Mechanisms of Protection of Catalase by NADPH

KINETICS AND STOICHIOMETRY*

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NADPH is known to be tightly bound to mammalian catalase and to offset the ability of the substrate of catalase (H₂O₂) to convert the enzyme to an inactive state (compound II). In the process, the bound NADPH becomes NADP⁺ and is replaced by another molecule of NADPH. This protection is believed to occur through electron tunneling between NADPH on the surface of the catalase and the heme group within the enzyme. The present study provided additional support for the concept of an intermediate state of catalase, through which NADPH serves to prevent the formation (rather than increase the removal) of compound II. In contrast, the superoxide radical seemed to bypass the intermediate state since NADPH had very little ability to prevent the superoxide radical from converting catalase to compound II. Moreover, the rate of NADPH oxidation was several times the rate of compound II formation (in the absence of NADPH) under a variety of conditions. Very little NADPH oxidation occurred when NADPH was exposed to catalase, H₂O₂, or the superoxide radical separately. That the ratio exceeds 1 suggests that NADPH may protect catalase from oxidative damage through actions broader than merely preventing the formation of compound II.

Interest in the disposal of reactive oxygen species stems from the growing evidence that these molecules are active or participating agents in mutagenesis and aging and in the cellular damage from a wide variety of environmental and endogenous stresses (1–3). One of the more highly studied cells under oxidative stress is the human erythrocyte with, and without, genetic impairment in maintaining NADP in the reduced state since NADPH had very little ability to prevent the superoxide radical from converting catalase to compound II. Moreover, the rate of NADPH oxidation was several times the rate of compound II formation (in the absence of NADPH) under a variety of conditions. Very little NADPH oxidation occurred when NADPH was exposed to catalase, H₂O₂, or the superoxide radical separately. That the ratio exceeds 1 suggests that NADPH may protect catalase from oxidative damage through actions broader than merely preventing the formation of compound II.

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Protection of Catalase by NADPH

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EXPERIMENTAL PROCEDURES

All incubations were with commercially available, extensively purified enzymes. Sigma was the source of the manganese superoxide dismutase from Escherichia coli. As with the studies of Kono and Fridovich (23), manganese superoxide dismutase was used for most of this study because, unlike Cu,Zn superoxide dismutase, it is not inactivated by H$_2$O$_2$. Roche Molecular Biochemicals (Mannheim, Germany) was the source of catalase from bovine liver, xanthine oxidase from bovine milk, Cu,Zn superoxide dismutase from bovine erythrocytes, glucose oxidase from Aspergillus niger, and glucose-6-phosphate dehydrogenase from yeast. The buffer used for most of this study was 0.1 M EDTA, 50 mM Na$_2$HPO$_4$, KH$_2$PO$_4$, pH 7.4 (Na-K phosphate buffer). A second buffer was KR-Tes, which is a Krebs-Ringer/Tes solution containing, in the following final millimolar concentrations: NaCl 119; KCl 4.7; CaCl$_2$ 2.5; KH$_2$PO$_4$ 1.2; MgSO$_4$ 1.2; and (sodium) Tes, pH 7.4, 22.6. Crystalline bovine liver catalase was dissolved in KR-Tes, concentrated on CF-25 ultrafiltration cones (Amicon), and then washed on the cones (12) with the buffer to be used in each experiment. All other enzymes were both dissolved and washed with the buffer to be used in each experiment.

