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REACTIONS OF BOVINE LIVER CATALASE WITH SUPEROXIDE RADICALS AND HYDROGEN PEROXIDE

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The oxidized intermediates generated upon exposure of bovine liver catalase to hydrogen peroxide (H_2O_2) and superoxide radical ($O_2^{\cdot-}$) fluxes were examined with UV-visible spectrophotometry. H_2O_2 and $O_2^{\cdot-}$ 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 (450-700 nm) regions showed that three oxidized intermediates, namely Compounds I, II and III, can be observed upon exposure of catalase to enzymatically generated H_2O_2 and $O_2^{\cdot-}$. Compound I is formed during the reaction of native enzyme with H_2O_2 and disappears in two ways: (i) via the catalytic reaction with H_2O_2 to restore native catalase and (ii) via the reaction with $O_2^{\cdot-}$ to form Compound II. At low H_2O_2 concentrations ($< 4.8 \times 10^{-9}$ M H_2O_2), Compound II reverts towards the native state mainly in a direct one-step reaction, whereas at higher H_2O_2 concentrations the pathway of Compound II back to the native enzyme involves Compound III. Formation of the latter from Compound II and H_2O_2 is irreversible and the rate constant of this reaction is $6.1 \pm 0.2 \times 10^4\ M^{-1}\ s^{-1}$. The formation of Compound III through the direct reaction of $O_2^{\cdot-}$ with native enzyme has also been observed. Depending on the experimental conditions, the inactivation of catalase by $O_2^{\cdot-}$ can be due to accumulation of Compound II ("slow" inhibition) or to the formation of Compound III ("rapid" inhibition) part of which leads to a dead end product. Formation of Compound III and of this dead end product are responsible for the irreversible inactivation in presence of an excess of H_2O_2.

KEY WORDS: Catalase, Superoxide, Hydrogen peroxide, Compound I, Compound II, Compound III.

Abbreviations Cpd, Compound, cat, catalase, GO, glucose oxidase, SOD, superoxide dismutase.

INTRODUCTION

In most eukaryotic organisms, the major part of the H_2O_2 produced in the course of oxidative cellular metabolism is localized in peroxisomes.[1] Peroxisomes are organelles with diameters ranging from 0.1 to 1.7 μm, bounded by a single membrane, containing catalase, and at least one H_2O_2-producing flavin oxidase,[2] as basic enzymatic constituents. Under normal physiological conditions, catalase controls the H_2O_2 concentration so that this does not reach toxic levels that could bring about oxidative damage inside peroxisomes and surrounding cytoplasm.[1]

Most of the catalases characterized so far can be classified as belonging to one of two types: typical catalases and catalase-peroxidases.[3,4,5] The typical catalases

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commonly isolated from animals,[6,7] plants[8] and microorganisms[9,10,11] resemble each other very closely. They are composed of four subunits of equal size,[12,13] each subunit being formed by a single polypeptide chain[14] with a high-spin Fe(III)-protoporphyrin IX as a prosthetic group.[15] The phenolate of a tyrosyl residue occupies the fifth coordination site of the heme iron and the sixth is vacant in the native resting enzyme.[16,17] The characteristics of the spatial organization of tetrameric catalases from *Penicillium vitale*,[9,18] bovine liver[18,19,20] and *Micrococcus lysodeikticus*[10] are known with a high resolution. These investigations showed that the positions and orientations of the heme are identical in these enzymes; it is located some 20 Å from the surface and is accessible via a largely hydrophobic channel. This structure helps to explain the marked preference of catalase for small substrates and the acquisition of activity toward larger substrates afforded by mild denaturation.[21] Other characteristics found in these typical catalases are the pH-independence of their catalytic activity in the range 5–10,[7,22] their stability in ethanol-chloroform[3] and a specific irreversible inhibition by 3-amino-1,2,4 triazole.[23] Catalase-peroxidases share several properties which distinguish them from the typical catalases.[3,4,5] They catalyze peroxidatic as well as catalatic reactions, their catalatic activity has a sharp pH dependence, they are inactivated by ethanol-chloroform, are not inhibited by 3-amino-1,2,4 triazole, and are more sensitive to temperature and H_2O_2 than the typical catalases. In addition, various atypical enzymes which do not belong to either class of catalases were isolated from various eukaryotic[24] and prokaryotic organisms.[25,26,27,28,29] The most intensely investigated catalases are the tetrameric ones with a heme group in the active site of each subunit. Among mammals, the best-known representative of the typical catalases is that of bovine liver.

H_2O_2 degradation by bovine liver catalase proceeds in two steps.[7,30] In the first step, the native ferrihemoproteins (free catalase) is oxidized by H_2O_2 into a spectroscopically distinct intermediate called Compound I:

$$\text{ferricatalase} + H_2O_2 \rightarrow \text{Compound I} + H_2O$$

There is now convincing evidence that this reaction occurs by removal of one electron from the iron atom with stabilization of the Fe(IV) state by an oxo atom derived from the peroxide to form an oxyferryl center, Fe(IV) = O, while the second electron is removed from a porphyrin orbital to form a π-cation radical.[31] In the second step, two electrons are transferred from an electron donor to form water and an oxidized product:

$$\text{Compound I} + HAOH \rightarrow \text{ferricatalase} + AO + H_2O$$

(where HAOH is a two-electron donor in which A is O, C=O or $H(CH_2)_nCH$ ($n = 1, 2$ or 3))

Substrate oxidation by Compound I is operationally divided into "catalatic" activity, when the electron donor is H_2O_2, and "peroxidatic" activity when the electron donor is an alcohol or formic acid.[32,33]

With certain other substrates (e.g. ascorbate[21]), a one-electron reduced form of Compound I, which is termed Compound II, is generated:

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

(where AH is a one-electron donor)

Compound II is spectrally distinct from free catalase and from Compound I. This Compound has been described by Chance as catalytically inactive.[34] It has been

suggested that this enzyme intermediate contains a Fe(IV)=O structure like Compound I, but no longer has a radical state.[35] Accumulation of Compound II may occur *"in vivo"* under abnormal conditions such as those associated with cell necrosis, tumor and prolongated hypoxia where the production of active hydrogen donors favourable to the formation of Compound II may occur.[36]

The catalase is also able to form an additional intermediate, namely Compound III, which does not participate in the normal "catalatic" or "peroxidatic" cycles and has been reported to be generally inactive towards most electron donors.[37] Compound III has properties similar to, but not identical with, the oxy Compounds of myoglobin and hemoglobin.[38] It can be generated via three procedures: (a) reduction of resting catalase followed by addition of O_2[39,40]; (b) addition of H_2O_2 to Compound II;[41,42] and (c) association of the resting enzyme with superoxide anion ($O_2^{\cdot-}$).[43,44] The first report of preparation of Compound III involved reaction of Compound II with excess H_2O_2 at 3°C.[41] On standing, Compound III was slowly converted back to Compound II. This led Keilin and Nicholls[37] to propose that H_2O_2 reacts with Compound II to produce Compound III in a reversible reaction. However, our understanding as to whether this reaction constitutes a true equilibrium or represents a dynamic feature of several consecutive reactions at steady-state has remained inconclusive.

Although the generation of oxyradicals has been evidenced in peroxisomes of animal[45] and plant cells,[1,46] there have been only a few papers written about the reaction of catalase with perhydroxyl radical ($HO_2^{\cdot}$) and its conjugate base, $O_2^{\cdot-}$. Earlier experiments with enzymatic sources of $O_2^{\cdot-}$ indicate that this oxyradical can inhibit the catalytic cycle.[47] Recently, the reaction of $O_2^{\cdot-}/HO_2^{\cdot}$ radicals with catalase was investigated by pulse and γ radiolysis techniques[40,44] These investigations showed that, in irradiated oxygen-saturated solutions of the bovine liver enzyme, all three catalase oxidized compounds can be generated. In the present communication, we confirm and extend the findings previously reported for bovine liver catalase by radiolysis, using enzymatic sources of $O_2^{\cdot-}$ and H_2O_2.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and had been purchased from Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany) or Sigma (St Louis, USA). Catalase from bovine liver (EC 1.11.1.6, 65000 U/mg), copper-zinc containing superoxide dismutase from bovine erythrocytes (EC 1.15.1.1., 5000 U/mg), 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) for at least 24 hours before use.

