Characterization of the Oxidase Activity of Mammalian Catalase*

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Catalase is a highly conserved heme-containing antioxidant enzyme known for its ability to degrade hydrogen peroxide into water and oxygen. In low concentrations of hydrogen peroxide, the enzyme also exhibits peroxidase activity. We report that mammalian catalase also possesses oxidase activity. This activity, which is detected in purified catalases, cell lysates, and intact cells, requires oxygen and utilizes electron donor substrates in the absence of hydrogen peroxide or any added cofactors. Using purified bovine catalase and 10-acetyl-3,7-dihydroxyphenoxazine as the substrate, the oxidase activity was found to be temperature-dependent and displays a pH optimum of 7–9. The $K_m$ for the substrate is $2.4 \times 10^{-4}$ M, and $V_{max}$ is $4.7 \times 10^{-5}$ M/s. Endogenous substrates, including the tryptophan precursor indole, the neurotransmitter precursor $\beta$-phenylethylamine, and a variety of peroxidase and laccase substrates, as well as carcinogenic benzidines, were found to be oxidized by catalase or to inhibit this activity. Several dietary plant micronutrients that inhibit carcinogenesis, including indole-3-carbinol, indole-3-carboxaldehyde, ferulic acid, vanillic acid, and epigallocatechin-3-gallate, were effective inhibitors of the activity of catalase oxidase. Difference spectroscopy revealed that catalase oxidase/substrate interactions involve the heme-iron; the resulting spectra show time-dependent decreases in the ferric heme of the enzyme with corresponding increases in the formation of an oxyferryl intermediate, potentially reflecting a compound II-like intermediate. These data suggest a mechanism of oxidase activity involving the formation of an oxygen-bound, substrate-facilitated reductive intermediate. Our results describe a novel function for catalase potentially important in metabolism of endogenous substrates and in the action of carcinogens and chemopreventative agents.

Mammalian catalase belongs to a family of Fe-protoporphyrin IX containing proteins that include a variety of cytochromes, globins, and peroxidases and is one of the best characterized antioxidant enzymes (1). As a homotetrameric heme-containing enzyme, it is known for its ability to convert hydrogen peroxide into water and oxygen (catalatic activity), and in the presence of low concentrations of hydrogen peroxide to oxidize low molecular weight alcohols (peroxidatic activity). The conversion of hydrogen peroxide to water by catalase is a two-step process whereby catalase heme Fe$^{3+}$ reduces one molecule of hydrogen peroxide to water, generating a covalent Fe$^{4+}$=O oxyferryl species and a porphyrin cation radical. This reaction intermediate, referred to as compound I, then oxidizes a second hydrogen peroxide molecule forming molecular oxygen and water (1–3) (see Fig. 1). The peroxidatic activity of catalase results from the ability of compound I to oxidize alcohols to aldehydes and water (4–6) (Fig. 1). Each catalase monomer binds one molecule of heme; the holoenzyme also binds two molecules of NADPH, although the precise role of this cofactor in enzymatic activity is unclear, because hydrogen peroxide provides both oxidative and reductive potential during catalysis. Recent studies suggest that NADPH may be important in maintaining catalase in an active state (7).

In mammalian cells, catalase is found at high concentrations in peroxisomes, along with a variety of oxidases and peroxidases (8). It has been suggested that the enzyme protects cells by removing hydrogen peroxide produced by flavin containing oxidases in the peroxisome, thereby preventing the accumulation of toxic levels of this reactive oxygen intermediate (9). However, hydrogen peroxide is important for an array of activities, including peroxidase-mediated metabolism, in cells, and potentially, without this reactive oxygen intermediate, cellular functioning would be limited. In addition, the $K_m$ for the catalatic activity of catalase is $>10$ mM, therefore, at low intracellular concentrations of hydrogen peroxide, this reaction is not kinetically favored, and it is assumed that peroxidases such as glutathione peroxidase or the recently discovered L-Cys peroxiredoxins effectively lower intracellular concentrations of hydrogen peroxide (10). In the present studies, we demonstrated that, in addition to the hydrogen peroxide degrading catalatic activity, mammalian catalases possess an oxidase activity. We found that several peroxidase substrates also function as substrates for mammalian catalase in the absence of hydrogen peroxide. This enzymatic activity is oxygen-dependent and inhibited by classic catalase inhibitors, including sodium azide and 3-amino-1,2,4-triazole as well as several dietary constituents thought to be antioxidants such as indole-3-carbinol and epigallocatechin-3-gallate, a constituent of green tea. Mammalian cells were found to contain endogenous substrates for the enzyme, in particular, indole, an intermediate in tryptophan biosynthesis and $\beta$-phenylethylamine, a neurotransmitter precursor. Taken together, these data suggest that the oxidase activity of catalase may be functionally important in cellular metabolism.

