Influence of the unusual covalent adduct on the kinetics and formation of radical intermediates in *Synechocystis* catalase-peroxidase: a stopped-flow and EPR characterization of the M275, Y249 and R439 variants

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Key words: KatG; *Synechocystis* PCC 6803 catalase-peroxidase; catalase activity; peroxidase activity; cyanide binding; compound I; compound II; compound III; tyrosyl radical; tryptophanyl radical.

Abbreviations: KatG, catalase-peroxidase; CcP, cytochrome c peroxidase; APX, ascorbate peroxidase; CD, circular dichroism; EPR, Electron Paramagnetic Resonance; HF EPR, high-field Electron Paramagnetic Resonance; *B. pseudomallei*, *Burkholderia pseudomallei*; *H. marismortui*, *Haloarcula marismortui*; *M. tuberculosis*, *Mycobacterium tuberculosis*. 
Abstract. Catalase-peroxidases (KatGs) are heme peroxidases with a catalatic activity comparable to monofunctional catalases. They contain an unusual covalent distal side adduct among the side-chains of W122, Y249 and M275 (Synechocysis KatG numbering). The known crystal structures suggest that Y249 and M275 could be in hydrogen-bonding distances to R439. In order to investigate the role of this peculiar adduct, the variants Y249F, M275I, R439A and R439N were investigated by electronic absorption steady-state and transient-state kinetic techniques and EPR spectroscopy combined with deuterium labeling. Exchange of these conserved residues exhibited dramatic consequences on the bifunctional activity of this peroxidase. The turnover number of catalase activity of M275I, Y249F, R439A and R439N are 0.6%, 0.17%, 4.9% and 3.14% of wild-type activity, respectively. By contrast, the peroxidase activity was unaffected or even enhanced, in particular for the M275I variant. As shown by the mass spectrometry and the EPR spectra the KatG-typical adduct is intact in both R439 variants as in the case of the wild-type enzyme, while in the M275I variant the covalent link exists only between Y249 and W122. In the Y249F variant the link is absent. The EPR studies showed that the radical species formed upon reaction of the Y249F and R439A/N variants with peroxoacetic acid are the oxoferryl-porphyrin radical, the tryptophanyl and the tyrosyl radicals, as in the wild-type enzyme. The dramatic loss in catalase activity of the Y249F variant allowed the comparison of the radical species formed with hydrogen peroxide and peroxoacetic acid. The EPR data strongly suggest that the sequence of intermediates formed in the absence of a one electron-donor substrate, is $\text{por}^{++}$ $\rightarrow$ Trp$^\bullet$ (or Trp$^{++}$) $\rightarrow$ Tyr$^\bullet$. The M275I variant did not form the Trp$^\bullet$ species due to the dramatic changes on the heme distal side, most probably induced by the repositioning of the remaining W122-Y249 adduct. The results are discussed with respect to the bifunctional activity of catalase-peroxidases.
INTRODUCTION

Catalase-peroxidases (KatGs) are present in bacteria and fungi and function primarily as hydrogen peroxide scavengers (1-3). According to sequence similarities they have been shown to be members of the class I of the superfamily of plant, fungal and bacterial peroxidases (4). In contrast to the other members of this group, as cytochrome c peroxidase (CcP) and ascorbate peroxidase (APX), they show a high catalase activity comparable with monofunctional catalases. Although the catalase activity is overwhelming, KatGs also show a substantial peroxidase activity with various one electron donors.

There are three crystal structures of KatGs available, namely of the archaeabacterium Haloarcula marismortui (5), of Burkholderia pseudomallei (6) and of Synechococcus PCC 7942 (PDB entry 1UB2), the latter being a cyanobacterial KatG with high homology to Synechocystis KatG. The structures show that the arrangement of the active site is similar to CcP and APX, containing the distal triad arginine, histidine, tryptophan and the proximal triad aspartate, tryptophan and histidine. The coordinates of these amino acids are virtually identical in all three enzymes.

An unprecedented covalent bond between the distal side tryptophan, tyrosine and methionine residues (in Synechocystis numbering W122, Y249 and M275) was proposed to be present in the H. marismortui enzyme (5). A mass spectrometric analysis of the tryptic peptides from recombinant wild-type KatG and the tryptophan, tyrosine and methionine variants of Synechocystis (7) and Burkholderia pseudomallei (8) confirmed that this novel covalent adduct really exists in solution and thus may be a common feature to all catalase-peroxidases. In Synechocystis KatG exchange of either W122 or Y249 prevents cross-linking, whereas exchange of M275 still allowed the covalent bond formation between W122 and Y249 (7).
The integrity of the covalent adduct appears to be important for the catalase but not the peroxidase activity of catalase-peroxidases. Upon exchange of the distal Trp (W122 in *Synechocystis*) the catalase activity was dramatically diminished in the *E. coli* (9) or even lost in the *Synechocystis* (10) enzymes, whereas the peroxidase activity was not affected in both cases. Moreover, upon exchange of the crosslinked Tyr residue (Y249 in *Synechocystis*) the bifunctional enzyme was totally converted to a monofunctional peroxidase (11). The Y249F variant shows spectral and kinetic features characteristic of most plant-type peroxidase intermediates, namely compound I and II (11, 12), thus different to wild-type KatG (10, 11). Additionally, the *B. pseudomallei* variant M264L (M275 in *Synechocystis*) showed a significantly reduced overall catalase but an intact peroxidase activity (8).

Interestingly, the Trp-Tyr-Met adduct appears to be hydrogen bonded to a neighbouring arginine residue (Arg439 in *Synechocystis*). In the *H. marismortui* and *Synechococcus* PCC 7942 KatG crystallographic structures, the corresponding arginine residue (Arg409) is in hydrogen-bonding distances to both Y218 and M244 (Figure 1A). Specifically, the NH1 and the NH2 groups of R409 form hydrogen bonds with the phenol oxygen of Y218 and the backbone oxygen of M244, respectively (Figure 1A). However, in the crystal structure of *B. pseudomallei* KatG the corresponding arginine (R426) points away from the covalent adduct in its predominant orientation (6) and no hydrogen bonds can be formed (Figure 1B). Only in its minor conformation (30%) the side chains of R426 are in a favorable position to form hydrogen bonds as in the case of *H. marismortui* (6).

The structural requirements that enable a peroxidase to disproportionate hydrogen peroxide are not really understood. The presence of the covalent adduct in KatGs as well as the arginine with the two possible orientations suggest that the region may have a, yet undefined, function in KatG catalysis. In order to investigate the role of M275 and R439 in the bifunctional activity of KatG and in the formation of the covalent adduct, the variants
M275I, R439A and R439N have been prepared and characterized by both steady-state and transient-state kinetic UV-Vis spectroscopy and mass spectrometry. In comparison to wild-type KatG, these variants exhibit dramatic differences in both the catalase and the peroxidase activities, as well as the spectral features and kinetics of interconversion between their redox intermediates. The radical intermediates formed by the M275I, R439A and R439N variants were also investigated by using EPR spectroscopy and compared to those formed by the Y249F variant. Only the M275I variant indirectly induced a significant change in the heme microenvironment so that the tryptophanyl radical intermediate was not formed. The same three radical intermediates previously identified in the wild-type enzyme (exchange-coupled oxoferryl-porphyrin, tryptophanyl and tyrosyl radicals) (13) were observed for the other variants. The possibility of using hydrogen peroxide for the radical formation allowed us to propose the sequence of radical formation.

EXPERIMENTAL PROCEDURES

Materials - Standard chemicals and biochemicals were obtained from Sigma Chemical Co. at the highest grade available. Cloning, expression and purification of wild-type KatG and the M275I and the Y249F variants from *Synechocystis* were described previously (11, 7, 14). Isotope labelling and purification of specifically deuterated KatGs was described recently by Ivancich et al. (13).

Mutagenesis of R439 - Oligonucleotide site-directed mutagenesis was performed using PCR-mediated introduction of silent mutations as described (10). A pET-3a expression vector that contained the cloned catalase-peroxidase gene from the cyanobacterium *Synechocystis* PCC 6803 (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'-GGC ACC CGG ATC CTT TAT G -3' containing a BamHI restriction site and 5'-AGT GCA GAC TAG TTC ATC CTT TAT G -3' containing a BamHI restriction site and 5'-AGT GCA GAC TAG TTC
GGA AAC G -3’ containing a SpeI restriction site. The internal 3’-primer was 5’- TGT GAG TAA GCT TAA ACC AGG CC -3’ and possessed a HindIII restriction site. The following mutant primers with the desired mutation and a silent mutation introducing a restriction site were constructed (point mutations italicized and restriction sites underlined): 5’- CTG GTT TAA GCT TAC CA4 CGA TCT AGG AC -3’ changed R439 to N; 5’- GTT TAA GCT TAC TCA CGC GGA TCT AGG AC -3’ changed R439 to A. The fragment defined by the BamHI and SpeI restriction sites was replaced by the new construct containing the point mutation. All constructs were sequenced to verify DNA changes using thermal cycle sequencing.