Electronic Spectra

Spectral properties of bovine liver catalase upon the addition of H_2O_2 and/or $O_2^{\cdot-}$ generating systems were monitored by recording UV-visible absorption with a Philips PU 8700 spectrophotometer. 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. Due to its molar absorption coefficient ($\varepsilon = 3.24 \times 10^5\,M^{-1}\,cm^{-1}$ per tretramer at 405 nm[48]) bovine liver catalase was used at a concentration of 3.10 μM. This was done by diluting an adequate amount of the dialyzed preparation in a Na_2HPO_4-KH_2PO_4 buffer (50 mM phosphate, pH 7). Potassium ferrocyanide, a one electron reductant of Compound I,[6] was used in some experiments, to create a large steady-state concentration of Compound II.

Catalase Assay

Catalase activity was assayed by a modification of the procedure of Del Rio *et al.*[49] as has been described previously.[50] The H_2O_2 concentration used in all the experiments was 10 mM. The activity was calculated in the linear phase of oxygen production which began 5 to 40 s after the start of the reaction, depending on the catalase sample.

Superoxide Dismutase Assay

Superoxide dismutase activity was estimated by the method of McCord and Fridovich[51] and was in agreement with the supplier's stated activity. Its concentration was calculated using a molar absorptivity of $1.03 \times 10^4\,M^{-1}\,cm^{-1}$ at 258 nm[51]

H_2O_2 Concentration of the Catalase Solution at Steady-state

In coupled enzymatic assay, the low levels of H_2O_2 maintained by the catalase action 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 equation (1):

$$[H_2O_2] = \frac{R\ \text{oxidase}}{k_1[\text{free cat}]} \qquad (1)$$

in which k_1 represents the rate constant for the reaction of free catalase with H_2O_2 ($k_1 = 1.7 \times 10^7\,M^{-1}\,s^{-1}$ [30]) and $R_{oxidase}$ represents the rate of production of H_2O_2 by the oxidase (for a complete development of this equation see reference 50). In this equation, $R_{oxidase}$ was estimated experimentally with a polarogaphic 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 405 nm (see below). All the assays were carried out in Na_2HPO_4-KH_2PO_4 buffer (50 mM phosphate, pH7) at 25°C in the presence of an excess of ethanol (2 mM, final concentration). Glucose-oxidase was used as a source of H_2O_2.

Free Catalase and Compound II Concentrations at Steady-state

The concentrations of free catalase and of Compound II present in the reaction mixtures were estimated by comparing the absorbance at 405 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 12 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 2, 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:

$$O.D._b = \varepsilon_{free}[free]_b + \varepsilon_{cpd\ I}[Cpd\ I]_b + \varepsilon_{cpd\ II}[Cpd\ II]_b \quad (2)$$

$$O.D._a = \varepsilon_{free}[free]_a + \varepsilon_{cpd\ II}[Cpd\ II]_a \quad (3)$$

$$[free]_a = [free]_b + [Cpd\ I]_b \quad (4)$$

$$[Cpd\ II]_a = [Cpd\ II]_b \quad (5)$$

$$[enzyme] = [free]_b + [Cpd\ I]_b + [Cpd\ II]_b \quad (6)$$

where O.D. is the absorbance of the reaction mixture at 405 nm and where [ ] and ε represent respectively the concentration and the molar absorptivity at 405 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 (2), (3), (4) and (5) yields:

$$[Cpd\ I]_b = [free]_a - [free]_b = \frac{O.D._b - O.D._a}{\varepsilon_{cpd\ I} - \varepsilon_{free}} \quad (7)$$

The ratio [free]/[Cpd I] 12 seconds after the addition of the glucose/glucose oxidase system was estimated to be 2.32. This ratio was calculated from variation of absorbance at 405 nm with respect to the native enzyme (Figure 1,A) using ε_{free} =

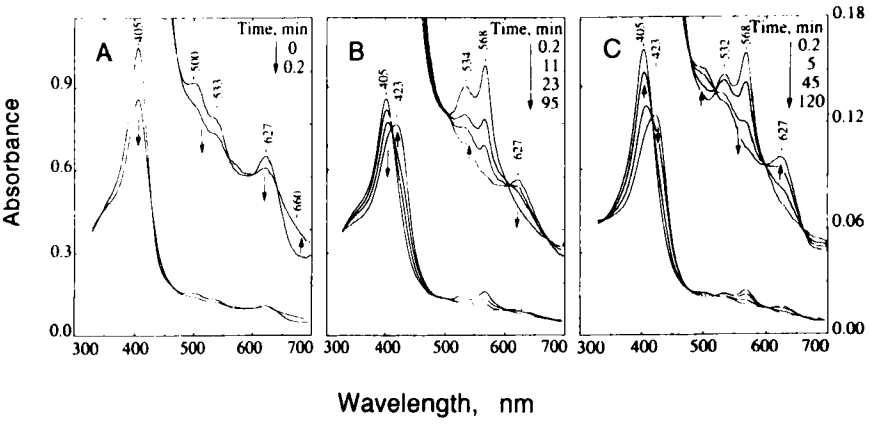

FIGURE 1 *Formation of Compound I and Compound II from native enzyme and decomposition of Compound II in presence of ethanol.* The reaction mixture consisted of bovine liver catalase (3.10 μM), glucose (10 mM), potassium ferrocyanide (100 μM) in 3 ml Na_2HPO_4-KH_2PO_4 buffer (50 mM in phosphate, pH 7). The temperature was 25°C. H_2O_2 was generated at a rate of 17 μmole L^{-1} min^{-1} by adding at 0 time glucose oxidase to the final concentration of 15 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 12 s; B) spectral changes monitored during the following 95 min; C) spectral changes monitored between 12 s and 120 min after the addition of ethanol. In this case, ethanol was added at a final concentration of 2 mM, 95 min after the addition of glucose oxidase.

$3.24 \times 10^5\,M^{-1}\,cm^{-1}$ per tetramer for ferricatalase[48] and $\varepsilon_{cpd\ I} = 2.19 \times 10^5\,M^{-1}\,cm^{-1}$ per tetramer for Compound I (determined from Figure 1A in reference 52). Supposing that, in the absence of ethanol, such ratio remained constant during the time course of the reaction and combining equations (4), (6) and (7) yields:

$$[\text{free}]_a = 3.32\,\frac{\text{O.D.}_b - \text{O.D.}_a}{\varepsilon_{cpd\ I} - \varepsilon_{free}} \tag{8}$$

and

$$[\text{Cpd II}]_a = [\text{enzyme}] - [\text{free}]_a \tag{9}$$

Compound III Concentration at Steady-state

The concentration of Compound III present in the reaction mixtures was evaluated from the absorbance at 420 nm using $\varepsilon_{free} = 2.65 \times 10^5\,M^{-1}\,cm^{-1}$ per tetramer for ferricatalase and $\varepsilon_{Cpd\ III} = 2.92 \times 10^5\,M^{-1}\,cm^{-1}$ per tetramer for Compound III. The ε_{free} and $\varepsilon_{Cpd\ III}$ at 420 nm were determined from the spectra of catalase solutions containing 100% native form and 100% Compound III respectively. This latter solution was prepared by a procedure similar to those described in the legend of Figure 6 except that xanthine oxidase was added at a final concentration of 9 nM. As further addition of xanthine oxidase caused no absorption change, it was considered that the reaction mixture contained 100% Compound III.