MATERIALS AND METHODS

Enzymes and Chemicals—Catechol (1,2-dihydroxybenzene), ferulic acid (4-hydroxy-3-methoxycinnamic acid), and 1,8-diaminonaphthalene were from Aldrich Chemical Co., 10-acetyl-3,7-dihydroxyphenoxazine, 2′,7′-dichlorofluorescein and resorufin (3H-phenoxazin-3-one)$^2$

$^2$ The abbreviations used are: resorufin, 3H-phenoxazin-3-one; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ATZ, 3-amino-1,2,4-triazole; DCFH, dichlorofluorescein; DMB, (dimethoxybenzidine) o-dianisidine; TBS, Tris-buffered saline with 0.1% Tween 20.
from Molecular Probes (Eugene, OR), and polyclonal anti-catalase antibodies from Abcam Ltd. (Cambridge, UK). Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad. n-Octyl-β-D-glucoside was from Calbiochem. Purified bovine and mouse liver catalase, twice crystallized bovine liver catalase, and all other chemicals were from Sigma, unless otherwise indicated. Purified bovine liver catalase was also obtained from Worthington Biochemical Corp. (Lakewood, NJ). Re-purified bovine liver and keratinocyte catalase were prepared as previously described (11).

Cell Culture—HA-1 and catalase-overexpressing OC5 Chinese hamster fibroblasts were obtained from Douglas Spitz at the University of Iowa (Iowa City, IA). All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 units/ml) and 5% CO₂ in a humidified incubator. All tissue culture reagents were obtained from Invitrogen. To prepare lysates, cells were scraped from culture dishes in 5 ml of phosphate-buffered saline and centrifuged (20000 × g, 5 min). Cell pellets were washed and resuspended in phosphate-buffered saline supplemented with 0.5% n-octyl-β-D-glucoside. After 1 min, cells were sonicated on ice for 30 s using a sonicator (ARTEK Systems Inc., Farmingdale, NY). Lysates were frozen at −70 °C until analysis.

Enzyme Assays—For standard catalase oxidase activity assays, reaction mixtures contained 50 mM phosphate buffer, pH 7.4, and 2.2 μM catalase in a reaction volume of 100 μl. For cell lysates, 100 μg of protein was added. The reaction was initiated by the addition of 20 nmol of 10-acetyl-3,7-dihydroxyphenoxazine. Unless otherwise indicated, all reactions were performed at room temperature for 10 min. Detection of the fluorescent product resorufin was quantified using an HTS 7000 Spectral changes in absorbance, when compared with control catalase, from the oxygen-depleted catalase mixture were transferred into cuvettes sealed under nitrogen. Difference spectra were recorded using oxygen-depleted catalase without substrate as a reference. Oxygen-depleted catalase was then re-aerated, and the difference spectra were recorded using re-aerated catalase without substrate as a reference.

**Kinetic Analysis**—Catalase (2.2 μM) was incubated with increasing concentrations of 10-acetyl-3,7-dihydroxyphenoxazine in reaction buffer, and fluorescence of resorufin product formed was measured every 30 s for 10 min. Resorufin was quantified using a standard curve. Lineweaver–Burke analysis was used to analyze reaction kinetics (12). A polarographic system fitted with a Clark oxygen electrode (Yellow

**Figure 1. The reactions of catalase.** Left panel, the hydrogen peroxide-degrading catalatic activity. Interaction of the catalase heme with a correctly positioned molecule of hydrogen peroxide results in the formation of a transient oxyferryl porphyrin centered radical (compound I). Binding of a second molecule of hydrogen peroxide results in breakdown of the intermediate state releasing molecular oxygen and water. Center panel, peroxidative activity of catalase. Interaction of catalase compound I with low molecular weight alcohols results in substrate oxidation via single electron transfer events. Under some circumstances catalatic or peroxidative activities may be interrupted by the formation of compound III, a catalytically inactive intermediate of catalase (not shown). Note that no net consumption of molecular oxygen occurs during the catalatic or peroxidative activities of the enzyme. Right panel, oxidase activity of catalase. Oxidation of the catalase heme with a strong reducing substrate such as benzidine (HB) and molecular oxygen results in formation of a compound II-like intermediate. In subsequent electron transfer the substrate is oxidized and the enzyme returns to its ground state. This model is consistent with the consumption of ~1 mol of oxygen for 2 mol of product formed. An incomplete reaction could potentially result in formation of radical centered intermediates and production of superoxide (dashed arrow).
Catalase Oxidase Activity

Springs Instruments, Yellow Springs, OH) was used to evaluate oxygen utilization by catalase oxidase. Reactions were run at 37 °C in 50 mM phosphate buffer, pH 7.4.

