Oxidation of Catalase by Singlet Oxygen*

(Received for publication, October 31, 1997, and in revised form, February 11, 1998)

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Different bands of catalase activity in zymograms (Cat-1a-Cat-1e) appear during Neurospora crassa development and under stress conditions. Here we demonstrate that singlet oxygen modifies Cat-1a, giving rise to a sequential shift in electrophoretic mobility, similar to the one observed in vivo. Purified Cat-1a was modified with singlet oxygen generated from a photosensitization reaction; even when the reaction was separated from the enzyme by an air barrier, a condition in which only singlet oxygen can reach the enzyme by diffusion. Modification of Cat-1a was hindered when reducing agents or singlet oxygen scavengers were present in the photosensitization reaction. The sequential modification of the four monomers gave rise to five active catalase conformers with more acidic isoelectric points. The pI of purified Cat-1a-Cat-1e decreased progressively, and a similar shift in pI was observed as Cat-1a was modified by singlet oxygen. No further change was detected once Cat-1e was reached. Catalase modification was traced to a three-step reaction of the heme. The heme of Cat-1a gave rise to three additional heme peaks in a high performance liquid chromatography when modified to Cat-1e. Full oxidation to Cat-1e shifted all peaks into a single one. Absorbance spectra were consistent with an increase in asymmetry as heme was modified. Bacterial, fungal, plant, and animal catalases were all susceptible to modification by singlet oxygen, indicating that this is a general feature of the enzyme that could explain in part the variety of catalases seen in several organisms and the modifications observed in some catalases. Modification of catalases during development and under stress could indicate in vivo generation of singlet oxygen.

Photosynthetic evolution of dioxygen into the atmosphere and its subsequent accumulation led to formation of an ozone layer in the stratosphere, which permitted the dispersal of microorganisms around earth by absorbing damaging ultraviolet radiation from the sun (1, 2). Atmospheric dioxygen also led to the evolution of adaptation mechanisms to live with a poisonous gas (3, 4).

The electron affinity of O2 makes it a reactive compound. Furthermore, dioxygen generates more reactive intermediates in its sequential univalent reduction to water. Likewise, singlet oxygen, excited states of O2, are highly reactive species. They arise upon absorption of radiation by O2, either directly or through prevalent cellular compounds such as tetrapyrrolys, flavins, pterins, chlorophylls, and retinoids. Reactive oxygen species (ROS)1 are inevitably produced in cells under aerobic or microaerobic conditions (4).

Primeval cells, which originated in an anoxic environment (2), had either to hide from O2, or to evolve mechanisms for efficient reduction of entering O2, disposal of ROS, and sequestration of transition metals, which participate in reducing agents or singlet oxygen scavengers were present in the photosensitization reaction. The sequential modification of the four monomers gave rise to five active catalase conformers with more acidic isoelectric points. The pI of purified Cat-1a-Cat-1e decreased progressively, and a similar shift in pI was observed as Cat-1a was modified by singlet oxygen. No further change was detected once Cat-1e was reached. Catalase modification was traced to a three-step reaction of the heme. The heme of Cat-1a gave rise to three additional heme peaks in a high performance liquid chromatography when modified to Cat-1e. Full oxidation to Cat-1e shifted all peaks into a single one. Absorbance spectra were consistent with an increase in asymmetry as heme was modified. Bacterial, fungal, plant, and animal catalases were all susceptible to modification by singlet oxygen, indicating that this is a general feature of the enzyme that could explain in part the variety of catalases seen in several organisms and the modifications observed in some catalases. Modification of catalases during development and under stress could indicate in vivo generation of singlet oxygen.

