Effect of Distal Cavity Mutations on the Formation of Compound I in Catalase-Peroxidases*

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Catalase-peroxidases have a predominant catalase activity but differ from monofunctional catalases in exhibiting a substantial peroxidase activity and in having different residues in the heme cavity. We present a kinetic study of the formation of the key intermediate compound I by probing the role of the conserved distal amino acid triad Arg-Trp-His of a recombinant catalase-peroxidase in its reaction with hydrogen peroxide, peroxoacetic acid, and m-chloroperbenzoic acid. Both the wild-type enzyme and six mutants (R119A, R119N, W122F, W122A, H123Q, H123E) have been investigated by steady-state and stopped-flow spectroscopy. The turnover number of catalase activity of R119A is 14.6%, R119N 0.5%, H123E 0.03%, and H123Q 0.02% of wild-type activity. Interestingly, W122F and W122A completely lost their catalase activity but retained their peroxidase activity. Bimolecular rate constants of compound I formation of the wild-type enzyme and the mutants have been determined. The Trp-122 mutants for the first time made it possible to follow the transition of the ferric enzyme to compound I by hydrogen peroxide spectroscopically underlining the important role of Trp-122 in catalase activity. The results demonstrate that the role of the distal His-Arg pair in catalase-peroxidases is important in the heterolytic cleavage of hydrogen peroxide (i.e. compound I formation), whereas the distal tryptophan is essential for compound I reduction by hydrogen peroxide.

Catalase-peroxidases (KatGs)1 cover a growing group of enzyme. They are components of the oxidative defense system of bacterial (1) and fungal (2, 3) cells and function primarily as catalases to remove hydrogen peroxide before it can damage cellular components. They are different from classical monofunctional catalases. On the basis of sequence similarities with fungal cytochrome c peroxidase (CCP) and plant ascorbate peroxidases (APX), KatGs have been shown to be members of class I of the superfamily of plant, fungal, and bacterial peroxidases (4). Despite striking sequence homologies between class I enzymes, there are dramatic differences in the catalytic activity and substrate specificity (5–7). The most interesting feature of bifunctional catalase-peroxidases is the overwhelming catalase activity with overall rate constants comparable with those of monofunctional catalases. In both CCP and APX, the catalase activity can be neglected. KatGs also function as broad specificity peroxidases, oxidizing various electron donors, including NAD(P)H (8–10), phenols, and anilines (7), whereas typical substrates for APX and CCP (ascorbate and cytochrome c, respectively) are extremely poor electron donors for KatGa (5–7, 11).

From both CCP and APX, the three-dimensional structures are known (12, 13) and exhibit highly conserved amino acid residues at the active site. They indicate the presence of a proximal histidine as well as the triad Arg-Trp-His at the distal side. Both physical characterization as well as sequence analysis suggest the presence of these residues also in catalase-peroxidases. At the moment there is no structural basis to understand the catalytic features of catalase-peroxidases. Thus, in developing ideas how protein structure modifies heme reactivity, class I peroxidases are an extremely exciting field of research, with KatGs being the least understood type of peroxidase.

The initial step in the catalytic mechanism of a peroxidase or catalase is heterolysis of the oxygen-oxygen bond of hydrogen peroxide. This reaction causes the release of one water molecule (15) and coordination of the second oxygen atom to the iron center (16). Two electrons are transferred from the enzyme to the coordinated oxygen atom, one from the iron and one from a second donor. In pea cytosolic APX, the porphyrin serves as the second donor (17), whereas, in CCP, the second donor is Trp-191 (18–20). Recently, we have shown that the spectrum of KatG compound I is reminiscent to APX compound I (7) with the important difference (and this is caused by the overwhelming catalase activity) that compound I formation of KatG could never be monitored with hydrogen peroxide. Instead of H₂O₂, it was necessary to use peroxoacetic acid (7).

The triad Arg-Trp-His is located near the peroxide binding site. Histidine is suggested to function as a general acid/base catalyst that assists in deprotonating the hydroperoxide and protonating the departing water (18), whereas distal arginine is proposed to stabilize the transition state for compound I formation by interacting with the developing negative charge on the oxygen atom being reduced during the heterolytic cleavage of the peroxide bond and to stabilize the resulting oxyferryl center of compound I (21). The role of distal tryptophan is least clear. In CCP the mutant W51F has been shown to be hyperactive (22).

