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CHARACTERIZATION OF HYDROGEN PEROXIDE AND SUPEROXIDE DEGRADING PATHWAYS OF *ASPERGILLUS NIGER* CATALASE: A STEADY-STATE ANALYSIS

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The oxidized intermediates generated upon exposure of *Aspergillus niger* catalase to hydrogen peroxide and superoxide radical fluxes were examined with UV-visible spectrophotometry. Hydrogen peroxide and superoxide radical were generated by means of glucose/glucose oxidase and xanthine/xanthine oxidase systems. Serial overlay of absorption spectra in the Soret (350-450 nm) and visible regions (450-700 nm) showed that the decomposition of hydrogen peroxide by the catalase of *Aspergillus niger* can proceed through one of two distinct pathways: (i), the normal "catalatic" cycle consisting of ferric catalase → Compound I → ferric catalase; (ii), a longer cycle where superoxide radical transforms Compound I to Compound II which is then converted to the resting ferric enzyme via Compound III. The latter sequence of reactions ensures that the catalase of *Aspergillus niger* restores entirely its activity upon exposure to low levels of superoxide radicals due to the actions of oxidases.

KEY WORDS: Catalase; *Aspergillus niger*; superoxide; hydrogen peroxide.

INTRODUCTION

Oxygen centered radicals and other reactive oxygen species are continuously produced in living organisms and are potentially damaging to cellular lipids, proteins and nucleic acids[1,2]. To reduce the levels of such deleterious oxygen species, living cells possess a battery of protective enzymes; the superoxide anion ($O_2^{\cdot-}$) is eliminated by superoxide dismutase[3], which catalyses its conversion to hydrogen peroxide (H_2O_2) plus oxygen, and hydrogen peroxide is removed by catalases[4], which convert it to water plus oxygen, and by peroxidases[5], which reduce it to water, using a variety of reductants available to the cell.

Catalases and peroxidases are related enzymes in so far as they have identical ferric protoporphyrin IX prosthetic groups[5,6]. Both enzymes catalyse the reaction by the same central pathway, the first step of which involves the two-electron oxidation of the enzyme by hydrogen peroxide to form a species referred to as Compound I[5,7]:

$$\textit{Peroxidase or catalase} + H_2O_2 \rightarrow \textit{Compound I} + H_2O$$

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This oxidation occurs by removal of one electron from the iron atom to form an oxyferryl center[8,9], $Fe^{IV} = O$, while the second electron is removed from a porphyrin orbital[9] or from an amino acid side-chain[10] to form an organic radical. Compound I of the peroxidases undergoes two sequential one-electron reductions with most of its substrates which regenerates the ferric enzyme[5,7]:

$$\textit{Compound I} + AH \rightarrow \textit{Compound II} + A^{\cdot}$$

$$\textit{Compound II} + AH \rightarrow \textit{Peroxidase} + A^{\cdot} + H_2O$$

(where AH represents a one-electron donor)

Compound II is a catalytic intermediate in which the $Fe^{IV} = O$ species remains intact but the porphyrin or amino acid radical has been reduced[11]. Hydrogen peroxide is not a good substrate for either Compound I or II of the peroxidase[5]. In contrast, Compound I of catalases efficiently ensures the two-electron oxidation of hydrogen peroxide to form O_2 and water, but reacts poorly with other substrates[4,7]:

$$\textit{Compound I} + H_2O_2 \rightarrow \textit{Catalase} + O_2 + H_2O$$

Compound II can be formed from catalase by one-electron reduction of Compound I with ferrocyanide or ascorbate[6]. However, it is not a catalytic intermediate in the two-electron reduction of Compound I by hydrogen peroxide. Moreover, compared to Compound II of horseradish peroxidase, it is a poor oxidant with respect to most substrates[4].

Both classes of enzymes have an appreciable affinity for the perhydroxyl radical ($HO_2^{\cdot}$) and its conjugate base, the superoxide anion ($O_2^{\cdot-}$). Several peroxidase types and the catalase from bovine liver are known to react rapidly with $O_2^{\cdot-}$, resulting in formation of Compound III, a form of the enzyme in which a superoxide anion is ligated to the heme iron[5,12,13,14]:

$$\textit{Peroxidase or catalase} + O_2^{\cdot-} \rightleftharpoons \textit{Compound III}$$

Alternatively superoxide radicals are removed with great efficiency by Compound I of horseradish peroxidase and bovine liver catalase[5,13,15]:

$$\textit{Compound I} + O_2^{\cdot-} \rightarrow \textit{Compound II} + O_2$$

Apparently, $HO_2^{\cdot}/O_2^{\cdot-}$ couple does not react with horseradish peroxidase Compound II[16] and, up to now, there is no reliable indication that it reacts with Compound II of catalases.

Several lines of evidence have recently indicated that myeloperoxidase[17,18], ovoperoxidase[19] and chloroperoxidase[20] catalyse the breakdown of $O_2^{\cdot-}$. So far, this "superoxide peroxidase activity" has never been reported with catalase. On the contrary, it has been observed that $O_2^{\cdot-}$ inhibits bovine liver catalase and that it does so in two distinct ways[21]. The first of these is a rapid process which can be prevented and reversed by superoxide dismutase. The second is a slow process, which can be prevented but not reversed by superoxide dismutase and which can be both prevented and reversed by ethanol[21]. It has been proposed that the two kinds of inhibition are due to the reaction of $O_2^{\cdot-}$ with ferric catalase and Compound I, to yield Compound III and Compound II respectively. Since Compounds II and III do not occur on the "catalatic" reaction pathway of catalase, it has been postulated that they represent inactive forms of the enzyme, and that their accumulation would be associated with inhibition[21].

In exploring the catalytic behaviour of a catalase from *Aspergillus niger*, we discovered that Compound II accumulation does not necessarily correlate with loss of enzyme activity. This led us to examine more closely the hydrogen peroxide and superoxide degrading pathways of those enzymes.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany) or Sigma (St. Louis, U.S.A.). Lyophilised catalase from *Aspergillus niger* (EC 1.11.1.6, 8500 U/mg solid, ratio A407/A280 = 0.82) was a preparation from Merck. This preparation was homogeneous, as checked by gel filtration chromatography, and was used directly. Copper/zinc containing superoxide dismutases from bovine erythrocytes (EC 1.15.1.1, 5000 U/mg solid), glucose oxidase from *Aspergillus niger* (EC 1.1.3.4, 250 U/mg solid, grade 1), cytochrome c from horse heart and xanthine oxidase from cow milk (EC 1.1.3.22, 1 U/mg) were products of Boehringer. The commercial preparation of catalase was dialyzed at 6°C against a Na_2HPO_4-KH_2PO_4 buffer (50 mM phosphate, pH 7.8) or against a phosphate-citrate buffer (32.2 mM Na_2HPO_4, 33.9 mM citric acid, pH 3.5) for at least 24 hours before use.

Electronic Spectra

Spectral properties of *Aspergillus niger* catalase upon the addition of H_2O_2 and/or O_2^- generating systems were monitored by recording UV-visible absorption with a Philips PU 8700 spectrophotometer. Due to its molar absorption coefficient ($\varepsilon = 3.6 \times 10^5\,M^{-1}\,cm^{-1}$)[22] *Aspergillus niger* catalase was used at a concentration of 3 μM. All the assays were carried out at 25°C with a volume of 3 ml in a cuvette having a light path of 1 cm. A phosphate-citrate buffer (32.2 mM Na_2HPO_4, 33.9 mM citric acid, pH 3.5) was used to generate high stationary levels of Compound II. In all the experiments in which O_2^- was generated, the catalase was dissolved in a Na_2HPO_4-KH_2PO_4 buffer (50 mM phosphate, pH 7.8). The latter type of experiments were performed at pH 7.8 rather than pH 3.5, because xanthine oxidase, the enzyme utilized to generate O_2^-, is ineffective at pH 3.5 but works efficiently at pH 7.8.

Catalase Assay

Catalase activity was assayed by a modification of the procedure of Del Rio *et al.*[23]. A WTW Clark oxygen electrode (model OXI 91) was standardized by setting 100% with air saturation in 100 ml of Na_2HPO_4-KH_2PO_4 buffer (50 mM phosphate, pH 7) at 30°C. A portion of 100 μl of stock H_2O_2 (10 M) was then added to the buffer and the mixture was stirred continuously. Nitrogen was allowed to bubble gently through the buffer until the electrode gave a reading of less than 20%. The rate of increase of O_2 concentration was then monitored with the Clark electrode coupled to a strip chart recorder. The reaction was initiated by the injection of 20 μl of the sample. The difference of the rates before (i.e. spontaneous reoxygenation from air) and after the sample addition was used to calculate the rate

of O_2 production due to catalase activity. The H_2O_2 concentration used in all the experiments was 10 mM.

Superoxide Dismutase Assay

Superoxide dismutase activity was estimated by the method of McCord and Fridovich and was in accord with the supplier's stated activity[3]. Its concentration was calculated using a molar absorptivity of $1.03 \times 10^4\,M^{-1}\,cm^{-1}$ at 258 nm[3].