Measurement of Catalase Oxidase Activity in Intact Cells or Cell Lysates—Cells were scraped from 80% confluent 6-well plates and centrifuged at 200 × g for 5 min. Pellets containing the cells were washed and resuspended in phosphate-buffered saline (10⁶ cells/ml). To begin the reactions with intact cells, 10-acetyl-3,7-di-hydroxyphenoxazine (200 μM final concentration) was added to the cells. Aliquots of the cell suspension (100 μl) were then placed in triplicate in wells of a black 96-well tissue culture plate (Costar, Corning, NY) and immediately analyzed on the fluorescence micro plate reader as described above.

RESULTS

Characterization of the Oxidase Activity of Catalase—Initially, 10-acetyl-3,7-di-hydroxyphenoxazine was used as the substrate to characterize the oxidase activity of catalase, because it is a non-fluorescent electron donor, which forms resorufin allowing for highly sensitive absorbance and fluorescence enzyme assays (see Reaction 1).

Oxidase activity was identified in purified catalases obtained from several different sources, including mouse and bovine liver, mouse and human keratinocytes, and hamster fibroblasts (Figs. 2 and 3A, and data not shown) (11). This activity was also detected in highly purified enzyme preparations, including twice crystallized bovine catalase and repurified bovine liver catalase (Fig. 3A) (11).

In absorbance assays the formation of a peak at 571 nm, corresponding to resorufin production, was detectable within 1 min (Fig. 2, left panel). Similar results were found in fluorescence assays (Fig. 3A). Using bovine liver catalase, the oxidase activity was further explored. Catalase oxidase activity was found to be linear with respect to time and protein concentration (Figs. 2 and 3A, inset) and was pH-dependent, with an optimum ranging from pH 7.0 to 9.0 (Fig. 3B). The rate of the reaction was also sensitive to temperature; in these experiments the rate of oxidase activity decreased when the incubation mix was placed on ice, and recovered when the mix was returned to room temperature (Fig. 3C and inset). Enzyme activity was inactivated by heating; inhibition of 50% of the enzyme activity occurred at ~52 °C (Fig. 3D). This is similar to the thermal stability of the catalatic activity of the enzyme (13). Deoxygenated and nitrogen-saturated reaction mixtures inhibited catalase oxidase activity (Fig. 2, left panel, inset) indicating that the reaction was oxygen-dependent. In addition, Fig. 4 shows that the catalase oxidase reaction readily consumes oxygen. Under these conditions, we found that 1.2 nmol of molecular oxygen/min/mg catalase was consumed for every 2.0 nmol of product formed.

The catalase oxidase activity was unaltered by the addition of hydrogen peroxide (100 nM to 10 mM) and/or ethanol (220 μM to 220 mM) to the reaction mix either before or after the addition of the substrate, indicating that the oxidation reaction was distinct from the peroxidase reaction (data not shown). Enzyme activity was also unaffected by the addition of cofactors including NADPH, NADP, NAD, NADH, FAD, or FMN (data not shown). Oxidation of 10-acetyl-3,7-di-hydroxyphenoxazine by catalase was reversible and saturable. The Michaelis constants (Kₘ for the substrate were 2.4 × 10⁻⁵ M and 2.5 × 10⁻⁵ M and the V_max 4.7 × 10⁻⁴ M/s and 5.9 × 10⁻⁵ M/s for bovine and mouse liver catalases, respectively. For the bovine liver catalase, the kcat for the reaction was 22 s⁻¹ and the catalytic efficiency (kcat/Km) was 9.0 × 10⁶ M⁻¹ s⁻¹. Taken together these data indicate that mammalian catalases exhibit a previously unrecognized oxidase activity that is independent of hydrogen peroxide.

In our next series of studies we determined if catalase oxidase activity was detectable in cultured cells. For these studies, we used a fibroblast cell line (HA-1) and a cloned variant adapted for resistance to hydrogen peroxide (OC5 cells) (14). OC5 cells express ~20-fold more catalase than the parental cells (15) (Fig. 5, inset). We found that 10-acetyl-3,7-di-hydroxyphenoxazine was readily oxidized in intact HA-1 and OC-5 cells as well as in cell lysates (Fig. 5). Significantly more activity was...
evident in cells overexpressing catalase and in lysates from these cells, a finding consistent with the increased catalase activity in these variants.

