Kinetics of Interconversion of Ferrous Enzymes, Compound II and Compound III of Wild-type Synechocystis Catalase-peroxidase and Y249F

PROPOSAL FOR THE CATALATIC MECHANISM*

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With the exception of catalase-peroxidases, heme peroxidases show no significant ability to oxidize hydrogen peroxide and are trapped and inactivated in the compound III form by H$_2$O$_2$ in the absence of one-electron donors. Interestingly, some KatG variants, which lost the catalatic activity, form compound III easily. Here, we compared the kinetics of interconversion of ferrous enzymes, compound II and compound III of wild-type Synechocystis KatG, the variant Y249F, and horseradish peroxidase (HRP). It is shown that dioxygen binding to ferrous KatG and Y249F is reversible and monophasic with apparent bimolecular rate constants of ($1.2 \pm 0.3$) $\times 10^5$ M$^{-1}$ s$^{-1}$ and ($1.6 \pm 0.2$) $\times 10^5$ M$^{-1}$ s$^{-1}$ (pH 7, 25 °C), similar to HRP. The dissociation constants ($K_d$) of the ferrous-dioxygen were calculated to be 84 μM (wild-type KatG) and 129 μM (Y249F), higher than that in HRP (1.9 μM). Ferrous Y249F and HRP can also heterolytically cleave hydrogen peroxide, forming water and an oxoferryl-type compound II at similar rates (($2.4 \pm 0.3$) $\times 10^5$ M$^{-1}$ s$^{-1}$ and ($1.1 \pm 0.2$) $\times 10^5$ M$^{-1}$ s$^{-1}$ (pH 7, 25 °C)). Significant differences were observed in the H$_2$O$_2$-mediated conversion of compound II to compound III as well as in the spectral features of compound II. When compared with HRP and other heme peroxidases, in Y249F, this reaction is significantly faster (($1.2 \pm 0.2$) $\times 10^5$ M$^{-1}$ s$^{-1}$). Ferrous wild-type KatG was also rapidly converted by hydrogen peroxide in a two-phasic reaction via compound II to compound III ($\sim 2.0 \times 10^5$ M$^{-1}$ s$^{-1}$), the latter being also efficiently transformed to ferric KatG. These findings are discussed with respect to a proposed mechanism for the catalatic activity.

Catalase-peroxidases (KatGs,1 EC 1.11.1.7) are bifunctional heme b-containing enzymes exhibiting an overwhelming catalatic activity and a substantial peroxidase activity with various one-electron donors. The catalatic activity of KatGs, i.e. the dismutation of hydrogen peroxide to oxygen and water, is unique for both heme peroxidase superfamilies (i.e. the superfamily of peroxidases from archaea, bacteria, fungi, and plants and the superfamily of animal peroxidases). Despite the availability of crystal structures (1–4), no structural bases for the high catalatic activity of KatGs has been assigned so far. Whether in KatGs compound I plays a similar role in the catalatic cycle as in monofunctional catalases (EC 1.11.1.6; see Fig. 1, Reaction 1 and 2) (5) is not clear at the moment. From a thermodynamic point of view, there is no barrier for the two-electron oxidation process, which oxidizes hydrogen peroxide to dioxygen. The standard reduction potential of the redox couple $\text{E}^{\*}^c$ (O$_2$/H$_2$O$_2$) is 280 mV (6), and normally, the oxidizing intermediates of heme peroxidases are strong oxidants (7). Similar to monofunctional catalases, compound I does not accumulate during H$_2$O$_2$ degradation but can be trapped by using organic peroxides (8). However, this intermediate exhibits only a low reactivity toward H$_2$O$_2$ (8).

Another peculiarity of KatGs regards the spectral features of compound II formed by one-electron reduction of compound I (see Fig. 1, Reaction 3), which in most heme peroxidases is a ferryl species with a red-shifted Soret band and two typical maxima in the visible region (7) and accumulates in the peroxidase cycle (see Fig. 1, Reactions 1, 3, and 4). Both in the absence and in the presence of one-electron donors, an accumulation of a ferryl-like compound II was never observed in wild-type KatGs, suggesting that the spectral features of KatG compound II could be similar to that of the ferric protein (8, 9).