H_2O_2 Concentration of the Catalase Solutions at the Steady-state

In coupled enzymatic assay, the low levels of H_2O_2 maintained by the action of catalase render virtually impossible the direct measurement of the steady-state concentration of this metabolite. Therefore, the steady-state concentrations in H_2O_2 of the catalase solutions were estimated indirectly using the following set of chemical equations:

$$\text{free catalase} + H_2O_2 \xrightarrow{k_1} \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + H_2O_2 \xrightarrow{k_2} \text{free catalase} + H_2O + O_2 \quad (2)$$

$$\text{Compound II} + H_2O_2 \xrightarrow{k_3} \text{Compound III} + H_2O \quad (3)$$

in which k_1, k_2 and k_3 represent respectively the rate constants for reaction 1, 2 and 3. Writing the material balance for H_2O_2 we obtain:

$$\frac{d}{dt}[H_2O_2] = R_{oxidase} - k_1[H_2O_2][\text{free cat}] - k_2[H_2O_2][\text{Cpd I}] - k_3[H_2O_2][\text{Cpd II}] \quad (4)$$

in which [ ] represents the concentration of the reactants and $R_{oxidase}$ represents the rate of production of H_2O_2 by the oxidase. All estimations of H_2O_2 at steady-state were carried out in the presence of an excess of ethanol (2 mM, final concentration). Under such conditions, Compound I is almost imperceptible. Since the rate constants for steps 1 and 2 are of the same order of magnitude (0.68 to 1.02×10^6 and 2.04×10^6 respectively[24]), it is justified to neglect the third term of the right member of equation (4). Moreover, under the conditions used in our experiments [free cat] and [Cpd II] are of the same order of magnitude (see table 3 & 4) and reaction 3 proceeds at a much lower rate than reaction 1. It is therefore justified to neglect also the last term of equation (4). Consequently, in the steady-state:

$$[H_2O_2] = \frac{R_{oxidase}}{k_1[\text{free cat}]} \quad (5)$$

In this equation, $R_{oxidase}$ was estimated experimentally with a polarographic oxygen electrode, taking into account that the oxidase produced 1 molecule of H_2O_2 for each molecule of O_2 consumed by the enzyme. The amount of free catalase present in the reaction mixture was measured spectrophotometrically at 407 nm (see below). Glucose-oxidase was used as a source of H_2O_2 and all the assays were carried out at 25°C and pH 7. However, as a first step in all experiments, Compound II was produced by adding glucose oxidase and glucose to the native enzyme at pH 3.5. This was done because preliminary experiments

showed that the stationary level of Compound II attained under such conditions was 3 times the level attained at neutral pH. The pH of the reaction mixture was readjusted to 7 immediately after injection of ethanol into the reaction mixture. The catalase from *Aspergillus niger* is an enzyme without activity optimum, which is as stable at pH 3.5 as at pH 7[25]. Blanks shown below confirm that, under our conditions, the activity and the stability of catalase are about the same at pH 3.5 and pH 7.8.

Free Catalase and Compound II Concentrations at the Steady-state

The concentrations of free catalase and of Compound II present in the reaction mixtures were estimated by comparing the absorbance at 407 nm before and after the addition of ethanol (2 mM, final concentration). As shown in the text, it can reasonably be assumed that in presence of a low flux of H_2O_2 due to the glucose oxidase action and during the 10 s following the addition of ethanol, the major part of Compound I was converted into the resting ferric enzyme and Compound II remained at a constant level (see Figure 3, experiment in which no additional glucose oxidase (GO) was added). Taking into account these two assumptions and using Beer's law, the following set of equations can be proposed:

$$O.D._b = \varepsilon_{free}[free]_b + \varepsilon_{cpd\ I}[Cpd\ I]_b + \varepsilon_{cpd\ II}[Cpd\ II]_b \quad (6)$$

$$O.D._a = \varepsilon_{free}[free]_a + \varepsilon_{cpd\ II}[Cpd\ II]_a \quad (7)$$

$$[free]_a = [free]_b + [Cpd\ I]_b \quad (8)$$

$$[Cpd\ II]_a = [Cpd\ II]_b \quad (9)$$

$$[enzyme] = [free]_b + [Cpd\ I]_b + [Cpd\ II]_b \quad (10)$$

where O.D. is the absorptivity of the reaction mixture at 407 nm and where [ ] and ε represent respectively the concentration and the molar absorptivity at 407 nm of the various catalase compounds. The indices a and b indicate that the measurements were performed before (=b) and after (=a) the addition of ethanol. Combining equations (6), (7), (8) and (9) yields:

$$[Cpd\ I]_b = [free]_a - [free]_b = \frac{O.D._b - O.D._a}{\varepsilon_{cpd\ I} - \varepsilon_{free}} \quad (11)$$

The ratio [free]/[Cpd I] 0.5 min after the addition of the glucose/glucose oxidase system was estimated to be 66/34 = 1.94 (table 1, line 2). Supposing that, in the absence of ethanol, such ratio remained constant during the time course of the reaction and combining equations (8), (10) and (11) yields:

$$[free]_a = 2.94\frac{O.D._b - O.D._a}{\varepsilon_{cpd\ I} - \varepsilon_{free}} \quad (12)$$

and

$$[Cpd\ II]_a = [enzyme] - [free]_a \quad (13)$$

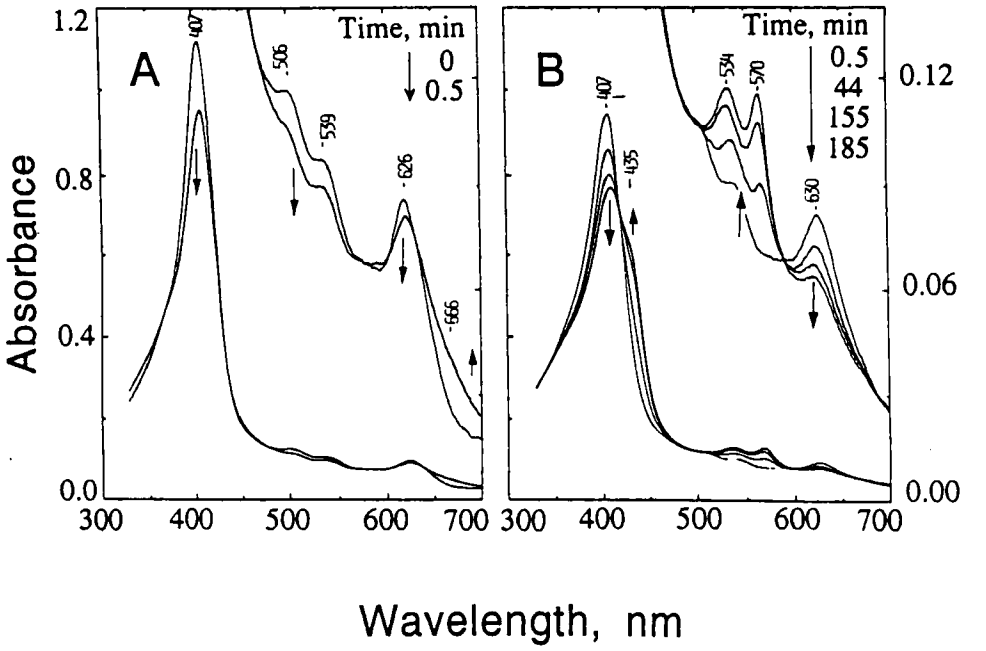

FIGURE 1 *Formation of Compound I and Compound II from native enzyme and H_2O_2*
The reaction mixture consisted of *Aspergillus niger* catalase (3μM), glucose (10 mM) in 3 ml of phosphate-citrate buffer (32.2 mM Na_2HPO_4, 33.9 mM citric acid, pH 3.5). The temperature was 25°C. H_2O_2 was generated at a rate of 4 μmole L^{-1} min^{-1} by adding at 0 time glucose oxidase to the final concentration of 2.5 nM. Air was bubbled through the cuvette to maintain oxygen at constant level during the duration of the experiment. Spectral changes are indicated by arrows. A) spectral changes observed during the first 0.5 min; B) spectral changes monitored during the following 185 min.

RESULTS

Formation of Compound I and of Compound II

Glucose oxidase and glucose were added to the native enzyme at pH 3.5 to expose it to a steady production of H_2O_2. Within 30 seconds following the addition of the oxidase, the Soret band height (407 nm) of the native enzyme was reduced by 15%, the absorbance at 506, 539, 626 nm decreased while an absorption band increased near 666 nm, indicating the formation of catalase Compound I (Figure 1a). Subsequent to the formation of Compound I, a shoulder appeared at 435 nm, together with new peaks at 534 and 570 nm, that are characteristic of Compound II (Figure 1,b)[28]. Comparison of the spectra recorded at different time intervals reveals well-defined isosbestic points at 420, 500 and 595 nm (Figure 1,b), indicating a lack of long-life intermediates in this conversion process. Table 1 presents the changes in each compound with time. The total activity of the preparation is also mentioned. The proportion of each constituent was evaluated from its molar extinction coefficient. This was known for ferricatalase[22] and was estimated to be $2.00 \times 10^5\ M^{-1}\,cm^{-1}$ at 407 nm for Compound I (determined from Figure 1 in reference 26) and $2.17 \times 10^5\ M^{-1}\,cm^{-1}$ at 435 nm for Compound II (see materials and methods). The results in Table 1 indicate that Compound II did not inhibit the

TABLE I
Evolution of different forms of Aspergillus niger catalase with time

Time (min)	Proportion of each Compound			Activity (mean ± C.I.[a]) (μmole O_2 s^{-1} mg^{-1})
	ferric	Compound I (%)	Compound II[b]	
0	100	0	0	44.8 ± 1.5
0.5	66	34	0	46.2 ± 3.7
155	33	17	50	44.4 ± 3.2
blank (180)	100	0	0	45.5 ± 3.2

[a] 95% confidence interval.
[b] see materials and methods for the evaluation of the amount of Compound II.