In the present work we undertook a detailed analysis of

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* This work was supported by Austrian Science Fund Grants P12374-MOB and P8208 and by Project 7554 of the Jubiläumsfond of the Austrian National Bank. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: KatG, catalase-peroxidase; PA, peroxoacetic acid; CPB, m-chloroperbenzoic acid; CCP, cytochrome c peroxidase; APX, ascorbate peroxidase; PCR, polymerase chain reaction.
compound I formation of recombinant catalase-peroxidase from the cyanobacterium *Synechocystis* PCC 6803. Our goal was to elucidate the role of the distal triad Arg-Trp-His in peroxide binding and cleavage of KatG and to ascertain similarities or differences to both APX and CCP. The wild-type enzyme and six mutants (R119A, R119N, W122F, W122A, H123Q, H123E) have been investigated by both steady-state and presteady-state kinetic analysis. The most exciting finding of this work is the role of tryptophan in catalase activity of KatG. The Trp-122 mutants lost their catalase activity completely but retained their peroxidase activity. The consequence was that, for the first time, in these two proteins compound I formation could be followed spectroscopically also with hydrogen peroxide. Mutation of distal arginine and histidine gave 10- to 10^4-fold decreases in catalase activity, indicating a role in O-O heterolysis of hydrogen peroxide. In contrast, their role in compound I formation with peroxoy acids was much less pronounced.

**EXPERIMENTAL PROCEDURES**

**Materials**

Materials were from the following sources: phenylmethylsulfonyl fluoride, hemin, pepstatin A, leupeptin, chloramphenicol, and amphicillin from Sigma; GFX PCR DNA and gel band purification kit, chelating Sepharose Fast Flow, and HiPrep® Sephacryl S-300 HR 16/60 gels from Amersham Pharmacia Biotech; the Centriprep-30 concentrators from Amicon; *Kpn I* from Stratagene; *Bam HI* and *Pst I* restriction enzymes, T4 DNA ligase, and alkaline phosphatase from Roche Molecular Biochemicals; and *Pfu* polymerases from Promega and Stratagene, respectively. All other chemicals were of the highest purity grade available.

**Methods**

**Mutagenesis**—Oligonucleotide site-directed mutagenesis was performed using PCR-mediated introduction of silent mutations as described (23). A pET-3a expression vector that contained the cloned catalase-peroxidase gene from the cyanobacterium *Synechocystis* PCC 6803 (7, 14) was used as the template for PCR. At first unique restriction sites were selected flanking the region to be mutated. The flanking primers were 5'-AAT GAT CAG GTA CCG GCC GAT AAA TG-3', containing a *Kpn I* restriction site and 5'-TGC ATA AAG GAT CCG GGT GC-3' containing a *Bam HI* restriction site. The internal 5'-primer was 5'-GCC GCG TGC ACG AAC TTA TCG CAT TG-3' and possessed a *Pst I* restriction site. The following primers were used with the desired mutation and a silent mutation introducing a restriction site were constructed (point mutations italicized and restriction sites underlined): 5'-AGT GCC TGC AGC GTG CCA GCC CAT AAC CAT TAA TCA TC-3' changed Arg-119 to Ala, 5'-AGT GCC TGC AGC GTG CCA GCC CAT CAT TCA TC-3' changed Arg-119 to Asn, 5'-AGT GCC TGC AGC GTG GAA GCC CAT AC-3' changed Trp-122 to Ala, 5'-AGT GCC TGC AGC GTG GCC CAT AC-3' changed Trp-122 to Phe, 5'-AGT GCC TGC AGC GTG GCC CAT AC-3' changed Trp-122 to Ala, 5'-AGT GCC TGC AGC GTG GCC CAT AC-3' changed Trp-122 to Phe, 5'-AGT GCC TGC AGC GTG GCC CAT AC-3' changed His-123 to Asn, and 5'-AGT GCC TGC AGC GTG GCC CAT AC-3' changed His-123 to Glu. The fragment defined by the *Kpn I* and *Bam HI* restriction sites was replaced by the new construct containing the point mutation. All constructs were sequenced to verify DNA changes using thermal cycle sequencing.

**Expression and Purification**—The mutant recombinant catalase-peroxidases were expressed in *Escherichia coli* BL21(DE3)pLysS and purified as described previously by Regelsberger et al. (7) and Jakobitsch et al. (14) using the same conditions as for the wild-type enzyme.

**Catalase Activity**—Catalase activity was determined in 67 mM phosphate buffer, pH 7.0. Protein concentrations were 1 μM in all experiments.

**Stopped-flow Measurements**—Transient-state measurements were performed using an Applied Photophysics instrument (Model SX-185MV) equipped with a 1-cm observation cell thermostated at 15 °C. Rate constants from experimental traces were calculated by the SpectralKinetic workstation version 4.38 interfaced to the apparatus. Conventional stopped-flow analysis was used to investigate the oxidation of peroxide by peroxidases and formation of compound I. At least three determinations of the pseudo-first-order rate constants, kₙₑᵣₙ, were measured for each substrate concentration, and the mean value was used to calculate the second-order rate constants. To allow calculation of pseudo-first-order rates, the concentrations of substrates were at least 5 times that of the enzyme. The slope of the linear plot of the pseudo-first-order rate constant versus substrate concentration was used to obtain the second-order rate constant for the reaction. All stopped-flow experiments were performed in 50 mM phosphate buffer, pH 7.0, with the exception of pH dependence studies on reaction rates for which the experimental traces were recorded in 50 mM citrate/ phosphate buffers at different pH values between 4.0 and 8.0. Reactions were also studied with a diode-array detector (model PD.1 from Applied Photophysics) as part of the stopped-flow machine, as well as in conventional steady-state spectroscopy using a Zeiss Specord S-10 diode-array spectrophotometer.