Spectroscopic studies. Optical spectra were recorded on a diode array spectrophotometer (Zeiss Specord S10) and a Hitachi U-3000 spectrophotometer equipped with a thermostatted cell holder. Circular dichroism studies were carried out using a JASCO J-600 spectropolarimeter. Far-UV (190 – 260 nm) experiments were carried out using protein concentrations of 1.5 µM and the path length of the cuvette was 1 mm. A good signal-to-noise ratio in the CD spectra was obtained by averaging twelve scans (resolution: 1 nm; bandwidth: 1 nm, response 16 s; scan speed 20 nm/min). The protein concentration was calculated from the known amino acid composition and absorption at 280 nm according to Gill and Hippel (15).

Steady-state kinetics. Catalase activity was determined polarographically in 50 mM phosphate buffer using a Clark-type electrode (YSI 5331 Oxygen Probe) inserted into a stirred water bath (YSI 5301B) at 30°C. Alternatively, the catalase activity was measured by continuously monitoring hydrogen peroxide concentration polarographically with a platinum electrode covered with a hydrophilic membrane and fitted to the Amperometric Biosensor Detector 3001 (Universal Sensors, Inc., U.S.A.). The applied electrode potential at pH 7 was 650 mV and the H2O2 electrode filling solution was prepared freshly half-daily. The electrode
was calibrated against known concentrations of hydrogen peroxide. All reactions were performed at 30°C and started by the addition of KatG. One unit of catalase is defined as the amount that decomposes 1 µmol of H₂O₂/min at pH 7 and 30°C. To cover the pH range 4-9 50 mM citrate-phosphate or 50 mM Tris/HCl buffers were used. Since the potential of hydrogen peroxide increases with decreasing pH the applied electrode potential had to be changed from 890 mV (pH 4) to 530 mV (pH 9).

Peroxidase activity was monitored spectrophotometrically using 1 mM H₂O₂ and 5 mM guaiacol (ε₄₇₀ = 26.6 mM⁻¹ cm⁻¹) or 1 mM o-dianisidine (ε₄₆₀ = 11.3 mM⁻¹ cm⁻¹) or 500 µM ascorbate (ε₂₉₀ = 2.8 mM⁻¹ cm⁻¹). Additionally, peroxidase activity was measured with peroxoacetic acid (1 mM) instead of H₂O₂. One unit of peroxidase is defined as the amount that decomposes 1 µmol of electron donor/min at pH 7 and 30°C.

**Transient-state kinetics.** Transient-state measurements were made using the model SX-18MV stopped-flow spectrophotometer from Applied Photophysics equipped with a 1-cm observation cell thermostated at 15°C. Calculation of pseudo-first-order rate constants (kₜₜ) from experimental traces at the Soret maximum was performed with the SpectraKinetic workstation v4.38 interfaced to the instrument. The substrate concentrations were at least five times that of the enzyme to allow determination of pseudo-first-order rate constants. Second-order rate constants were calculated from the slope of the linear plot of pseudo-first-order rate constants versus substrate concentration. To follow spectral transitions a photodiode array accessory (model PD.1 from Applied Photophysics) connected to the stopped-flow machine together with the XScan Diode Array Scanning v1.07 software was utilized. The kinetics of oxidation of ferric KatG variants to compound I by peroxoacetic acid or hydrogen peroxide or the formation of the cyanide complex were followed in the single mixing mode. Ferric KatG and either peroxide or cyanide were mixed to give a final concentration of 1 µM.
enzyme and 5-250 μM peroxide or 20-500 μM cyanide. The first data point was recorded 1.5 ms after mixing and 2000 data points were accumulated.

Sequential-mixing stopped-flow analysis was used to measure compound I reduction by one-electron donors. In the first step the enzyme was mixed with peroxyacetic acid or H₂O₂ and, after a delay time where compound I was built, the intermediate was mixed with the electron donors aniline, ascorbate or o-dianisidine. All stopped-flow determinations were measured in 50 mM phosphate buffer, pH 7.0 and 15°C, and at least three determinations were performed per substrate concentration. The temperature (15°C) was used in order to allow comparison of the transient-state kinetic data with those obtained with other variants of *Synechocystis* KatG.

**Mass spectrometry** - Molecular masses were determined on a LC/ESI-MS apparatus (Waters Micromass Q-TOF Ultima Global) as previously reported (7). The proteolytic digest patterns were compared with virtual digests performed by the PeptideMass software, which is available via the ExPASy World Wide Web server, at the URL address [http://www.expasy.ch/www/tools.html](http://www.expasy.ch/www/tools.html).

**EPR spectroscopy** - Conventional 9-GHz EPR measurements were performed using a Bruker ER 300 spectrometer with a standard TE₁₀₂ cavity equipped with a liquid helium cryostat (Oxford Instrument) and a microwave frequency counter (Hewlett Packard 5350B). Typically, EPR samples were prepared by mixing manually 0.5-1.0 mM native enzyme (100 mM TRIS/maleate buffer, pH 8.0, pH 7.4 or pH 5.6) with an excess of peroxyacetic acid (10 fold) or hydrogen peroxide (5 and 10 fold), directly in the 4 mm-EPR tubes kept at 0°C. The reaction was stopped by rapid immersion of the EPR tube in liquid nitrogen after 5 s. The home-built high-field EPR spectrometer (95-285 GHz) has been described elsewhere (16).
RESULTS

Electronic absorption and circular dichroism spectra – The UV-Vis absorption spectra of recombinant wild-type KatG and the three variants exhibited the typical bands of a heme b-containing peroxidase in the visible and near ultraviolet region. The Soret peak was at 407 nm (in R439A at 408 nm) together with two bands around 510 and 638 nm (CT1) suggesting the presence of a dominating five-coordinate high-spin heme coexisting with a low portion of six-coordinate high-spin heme (17, 18). The variant M275I has a small shoulder at about 368 nm which could indicate the presence of some free heme. This fits well with the so-called purity number (Reinheitszahl, i.e. the ratio $A_{406}/A_{280}$), which was 0.41-0.48 for the M245I variant, whereas 0.56-0.64 for wild-type KatG, 0.52-0.57 for R439A and 0.46-0.52 for R439N, respectively. The yield of expression varied in the range of 30 mg to 60 mg per liter E. coli cell culture. The spectral features of Y249F have been described recently (11, 18).

The CD spectra of the recombinant enzymes were typical for proteins composed predominantly of $\alpha$-helices (not shown). Very little difference was observed between the CD spectra of wild-type and the three variants indicating that there was no large scale conformational change in the structure. If conformational changes did occur, they must be very localized and minimal and thus went undetected by CD.

Mass spectrometry – Recently it has been demonstrated by Jakopitsch et al. (7) that exchange of either W122 or Y249 in Synechocystis KatG prevents formation of the KatG-specific distal side covalent adduct, whereas exchange of M275 still allowed bond formation between W122 and Y249. In this work, we have investigated the impact of exchange of R439 on the formation of the adduct. If the covalent link between W122, Y249 and M275 exists in solution, a peptide of [MH]$^+$ with the monoisotopic mass of 7096.34 Da should have been detectable after tryptic digestion and LC/ESI-MS analysis. Figure 2 shows the isotopic
pattern of the [M5H]5+ species (1420.07 Da), found for both wild-type KatG and the R439N variant, both not for M275I. This clearly rules out that R439 takes part in the formation and/or integrity of the covalent bonds. Structural information obtained by EPR spectroscopy on the native R439 variants confirm these results (see below).

Catalase and peroxidase activity – Wild-type KatG shows an overwhelming catalase activity ($k_{\text{cat}}$ of 3500 s$^{-1}$). Upon exchange of Y249 by phenylalanine the enzyme was totally converted from a bifunctional peroxidase to a monofunctional peroxidase (11). Exchange of both M275 and R439 had also a dramatic influence on the catalatic activity (Table 1). The $k_{\text{cat}}$ value of the M275I was only 0.6 % of wild-type and the arginine variants retained only 3 – 5 % of wild-type activity. There was no difference in the pH profile of catalase activity between wild-type and mutant proteins (maximum at pH 6.5).

The peroxidatic activity of R439A, R439N and Y249F (11) variants towards the aromatic donors o-dianisidine and guaiacol was similar to that of wild-type KatG (Table 1) as was the maximum of peroxidase activity (pH 5.5). By contrast, M275I showed a substantial increase in peroxidase activity. Compared to wild-type KatG, the peroxidase activity for the M275I variant was 8 times higher with o-dianisidine and even about 30 times higher with guaiacol (Table 1). Interestingly, with both aromatic electron donors the pH maximum shifted to pH 7.0.

Since in wild-type KatG an overwhelming catalase activity strongly favors $\text{H}_2\text{O}_2$ disproportionation over the oxidation of one-electron donors, using peroxoacetic acid instead of hydrogen peroxide allowed us to directly compare the peroxidase activity of wild-type KatG and the variants. The peroxidase activity of the variants was similar to that of wild-type KatG when peroxoacetic acid was used to initiate the peroxidase reaction as shown in Table 1.
The typical substrate for ascorbate peroxidase (APX), namely ascorbate, is a very poor electron donor for catalase-peroxidases and therefore it is not possible to detect ascorbate oxidation mediated by native KatG (11, 14). However, M275I and both R439 variants, exhibited a reasonable ascorbate-peroxidase activity, which dramatically increased upon decreasing pH (not shown). By contrast, ascorbate peroxidase activity of Y249F was negligible.