Incubation at pH 3.5 and assay conditions were as described in the legend of Figure 1. The proportion of each constituent was evaluated from its molar extinction coefficient and the variation of absorbance with respect to the native enzyme. The global activity of the preparation was estimated with a polarographic oxygen electrode as described in the text. A blank, in which glucose was omitted, was made to evaluate the loss of activity in the absence of H_2O_2 during the time course of the experiment.

conversion of hydrogen peroxide by catalase: the activity of the preparation which contained about 50% of Compound II was found to be comparable to that of the catalase solution before its exposure to H_2O_2. This observation contrasts with claims in several reports that catalase Compound II is an inactive form of catalase[6,27].

Reaction of Compound I and of Compound II with Ethanol

Compounds I and II were produced as described previously by adding glucose oxidase and glucose to the native enzyme at pH 3.5. The addition of 2 mM ethanol 0.5 min after the start of the reaction resulted in quick reversion of part of Compound I to the native form (Figure 2,a). The addition of 2 mM ethanol 138 minutes after the start of the reaction caused the accumulation of Compounds I and II to be reversed (Figure 2,b and c). During the first 2 minutes following the addition of ethanol, the Soret intensity increased quickly (Figure 2,b) and the spectra displayed isosbestic points identical to those reported in Figure 2,a. Scans of the two spectra in differential mode (not shown) were similar, indicating that Compound I was converted into resting ferric enzyme. The subsequent spectra (Figure 2,c) showed that Compound II slowly reverted to the native state with no apparent intermediate (isosbestic points at 420, 462, 519, 586 nm).

The spectra of ferricatalase and Compound I are essentially isosbestic at 435 nm[28]. Therefore, the kinetics of the appearance and of the decomposition of Compound II upon exposure of the enzyme to a flux of H_2O_2 were measured at this wavelength (Figure 3). After addition of glucose oxidase, the signal rose for 10 hours, until a constant steady-state concentration of Compound II was reached (Figure 3,a). The addition of ethanol to the above mixture induced the same spectral changes as those shown in Figure 2,b, indicating that Compound I was present and thus that H_2O_2 was still generated (It will be shown below that Compound I is unstable in absence of H_2O_2). This addition (see arrow) resulted in a drop in absorbance at 435 nm (Figure 3,a).

These experiments were repeated by adjusting the pH at 7 immediately after addition of ethanol; this pH was chosen in order to allow comparison with

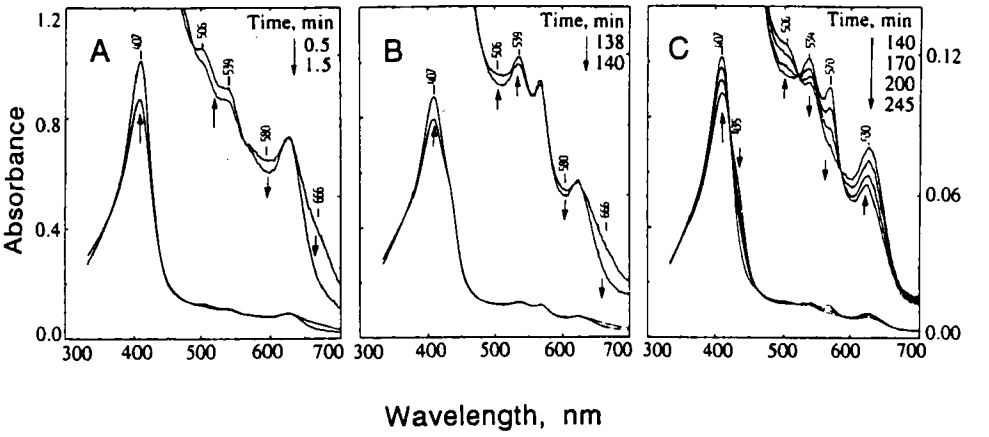

FIGURE 2 *Influence of ethanol on Compound I and Compound II*
Incubation at pH 3.5 and assay conditions were as described in the legend of Figure 1. The reaction was initiated by the addition of ethanol at a final concentration of 2 mM. Spectral changes are indicated by arrows. Scans were taken at the intervals indicated. A) ethanol was added 0.5 min after adding glucose oxidase; B & C) ethanol was added 138 min after adding glucose oxidase.

experiments performed in the presence of superoxide dismutase (SOD), described below (SOD is inactive at pH 3.5 but works efficiently at pH 7). Typical decay curves obtained after addition of ethanol and readjustment of the pH of the reaction mixture to 7 (see arrow) are illustrated in Figure 3,b. Results obtained when air (filled circles) or N_2 (open circles) was bubbled through the cuvette during the incubation with ethanol are given together for comparison. In the absence of O_2, i.e., when glucose oxidase failed to produce H_2O_2, the absorbance at 435 nm remained at a constant level for several minutes. This result suggests participation of H_2O_2 in the frequently cited "spontaneous" disappearance of Compound II that accompanies a decrease in the concentration of Compound I. Moreover, this observation also constitutes evidence that under the conditions used in our experiments, ethanol failed to react at an appreciable rate with Compound II.

Reaction of Compound II with H_2O_2 and Formation of Compound III

The involvement of H_2O_2 in the decomposition process of Compound II was further tested by the addition of increasing concentrations of glucose oxidase immediately after the injection of ethanol in the above reaction mixture. Typical traces, in which the initial absorbance changes at 435 nm were monitored in the presence of varying amounts of glucose oxidase, are shown in Figure 3,b (filled symbols). As the initial variations in absorbance were of simple exponential character (not shown), the first-order rate constants of the reaction (k_{obs}) were determined. A plot of k_{obs} as a function of the initial concentration in H_2O_2 at the steady-state (see "Materials & Methods" for calculation of H_2O_2 concentrations at the steady-state) gave a straight line (Figure 4). A second-order rate constant of $1.30 \pm 0.04 \times 10^3\,M^{-1}s^{-1}$ for the reaction of Compound II with H_2O_2 was estimated from the slope of the line using a linear least-squares fit to the data. The zero ordinate intercept indicates that, apart from the reaction of Compound II with H_2O_2, there were no other appreciable reactions occurring. Similar results were

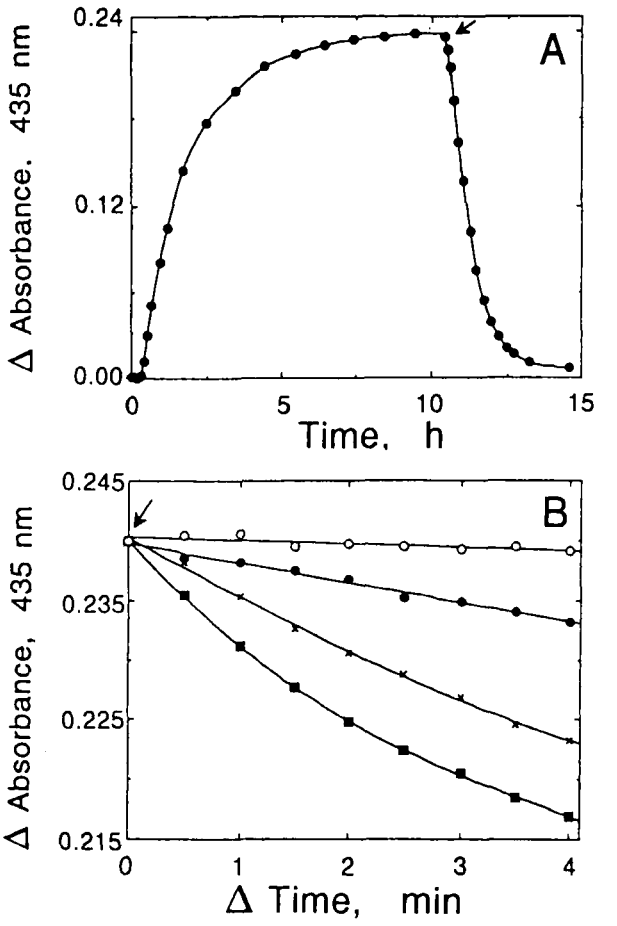

FIGURE 3 *Process of the formation of Compound II and its disappearance in presence of ethanol* A) Incubation at pH 3.5 and assay conditions were as described in the legend of Figure 1, except that H_2O_2 was generated at a rate of 1 μmole L^{-1} min^{-1} by adding at zero time glucose oxidase to a final concentration of 0.625 nM. Ethanol was added at a final concentration of 2 mM, 10 hours after adding glucose oxidase (see arrow). Compound II was monitored at 435 nm. B) Compound II was generated by the addition of 0.625 nM glucose oxidase and 10 mM glucose to 6 μM *Aspergillus niger* catalase in 1.5 ml of citrate-phosphate buffer (0.68 mM citric acid, 0.65 mM Na_2HPO_4, pH 3.5). 10 hours after the addition of glucose oxidase, the pH of the reaction mixture was readjusted to 7 by adding 1.5 ml of a Na_2HPO_4-KH_2PO_4 buffer (100 mM phosphate, pH 7) and ethanol was added at a final concentration of 2 mM (see arrow). ○, results obtained when N_2 was bubbled through the cuvette during incubation with ethanol; ●, no additional GO added; X, 30 nM GO; ■, 60 nM GO.

obtained when the rate of H_2O_2 production was increased by elevating the level of glucose or of oxygen in the reaction mixture, or by carrying out the experiments in the presence of 1 μM superoxide dismutase (not shown). This latter experiment shows that $O_2^{\cdot-}$ plays no role in the decomposition process of Compound II depicted in Figure 3,b.

