments (10 peptides) are shown along with the heme in yellow.

Fig. 6. Structure of *Ricinus* MS based on alignment with the structure of MS from *Bacillus anthracis*. **A.** The entire structure, black segments representing peptides that contained oxidized residues after the tissue had be exposed to H_2O_2. **B.** Illustrates only the oxidized (black) segments (8 peptides) of MS. Mg ions are shown in yellow.

Figure 1

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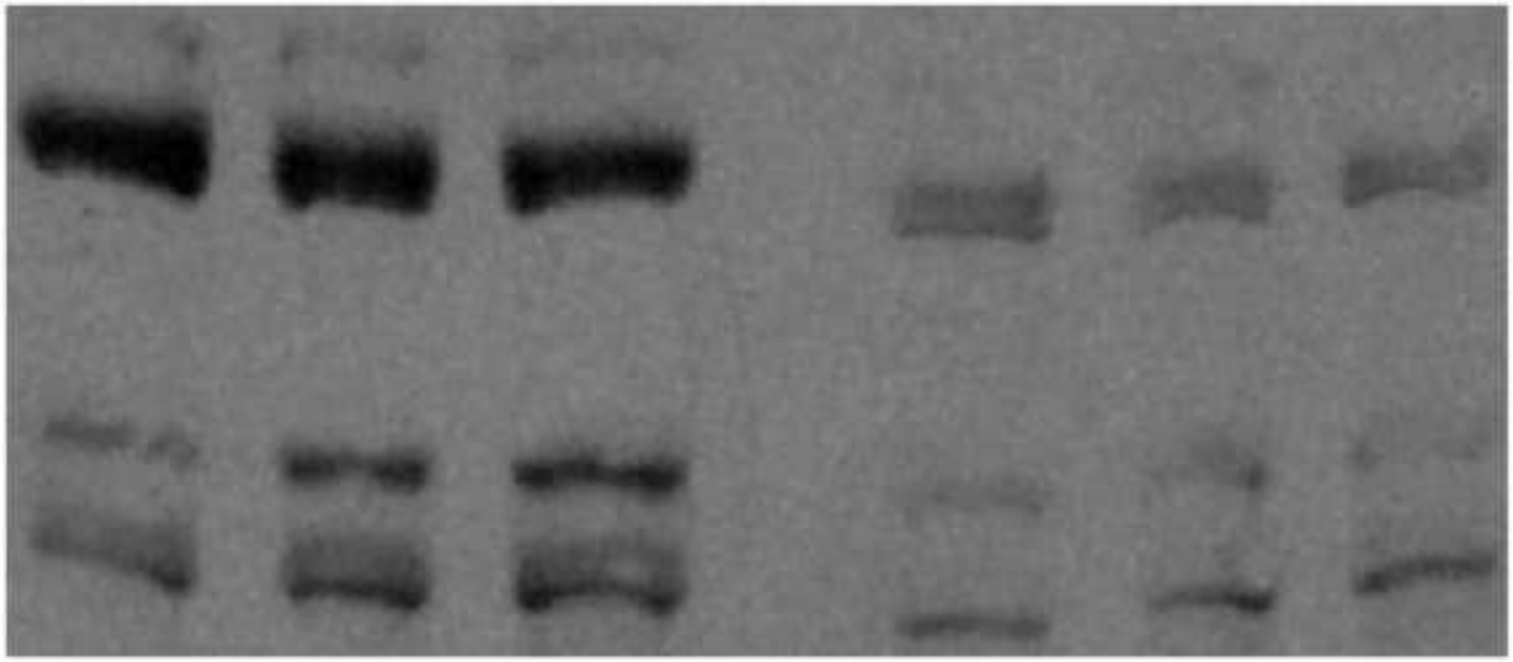