**RESULTS**

Recently, we reported a high level expression in *E. coli* of a recombinant form of a homodimeric catalase-peroxidase from the cyanobacterium *Synechocystis* PCC 6803. Both physical and preliminary kinetic characterization revealed its identity with the wild-type protein (7, 14).

**Spectral Properties**—Fig. 1 depicts CD spectra of ferric native recombinant KatG and of all six mutants investigated in this study. The far-UV CD spectra give a measure of the protein secondary structure. The signal obtained for catalase-peroxidase was characteristic of a protein composed primarily of α helices. Very little difference was observed between the CD spectra of wild-type and the six mutant proteins, indicating that there was no large scale conformational change in the structure. If conformational changes did occur, they must have been very localized and unobserved by the CD method used. Since the α-helical content seemed to be insensitive to the amino acid substitution at the distal Arg, Trp, or His, we assumed that the mutants were folded properly, as is the wild-type enzyme.

**Peroxidase Assay**—Peroxidase activity was monitored spectrophotometrically using 1 mM H₂O₂ and either 1 mM oxidized dianisidine (ε₄₆₀ = 11.3 mM⁻¹ cm⁻¹) or 20 mM pyrogallol (ε₄₃₀ = 2.47 mM⁻¹ cm⁻¹) following the oxidation rate in 67 mM phosphate buffer, pH 7.0. One unit of peroxidase is defined as the amount that decomposes 1 μmol of electron donor/min at pH 7 and 25 °C.

![Fig. 1. Circular dichroism spectra of wild-type (thick line) and six mutants at the distal heme region (thin lines) of *Synechocystis* PCC 6803 catalase-peroxidase. Molar ellipticities (θ) in the far-UV region are plotted versus wavelength. Samples were prepared in 50 mM phosphate buffer, pH 7.0, at a protein concentration of 1 μM.](image-url)
ferric peroxidase in the visible and near ultraviolet region. Fig. 2A shows the Soret peak to be at 406 nm and the two charge-transfer bands at 500 (CT2) and 631 nm (CT1). Both the Soret and the CT1 band suggested the presence of a five-coordinate high spin heme coexisting with a six-coordinate high spin heme. The $A_{406}/A_{320}$ ratio (i.e. Reinheitszahl) varied between 0.63 and 0.65 and was similar to that of wild-type KatG (6). The spectral parameters of the mutants are summarized in Table I. Mutations of Arg, Trp, and His caused shifts of 1–4 nm at the Soret region and more pronounced shifts at both CT1 and CT2. Mutation of histidine decreased the $k_{cat}$ value by a factor of 3000–4000, whereas the $k_{cat}$ value of catalase activity was 14.6% for R119A and 0.5% for R119N compared with the wild-type protein. The catalase activity of the variants was measured polarographically and was proportional to hydrogen peroxide concentrations in the range 2–20 mM. Corresponding to the effect of mutation on catalytic activity, the concentration of the mutants in the assays had to be 30–300 nM (per heme). At higher peroxide levels (>20 mM), the reaction rate again lost proportionality to $H_2O_2$ concentration. Generally, inactivation of mutants at high $H_2O_2$ concentration was more dramatic than inactivation of the wild-type protein.

Both the wild-type protein and the Arg-119 and His-123 variants exhibited only a small decrease in absorbance (hypochromicity) of −0.2% at the Soret region. Assuming that compound I would give a Soret absorbance −60% of that of the ferric enzyme (7), these hypochromicities fitted a steady-state compound I concentration between 0% and 4%. This calculation was based on a hypothetical catalase reaction mechanism involving Reactions 1 (a and b) and 2 (a and b) in Fig. 6. During catalase activity the ratio of free enzyme to compound I is a constant determined by the rate constants of compound I formation, $k_{1(app)}$ and compound I reduction, $k_{2(app)}$. From [KatG]/[compound I] = $k_{2(app)}/k_{1(app)}$, it follows that during catalase activity $k_{2(app)} >> k_{1(app)}$. Our experiments demonstrated that mutation of distal Arg and His did not alter this ratio. On the contrary, mutation of distal Trp dramatically shifted this ratio. Addition of hydrogen peroxide to both catalytically inactive mutants W122F and W122A led to compound I accumulation. Apparently, in the Trp-122 variants, Reaction 2 (a and b) was blocked.

The peroxidatic activity of the Arg-119 and His-123 mutants was reduced proportionally to the catalase activity. In contrast, in both Trp-122 variants, the peroxidatic to catalatic ratio dramatically increased. Although having lost the catalase activity, the specific peroxidase activity of W122F was still 0.5 units/mg (1 mM $H_2O_2$, 5 mM o-dianisidine) and 0.83 units/mg (1 mM $H_2O_2$, 20 mM pyrogallol).