Upon direct addition of excess H_2O_2 in place of ethanol to the above mixture at pH 3.5, catalase Compound II rapidly reverts to the native form. The dependence

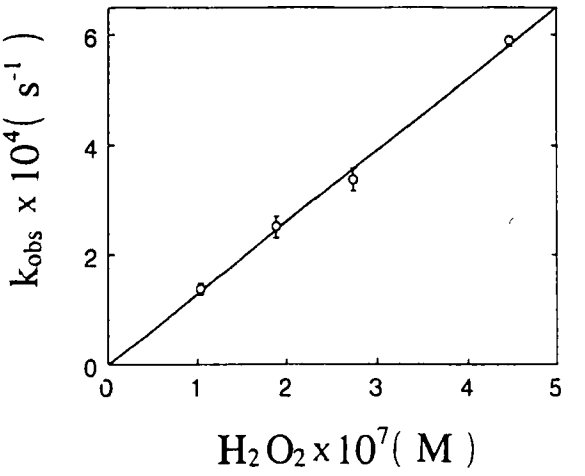

FIGURE 4 *Plot of k_{obs} versus H_2O_2 concentration at steady-state for the decomposition of Compound II*

Experiments were conducted as described in the legend to Figure 3,b with various amounts of glucose oxidase. The k_{obs} were obtained from the initial exponential changes in absorbance at 435 nm occuring within 30 s after mixing. Each determination of k_{obs} is the mean of four traces. The line is a linear least-squares fit of the data.

of the degree of conversion on the amount of H_2O_2 used in the assay is shown in Figure 5,a. Spectra were taken within 6 s following the addition of H_2O_2; they displayed isosbestic points at 420, 500 and 595 nm, indicating a lack of long-life intermediates in this conversion process. In order to analyze further the mechanism of the disappearance of Compound II, the reaction was repeated at 3°C. Catalase Compound II was prepared at 25°C as before, and was cooled down before the reaction. The addition of excess H_2O_2 to the mixture at 3°C resulted in the formation of species similar to catalase Compound III, with absorption maxima at 412, 549 and 589 nm[28]. The dependence of the conversion of Compound II to Compound III on H_2O_2 concentration initially present in the cuvette is shown in Figure 5,b. As before, spectra were taken within 6 s after addition of H_2O_2. They displayed isosbestic points at 425, 485, 553, 578 and 601 nm. On standing, Compound III was rapidly converted to its native state (Figure 5,c) with no apparent intermediate involved (isosbestic points at 413, 465, 540, 560, 573 and 594 nm). The decrease of absorbance at 425 nm (not shown in detail), indicates a half-time for the reaction of about 1 minute.

Long-Life Intermediate Generated in Presence of O_2^-

The aerobic xanthine oxidase reaction, which is known to generate large amounts of urate, H_2O_2 and O_2^-, was used as the source of this radical. Its effect on the oxidoreduction states of catalase was monitored spectrophotometrically. The data were presented as the difference between spectra because changes were too weak to provide clear information in direct mode. The difference between spectra of Compound I and ferric catalase and between spectra of Compound II and Compound I are shown in Figure 6,a, for comparison. These spectra were obtained from separate experiments performed as above (see Figure 1). The scans following

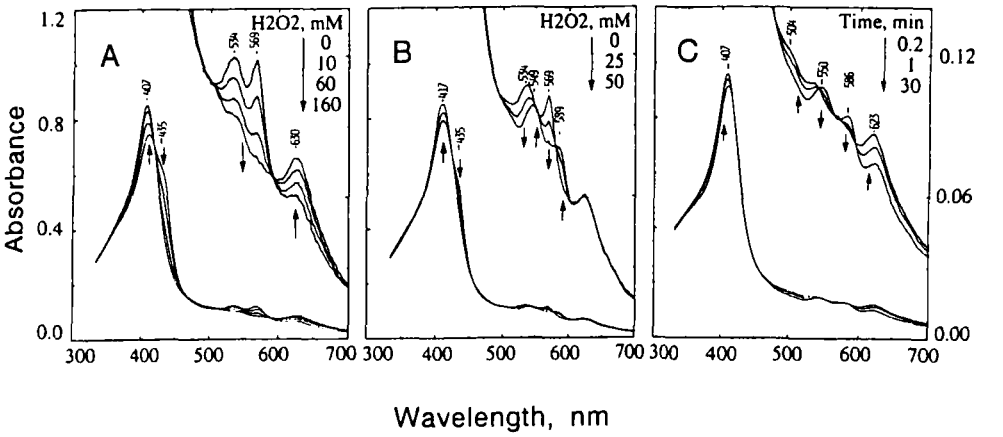

FIGURE 5 *Influence of excess H_2O_2 on Compound II*

Incubation at pH 3.5 and assay conditions were as described in the legend of Figure 1. The reaction was initiated by the addition of H_2O_2, 5 hours after adding glucose oxidase. Spectral changes are indicated by arrows. A) Spectral changes from Compound II to ferric enzyme at 25°C; B) spectral changes from Compound II to Compound III at 3°C; in both cases, H_2O_2 was added stepwise as indicated and each spectrum was recorded 6 s after addition of H_2O_2. C) Decomposition of Compound III at 3°C; in this case, Compound III was prepared as in B by the addition of H_2O_2 at a final concentration of 50 mM and scans were taken at the time intervals indicated.

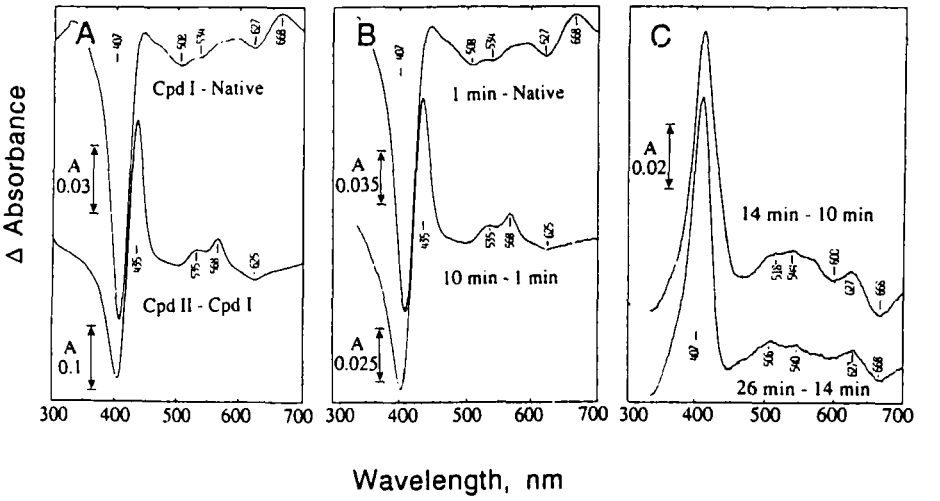

FIGURE 6 *Influence of O_2^- on the states of A. niger catalase*

A) Difference between spectra performed as described in Figure 1 from separate experiments. B) & C) Difference between spectra recorded at different time intervals after the addition of xanthine oxidase to the final concentration of 0.4 nM; the reaction mixtures consisted of *Aspergillus niger* catalase (3 μM), xanthine (0.1 mM) in 3 ml Na_2HPO_4-KH_2PO_4 buffer (50 mM in phosphate, pH 7.8) at 25°C.

the addition of xanthine oxidase showed the formation of two distinct spectral intermediates (Figure 6,b and c). The first species was formed rapidly and the spectral variation clearly resembles the difference between the spectra of Compound I and ferricatalase. The second species was formed more slowly and the spectral variation resembles the difference between the spectra of Compound II and Compound I. Surprisingly, none of the spectra observed had the twin absorption peaks at

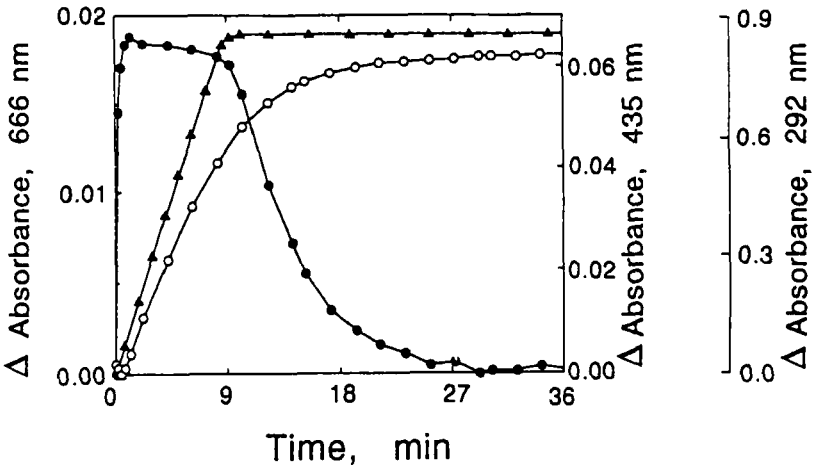

FIGURE 7 *Time course for the formation and disappearance of Compound I and for Compound II and urate accumulation upon addition of the O_2^- generating system.*

Incubation at pH 7.8 and assay conditions are described in the legend of Figure 6. Urate appearance (▲) was monitored at 292 nm while Compound I (●) and Compound II (○) were followed at 668 and 435 nm respectively.