The solution of each enzyme was assayed for protein concentration by the method of Lowry (24, 25). The activity of catalase was determined from the first-order rate constant of the rate of disappearance of H$_2$O$_2$, at an initial concentration of 10 mM, as measured by absorbance at 240 nm with a recording spectrophotometer (25). By this assay, the bovine liver catalase was found to be similar to previously published spectra (22, 30). The spectra for ferricatalase, compound I, and compound II were confirmed in the following manner. One ml of 100 mM potassium Chex buffer, pH 6.6, was added to 0.5 ml of the bovine liver catalase from the bottle (20 mg/ml) to dissolve all enzyme crystals. The dissolved enzyme was further diluted in 50 mM Na-K phosphate buffer, pH 6.6, to a concentration of about 10 $\mu$M. One ml of enzyme preparation was then transferred to a cuvette. After a preincubation period of 5 min at 37 °C, the spectrum of ferricatalase was obtained. After the first spectrum was obtained, 5 ml of 3% peracetic acid were added to the cuvette, and the spectrum was immediately re-determined (compound I). One microliter of 60 mM potassium ferricyanide was then added to the same reaction mixture, and after 10 min of incubation at 37 °C, the spectrum was determined again (compound II). The absorbances of equimolar concentrations of ferricatalase (the resting or free form of catalase) and compounds I, II, and III at intervals of 1 nm between 350 and 750 nm. The plots from these absorbances were similar to previously published spectra (22, 30). The spectra for ferri- catalase, compound I, and compound II were confirmed in the following manner. One ml of 100 mM potassium Chex buffer, pH 6.6, was added to 0.5 ml of the bovine liver catalase from the bottle (20 mg/ml) to dissolve all enzyme crystals. The dissolved enzyme was further diluted in 50 mM Na-K phosphate buffer, pH 6.6, to a concentration of about 10 $\mu$M. One ml of enzyme preparation was then transferred to a cuvette. After a preincubation period of 5 min at 37 °C, the spectrum of ferricatalase was obtained. After the first spectrum was obtained, 5 ml of 3% peracetic acid were added to the cuvette, and the spectrum was immediately re-determined (compound I). One microliter of 60 mM potassium ferricyanide was then added to the same reaction mixture, and after 10 min of incubation at 37 °C, the spectrum was determined again (compound II). The absorbances of equimolar concentrations of the four forms of catalase were used to determine the amount of each form in H$_2$O$_2$-treated catalase by a least squares method, as follows. The minimum was found for the sum (from 501 to 750 nm) of the squares of the absorbance of each form of catalase. The formation of each form of catalase was determined by the rate of formation of O$_2$; the rate of formation of O$_2$ was determined by the rate of reduction of cytochrome c by O$_2$. The factor $k_2/k_3$ provided a means for correcting for the small fraction of O$_2$ that undergoes spontaneous dismutation before the O$_2$ can reduce cytochrome c. The rates of generation of O$_2$, as measured by the rate of reduction of cytochrome c, were similar whether catalase (20 $\mu$M) was present or absent. At pH 7.4 and 37 °C, the xanthine oxidase had an average specific activity of 115 $\mu$mol min$^{-1}$ per $\mu$mol of enzyme. The total rate of production of H$_2$O$_2$ by xanthine oxidase was determined from the rate of production of urate, as measured by the rate of increase in absorbance at 295 nm (29), and was confirmed with the assay for H$_2$O$_2$ of Green and Hill (26) on incubations containing xanthine oxidase, xanthine, and superoxide dismutase. The rate of oxidation of NADPH by ferricytochrome c by O$_2$ was determined from the amount of the peroxynitrite formed, as described previously (12). All reaction mixtures had a final volume of 1.0 ml. All incubations were at pH 7.4 and 37 °C except for one experiment in which the conditions were otherwise stated. The reaction components were at the final concentrations indicated within parentheses when the concentrations were not specified and were as follows: catalase, and, when present, xanthine oxidase, xanthine (100 $\mu$M), superoxide dismutase, NADPH (2 $\mu$M), glucose 6-phosphate (1 $\mu$M), glucose-6-phosphate dehydrogenase (10 $\mu$g/ml), glucose (5 $\mu$M).

For following the kinetics of reactions, readings of absorbance from the spectrophotometer were taken on each group of cuvettes at intervals of 1 min per group and were automatically stored in a computer for later statistical analysis. For obtaining spectra of catalase, absorbance readings were taken at intervals of 1 nm in the range 350-750 nm. The spectra were obtained with a Beckman DU-7 spectrophotometer at a recording speed of 1,200 nm min$^{-1}$. Prof. Peter Nicholls kindly provided the absorbances of equimolar concentrations of ferricatalase (the resting or free form of catalase) and compounds I, II, and III at intervals of 1 nm between 350 and 750 nm. The plots from these absorbances were similar to previously published spectra (22, 30). The spectra for ferricatalase, compound I, and compound II were confirmed in the following manner. One ml of 100 mM potassium ferricyanide was added to the same reaction mixture, and after 10 min of incubation at 37 °C, the spectrum was determined again (compound II). The absorbances of equimolar concentrations of the four forms of catalase were used to determine the amount of each form in H$_2$O$_2$-treated catalase by a least squares method, as follows. The minimum was found for the sum (from 501 to 750 nm) of the squares of the absorbance of each form of catalase. The formation of each form of catalase was determined by the rate of formation of O$_2$; the rate of formation of O$_2$ was determined by the rate of reduction of cytochrome c by O$_2$. The rate of generation of O$_2$, as measured by the rate of reduction of cytochrome c, were similar whether catalase (20 $\mu$M) was present or absent. At pH 7.4 and 37 °C, the xanthine oxidase had an average specific activity of 115 $\mu$mol min$^{-1}$ per $\mu$mol of enzyme. The total rate of production of H$_2$O$_2$ by xanthine oxidase was determined from the rate of production of urate, as measured by the rate of increase in absorbance at 295 nm (29), and was confirmed with the assay for H$_2$O$_2$ of Green and Hill (26) on incubations containing xanthine oxidase, xanthine, and superoxide dismutase. The rate of oxidation of NADPH by ferricytochrome c by O$_2$ was determined from the amount of the peroxynitrite formed, as described previously (12). All reaction mixtures had a final volume of 1.0 ml. All incubations were at pH 7.4 and 37 °C except for one experiment in which the conditions were otherwise stated. The reaction components were at the final concentrations indicated within parentheses when the concentrations were not specified and were as follows: catalase, and, when present, xanthine oxidase, xanthine (100 $\mu$M), superoxide dismutase, NADPH (2 $\mu$M), glucose 6-phosphate (1 $\mu$M), glucose-6-phosphate dehydrogenase (10 $\mu$g/ml), glucose (5 $\mu$M).