**Compound I Formation**—The initial event in the catalytic mechanism of a peroxidase or catalase is a two-electron oxidation of the enzyme by hydrogen peroxide to an intermediate called compound I (Reaction 1, a and b). Compound I of peroxidases normally exhibits the same Soret band maximum as the corresponding native peroxidase but a hypochromicity of about 40–50%. We have recently demonstrated that bifunctional catalase-peroxidases exhibit similar spectral changes upon addition of peroxides (7). In contrast to its homologous members of catalase kinetics in a Michaelis-Menten way (and therefore the definition of $K_m$ and $k_{cat}$ should be avoided), we have calculated apparent $K_m$ and $k_{cat}$ values as has been done in the literature in recent years (5–7, 11, 14). With wild-type KatG (1 mM heme), there was a linear correlation in a Lineweaver-Burk plot between 50 μM and 10 mM hydrogen peroxide allowing determination of both apparent $K_m$ and $v_{max}$. At $H_2O_2$ concentrations higher than 10 mM, a progressive inhibition of enzyme activity was seen. For the wild-type protein, the corresponding apparent $k_{cat}$ value with $H_2O_2$ as the sole substrate was determined to be 3500 s$^{-1}$ and the apparent $K_m$ was 4.9 mM. The $k_{cat}/K_m$ ratio was $7.1 \times 10^5$ M$^{-1}$ s$^{-1}$.

Table I contains some of the kinetic constants for KatG and the investigated mutants. It shows how the mutations decrease the affinity for hydrogen peroxide as well as the turnover rates and thus confirms that the changed residues are involved in catalase activity of KatG. Interestingly, both Trp-122 mutants lost their catalase activity completely. Mutation of Arg-119 and His-123 gave partially active forms. Mutation of histidine decreased the $k_{cat}$ value by a factor of 3000–4000, whereas the $k_{cat}$ value of catalase activity was 14.6% for R119A and 0.5% for R119N compared with the wild-type protein. The catalase activity of the variants was measured polarographically and was proportional to hydrogen peroxide concentrations in the range 2–20 mM. Corresponding to the effect of mutation on catalytic activity, the concentration of the mutants in the assays had to be 30–300 nM (per heme). At higher peroxide levels (>20 mM), the reaction rate again lost proportionality to $H_2O_2$ concentration. Generally, inactivation of mutants at high $H_2O_2$ concentration was more dramatic than inactivation of the wild-type protein.

Compound I Formation in Catalase-Peroxidases

![Absorption spectra and stopped-flow measurements of wild-type catalase-peroxidase from Synechocystis PCC 6803. A, spectra of 20 μM ferric enzyme (1) and compound I (2) formed by mixing 20 μM enzyme with 200 μM peroxoacetic acid and waiting for 20 s. B, plot of pseudo-first-order rate constants between wild-type enzyme and peroxoacetic acid. The inset shows a typical stopped-flow time trace of 1 μM enzyme with 100 μM peroxoacetic acid monitored at 406 nm and 15 °C in 50 mM phosphate buffer, pH 7.0.](image-url)
Compound I Formation in Catalase-Peroxidases

class I peroxidases, APX and CCP, monitoring of compound I in KatGs is impossible because of its high catalase activity ($k_{1(app)} \gg k_{1(obs)}$). Consequently, peroxo acids had to be used to form a stable KatG compound I.

From the single-mixing stopped-flow experiments on rates of compound I formation using excess peroxy acids, the following results were obtained. Reaction of wild-type KatG with both peroxoacetic acid (PA) and m-chloroperbenzoic acid (CPB) exhibited single exponential curves, indicating pseudo-first-order kinetics (Fig. 2B, inset). Plots of the first-order rate constants, $k_{obs}$, versus peroxide concentration were linear with very small intercepts ($<0.5$ s$^{-1}$) (Fig. 2B). These small intercepts fitted well with the observation that wild-type compound I was stable for more than 30 s, even in the presence of excess PA. With PA and CPB the rate constants for compound I formation, $k_{1(app)}$, were $(3.9 \pm 0.4) \times 10^4$ M$^{-1}$ s$^{-1}$ and $(5.3 \pm 0.2) \times 10^4$ M$^{-1}$ s$^{-1}$, respectively (pH 7.0, 15°C). Fig. 2A shows the spectrum of KatG compound I produced by mixing 20 mM recombinant enzyme with 200 mM peroxoacetic acid. Its spectrum was distinguished from the resting state by a 40% hypochromicity at 406 nm and two distinct peaks at 604 and 643 nm. Isosbestic points between compound I and the resting enzyme were determined to be at 357, 430, and 516 nm.