RESULTS

Formation and Decomposition of Compound II

Glucose oxidase and glucose were added to a solution containing the native enzyme at pH 7 and an excess of ferrocyanide. This resulted in the immediate decrease in absorbance at 405 nm of the Soret band and a red shift of the α-band of the native enzyme to 660 nm, which are associated with the formation of Compound I (Figure 1A)[42]. During the following 95 min, increased absorption appeared in the region of 423, 534 and 568 nm that are characteristic of Compound II (Figure 1B)[42]. The spectrum displayed isosbestic points at 414, 477, 500, 608 and 675 nm, suggesting a direct conversion without long-life intermediates (Figure 1B). The halftime for the formation of Compound II is 35 min, and its concentration reached a maximum, involving 95% of the catalase haematins after about 4 hours.

To determine the stability of Compound II, 2 mM ethanol was added which decreased the concentration of residual Compound I instantly to very nearly zero (not shown) because of the peroxidatic reaction between Compound I and ethanol. This reduction in the concentration of Compound I caused Compound II to revert slowly to the native form with a half time of 20 min (Figure 1C). The spectra presented in Figure 1C displayed isosbestic points at 419, 479, 520, 600 and 661 nm, suggesting a direct conversion without long-life intermediates.

Involvement of H_2O_2 in Compound II Decomposition

That H_2O_2 was involved in the Compound II decomposition process was tested by the addition of increasing concentrations of glucose oxidase immediately after the

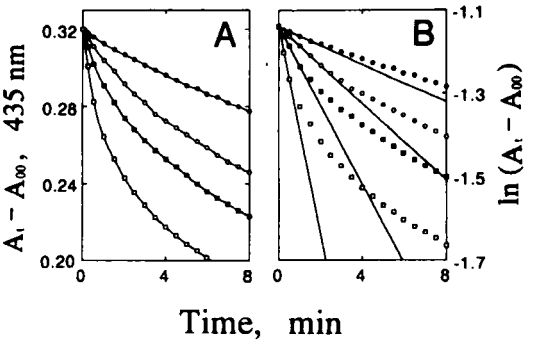

FIGURE 2 *Rate of disappearance of C'ompound II at increasing glucose oxidase concentration.* Incubation at pH 7 and assay conditions were as described in the legend of Figure 1C except that an additional amount of glucose oxidase was added to the reaction mixture immediately after the injection of ethanol. A) Compound II conversion curves monitored at 435 nm; B) conversion curves plotted according to equation 10 in the text. ●, no additional GO added; ○, 30 nM GO; ■, 60 nM GO; □, 150 nM GO.

injection of ethanol in the above reaction mixture. The kinetics of the decomposition of Compound II were followed at 435 nm since the spectra of ferricatalase and Compound I are essentially isosbestic at this wavelength.[34] Typical traces where the initial absorbance changes at 435 nm are monitored in the presence of varying amounts of glucose oxidase are shown in Figure 2A. Figure 2B shows the natural log of the absorbance plotted against time according to

$$\ln (A_t - A_{oo}) = -k_{obs} t + \ln(A_o - A_{oo}) \quad (10)$$

where A_o, A_t, and A_{oo} are the absorbances at times o, t, and 120 min. During the first 40 s following the addition, the replotted lines (Figure 2B) for each glucose

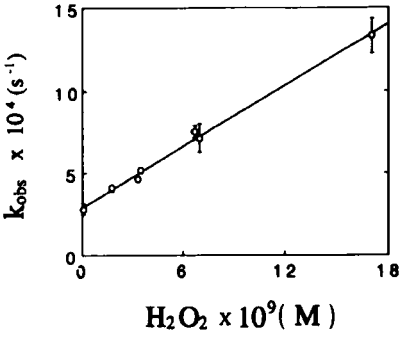

FIGURE 3 *Plot of k_{obs} versus H_2O_2 concentration at steady-state for the decomposition of Compound II.* Experiments were conducted as described in the legend to Figure 2 with various amounts of glucose oxidase. The k_{obs} were obtained from initial changes in absorbance at 435 nm occurring within 40 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.

oxidase concentration were linear, indicating that the initial reaction kinetics is first order. The slope of the lines allowed estimation of the rate constants (k_{obs}). A plot of k_{obs} as a function of the H_2O_2 initial concentration at steady-state is presented in Figure 3 (see material & methods for calculation of H_2O_2 concentrations at steady-state). A linear dependence of k_{obs} on H_2O_2 concentration was observed with a clear ordinate intercept. Similar results were obtained when the rate of H_2O_2 production was increased by elevating the level in glucose or in oxygen of the reaction mixture or by carrying the experiments in the presence of 1 μM superoxide dismutase (SOD) (not shown). The data of Figure 3 suggest that the decomposition of Compound II can proceed through one of two distinct reaction paths: the linear dependence of k_{obs} on H_2O_2 concentration shows that H_2O_2 is involved in the frequently cited "spontaneous" disappearance of Compound II that accompanies a decrease in concentration of Compound I. A non-zero intercept is a good indication that Compound II decomposition proceeds at an appreciable rate even in absence of H_2O_2. A second-order rate constant of $6.1 \pm 0.2 \times 10^4 M^{-1} s^{-1}$ for the reaction of Compound II with H_2O_2 was estimated from the slope of the line whereas a first-order rate constant of $2.9 \pm 0.2 \times 10^{-4} s^{-1}$ was estimated from the intercept for the reaction occuring in absence of H_2O_2.

Reaction of Compound II with H_2O_2 and Formation of Compound III

In order to further analyze 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

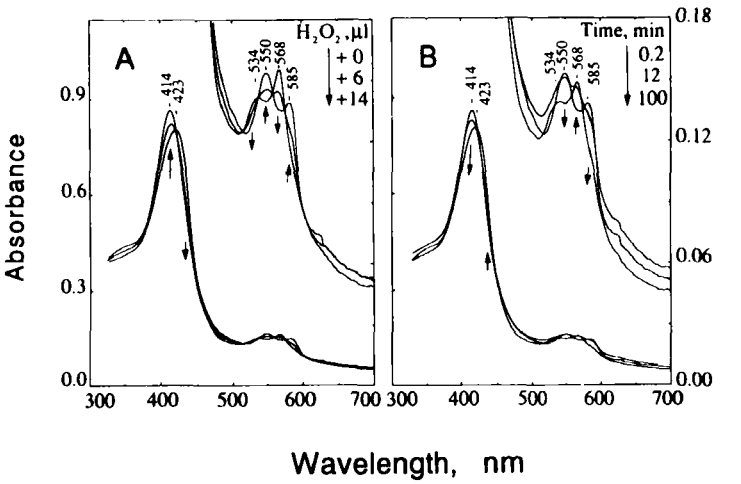

FIGURE 4 *Formation of Compound III from Compound II and H_2O_2 and spontaneous decomposition of Compound III* Incubation at 25°C and assay conditions were as described in the legend of Figure 1. 95 min after adding glucose oxidase, the incubation medium was cooled down and the reaction was initiated by the addition of 30% H_2O_2 solution. Spectral changes are indicated by arrows. A) Spectral changes from Compound II to Compound III at 3°C; H_2O_2 was added stepwise as indicated (µl 30% H_2O_2 solution) and each spectrum was recorded 6 s after addition of H_2O_2; B) spontaneous decomposition of Compound III at 3°C; in this case, Compound III was prepared as in A) by addition of 20 µl 30% H_2O_2 solution and scans were taken at the time intervals indicated.

the mixture at 3°C resulted in the formation of species similar to catalase Compound III[42], with absorption maxima at 414, 550 and 584 nm. 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 4A. Spectra were taken within 6 s after addition of H_2O_2. They displayed isosbestic points at 454, 521, 537, 560 and 578 nm, indicating a lack of long-life intermediates in this conversion process. At 3°C, Compound III was stable for about 5 minutes after its formation. After this time, Compound II was slowly formed again (Figure 4B). This observation has led some authors[37] to propose that the formation of Compound III from Compound II and H_2O_2 is a reversible reaction. However, successive scans made during the course of the conversion (Figure 4B) showed a lack of well defined isosbestic points, suggesting a complex reversion pattern. Moreover, as Compound III is known to decompose spontaneously to the ferric form,[39,44] the apparent equilibrium between Compound II and III might represent a dynamic feature of several consecutive reactions rather than a true equilibrium.