RESULTS

Steady-state Concentrations of H$_2$O$_2$ and O$_2$—The experimental conditions were set as follows so as to be biologically realistic: the pH was 7.4; the temperature was 37 °C; and the catalase concentration (2–3 $\mu$M) was similar to that of human erythrocytes (11). The rates of H$_2$O$_2$ and O$_2$ generation were 2–15 nmol ml$^{-1}$ min$^{-1}$, which are similar to the estimated rate of H$_2$O$_2$ generation in the human erythrocyte under resting
conditions and under peroxidative stress, respectively (9). Under these conditions, the concentrations of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) were too low to be determined by present methods, but they could be determined indirectly. Whether generated by glucose oxidase or xanthine oxidase, \( \text{H}_2\text{O}_2 \) rises to a steady-state concentration at which the rate of removal in the presence of catalase (Reaction 1) equals the rate of generation.

\[
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]  
(Reaction 1)

The bovine liver catalase was found to have a specific activity (see "Experimental Procedures") that would result in a steady-state nanomolar concentration of \( \text{H}_2\text{O}_2 \) of 0.7 \( V/C \), in which \( V \) is the total rate of generation of \( \text{H}_2\text{O}_2 \) in \( \text{nmol ml}^{-1} \text{ min}^{-1} \) and \( C \) is the micromolar concentration of the catalase.

When \( \text{O}_2^- \) was generated by incubation of 40 \( \text{nm} \) xanthine oxidase with xanthine (100 \( \mu\text{M} \)), the xanthine oxidase produced uric acid at an average rate of 8.8 \( \text{nmol ml}^{-1} \text{ min}^{-1} \) but \( \text{O}_2^- \) at an average rate of only 4.6 \( \text{nmol ml}^{-1} \text{ min}^{-1} \). This result indicated that 74% of the oxidation of xanthine by xanthine oxidase under these conditions was leading to the direct production (29) of \( \text{H}_2\text{O}_2 \) (Reactions 2 and 3), whereas the remainder was resulting in the production of \( \text{O}_2^- \) (Reaction 4).

\[
\text{Xanthine} + \text{enzyme} \rightarrow \text{uric acid} + \text{enzyme-H}_2
\]  
(Reaction 2)

\[
\text{Enzyme-H}_2 + \text{O}_2 \rightarrow \text{enzyme} + \text{H}_2\text{O}_2
\]  
(Reaction 3)

\[
\text{Enzyme-H}_2 + 2\text{O}_2 \rightarrow \text{enzyme} + 2\text{H}^+ + 2\text{O}_2^-
\]  
(Reaction 4)

Results of the cytochrome \( c \) assay indicated that 1 \( \mu\text{M} \) manganese superoxide dismutase reduced the net rate of generation of \( \text{O}_2^- \) from 40 \( \text{nm} \) xanthine oxidase (Reaction 5) by 97%.

\[
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2
\]  
(Reaction 5)

\( \text{O}_2^- \) was assumed to reach a steady-state concentration at which the rate of removal of \( \text{O}_2^- \), whether by superoxide dismutase or spontaneous dismutation (Reaction 5), equaled the rate of generation of \( \text{O}_2^- \). The second-order spontaneous dismutation rate of \( 4.8 \times 10^2 \text{ M}^{-1} \text{ s}^{-1} \) at room temperature (31) was calculated to be \( 6.0 \times 10^2 \text{ M}^{-1} \text{ s}^{-1} \) after adjustment to 37 °C (32), indicating that a rate of \( \text{O}_2^- \) production of 4.6 \( \text{nmol ml}^{-1} \text{ min}^{-1} \) should result in a steady-state concentration of 360 \( \text{nm} \) for \( \text{O}_2^- \) in the absence of superoxide dismutase. As expected from Reactions 2–5, the production of \( \text{H}_2\text{O}_2 \) by xanthine oxidase in the presence of manganese superoxide dismutase reached a steady-state rate similar to the rate of production of uric acid. For 40 \( \text{nm} \) xanthine oxidase and 2 \( \mu\text{M} \) catalase, the concentration of \( \text{H}_2\text{O}_2 \) would have been 3 \( \text{nm} \).