* This work was supported in part by Grant IN-208994 from Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México, and Grant 2246P-N9508 from Consejo Nacional de Ciencia y Tecnología, Mexico. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviation used are: ROS, reactive oxygen species; PB, phosphate buffer; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; Me2SO, dimethyl sulfoxide; 5-ASA, 5-acetyl salicylic acid.
Catalases are prevalent in most organisms (4). Many microorganisms have more than one catalase, and in some a catalase is related to cell differentiation (32–45). Two catalase genes have been cloned in Aspergillus nidulans, one expressed only for asexual spores (conidia) (39, 40). A third catalase activity has been recently found.2 Neurospora crassa has two3 or three (46) catalase genes. The cat-1 gene mapped to complementation group IIIR has been cloned and partially sequenced (72). It codes for a homotetramer of 320-kDa molecular mass. Cat-1 is present in the whole vegetative life cycle of N. crassa.3 The cat-2 gene, assigned to complementation group VIIR, has not been cloned. It is expressed under stress conditions and transiently during the formation of conidia.3

Catalase-specific activity increases stepwise with each morphogenetic transition of the N. crassa conidiation process.3 Cat-1 was modified in these transitions and under stress conditions.3 Since we have demonstrated enzyme inactivation due to ROS-specific oxidation under cell differentiation and stress conditions (24, 25), it was important to determine if antioxidant enzymes such as catalases were also vulnerable to in vivo alteration by ROS. As predicted (15, 16), these enzymes are susceptible to oxidation by ROS but not affected in their activity.

We show that Cat-1 was oxidized through a sequential reaction of the four monomers with singlet oxygen, giving rise to active catalase conformers with more acidic isoelectric points. Modification could be traced to a three-step reaction of the heme with singlet oxygen. Catalases from different organisms were similarly modified by singlet oxygen, indicating a general feature of the enzyme that could explain in part the variety of catalases seen in several organisms (47–51) and the observed modifications in some catalases (52–55).

EXPERIMENTAL PROCEDURES

Strains and Cultures—N. crassa, wild type strain 74-OR23-1A from the Fungal Genetic Stock Center, was grown at 30 °C in slants of agar minimal medium of Vogel, supplemented with 1.5% sucrose. A. nidulans, wild type strain FGSC26 (bia1; tea1; Fungal Genetic Stock Center), was grown for 18 h at 200 rpm, from a inoculum of conidia (10^6/ml), in Kafer's minimal nitrate medium, supplemented with 2.5 μg/liter betin and 1% glucose. Saccaromyces cerevisiae, wild type strain S288C, was grown in yeast extract/peptone/dextrose liquid medium at 30 °C for 40 h at 250 rpm. Streptomyces coelicolor, wild type strain A3(2) J801, was grown 10 days at 200 rpm, from an inoculated Hopwood's minimal medium with an initial culture, grown from spores, in the same medium.

Catalase Activity—Catalase activity was measured by determining the initial rate of dioxygen production with a Clark microelectrode (56). Reaction was started by injecting catalase, usually 5 μl or less, into a sealed chamber filled with 2 ml of 10 mM H_2O_2 in 10 mM phosphate buffer (PB), pH 7.8 (adjusted by mixing NaH_2PO_4 and KH_2PO_4 solutions). Units are defined as micromoles of O_2 produced per min per mg of protein under these conditions. Activity was measured in samples just before loading them on a gel for electrophoresis.

Catalase activity in polyacrylamide gels was determined by incubating the gel after electrophoresis, 5 min in 5% methanol and then, after rinsing three times with tap water, 10 min in 10 mM H_2O_2. The gel, rinsed with tap water, was incubated in a 1/1 mixture of freshly prepared 2% potassium ferricyanide and 2% ferric chloride. Blue color developed in the gel except at zones where H_2O_2 was decomposed by catalase (57). Staining was stopped by soaking the gel in a 10% acetic acid and 5% methanol solution.

Purification of Catalase—N. crassa, A. nidulans, S. cerevisiae, and S. coelicolor cells were homogenized 5 times for 30 s in a Bead-Beater with glass beads (710–1180 μm for the fungi and 150–212 μm for the bacterium) at a ratio of 1 g of dry weight per 7.5 ml of 20 mM Hepes, pH 7.2, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.1 mM deferoxferrioxamine B mesylate (Desferal). Sunflower or maize seeds were ground in the same buffer in a mortar.