**Cyanide binding** – Cyanide is often used to probe the accessibility to the distal heme cavity and thereby simulating the H₂O₂-mediated formation of compound I. Upon cyanide binding to heme proteins, a conversion of the ferric iron from high-spin to low-spin state is observed, as in the case of wild-type KatG and the variants, that was also accompanied by a shift of the Soret band to 422 nm and the appearance of a new prominent peak around 540 nm (Figure 3). The cyanide complex of wild-type KatG and the arginine variants exhibited a small hypochromicity at the Soret absorbance (Figure 3B), whereas in case of the M275I variant a small hyperchromicity was observed (Figure 3A). Under pseudo-first order conditions, the observed rate constant of cyanide binding to the ferric proteins linearly increased with the concentration of cyanide (Figures 3C and 3D). From the slope of the plots the apparent second-order rate constant \(k_{on}\) for cyanide binding were calculated to be \((3.2 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}\) for M275I, \((7.6 \pm 0.3) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}\) for R439A and \((6.2 \pm 0.2) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}\) for R439N, respectively, very similar to native KatG (14) (Table 2). The finite intercept of these plots, 1.4 s\(^{-1}\) for M275I, 1.9 s\(^{-1}\) for R439A and 3.1 s\(^{-1}\) for R439N represent \(k_{off}\). From the \(k_{off}/k_{on}\) ratios the dissociation constants were calculated to be 4.4 µM for M275I, 2.5 µM for R439A and 5.1 µM for R439N were calculated. The dissociation constants of these variants decreased substantially as compared to the wild-type enzyme (15.8 µM).
**Compound I formation.** The absorption spectrum of compound I formed by the treatment of catalase-peroxidases with hydrogen peroxide cannot be readily observed because of the overwhelming catalase activity of these enzymes. In contrast, by using organic peroxides such as peroxoacetic acid, it is possible to observe the 40-50% hypochromicity of the Soret peak at 407 nm, characteristic for the absorption spectrum of the oxoferryl-porphyrin radical species (conventional compound I). However, and similar to previous work with variants of Y249 (11) or W122 (10), exchange of either M275 or R439 allowed us to monitor the enzyme oxidation at 407 nm also by using hydrogen peroxide. This fits well with the decreased overall catalatic activity of these variants. In the R439N and R439A variants the reaction of the ferric enzyme with small excess of hydrogen peroxide or peroxoacetic acid was monophasic. Compound I formed by peroxoacetic acid was stable, whereas compound I formed with hydrogen peroxide decayed slowly back to the ferric enzyme after depletion of hydrogen peroxide. During this turnover the Soret band remained at 407 nm (Figure 4A). The inset to Figure 4A depicts a typical time trace at 407 nm of the reaction between 5 µM R439A and 10 µM H₂O₂. Compound I was stable for about 200 ms and finally slowly decayed within 4 seconds to the ferric state. The higher excess of hydrogen peroxide was added the longer the compound I spectrum predominated, indicating that in R439A compound I reduction was rate-limiting in enzyme turnover. The R439N variant exhibited the same behaviour (data not shown). The plot of the pseudo-first-order rate constants versus hydrogen peroxide concentrations (5 – 20 µM) was linear (Figure 4B) and the $k_{app}$ was calculated to be (2.3 ± 0.4) × 10⁶ M⁻¹ s⁻¹ for the R439A and (1.5 ± 0.2) × 10⁶ M⁻¹ s⁻¹ for R439N at pH 7.0. The rate constants for compound I formation with peroxoacetic acid were (7.1 ± 0.2) × 10⁵ M⁻¹ s⁻¹ (R439A) and (4.4 ± 0.3) × 10⁵ M⁻¹ s⁻¹ (R439N) at pH 7.0, which is at least 10-fold higher than the reaction of the wild-type enzyme (Table 2).
The most interesting finding was the formation of a new intermediate prior to formation of (conventional) compound I when a higher excess (starting at 20 fold excess) of either hydrogen peroxide or peroxoacetic acid was used in both arginine variants (Figure 4C). After mixing of 5 µM R439A with 100 µM hydrogen peroxide the first spectrum which could be recorded with the stopped-flow apparatus (1.3 ms) showed absorption bands at 414 nm, 542 nm and 575 nm (bold line in Figure 4C). The very rapid formation of this new intermediate excluded rate determination with stopped-flow experiments. The intermediate was formed independent of the type of peroxide used and was transformed to the typical compound I species within 50 ms (Figure 4D). There was a clear monophasic transition from this fast appearing species to compound I with isosbestic points at 395 nm and 520 nm. Compound I built with hydrogen peroxide dominated for seconds (depending on the H₂O₂ concentration) and finally, decayed to the ferric enzyme with clear isosbestic points at 360 nm, 430 nm and 526 nm (Figure 4E). With peroxoacetic acid the latter reaction was not observed and compound I was stable.

Figure 5 shows the spectral changes when 5 µM M275I were mixed with 250 µM hydrogen peroxide. Compound I formation was finished after 200 ms (bold line) but it was not stable. It decayed to an intermediate with absorption bands at 414 nm and 542 nm and 575 nm (grey line), very similar to (i) the spectral features of the first intermediate observed upon mixing of the R439 variants with excess H₂O₂ (see above) or (ii) to the recently observed intermediate which formed upon mixing the Y249F variant with excess (more than 10 fold) hydrogen peroxide (11). It was not possible to monitor a pure oxoferryl-type compound II spectrum of the M275I variant, not even by using a low excess of hydrogen peroxide. This behaviour differs substantially from our findings on the Y249F variant (11), which showed a clear transition of conventional compound I via an oxoferryl-type compound II spectrum to a redox intermediate with absorption peaks at 414 nm, 542 nm and 576 nm.
when ferric $Y_{249F}$ was incubated with a 10-fold (or higher) excess of hydrogen peroxide. The reaction of 5 µM $M_{275I}$ with 250 µM peroxoacetic acid is shown in Figure 5B. A conventional compound I was observed within 200 ms and remained relatively stable. The spectrum of the intermediate monitored after 10 s (Figure 5B, grey spectrum) was slightly red-shifted compared to conventional compound I. The plots of the pseudo-first-order rate constants of formation of conventional compound I versus different concentrations of hydrogen peroxide (Figure 5C) and peroxoacetic acid (Figure 5D) were linear and the calculated rate constants for compound I formation were $(1.2 \pm 0.1) \times 10^5$ M$^{-1}$ s$^{-1}$ and $(3.1 \pm 0.1) \times 10^5$ M$^{-1}$ s$^{-1}$, respectively (Table 2). Thus, the peroxoacetic acid mediated reaction was similar with both R439 and M275 variants, whereas the hydrogen peroxide mediated reaction was about ten times slower with M275I. Additionally, the hypochromicity of the conventional compound I spectrum of M275I was lower (compare Figures 4C and 5A). For comparison, the corresponding rate constants for W122F and Y249F were $8.2 \times 10^4$ M$^{-1}$s$^{-1}$ (10) and $1.1 \times 10^7$ M$^{-1}$ s$^{-1}$ (11) with hydrogen peroxide and $1.8 \times 10^5$ M$^{-1}$s$^{-1}$ (10) and $8.0 \times 10^6$ M$^{-1}$ s$^{-1}$ (11) with peroxoacetic acid, respectively. In the M275I variant compound I formation mediated by peroxoacetic acid is faster than in wild-type KatG, comparable to the W122F (10) variant but slower than in Y249F (11).

Compound I reduction - A typical plant peroxidase type compound II (peaks at 418 nm, 535 nm and 560 nm) was observed when one electron donors were added to a preformed compound I in the Y249 variants (11). By contrast, both R439 variants exhibit a similar behaviour like wild-type KatG. Compound I reduction with ascorbate was measured at 407 nm and showed a biphasic behaviour with a fast initial phase followed by a slower second phase. Figure 6 shows the spectral changes after adding 2 mM ascorbate to a preformed
compound I of R439N (2 µM). Compound I is bold, the spectrum of the intermediate at the end of the fast initial phase (150 ms) is a thin line, and the spectrum of the final intermediate at the end of the slow phase (1.5 sec) is in grey. The inset of Figure 6A shows the corresponding time trace at 407 nm indicating the times at which spectra were taken. Similar to wild-type KatG the Soret band remained at 407 nm in the time course of these transitions. Table 2 summarizes the rate constants determined for compound I reduction with different one electron donors measured at 15°C. The rates were obtained by fitting the first exponential phase of the reaction (Figure 6B) and plotting the pseudo-first order rate constants versus substrate concentration (Figure 6C). In a typical experiment 4 µM of ferric enzyme was mixed with either 200 µM hydrogen peroxide or peroxoacetic acid and after a delay time of 200 – 400 ms one electron donors were added. The obtained apparent bimolecular rate constants were independent of the nature of the peroxide used in compound I formation (Table 2). The hierarchy in reactivity towards the three investigated electron donors (ascorbate < aniline << o-dianisidine) was not changed compared to wild-type KatG, but the apparent bimolecular rate constants were significantly increased (Table 2).

