Nitric Oxide Converts Catalase Compounds II and III to Ferricatalase

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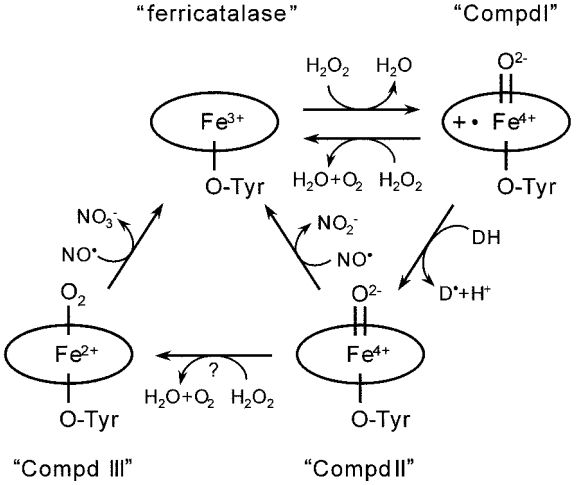
Catalase is an antioxidant enzyme that disproportionates H_2O_2 to water and oxygen: one molecule of H_2O_2 oxidizes the heme of catalase to the level of Compound I, a ferryl oxo species with a porphyrin π-cation radical, and a second molecule reduces Compound I back to ferricatalase (see Scheme 1). As such catalase protects cellular components from oxidative damages caused by H_2O_2.

Under certain conditions catalase forms off-pathway intermediates. Compound I can be reduced to Compound II by an appropriate one-electron donor such as ferrocyanide[1] and nitrite[2]. Compound II is further converted to Compound III, a Fe^{2+}-O_2 species, by excess H_2O_2[1] or to ferricatalase by nitrite.[2] Compounds II and III are presumed to be inactive and sometimes lead to irreversible loss of the enzyme activity.

Cells produce nitric oxide ($NO^{\bullet}$) under normal and pathological conditions. Although $NO^{\bullet}$ is an important biological regulator, its overproduction is responsible for the nitrosative stress that often leads to cell death. As a strong π acceptor, $NO^{\bullet}$ binds to both ferric and ferrous states of various heme enzymes such as peroxidases[3] and catalase,[4,5] blocking the active site to inhibit the enzymes. In a previous work, we showed that the Fe^{3+}-$NO^{\bullet}$ catalase was rapidly converted to the active ferricatalase by the reaction with superoxide anion radical ($O_2^{\bullet-}$)[6]. Brunelli *et al*.[5] showed that H_2O_2 oxidized the bound $NO^{\bullet}$ to nirite regenerating ferricatalase.

$NO^{\bullet}$ interacts with hemes at other redox states as well. Glover *et al*.[7] showed that $NO^{\bullet}$ acted on horseradish peroxidase Compounds I and II as a one-electron reductant converting them to Compound II and ferricatalase, respectively. Interestingly the rate of reaction with Compound II was much faster than that with Compound I. Abu-Soud and Hazen[8] demonstrated that $NO^{\bullet}$ accelerated the formation and decay of Compound II of myeloperoxidase. In this study we show that $NO^{\bullet}$ reduces both Compound II and III of catalase to ferricatalase restroring the enzyme activity.



Scheme 1. A proposed mechanism for the conversion by $NO^{\bullet}$ of catalase Compounds II and III to ferricatalase. Two-electron oxidation of ferricatalase by H_2O_2 generates the catalytically active Compound I, a ferryl oxo species with porphyrin π-cation. Filling an electron in the π-cation radical by a one-electron reductant (DH) such as ferrocyanide produces Compound II, another inactive species. Compound II is reduced to ferricatalase by oxidizing $NO^{\bullet}$ to nitrite. Compound III, produced by addition of excess H_2O_2 to Compounds II, can also be converted to ferricatalase by $NO^{\bullet}$, the latter being oxidized to the level of nitrate.

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Results ad Discussion

The upper panel of Figure 1 shows the spectral changes accompanying the conversion of catalase Compound II to ferricatalase by $NO^{\bullet}$. Addition of ferrocyanide (100 μM), a one-electron donor, to ferricatalase (7 μM) under the turnover condition generated Compound II, a ferryl oxo heme, which is characterized by the absorption at 424, 534, and 567 nm (spectrum a). H_2O_2 was supplied continuously by the glucose oxidase (75 nM)/glucose (10 mM) system. Addition of an aliquot of $NO^{\bullet}$-saturated buffer to Compound II resulted in the decay of Compound II absorption with a concomitant increase in the absorption bands (405 and 625 nm) of ferricatalase. A set of isosbestic points indicates that Compound II was converted to ferricatalase without detectable intermediates.