In contrast to other heme peroxidases, KatG compound III does not accumulate even under high concentrations of H$_2$O$_2$. In Mycobacterium KatG, 400 mM H$_2$O$_2$ had to be added to ferric KatG to monitor the formation of compound III spectroscopically, but the observed intermediate was unstable and rapidly decayed to the ferric enzyme (10). Normally, peroxidase compound III is a ferrous-dioxyl/ferric-superoxide complex (similar to oxyhemoglobin or oxymyoglobin) formed by three different routes (7): (a) the oxygenation of ferrous peroxidase (see Fig. 1, Reaction 6); (b) the reaction of ferric enzyme with superoxide anion (see Fig. 1, Reaction 8); and (c) the reaction of (ferryl)-compound II with excess hydrogen peroxide (see Fig. 1, Reaction 11). Recently, the rate of reaction between ferric Mycobacterium KatG and superoxide was determined by pulse radiolysis to be $5.5 \times 10^5$ M$^{-1}$ s$^{-1}$ (11). However, the reactivity of ferrous KatG is completely unknown. Neither its reactivity toward dioxygen (see Fig. 1, Reaction 6) nor its two-electron oxidation reaction to compound II mediated by H$_2$O$_2$ (see Fig. 1, Reaction 10) (12) nor the transition of compound II to compound III has been investigated so far, although the knowledge of these reactions could help to understand the KatG-specific high catalase activity. This is most obvious when looking at the...
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variants Y249F of Synechocystis KatG (13) or Y229F of Mycobacterium KatG (14). These tyrosines are part of a KatG-specific covalent link at the distal heme side, formed among the side chains of conserved tryptophan, tyrosine and methionine. Most interestingly, disruption of this covalent link by exchange of these tyrosines converted the bifunctional KatGs to typical monofunctional heme peroxidases. The catalase activity was dramatically decreased, the formation of (conventional) compound I could be followed even with equimolar $\text{H}_2\text{O}_2$, and a typical (ferryl-)compound II was formed, which was easily transformed to compound III (13, 14).

These findings motivated us to perform the following comparative kinetic investigation of the reaction of ferrous wild-type KatG from Synechocystis, the variant Y229F, and horse-radish peroxidase, respectively, with both dioxygen and hydrogen peroxide as well as of the interconversion of compound II to compound III. By using the stopped-flow technique, we were able to calculate the apparent bimolecular rate constants of the transitions ferrous protein $\rightarrow$ compound II, and compound II $\rightarrow$ compound III and detect significant differences between the three investigated protein species.

**EXPERIMENTAL PROCEDURES**

Materials—Hydrogen peroxide, obtained as a 30% solution from Sigma, was diluted, and the concentration was determined using $e_{240} = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$ (15). Sodium dithionite was from Aldrich and was used either directly or from a freshly prepared anaerobic stock solution. All experiments were performed at 25 °C and 50 mm phosphate buffer, pH 7.0. All solutions were made anaerobic by flushing with nitrogen gas ($\text{O}_2 < 3 \text{ ppm}$) and stored in a glove box (Meca-Plex, Neugebauer) at 4 °C. To achieve a defined oxygen concentration, oxygen-saturated 50 mM phosphate buffer (100% oxygen saturation at 25 °C) was bubbled with nitrogen for different periods, and the oxygen concentration was measured polarographically. Oxygenated buffer solutions were transported to the stopped-flow device in gas-tight syringes with no gas head space to avoid loss of oxygen or contact with oxygen.