Spectral Analysis of Catalase Oxidase—The reduced and oxidized intermediate states of catalase can be identified by specific spectral characteristics (16, 17). Interconversion of these different states provides important insights into the reaction mechanism. Catalase reaction intermediates are identified based on changes in the Soret bands of heme, as well as in the visible spectrum. Therefore, we next examined the spectral characteristics of the catalase oxidase activity using difference spectrophotometry. Difference spectroscopy allowed us to characterize the catalase oxidase reaction mechanism and to measure the kinetics of enzyme-substrate complex and product formation. As indicated above, with 10-acetyl-3,7-dihydroxyphenoxazine as the substrate, formation of resorufin was evident in the difference spectra (Fig. 2, left panel). We also found that substrate binding to catalase caused marked shifts in the heme absorption characteristics of the enzyme. Thus, the binding of 10-acetyl-3,7-dihydroxyphenoxazine to bovine liver catalase resulted in a time-dependent formation of a trough at ~403 nm and a peak at around 425 nm (Fig. 2, right panel). The optimum for this peak increased to 434 nm over the 20-min assay period. For mouse liver catalase, the trough was observed at ~404 nm, and the peak at around 432 nm (data not shown). The decrease in the Soret peak of catalase at 404 nm is the apparent result of a decrease in ferric heme with a corresponding increase in an oxyferryl species as shown by the time-dependent appearance of the peak at 434 nm (17). We suggest that this species is compound II or compound II-like (17). Over the time period examined, it appears that substrate oxidation by catalase continually leads to the formation of this intermediate. These data are consistent with our observation that the oxidase reaction utilizes molecular oxygen (Fig. 4). They also indicate that the catalase oxidase activity involves alterations in the oxidation state of the heme-iron moiety.

Identification of Catalase Oxidase Substrates and Inhibitors—Previous studies have demonstrated that halides inhibit the activity of catalase as well as a number of oxidases (18–21). NaF was found to be a highly effective inhibitor of catalase oxidase activity. Kinetic analysis revealed that NaF was an uncompetitive inhibitor with a $K_i$ of $7.5 \times 10^{-4} \text{M}$ (Fig. 6A and TABLE ONE). Therefore, NaF appears to function by binding directly to the enzyme-substrate complex, but not to free catalase. This binding would render catalase oxidase catalytically inactive, without affecting its affinity for the substrate. It should be noted that NaF by itself did not alter the difference spectrum of catalase indicating that its action is not dependent on changes in catalase heme (data...
Interestingly, the classic catalase inhibitors sodium azide
and 3-aminotriazole were found to be competitive inhibitors of catalase
oxidase activity, but only at high concentrations ($K_i = 9.9 \times 10^{-3}$ M and
$7.1 \times 10^{-2}$ M, respectively; data not shown).

Because 10-acetyl-3,7-dihydroxyphenoxazine is also a peroxidase
substrate (22), we next tested other peroxidase substrates, including
catechol, DCFH, and pyrogallol, as substrates for catalase oxidase
activity (23, 24) (see TABLE ONE for structures). Catalase readily oxidized all
three of these compounds (Fig. 7). These data indicate that catalase has
broad substrate specificity, a finding consistent with similar reports on
the substrate specificity of peroxidases (25).

We next determined if the peroxidase/catalase oxidase substrates
compete with 10-acetyl-3,7-dihydroxyphenoxazine for catalase
activity. All three compounds were found to readily inhibit the oxida-
tion of 10-acetyl-3,7-dihydroxyphenoxazine (TABLE ONE). Kinetic analysis of these reactions revealed that these catalase ox-
idase substrates were competitive inhibitors of the reaction (Fig. 6B
and data not shown). DCFH was the most effective inhibitor ($K_i =
3.2 \times 10^{-5}$ M) (TABLE ONE), followed by catechol ($K_i = 5.9 \times 10^{-4}$
M), and pyrogallol ($K_i = 1.7 \times 10^{-3}$ M). Other peroxidase substrates,
including 5-bromo-4-chloro-3-indolyl phosphate ($K_i = 5.7 \times 10^{-5}$
M), $o$-dianisidine ($K_i = 9.9 \times 10^{-3}$ M), $\beta$-phenethylamine ($K_i = 2.2 \times
10^{-4}$ M), luminol ($K_i = 7.7 \times 10^{-4}$ M), and 2,2'-azino-bis(3-ethylben-
zhiazoline-6-sulfonic acid) (ABTS, $K_i = 8.1 \times 10^{-4}$ M) were also
effective competitive inhibitors of the reaction (TABLE ONE and
not shown). $\beta$- Phenethylamine is of interest because it is a neuro-
transmitter precursor (26).