Human catalase was extracted from blood clots, homogenized in a Potter-Elvehjem homogenizer in the same buffer. Cell extracts were centrifuged 20 min at 6,000 × g and 4 °C. Supernatants were heated 5 min at 60 °C and frozen at −20 °C. After thawing, extracts were centrifuged as before, and the resulting supernatants were used for catalase determinations. These supernatants contained usually 85% of the initial catalase activity. Different catalase activities were separated by electrophoresis and electrolution (see below). Electroluted catalases from N. crassa, analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions, showed a high degree of purification judged by the heavy 80,000-kDa catalase band stained with Coomassie Brilliant Blue, and a low number of faint bands in the gel.

For heme extraction, Cat-1 from N. crassa condensing aerial mycelium was purified to near homogeneity with a method to be described in detail elsewhere. Briefly, the cell extract was frozen and thawed twice, and after centrifugation, the supernatant was precipitated with acetone. The precipitate was resuspended in the same buffer and centrifuged, and the supernatant was fractionated with ammonium sulfate. The 35% ammonium sulfate precipitated fraction, containing most of the activity, was resuspended in 3 ml of 0.5 mM ammonium sulfate and 50 mM PB (solution PS), and then 1 volume of phenyl-Sepharose CL-4B, equilibrated in the same solution, was added. The resulting sludge was stirred for 30 min at room temperature, washed with 50 ml of solution PS, and then loaded onto a small column. The enzyme was eluted with 20 ml of 50 mM PB and 20 ml of 10 mM PB. Fractions having the activity were pooled and concentrated with an Amicon ultrafiltration cell, holding a YM30 filter, and applying N_2 at a pressure of 25 p.s.i. The filter was washed with 1 volume of 10 mM PB which was added to the concentrated enzyme.

Electrophoresis—Two types of PAGE were used, minigels and preparative gels. Minigels were used for detection of catalase activity bands. They were 8 × 9 cm and 0.75 mm thick, 8% polyacrylamide gel, 0.2% bis-acrylamide, made according to the Laemmli procedure (58), but without adding β-mercaptoethanol or boiling the samples. Gels were run at 150 V for 2.5 h and immediately after electrophoresis stained for catalase activity.

For the preparative procedure a supernatant of a cell extract was loaded on a 12 × 16 cm, 2 mm thick, 5.5% polyacrylamide gel, 0.15% bis-acrylamide, having cast a single pit. Electrophoresis was run at 200 V for 4 h, at 4 °C. After electrophoresis, a slice from one side of the gel was stained for catalase activity and used to cut out slices of the gel containing the different catalases. These were then electroeluted using a “Little Blue Tank” from ISCO. Gel slices in pieces were loaded into microtraps containing electrophoresis buffer diluted 10 times. Electroelution was done at 3 W for 3 h at 4 °C. Before recovering the enzyme, electric current was inverted for 10 s. Electrophoresed catalases were stored in aliquots at −20 °C.

Electrofocusing of catalases was done in a 14 × 16 cm, 5.5% polyacrylamide gel, 0.75 mm thick, containing the following concentration and pH ranges of ampholytes, 0.75% of pH 3.5–10.0, 5% of pH 4.0–6.6, 1.7% of pH 5.0–7.0, and 0.8% of pH 2.5–5.0. Acetic acid, 20 mM, was used for acidic ferricyanide and 20 mM sodium bicarbonate as catalyst. A 1% solution of pH 3.5–10.0 and 5% of pH 4.0–6.5 ampholytes in 15% glycerol was added to samples in a ratio 1.3, and these were loaded onto the gel after a prerun of 15 min at 20 W. Electrophoresis was set at 25 W, for 3 h at 25 °C. The gel was then stained for catalase activity.

To determine isoelectric point of the different catalase activities, a lateral strip from top to bottom of the gel was cut into 2-mm slices, each slice was incubated overnight at 22 °C in 1 ml of distilled water, and the pH of each sample was determined.

Photosensitization Reactions—Two systems were used. 1) Catalase (770 μg/ml) in 10 mM PB containing 10 mM uric acid was subjected to oxidation by singlet oxygen generated with riboflavin (1.37 mM) and a fluorescent light source (0.4 W/cm²). A 96-well tissue-culture plate with 30 μl/well was illuminated for 4 h at room temperature. Riboflavin was eliminated by filtration and washing with 15 ml of 50 mM PB through Millipore Ultrafree-CL cellulose (5,000 nominal molecular weight limit) and the enzyme was used for heme extraction and analysis. 2) A drop of 15 μl of purified catalase (40 units) in electrophoretic buffer (2.5 mM Tris, pH 8.9, 19 mM glycine) was suspended from a micropipette tip in a 2-ml vial in close vicinity to a filter paper impregnated with a photoactive dye, usually rose Biscillin, acidine orange, methylene blue, or toluidine blue were also used. Illumination was done for 3 h with an incandescent light bulb (100 W) at 40 cm distance (see Fig. 4A).