To discriminate between both hypothesis, an excess of ethanol was added to the above reaction mixture 5 minutes after the injection of H_2O_2. The assay was carried out in the presence of ethanol in order to maintain at very nearly zero the concentration in Compound I which issued from the reaction of ferricatalase with H_2O_2. Spectra of the resulting solution were determined at different time intervals after mixing and are presented in differential mode in Figure 5A. The differential spectra obtained in absence of ethanol from a separate experiment are shown in Figure 5B for comparison. In the presence of ethanol, none of the spectra showed an increase of absorption peaks at 534 and 568 nm that are characteristic of Compound II (Figure 5A). In contrast, both peaks were apparent in the absence of the

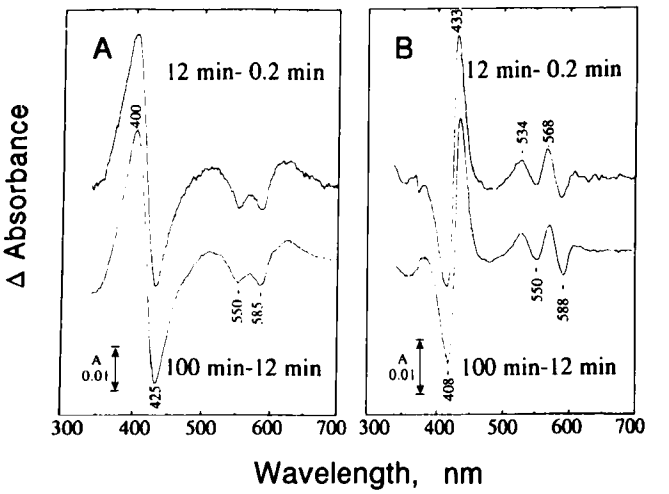

FIGURE 5 *Spontaneous decomposition of Compound III in presence and in absence of ethanol* Compound III was prepared at 3°C as described in the legend of Figure 4 by addition of H_2O_2 at a final concentration of 66 mM, 95 min after the addition of glucose oxidase into the incubation medium. A) Difference between spectra recorded at different time intervals after the addition of ethanol to the final concentration of 2 mM; in this case, ethanol was added 5 minutes after adding H_2O_2; B) difference between spectra obtained in absence of ethanol.

alcohol (Figure 5B). It is thus clear that ethanol, by maintaining a low level in Compound I, inhibited Compound II formation. This observation provides a conclusive evidence that under the conditions used in our experiments, Compound II reacted with H_2O_2 in an irreversible way.

Reaction of Ferricatalase with O_2^- and Formation of Compound III

Compound III of bovine liver catalase can also be directly formed by a reaction of the ferric enzyme with O_2^-:[43,44] the addition of xanthine oxidase to a solution containing the native enzyme, xanthine and ethanol at 3°C and pH 7 resulted in the formation of Compound III-like species with absorption maxima at 410, 547 and 584 nm (Figure 6A). The assay was carried out in the presence of ethanol to maintain at very nearly zero the concentration of Compound I issued from the reaction of ferricatalase with H_2O_2. This reaction was inhibited in the presence of 1 μM superoxide dismutase (data not shown). When all the xanthine was consumed, Compound III rapidly reverted to resting enzyme (Figure 6B). The difference spectra during Compound III decay toward resting enzyme are presented in Figure 7. The spectral changes for the formation and reversion of Compound III showed the same isosbestic points in the visible region at 413, 485, 531, 600 and 657 nm, suggesting that both the forward and reverse reactions occured in a single reversible step. Moreover, the spectra of Figure 7 are essentially identical to the spectra presented in Figure 5A, indicating that in both cases, Compound III reverted to native resting form through the same path.

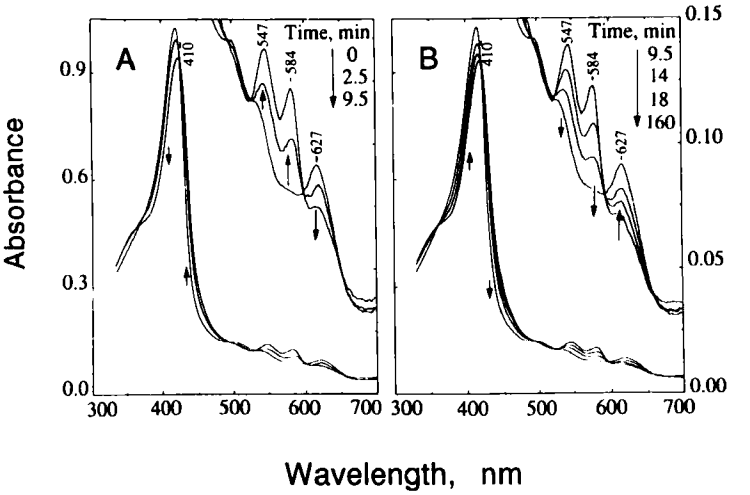

FIGURE 6 *Formation of Compound III from native enzyme and O_2^- and spontaneous decomposition of Compound III* The reaction mixture consisted of bovine liver catalase (3.10 μM), xanthine (300 μM), ethanol (2 mM) in 3 ml Na_2HPO_4-KH_2PO_4 buffer (50 mM in phosphate, pH 7) at 3°C. The reaction was initiated by the addition of xanthine oxidase at a final concentration of 3.3 nM. A) Spectral changes observed during the first 9.5 min; B), spectral changes monitored during the following 160 min.

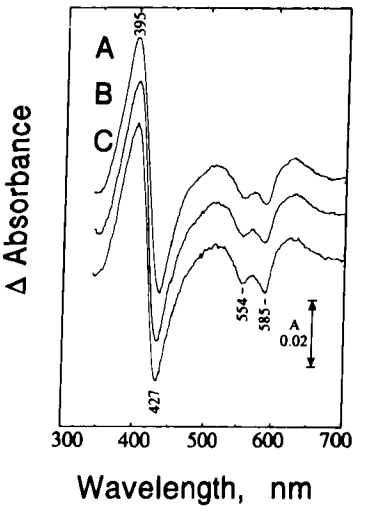

FIGURE 7 *Kinetic difference spectra for Compound III decay toward native enzyme* Assay conditions were as described in the legend of Figure 6. Difference spectra represent changes in absorbance occuring between 9.5 and 160 min after the addition of xanthine oxidase. A) spectral changes recorded between 9.5 and 14 min; B) between 14 and 18 min and C) between 18 and 160 min.

Reaction of Compound I with O_2^-

The reaction of Compound I with O_2^- was investigated by incubating the native enzyme with the xanthine-xanthine oxidase system at 25°C and in the absence of ethanol. The data are presented as the difference between spectra because the changes are too small to provide unambiguous 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 8A, for comparison. These spectra were obtained from separate experiments performed as above (see Figure 1).