Catalase Activity, dA_{240}/min*mg protein

630 ± 32.0 445 ± 20.5

Figure 2

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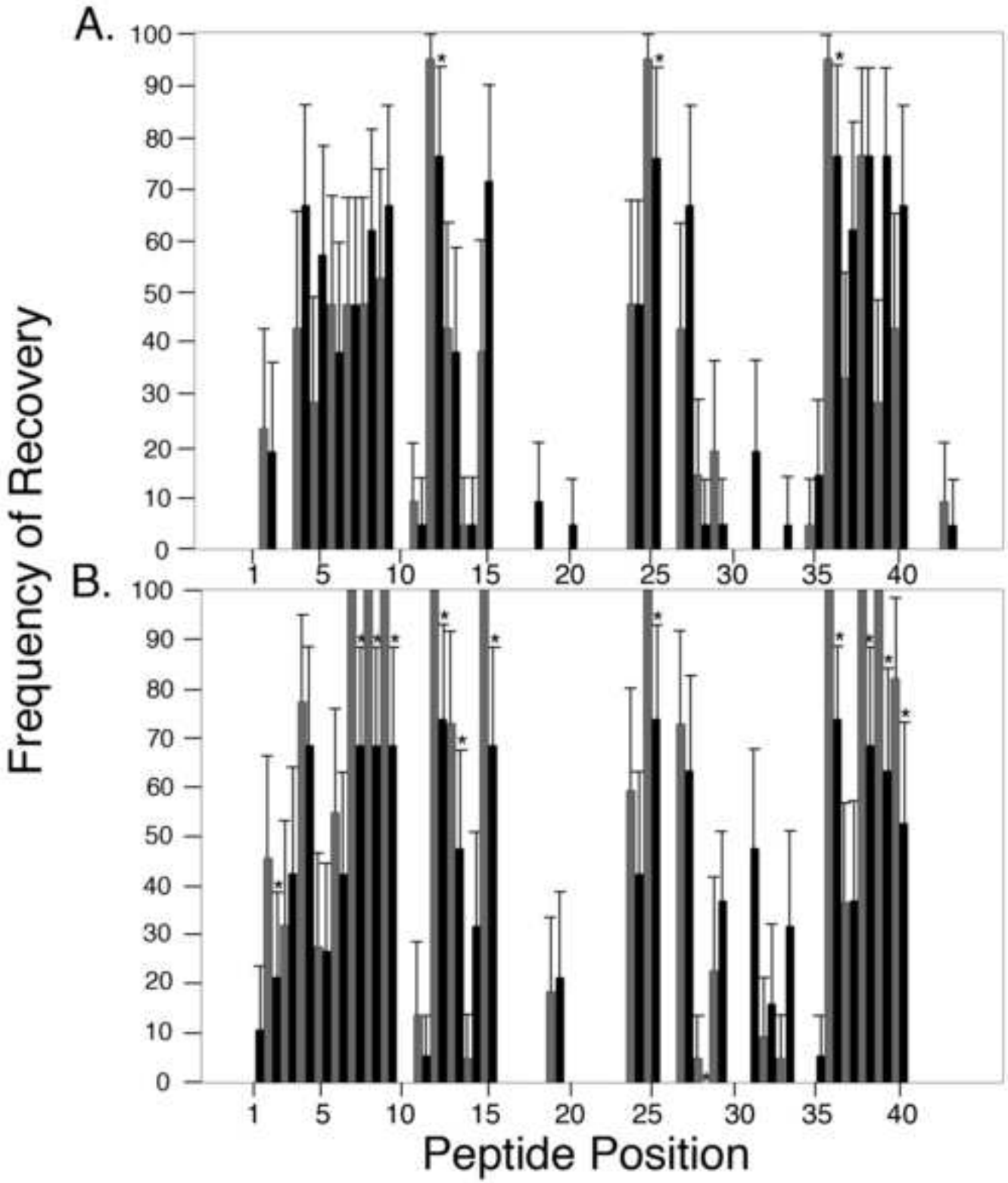