**Determination of Compounds I, II, and III**—Changes in the absorbance at 435 nm are traditionally used to follow the kinetics of compound II formation and removal. Changes at this wavelength, however, also reflect changes in the concentration of compound III. Computerized analysis of the absorption spectrum of bovine liver catalase at various times in the incubation gave the percentage of catalase that was in each of the four states of the enzyme (see "Experimental Procedures") and thereby revealed the extent to which absorbance changes at 435 nm were essentially measures of compound II alone. At a xanthine concentration of 100 \( \mu\text{M} \), the generation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) by 40 \( \text{nm} \) xanthine oxidase ended between 10 and 20 min after the start of the reaction, when the xanthine was depleted (Fig. 2). During this exposure, the percentage of catalase in the native state (ferricatalase) fell to a minimum of 49%. The percentage as compound III rose initially more rapidly than did compound II but reached a maximum of only 9% (Fig. 2). In contrast, compound II rose steadily to a maximum of 33% at 20–24 min. The increase in absorbance at 435 nm provided an estimate of the combined percentage of compound II and compound III (see "Experimental Procedures"), and this estimate was in general agreement with the combined percentage as determined by the computerized analysis of the absorption spectrum from 501 to 750 nm (Fig. 2). This agreement served to confirm that the difference in molar (heme) specific absorbance between ferricatalase (or compound I) and compound II at 435 nm was 32,000, that compound III had a similar molar specific absorbance at 435 nm, and that the bovine liver catalase used in this study had four functional heme groups. This information, in turn, allowed comparison of the loss in activity of bovine liver catalase and the formation of compounds II by both \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \). In results not given, this comparison confirmed that compound II has no catalase activity at pH 7.4 and at 37 °C, as had been demonstrated earlier under other conditions by Chance (33).

The percentage of catalase in each of its four forms was determined at intervals for a variety of reactions. Table I gives the results only at either 8 or 60 min of reaction time. The ratio of compound I to ferricatalase was lower when the catalase was exposed to the action of xanthine oxidase (Table I, row e) than when it was exposed to the action of glucose oxidase (Table I, rows a and c). The percentage as compound II was lower in the four reactions containing NADPH than in the corresponding reactions without added NADPH. At 8 min, less compound III was present in those reactions in which only \( \text{H}_2\text{O}_2 \) was generated (Table I, rows a, b, f, and h) than in those reactions in which \( \text{O}_2^- \) was also generated (Table I, rows e, g, and i). The presence of ethanol (2 \( \text{m} \)) essentially eliminated the presence of compounds I and II but allowed the generation of some compound III (Table I, row i).

**Kinetics of the Formation and Disappearance of Compound II**—Results of the previous section indicated that changes in the absorbance of catalase at 435 nm were essentially those from compound II except during the 1st min after the generation of \( \text{O}_2^- \) when a small amount of compound III was formed. The formation of compound II was followed by recordings of the absorbance at 435 nm of 2 \( \mu\text{M} \) catalase during incubation under the various conditions described in the legend of Fig. 3. Drops in absorbance at the start of the reaction at 5 min were caused by the slight dilution resulting from the addition of the starting solution. The absorbance increases at 435 nm, such as those of