When M275I compound I (formed by mixing 5 µM M275I with 50 µM hydrogen peroxide; bold spectrum in Figure 7A) was allowed to react with 500 µM ascorbate, a redox intermediate was formed within 1 s with absorbance maxima at 414 nm, 542 nm and 575 nm (Figure 7A). This transient intermediate (bold spectrum in Figure 8B) was reduced back to the ferric enzyme. Both transitions exhibited clear isosbestic points at 400 nm (compound I reduction; Figure 7A) and 415 nm (formation of ferric M275I; Figure 7B). The spectral features of the observed intermediate are very similar to those obtained when compound I of M275I formed by hydrogen peroxide decayed in the absence of an one-electron donor (Figure 7E). However, this transition was slow compared to compound I reduction mediated by ascorbate. Figure 7C depicts the time traces at 407 nm obtained after reaction of
compound I (preformed upon reaction of 5 µM M275I with 50 µM hydrogen peroxide), with pure buffer and different concentrations of ascorbate. The time traces were biphasic and both phases depended on the amount of added ascorbate. The more ascorbate was added the faster were both phases. In the absence of ascorbate the second phase of reaction did not occur (bold time trace in Figure 7C). Fitting the first exponential phase and plotting the pseudo-first order rate constants versus ascorbate concentrations yielded apparent bimolecular rate constants of $2.2 \times 10^4$ M$^{-1}$ s$^{-1}$ (compound I preformed with hydrogen peroxide, Figure 7D) and $1.6 \times 10^4$ M$^{-1}$ s$^{-1}$ (compound I preformed with peroxoacetic acid, not shown), respectively. From the second phase (formation of ferric M275I) rate constants could be estimated to be in the in the range of $10^1$ to $10^2$ M$^{-1}$ s$^{-1}$. Regarding aniline as electron donor for M275I compound I, the determined rate constant was similar to wild-type KatG, but the o-dianisidine mediated reaction was too fast to be measured in the stopped-flow apparatus.

As mentioned above, compound I formed upon oxidation of M275I by H$_2$O$_2$, was not stable and decayed to an intermediate with absorption maxima at 414 nm, 542 nm and 575 nm (Figure 7E) very similar to that obtained by addition of ascorbate to M275I compound I. In the absence of ascorbate, the intermediate with a maximum of the Soret band at 414 nm was stable for more than 10 seconds, but it was transformed to ferric M275I when adding ascorbate (Figure 7F). The kinetics of this transition was very similar to that described in Figure 7C, underlining that it is a redox-active intermediate and not compound III.

Characterization of the Y249F, M275I, R439A and R439N variants of KatG by Electron Paramagnetic Resonance spectroscopy: the native (ferric) enzyme - We have previously reported the low temperature (4K) 9-GHz EPR signal of the wild-type *Synechocystis* catalase-peroxidase in the native state (13). The two main resonances observed at $g^\text{eff} \perp \approx 6$ and $g^\text{eff} \parallel \approx 2$ are characteristic of pentacoordinated heme iron enzymes ($S = 5/2$) in the high-spin state. The
EPR spectrum of the wild-type enzyme (Figure 8, top) was assigned to two predominant Fe(III) high-spin species, represented by the superposition of an axial signal (effective g-values of \(g_\perp = 5.93\) and \(g_\parallel = 1.99\)) and a rhombically distorted (\(g_x = 6.57, g_y = 5.10\) and \(g_z = 1.97\)) signals (13). The g-values directly measured from the EPR spectrum of the native enzyme are very sensitive to structural changes in the heme environment. Accordingly, we have previously used the high sensitivity of the native (ferric) enzyme EPR spectrum to monitor small changes in the geometry of the heme site, induced by the different mutations and/or pH variations (13). Specifically, the relative contribution of the two Fe(III) high-spin signals showed a marked dependence on the pH values when varying the pH between 5.6 and 8.3 (Figure 8, top). In the case of the mutations on the catalytically-essential tryptophan and arginine residues of the heme distal site (W122F and R119A) a predominantly axial EPR signal with no pH dependence was observed for the ferric enzymes. This effect was correlated to the structural changes originated by the disruption of the extensive H-bonding network on the heme distal side (13).

Figure 8 shows the EPR spectra of the Y249F, M275I, R439A and R439N variants all in the native state and at two different pH values (5.6 and 8.3). The EPR spectra of all these variants were similar to that of wild-type enzyme at basic pH, i.e. dominated by two main EPR signals with effective g-values of \(g_\perp = 5.93\) and \(g_\parallel = 1.99\) for the axial signal and \(g_x = 6.57, g_y = 5.10\) and \(g_z = 1.97\) for the rhombically distorted signal. The arginine variants showed not only the same ferric spectrum as the wild-type enzyme, but also the same pH dependence of the EPR signals. In contrast, a significant difference was observed for the Y249F and M275I variants: a) no pH dependence for the Y249F variant (see Figure 8, middle); b) the inversion of the pH dependence on the M275I ferric signals as compared to the wild-type enzyme (see Figure 8, top). Similar distinct differences were previously reported for the proximal tryptophan (W341F) and the distal histidine (H123Q) variants,
respectively (13). The M275I and R439A/N variants also showed a higher proportion of at
least one type of low-spin ferric species, as it was previously observed in other distal side
mutations [see Figure 1 in Ivancich et al. (13)].

The EPR characterization of the radical intermediates. EPR spectroscopy was further
used to precisely characterize the electronic structure of the radical intermediates formed by
the Y249, M275 and R439 variants of Synechocystis catalase-peroxidase. We have previously
shown that in the wild-type enzyme three radical intermediates, with distinct EPR spectra,
were formed upon reaction with peroxoacetic acid (13). Specifically, an oxoferryl-porphyrin
radical \([\text{Fe(IV)=O por}^\bullet]\), typical for monofunctional catalases and peroxidases, was partially
trapped in the time course of 10 s (the mixing being performed at 0°C), as well as a tyrosyl
(\(\text{Tyr}^\bullet\)) and a tryptophanyl (\(\text{Trp}^\bullet\)) radical species. The 9-GHz EPR spectrum of the M275I,
R439A and R439N variants after reaction with peroxoacetic acid, showed the broad axial
signal (total width of ca. 2000 G) characteristic of the exchange-coupled porphyrin \(\pi\)-radical
with effective g-values of \(g_\perp = 2.35\) and \(g_\parallel = 2.00\) (data not shown). Such a broad EPR signal
was previously observed for the Y249F variant (11) and the native enzyme (13). It is of note
that the exchange-coupled oxoferryl-porphyrin radical species is the so-called (conventional)
compound I intermediate in catalases and peroxidases, which exhibits a characteristic
electronic absorption spectrum (e.g. see Figures 4 and 5).

A narrow signal (less than 100 G full width) centered at \(g \approx 2\), indicative of an isolated
protein-based radical, was also detected in the 9 GHz-EPR spectra of the Y249F, M275I,
R439A, and R439N variants. Figure 9 (solid traces) shows the comparison of these radical
species together with that of the wild-type enzyme. The EPR signals of the radical species
formed after treatment of the Y249F and R439A/N variants with a 10-fold excess of
peroxoacetic acid (Figure 9, middle) were identical to that observed for the wild-type enzyme
We have previously demonstrated that when two protein-based radicals contribute to the 9 GHz EPR spectrum as in the case of the *Synechocystis* catalase-peroxidase, it is necessary to use deuterium labeling experiments combined with multifrequency EPR spectroscopy to unequivocally identify the chemical nature of the radical(s) formed (13). The rationale of these experiments was previously described in an extensive manner (13) and will not be repeated here. The key aspect is that the 9 GHz EPR spectrum of a protein-based radical is dominated by the protons hyperfine couplings (given by the interactions of the protons with the electron spin of the radical), thus, a measurable change in the 9 GHz EPR spectrum of the Tyr• (or Trp•) occurs when the enzyme contains fully-deuterated Tyr (or Trp) residues instead of the normal (protonated) amino acids [see Figure 4 in Ivancich et al. (13)].

Figure 10 (top) shows the deuterium labeling experiments on the Y249F variant. The EPR spectrum of the protein-based radical formed upon treatment of the Y249F variant with peroxyacetic acid was identical to the wild-type spectrum obtained under the same conditions. The spectral changes on the EPR signal upon perdeuteration of either tyrosine or tryptophan residues in the Y249F variant were also the same as those observed in the native enzyme [see Figure 4 in (13)]. In addition, the high-field (285 GHz) EPR spectrum of the Y249F sample confirmed the presence of both the tyrosyl and the tryptophanyl radicals (data not shown) as in the case of wild-type enzyme (13).

A different situation was observed for the M275I variant upon treatment with peroxyacetic acid. The 9 GHz EPR spectra of this variant (Figure 9, bottom) was clearly narrower than that of the wild-type enzyme, obtained in the same conditions. In addition, the g-values accurately measured from the high-field EPR spectrum of the M275I variant well agreed with a Tyr•-only spectrum. Taken together, these facts constituted strong evidence for the formation of only the Tyr• species in the M275I variant. The same narrower radical
spectrum as well as the g-values (resolved from the HF EPR spectrum) in agreement with a Tyr•-only species, were observed for all three distal-side variants, i.e. W122F, H123Q and R119A (13).