Interestingly, another aromatic amine that is a peroxidase sub-
strate, 4-dimethylaminoantipyrine (27), was found to be non-competitive
inhibitor of catalase oxidase activity ($K_i = 9.9 \times 10^{-4}$ M), when
10-acetyl-3,7-dihydroxyphenoxazine was used as the substrate (Fig. 6C
and TABLE ONE). This suggests that it has equal affinity for catalase
and the catalase-substrate complex, where the presence of bound sub-
strate does not affect the binding of inhibitor and vice versa. These data
indicate that the peroxidase substrates can function to inhibit catalase oxidase by distinct mechanisms. Moreover, their mechanism of inhibition of catalase oxidase is distinct from NaF.

O-Dianisidine is a benzidine analog that is a well recognized chemical carcinogen (28). Structurally related carcinogens, including benzidine, 3′,2′-dimethyl-4-aminobiphenyl, 4,4′-diamino-3,3′-dimethylbiphenyl (o-tolidine), and 3,3′-diaminobenzidine, like o-dianisidine, were all effective inhibitors of 10-acetyl-3,7-dihydroxyphenoxazine oxidation by catalase (TABLE TWO, Fig. 8, left panel, and data not shown). These benzidines were all competitive inhibitors with respect to 10-acetyl-3,7-dihydroxyphenoxazine. Benzidine was the most active with a $K_i$ of 2.1 $\times$ 10^{-5} M (TABLE TWO). These data further demonstrate the broad substrate specificity of catalase oxidase and implicate the enzyme activity as potentially important for activating carcinogens. It is of interest that the $K_i$ values of the different benzidines varied ~50-fold. This was related to the electron donating aromatic substitutions, which appear to interfere with activity of benzidines in the catalase oxidase assay.

Similar to the substrate 10-acetyl-3,7-dihydroxyphenoxazine, the benzidines also altered the heme spectra in catalase (Fig. 8, right panel, and data not shown). Difference spectroscopy analysis of the reaction of o-dianisidine with the enzyme revealed a time-dependent decrease in ferric heme, indicated by the formation of a trough at 402 nm, and the formation of an oxyferryl species, indicated by a peak that increased over time at 434 nm (Fig. 8, right panel). Over time, o-dianisidine generated additional absorption peaks in the range of 530–540 nm and 560–580 nm, accompanied by the appearance of an isosbestic point at ~408 nm, characteristic of formation of compound II (Fig. 8, right panel inset) and possibly, compound III (17). These data indicate that the catalase oxidase reaction with structurally distinct substrates is likely to occur via generally similar reaction mechanisms.

ABTS and pyrogallol are also known substrates for laccases (29, 30), a class of fungal enzymes, which use molecular oxygen to oxidize phenols and aryl amines (18). Similar to peroxidase substrates, the laccase substrates 1,8-diaminonaphthalene and ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid), and the related vanillic acid (4-hydroxy-3-methoxy benzoic acid) also inhibit 10-acetyl-3,7-dihydroxyphenoxazine oxidation. All were competitive inhibitors with respect to 10-acetyl-3,7-dihydroxyphenoxazine with $K_i$ values of 1.1 $\times$ 10^{-4} M, 1.3 $\times$ 10^{-6} M, and 2.3 $\times$ 10^{-4} M, respectively (TABLE ONE and not shown). Taken together, our findings demonstrate that a spectrum of endogenous, as well as dietary phenols and aryl amines, are substrates and/or inhibitors for the oxidase activity of catalase.

**DISCUSSION**

Our data demonstrate that mammalian catalase possesses a previously unrecognized oxidase activity. This activity was identified in catalases isolated from diverse sources, including bovine and mouse liver, mouse and human keratinocytes, fibroblasts, as well as in highly purified preparations of the enzyme, including twice-crystallized catalase and re-purified catalase derived from bovine liver. In addition, this activity could also be detected in intact cells and cell lysates. That intact cells possess this activity suggests that it may play a role in cellular metabolism. This is supported by our discovery that there are at least two endogenous substrates/inhibitors of catalase oxidase activity (see further below). Our findings that the oxidase activity exhibited unique substrate and cofactor requirements, as well as reaction mechanisms, demonstrate that catalase oxidase activity is distinct from its previously recognized catalatic and peroxidatic activities. In this regard, the new enzymatic activity, which was oxygen-dependent, did not require the addition of hydrogen peroxide or any additional cofactors, similar to the oxidase activity found in catalase-peroxidase KatG (33). In addition, unlike the catalatic activity of catalase, which does not strictly follow classic Michaelis-Menten kinetics, presumably due to the high reaction rate and inactivation of the enzyme at high hydrogen peroxide concentrations (13, 34), catalase oxidase activity was readily saturable and reversible. The apparent affinity of the enzyme for 10-acetyl-3,7-dihydroxyphenoxazine ($K_m = 2.4 \times 10^{-4} M$) is significantly greater than for its catalatic metabolism of hydrogen peroxide ($K_m = 2.5 \times 10^{-2} M$).