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**FIG. 2. Percentages of four forms of catalase during exposure to \( \text{O}_2^- \) generated by xanthine oxidase.** The reaction was at 37 °C with bovine liver catalase (3 \( \mu\text{M} \)) and Na-K phosphate buffer (50 m\( \text{M} \)), pH 7.4. The results are averages of incubations at 3 different times. The presence of xanthine (100 \( \mu\text{M} \)) and xanthine oxidase (40 \( \text{nm} \)) caused the generation of \( \text{O}_2^- \) at a rate of 4.2 \( \text{nmol ml}^{-1} \text{ min}^{-1} \) and \( \text{H}_2\text{O}_2 \) at a total rate of 8.0 \( \text{nmol ml}^{-1} \text{ min}^{-1} \). Percentages of the four forms of catalase were determined by a least squares method (see "Experimental Procedures") from absorbances from 501 to 750 nm. ×, the combined percentage of compound II and compound III, as determined by changes in absorbance at 435 nm.
Catalase, at a final concentration of 3 μM, was exposed at pH 7.4 and 37 °C to glucose oxidase (Gox), 6.7 nM, generating H₂O₂ at a rate of 14.8 nmol ml⁻¹ min⁻¹, or to xanthine oxidase (Xox), 40 nM, generating H₂O₂ at a rate of 6.8 to 6.9 nmol ml⁻¹ min⁻¹, and O₂ at an average rate of 4.1 nmol ml⁻¹ min⁻¹. Other final concentrations were as follows: manganese superoxide dismutase (SOD), 1 μM; NADPH, 2 μM; and ethanol, 2 mM. NADPH was kept in the reduced state by the presence of glucose-6-phosphate dehydrogenase and glucose 6-phosphate (see “Experimental Procedures”). The reaction time was 8 min except for that of rows c and d which was 60 min.

| Reaction             | Ferricatalase | Compound I       | Compound II      | Compound III     | Compound I/ ferricatalase (ratio) |
|----------------------|---------------|------------------|------------------|------------------|----------------------------------|
| (a) Gox              | 55.2          | 37.6             | 6.7              | 0.5              | 0.68                             |
| (b) Gox/NADPH       | 63.8          | 38.1             | -0.1             | -1.8             | 0.60                             |
| (c) Gox (60 min)    | 40.2          | 24.9             | 32.1             | 2.8              | 0.62                             |
| (d) Gox/NADPH (60 min) | 57.2         | 38.3             | 4.6              | -0.1             | 0.67                             |
| (e) Xox             | 54.7          | 20.4             | 20.9             | 4.3              | 0.37                             |
| (f) Xox/SOD         | 60.5          | 31.4             | 8.5              | -0.4             | 0.52                             |
| (g) Xox/NADPH       | 58.8          | 27.3             | 11.8             | 2.1              | 0.46                             |
| (h) Xox/NADPH/SOD   | 66.1          | 31.7             | 2.3              | -0.1             | 0.48                             |
| (i) Xox/ethanol     | 98.1          | -1.0             | 0.6              | 2.3              | (0)                              |

The difference between the two curves in Fig. 3B was similar to the difference between the two curves in Fig. 3C, indicating that NADPH had very little effect on the rate of generation of compound II that could be attributed to O₂ alone. In five experiments of the type shown in Fig. 3, B and C, NADPH decreased the difference between the VIIs (with and without superoxide dismutase) by an average of 11 ± 12% (±S.E. of the mean). Results similar to those of Fig. 3, B and C, were obtained with 40 nM xanthine oxidase and Cu,Zn superoxide dismutase and, at a 5-fold slower rate of O₂ generation, with 8 nM xanthine oxidase and manganese superoxide dismutase (results not given). In contrast to the large effect of NADPH in lowering the rate of compound II formation by H₂O₂, NADPH only mildly affected the rate of decay of compound II (Table II).

**Fig. 3. Changes in absorbance of catalase at 435 nm during exposure to O₂ and H₂O₂.** The conditions were those of rows a–h of Table I except that the catalase concentration was 2 μM. A, C, blank in which glucose was omitted. For purposes of graphic clarity, the blanks of B and C, which resembled that of A, were omitted, and the absorbances of the lower two curves of A and the lower curve of B and C were decreased by 0.01. SOD, superoxide dismutase.

![Graph showing changes in absorbance of catalase at 435 nm](image)

The calculation of Equation 1

\[
\text{[compound II]} = \frac{V_{f}(1 - e^{-kt})}{k}
\]