The scans following the addition of xanthine oxidase to native enzyme showed the formation of two distinct spectral intermediates (Figure 8B 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. None of the spectra observed had the twin absorption peaks at 550 and 585 nm that are characteristic of Compound III, in accordance with the poor stability of this intermediate at 25°C. When all the xanthine was consumed (9 minutes), Compound I rapidly reverted to a resting enzyme as was shown by a spectral variation (Figure 8C) which is the inverse of the difference between Compound I and ferric catalase. The experiment was carried out under conditions where a large amount of H_2O_2 was generated due to spontaneous O_2^- dismutation. Thus, it seems likely that Compound I originated from the reaction of ferricatalase with H_2O_2, and was subsequently converted into Compound II by reacting with O_2^-. 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

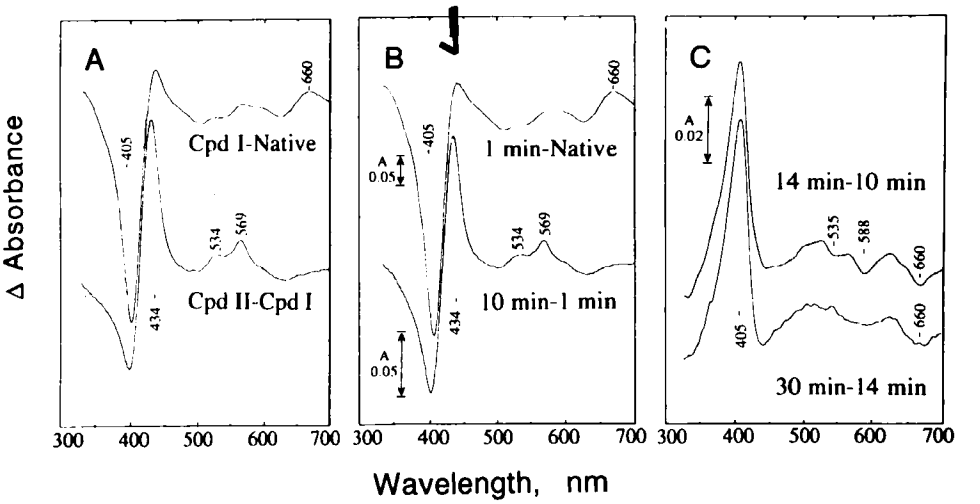

FIGURE 8 *Formation of Compound I and of Compound II from native enzyme and* O_2^- The reaction mixtures consisted of bovine liver catalase (3.10 μM), xanthine (70 μM) in 3 ml Na_2HPO_4-KH_2PO_4 buffer (50 mM in phosphate, pH 7) at 25°C. A) Difference between spectra from separate experiments performed as described in Figure 1; B) & C) difference between spectra recorded at different time intervals after the addition of xanthine oxidase to the final concentration of 0.4 nM.

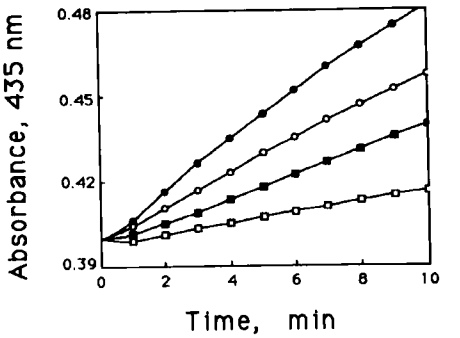

FIGURE 9 *Rate of appearance of Compound II at increasing superoxide dismutase concentration* Incubation at pH 7 and assay conditions were as described in the legend of Figure 8 except that superoxide dismutase was added to the reaction mixture before the injection of 0.4 nM xanthine oxidase. ●, no SOD added; ○, 0.2 nM SOD; ■, 0.4 nM SOD; □, 1 μM SOD.

changes at 435 nm are monitored in the presence of increasing amounts of SOD are shown in Figure 9. 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.

Lack of Reaction of Compound II with O_2^-

To investigate the reaction of Compound II with O_2^-, the former compound was produced by adding glucose oxidase and glucose to a solution containing the native

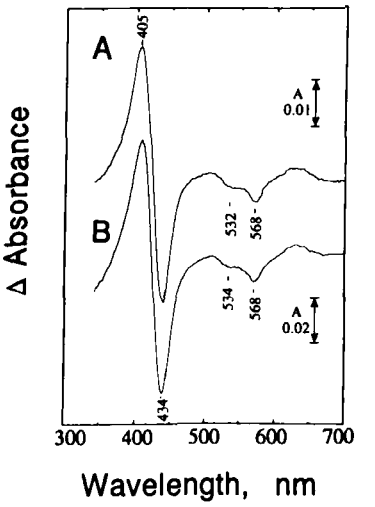

FIGURE 10 *Influence of O_2^- and ethanol on Compound II decomposition* Compound II was generated by the addition of 15 nM glucose oxidase, 10 mM glucose and 100 μM potassium ferrocyanide to 6.20 μM bovine liver catalase in 1.5 ml of Na_2HPO_4-KH_2PO_4 buffer (50 mM in phosphate, pH 7). The temperature was 25°C. 95 min after the addition of glucose oxidase, the volume of the reaction mixture was adjusted to 3 ml by adding 1.5 ml of a Na_2HPO_4-KH_2PO_4 buffer (50 mM phosphate, pH 7) containing 140 μM xanthine. The reaction was initated immediately after the volume adjustment by the addition of 2 mM ethanol (A) or by the simultaneous addition of 2 mM ethanol and 0.66 nM xanthine oxidase (B). In both cases, difference spectra represent changes in absorbance occurring between 30 s and 8 min after the start of the reaction.

enzyme and an excess of ferrocyanide (see Figure 1). After about 95 min, xanthine and ethanol were added together in the above reaction mixture. The spectral variation observed between 30 s and 8 min after the addition of xanthine and ethanol is presented in Figure 10A. It is typical of a difference between the spectra of Compound II and native form presented in Figure 1C, indicating that part of Compound II was converted into resting ferric enzyme as a result of the removal of Compound I. Similar changes in spectra were obtained when xanthine oxidase was added immediately after the injection of ethanol and of xanthine in the above reaction mixture (Figure 10B). However, in presence of the O_2^- generating system, the conversion of Compound II to native enzyme was accelerated.

To discover the transients responsible for this increase, the above experiment was repeated in the presence of SOD. The kinetics of decomposition of Compound II were measured at 435 nm immediately after the injection of the O_2^- generating system in the above reaction mixture. As the initial variations in absorbance were of simple exponential character, the first-order rate constants of the reaction were determined. The data of Table 1 summarize a series of such experiments. Data obtained in absence of xanthine oxidase and/or of SOD are given for comparison. The results in Table 1 confirm that the decay with the O_2^- generating system is significantly greater than the decay with ethanol alone. However, the presence of SOD in the reaction mixture did not change significantly the rate of Compound II decomposition even when the concentration of the O_2^- scavenger was increased to

TABLE 1

Influence of O_2^- on the rate of conversion of Compound II into native catalase Incubation and assay conditions were as described in the legend of Figure 10 except that superoxide dismutase was added to the reaction mixture before the injection of xanthine oxidase and ethanol. Results obtained in the absence of superoxide dismutase or in the absence of xanthine oxidase are given for comparison

Xanthine oxidase (nM)	Superoxide dismutase (μM)	Rate constants (mean ± C.I.[a]) ($10^4 \times k_{obs}, s^{-1}$)
0	0	3.2 ± 0.2
0.66	0	7.9 ± 0.7
0.66	1.1	7.6 ± 0.7

[a] 95% confidence interval

1.1 μM. It is thus clear that, under the conditions used in Figure 10, O_2^- plays no role in Compound II decomposition. Therefore, the acceleration of Compound II decay can reasonably be attributed to an increase in the level of H_2O_2 associated with the xanthine oxidase action.

Lack of Reaction of Compound II with Ethanol and with Ferrocyanide

To investigate the effect of ethanol and of ferrocyanide on Compound II decomposition, Compound II was produced as previously (shown in Figure 8) by incubating the native enzyme with the xanthine-xanthine oxidase system at 25°C. After 30 min, when all the xanthine was consumed and when all the Compound I was reconverted into ferric form (see Figure 8), either ethanol or ethanol and ferrocyanide were injected in the reaction mixture. The rate of decomposition of Compound II was then determined at 435 nm. As the initial variations in absorbance were of simple exponential character, the first-order rate constants of the reaction were determined (data presented in Table 2). The results in table 2 show that Compound II decay rates are similar in absence and in presence of ethanol or ferrocyanide. It is thus clear that neither ethanol, nor ferrocyanide plays a role in the Compound II decomposition process.