549 and 589 nm that are characteristic of Compound III[28]. When all the xanthine was consumed (10 minutes), catalase Compound I rapidly reverted to a resting enzyme as shown by a spectral variation which is the inverse of the difference between Compound I and ferric catalase (Figure 6,c). The experiment was carried out under conditions where a large amount of H_2O_2 was generated due to the direct action of xanthine oxidase and to the spontaneous O_2^- dismutation. It seems thus likely that Compound I originated from the reaction of ferricatalase with H_2O_2, which in turn was converted into Compound II by reacting with O_2^-. Valuable support for the proposed scheme is given by a close observation of spectral changes of catalase upon the addition of xanthine oxidase to the xanthine-catalase mixture (Figure 7). Bearing in mind that, at 668 nm, ε (native) = ε (Compound II) and at 435 nm ε(native) = ε(Compound I)[28], one has to ascribe the absorption fluctuations at the two wavelengths to a variation of the concentrations of Compound I and Compound II respectively. Kinetic analysis showed that the appearance of Compound I was rapid (maximum heme occupancy by H_2O_2 within 3 minutes) and preceded the slower formation of Compound II (Figure 7). Moreover, Compound II reached a stationary level when Compound I was entirely reconverted to native enzyme, suggesting that the presence of Compound I is a prerequisite to Compound II formation (Figure 7). The ascending part of the curve at 435 nm (Figure 7) corresponded to the active O_2^--producing phase as revealed when the time course of urate production was monitored at 292 nm. The plateau began when all the xanthine had been consumed by xanthine oxidase; this indicated that, as opposed to Compound I, Compound II is stable for several minutes in the absence of O_2^- and H_2O_2.

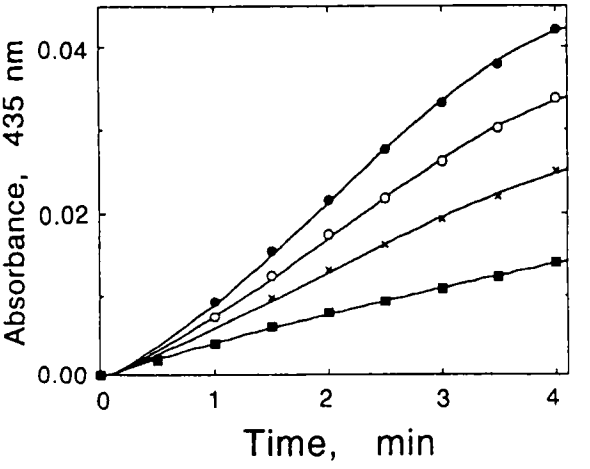

FIGURE 8 *Rate of appearance of Compound II at increasing superoxide dismutase concentration.* Incubation at pH 7.8 and assay conditions were as described in the legend of Figure 6 except that superoxide dismutase was added to the reaction mixture before the injection of 0.4 nM xanthine oxidase. ○, 2.8 nM SOD; X, 5.6 nM SOD; ■, 1.1 μM SOD. Results obtained in absence of SOD (●) are given for comparison.

Reaction of Compound I and Compound II with O_2^-

The hypothesis that O_2^- was responsible for the conversion of Compound I to Compound II has been tested by the addition of superoxide dismutase to the above reaction mixture. Typical traces, where the initial absorbance changes at 435 nm are monitored in the presence of increasing amounts of SOD, are shown in Figure 8. In the presence of the O_2^- scavenger, the conversion of Compound I to Compound II was considerably slowed down. This indicates that superoxide radicals take part in the reaction. To investigate the reaction of Compound II with O_2^-, the former was produced by adding glucose oxidase and glucose to the native enzyme at pH 3.5 (see Figure 1). After about 5 hours, the pH of the reaction mixture was adjusted to pH 7.8 and 2 mM ethanol was added. The change in absorption spectra occurring between 30 s and 8 minutes after the injection of ethanol is presented in Figure 9a. The spectral variation clearly resembles the opposite of the difference between the spectra of Compound II and native form (see Figure 6a for comparison); this indicates that part of Compound II was converted into resting ferric enzyme during this time interval. Similar changes in spectra were obtained when xanthine and xanthine oxidase were added immediately after the injection of EtOH in the above reaction mixture (Figure 9b). However, in the presence of the O_2^- generating system, the rate of Compound II decomposition was increased. To discover the transients responsible for this increase, the experiment of Figure 9b was repeated in the presence of increasing concentrations of SOD. Plots of absorbance at 435 nm (Figure 10) indicated that the presence of the O_2^- scavenger in the reaction mixture reduced the rate of Compound II decomposition. It is thus clear that the acceleration of Compound II decomposition is due, at least in part, to the reaction of O_2^- with Compound II itself or with some intermediate (Compound III?) generated in the conversion path of Compound II to the native form.

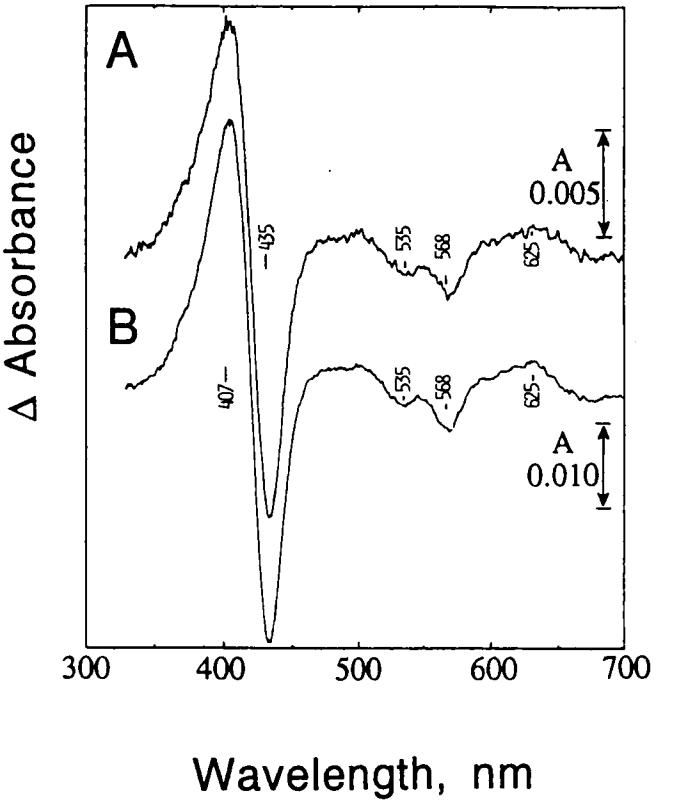

FIGURE 9 *Influence of O_2^- and ethanol on Compound II decomposition process.*
Compound II was generated by the addition of 0.625 nM glucose oxidase and 10 mM glucose to 6 μM *Aspergillus niger* catalase in 1.5 ml of citrate-phosphate buffer (0.68 mM citric acid, 0.65 mM Na_2HPO_4, pH 3.5). 5 hours after the addition of glucose oxidase, the pH of the reaction mixture was readjusted to 7.8 by adding 1.5 ml of a Na_2HPO_4-KH_2PO_4 buffer (100 mM phosphate, pH 7.8) containing 0.2 mM xanthine. The reaction was initiated immediately after the pH adjustment by the addition of 2 mM ethanol (A) or by the simultaneous addition of 2 mM ethanol and 0.4 nM xanthine oxidase (B). In both cases, difference spectra represent changes in absorbance occuring between 30 s and 8 min after the start of the reaction.

Effect of O_2^- on Catalase Activity

The influence of O_2^- on catalase activity was investigated by incubating the enzyme with the xanthine/xanthine oxidase system. After 2 hours of exposure to the flux of O_2^-, the proportion of each constituent was evaluated with a spectrophotometer (Table 2). The global activity of the preparation was then evaluated and compared with the activity of a blank in which xanthine oxidase was omitted. The results in Table 2 demonstrate that catalase activity was not affected by prolonged exposure of the enzyme to a flux of O_2^-: the activity of the preparation exposed for 2 hours to the flux of O_2^- was found to be comparable to that of catalase solution before its exposure to O_2^-. The data in the table also indicate that the oxyradical induced the enrichment of the catalase preparation in Compound II. Taken together, these elements confirm that Compound II is an "active" form of catalase.