for a reaction in which the formation of compound II, at a constant rate of \( V_{f} \), is offset by the first-order decay of compound II, with a decay constant of \( k \). The visual curve-fitting method was used to determine provisional values for \( V_{f} \) and \( k \), then the final values were determined from iteration for a least squares fitting of the increases in absorbances at 435 nm between 1 and 9 min after the start of the reaction. These calculations indicated that NADPH reduced the rate of compound II formation (\( V_{f} \)) from H₂O₂ by an average of 82%, when the H₂O₂ was generated by glucose oxidase in three experiments of the type shown in Fig. 3A, and by an average of 83% when the H₂O₂ was generated in five experiments by xanthine oxidase in the presence of superoxide dismutase, as in Fig. 3, B and C. The difference between the two curves for the xanthine oxidase reaction, with and without superoxide dismutase (Fig. 3B), was considered to represent the contribution to the rate of formation of compound II that was attributable only to O₂. The difference between the two curves in Fig. 3B was similar to the difference between the two curves in Fig. 3C, indicating that NADPH had very little effect on the rate of generation of compound II that could be attributed to O₂ alone. In five experiments of the type shown in Fig. 3, B and C, NADPH decreased the difference between the VIIs (with and without superoxide dismutase) by an average of 11 ± 12% (±S.E. of the mean). Results similar to those of Fig. 3, B and C, were obtained with 40 nM xanthine oxidase and Cu,Zn superoxide dismutase and, at a 5-fold slower rate of O₂ generation, with 8 nM xanthine oxidase and manganese superoxide dismutase (results not given). In contrast to the large effect of NADPH in lowering the rate of compound II formation by H₂O₂, NADPH only mildly affected the rate of decay of compound II (Table II).

**Table I**

| Reaction          | Ferricatalase | Compound I | Compound II | Compound III | Compound I/ ferricatalase (ratio) |
|-------------------|---------------|------------|-------------|--------------|---------------------------------|
| (a) Gox           | 55.2          | 37.6       | 6.7         | 0.5          | 0.68                            |
| (b) Gox/NADPH     | 63.8          | 38.1       | -0.1        | -1.8         | 0.60                            |
| (c) Gox (60 min)  | 40.2          | 24.9       | 32.1        | 2.8          | 0.62                            |
| (d) Gox/NADPH (60 min) | 57.2        | 38.3       | 4.6         | -0.1         | 0.67                            |
| (e) Xox           | 54.7          | 20.4       | 20.9        | 4.3          | 0.37                            |
| (f) Xox/SOD       | 60.5          | 31.4       | 8.5         | -0.4         | 0.52                            |
| (g) Xox/NADPH     | 58.8          | 27.3       | 11.8        | 2.1          | 0.46                            |
| (h) Xox/NADPH/SOD | 66.1          | 31.7       | 2.3         | -0.1         | 0.48                            |
| (i) Xox/ethanol   | 98.1          | -1.0       | 0.6         | 2.3          | (0)                             |

\[ V_{f}(1 - e^{-kt})/k \]  

\[ \text{for a reaction in which the formation of compound II, at a constant rate of } V_{f}, \text{ is offset by the first-order decay of compound II, with a decay constant of } k. \text{ The visual curve-fitting method was used to determined provisional values for } V_{f} \text{ and } k, \text{ then the final values were determined from iteration for a least squares fitting of the increases in absorbances at 435 nm between 1 and 9 min after the start of the reaction. These calculations indicated that NADPH reduced the rate of compound II formation (} V_{f} /\text{ from H}_{2}O_{2} \text{ by an average of 82%, when the H}_{2}O_{2} \text{ was generated by glucose oxidase in three experiments of the type shown in Fig. 3A, and by an average of 83% when the H}_{2}O_{2} \text{ was generated in five experiments by xanthine oxidase in the presence of superoxide dismutase, as in Fig. 3, B and C. The difference between the two curves for the xanthine oxidase reaction, with and without superoxide dismutase (Fig. 3B), was considered to represent the contribution to the rate of formation of compound II that was attributable only to O}_{2}. \text{ The difference between the two curves in Fig. 3B was similar to the difference between the two curves in Fig. 3C, indicating that NADPH had very little effect on the rate of generation of compound II that could be attributed to O}_{2} \text{ alone. In five experiments of the type shown in Fig. 3, B and C, NADPH decreased the difference between the VIIs (with and without superoxide dismutase) by an average of 11 ± 12% (±S.E. of the mean). Results similar to those of Fig. 3, B and C, were obtained with 40 nM xanthine oxidase and Cu,Zn superoxide dismutase and, at a 5-fold slower rate of O}_{2} \text{ generation, with 8 nM xanthine oxidase and manganese superoxide dismutase (results not given). In contrast to the large effect of NADPH in lowering the rate of compound II formation by H}_{2}O_{2}, \text{ NADPH only mildly affected the rate of decay of compound II (Table II).} \]
TABLE II
Rate constants for disappearance of compound II

| Additions                | 10^4 × k (s^-1) |
|-------------------------|-----------------|
| (a) None                | 7.39 ± 0.43     |
| (b) NADPH, 2 μM         | 10.33 ± 1.50    |
| (c) Ethanol, 2 mM       | 9.37 ± 0.62     |

Significance of the differences (degrees of freedom) were as follows: a versus c, t = 5.9, p < 0.001; a versus b, t = 4.2, p < 0.005; b versus c, t = 1.3, not significant.