However, hydrogen peroxide is metabolized much more efficiently than 10-acetyl-3,7-dihydroxyphenoxazine ($k_{cat} = 4.0 \times 10^9 M^{-1} s^{-1}$ versus $9.0 \times 10^8 M^{-1} s^{-1}$, respectively). Our studies indicate that the rate of oxidation of 10-acetyl-3,7-dihydroxyphenoxazine is similar to the metabolism of many carcinogens by P450 enzymes (35). It is important...
### TABLE ONE

**Ability of various electron donors to inhibit catalase oxidase activity**

| Inhibitor**a** | Structure | $K_i$ (M) |
|----------------|-----------|-----------|
| 2',7'-dichlorofluorescein | ![Structure](image1.png) | $3.2 \times 10^{-5}$ |
| 5-bromo-4-chloro-3-indolyl-phosphate | ![Structure](image2.png) | $5.7 \times 10^{-5}$ |
| 1,8-diaminonaphthalene | ![Structure](image3.png) | $9.9 \times 10^{-5}$ |
| 2-phenethylamine | ![Structure](image4.png) | $2.2 \times 10^{-4}$ |
| indole | ![Structure](image5.png) | $4.3 \times 10^{-4}$ |
| 1,2-dihydroxybenzene (catechol) | ![Structure](image6.png) | $6.0 \times 10^{-4}$ |
| sodium fluoride | NaF | $7.5 \times 10^{-4}$ |
| 5-amino-2,3-dihydro-1,4-phthalazine-dione (luminol) | ![Structure](image7.png) | $7.7 \times 10^{-4}$ |
| 2,2'-azino-bis [3-ethyl benzthiazoline-6-sulfonic acid] | ![Structure](image8.png) | $8.1 \times 10^{-4}$ |
| 4-dimethylaminoantipyrine | ![Structure](image9.png) | $9.9 \times 10^{-4}$ |
| 1,2,3-trihydroxybenzene (pyrogallol) | ![Structure](image10.png) | $1.7 \times 10^{-3}$ |
| salicylhydroxamic acid | ![Structure](image11.png) | $1.8 \times 10^{-3}$ |

**a** Compounds were evaluated as inhibitors of bovine liver catalase oxidase using 10-acetyl-3,7-dihydroxyphenoxazine as the substrate as described under "Materials and Methods."
to note that 10-acetyl-3,7-dihydroxyphenoxazine is an artificial substrate, and it is likely that better substrates will be identified, including endogenous metabolites.

The reaction mechanisms for the catalase catalatic and peroxidatic reactions have been studied extensively. In native catalase, protoporphyrin-bound iron exists in a high spin ferric state ($Fe^3+$). Mechanistically, both the catalatic and peroxidatic reactions are believed to be similar; in a common first step, interaction of the enzyme with a molecule of hydrogen peroxide leads to the formation of compound I (Fig. 1, left panel). This heme-based intermediate is formed by electron donation, generating an oxyferriyl species ($Fe^{4+}=O$) and a porphyrin $\pi$-cation radical. In the second step of the catalatic reaction, two electrons derived from an additional molecule of hydrogen peroxide are used to convert the enzyme from the compound I state back to the resting ferric state, leading to the release of oxygen and the formation of water. Alternatively, catalase compound I may oxidize small molecular weight alcohols mediating the peroxidatic reactions of catalase (Fig. 1, center panel). In addition, under certain circumstances, for example in the presence of excess hydrogen peroxide, compound I, in a single electron transfer, may be converted to compound II, a porphyrin-oxyferryl moiety. In subsequent reactions, compound II can react either with another molecule of hydrogen peroxide to form inactive compound III (oxyferrous catalase), or with single electron donors, including NADPH and can be reduced back to the ferric state (Fig. 1, see more below). In fact, it is thought that catalase-bound NADPH may prevent, as well as reverse, the accumulation of compound II (7, 36). Difference spectroscopy has been used to analyze both the catalatic and alcohol peroxidase activities of catalase. Spectroscopic analysis of the catalase oxidase activity indicates that the reaction involves heme iron. A mechanism mediated by single electron transfers leading to the formation of compound II, through interactions of heme iron and molecular oxygen, is consistent with our findings (Fig. 1, right panel). This is supported by our observation that, in a manner similar to that observed in heme peroxidases, initiation of catalase oxidase activity resulted in formation of a clearly defined isosbestic point at about 408 nm (37) (Fig. 2, right panel). In this scheme, endogenous or exogenous single electron donors present in cells presumably convert the enzyme back to the ferric state. Our findings that NADPH does not affect 10-acetyl-3,7-dihydroxyphenoxazine-induced compound II formation are significant, because they suggest that the regulation of catalase oxidase activity is independent of this cofactor. Consumption of oxygen during the reaction and the marked persistence of the compound II spectra we observed, tempt one to speculate that the oxidase activity of catalase, like the peroxidase activity, may involve sequential single electron transfer events. In addition, our data suggest that, in the presence of oxygen, rapid events involving donation of an electron by the substrate and interaction of molecular oxygen with the heme iron result in the formation of an oxyferryl moiety and a substrate-centered cationic radical, stabilization of this radical and accumulation of a compound II-like intermediate. We speculate that, in a less facile reaction, potentially mediated by a hydride transfer, the substrate is oxidized and the enzyme returned to the resting state. Our finding that 1.2 mol, rather than 2 mol, of oxygen was consumed for each mole of product produced, supports such a mechanism. This mechanism may compete with an alternative path of incomplete reactions, potentially mediated by the formation of radical-centered intermediates. However, at the present time, the precise mechanism of the oxidase reaction is unclear, because the structures of the reaction intermediates and products are unknown.