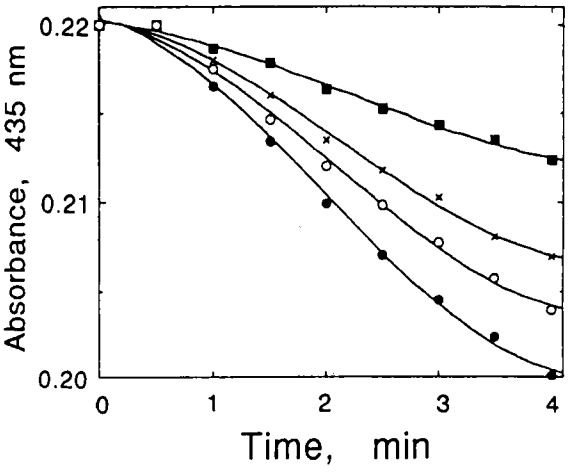

FIGURE 10 *Rate of disappearance of Compound II at increasing superoxide dismutase concentration.* Incubation and assay conditions were as described in the legend of Figure 9 except that superoxide dismutase was added to the reaction mixture before the injection of xanthine oxidase and ethanol. ○, 2.8 nM SOD; X, 5.6 nM SOD, ■, 1.1 μM SOD. Results obtained in absence of SOD (●) are given for comparison.

DISCUSSION

Catalase from *Aspergillus niger* (ANC) shares many common structural and mechanistic features with bovine liver catalase, the most extensively studied variety of the enzyme; both enzymes have been shown to consist of four subunits[4,22], each subunit containing a protohemin prosthetic group[4,29] with the same proximal tyrosine ligation[30]. Moreover, preliminary characterization of the native ferric enzyme and of the oxidized intermediate Compound I indicates that oxidation states and catalytic cycle of ANC are similar to bovine liver catalase[22,24]. However, the fungal catalase has been shown to be significantly more stable than the beef liver enzyme when subjected to high concentrations of peroxides[25,31]. In addition,

TABLE 2
Effect of O_2^- on catalase activity

Time (min)	Proportion of each Compound			Activity (mean ± C.I.[a]) (μmole O_2 s^{-1} mg^{-1})
	ferric	Compound I (%)	Compound II	
0	100	0	0	45.1 ± 1.2
120	39	20	41	44.3 ± 3.5
blank (120)	100	0	0	46.2 ● 2.0

[a] 95% confidence interval.

Incubation at pH 7.8 and assay conditions were as described in the legend of Figure 6 except that O_2^- was generated by adding xanthine oxidase to the final concentration of 0.02 nM. The proportion of each constituent and the global activity of the preparation were estimated as in table 1. A blank, in which xanthine oxidase was omitted, was made to evaluate the loss of activity in absence of O_2^- during the time course of the experiment.

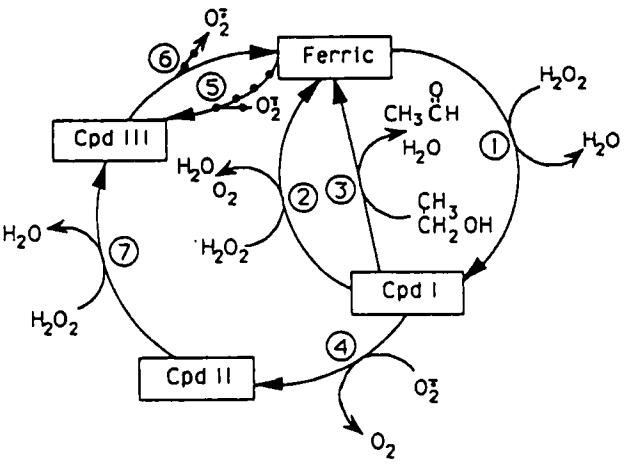

FIGURE 11 *Relationships between the oxidized intermediates of catalases.*
The normal "catalatic" cycle includes ferric catalase → Compound I → ferric catalase (reactions 1 and 2). Reactions 1 and 3 denote the "peroxidatic" cycle which involves a direct $2e^-$ transfer from ethanol to Compound I. $O_2^{\cdot-}$ leads to the formation of Compound II and Compound III (reactions 4 and 5). The former Compound is reconverted to the resting ferric enzyme via Compound III (reactions 7 and 6). Solid arrows: reactions that have been observed in some mammalian and bacterial catalases[4,6] and in *A. niger* catalase (this work); spotted arrows: reactions that, until now, have only been observed in bovine liver catalase (step 5)[13,14] or in some peroxidases (release of $O_2^{\cdot-}$ during step 6)[33,34].

Kikuchi-torii *et al.*[22,26] have found that upon prolonged exposure of ANC to high concentrations of methyl hydroperoxide, only Compound I was formed, and Compound II was not detected, in contrast to mammalian catalases[28,32].

These unusual properties of the fungal catalase encouraged us to study the formation and decomposition of ANC oxidized intermediates in greater detail. In the following discussion, only the substrates investigated in the present study (hydrogen peroxide, superoxide radical and ethanol) are taken into consideration. The interrelationships between the different states of catalases from various origins are illustrated in Figure 11.

Oxidized Intermediate Generated in Presence of Hydrogen Peroxide - Compound I

The simple sequence of consecutive reactions proposed some time ago by Chance[36] for catalytic decomposition of hydrogen peroxide still seems to represent the most accurate description of the kinetics of catalases in the "catalatic" function; a molecule of hydrogen peroxide reacts with native enzyme to form a spectroscopically distinct intermediate, Compound I (reaction 1), which reacts with a second molecule of hydrogen peroxide to give native enzyme, water, and oxygen (reaction 2). At any moment, only a fraction of the catalase heme is bound to H_2O_2 in the form of Compound I, and that fraction reaches a maximal level determined by the ratio of the rate constants of reactions 1 and 2[35]. Ratios of Compound I concentration to total catalase concentration range from 0.3 to 0.5 with mammalian catalases[37]. An alternative substrate for this second part of the cycle is ethanol, which reacts with Compound I to form ferricatalase, acetaldehyde,

and water (reaction 3). Although the rate constant is much greater with H_2O_2 than with ethanol[35], the latter can be added to a concentration that is several orders of magnitude higher than that of H_2O_2. Under these conditions Compound I is almost imperceptible, whereas detectable amounts of Compound I are present when catalase has H_2O_2 as the only substrate.

The results obtained here with a fungal catalase are in complete agreement with those obtained earlier with mammalian enzymes; from known molar extinction coefficient of ferricatalase and Compound I, the degree of conversion into the latter was estimated to be 0.34 in presence of a steady production of H_2O_2. The addition of an excess of ethanol to this system resulted in a quick reversion of part of Compound I to the native form, showing that this complex is peroxidatically active towards ethanol.

Oxidized Intermediates Generated in Presence of Superoxide Radicals

While catalase is primarily known for its catalytic decomposition of hydrogen peroxide, it also reacts with superoxide anions and perhydroxyl radicals[12,13,14]. This leads to the formation of two different intermediates, Compound II and Compound III.

Formation and decomposition of Compound II. The formation of Compound II from mammalian catalases in the presence of peroxide slowly generated by glucose oxidase was first observed by Chance in 1950[27]. It is formed from a one electron reduction process of Compound I but the nature of the substances which reduce Compound I in the above system is still poorly understood. According to Chance's data, preparation of mammalian catalase partially converted into Compound II exhibited a lower activity than the same preparation of enzyme in native form[26]. It was thus generally considered that the formation of Compound II inhibits the destruction of hydrogen peroxide by catalase. However, inhibition upon exposure to H_2O_2 generated by glucose oxidase was never complete but reached a steady-state at between 60 and 80% inhibition[27,38]. A steady concentration in Compound II of similar amplitude was observed under the same conditions, strengthening further the idea that Compound II was catalytically inactive. That a steady-state level of catalase activity was achieved, rather than complete inhibition, was interpreted as the sign that Compound II was reactivated by one electron transfer from single-electron donor in the redox system. In accordance with this view, a decrease in the concentration of Compound I due to the addition of ethanol was found to favor Compound II decomposition and thereby shifted the equilibrium toward a higher level of steady-state activity[27]. It has been proposed that the reducing substance leading to Compound II decomposition must be due to the presence of reducing groups within the catalase molecule. However, as with the formation of Compound II, the nature of the reducing substance(s) leading to the decomposition of Compound II in the above system is unclear. Evidence has recently been presented showing that superoxide radicals can reduce Compound I into Compound II (reaction 4, Figure 11)[13]. As Compound II was considered to be catalytically inactive, it has been postulated by Kono and Fridovich[21] that the above reaction leads to an irreversible inhibition of catalase activity.