The sequential mixing stopped-flow apparatus (Model SX-18MV) and the associated computer system and software were from Applied Photophysics. All reactions were followed by either using a monochromator or using the diode-array detector (Applied Photophysics PD.1) attached to the stopped-flow machine. All tubes of the stopped-flow were flushed several times with a nitrogen-bubbled dithionite solution to remove free oxygen from the system. Final enzyme concentration varied typically from 25 °C corresponds to $1262 \text{ M}^{-1} \text{ cm}^{-1}$ (15). Sodium dithionite was from Aldrich and was used either directly or from a freshly prepared anaerobic stock solution. All experiments were performed at 25 °C and 50 mm phosphate buffer, pH 7.0. All solutions were made anaerobic by flushing with nitrogen gas ($\text{O}_2 < 3 \text{ ppm}$) and stored in a glove box (Meca-Plex, Neugebauer) at 4 °C. To achieve a defined oxygen concentration, oxygen-saturated 50 mM phosphate buffer (100% oxygen saturation at 25 °C) was bubbled with nitrogen for different periods, and the oxygen concentration was measured polarographically. Oxygenated buffer solutions were transported to the stopped-flow device in gas-tight syringes with no gas head space to avoid loss of oxygen or contact with oxygen.

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The $\text{H}_2\text{O}_2$-mediated transition of compound II to compound III was either monitored in the $\text{H}_2\text{O}_2$-mediated reaction sequence ferrous peroxidase $\rightarrow$ compound II $\rightarrow$ compound III (in the case of wild-type KatG) by using the conventional mode and the diode array detector or monitored in the sequential stopped-flow mode by preforming compound II (in the case of Y229F and HRP) and following its reaction with $\text{H}_2\text{O}_2$ as absorbance increases at 414 nm. In a typical experiment, Y229F was mixed with equimolar $\text{H}_2\text{O}_2$, and finally, after a delay time of 7 s, with hydrogen peroxide. The final concentrations and conditions are as follows: 1 μM Y229F, 10–150 μM $\text{H}_2\text{O}_2$, 50 mm phosphate buffer, pH 7.0, and 25 °C.

All reactions were also analyzed by using the Pro-K simulation program from Applied Photophysics. Time traces were fitted using the single-exponential equation of the Applied Photophysics software, and from the slopes of the linear plots of the $k_{\text{off}}$ values versus substrate concentration, the apparent second-order rate constants were obtained.

**RESULTS AND DISCUSSION**

Reaction of Ferrous Wild-type KatG and the Y229F Variant with Dioxygen—Even in the presence of a high excess of hydrogen peroxide, it is not possible to monitor spectroscopically the formation of compound III (oxoferric peroxidase) of Synechocystis wild-type KatG (Fig. 1, Reaction 6). By contrast, only a minor excess (10-fold) of $\text{H}_2\text{O}_2$ led to the formation of compound III in the variant Y229F, as indicated by the appearance of absorption bands at 414, 542, and 576 nm (13). To understand these significant differences and eventually to relate them to the dramatic differences in enzymatic properties (Y229F lost the catalase activity), it is necessary to investigate pathways of compound III formation and transition. Thus, the ferrous forms of both wild-type KatG and Y229F were formed and probed for their reactivity with dioxygen.

A good spectrum of ferrous Synechocystis wild-type KatG has its peak maxima at 438 nm and in the visible region at 558 nm with a shoulder around 580 nm (Fig. 2A, first spectrum). Ferrous Y229F exhibited similar spectral features (Fig. 3A, first spectrum). The addition of dioxygen to ferrous wild-type KatG resulted in the rapid formation of compound III with absorbance maxima at 414, 548, and 578 nm. Fig. 2A demonstrates the spectral transition with clear isosbestic points at 391, 427, 473, 552, 573, and 590 nm. The reaction was monophasic, and the observed pseudo-first-order rate constants ($k_{\text{off}}$) were determined by fitting the absorbance (414 nm) versus time curves to a single exponential function (Fig. 2B). The observed pseudo-first-order rate constants were directly proportional to the initial oxygen concentrations and allowed the determination of the apparent second-order rate constant for oxygen binding ($k_{\text{on}}$) from the slope of the plot (Fig. 2C). With $(1.2 \pm 0.3) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (pH 7.0 and 25 °C), the rate constant is about twice as much as that determined for horseradish peroxidase $(5.3 \pm 0.4) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.0 and 25 °C (17). However, the $k_{\text{off}}$ values are different. In HRP, $k_{\text{off}}$ was reported to be $<0.1 \text{ s}^{-1}$ (17), resulting in $K_D = k_{\text{off}}/k_{\text{on}} = 1.9 \mu\text{M}$ (17), whereas $k_{\text{off}}$ in the case of wild-type KatG is $10 \text{ s}^{-1}$ (Fig. 2C, intercept), resulting in a $K_D$ value of 84 μM. As a consequence of this high dissociation rate constant, the ferrous-dioxy complex is unstable.