Interestingly, as observed with the activities of many peroxidases, the oxidase activity of catalase exhibits broad substrate specificity. Thus, three structurally diverse substrates, in addition to 10-acetyl-3,7-dihydroxyphenoxazine, were identified: catechol, DCFH, and pyrogallol. These substrates were found to be competitive inhibitors of catalase oxidase, with respect to 10-acetyl-3,7-dihydroxyphenoxazine, suggesting that they function by similar mechanisms. Because resorufin, the product of 10-acetyl-3,7-dihydroxyphenoxazine oxidation, is intensely fluorescent, it allowed us to rapidly screen and identify inhibitors of

### TABLE TWO

| Inhibitor          | Structure | $K_i$ (M) |
|--------------------|-----------|-----------|
| benzidine          |           | $2.1 \times 10^{-5}$ |
| 3,2'-dimethyl-4-aminobiphenyl | | $6.8 \times 10^{-5}$ |
| 3,3'-dimethoxybenzidine (o-dianisidine) | | $9.9 \times 10^{-5}$ |
| 4,4'-diamo-3,3' dimethylbenzophenyl (o-tolidine) | | $2.5 \times 10^{-4}$ |
| 3,3'-diaminobenzidine | | $9.9 \times 10^{-4}$ |

1 Benzidine and various analogs were evaluated as inhibitors of bovine liver catalase oxidase as described under "Materials and Methods."
catalase oxidase, some of which may also be substrates of the enzyme. Almost all inhibitors, which included a number of structurally diverse compounds many of which are also peroxidase substrates, were found to be competitive, with respect to 10-acetyl-3,7-dihydroxyphenoxazine. These include 5-bromo-4-chloro-3-indoyl phosphate, luminol, and 1,8-diaminonaphthalene, a laccase substrate. Laccases, which transfer electrons from a substrate to oxygen, are copper-containing monofunctional oxidases (p-diphenol:oxygen oxidoreductase) that, similar to peroxidases, are also known to exhibit broad substrate specificity (18).

These findings raise questions about the nature of substrate binding to catalase. Although the precise site on the catalase structure for its oxidase activity is unknown, the substrate-binding regions of many peroxidases and catalase-peroxidases have been well characterized (38, 39). These enzymes generally have a substrate binding pocket large and varied enough to facilitate the binding of many diverse substrates. It is possible that catalase has a similar pocket that allows it to mediate the oxidation of the broad range of substrates for the oxidase reaction. Based on the crystal structure of bovine liver catalase, we have identified one such region. This potential pocket is adjacent to the β-barrel region of catalase, abutting the heme environs and defined by Phe81, Phe131, and Trp141 (Fig. 9). In this regard, this particular region is thought to be occupied by large inhibitors of the catalase catalatic reaction, including salicylic acid (39). However, compounds such as the sterically bulky 4-dimethylaminopyridine are unlikely to fit well into this pocket, as evidenced by the fact that it inhibits catalase oxidase by a distinct mechanism. Of particular interest was our finding that 3-aminotriazole is a competitive inhibitor of the catalase oxidase reaction. Following binding to catalase, 3-aminotriazole is thought to covalently modify the enzyme via an oxidized intermediate (38, 39). Based on alterations in localized hydrogen bonding, a binding pocket for 3-aminotriazole in the main access channel for peroxide, localized between His74 and Asn348 in the crystal structure, has been identified (38). The potential binding pocket for oxidase substrates that we have defined overlaps this region. Our findings, in conjunction with the observation that a compound II-like state is stabilized during catalase oxidase activity, suggest that substrate oxidation is mediated by formation of an oxyferryl intermediate and the resultant radical.