The dramatic reduction, or almost complete lost, of the catalase activity in the Y249F, M275I and R439A/N variants (Table 1) allowed us to compare the EPR protein-based radical signals obtained by the reaction of these enzymes with hydrogen peroxide and peroxoacetic acid. For all three variants, the EPR spectra of the samples treated with hydrogen peroxide (Figure 9, dotted trace) were narrower than those obtained by using peroxoacetic acid (Figure 9, solid traces). Deuterium labeling experiments were necessary for discriminating the type of radical detected, Tyr• or Trp•, as a function of the substrate type and concentration used for each variant. Figure 10 shows the spectra obtained upon treatment of the Y249F sample with 5-fold excess peroxoacetic acid (top) as well as with 5-fold (middle) and 15-fold (bottom) excess of hydrogen peroxide. As mentioned above, both the trytophanyl and the tyrosyl radicals were detected upon treatment of the Y249F variant with peroxoacetic acid. The spectra of the Y249F samples treated with 5- and 10-fold excess hydrogen peroxide were both narrower than the EPR spectrum obtained after treatment with peroxoacetic acid (compare the three control spectra in Figure 10). The absence of spectral differences between the non-deuterated (control, solid trace) and the perdeuterated-Trp (dotted trace) samples of the Y249F variant, both treated with 5-fold (or 15-fold) hydrogen peroxide, and the evident changes in the EPR spectrum of the perdeuterated-Tyr sample (dashed trace) as compared to the control spectrum clearly demonstrated that only the tyrosyl radical could be detected when using hydrogen peroxide as substrate. The same results were observed for the R349A and R349N variants (see Figure 9, top), i.e. the Trp• and Tyr• were formed with peroxoacetic acid, but only the Tyr• was detected upon treatment with hydrogen peroxide. In contrast, only
the Tyr species was detected in the M275I variant using both peroxoacetic acid and hydrogen peroxide as substrate (Figure 9, bottom).

**DISCUSSION**

*Structural changes induced by the mutations related to the crosslinked adduct of* *Synechocystis catalase-peroxidase.* The structures of KatGs (5, 6) and mass spectrometric analysis (7, 8) have revealed a unique covalent linkage among the side chains of a tryptophan, a tyrosine and a methionine on the distal heme side (W122, Y249 and M275 in *Synechocystis* KatG). Substrates entering the two putative channels in KatG (6) could come in contact with this unprecedented adduct. Hydrogen peroxide most probably enters the distal side cavity through a channel similarly to, but longer and more restricted than, the access route in (monofunctional) peroxidases and comes in contact with the active-site residues Arg, His and Trp (5, 6), the latter (W122) being part of this unusual adduct (Figure 1). A second U-shaped access route to the core of the protein leading to the methionine which is also part of this covalent linkage (M275), has been also postulated (6). In addition, the structure of *B. pseudomallei* KatG showed that a conserved arginine (R426, corresponding to R439 in *Synechocystis*) exists in two orientations (6): in the predominant form (Figure 1B) the guanidinium group is located on the surface at the bottom of the U-shaped channel, whereas in its minor orientation the Arg side chain is in H-bonding distance to tyrosine and methionine, both residues being part of the covalent adduct. These findings clearly underline the importance of investigating the role of these residues in the bifunctional activity of catalase-peroxidases.

Mass spectrometric analysis of the tryptic peptides from recombinant *Synechocystis* wild-type KatG and the variants W122F, Y249F, M275I, R439A and R439N have demonstrated that exchange of either W122 or Y249 prevents the formation of the covalent adduct, whereas
exchange of M275 still allowed bond formation between W122 and Y249 (7). In this work, we have demonstrated that both R439 variants do not affect the linkage between W122, Y249 and M275 existing in the wild-type enzymes in solution. Moreover, the EPR spectra of the R439 variant in frozen solution further confirmed that no H-bond was formed between R439 and the amino acids Y249 and M275 since the ferric spectrum of this variant turned out to be identical to that of the wild-type enzyme. As previously shown, the EPR spectrum of the ferric KatG is extremely sensitive to small changes in the microenvironment of the heme site (13). Accordingly, measurable changes on the ferric EPR spectrum (and/or on the pH dependence) of the R439 variant should have been detected if R439 would be a H-bond donor to Y249 and M275.

As observed from the electronic absorption spectra in solution, some of the variants show an increase of the 6-coordinated low-spin species at the expense of the native 5-coordinate high-spin heme (17). The g-values and the rhombicity of the low-temperature ferric EPR spectrum of heme enzymes constitute an excellent marker of the coordination number and spin state of the heme iron. Accordingly, we have used EPR spectroscopy to monitor possible changes induced by the mutations. Specifically, the R439 variants showed that the only difference with the wild-type enzyme is the contribution of a higher proportion (about 20% as compared to 5% in the wild-type KatG) of low-spin hexacoordinated species, but the overall pattern of a pentacoordinated high-spin species is conserved.

It is important to note that exchange of the indole group of W122 has an important impact on the distal H-bonding network (17) by specifically inducing the disruption of the extensive H-bonding network (13), without affecting the overall heme architecture (17). As a consequence, the binding of the anionic ligand fluoride is impaired (17). This is in contrast to the exchange of Y249, which as shown by our previous RR studies weakens the heme binding, most probably as a result of a readjustment of the KatG-typical (Y249 containing)
LL1 loop at the heme edge (18), but only slightly affects the binding of fluoride to the Fe atom as well as its H-bond interaction with W122 (18). The EPR studies in this work specifically showed that the effect of the mutation on Y249 is less dramatic than that induced by the W122 variant (13). It can be explained by a milder repositioning of the distal Trp induced by the disruption of the covalent link between W122 and Y249, although the extensive H-bonding network remained intact (as inferred from the formation of the Trp radical, see below). This suggests that the absence of the covalent bonds between W122 and Y249 does not significantly perturb the relative orientation of the W122 and the heme.

However, exchange of M275 induced a more dramatic change in the microenvironment of the heme active site, as seen from the changes on the ferric EPR spectrum in which the ratio of the two pentacoordinated high-spin species with different rhombicity was inversed as compared to the wild-type enzyme (Figure 8). A similar dramatic effect was observed when the distal histidine (H123) was exchanged to a glutamine (13). The exchange of M275 still allowed the formation of an adduct involving W122 and Y249 (7) that would undergo a dramatic repositioning induced by the absence of the covalent link between M275 and the partially-formed adduct. Mutation of M275 still allows fluoride binding to some extent (18). Similarly, the kinetics of cyanide binding to ferric M275I and both R439 variants closely resembles to that of the wild-type protein, indicating that its access through the main channel is not altered in these variants.

Effect on the chemical nature of the radical intermediates in KatG induced by the variants related to the crosslinked adduct. We have previously shown that the strategy of combining site-directed mutagenesis and multifrequency EPR spectroscopy is a strong requirement to unequivocally identify the radical intermediates in *Synechocystis* catalase-peroxidase (13). In a further effort to understand the possible role of the amino acid residues
involved in the unprecedented crosslink in catalase-peroxidases, we have used this approach and have included steady-state and presteady-state kinetics studies. The predominant heme bands in the electronic absorption spectrum of the catalase-peroxidase both for the native enzyme and the reactive intermediates, so-called compound I and compound II (see Figures 4 & 7), make impossible to monitor the formation of protein-based radical intermediates in catalase-peroxidases by using the UV-Vis absorption spectrum. The absorption bands of a tyrosyl radical would be masked by the Soret heme bands of the compounds I and II (406-418 nm) and that of a tryptophanyl radical will be underneath the α and β heme bands at ca. 500-580 nm. Accordingly, it is of great interest to combine the information obtained by the UV-Vis spectrum with the EPR spectrum to be able to define the radical species formed under different conditions (19).