Figure 3

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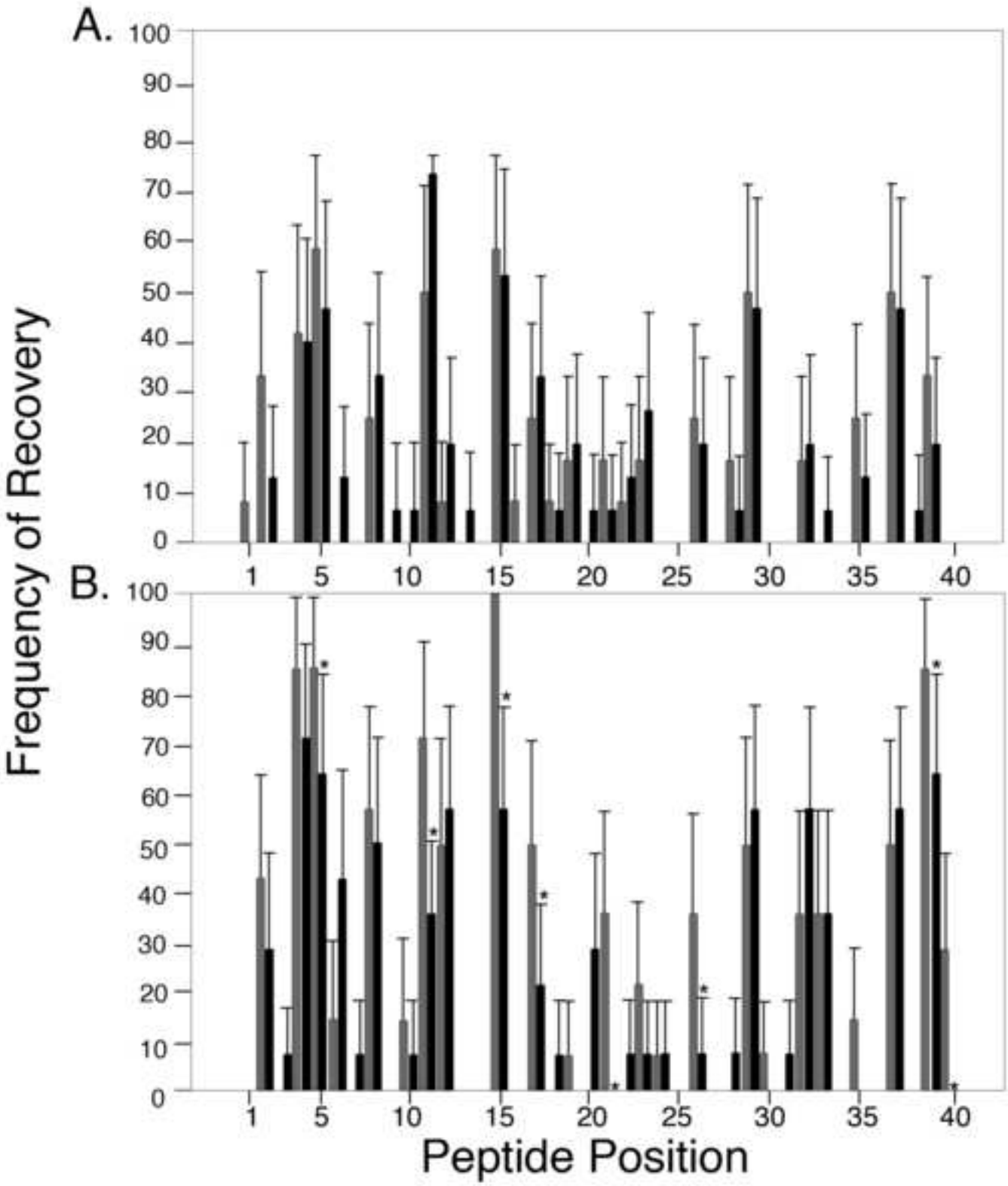

