Catalase-peroxidases (KatGs) are heme peroxidases with a catalatic activity comparable to monofunctional catalases. They contain an unusual covalent distal side adduct with the side chains of Trp122, Tyr249, and Met275 (Synechocysis KatG numbering). The known crystal structures suggest that Tyr249 and Met275 could be within hydrogen-bonding distance to Arg439. 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 numbers of catalase activity of M275I, Y249F, M275I, R439A, and R439N were 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 mass spectrometry and EPR spectra, the KatG typical adduct is intact in both Arg439 variants, as is the case of the wild-type enzyme, whereas in the M275I variant the covalent link exists only between Tyr249 and Trp122. In the Y249F variant, the link is absent. EPR studies showed that the radical species formed upon reaction of the Y249F and R439A/N mutants with peroxoacetic acid are the oxoferrylporphyrin 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 por+ → Trp− (or Trp+ → Tyr). The M275I variant did not form the ‘Trp’ species because of the dramatic changes on the heme distal side, most probably induced by the repositioning of the remaining Trp122–Tyr249 adduct. The results are discussed with respect to the bifunctional activity of catalase-peroxidases.
but numbers in parentheses coordinates deposited in the Protein Data Bank (accession code 1ITK).

acid numbering is for 1BKG) showing the major conformation of arginine. No hydrogen bonds to form hydrogen bonds, as in the case of 1ITK. The figure was constructed using the Adaptive Molecular Dynamics (AMD) program (10, 11). Additionally, the B. pseudomallei variant M264L (Met275 in Synechocystis) showed a significantly reduced over:

The Trp-Tyr-Met adduct appears to be hydrogen-bonded to a neighboring arginine residue (Arg429 in Synechocystis) and the corresponding arginine (Arg409) is in hydrogen-bonding distance to both Tyr218 and Met244 (Fig. 1A). Specifically, the NH1 and the NH2 groups of Arg409 form hydrogen bonds with the phenol oxygen of Tyr218 and the backbone oxygen of Met244, respectively (Fig. 1A). In the crystal structure of B. pseudomallei KatG, the corresponding arginine (Arg426) points away from the covalent adduct in its predominant orientation (6), and no hydrogen bonds can be formed (Fig. 1B). Only in its minor conformation (30%) are the side chains of Arg426 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 Met275 and Arg429 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 with 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 oxoferrylporphyrin, tryptophanyl, and tyrosyl radicals) (13) were observed for the other variants. The possibility of using hydrogen peroxide for radical formation allowed us to predict the sequence of radical formation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Standard chemicals and biochemicals were obtained from Sigma 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 labeling and purification of specifically deuterated KatGs were described recently by Ivancich et al. (13).

**Mutagenesis of Arg429**—Oligonucleotide site-directed mutagenesis was performed using PCR-mediated introduction of silent mutations as described (10). A pET-3a expression vector that contains 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′-GCC ACC CCG ATC CTT TAT G-3′ containing a BamHI restriction site and 5′-AGT GCA GAC TAG TTC GGA AAC G-3′ containing an SpeI restriction site. The internal 3′ primer was 5′-TGT GAG TTA 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 GCTT TACA CCA CAG AAG TCT AGG AC-3′ changed Arg439 to N; 5′-CGC ACC CGG ATC CTT TAT G-3′ changed Arg439 to Ala.

**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-400 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 Hilderbrand (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.). The applied electrode potential at pH 7 was 650 mV, and the H2O2 electrode-filling solution was prepared fresh each half-day. 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

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**Fig. 1.** Distal site structure of catalase-peroxidases. A, crystal structure from *H. marismortui*. The figure was constructed using the coordinates deposited in the Protein Data Bank (accession code 1ITK). The cross-linking between arginine, tyrosine, and methionine are shown. B, crystal structure from *B. 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.

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**EXPERIMENTAL PROCEDURES**

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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 4.9) to 530 mV (pH 9).