In the wild-type Synechocystis catalase-peroxidase, the intermediate referred as conventional compound I in the description of the steady-state and kinetic results and identified by the expected electronic absorption spectrum of the oxoferryl-porphyrin radical species, was shown to actually be the superposition of an oxoferryl-porphyrin radical, a tryptophanyl radical and a tyrosyl radical. These three radical species could be discern from the multifrequency EPR spectroscopy studies on the enzyme (13). As mentioned before, the Tyr• and Trp• bands were certainly masked by the predominant heme bands in the electronic absorption spectrum. As in the case of the wild-type enzyme, all variants studied in this work showed a broad EPR signal assigned to the exchange-coupled oxoferryl-porphyrin radical species, the compound I, in good agreement with their electronic absorption spectra (see Figure 6 in ref. 11, and Figures 4 and 5 in this work). A narrow radical signal was also detected for the Y249F and R439A/N variants upon reaction with peroxoacetic acid. Deuterium labeling experiments proved that this narrow EPR signal had the contribution of both the tryptophanyl and the tyrosyl radicals as in the case of wild-type enzyme.
Interestingly, the relative proportion of Trp• was lower in the Y249F variant as compared to the wild-type and the R439A/N variant, as judged from the high field EPR spectrum. A similar situation was previously reported for the proximal tryptophan variant (W341F) and explained by the fact that only a mild repositioning of the residues involved in the extensive hydrogen-bond network have been induced by the mutation of the proximal tryptophan residue via the previously proposed existing connection (17) between the heme proximal and distal sides. The behavior of the ferric heme EPR spectrum of the Y249F variant (Figure 8) being the same as that of the W341F variant (13), the relatively lower yield of Trp• in the Y249F variant can be as well attributed to a mild repositioning of the distal tryptophan residue (W122) induced by the absence of the covalent link between W122 and Y249 in this variant (7), without disruption of the extensive hydrogen-bond network. A different situation was observed when exchanging the M275 residue in *Synechocystis* KatG. The mutation induced a more dramatic change in the microenvironment of the heme site, as seen from the behavior of the ferric heme EPR spectrum (Figure 8). Most probably, the exchange of the M275 by an isoleucine induced a substantial repositioning of the remaining W122-Y249 adduct (7) that in this case, induced severe modifications on the extensive hydrogen-bond network. This proposal is substantiated by the fact that the tryptophanyl radical was not formed in the M275I variant (see below). Accordingly, upon enzyme reaction with peroxyacetic acid, and similarly to the situation previously observed for the H123Q variant (13), only the oxoferryl-porphyrin radical intermediate and the tyrosyl radical species were formed in the M275I variant. Moreover, based on the comparison of bimolecular rate constants of the compound I formation upon reaction with hydrogen peroxide, the possibility that the tryptophanyl radical was indeed formed but not detected in the mixing times used for our EPR experiments could be clearly ruled out. Specifically, the rate for compound I formation of the M275I variant was the lowest as compared to those of the R439A, R439N
and Y249F variants (see Table 2) and both protein-based radicals were detected for the R439A/N and Y249F variants upon reaction with peroxoacetic acid.

The results reported previously for the mutation of the crosslinked tyrosine residue in *M. tuberculosis* KatG (Y229) indicate that there are differences both on the kinetics of compound II formation and on the ferric EPR signal of this tyrosine variant between the *Synechocystis* and the *M. tuberculosis* enzymes. Specifically, a more dramatic change in the ferric EPR spectrum of the Y229F variant as compared to the wild-type *M. tuberculosis* enzyme was observed by Magliozzo and coworkers (see Figure 2 in ref. 21). This may indicate that the positioning of the distal side residues are somewhat different in the *Synechocystis* and *M. tuberculosis* catalase-peroxidases. In contrast, the tyrosyl radical EPR signal was detected for both enzymes, confirming that the crosslinked tyrosine residue is not the site for the Tyr$^*$ formation neither in *Synechocystis* nor in *M. tuberculosis* KatGs.

The use of a high excess (10 fold or more) of hydrogen peroxide on the Y249F and M275I variants resulted in the formation of an intermediate with absorption bands at 414 nm, 542 nm, and 575 nm attributed to a compound III-type species. Since this intermediate has been proposed based on the changes in the absorption spectrum, it is not clear which is the actual electronic structure of such intermediate. In the case of horseradish peroxidase, it has been proposed that there might be several ways of obtaining the so-called compound III (for a review, see ref. 31). The one possibility that seems to apply to the case of the Y249F and M275I variants is that such an intermediate forms from compound II and an excess of hydrogen peroxide. The proposed resonant structures for compound III are the oxyferrous and the ferric-superoxide ion complexes. There is no precedent of the ferric-superoxide EPR spectrum but, more important it could in principle be an EPR silent species. The corresponding EPR spectrum obtained for the M275I variant with 10 fold-excess hydrogen
peroxide was slightly narrower than the one obtained by using equimolar or 5 fold-excess hydrogen peroxide (see Figure 10). Deuterium labeling experiments clearly showed that the spectrum also originated from a tyrosyl radical species. Thus, the observed Tyr• spectrum could at most account for the shift of the Soret band but not for the α and β bands observed in the absorption spectrum. More work has to be done in order to understand the, possibly, apparent mismatch between the electronic absorption spectrum and the EPR spectrum of the intermediate obtained with high excess of hydrogen peroxide in the Y249F and M275I variants.

The dramatic decrease, or even suppression of the catalase activity in the R439A, R439N, and Y249F variants (see Table 2) allowed us to compare the radical intermediates formed upon enzyme reaction with hydrogen peroxide and with peroxoacetic acid. It is well known that the reaction of catalases with peroxoacetic acid results in a much more stable oxoferryl-porphyrin radical intermediate than when using hydrogen peroxide. Then, taking into account that the oxoferryl-porphyrin radical intermediate is the first radical species to be formed, it is not surprising that both protein-based radical species (Trp• and Tyr•) could be detected upon enzyme reaction with peroxoacetic acid in the wild-type as well as the Y249F and R439A/N variants of KatG (Figure 9). In contrast, the enzyme reaction with hydrogen peroxide resulted in a much less stable oxoferryl-porphyrin radical intermediate thus the radical migration to the amino acid residues was also faster. This could explain the fact that only the Tyr• species was detected for the Y249F and R439A/N variants, when using hydrogen peroxide. This findings strongly suggest that the sequence for the formation of the radical species in Synechocystis KatG is the following: Por••, Trp• and Tyr•. Moreover, the variants studied in this work reinforce our previous proposal related to the key role of the extensive H-bonding on the heme distal side on the formation of the tryptophanyl radical.
**Effect on the catalytic activity of Synechocystis KatG.** The exchange of W122 (10), Y249 (11), M275 and R439 dramatically influenced the catalase but not the peroxidase activity in *Synechocystis* KatG. Assuming that the first enzymatic step in KatG turnover, namely the oxidation of the ferric enzyme by hydrogen peroxide to compound I, is common to both the peroxidatic and the catalytic cycle, it can be concluded that these manipulations mainly affect the two-electron reduction of compound I (i.e. oxygen release from H₂O₂) but not its formation (i.e. enzyme oxidation by H₂O₂). At first sight this hypothesis is strikingly simple and strengthened by the fact that in these variants formation of conventional compound I (hypochromicity at the Soret band caused by formation of an oxoferryl-porphyrin radical species) can be observed also with H₂O₂, whereas in wild-type KatG hypochromicity is observed only with organic peroxides. Under the assumption that in the catalatic turnover compound I formation (k₁) is slower than its reduction (k₂) then one has to assume that the mutations (e.g. of W122, Y249 or M275) mainly affect k₂. But this scenario appears to be much too simplified since many of our findings would remain unexplained: (i) Wild-type compound I produced with peroxoacetic acid has the typical spectral features of an oxoferryl porphyrin π-cation radical species but reacts extremely slow with H₂O₂ as demonstrated by the sequential-stopped flow technique (10); (ii) in wild-type *Synechocystis* catalase-peroxidase, the chemical nature of the intermediate referred as conventional compound I was shown to be the superposition of the oxoferryl porphyrin π-cation radical, the tryptophanyl radical and the tyrosyl radical as demonstrated by our recent multifrequency EPR spectroscopy study (13); (iii) the observation of redox intermediates with unique features in their room-temperature electronic absorption spectrum suggesting the existence of alternative electronic structures of compound I (Figures 4 & 5) and compound II (Figures 6 & 7) as will be discussed below.
In most peroxidases the dominating electronic structure of compound I is the oxoferryl porphyrin π-cation radical species. However, intramolecular electron transfer between the oxoferryl porphyrin radical and a protein residue (Tyr or Trp) can occur, e.g. formation of tryptophan radicals in cytochrome c peroxidase (22) and lignin peroxidase (23) or a tyrosyl radical in turnip peroxidase (24). There is a correlation between the standard reduction potential of the ferric/ferrous couple and the stability of high-valent porphyrin species (25). In case of catalase-peroxidases the standard reduction potential of the ferric/ferrous couple of *M. tuberculosis* KatG was determined to be -50 mV (26), which is much more positive than that of other plant-type peroxidases (27). This fits with the observation that *Synechocystis* KatG is able to oxidize chloride (27) a two-electron oxidation reaction which needs a redox potential of the KatG couple compound I/native enzyme to exceed 1.1 V. But regarding the catalatic reactivity (i.e. O₂ production) it is not a thermodynamic requirement since $E^{∞^o} (O₂/H₂O₂) = 280$ mV (28).

Nevertheless, the classical compound I formed by mixing of wild-type KatG with peroxoacetic acid reacts only very slowly with H₂O₂. Since the protein architecture is unchanged, it is thus tempting to speculate about the existence of an alternative compound I, which is in rapid equilibrium with the oxoferryl porphyrin π-cation radical species and has a higher reactivity towards H₂O₂ thereby being responsible for molecular oxygen release and regeneration of the enzyme in its resting state. In wild-type KatG this reaction is fast thus preventing observation of conventional compound I even by use of the stopped-flow technique since the equilibrium is shifted totally towards the catalatically competent intermediate. However, upon mutation of distinct amino acids in the heme cavity, the formation and/or stability of this redox intermediate or its reactivity towards H₂O₂ is impaired and the conventional compound I can accumulate even in the presence of H₂O₂.
The electronic structure and the visible spectrum of this postulated catalytically reactive compound I is unknown as is its contribution to the observed spectral transitions in the Figures 4C and 4D as well as 5A. The spectrum of the intermediate, which was observed upon incubation of both R439 variants with excess hydrogen peroxide (bands at 414 nm, 542 nm and 572 nm; Figure 4C and 4D) was very similar to that of the hexacoordinated low-spin species obtained upon mixing of hydroxylamine with *Arthromyces ramosus* peroxidase (29), which was interpreted as a structural model for compound 0, normally being difficult to observe since its formation is followed by the rapid heterolytic cleavage of hydrogen peroxide.