Structure-activity analysis of the oxidation of the benzidine derivatives by catalase provides additional insights into the reaction mechanism. It was unexpected that there was an inverse relation between the activity of these compounds and electron donating substituents on their aromatic rings. We speculate that electron-donating moieties limit polarity induced by the presence of the benzidine in the substrate-binding region of catalase, a potentially important factor in aligning the substrates with the appropriate amino acids regulating the oxidation reaction. Taken together, our findings are consistent with binding of catalase oxidase substrates to a pocket adjacent to the β-barrel region where the electronegative substrates are optimally oriented through interaction with Arg74, Arg111, Arg364, and Phe131 (Fig. 9).

Also of interest is our finding that various benzidines and several dietary constituents, some of which are anti-carcinogens, are active in the catalase oxidase assay. Many of these compounds, including benzo-3,4-dihydric, are also peroxidase substrates. It is well recognized that peroxidases have the capacity to activate chemical carcinogens such as benzidine (40). One electron oxidation of benzidines can generate reactive imines, which react further to form carcinogenic azobis derivatives (40, 41). Currently, it is unknown if catalase oxidation of benzidines results in the formation of carcinogenic derivatives. The activity of indole derivatives, catechol, vanillic, and ferulic acid, as well as epigallocatechin-3-gallate, is presumably dependent on oxidizable ring substitutions and/or ring nitrogens. Of interest is our finding that several dietary constituents known to inhibit the carcinogenic process,

![FIGURE 8. Effects of o-dianisidine on catalase. Left panel, inhibition of 10-acetyl-3,7-dihydroxyphenoxazine oxidation by o-dianisidine. Catalase oxidase activity was measured in the absence and presence of increasing concentrations of o-dianisidine. Right panel, catalase difference spectra. Catalase (2.2 μM) was added to the reference and sample cuvettes. Difference spectra were generated at 1-min intervals immediately after the addition of 10 μM o-dianisidine to the sample cuvette. Inset, difference spectra of compound II. Catalase compound II was formed by adding peroxoacetic acid and potassium ferrocyanide to the sample cuvette (12).](http://www.jbc.org/)

![FIGURE 9: Potential site for catalases oxidase substrates. A proposed binding site (A) for catalase oxidase substrates was identified in the crystallographic structure of catalase.](http://www.jbc.org/)
including ferulic acid, indole-3-carbinol, and the green tea constituent epigallocatechin-3-gallocate (42–44), were active in the catalase oxidase assay. One could speculate that, if catalase oxidase is important in activating carcinogens such as benzidine, the ability of the dietary constituents to interfere in this process may be important in their anti-cancer activity.

Two endogenous metabolites were active in our catalase oxidase assay, the tryptophan precursor, indole, and the neurotransmitter precursor, β-phenethylamine. Additional, as yet unidentified metabolites, were also identified in acid-soluble extracts of fibroblasts and keratinocytes that inhibit oxidation of 10-acetyl-3,7-dihydroxyphenoxazine by catalase.3 These compounds may be substrates and/or regulators of catalase oxidase and suggest that the enzyme is likely to be functionally important in cellular metabolism. It remains to be determined if dietary components active in the catalase oxidase assay will modulate the action of endogenous metabolites in cells.

In summary, we have characterized a novel oxidase activity of mammalian catalase. This oxidase activity, which is distinct from the catalatic and peroxidatic activities of the enzyme, utilizes diverse substrates and inhibitors, including carcinogens, anticarcinogens, and endogenous ligands. The oxidase reaction requires molecular oxygen and appears to occur via single electron transfers through a compound II-like intermediate. Of interest are recent crystallographic studies demonstrating dioxygen bound to heme in Helicobacter pylori catalase (45), a finding that suggests a possible mechanism of oxygen utilization in the mammalian catalase oxidase reaction. A previous assumption has been that catalase functions only to remove excess cellular hydrogen peroxide and/or to metabolize small molecular weight electron donors such as ethanol. Our studies indicate that the enzyme possesses broader functions, possibly in metabolism and/or detoxification reactions. Further studies are needed to more precisely characterize the site and reaction mechanism for the oxidase reaction in catalase and to define the role of this enzymatic activity in cellular metabolism.

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