Home Extraction and HPLC Analysis—The heme of purified Cat-1 (770 μg) was extracted under dim light with acid acetone. Catalase preparations used for heme extraction were immediately after purification, or after 1 or 3 weeks of storage, and after 1 week of storage modified.

2 L. Kawasaki and J. Aguirre, personal communication.
3 F. Lledia and W. Hansberg, submitted for publication.
by photosensitization with riboflavin and light. The acetone extract was dried with a stream of N₂. Heme was dissolved in 50 μl of acetonitrile containing 0.5% trifluoroacetic acid, and a 0.5% trifluoroacetic acid solution was slowly added to obtain a acetonitrile/water proportion of 60/40.

Heme was separated in a HPLC, using a Waters Delta-Pack HPI C₁₈, 300 Å column (WAT035571) and a water/acetonitrile gradient (curve 7) of 40 to 100% acetonitrile, containing 0.1% trifluoroacetic acid.

RESULTS

Catalase Modification Is Oxygen-dependent—The electrophoretic mobility of purified Cat-1a changed during storage due to a gain in negative charges, giving rise to the modified Cat-1b to Cat-1e. In vivo, Cat-1c and Cat-1e appeared or became prominent during the recurrent hypoxantinase state of the condensation process and under stress conditions. We considered that ROS could react with Cat-1a in vivo and in vitro to give Cat-1c and Cat-1e. Indeed, there was a modification of Cat-1a when stored for 1 week under air or pure dioxygen (Fig. 1, lane 3 and 4), respectively, and no modification was observed when stored under argon (Fig. 1, lane 2).

Catalase Is Modified by Singlet Oxygen—Cat-1a, incubated in the presence of riboflavin and light, exhibited a time-dependent shift in electrophoretic mobility to Cat-1e (Fig. 2A). Cat-1a mobility did not change with light alone (lane 2), nor did its specific activity after 24 h of intense illumination (not shown). Photosensitization reactions give rise to singlet oxygen (mainly ¹O₂), but in presence of a reducing agent the main product is superoxide ion. Reducing agents, such as thiols, clearly inhibited the shift from Cat-1a to Cat-1e, but other reducing agents, dimethyl sulfoxide (Me₂SO) and NADPH, were less effective (Fig. 2B). Added superoxide dismutase did not affect the change in mobility brought about by photosensitization with riboflavin alone (Fig. 2, compare lane 5 of A with lane 1 of B). These results suggest that singlet oxygen caused the Cat-1a modification.

In fact, the singlet oxygen scavengers histidine, tryptophan, tyrosine, and 5-amino salicylic acid (5-ASA) effectively inhibited Cat-1a modification; 1,4-diazabicyclooctane and Trolox, a water-soluble α-tocopherol derivative, were less effective (Fig. 2C). These results confirmed that singlet oxygen caused the changes in electrophoretic mobility of Cat-1a.

A hydroxyl radical may be formed under our experimental conditions. However, this species did not change the mobility of Cat-1a, but caused instead enzyme inactivation in about 3 h. To avoid Cat-1a inactivation by hydroxyl radical, uric acid was used in the experiments shown in Fig. 2. Uric acid is a good hydroxyl radical scavenger and does not react significantly with singlet oxygen (4). In the absence of uric acid the enzyme was inactivated (Fig. 2B, lane 2).