Binding of dioxygen to ferrous Y229F (absorption bands at 438 and 558 nm) followed similar kinetics. The transition to compound III (absorption bands at 414, 545, and 578 nm) was also monophasic with clear isosbestic points at 391, 427, 475, 550, and 586 nm, respectively (Fig. 3A). The observed first-order rate constants were determined at 414 nm (compound III formation) and at 440 nm (oxidation of the ferrous enzyme) and plotted against the concentration of oxygen, yielding an apparent bimolecular rate constant of $(1.6 \pm 0.2) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at 414 nm at pH 7.0 and 25 °C (Fig. 3C). Similar to wild-type KatG, the finite intercept of 20 s$^{-1}$ indicates reversible dioxygen binding. The calculated dissociation constant, $K_D$, is 129 μM at pH 7.0.

HRP compound III reverts over a period of minutes to the native ferric state ($k_{\text{decay}} = 8.2 \times 10^{-3} \text{ s}^{-1}$) according to Reaction 9 in Fig. 1, and the transition is independent of oxygen concentration (17). Most interestingly, wild-type KatG compound III is much more unstable. At pH 7.0 the decay rate was determined to be 2.5–3.6 s$^{-1}$, which is about 350 times higher than that of HRP compound III. By contrast, conversion of...
Y249F compound III to the ferric state was about 40 times slower ($0.07 \text{ s}^{-1}$) than that of wild-type KatG compound III.

From these findings, the following conclusion can be drawn. The kinetics of compound III formation by binding of dioxygen to the ferrous proteins as well as the spectral features of compound III are similar in wild-type KatG, Y249F, and HRP. However, there is a significant difference in the stability of the oxyperoxidases. In HRP, compound III is very stable; it neither decays to ferric HRP-releasing superoxide ($k_{\text{decay}} = 2.5 \times 10^{-3} \text{ s}^{-1}$) (Fig. 1, Reaction 9) nor dissociates to ferrous HRP-releasing dioxygen (indicated by the low $k_{\text{off}}$ value of 0.1 s$^{-1}$ (17) (Fig. 1, Reaction 7)) at reasonable rates. By contrast, KatG compound III is very unstable, and both pathways are more than 2 orders of magnitude faster ($k_{\text{off}}$ of $10 \text{ s}^{-1}$ and $k_{\text{decay}}$ of 2.5–3.6...
Due to the instability of Y249F, the reaction was biphasic with single-exponential fit of the reaction of 1 μM ferrous Y249F with 50 μM hydrogen peroxide. Abs, absorbance. C, a plot of the pseudo-first-order rate constants versus hydrogen peroxide concentration.

The high dissociation rate of Y249F prevents the formation of the KatG-typical covalent adduct (18) but only slightly affects the binding and dissociation of fluoride (19) and dioxygen to the ferric protein. However, it decelerates compound III decay via Reaction 9 (Fig. 1).