When PA or CPB was added to R119A and R119N, a similar exponential decrease of absorbance at the Soret maximum was observed. However, the exponential phase (representing ~90% of total hypochromicity) was followed by a relatively slow decrease in absorbance of this mutant, and this decrease could be attributed to a decay of compound I. The pseudo-first-order rate constants, $k_{obs}$, were calculated from the first exponential phase. The $k_{1(app)}$ values determined from plots of these first-order rate constants, $k_{obs}$, versus peroxide concentration are summarized in Table II. There was a distinct influence of mutations of Arg-119 on the reaction with PA, whereas the reactivity toward CPB seemed to be unaffected. With PA the corresponding values were 0.2 s$^{-1}$ and 0.6 s$^{-1}$ with R119N and 0.6 s$^{-1}$ with R119A, whereas with CPB the corresponding values were 3.6 and 4.5 s$^{-1}$, respectively. This fitted well with the observation that compound I always caused a faster decrease in absorbance during the second (linear) phase than PA.

The over all catalase activity of recombinant wild-type KatG showed a significant pH dependence, with a maximum activity at pH 6.5 (Fig. 4A). The apparent bimolecular rate constant for the reaction between wild-type KatG and PA as well as between the W122F mutant and hydrogen peroxide depended on pH and exhibited a similar bell-shaped pattern with a maximum at pH 6 (Fig. 4, B and C). Since both peroxides caused a similar dependence on pH, it is reasonable to assume that two enzyme ionizations influence the formation of compound I.

Decay of Compound I—Inactivation of mutants at high hydrogen peroxide concentrations was more pronounced than inactivation of the wild-type protein. This correlated with compound I stability. Wild-type compound I was stable for more than 30 s, whereas in the mutants formation of compound I was followed by a slow phase of spectral transition. At low hydrogen...
peroxide concentrations (<100 µM), a very slow decay of compound I back to the ferric enzyme was observed (data not shown), whereas at higher peroxide concentrations, a red shift of the spectrum occurred. The spectrum shown in Fig. 5 A was recorded 4 s after W122F was mixed with 50 mM H₂O₂. The absorbance maxima were at 417, 544, and 578 nm, respectively. Longer incubation times resulted in a continuous decrease of absorbance, indicating heme destruction (Reaction 5 in the scheme of Fig. 6). With PA and above all with CPB, these spectral transitions were faster.

Compound I suffered a completely different fate when a one-electron donor was offered. Addition of ascorbate to wild-type compound I resulted in the formation of an intermediate with a Soret maximum at 407 nm and a distinct peak at 626 nm (Fig. 5 B, spectrum 3). The isosbestic points between this intermediate and the ferric enzyme were at 345, 426, 489, and 531 nm. Recently, we have proposed that this intermediate is KatG compound II, which is still one oxidizing equivalent above that of the native enzyme (containing an oxidized amino acid residue, which is not electronically coupled to the heme) (7). Upon addition of ascorbate to W122F compound I, an intermediate with a Soret absorbance between that of compound I and the ferric protein accumulated (Fig. 5 C, spectrum 3). Recently, we have shown that ascorbate is a very poor substrate for both wild-type and recombinant KatG from Synechocystis PCC 6803 (8). On the contrary, preliminary (unpublished) studies about the peroxidase activity have shown that the W122F mutant exhibited an enhanced peroxidase activity with ascorbate compared to the wild-type protein.

### Table II

|                  | WT   | R119A | R119N | W122F | W122A | H123Q | H123E |
|------------------|------|-------|-------|-------|-------|-------|-------|
| **k<sub>app</sub> µ<sup>-1</sup>s<sup>-1</sup>** |      |       |       |       |       |       |       |
| Hydrogen peroxide| ND   | ND    | ND    | 8.2 × 10<sup>4</sup> | 8.8 × 10<sup>4</sup> | ND    | ND    |
| Peroxoacetic acid| 3.9 × 10<sup>4</sup> | 1.9 × 10<sup>3</sup> | 4.5 × 10<sup>2</sup> | 1.8 × 10<sup>5</sup> | 2.1 × 10<sup>5</sup> | ND    | 1.1 × 10<sup>5</sup> |
| m-Chloroperbenzoic acid | 5.3 × 10<sup>4</sup> | 5.0 × 10<sup>3</sup> | 1.4 × 10<sup>4</sup> | 7.3 × 10<sup>4</sup> | 9.0 × 10<sup>5</sup> | 7.3 × 10<sup>4</sup> | 7.5 × 10<sup>5</sup> |
DISCUSSION

The availability of significant amounts of fully intact recombinant catalase-peroxidase and properly folded mutants for the first time permitted a comprehensive study of the role of active site residues in compound I formation of a catalase-peroxidase. The amino acid triad Arg-Trp-His is found at the distal heme site of all members of class I peroxidases (CCP, APX, and KatG); however, these three peroxidases exhibit dramatic differences in enzyme reactivities. The key intermediate in the peroxidase cycle is compound I, and its kinetic and spectral investigation is a prerequisite for understanding the bifunctional behavior of catalase-peroxidases.