Our data clearly indicate, on the contrary, that Compound II from ANC was rapidly converted into native resting ferric enzyme by excess H_2O_2, without loss of

activity of the preparation. *Aspergillus niger* Compound II may thus be considered as an "active form" of catalase. Moreover, analysis of the visible region of the spectra of ANC shows that the appearance of Compound I preceded the slower formation of Compound II. We have also observed that Compound II reached a stationary level when Compound I was entirely reconverted to native enzyme. These results indicate that the presence of Compound I is a prerequisite to the formation of Compound II and thus, that ANC Compound II is formed through a pathway similar (reaction 1 + 4, Figure 11) to those proposed earlier for the mammalian enzyme. Unlike the bovine liver corresponding compound, ANC Compound II was stable for several minutes in the absence of O_2^- and H_2O_2. This suggests that, in contrast to the bovine liver enzyme, the preparation of fungal catalase was free of endogenous one electron-donors. On the other hand, spectral evidence has been obtained showing that in the presence of the O_2^- generating system, the rate of Compound II decomposition toward the native form was increased. It has also been observed that the addition of superoxide dismutase in the above reaction mixture reduced the rate of Compound II decomposition. Moreover, no intermediate was detected during the decomposition process of Compound II into the native form. Consequently we propose that, with Compound II, O_2^- acts as a one electron reducing agent:

$$\textit{Compound II} + O_2^- + 2H^+ \rightarrow \textit{catalase} + O_2 + H_2O$$

An alternative explanation suggesting that O_2^- could react with some intermediate generated in the conversion path of Compound II to the native form is presented below.

Formation of Compound III. Compound III of catalases and peroxidases can be formed via three reaction paths: a) by a reduction of the native enzyme followed by oxygen addition to form a ferrous oxygen adduct, Fe^{2+}-O_2[39,33], b) by excess H_2O_2 reacting with Compound II and leading to a ferryl-hydrogen peroxide adduct, Fe^{4+}-H_2O_2[28,33,40], c) by reaction of the native ferric enzyme with superoxide leading to a ferri-superoxide adduct, Fe^{3+}-O_2^-[5,13,14,33,41]. The spectral features of Compounds III prepared via all three techniques are almost identical, suggesting that the corresponding adducts possess similar structures[42].

Two reports[14,43] confirmed the occurrence of the latter reaction in mammalian catalases and proposed that, under conditions when [catalase] $\gg$ [O_2^-], O_2^- disappears mainly by reaction 5. However, when conditions were such that [O_2^-]/[catalase] ~ 2-4, the authors observed the formation of Compound I and III. Moreover, a recent report of Gebicka *et al.*[13] demonstrated that in irradiated oxygen-saturated solutions of bovine liver catalase, all three catalase compounds were observed after the radiolysis pulse. A careful analysis of absorption changes occuring after the pulse led those authors to propose that Compound I disappears in two ways: i) via the catalytic reaction with H_2O_2 to restore ferric catalase (reaction 2) and ii) via the reaction with O_2^- to form the relatively stable Compound II (reaction 4). Part of the O_2^- formed after the pulse reacted with native catalase to form unstable Compound III (reaction 5), which was converted back to ferric catalase over a period of minutes after the pulse (reaction 6). As stated in the introduction, Kono and Fridovich[21] postulate that formation of Compound III leads to a rapid and reversible inhibition of mammalian catalase activity.

The data obtained here with a fungal catalase show, on the contrary, that

Compound II is the only long life intermediate generated in presence of $O_2^{\cdot-}$. The present study reveals also that in absence of H_2O_2 and of $O_2^{\cdot-}$, Compound II fails to convert back into native form. Moreover, Compound II is rapidly converted into native resting ferric enzyme by excess H_2O_2. Successive scans made during the course of the conversion enabled us to detect Compound III. Taken together, these observations indicate that Compound II is converted to native resting enzyme via the transient formation of Compound III. Therefore it is concluded that, under the conditions used, ANC Compound III appears mainly by reactions 1 + 4 + 7 (Figure 11) rather than by reaction 5 as originally observed by Shimizu *et al.*[14] for mammalian catalases. These authors used pulse radiolysis experiments to confirm reaction 5 when conditions were such that $O_2^{\cdot-}$ was in excess of H_2O_2. In contrast, the present experiments were carried out under conditions where the concentration of H_2O_2 can be reasonably assumed to be in excess of that of $O_2^{\cdot-}$. It is thus possible that the disparities observed between the fungal and mammalian catalases are due to differences in experimental conditions.

Decomposition of Compound III. Most of the cases investigated show that Compound III from hemoproteins tends to revert spontaneously to native resting enzyme in a single step, with concomitant release of stoichiometric amounts of superoxide[33,34]. However, the addition of superoxide dismutase to Compound III of several peroxidases considerably slows down conversion of this oxidized intermediate to the native form[44,45]. This suggests that superoxide may also react with Compound III:

$$\textit{Compound III} + O_2^{\cdot-} + 2H^+ \rightarrow \textit{peroxidase} + O_2 + H_2O_2$$

Reaction of $O_2^{\cdot-}$ with Compound III has also been demonstrated in oxyhemoglobin, the dioxygen complex of ferrous hemoglobin, similar to Compound III[46,47]. This reaction was found to occur at high rates ($k_{app} = 6.5 \times 10^6\ M^{-1}\ s^{-1}$).

The data obtained with the fungal catalase suggest a direct conversion of Compound III to native enzyme with no intermediate involved. On the other hand, we have presented evidence showing that SOD, when present in the reaction mixture, markedly reduces Compound II decay rate. This evidence could be a sign that an equilibrium exists between Compound II and Compound III. In this hypothesis, $O_2^{\cdot-}$ could accelerate Compound II decay by reacting with Compound III and, in so doing, could shift the equilibrium between Compound II and Compound III:

$$\textit{Compound II} + H_2O_2 \rightleftharpoons \textit{Compound III} + H_2O$$

$$\textit{Compound III} + O_2^{\cdot-} + 2H^+ \rightarrow \textit{catalase} + O_2 + H_2O_2$$

The existence of such an equilibrium has already been documented in horseradish peroxidase[48] and in bovine liver catalase[49]. However, we have obtained no clear indication that the reaction between ANC Compound II and H_2O_2 could be reversible. Therefore, ANC Compound II might be converted to the native resting form by reacting directly with $O_2^{\cdot-}$ as proposed above (see "Formation and Decomposition of Compound II"). The data presented in this work do not allow us to discriminate between the above two hypotheses which will thus not be presented in Figure 11.

Inhibition of Aspergillus niger Catalase by Superoxide Radicals

The possibility that $O_2^{\cdot-}$ might inhibit catalase was first investigated by Kono and Fridovich in 1982[21]. This was explored by measuring the activity of a mammalian catalase after its exposure for a varying time interval to a flux of $O_2^{\cdot-}$ generated by xanthine oxidase. According to these authors, two distinct types of inhibition could be distinguished under these conditions. One of these was rapidly established and could be rapidly reversed by the addition of superoxide dismutase. The second developed slowly and was reversed by ethanol, but not by superoxide dismutase. The rapid inhibition was reportedly due to the formation of unstable Compound III (reaction 5) while the slow inhibition was attributed to the production of Compound II through the reduction of Compound I by superoxide (reaction 4).

The spectroscopic analysis described in this paper shows that in the presence of enzymatically generated $O_2^{\cdot-}$, another H_2O_2 consuming pathway different from the "catalatic" cycle proposed earlier by Chance[36] may be activated (reaction 1 + 4 + 7 + 6 rather than 1 + 2). The rate constant for reaction 7 has been estimated to be $1.30 \pm 0.04 \times 10^3$ $M^{-1}s^{-1}$ (see Figure 4). This value is up to 3 orders of magnitude lower than the rate constant for the reaction of H_2O_2 with Compound I ($k_2 = 2.04 \times 10^6$)[24]. The decomposition process of H_2O_2 must then proceed less rapidly through steps 1 + 4 + 7 + 6 than via steps 1 + 2 which indicates that the reaction of $O_2^{\cdot-}$ with the catalase of *A. niger* may lead to a significant decrease in its H_2O_2 degrading capability. However steps 7 + 6 would provide a reaction path to ensure that the enzyme restores entirely its activity upon exposure to low levels of the oxy-radical. In accordance with this view, our data indicates that ANC Compound II was rapidly converted into the native resting ferric form of the enzyme by excess H_2O_2, without loss of activity of the preparation. It is thus clear that the catalase from *A. niger* was not irreversibly inactivated by low levels of $O_2^{\cdot-}$ due to the action of oxidases or by excess H_2O_2 used in the activity test. Whether this property constitutes a particularity of catalase from *A. niger* or could be found in catalases from other origins remains to be investigated.

Acknowledgements

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Abbreviation List

ANC, *Aspergillus niger* catalase; Cpd, Compound; GO, glucose oxidase; SOD, superoxide dismutase.