FIG. 5. Lag in compound II formation caused by added NADPH. The reaction mixtures were incubated at 35 °C and contained, in the following final concentrations: potassium phosphate buffer, pH 6.5, 10 mM; beef liver catalase, 1.0 μM; glucose oxidase, 2 mM; glucose, 4 mM; and NADPH at (curve a) 0.0, (curve b) 3.3, (curve c) 6.6, and (curve d) 10.0 μM. Arrow, start of the reactions by the addition of glucose.

FIG. 4. The amounts of compounds (Cpdx) II and III formed, and NADPH oxidized, over 10 min in reactions in which H₂O₂ or O₂⁻, and H₂O₂ were generated. Glucose oxidase (Gox) was present at a final concentration of 4.1 μM and generated H₂O₂ at a rate of 7.2 nmol ml⁻¹ min⁻¹. Xanthine oxidase (Xox) was present at a final concentration of 40 μM and generated H₂O₂ and O₂⁻ at rates of 7.9 and 4.0 nmol ml⁻¹ min⁻¹, respectively. Other final concentrations were catalase, 2 μM; manganese superoxide dismutase (SOD), 1 μM; ethanol, 2 mM; and NADPH 2 μM. The amount of NADPH oxidized was determined from the amount of 6-phosphogluconate generated. The estimates for the amount of NADPH oxidized were corrected for the formation of NADP⁺ from the NADP⁺ (2 μM) initially present and for the amount of 6-phosphogluconate generated in 10 min in blank reactions (averaging 0.8 nmol ml⁻¹ in the blank consisting of catalase without glucose oxidase and 1.2 nmol ml⁻¹ in the blank consisting of xanthine and xanthine oxidase without catalase). The number above each column is the standard deviation based on the number of replicates shown in parentheses.

only ferricatalase and compound III were present when ethanol was added to the reaction mixture. The ability of NADPH to modify the direct formation of compound III from ferri- catalase by O₂⁻ (step 4 of Fig. 1) was therefore evaluated by observing the effect on catalase of the xanthine oxidase reaction in the presence of ethanol (Fig. 4). Before conversion to nmol ml⁻¹ in 10 min, the increase in absorbance at 435 nm was corrected for the drop in absorbance resulting from the addition of the starting solution of xanthine, as observed in a control solution to which an equivalent volume was added as water. Although low, the concentration of compound III was less when NADPH was present. Without added NADPH, the concentration of compound III reached a (heme) concentration 0.103 ± 0.027 μM (mean ± S.D.) at 10 min, corresponding to 1.3% of the catalase. The concentration of compound III at 10 min with added NADPH was 0.027 ± 0.021 μM. The difference was significant (4 degrees of freedom, t = 3.9) at the level of 0.01 < p < 0.025. A second method for determining the stoichiometry between...
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This scheme replaces the reactions shown on the right in Fig. 1. Except for step 3, the representations and reactions are those of Hillar et al. (17). AH represents a postulated oxidizable amino acid near the heme group (17). An intermediate, formed by step 3a, is reduced by NADPH via electron tunneling.

**Support for the Intermediate State**—The present authors (12) have said that NADPH both decreases the formation of compound II and increases the rate of removal of compound II. In contrast, Hillar et al. (16, 17) state that NADPH decreases the rate of formation of compound II but does not increase its rate of removal. Both actions of NADPH were observed in the present study, but decreasing the rate of formation of compound II was the predominant action. NADPH only mildly increased the rate of spontaneous decay of compound II (Table II). The decay constant of compound II in the presence of NADPH was 1.4 times the constant without NADPH (Table II). The least squares fitting of the absorbances of Fig. 3A (upper curve) to Equation 1 provided an estimate of the decay constant and initial rate of increase in concentration of compound II. Equation 1 revealed that the concentration of compound II at 10 min of reaction time would have been reduced by only 12% by increasing the decay constant by a factor of 1.4. In contrast, NADPH actually decreased the concentration by 80% (Fig. 3A).