With CCP and APX, the reaction between the resting enzyme and hydrogen peroxide could be followed by conventional stopped-flow spectroscopy. For the transient-state kinetics of compound I formation of pea cytosolic APX, two second-order rate constants were published, namely $8.3 \times 10^7$ m$^{-1}$ s$^{-1}$ (26) and $4.0 \times 10^7$ m$^{-1}$ s$^{-1}$ (27). The rate for compound I formation of yeast CCP with hydrogen peroxide was shown to be in the range of $10^7$ to $10^8$ m$^{-1}$ s$^{-1}$ (28–30). In contrast, we failed in assessing the formation of KatG compound I by adding hydrogen peroxide even by rapid-scanning stopped-flow spectroscopy. The characteristic spectral transitions that occur when APX (24) and CCP (15) are mixed with H$_2$O$_2$ were not observed. This could be caused by one of the following two reasons: (i) compound I is not the intermediate in catalase reaction, or (ii) the catalase reaction cycle of a KatG is similar to monofunctional catalases, thus involving Reactions 1 (a and b) and 2 (a and b) with $k_{2(app)} \gg k_{1(app)}$ (Fig. 6). The results presented in this paper give substantial evidence that the catalase reaction of KatG involves compound I. First, the spectrum of the intermediate formed upon addition of peroxoacetic acid to the wild-type protein is reminiscent of what occurs with other peroxidases when PA is added (Fig. 2A). Second, the mutants W122F and W122A for the first time allowed us to monitor the direct reaction of a catalase-peroxidase with hydrogen peroxide, resulting in an enzyme intermediate with spectral features also similar to the classical plant peroxidase compound I (including APX) (Fig. 3A), which has been shown to contain two oxidizing equivalents (13) and forms a porphyrin $\pi$-cation radical in combination with an iron(IV) center (17, 27). Since the Trp mutants completely lost their catalase activity but retained their peroxidase activity, it is reasonable to assume that Trp-122 is the crucial residue in compound I reduction by H$_2$O$_2$ and W122A and W122F exhibited a similar reactivity toward H$_2$O$_2$, but extend significantly different side chain volumes into the cavity, one could speculate that the missing indole-ferryl interaction is likely to be responsible for the reduced reactivity of both Trp-122 mutants toward H$_2$O$_2$ and not changes in the distal cavity volume. From mutational analysis of CCP reactivity, it is known that mutations at distal Trp (Trp-51, CCP numbering) significantly affected the coordination and functional properties of the enzyme (31–34). Trp-51 in CCP forms part of the distal active-site cavity near the open coordination position of the heme iron. In CCP the tryptophan side chain is in van der Waals contact with the heme and forms
a hydrogen bond with one of the water molecules, which is displaced on binding H$_2$O$_2$ (12). Other peroxidases such as lignin peroxidase (35) and horseradish peroxidase (36) have a Phe in this position. Studies on the CCP variants W51F and W51A have shown that the bimolecular rate constants of compound I formation were relatively unaffected by these mutations. This seems to be a further discrepancy between CCP and catalase-peroxidases.

The distal histidine is highly conserved in peroxidases and has been considered to play a major role as a general acid-base catalyst for the peroxidase reaction cycle (12, 17, 18, 37). Poulos and Kraut (37) proposed that the distal His assists in the formation of the initial Fe-OOH complex by deprotonating an approaching H$_2$O$_2$ as a base and the following heterolytic cleavage of the O-O bond by protonating the departing distal oxygen as an acid. Recent studies with mutant peroxidases have been conducted to investigate the role of the distal His. The replacement of His-52 by Leu in CCP has profound effects on the rate of compound I formation, the apparent bimolecular rate constant being decreased by 5 orders of magnitude relative to the value for native enzyme (38). In horseradish peroxidase, the mutants in which the distal His was replaced by Ala, Val, and Leu also exhibited extremely low reactivity to H$_2$O$_2$ (39, 40).

Similar to the wild-type KatG, a direct monitoring of compound I formation with hydrogen peroxide was impossible in the Arg-119 and His-123 mutants because reduction of compound I apparently seemed to be much faster than its production ($k_{3(app)} > k_{1(app)}$). Thus, the significantly reduced overall catalase activities of these mutants unequivocally demonstrate that in catalase-peroxidases the distal His and Arg exhibit a similar role, which can be described by the Poulos-Kraut mechanism (37). Going from H$_2$O$_2$ to PA and CPB, the role of distal His and Arg became less pronounced in compound I formation.