TABLE 2

Influence of ethanol and of ferrocyanide on the rate of conversion of Compound II into native catalase Incubation and assay conditions were as described in the legend of Figure 8 except that ethanol and ferrocyanide were added to the reaction mixture 30 min after the addition of xanthine oxidase. The result obtained in the absence of ethanol and ferrocyanide is given together for comparison

Ethanol (mM)	Ferrocyanide (μM)	Rate constants (mean ± C.I.[a]) ($10^4 \times k_{obs}, s^{-1}$)
0	0	2.7 ± 0.2
2	0	2.8 ± 0.7
10	0	2.9 ± 1.0
2	100	2.8 ± 0.2

[a] 95% confidence interval

Role of Compound II in Inactivation

In order to investigate if the formation of Compound II inhibits the destruction of hydrogen peroxide by catalase, Compound II was generated as above (see Figure 1) by adding glucose oxidase and glucose to a solution containing the native enzyme and an excess of ferrocyanide. After 2 hours of exposure to the flux of H_2O_2, an excess of ethanol (2 mM) was added to reduce Compound I instantly to very nearly zero and the proportion of each constituent was measured with a spectrophotometer (Table 3). After suitable dilution, samples were also tested for catalase activity with a polarographic oxygen electrode. The results in Table 3 (lines 1 & 2) show a loss of activity related to the formation of Compound II. No direct proportionality was observed between the number of haematins in form of Compound II and the extent of inactivation: a decrease of the number of free haematins by about 88% caused a reduction of the catalase activity by only 31% (Table 3, line 2).

When the activity measurements were performed 2 hours after the injection of the alcohol, the data of Table 3 (line 3) also indicates that this inhibition can be entirely reversed. The spectroscopic measurements undertaken under such conditions demonstrate that ethanol and a low level of H_2O_2 due to the glucose oxidase action caused the conversion of the major part of Compound II into ferric form (Table 3, line 3).

Effect of O_2^- on catalase

The influence of O_2^- on catalase activity was investigated by incubating the enzyme with the xanthine/xanthine oxidase system at 25°C. After 6 hours of exposure to the flux of O_2^-, an excess of ethanol (2 mM) was added and the proportion of each constituent was evaluated as above (see Table 3). The global activity of the preparation was then estimated with a polarographic oxygen electrode and compared with the activity of a blank in which xanthine oxidase was omitted.

The results in Table 4 (lines 1 & 2) indicate that the oxyradical induced the enrichment of the catalase preparation in Compound II and a resulting loss of activity:

TABLE 3

Role of Compound II in inactivation of catalase Incubation at 25°C and assay conditions were as described in the legend of Figure 1. The initial proportion of each constituent was evaluated by comparing the absorbance of the various reaction mixtures at 405 nm before and after the addition of 2 mM ethanol (see materials and methods). The global activity of the preparation was estimated with a polarographic oxygen electrode as described in the text

Incubation times		Initial proportion of each Compound		Activity (mean ● C.I.[a])	
with glucose/glucose oxidase system (min)	with ethanol	ferric (%)	Compound II	(μmole O_2 s^{-1} mg^{-1})	(%)[b]
0	0.2	100	0	564 ± 35	100 ± 6
120	0.2	12	88	389 ± 27	69 ± 5
240	120	95	5	537 ± 42	95 ± 7

[a] 95% confidence interval

[b] Percentage of catalase activity was calculated from the rate of oxygen production at 0 time.

TABLE 4

Effect of O_2^- on catalase activity Incubation at 25°C and assay conditions were as described in the legend of Figure 8. The initial proportion of each constituent was evaluated by comparing the absorbance of the various reaction mixtures at 405 nm before and after the addition of 2 mM ethanol (see materials and methods). The global activity of the preparation was estimated with a polarographic oxygen electrode as described in the text

Incubation times		Initial proportion of each Compound		Activity (mean ± C.I.[a])	
with xanthine xanthine oxidase system (min)	with ethanol	ferric (%)	Compound II (%)	(μmole O_2 s^{-1} mg^{-1})	(%)[b]
0	0.2	100	0	575 ● 52	100 ● 9
360	0.2	42	58	449 ± 67	78 ± 12
480	120	93	7	599 ● 50	104 ± 9

[a] 95% confidence interval

[b] Percentage of catalase activity was calculated from the rate of oxygen production at 0 time.

the activity of the preparation which contained about 58% of the Compound II was 22% lower than that of the catalase solution before its exposure to O_2^-. Moreover, when activity was tested 2 hours after the injection of the alcohol, no activity loss was observed (Table 4, line 3). As shown in Table 3, the spectroscopic measurements undertaken under such conditions reveal that ethanol caused the conversion of the major part of Compound II into ferric form and therefore that the enzyme was not irreversibly inactivated by low level in O_2^- and H_2O_2. The data of Table 4 show also clearly that if a higher concentration of H_2O_2 is injected into the reaction mixture containing Compound II, an appreciable loss in activity occurs (Table 4, line 2). Taken together, these elements indicate that a part of Compound II or of some intermediate generated along the conversion path to the native form is irreversibly inactivated by the excess of H_2O_2 used in the activity test.

Role of Compound III in Inactivation

In the presence of excess H_2O_2, Compound II is unstable and may be rapidly converted to the resting form via Compound III (see Figures 4 and 5). It is thus possible that a transient accumulation of Compound III is responsible for the loss of activity observed in Tables 3 & 4. To investigate this possibility, Compound III was generated as above (see Figure 6) by adding xanthine oxidase to a solution containing the native enzyme, xanthine and ethanol at 3°C. As before, the assay was carried out in the presence of 2 mM ethanol to maintain to very nearly zero the concentration in Compound I which issued from the reaction of ferricatalase with H_2O_2. When all the xanthine was consumed, the proportion of each constituent was evaluated, from the known molar extinction coefficient of fericatalase and Compound III, and samples were tested for catalase activity with a polarographic oxygen electrode (Table 5).

The results in Table 5 demonstrate that a loss of activity was related to the formation of Compound III (Table 5, lines 1 & 2). Moreover, when activity was tested 2 hours after the injection of xanthine oxidase no activity loss was observed (Table 5, line 3). The spectroscopic measurements undertaken under such conditions

TABLE 5

Role of Compound III in inactivation of catalase Incubation at 3°C and assay conditions were as described in the legend of Figure 6. The initial proportion of each constituent was evaluated by comparing the absorbance of the various reaction mixtures at 420 nm (see materials and methods). The global activity of the preparation was estimated with a polarographic oxygen electrode as described in the text

Incubation times	Initial proportion of each Compound		Activity (mean ± C.I.[a])	
with xanthine/xanthine oxidase system + ethanol (min)	ferric (%)	Compound III (%)	(μmole O_2 s^{-1} mg^{-1})	(%)[b]
0	100	0	553 ± 77	100 ± 14
9.5[c]	49	51	455 ± 17	82 ± 3
120[c]	100	0	581 ± 10	105 ± 2

[a] 95% confidence interval
[b] Percentage of catalase activity was calculated from the rate of oxygen production at 0 time.
[c] All the xanthine was consumed 9.5 min after the injection of xanthine oxidase.

demonstrates that during this time interval, Compound III was entirely reconverted into native forin (Table 5, line 3). The data in Table 5 (lines 1 & 2) show also that the reduction in activity associated with the formation of Compound III was of similar magnitude to those observed in the presence of Compound II (see Table 4, lines 1 & 2 for comparison): the activity of the preparation which contained about 51% of the Compound III was 18% lower to that of the catalase solution before its exposure to O_2^-. Since the presence of excess H_2O_2 results in the conversion of Compound II to resting form via Compound III (see Figures 4 and 5), these data demonstrate that it is Compound III-rather than Compound II-which is responsible for the loss of activity observed in Tables 3 and 4.