We measured the time-dependent increase of the absorption band at 625 nm, which is unique to ferricatalase, to estimate the rate of ferricatalase formation as shown in the lower panel of Figure 1. Addition of $NO^{\bullet}$ to Compound II (point a) caused a rapid increase of the absorption at 625 nm accompanied by a slow phase. The conversion was quantitative as the amplitude of DA_{625} is proportional to the concentration of added $NO^{\bullet}$. A similar biphasic kinetics was obtained for the reaction of $NO^{\bullet}$ with Compound II of horseradish peroxidase.[7]

We next examined the reaction of Compound III with $NO^{\bullet}$

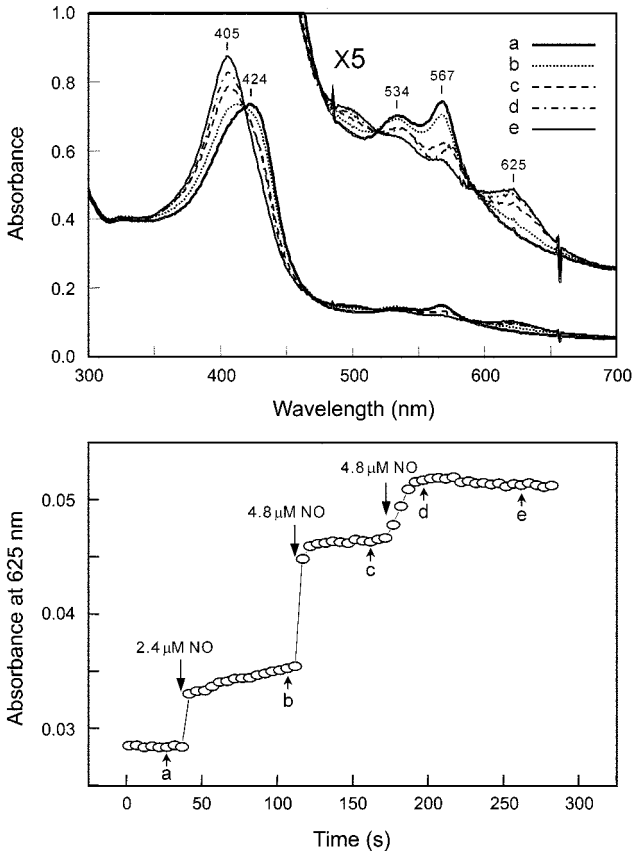

Figure 1. Conversion of catalase Compound II to ferricatalase. Catalase Compound II (spectrum a) was produced by incubating ferricatalase (7 μM) in 50 mM KP_i (pH 7.0) with H_2O_2 (generated by 10 mM glucose and 75 nM glucose oxidase) and 100 μM ferrocyanide for 90 min. The lower panel shows the increase in the absorbance at 625 nm due to the formation of ferricatalase upon addition of NO$^\bullet$ to Compound II. Spectra obtained at times a-e in the lower panel are plotted in the upper panel.

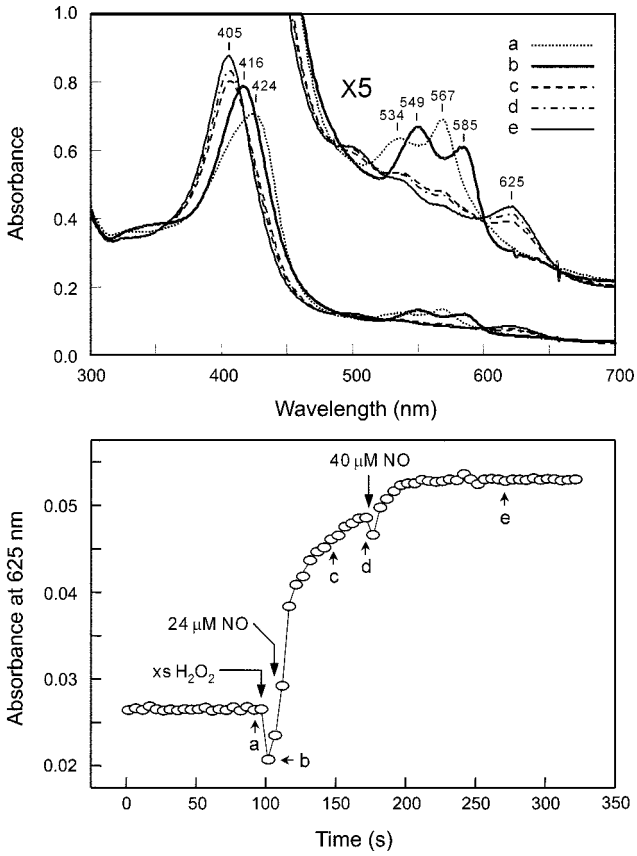

Figure 2. Conversion of catalase Compound III to ferricatalase. Catalase Compound III (spectrum b) was produced by adding 40 mM H_2O_2 to Compound II (spectrum a). The lower panel shows the increase in the absorbance at 625 nm due to the formation of ferricatalase upon addition of NO$^\bullet$ to Compound III. Spectra obtained at times a-e in the lower panel are plotted in the upper panel.