References

1. B. Halliwell (1991) Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *The American Journal of Medicine*, **91**, 14S-22S.
2. B. Halliwell and J.M.C. Gutteridge (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal*, **219**, 1-14.
3. J.M. McCord and I. Fridovich (1969) Superoxide dismutase. An enzymatic function for

erythrocuprein (hemocuprein). *Journal of Biological Chemistry*, **244**, 6049-6055.
4. P. Nicholls and G.R. Schonbaum (1963) Catalases. In *The enzymes* (eds. P.D. Boyer, H. Lardy and K. Myrback), Academic Press, Orlando FL., Vol. 8, pp. 147-225.
5. H.B. Dunford and J.S. Stillman (1976) On the function and mechanism of action of peroxidases. *Coordination Chemistry Reviews*, **19**, 187-251.
6. A. Deisseroth and A.L. Dounce (1970) Catalase: physical and chemical properties, mechanism of catalysis, and physiological role. *Physiological Reviews*, **50**, 319-375.
7. B. Chance, L. Powers, Y. Ching, T. Poulos, I. Yamazaki and K.G. Paul (1984) X-Ray absorption studies of intermediates in peroxidase activity. *Archives of Biochemistry and Biophysics*, **235**, 596-611.
8. H.B. Dunford (1991) Horseradish peroxidase: structure and kinetic properties. In *Peroxidases in Chemistry and Biology* (ed. J. Everse, K.E. Everse and M.B. Grisham), CRC Press, Boca Raton, USA, pp. 1-24.
9. W.J. Chuang and H.E. Van Wart (1992) Resonance Raman spectra of horseradish peroxidase and bovine liver catalase Compound I species. Evidence for predominent $^2A_{2u}$ π-cation radical ground state configurations. *Journal of Biological Chemistry*, **267**, 13293-13301.
10. J.E. Erman, L.B. Vitello, J.M. Mauro and J. Kraut (1989) Detection of an oxyferryl porphyrin π-cation radical intermediate in the reaction between hydrogen peroxide and a mutant cytochrome c peroxidase. Evidence for Tryptophan-191 involvement in the radical site of Compound I. *Biochemistry*, **28**, 7992-7995.
11. W.J. Chuang, J. Heldt and E.W. Wart (1989) Resonance Raman spectra of bovine liver catalase Compound II. Similarity of the heme environment to horseradish peroxidase Compound II. *Journal of Biological Chemistry*, **264**, 14209-14215.
12. B.H. Bielski and D.E. Cabelli (1991) Highlights of current research involving superoxide and perhydroxyl radicals in aqueous solutions. *International Journal of Radiation Biology*, **59**, 291-319.
13. L. Gebicka, D. Metodiewa and J.L. Gebicki (1989) Pulse radiolysis of catalase in solution. I. Reactions of O_2^- with catalase and its Compound I. *International Journal of Radiation Biology*, **55**, 45-50.
14. N. Shimizu, K. Kobayashi and K. Hayashi (1984) The reaction of superoxide radical with catalase. Mechanism of the inhibition of catalase by superoxide radical. *The Journal of Biological Chemistry*, **259**, 4414-4418.
15. B.H. Bielski and J.M. Gebicki (1974) Study of peroxidase mechanisms by pulse radiolysis III. The rate of reaction of O_2^- and HO_2 radicals with horseradish peroxidase Compound I. *Biochimica et Biophysica Acta*, **364**, 233-235.
16. B.H.J. Bielski, D.A. Comstock, A. Haber and P.C. Chan (1974) Study of peroxidase mechanisms by pulse radiolysis. II. Reaction of horseradish peroxidase Compound I with O_2^- *Biochimica et Biophysica Acta*, **350**, 113-120.
17. R.F. Rest and J.K. Spitznagel (1977) Subcellular distribution of superoxide dismutase in human neutrophils. Influence of myeloperoxidase on the measurement of superoxide dismutase activity. *Biochemical Journal*, **166**, 145-153.
18. R.A. Cuperus, A.O. Muijsers and R. Wever (1986) The superoxide dismutase activity of myeloperoxidase; formation of Compound III. *Biochimica et Biophysica Acta*, **871**, 78-84.
19. J.W. Heinecke and B.M. Shapiro (1990) Superoxide peroxidase activity of ovoperoxidase, the cross-linking enzyme of fertilization. *The Journal of Biological Chemistry*, **265**, 9241-9246.
20. D. Metodiewa, M. Pickard and H.B. Dunford (1989) The reactions of chloroperoxidase in the presence of xanthine/xanthine oxidase. *Biochemical and Biophysical Research Communications*, **159**, 1086-1092.
21. Y. Kono and I. Fridovich (1982) Superoxide radical inhibits catalase. *The Journal of Biological Chemistry*, **257**, 5751-5754.
22. K. Kikuchi-Torii, S. Hayashi, H. Nakamoto and S. Nakamura (1982) Properties of *Aspergillus niger* catalase. *The Journal of Biochemistry*, **92**, 1449-1456.
23. L.A. Del Rio, M.G. Ortega, A.L. Lopez and J.L. George (1977) A more sensitive modification of the catalase assay with the Clark oxygen electrode. *Analytical Biochemistry*, **80**, 409-415.
24. F.E. Hammer (1960) Purification and characterization of catalase from *Aspergillus niger*. *Proceedings of the Annual Meeting of the American Chemical Society*, 17C-18C.
25. D. Scott and F. Hammer (1960) Properties of *Aspergillus* catalase. *Enzymologia*, **22**, 229-237.
26. K. Kikuchi, Y. Kawamura-Konishi and H. Suzuki (1992) The reaction of *Aspergillus niger* catalase with methyl hydroperoxide. *Archives of Biochemistry and Biophysics*, **296**, 88-94.
27. B. Chance (1950) The reactions of catalase in the presence of the notatin system. *Biochemical Journal*, **46**, 387-402.

28. B. Chance (1952) The spectra of the enzyme-substrate complexes of catalase and peroxidase. *Archives of Biochemistry and Biophysics*, **41**, 404–415.
29. H. Gruft, R. Ruck and J. Traynor (1978) Properties of a unique catalase isolate from *Aspergillus niger*. *Canadian Journal of Biochemistry*, **56**, 916–919.
30. K.D. Sharma, L.A. Andersson, T.M. Loehr, J. Terner and H.M. Goff (1989) Comparative spectral analysis of mammalian, fungal, and bacterial catalases. Resonance Raman evidence for iron-tyrosinate coordination. *The Journal of Biological Chemistry*, **264**, 12772–12779.
31. P.T. Vasudevan and R.H. Weiland (1990) Deactivation of catalase by hydrogen peroxide. *Biotechnology and Bioengineering*, **36**, 783–789.
32. B. Chance and D. Herbert (1950) The enzyme-substrate Compounds of bacterial catalase and peroxides. *Biochemical Journal*, **46**, 402–414.
33. H. Wariishi and M.H. Gold (1990) Lignin peroxidase Compound III. Mechanism of formation and decomposition. *The Journal of Biological Chemistry*, **265**, 2070–2077.
34. G. Rotilio, G. Falcioni, E. Fioretti and M. Brunori (1975) Decay of oxyperoxidase and oxygen radicals: a possible role for Myeloperoxidase. *Biochemical Journal*, **145**, 405–407.
35. B. Chance, H. Sies and A. Boveris (1979) Hydroperoxide metabolism in mammalian organs. *Physiological Reviews*, **59**, 527–605.
36. B. Chance (1947) An intermediate Compound in the catalase-hydrogen peroxide reaction. *Acta Chemica Scandinavica*, **1**, 236–267.
37. B. Chance, D.S. Greenstein and F.J.W. Roughton (1952) The mechanism of catalase action. I. Steady-state analysis. *Archives of Biochemistry and Biophysics*, **37**, 301–321.
38. H.N. Kirkman, S. Galiano and G.F. Gaetani (1987) The function of catalase-bound NADPH. *The Journal of Biological Chemistry*, **262**, 660–666.
39. N. Shimizu, K. Kobayashi and K. Hayashi (1988) Studies on the equilibria and kinetics of the reactions of ferrous catalase with ligands. *The Journal of Biochemistry*, **104**, 136–140.
40. D. Keilin and P. Nicholls (1958) Reactions of catalase with hydrogen peroxide with hydrogen peroxide and hydrogen donors. *Biochimica et Biophysica Acta*, **29**, 302–307.
41. Y. Sawada and I. Yamazaki (1973) One-electron transfer reactions in biochemical systems. VIII. Kinetic study of superoxide dismutase. *Biochimica et Biophysica Acta*, **327**, 257–265.
42. M. Mylrajan, K. Valli, H. Wariishi, M.H. Gold and T.M. Loehr (1990) Resonance Raman spectroscopic characterization of Compound III of lignin peroxidase. *Biochemistry*, **29**, 9617–9623.
43. K. Kobayashi, N. Shimizu and K. Hayashi (1986) The reactions of superoxide radical with catalase. In *Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine* (ed. G. Rotilio), Elsevier Science Publishers B.V., Amsterdam., pp. 228–230.
44. D. Cai and M. Tien (1992) Kinetic studies on the formation and decomposition of Compounds II and III. *The Journal of Biological Chemistry*, **267**, 11149–11155.
45. H. Jenzer and H. Kohler (1986) The role of superoxide radicals in lactoperoxidase-catalysed H_2O_2-metabolism and in irreversible enzyme inactivation. *Biochemical and Biophysical Research communications*, **139**, 327–332.
46. A. Mal, A. Nandi and I.B. Chatterjee (1991) Haemoglobin: a scavenger of superoxide radical. *Journal of Biosciences*, **16**, 43–53.
47. H.C. Sutton, P.B. Roberts and C.C. Winterbourn (1976) The rate of reaction of superoxide radical ion with oxyhaemoglobin and methaemoglobin. *Biochemical Journal*, **155**, 503–510.
48. R. Nakajima and I. Yamazaki (1987) The mechanism of oxyperoxidase formation from ferryl peroxidase and hydrogen peroxide. *The Journal of Biological Chemistry*, **262**, 2576–2581.
49. D. Keilin and P. Nicholls (1958) Reactions of catalase with hydrogen peroxide and hydrogen donors. *Biochimica et Biophysica Acta*, **29**, 302–307.

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