DISCUSSION

Regeneration of Ferric Catalase from Compound II

The catalase can exist in various oxidation states. There are three known oxidized intermediates, namely Compound I, Compound II and Compound III. These intermediates are interconvertible upon the addition of suitable oxidants and reductants to the ferric (resting) form of the enzyme, as shown in Figure 11A. Earlier experiments of Gebika *et al.*[44] showed that, in irradiated oxygen-saturated solutions of bovine liver catalase, all three catalase oxidized intermediates can be observed after the radiolysis pulse, as summarized in Figure 11B. A careful analysis of absorption changes occurring after the pulse led those authors to propose that Compound I is formed in the reaction of catalase with H_2O_2 (reaction 1) which 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 3). Part of the O_2^- formed after the pulse reacted also with native catalase to form an unstable Compound III (reaction 4), which was converted back to ferric catalase over a period of minutes after the pulse (reaction 5).

The data presented in this study both confirm and extend the analysis of Gebicka *et al.*:[44] In particular, the five reactions already evidenced by those authors in the

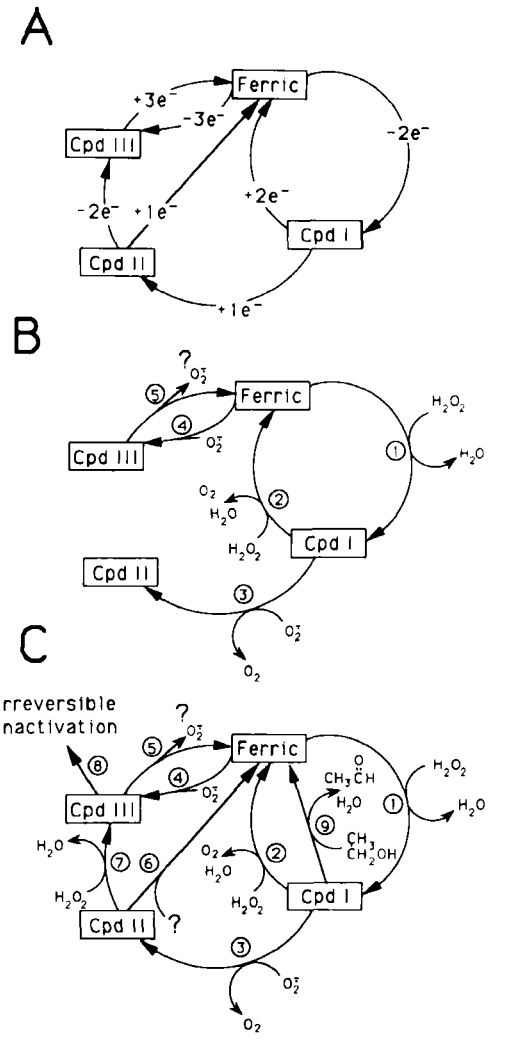

FIGURE 11 *Reactions of bovine river catalase with superoxide radicals, hydrogen peroxide and ethanol* A) Redox states of catalase: resting form (ferric) and three oxidized forms (Cpd I, Cpd II and Cpd III); B) reaction pathways deduced from pulse radiolysis experiments; C) reaction pathways derived from experiments in which O_2^- and H_2O_2 are generated by an oxidizing system. Note that Compound II is less stable in C) than in B). As reported in this paper, Compound II is reconverted to the resting ferric enzyme by a direct one-step reaction (reaction 6) or via Compound III (reactions 7 + 5). The nature of the reducing substance(s) leading to the one-step decomposition of Compound II (reaction 6) is still poorly understood. The Compound III pathway is combined with irreversible inactivation whenever excess H_2O_2 is present (reaction 8). Until now, release of O_2^- during step 5 has only been observed in some peroxidases.[53,54]. Ethanol reacts with Compound I by direct $2e^-$ transfer (reaction 9).[6,30] Rate constants from various above mentionned reactions measured at 25°C and pH ranging from 7 to 7.5: $k_1 = 1.7 - 3.0 \times 10^7\ M^{-1}\ s^{-1}$[6,30,44]; $k_2 = 1.0 - 2.6 \times 10^7\ M^{-1}\ s^{-1}$[6,30]; $k_3 = 5.0 \times 10^6\ M^{-1}\ s^{-1}$[44]; $k_4 = 2.0 - 3.0 \times 10^5\ M^{-1}\ s^{-1}$[43,44]; $k_5 = 1.1 \times 10^{-2}\ s^{-1}$[44]; $k_6 = 2.7 - 3.2 \times 10^{-4}\ s^{-1}$ [This study]; $k_7 = 6.1 \times 10^4\ M^{-1}\ s^{-1}$ [This study]; $k_9 = 0.2 - 1.0 \times 10^3\ M^{-1}\ s^{-1}$[6,30].

presence of radiolytically generated O_2^- (reactions 1 to 5) were also observed under our experimental conditions. However, our spectroscopic analysis indicates that under the conditions used in our experiments, Compound II is less stable than originally observed by Gebika *et al.*;[44] it is shown here, that Compound II can revert toward the native resting form in two distinct ways as depicted in Figure 11C: (i) by a direct one-step reaction which takes place in the absence of H_2O_2 (reaction 6) or (ii) via the reaction with H_2O_2 to form Compound III (reaction 7), which is then rapidly converted back to the resting enzyme (reaction 5).

The existence of steps 6 & 7 is well documented for various peroxidases[55] and a few data have been reported for catalases. The rate constant measured here for step 6 ($2.7 - 3.2 \times 10^{-4}\,s^{-1}$) accords well with those previously published.[58,59] Some published data tend to suggest that the reaction of Compound II with H_2O_2 producing Compound III (reaction 7) could be reversible for mammalian catalases.[37] In contrast with this proposal, it is demontrated here that reaction 7 is *irreversible* for bovine liver catalase (Figure 5). The rate constant of reaction 7 has been estimated for the first time in a mammalian catalase; the experimental value obtained ($6.1 \times 10^4\,M^{-1}\,s^{-1}$) is one order of magnitude higher than the only rate constant published so far and measured under similar conditions for the *Aspergillus niger* catalase ($1.30 \times 10^3\,M^{-1}\,s^{-1}$)[50].

One of the most striking features of the data presented in this study is that an appreciable fraction of Compound II of bovine liver catalase reverts towards the native form via Compound III (reaction 7 + 5), even in the presence of low H_2O_2 levels: The data presented in Figure 3 indicate that, in the presence of 5×10^{-9} M H_2O_2, this path is responsible for about 50% of Compound II decomposed into the native form. In aerobic cells the level of H_2O_2 has been shown to be regulated at concentrations in that range.[30] Therefore, "*in vivo*", the occurrence of step 7 + 5 as a pathway of decomposition of bovine liver catalase Compound II could be as significant as step 6. This conclusion stands in contrast with the current dominant view that, in the presence of low stationary levels of H_2O_2, step 6 is the only way of Compound II decomposition.

Role of Compounds II and III in Irreversible Inactivation upon high H_2O_2 concentration

The data presented here clearly indicate that, contrary to observations reported by Chance,[34] no direct proportionality exists between the number of haematins in the form of Compound II and the extent of inactivation. At high H_2O_2 concentrations, the path involving Compound III (reaction 7 + 5) is the main process responsible for the conversion of Compound II into the native form (Figure 3 & Figure 4A). On the other hand, the substitution of native catalase by Compound III gives the same activity loss as the substitution by Compound II (Tables 4 & 5). Taken together, these observations indicate that Compound III – rather than Compound II – is the intermediate which undergoes irreversible inactivation in the presence of an excess of H_2O_2.