**Reaction of Ferrous HRP, Wild-type KatG, and the Y249F Variant with Hydrogen Peroxide**—The high dissociation rate constant of the ferrous-dioxy complex of KatG implies the presence of ferrous KatG in steady state. Besides binding dioxygen, a ferric heme peroxidase can also mediate the heterolytic electron oxidation of HRP with H2O2 are very similar (not shown). The calculated apparent bimolecular rate constant was determined to be \((1.1 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{s}^{-1}\) (pH 7.0, 25 °C) with an intercept of 0.3 s⁻¹. These findings underline the high similarity between the KatG variant Y249F and HRP. Not only are the kinetics of compound I and compound II formation and the UV-Vis spectra nearly identical, but in addition, reduction of both redox intermediates with ascorbate and tyrosine follow the same kinetics (13), apart from the most important fact that the catalytic activity of both enzymes is negligible.

Completely different is the \(\text{H}_2\text{O}_2\)-mediated oxidation of wild-type ferrous KatG. In contrast to the Y249F, no ferryl-type compound II is formed. This fits well with previous observations that wild-type KatG compound II exhibits spectral features similar to ferric KatG (8, 9, 13). Fig. 5A shows the spectral changes obtained in the reaction of 3 μM ferrous wild-type KatG with 50 μM hydrogen peroxide. The reaction again exhibited a clear isosbestic point at 421 nm. The bold spectrum depicts a spectrum similar (but not identical) to the ferric enzyme in the Soret region (408 nm) but not at higher wavelength (bands at 540 and 580 nm), indicating that some portion of the protein was already in the compound III state. The bold spectrum was selected 880 ms after mixing ferrous wild-type KatG with \(\text{H}_2\text{O}_2\). It finally converted to the spectrum of the ferric state (data not shown).

![Fig. 4. Reaction of ferrous Y249F with hydrogen peroxide (compound II formation). A, spectral changes after mixing of 2 μM ferrous Y249F with 100 μM hydrogen peroxide. Spectra were taken after 1, 6, 11, 26, and 86 ms (bold line). The conditions were as follows: 50 mM phosphate buffer, pH 7.0; 25 °C. B, a typical time trace at 440 nm with single-exponential fit of the reaction of 1 μM ferrous Y249F with 50 μM hydrogen peroxide. Abs, absorbance. C, a plot of the pseudo-first-order rate constants versus hydrogen peroxide concentration.](http://www.jbc.org/)

![Fig. 5. Reaction of ferrous wild type KatG with hydrogen peroxide. A, spectral changes due to mixing of 3 μM ferrous enzyme with 50 μM hydrogen peroxide. Spectra were taken at 1, 24, 80, 260, and 880 ms. The conditions were as follows: 50 mM phosphate buffer, pH 7.0; 25 °C. B, a typical time trace at 440 nm with single-exponential fit of the reaction of 1 μM ferrous wild type KatG with 100 μM hydrogen peroxide (final concentrations). Abs, absorbance. C, a plot of the pseudo-first-order rate constants versus hydrogen peroxide concentration. D, spectral changes obtained after the reaction of 3 μM ferrous KatG with 300 μM hydrogen peroxide. Subsequent spectra were taken after 1, 6, 19, and 42 ms (bold line) and 4 s. The conditions were as in panel A.](http://www.jbc.org/)
The elucidation of the kinetics of the reaction of ferrous wild-type KatG with H\textsubscript{2}O\textsubscript{2} faced several problems. Only with low concentrations of H\textsubscript{2}O\textsubscript{2} (<50 \muM) could a shift in the Soret region from 438 to 408 nm be observed, whereas at higher concentrations, the spectral features of compound III began to dominate within milliseconds (compare Fig. 5, A and D), suggesting that the reaction sequence is also ferrous wild-type KatG \rightarrow compound II \rightarrow compound III. The slowest rate in this transition was estimated to be $\sim 2.0 \times 10^5$ m\textsuperscript{-1} s\textsuperscript{-1}. With respect to the spectral features of compound II, this investigation underlined that significant differences exist between wild-type KatG and Y249F, the latter corresponding to a typical ferryl-type (low spin) compound II like in HRP, whereas the spectral features of wild-type compound II suggests the presence of a high spin compound II (ferric protein plus protein radical). It has to be noted that the unexpected high rate of compound II to compound III conversion was very similar to the rate reported for the H\textsubscript{2}O\textsubscript{2}-mediated transition of compound II to compound III of the monofunctional bovine liver catalase ($6.1 \times 10^4$ m\textsuperscript{-1} s\textsuperscript{-1}) (23).