However, this rapidly formed intermediate of both R439 variants was also similar to the intermediate, which was observed when the classical compound I of M275I decayed (414 nm, 542 nm and 575 nm; Figure 5A) and to the intermediate, which was formed from Y249F compound I via compound II in the absence of one-electron donors (11). In case of both R439 variants the appearance of this intermediate was monitored spectroscopically before the classical compound I, whereas in case of M275I it derived from conventional compound I and in both cases the spectral interconversion was monophasic suggesting an equilibrium between conventional compound I and this intermediate. Whether this intermediate plays a role in the catalytic cycle of wild-type KatG is not clear at the moment.

Interesting was the observation that the kinetics of M275I compound I transition in the absence of one-electron donors depends on the nature of the peroxide used in compound I formation (compare Figures 5A and 5B). Though exhibiting compound III-like spectral features, the formed intermediate reacts with one-electron donors excluding that it is the oxygenated ferrous peroxidase (i.e. compound III), which should not be redox active. Furthermore, the spectrum is very similar to the spectrum of a ferryl-protein radical cation
compound I observed when the horseradish peroxidase variant H42L was incubated with hydrogen peroxide (30).

In contrast to the two-electron reduction of compound I, the one-electron reduction does not depend on the electronic structure of this compound. This is best demonstrated by comparison between wild-type KatG and Y249F. In Y249F a conventional pure oxoferryl-compound II is seen (11), whereas in wild-type KatG the spectral features of compound II are similar to that of the ferric enzyme, indicating that the oxoferryl-center in the conventional compound I could be first reduced thereby producing the ferric enzyme, a protein radical and releasing water. The nature of the peroxide used in enzyme oxidation does not influence this reactivity. Also with most of the mutants investigated until now (including both R439 variants) a significant red-shift of the Soret band was never observed in the peroxidase cycle. There are two important exceptions, namely Y249F and M275I, both residues being part of the KatG-specific covalent adduct. Comparison of the steady-state catalase and peroxidase activity data suggest that the binding and oxidation pattern of one-electron donors is different from that of H₂O₂, though one has to keep in mind that artificial peroxidase substrates were used since the endogenous donor is still unknown. If one-electron donors and H₂O₂ would compete for the same compound I species and bind at the same site the dramatic decrease in catalase activity had to be accompanied by a dramatic increase in peroxidase activity. In reality the increase in peroxidase activity is much smaller with the exception M275I. This is best underlined by Y249F which completely lost the catalase activity but still has a peroxidase activity similar to wild-type KatG.

In Y249F both compound I and compound II spectra exhibit spectral features typical for plant-type peroxidases but different to wild-type KatG (11). By contrast in wild-type KatG and R439A and R439N the Soret band of the compound II spectra is still at 406 nm (Figure
6A), whereas compound II formed from M275I classical compound I (preformed with H$_2$O$_2$) upon addition of one-electron donors has absorption bands at 414 nm, 542 nm and 575 nm, very similar to the intermediate formed from classical M275I compound I in the absence of one-electron donors. Two alternative structures of KatG compound II could exist similar to cytochrome $c$ peroxidase compound II (28), namely a ferryl form and a protein radical form. In contrast to the reactivity with H$_2$O$_2$, the compound I reduction to compound II by one-electron donors does not depend on the nature of the organic peroxide used in compound I formation (Table 2). Under the assumption that the protein radical compound II has spectral features similar to ferric KatG and taken into account that wild-type KatG and most of the mutants do not show a red-shift in enzyme turnover, it could be concluded that this compound II species is the dominating species in the peroxidatic cycle. As mentioned above, only with Y249F and M275I a red-shift was observed, in case of Y249F exhibiting the typical oxoferryl-spectral features, whereas in M275I clearly more work is needed to understand the correlation between its electronic structure and both UV-Vis and EPR data.

Interestingly, M275I is the first KatG variant which showed a significantly increased overall peroxidase activity (Table 1). In addition to R439A/N it shows also ascorbate oxidation at reasonable rates, a reaction which was so far not observed with wild-type KatG or other variants. This suggests that small peroxidase substrates could enter the enzyme through the U-shaped channel and that their access has been facilitated by exchange of M275 and R439 both being part of or adjacent to the U-shaped channel. This is strengthened by the observation that compound I reduction rates were significantly enhanced in M275I and both R439 variants and that the pH-profile was affected in case of M275I mediated oxidation of aromatic peroxidase substrates.
Summing up, the unique covalent adduct plays a central role in enzyme stability and reactivity and its manipulation strongly affects the electronic nature of the redox intermediates. The contribution to protein stability is mainly by anchoring a KatG-specific loop to the molecular surface via Y249 and by participation in the extensive hydrogen-bond network on the distal side of the heme, involving W122, H123, R119, several structural waters, the heme 6-propionate group and W106, the latter being the site of the tryptophanyl radical (13). Disruption of this network by mutagenesis mainly affects H₂O₂ oxidation but not H₂O₂ reduction during compound I formation. Concomitantly, the tryptophanyl radical (on W106) is not formed when the extensive H-bonding network is disrupted. Access for hydrogen peroxide to the heme occurs through the main but narrow channel. Peroxidatic substrates most probably can donate electrons by penetrating the second U-shaped channel in the vicinity of the covalent adduct. This demonstrates that catalase-peroxidases are unique systems to study and understand the role of electron pathways in peroxidases and catalases and to understand the mechanism of catalase activity of a heme protein, which is far from being understood even in the case of monofunctional catalases (28, 31, 32).

REFERENCES


LEGENDS TO FIGURES

**Figure 1:** Distal site structure of catalase-peroxidases. (A) Crystal structure from *Haloarcula marismortui*. The figure was constructed using the coordinates deposited in the Protein Data Bank (accession code 1ITK). The crosslinking between arginine, tyrosine and methionine are shown. (B) Crystal structure from *Burkholderia pseudomallei* (PDB accession code 1BKG) showing the major conformation of arginine. No hydrogen bonds are possible between arginine and tyrosine and methionine. The amino acid numbering is for *Haloarcula* and *Burholderia* KatG, respectively, but numbers in parentheses denote numbering for *Synechocystis* KatG.

**Figure 2.** Mass spectrometric analysis of peptides obtained by tryptic digestion of wild-type *Synechocystis* KatG and the variants M275I and R439N. Peptides in the mass/charge range of 1400 – 1440 Da are shown. Spectra were normalized to the same intensity (500 counts). Wild-type KatG and the variant R439N show the isotopic pattern of \([M5H]^+\) deriving from \([MH]^+\) = 7096.34 Da, which corresponds to the monoisotopic mass of the covalently linked peptides. No covalently linked fragment was detected in the M275I variant.

**Figure 3:** Cyanide binding to the ferric forms of the variants M275I and R439N. (A) UV-Vis spectra of the ferric form and cyanide complex (bold) of M275I. Conditions: Ferric proteins (6 µM) were mixed with 10 mM cyanide in 50 mM phosphate buffer, pH 7.0 and 25°C. The region between 470 and 700 nm has been expanded 6-fold. (B) UV-Vis spectra of the ferric form and cyanide complex (bold) of R439N. (C) Pseudo-first-order rate constants for the formation of the cyanide complex of M275I. Conditions: 1 µM ferric enzyme, 50 mM
phosphate buffer, pH 7.0 and 15°C. (D) Pseudo-first-order rate constants for the formation of the cyanide complex of R439N. Conditions as in (C).