Modification in electrophoretic mobility of Cat-1a was due to an increase in net negative charges. Thus, we compared the isoelectric point of purified Cat-1a to Cat-1e with the isoelectric point of the Cat-1a modified by photosensitization with riboflavin. Cat-1a to Cat-1e were purified from a nondenaturing gel; Cat-1b was the enzyme electrodeth from the zone of the gel between Cat-1a and Cat-1c. The pl of purified catalases decreased progressively from Cat-1a (pI = 5.45) to Cat-1e (pI = 5.25) (Fig. 3A, lanes 1–4), corresponding to a 0.05 change between successive conformers with the exception of Cat-1c. Illuminated Cat-1a with riboflavin showed a similar modification in pl (Fig. 3A, lanes 5–8). No further modification was observed on continuing the photosensitization reaction once the pl of Cat-1e was reached (lane 8 and 9). Purified Cat-1c exhibited two very different isoelectric points, 5.80 and 4.72 (Fig. 3A, lane 3). A possible explanation for these results is offered in Fig. 3B (see below).

Results shown up to now indicate the participation of singlet oxygen in the modification of Cat-1a. However, the excited state of the photosensitizer or a degradation product from it could react with the enzyme. Hartman et al. (59) showed that singlet oxygen is the only ROS that can diffuse through air. Thus, we incubated Cat-1a with a source of singlet oxygen at a distance of less than 2 mm (Fig. 4A). Under these conditions, photoactive compounds changed the electrophoretic mobility of Cat-1a. Rose Bengal was more effective than acridine orange, methylene blue, riboflavin, and toluidine blue in Fig. 4B. Differences were due to the incandescent light source used, because in similar experiments, using a halogen light source at the same light intensity, the sample containing toluidine blue was the most effective (not shown).

Catalases from Different Organisms Are also Modified by Singlet Oxygen—A shift in electrophoretic mobility was observed when Cat-2 of N. crassa was subjected for 3 h to a source of singlet oxygen (Fig. 5A, lanes 3–6). This modified Cat-2 coincided with the mobility of Cat-2' found in conidia and the one formed during storage of purified Cat-2 (not shown). Purified enzymes from other fungi, catalase A from A. nidulans conidia (Fig. 5B) and catalase T from S. cerevisiae (Fig. 5C) were also susceptible to modification by singlet oxygen (lanes 3–6). Purified HPII from E. coli showed a similar modification with riboflavins and light (Fig. 5D, lanes 3–6). Modification of
these catalases was impaired by histidine or 5-ASA (Fig. 5, A–D, lanes 7 and 8). A partially purified cell extract from S. coelicolor showed up to five bands of catalase activity. The band with the lowest mobility shifted to a higher mobility when illuminated in the presence of riboflavin (Fig. 5E, lanes 3–6); this shift was impaired by His or 5-ASA (lanes 7 and 8). It is noted that a band with intermediate mobility appeared with incubation; the appearance of this band was still present in the sample with His (lane 7). None of these catalases were photoinhibited (Fig. 5, A–E, lanes 1 and 2). In contrast, the commercial bovine liver catalase and purified human erythrocyte catalase were photoinactivated (lanes 1 and 2 in Fig. 5, F and G, respectively) (60, 61) and modified by singlet oxygen (lanes 3–6). However, the modification by singlet oxygen did not increase this inactivation (compare lanes 2 and 6). Catalases in a partially purified cell extract from Helianthus annuus and Zea mays seeds were modified by singlet oxygen (Fig. 5, H and I, respectively). Because these catalases were photoinactivated, the extracts were illuminated only for 5–30 min (lanes 2–8) (60). Illumination alone of the sunflower cell extract was sufficient to cause a shift in catalase electrophoretic mobility (Fig. 5H, lanes 1 and 2), but this change in mobility was more prominent in the presence of singlet oxygen (lanes 3–6). 5-ASA inhibited the sunflower catalase activity but not the Z. mays enzyme (Fig. 5, H and I, lane 8). His was effective in preventing the modification in both plant catalases (lane 7). In summary, the modification by singlet oxygen giving rise to more acidic
active conformers seems to be a universal characteristic of catalases.