Relevance for Catalase Activity and Proposal of the Catalatic Mechanism—In monofunctional catalases, the catalatic pathway is thought to include Reactions 1 and 2 (Fig. 1). Depending on experimental conditions, inactivation of catalase activity of monofunctional catalases is assumed to occur due either to accumulation of a (ferryl-like) compound II ("slow" inhibition) or to formation of compound III ("rapid" inhibition) at higher peroxide concentrations. The catalatic mechanism of KatG is still under discussion. Assuming a mechanism similar to monofunctional catalases is one working hypothesis; however, many findings hold against it as follows. (i) Wild-type compound I produced with peroxyacetic acid reacts extremely slow with H\textsubscript{2}O\textsubscript{2} as demonstrated by the sequential stopped-flow technique (8, 9). (ii) In wild-type Synchocystis catalase-peroxidase, the chemical nature of the intermediate referred to as conventional compound I was shown to be the superposition of the oxoferryl porphyrin \pi-cation radical, the tryptophanyl radical, and the tyrosyl radical as demonstrated by our recent multi-frequency EPR spectroscopy study (24). (iii) The observation of redox intermediates with unique features in their room temperature electronic absorption spectrum suggests the existence of alternative electronic structures of compound I (13, 24).

An alternative mechanism could be very similar to that proposed for the human peroxidases myeloperoxidase (20) and lactoperoxidase (25), which includes Reactions 6, 10, and II in Fig. 1. When compared with KatG, the catalatic activity of myeloperoxidase and lactoperoxidase is low, but the present study revealed important differences in the rates of the H\textsubscript{2}O\textsubscript{2} mediated oxidation of compound II to compound III. In KatGs, the rate constant for this reaction is $1.2 \times 10^4$ m\textsuperscript{-1} s\textsuperscript{-1} and therefore at least 2 orders of magnitude faster when compared with myeloperoxidase ($78$ m\textsuperscript{-1} s\textsuperscript{-1}) (20) or lactoperoxidase (70 m\textsuperscript{-1} s\textsuperscript{-1}) (25). Another important fact is that wild-type KatG compound III does not accumulate during hydrogen peroxide degradation even upon incubation of the ferric enzyme with high H\textsubscript{2}O\textsubscript{2} concentrations. By contrast, lactoperoxidase and thyroid peroxidase compound III can already be formed by the addition of 200 \muM hydrogen peroxide to 1.7 \muM ferric enzyme (26), and in the KatG variant Y249F, a 10-fold excess of H\textsubscript{2}O\textsubscript{2} already converts compound II to compound III (13). Nevertheless, in the absence of H\textsubscript{2}O\textsubscript{2}, we succeeded in forming pure KatG compound III by following the oxygen binding to ferrous...
KatG and could demonstrate that the spectral features of wild-type KatG, Y249F, and HRP compound III are very similar but that there are substantial differences in the stability of the corresponding dioxygen complexes. Furthermore, the two-electron oxidation of ferrous KatG by H$_2$O$_2$ unequivocally demonstrated significant differences between wild-type KatG and Y249F (or HRP). In wild-type KatG (and in contrast to Y249F and HRP), no oxoferryl-type compound II accumulates in detectable concentrations, which confirmed earlier observations in the reaction between compound I preformed with peroxoacetic acid and one-electron donors (9, 10, 13), which suggested that KatG compound II could be a protein radical species with spectral features similar to ferric KatG.