**Figure 4:** Reaction of ferric R439A with hydrogen peroxide. (A) Spectra obtained after mixing of 5 µM R439A with 10 µM hydrogen peroxide. Compound I is formed without any detectable intermediates. The inset shows the time trace at 407 nm for this reaction. Compound I is not stable and decays back to the ferric enzyme. Conditions: 50 mM phosphate buffer, pH 7.0 and 25°C. (B) Plot of the pseudo-first-order rates against hydrogen peroxide concentration. The slope yielded the apparent second-order rate constant for the reaction of ferric enzyme with hydrogen peroxide. The inset shows the reaction of 1 µM R439A with 15 µM hydrogen peroxide followed at 407 nm. The reaction was fitted using a single exponential equation. Conditions: 1 µM ferric R439A, 5 – 20 µM hydrogen peroxide, 50 mM phosphate buffer, pH 7.0 and 15°C. (C) Spectral changes upon reaction of 5 µM R439A with 100 µM hydrogen peroxide. Thin line, ferric spectra; bold line, reaction intermediate formed within 1.3 ms exhibiting absorbance maxima at 414 nm, 542 nm and 575 nm; grey line, compound I (spectra taken after 47 ms). Conditions as in (A). (D) Transformation of the new intermediate to compound I. First spectra (bold line) was taken 2 ms after mixing 5 µM R439A with 100 µM hydrogen peroxide. Subsequent spectra were taken after 6.4 ms, 12 ms, 19 ms, 47 ms. After 47 ms compound I was reached. The reaction showed clear isosbestic points at 395 nm and 520 nm. Conditions as in (A). (E) Decay of 5 µM compound I (preformed with 100 µM hydrogen peroxide) of R439A to ferric enzyme. Compound I was stable for 2.5 s (bold line). Subsequent spectra were taken at 3.5 s, 5 s, 7 s and 10 s. Isosbestic points were at 360 nm, 430 nm and 526 nm.
**Figure 5**: Compound I formation and decay in the variant M275I. (A) Spectral changes observed after mixing of 5 µM ferric M275I (thin line) with 250 µM hydrogen peroxide. Compound I formation was completed after 200 ms (bold line) and decayed to an intermediate with compound III-like absorbance bands (grey line, spectra after 10 s) with peaks at 414 nm, 542 nm and 575 nm. Conditions: 50 mM phosphate buffer, pH 7.0 and 25°C. (B) Spectral changes obtained after mixing of 5 µM ferric M275I (thin line) with 250 µM peroxoacetic acid. Bold line depicts compound I (200 ms). Compound I also decayed but after 10 s (grey line) only a slight shift was observed. Conditions as in (A). (C) Pseudo-first order rate constants for the reaction of 1 µM M275I with 10 – 150 µM hydrogen peroxide plotted against the concentration of hydrogen peroxide. Conditions: 50 mM phosphate buffer, pH 7.0 and 15°C. (D) Pseudo-first order rate constants for the reaction of 1 µM M275I with 20 – 200 µM peroxoacetic acid plotted against the concentration of peroxoacetic acid. Conditions as in (C).

**Figure 6.** Compound I reduction of the *Synechocystis* KatG variant R439N. (A) Spectral changes upon addition of ascorbate to R439N compound I formed with hydrogen peroxide. Final concentrations: 2 µM R439N compound I and 2 mM ascorbate. First spectrum (1, bold) is that of R439N compound I taken 1.3 ms after adding ascorbate. Second spectrum (2, thin) is taken after 150 ms and third spectrum (3, grey line) is taken after 1.5 s. The inset shows the corresponding time trace at 407 nm and 25°C (50 mM phosphate buffer, pH 7.0) with numbers indicating times of spectrum selection. (B) Time trace (407 nm) and single-exponential fit of compound I reduction. 1 µM of compound I formed with hydrogen peroxide was mixed with 2 mM ascorbate (50 mM phosphate buffer, pH 7.0 and 15°C). (C) Pseudo-first-order rate constants for R439N compound I reduction plotted against ascorbate concentration. Conditions as in (B).
Figure 7. Kinetics of reduction of M275I compound I. (A) Spectral changes upon addition of ascorbate to compound I preformed with ten-fold excess of hydrogen peroxide. Final concentrations: 5 µM compound I and 500 µM ascorbate. The bold line indicates compound I and subsequent spectra were taken at 1.3 ms, 45 ms, 127 ms, 536 ms and 1.7 sec. (B) Continuation of (A). Spectra were taken at 2.1 sec (bold line), 3.1 sec, 4.3 sec, 6.2 sec, 8.7 sec. (C) Time traces at 407 nm for compound I reduction. Biphasic time traces showing the dependence on the concentrations of ascorbate used: bold line, reaction without ascorbate; thin line, 100 µM ascorbate; dashed line, 500 µM ascorbate; grey line, 2 mM ascorbate. (D) Pseudo-first-order rate constants for compound I reduction of M275I plotted against ascorbate concentration. Conditions: 1 µM M275I, 50 µM hydrogen peroxide, 50 µM – 1000 µM ascorbate, 50 mM phospat buffer, 15°C. Pseudo-first-order rate constants were obtained by fitting the first exponential phase of reaction. (E) Decay of M275I compound I (bold spectrum) in the absence of an one-electron donor. Spectra were taken 500 ms, 1 s, 2 s, 10 s after mixing with buffer. The intermediate with the maximum at 414 nm and peaks at 542 nm and 575 nm was formed completely after 4 sec and was stable for at least 10 sec. (F) Reaction of this intermediate (bold spectrum) with 5 mM ascorbate. Spectra were taken 1 s, 1.2 s and 2 s after addition of ascorbate.

Figure 8. The 9-GHz EPR spectra of the Synechocystis catalase-peroxidase (wild-type and M275I, Y249F, R439N variants) in the ferric (native) state. In each case, the spectra recorded at pH 5.6 (dashed trace) and 8.3 (solid trace) are shown. The inset shows an expansion of the g₀ component of all the EPR spectra. Experimental conditions: temperature, 4 K; modulation frequency, 5 G; microwave power, 1 mW; modulation frequency, 100 kHz.
Figure 9. The 9-GHz EPR spectra of the protein-based radicals in wild-type and variants of *Synechocystis* catalase-peroxidase formed upon treatment with 10-fold excess of peroxoacetic acid (solid trace) as well as 5-fold excess of hydrogen peroxide (dashed trace). Experimental conditions: temperature, 60 K; modulation frequency, 0.5 G; microwave power, 0.05 mW; modulation frequency, 100 kHz.

Figure 10. Comparison of the 9-GHz EPR of the protein-based radicals formed in the Y249F variant of *Synechocystis* catalase-peroxidase upon treatment with 5 fold-excess of peroxoacetic acid (top) as well as 5 fold-excess (middle) and 15 fold-excess (bottom) of hydrogen peroxide. In each case, the spectra obtained for samples containing perdeuterated tyrosines (dotted trace) and perdeuterated tryptophans (dashed trace) are superimposed to those of the control (non deuterated) samples. The expected spectral changes on the EPR signal of the samples containing perdeuterated-Trp or perdeuterated Tyr residues upon treatment with peroxoacetic acid (in particular, on the peak-to-trough width shown by the bars (solid trace for the control and dashed trace for the perdeuterated-Trp, respectively) clearly demonstrate that both the tryptophanyl and the tyrosyl radicals were detected in this case. In contrast, in the time scale of our experiments only the tyrosyl radical was detected upon reaction with hydrogen peroxide (same EPR signal for the control and perdeuterated-Trp samples). Experimental conditions are the same as those in Figure 9, except for the sample concentration.
Table 1. Apparent $K_m$ and $k_{cat}$ values for the catalase activity of wild-type and variant catalase-peroxidases from *Synechocystis* PCC 6803. Also given are specific peroxidase activities (units per mg protein). Reaction conditions: 50 mM phosphate buffer, pH 7, and 30°C. For catalase and peroxidase assays as well as unit definition see Experimental Procedures.

<table>
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<tr>
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<th>Wild-type</th>
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Table 2. Bimolecular rate constants of compound I formation, cyanide binding and compound I reduction for wild-type *Synechocystis* KatG and the variants M275I, R439A and R439N. Conditions: 50 mM phosphate buffer, pH 7, and 15°C. Compound I was formed with either peroxoacetic acid\(^\text{A}\) or hydrogen peroxide\(^\text{B}\). For details see Materials and Methods. n.d., not detectable

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
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<th>R439A</th>
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<td>(\times 10^4) M(^{-1}) s(^{-1})</td>
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</table>
Figure 1
Figure 2
Figure 3
Figure 4
Fig 5

(A) Absorbance vs. Wavelength (nm)

(B) Absorbance vs. Wavelength (nm)

(C) $k_{\text{obs}}$ vs. Hydrogen Peroxide (µM)

(D) $k_{\text{obs}}$ vs. Peroxoacetic Acid (µM)

For Hydrogen Peroxide:

$y = 0.1199x + 0.7299$

For Peroxoacetic Acid:

$y = 0.3075x + 1.9843$
Figure 6

(A) 

![Graph showing absorbance over wavelength with data points and a linear regression equation: $y = 13.389x + 4.303$.](https://example.com/graph.png)

(B) 

![Graph showing absorbance over time with a linear regression equation: $y = 13.389x + 4.303$.](https://example.com/graph.png)

(C) 

![Graph showing absorbance over time with a linear regression equation: $y = 13.389x + 4.303$.](https://example.com/graph.png)
Fig. 7
\[ g_{\perp}^{\text{eff}} = 5.93 \]

\[ g_{||}^{\text{eff}} = 1.99 \]

\begin{align*}
\text{pH 8.3} & \quad \text{wild type} \\
\text{pH 5.6} & \\
M275I & \\
Y249F & \\
R439N & \\
R439A &
\end{align*}

Fig. 8
Wild type ($W^*, Y^*$)  
R439A  
Y249F  
M275I  

10 fold peroxoacetic acid  
5 fold $H_2O_2$
Fig. 10

Y249F

+ 5 fold peroxoacetic acid

+ 5 fold $\text{H}_2\text{O}_2$

+ 15 fold $\text{H}_2\text{O}_2$
Influence of the unusual covalent adduct on the kinetics and formation of radical intermediates in synechocystis catalase-peroxidase: A stopped-flow and EPR characterization of the M275, Y249 and R439 variants

Christa Jakopitsch, Anabella Ivancich, Florian Schmuckenschlager, Anuruddhika Wanasinghe, Gerald Poeltl, Paul G. Furtmueller, Florian Rueker and Christian Obinger

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