The Heme of Cat-1a Is Modified by Singlet Oxygen—Heme from Cat-1a was isolated with acid acetone and analyzed by HPLC. The main heme peak from Cat-1a (peak a in Fig. 6A) migrated together with a protoporphyrin IX standard. Three additional smaller peaks were observed: two peaks eluted few minutes earlier (b and c), indicating less hydrophobic porphyrins, and one (peak d) migrated close to unmodified heme. In Cat-1a, modified during 1 week of storage to give mainly Cat-1c (Fig. 6B), the main heme peak decreased and the other three increased (Fig. 6B). This was more evident after storage for 3 weeks (Fig. 6C). When the 1-week stored catalase was modified by photosensitization with riboflavin, peaks a, b, and c disappeared, and peak d increased (Fig. 6D). There was no detectable change in the polypeptides from the acid acetone extracts when analyzed by sodium dodecyl sulfate-PAGE (Fig. 7B). Thus, heme from the Cat-1a, modified during storage or by singlet oxygen, changed by a three-step reaction: the unmodified heme peak a gave rise to b, c, and d; the fully modified heme presented only peak d.

Cat-1 Modification Results in an Increase in Heme Asymmetry—Heme absorbance spectra from Cat-1a and Cat-1e were run in the absence or presence of imidazole. Heme extracted from Cat-1a showed a Soret peak at 369 nm and the usual small peak at 592 nm (Fig. 8A). Imidazole shifted the Soret peak to 408 nm, but its absorbance was low as compared with the hemin standard when shifted from 395 to 412 nm with imidazole (Fig. 8C). Hexa-coordination of the Fe(III)protopheme IX (heme b) shifts the Soret peak from a high spin (395 nm) to a low spin (412 nm) with an increase in absorbance. The different Soret peaks and the low absorbance at 408 nm indicates that heme in Cat-1a is not heme b. Cat-1e, modified by photosensitization, showed a Soret peak at 363 nm that shifted to 408 nm with imidazole, but its absorbance was even smaller than the one of the unmodified heme (Fig. 8B). Absorbance ratio of the Soret peaks (+imidazole/−imidazole) was 1.45 for the unmodified heme and 2.65 for the modified one. These results are consistent with an asymmetric heme in Cat-1a, and an increase in asymmetry with heme modification.

DISCUSSION

Singlet Oxygen Modified Cat-1a—Modification of purified Cat-1a was dependent on O₂; no modification occurred under argon. Singlet oxygen generated by photosensitization reactions brought about a rapid sequential shift in electrophotoretic mobility of purified Cat-1a, similar to the ones observed in vivo.² Modification of catalase by photosensitization reactions was hindered by reducing agents, such as thiols, conditions in which the main product is superoxide instead of singlet oxygen. Singlet oxygen scavengers also impaired the modification of catalase. The best singlet oxygen scavengers were 5-ASA, histidine and tryptophan; the last two have the highest second-order rate constant for reaction with singlet oxygen (4). Besides reacting with singlet oxygen, 5-ASA could bind to the enzyme and hinder the access of dioxygen to the heme; in fact, we have observed inhibition of Cat-1a by 5-ASA.³ The electrophotoretic mobility of Cat-1a did not change with light alone, even when its heme was excited for 2 h with 400 nm monochromatic light of a fluorometer (not shown). Modification of Cat-1a was still observed when the photosensitization reaction was separated from the enzyme by an air barrier, demonstrating that singlet oxygen generated by photosensitization was a prerequisite for modification. On the contrary, inhibition of Cat-1c by 5-ASA hindered the modification of Cat-1c (Fig. 9B), similar to what we observed in vivo.⁴ This observation indicates that Cat-1c modification requires the continuous presence of 5-ASA and air in the enzyme solution. Moreover, the modification of Cat-1c by singlet oxygen was also hindered by reducing agents, such as thiols. In conclusion, while singlet oxygen generated in solution could modify Cat-1c, the presence of catalase and oxygen (air) were necessary for the modification process. This indicates that the modification of Cat-1c by singlet oxygen requires a multi-step reaction, which could involve the reaction of singlet oxygen with the enzyme, followed by the reaction of the resulting product with oxygen and finally the subsequent reaction with the enzyme.

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oxygen was in fact the oxidizing agent. Dioxygen in its basal triplet state, albeit in an infrequent reaction, would be the reacting species during storage of catalase, producing the same modification of catalase. The reaction would be orders of magnitude more frequent with dioxygen in an activated singlet state, producing a rapid modification of catalase.