Reconsideration of our results at pH 6.5 (12) in the same manner indicates that the action of NADPH at that pH was also one of preventing the formation of compound II by \( \text{H}_2\text{O}_2 \), rather than increasing the rate of removal of compound II. That NADPH prevents the formation of compound II by \( \text{H}_2\text{O}_2 \), rather than increases the decay of compound II, is supported also by the unexpected finding, in the present study, that NADPH has a limited ability to offset the formation of compound II that arises from the primary action of \( \text{O}_2^\cdot \). Had the protective action of NADPH been due largely to increasing the rate of removal of compound II, then NADPH should have provided protection against both \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^\cdot \). The results in Table II show that NADPH, unlike ethanol, does not prevent the formation of compound II by lowering the concentration of compound I. By documenting that NADPH prevents the formation of compound II, rather than increases the rate of removal of compound II, the present study strengthens the claim of an intermediate state of bovine liver catalase, between compound I and compound II. It is difficult to explain the preventive role of NADPH without assuming the presence of such an intermediate.

**Modification and Expansion of the Model**—If the mechanism of an intermediate, as proposed by Hillar et al. (17), is accepted, then the findings of the present study would require that \( \text{O}_2^\cdot \) reacts directly with compound I to produce compound II, bypassing the intermediate in the process (Fig. 6). Hillar et al. (16, 17) found that ferrocyanide increased the rate of formation of compound II through what they assumed to be the one-electron reduction of the intermediate by ferrocyanide at step 3b (see Fig. 6). Since they found that NADPH greatly reduced the rate of compound II formation by ferrocyanide, it is unlikely that the action of \( \text{O}_2^\cdot \) is at step 3b. In their two articles on the concept of an intermediate, and in support of that concept, Hillar et al. (16, 17) claimed that the rate of oxidation of NADPH was similar to the rate at which compound II was formed in the absence of NADPH. When corrected for the molar specific absorbances of NADPH and compound II, however, the rate of NADPH oxidation can be shown to be approximately 3 times the rate of compound II formation in their first article (16) and even higher in their second report (17). Fig. 5, in fact, is a repeat of the experiment of Hillar and Nicholls (16) and reveals a ratio of 3.9. When the rate of NADPH oxidation is measured by the rate at which 6-phosphogluconate is formed by the NADPH-generating system, the ratio of NADPH oxidation to compound II formation is 3 to 4 at pH 7.4 (Fig. 4) and pH 6.5 (12) and at different rates of \( \text{H}_2\text{O}_2 \) generation (12). Thus, a ratio of 3 or more has been demonstrated at two pH values, by two methods, and at different rates of \( \text{H}_2\text{O}_2 \) production. As indicated in the legend of Fig. 4, very little NADPH was oxidized when \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^\cdot \) were generated in the absence of catalase. For detection of an effect of substances of low molecular weight, such as ions of trace metals that might accompany the catalase, these control incubations contained an ultrapure of the catalase. The volume of the ultrapure was the same as the volume of catalase solution added to the incubation mixtures. Moreover, these control incubations provide an overestimate of the ability of \( \text{H}_2\text{O}_2 \) to oxidize NADPH in the absence of catalase, since the \( \text{H}_2\text{O}_2 \) would reach concentrations well above those in the presence of catalase. Very little oxidation of NADPH occurred when catalase was exposed to \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^\cdot \) in the presence of ethanol (Fig. 4) and therefore at extremely low concentrations of compound I (Table I). Reversibility of step 3a of Fig. 6 could cause the rate of NADPH oxidation to exceed the rate at which compound II would be formed in the absence of NADPH. Specifically, the reversibility would need to result in a relatively rapid interconversion of compound I and the intermediate. Computer simulations of the schemes of Figs. 1 and 6, however, indicate that this reversibility would cause the curve for compound II formation to have two distinct slopes and therefore to fail to fit Equation 1. Although the steady-state concentration of compound III is low, the concentration is even lower in the presence of NADPH, even under conditions when essentially no compound I or compound II is present (Table I and Fig. 4).

Almarsson et al. (18) point out that compound I is an oxidant and also an unstable species, tending to engage in the side reaction of becoming compound II when the encounter between compound I and \( \text{H}_2\text{O}_2 \) is delayed. Such a delay occurs under physiological conditions, when the rate of generation of \( \text{H}_2\text{O}_2 \) is low. That compound I is a strong oxidant is underscored by the fact that compound I oxidizes \( \text{H}_2\text{O}_2 \) in the normal cycle of \( \text{H}_2\text{O}_2 \) disposal (Fig. 1). Compound I leads to the oxidation of a reductant within the structure of catalase, the so-called endogenous donor, causing compound I to become compound II. We wish to
suggest that NADPH may protect catalase from oxidative damage through actions broader than merely preventing the formation of compound II. NADPH may lead to the reduction of oxidizing states and internal groups of catalase other than the intermediate, possibly including a small percentage of compound I, itself. This broader action of NADPH could account for the oxidation of NADPH at a rate exceeding by severalfold the rate at which compound II would otherwise be formed.

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