Compound III has been proposed as an intermediate in the reaction leading to irreversible inactivation of other hemoproteins such as lactoperoxidase,[57,60] horseradish peroxidase[41] and lignin peroxidase.[53,61] Nevertheless, it is the very first time that the Compound III of catalases is clearly demonstrated to be a "fragile" intermediate, much more susceptible than Compound II to undergo irreversible inactivation.

Little is known about the exact mechanism of inactivation of Compound III of peroxidase by H_2O_2. In particular, it is not yet clear whether H_2O_2 or a secondary oxygen toxic species, possibly generated at close proximity to the catalytic active site, is the deactivating agent. This latter alternative has received support from a recent investigation of the inactivation mechanism of lactoperoxidase by excess H_2O_2, using ESR[62]. The results indicated that, in a system containing Compound III of lactoperoxidase and high concentrations of H_2O_2, superoxide and hydroxyl radicals are indeed generated . The molecular mechanism was considered to involve the ferrous enzyme, which - in lactoperoxidase - is an intermediate in the conversion of Compound III back to the native enzyme.[62,56]

Inhibition of Catalases by Superoxide Radicals

The possibility that O_2^- inhibits catalase was first investigated by Kono and Fridovich in 1982.[47] This was explored by measuring the activity of a bovine catalase after exposure for varying time intervals to a flux of O_2^- generated by xanthine oxidase. According to these authors,[47] superoxide inhibits catalase, both by a rapid reaction which is prevented and reversed by superoxide dismutase and by a slow reaction which is prevented by superoxide dismutase or ethanol and reversed by ethanol only. The "rapid" inhibition of catalase by O_2^- was observed upon simultaneous exposure of the enzyme to a high excess of H_2O_2 (10^{-2} M) and a flux of O_2^-. In contrast, the "slow" inhibition was evidenced after prolonged exposure to low H_2O_2 and O_2^- concentrations. The "rapid" inhibition was reportedly due to the formation of an unstable Compound III (reaction 4, Figure 11B) while the "slow" and irreversible inhibition was attributed to the production of Compound II as a dead-end product, through the reduction of Compound I by O_2^- (reaction 3, Figure 11B). An univalent reduction of Compound II by ethanol has been invoked to explain both the prevention and the reversal of the "slow" inhibition of catalase by this alcohol.[47]

If H_2O_2 is continuously generated by an oxidizing system, previous investigations have shown that a steady state in the concentrations of both native enzyme and Compound I is obtained for several minutes.[34,59] The reaction velocities which have been obtained in presence of large excesses of hydrogen peroxide (up to 50 mM) also agree with steady-state concentrations of the two forms under most conditions.[63] In the steady state, only a fraction of the catalase heme is 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. Ratios of Compound I concentration to total catalase concentration range from 0.3 to 0.5 with mammalian catalases.[44,30] The experiments of Kono & Fridovich[47] were carried out under conditions where the concentration of H_2O_2 exceeds that of O_2^-. Under such conditions the "catalatic" cycling (steps 1 + 2) is several orders of magnitude faster than conversion rates among the other enzyme intermediates. The kinetic data obtained recently from irradiation experiments of oxygenated solutions of bovine liver catalase[44] indicate that the rate constant for the reaction of O_2^- with Compound I (reaction 3) is one order of magnitude higher than the rate constant for the reaction of O_2^- with native enzyme (reaction 4).[44] Therefore, in a large gamut of H_2O_2 concentrations, reaction 3 proceeds faster than reaction 4 and O_2^- must be mainly trapped by Compound I to yield Compound II rather than by the native enzyme to yield Compound III as initially proposed by Kono & Fridovich.[47]

The spectroscopic analysis presented in this paper shows that Compound I and

Compound II are the main detectable intermediates in the presence of low stationary levels of H_2O_2 and of $O_2^{\cdot-}$ (Figure 8, Table 4). In contrast, Compound III can be observed when high concentrations of H_2O_2 are added to the reaction mixture (Figure 4A). The rate constant obtained here for steps 6 & 7 indicate that, at low H_2O_2 concentrations ($< 10^{-8}$ M H_2O_2), the decomposition process of Compound II proceeds slowly, whatever the reaction path (half time > 20 min). Those rate constants also indicate that, in the presence of the concentration of peroxide used by Kono & Fridovich to monitor catalase activity (10^{-2} M H_2O_2), the decomposition process of Compound II must proceed rapidly (half time < 1 min) through reactions 7 + 5. It is thus concluded that the "slow" inhibition of catalase by $O_2^{\cdot-}$ is due to the progressive appearance of a stationary level of Compound II. Under such experimental conditions, reaction 6 and/or 7 are the rate-limiting steps of the Compound II decomposition process which proceeds slowly. In contrast, the "rapid" inhibition occurring in the presence of a high excess of H_2O_2 is due to transient accumulation of an appreciable amount of the enzyme in the form of Compound III (originating mainly from steps 3 + 7, Figure 11C), part of which leads to a dead end product (reaction 8, Figure 11C), the other part being rapidly converted back to the native enzyme (reaction 5, Figure 11C). In both kinds of inhibition, the accumulation of an oxidized Compound temporarily removes part of the enzyme from the pathway by which most of the O_2 is being formed (steps 1 + 2, Figure 11C) and hence the formation of this Compound leads to a decrease of catalase activity.

The data presented in Table 4 show that the inhibition caused by the accumulation of Compound II can be largely reversed by the addition of ethanol. The results in Table 2 indicate that Compound II decay rates are similar in the absence and in the presence of ethanol. This demonstrates that, in contrast to the conclusion of Kono & Fridovich,[47] reactivation by ethanol is not due to univalent reduction of Compound II by the alcohol. The spectroscopic measurements reported above reveal that ethanol causes the quick reversion of the major part of Compound I to the native form (reaction 9, Figure 11C). We have also observed that the decrease in the concentration of Compound I due to the addition of ethanol shifts the equilibrium between various forms towards a higher level of native enzyme. This phenomenon had already been observed by several authors;[34,37,59] it is attributed to the fact that the removal of Compound I by ethanol strongly inhibits reaction 3 (Figure 11) which leads to a drop in the level of Compound II. That the "slow" inhibition of catalase by $O_2^{\cdot-}$ is both prevented and largely reversed by ethanol is thus due to the lower level of Compound II obtained in the presence of alcohol and to the resulting decrease of irreversible inactivation of catalase (inhibition of steps 7 + 8, Figure 11C).

In summary, the kinetic data obtained recently from irradiation experiments of oxygenated solutions of bovine liver catalase[44] strongly suggest that the "rapid" inhibition of catalase by superoxide radicals has the same initiating step as the "slow" inhibition, e.g. the reaction of Compound I with $O_2^{\cdot-}$ (reaction 3, Figure 11). The data presented in this study demonstrate that the two kinds of inhibition differ from each other by two striking features: i) the nature of the oxidized intermediate which accumulates in the system (Compound II in "slow" inhibition or Compound III in "rapid" inhibition) and ii) the rate at which those intermediates are converted towards the native form (half time > 20 min in "slow" inhibition or half time < 1 min in "rapid" inhibition).

CONCLUSIONS

The data presented in this work clearly substantiate the existence of reaction paths which ensure that the catalase of bovine liver restores entirely its activity upon exposure to low levels of H_2O_2 and O_2^-. Moreover, it is shown here that the excess of H_2O_2 used in the activity test is the primary factor responsible for the apparent "irreversible" inactivation of mammalian catalase occurring upon prolonged exposure of the enzyme to O_2^-. Altogether, it may be reasonably postulated that under the conditions normally encountered "*in vivo*" (i.e. in absence of high excess H_2O_2) the bovine liver catalase is not irreversibly inactivated by the low levels of O_2^- due to the action of oxidases.

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