(see Fig. 2). Compound III containing a Fe^{2+}-O_2 heme was prepared by adding excess H_2O_2 to Compound II (spectrum a) as described by Lardinois.[1] We observed evolution of a gas which is presumed to be oxygen (see Scheme 1). Absorption bands at 416, 549, and 584 nm (spectrum b) clearly identify Compound III as the major product. Addition of NO$^\bullet$ to Compound III resulted in the formation of ferricatalase as evidenced by the absorption bands at 405 and 625 nm (spectra c-e). Isosbestic points were not so clear as those in the conversion of Compound II to ferricatalase suggesting that a third species could be involved in the reaction. It is highly probable that the regenerated ferricatalase reacted with the excess H_2O_2 performing a normal catalytic function so that the spectra had contributions from Compound I.

The lower panel of Figure 2 shows the increase in the absorbance at 625 nm during the conversion of Compound III to ferricatalase. Comparing with the lower panel of Figure 1, one can notice that the conversion rate was much slower than that for Compound II. Accordingly a higher concentration of NO$^\bullet$ was needed to see the same increase in A_{625} since oxygen can compete with Compound III for the added NO$^\bullet$.

Both Compounds II and III are known to be inactive. At low H_2O_2 concentrations, Compound II spontaneously decays back to ferricatalase. At higher H_2O_2 concentrations, Compound II is converted to Compound III, a fraction of which becomes irreversibly inactivated.[1,9] Although the conversion of Compounds II and III to ferricatalase is manifest in the spectral changes, it is worth confirming that NO$^\bullet$ restores the enzyme activity. Under the conditions detailed in Experimental section, the activity of Compound II was 82 ± 3% of the native enzyme without NO$^\bullet$ treatment. This means that most of Compound II decayed back to ferricatalase upon dilution of the reaction mixture to obtain a 5 nM catalase solution which is appropriate for the activity assay. Addition of NO$^\bullet$, however, raised the activity further up to 94 ± 2% demonstrating that NO$^\bullet$ converted residual Compound II to the active ferricatalase. About 6% of the enzyme seemed to be irreversibly inactivated. Similarly, Compound III without NO$^\bullet$ treatment showed a relative activity of 65 ± 3%, which was increased to 82 ± 2% when NO$^\bullet$ was added to convert Compound III to ferricatalase. Irreversible inactivation was ~18% for Compound III, which is much larger than that for Compound II, in agreement with

a previous observation.[1]

In conclusion, $NO^{\bullet}$ converted catalase Compounds II and III to ferricatalase restoring the enzyme activity. Compound II reacted with $NO^{\bullet}$ at a much faster rate than Compound III. A proposed mechanism is summarized in Scheme 1.

Experimental Section

Bovine liver catalase was purchased from Roche Applied Science (Mannheim, Germany) and dialyzed extensively against 50 mM KPi (pH 7.0). Concentration of catalase (per heme) was estimated by using $\varepsilon_{405} = 1.49 \times 10^5\ cm^{-1}\ M^{-1}$ for ferricatalase.[4] $NO^{\bullet}$ was generated by acidifying aqueous nitrite solution and gaseous $NO^{\bullet}$ was bubbled through a deoxygenated buffer to obtain $NO^{\bullet}$-saturated solution.[10] All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Catalase activity was measured in a 50 mM KP_i solution (pretreated with Chelex 100) containing 0.5 mM diethylenetriaminepentaacetic acid (DTPA). An enzyme solution was diluted with the assay buffer to obtain 5 nM catalase and 500 μM H_2O_2 was added to initiate the enzyme reaction. Absorption of H_2O_2 at 240 nm was measured as a function of time on a Hewlett-Packard HP-8483 diode array spectrophotometer.

Compounds II and III were prepared according to Lardinois.[1] Briefly, ferricatalase (7 μM) was allowed to turn over H_2O_2 generated by 10 mM glucose and 75 nM glucose oxidase. Addition of 100 μM ferrocyanide to the reaction mixture slowly accumulated Compound II, which was monitored spectrophotometrically. In 90 min almost all catalase was converted to Compound II, which was identified by the absorption bands at 424, 534, and 567 nm. Compound III was prepared by adding 40 mM H_2O_2 to Compound II. A full conversion was confirmed by the absorption bands of Compound III at 416, 549, and 584 nm.

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