Whether Trp plays a role in compound I formation is not clear. It is known from studies with horseradish peroxidase that small structural perturbations around the distal His can seriously decrease the peroxidase activity (41). One can speculate that the exchange of distal Trp caused similar structural perturbations of distal His and finally a decreased reactivity toward hydrogen peroxide. It is much easier to relate an important role in the two-electron reduction of compound I by hydrogen peroxide (Reaction 2) to distal Trp. The present work demonstrates that the indole ring of distal Trp is essential for the catalase activity of these bifunctional enzymes. Interestingly, in both CCP and APX this indole ring is also present but, nevertheless, these homologous enzymes exhibit no catalase activities. The actual structural differences between CCP and APX with catalase-peroxidase are unknown because at the moment the crystal structure of a KatG protein is not available.

Our experiments have also demonstrated that the mechanism of H$_2$O$_2$ degradation in bifunctional catalase-peroxidases and monofunctional catalases is different. Fig. 6 shows a schematic summary of our findings. Reaction of ferric KatG with hydrogen peroxide leads to a two-electron oxidation of the ferric enzyme to compound I (Reaction 1, a and b). Distal His and Arg seem to play a similar role as in other peroxidases. In monofunctional catalases, the equivalent function of Arg is taken over by Asn (42). Compound I is involved in both the catalase cycle and the peroxidase cycle. In the catalase cycle, a second H$_2$O$_2$ molecule is used as a reducing agent of compound I (Reaction 2 (a and b) in Fig. 6). In this two-electron reduction step of the enzyme, the indole ring of Trp is essential. Its actual role is not clear at the moment, but one could speculate that the indole nitrogen is involved in both the binding and finally oxidation of the second H$_2$O$_2$ molecule. In monofunctional catalases, again His and Asn are necessary to catalyze this step (42).

Based on our experiments with the Trp mutants in the absence of one-electron donors, we have included a third pathway for KatG compound I in the scheme of Fig. 6 (Reaction 5), which shows that compound I can also decay to an intermediate (designated X in the scheme). This intermediate was formed by long-time exposure of the Trp mutants with hydrogen peroxide or by addition of high concentrations of H$_2$O$_2$. It seems to be a stable end product but leads to irreversible enzyme inactivation and finally heme destruction.

In the peroxidatic cycle, compound I is reduced in two one-electron steps via compound II back to the ferric enzyme (Reactions 3 and 4). Recently, based on its spectral features, we have proposed that the single oxidizing equivalent in KatG compound II is contained on an amino acid that is not electronically coupled to the heme (7). We have also shown that ascorbate is a very poor substrate for cyanobacterial catalase-peroxidases (5–7, 11). In the system KatG/PA/ascorbate compound II accumulated (11). Interestingly, for the Trp-122 mutants, ascorbate seemed to be a better substrate underlying preliminary experiments, which indicated that the distal Trp is not involved in the one-electron reduction of compound I (i.e. in the peroxidase activity). This fitted well with our observation that the spectrum of the accumulating intermediate formed upon addition of ascorbate to W122F compound I was a mixture of compound I and a compound II (assuming similar spectral features of Trp-122 compound II and wild-type compound II) (Fig. 5C, spectrum 3). The actual ratio of [compound II]/[compound I] in Trp-122 during ascorbate oxidation suggested that $k_{3(app)} = 2 \times k_{4(app)}$, whereas in the wild-type KatG $k_{3(app)} > k_{4(app)}$.

Summing up, nature has evolved two heme enzymes that function primarily in hydrogen peroxide degradation: monofunctional catalases and catalase-peroxidases. Both types of enzyme use hydrogen peroxide as oxidant and reductant, thereby producing water and molecular oxygen. Our work has shown that in the two classes different active-site residues are involved; catalase-peroxidases use the distal His-Arg pair in the oxidation stage and Trp in the reduction stage, whereas monofunctional catalases utilize the distal His-Asn pair for both stages (42).

In further experiments we plan to perform a comprehensive EPR and resonance Raman study of the mutants as well as of the postulated KatG intermediates. We shall also look at the effect of the distal-site mutations on the kinetics of both compound I and compound II reduction by typical one-electron donors.