**Activity of the Modified Catalase**—Cat-1 did not change its specific activity when modified by singlet oxygen. A similar $K_4$ of Cat-1a and Cat-1c and inhibition by 5-ASA were detected. Two reports suggested that catalase activity from beef liver was inhibited by singlet oxygen, formed from photosensitization with tetrasulfonated metallophthalocyanines (62) and by rose Bengal (63). Both studies report increased inactivation of catalase, producing the same absorbance spectra with imidazole suggested a different heme. Modification of Cat-1a to Cat-1c diminished this heme peak giving rise to two more hydrophilic peaks and a slightly more hydrophobic one. Cat-1e, the completely modified enzyme, showed only the most hydrophobic heme peak. These results indicate that the heme was modified by dioxygen in what seems to be a three-step reaction. Modification probably increased the asymmetry of the heme as suggested by the absorbance spectra. Breakage of the $\beta$-bridge between pyrroles II and III, as seen in 35% and 50% of the heme molecules of *P. mirabilis* catalase (55) and the bovine catalase (31), is not compatible with the shift of the Soret peak observed in the presence of imidazole.

Singlet oxygen is a reactive species that could hydroxylate the porphyrin ring. Hydroxylation of heme by singlet oxygen would explain the two less hydrophobic peaks in the HPLC. The heme $b$ incorporated into the HPPI of *Escherichia coli* is hydroxylated spontaneously into cis-heme $d$ without a change in activity (52). A catalase from *Penicillium vitale* has a similar heme $d$ (53). The heme of a *N. crassa* catalase, probable Cat-1, which is the main activity, was described as a chlorin (67). Thus, hydroxylation of heme by singlet oxygen could be part of the modification reaction observed in the heme of Cat-1c and Cat-1e. Besides hydroxylation, another heme modification introducing negative charges is required to explain changes in pl. The exact reaction of singlet oxygen with heme is under current investigation.

**Other Possible Sources for Cat-1a Modification**—Three different catalase genes were reported for *N. crassa* based on catalase electrophoretic mobility and its segregation after crossing different wild type strains (46). The authors assumed no post-translational modification of catalases. In view of our results showing a change in electrophoretic mobility by reaction with singlet oxygen, arguments for three catalase genes are questionable. Because two of the presumed catalases were shown to map very close to each other in the right arm of chromosome III (46), they could be two forms of the same catalase, probably Cat-1a and Cat-1c. Deletion of the *cat-1* gene should give a definitive answer.

A post-translational modification of a methionine to methionine sulfone has been detected in close proximity of the active...
site of the P. mirabilis PR catalase (54). This modification could be related to an oxidative modification of methionine by singlet oxygen, since methionine is one of the most reactive amino acids with singlet oxygen (4). Because most other catalases have a valine in place of the methionine sulfone of the P. mirabilis catalase, this modification is probably exceptional. N. crassa Cat-1 also has a valine residue in this position (72).

Deamination of glutamine and asparagine is often a cause of increased electrophoretic mobility in proteins (68). We have incubated Cat-1a in different alkaline buffers where deamination have been observed. However, modification of Cat-1a during storage was actually lower in alkaline buffers as compared with acid ones. We have discarded electrophoretic mobility changes due to modification of Cat-1 cysteines, by reaction of thiols and disulfides with Cat-1a, and also modification of methionine sulfoxide, by incubating Cat-1a in acidic Me$_3$SO (69). A change due to partial proteolysis was also dismissed because no protease activity could be detected in the purified Cat-1a after 18 h in the presence of azocasein (70). Modification of the polypeptide is improbable since the modified and unmodified polypeptide activity could be detected in the purified Cat-1a after proteolysis of sunflower cotyledons during growth after germination and ent pI (49). Eight charge isoforms appeared in the peroxisomes of B. subtilis, the site of the heme spectra.

Dr. Mario Rivera, Oklahoma State University, for help in the interpretation of the heme spectra.

Acknowledgements—We thank Dr. Jesús Aguirre and Dr. Armando Gómez-Puyou for critically reading the manuscript. We are indebted to Dr. Mario Rivera, Oklahoma State University, for help in the interpretation of the heme spectra.
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