The present data also suggest that in wild-type KatG, oxoferryl (compounds I and II) and dioxygen adducts (compound III) are destabilized when compared with Y249F and HRP. One significant structural difference between wild-type KatG and Y249F is that in the variant, the KatG-specific covalent adduct (which includes Tyr-249, Met-275, and Trp-122) at the distal heme side is absent (18). We now know three striking consequences; the catalase activity in Y249F is lost (whereas the peroxidase activity is unaffected), and in the presence of H$_2$O$_2$, an oxoferryl-type compound II is formed and easily converts to compound III (13, 24). By contrast, in the presence of the covalent link, the catalase activity is high, and neither an oxoferryl-type compound I nor II accumulates, nor does compound III. Based on these data, we propose the following mechanism that has the stoichiometry of a classical catalatic activity (2 H$_2$O$_2$ $\rightarrow$ O$_2$ + 2 H$_2$O).

\[ \text{R-PorFe}^{	ext{II}} + 2 \text{H}_2\text{O}_2 \rightarrow \text{R-PorFe}^{	ext{III}} + \text{O}_2 + 2 \text{H}_2\text{O}. \]

**REACTION I**

\[ ^{\prime}\text{R-PorFe}^{	ext{III}} + \text{H}_2\text{O}_2 \rightarrow \text{R-PorFe}^{	ext{IV}} + \text{O}_2 \]

**REACTION II**

\[ ^{\prime}\text{R-PorFe}^{	ext{III}} + \text{O} \rightarrow \text{R-PorFe}^{	ext{III}} - \text{O} \]

**REACTION III**

In the first reaction, the ferric enzyme is oxidized to compound I, thereby reducing H$_2$O$_2$ to water. Recent EPR experiments have shown the contribution of three different oxoferryl species obtained by reaction of wild-type KatG with peroxoacetic acid, namely an exchange-coupled porphyrin radical, a tryptophan, and a tyrosyl radical (13, 24). Upon using H$_2$O$_2$ in enzyme oxidation, neither the accumulation of the classical compound I (porphyrin radical-type, which typically shows a hypochromicity in the Soret peak) nor the accumulation of a protein radical species (which should exhibit an oxoferryl-type compound II spectrum like in cytochrome c peroxidase (27)) could be ever observed spectroscopically (in contrast to Y249F). This suggests a rapid conversion of compound I by the second hydrogen peroxide molecule. Based on previous findings that wild-type compound I, which accumulates with peroxoacetic acid and exhibits a 40–50% hypochromicity in the Soret region, reacts only very slowly with H$_2$O$_2$, we proposed that the protein radical form has a higher reactivity toward H$_2$O$_2$ (24). Mechanistically, the reaction of this species with H$_2$O$_2$ would be similar to that of an oxoferryl compound II with H$_2$O$_2$, namely displacement of the ferryl oxygen by both oxygen atoms deriving from hydrogen peroxide as has been demonstrated for the compound II to compound III conversion of lactoperoxidase by isotopic labeling (28). The present study has demonstrated that this reaction is at least 2 orders of magnitude faster in KatGs ($k_{\text{app}}$ of 1.2 $\times$ 10$^5$ m$^{-1}$ s$^{-1}$) when compared with other heme peroxidases (e.g. HRP 20 m$^{-1}$ s$^{-1}$ (21) or myeloperoxidase 78 m$^{-1}$ s$^{-1}$ (20)). The reaction product would be a ferrous-dioxyferric-superoxo complex containing a protein radical site when H$_2$O$_2$ reacts with the protein radical/oxoferryl-type compound I instead of oxoferryl-type compound II. Storing a one-oxidation equivalent could guarantee that (i) finally dioxygen and not superoxide is released when ferric KatG is formed and (ii) that the dioxygen adduct is much more unstable when compared with the typical oxymyoglobin-like compound III. As a consequence, this species does not accumulate, and the enzyme cycles rapidly. In this mechanism, the dioxygen species is attached to the heme iron before being released, which is in contrast to the proposed catalatic mechanism of monofunctional catalases (5). Although this proposed mechanism is very speculative at the moment, it is based on significant differences between wild-type KatG and Y249F (or HRP) presented in this work. It provides a basis for further investigations that will help to understand the extraordinary bifunctional activity of catalase-peroxidases.

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