REFERENCES

1. Loewen, P. C. (1997) in Oxidative Stress and the Molecular Biology of Antioxidant Defenses (Scandolios, J. G., ed.) pp. 273–308, Cold Spring Harbor Press, Cold Spring Harbor, NY
2. Fraaije, M. W., Roubroeks, H. P., Hagen, W. R., and Van Berkel, W. J. H. (1996) Eur. J. Biochem. 235, 192–198
3. Levy, E., Eyal, Z., and Hochman, A. (1992) Arch. Biochem. Biophys. 296, 321–327
4. Weidner, K. G. (1992) Curr. Opin. Struct. Biol. 2, 388–393
5. Obinger, C., Regelsberger, G., Strasser, G., Burner, U., and Peschek, G. A. (1997) Biochem. Biophys. Res. Commun. 235, 545–552
6. Regelsberger, G., Obinger, C., Zeder, R., Altmann, F., and Peschek, G. A. (1999) FEBS Lett. 470, 1–12
7. Regelsberger, G., Jakopitsch, C., Engleder, M., Rüker, F., Peschek, G. A., and Obinger, C. (1999) Biochemistry 38, 10480–10488
8. Marcinkevičiene, J. A., Maglozer, R. S., and Blanchard, J. S. (1995) J. Biol. Chem. 270, 22280–22285
9. Nagy, J. M., Cass, A. E. G., and Brown, K. A. (1997) J. Biol. Chem. 272, 31265–31277
10. Johnson, K., Froland, W. A., and Schultz, P. G. (1997) J. Biol. Chem. 272, 2834–2840
11. Obinger, C., Regelsberger, G., Furtmüller, P. G., Jakopitsch, C., Rüker, F., Pircher, A., and Peschek, G. A. (1999) Free Radical Res. 31, S243–S249
12. Finzel, B. C., Poulos, T. L., and Kraut, J. (1984) J. Biol. Chem. 259, 13027–13030
13. Patterson, W. R., and Poulos, T. L. (1995) Biochemistry 34, 4331–4341
14. Jakopitsch, C., Rüker, F., Regelsberger, G., Dockal, M., Peschek, G. A., and Obinger, C. (1999) Biol. Chem. 380, 1097–1096
15. Schonbaum, G. R., and Lo (1972) J. Biol. Chem. 247, 3353–3360
16. Hager, L. P., Doubek, D. L., Silverstein, R. M., Harges, J. H., and Martin, J. C. (1972) J. Am. Chem. Soc. 94, 4364–4366
17. Patterson, W. R., Poulos, T. L., and Goodin, D. B. (1995) Biochemistry 34, 4342–4345
18. Erman, J. E., Vitello, L. B., Mauro, J. M., and Kraut, J. (1989) Biochemistry 28, 7992–7995
19. Scholes, C. P., Liu, Y., Fishel, L. A., Farnum, M. A., Mauro, J. M., and Kraut, J. (1989) Isr. J. Chem. 29, 85–92
20. Sivaraja, M., Goodin, D. B., Mauk, A. G., Smith, M., and Hoffman, B. A. (1989) Science 245, 738–740
21. Vitello, L. B., Erman, J. E., Miller, M. A., Wang, J., and Kraut, J. (1993) Biochemistry 32, 9807–9818
22. Roe, J. A., and Goodin, D. B. (1993) J. Biol. Chem. 268, 20037–20045
23. Kohli, R. M. (1998) BioTechniques 25, 184–188
24. Beers, R. F., and Sizer, I. W. (1952) J. Biol. Chem. 195, 133–140
25. Nelson, D. P., and Kiesow, L. A. (1972) Anal. Biochem. 49, 474–478
26. Mandelman, D., Jamal, J., and Poulos, T. L. (1998) Biochemistry 37, 17610–17617
27. Marquez, L. A., Quitarino, M., Zilinskas, B. A., and Dunford, H. B. (1996) FEBS Lett. 389, 155–156
28. Balny, C., Anni, H., and Yonetani, T. (1987) FEBS Lett. 221, 349–354
29. Loo, S., and Erman, J. E. (1975) Biochemistry 14, 3467–3470
30. Ohlsson, P. I., Yonetani, T., and Wold, S. (1986) Biochim. Biophys. Acta 874, 160–166
31. Goodin, D. B., Mauk, A. G., and Smith, M. (1987) J. Biol. Chem. 262, 7719–7724
32. Wang, J. M., Mauro, J. M., Edwards, S. L., Oatley, S., Fishel, L. A., Ashford, V. A., Xuong, N. H., and Kraut, J. (1990) Biochemistry 29, 7160–7173
33. Smulevich, G., Wang, Y., Mauro, J. M., Wang, J., Fishel, L. A., Kraut, J., and Spire, T. G. (1990) Biochemistry 29, 7174–7180
34. Goodin, D. B., Davidson, M. G., Roe, J. A., Mauk, A. G., and Smith, M. (1991) Biochemistry 30, 4953–4962
35. Edwards, S. L., Raag, R., Wariishi, H., Gold, M. H., and Poulos, T. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 759–754
36. Henriksen, A., Welinder, K. G., and Gajhede, M. (1998) J. Biol. Chem. 273, 2241–2248
37. Poulos, T. L., and Kraut, J. (1980) J. Biol. Chem. 255, 8199–8205
38. Erman, J. E., Vitello, L. B., Miller, M. M., Shaw, A., Brown, K. A., and Kraut, J. (1993) Biochemistry 32, 9798–9806
39. Newmyer, S. L., and Ortiz de Montellano, P. R. (1995) J. Biol. Chem. 270, 19430–19438
40. Rodriguez-Lopez, J. N., Smith, A. T., and Thorneley, R. N. F. (1996) J. Bioorg. Chem. 1, 136–142
41. Tanaka, M., Ishimori, K., Mukai, M., Kitagawa, T., and Morishima, I. (1997) Biochemistry 36, 9889–9898
42. Fita, I., and Rossmann, M. G. (1985) J. Mol. Biol. 185, 21–37