Figure 4

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Figure 5

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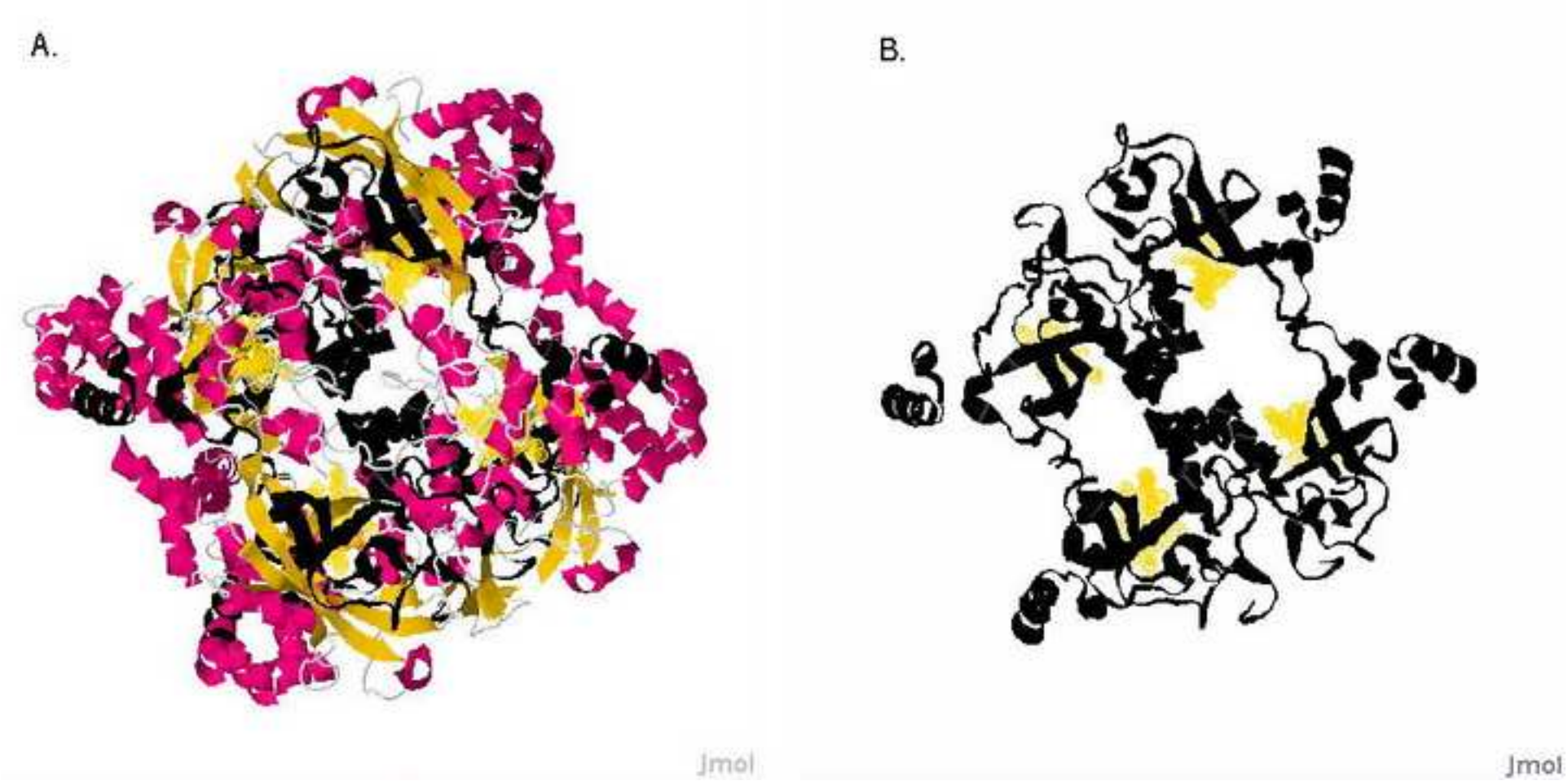

Figure 6

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