Peroxidase activity was monitored spectrophotometrically using 1 mM H$_2$O$_2$ and 5 mM guaiacol ($\epsilon_{320} = 26.6 \text{ m}^{-1} \text{ cm}^{-1}$) or 1 mM o-dianisidine ($\epsilon_{384} = 11.3 \text{ m}^{-1} \text{ cm}^{-1}$) or 500 $\mu$M ascorbate ($\epsilon_{280} = 2.8 \text{ m}^{-1} \text{ cm}^{-1}$). Additionally, peroxidase activity was measured with peroxyacetic acid (1 mM) instead of H$_2$O$_2$. One unit of peroxidase is defined as the amount that decomposes 1 $\mu$mol 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 thermostatted at 15 °C. Calculation of pseudo-first order rate constants ($k_{\text{obs}}$) from experimental traces at the Soret maximum was performed with the SpectraKinetic workstation v.4.38 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 peroxyacetic 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 mM enzyme and 5–250 $\mu$M peroxide or 20–500 $\mu$M 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 H$_2$O$_2$ 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 five independent determinations were performed per substrate concentration. The temperature (15 °C) was used 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 (www.expasy.ch/www/tools.html).

**EPR Spectroscopy**—Conventional 9-GHz EPR measurements were performed using a Bruker ER 300 spectrometer with a standard TE$_{202}$ 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, or 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–via 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 for R439A and 406 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 partial amount 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 $A_{407}/A_{280}$), which was 0.41–0.48 for the M245I variant and 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 30–60 mg per liter of 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 Trp$^{122}$ or Tyr$^{249}$ in Synechocystis KatG prevents formation of the KatG-specific distal side covalent adduct, whereas exchange of Met$^{275}$ still allowed bond formation between Trp$^{122}$ and Tyr$^{249}$. In this work, we have investigated the impact of exchange of Arg$^{439}$ on the formation of the adduct. If the covalent link between Trp$^{122}$, Tyr$^{249}$, and Met$^{275}$ 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. Fig. 2 shows the isotopic pattern of the [MH]$^+$ species (1420.07 Da) found for both wild-type KatG and the R439N variant, both not for M275I. This clearly rules out the hypothesis that Arg$^{439}$ takes part in the formation and/or integrity of the covalent bonds. Structural information obtained by EPR spectroscopy on the native Arg$^{439}$ variants confirms 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 Tyr$^{249}$ by phenylalanine the enzyme was totally converted from a bifunctional peroxidase to a monofunctional peroxidase (11). Exchange of both Met$^{275}$ and Arg$^{439}$ also had a dramatic influence on the catalytic activity (Table I). 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 peroxidase activity of R439A, R439N, and Y249F (11) variants toward the aromatic donors o-dianisidine and guaiacol was similar to that of wild-type KatG (Table I) as was the maximum of peroxidase activity (pH 5.5). By contrast, M275I showed a substantial increase in peroxidase activity. Compared with 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 I). Interestingly, with both aromatic electron donors the pH maximum shifted to pH 7.0.

Because in wild-type KatG, an overwhelming catalase activity strongly favors H$_2$O$_2$ disproportionation over the oxidation of one-electron donors, using peroxyacetic 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 peroxyacetic acid was used to initiate the peroxidase reaction as shown in Table I.

The typical substrate for 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 Arg$^{439}$ variants, exhibited a reasonable ascorbate-peroxidase activity, which dramatically increased with decreasing pH (not shown). By contrast, the 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 H$_2$O$_2$-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 (Fig. 3). The cyanide complex of wild-type KatG and the arginine variants exhibited a small hypochromicity at the Soret absorbance (Fig. 3B), whereas in the case of the M275I variant a small hyperchromicity was observed (Fig. 3A).

Under pseudo-first order conditions, the observed rate constant of cyanide binding to the ferric proteins linearly increased with the concentration of cyanide (Fig. 3, C and D). From the slope of the plots the apparent second order rate constant (k_{on}) for cyanide binding was calculated to be $(3.2 \pm 0.2) \times 10^5 M^{-1} s^{-1}$ for M275I, $(7.6 \pm 0.3) \times 10^5 M^{-1} s^{-1}$ for R439A and $(6.2 \pm 0.2) \times 10^5 M^{-1} s^{-1}$ for R439N, respectively, very similar to native KatG (14) (Table II). 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 $\mu$M for M275I, 2.5 $\mu$M for R439A, and 5.1 $\mu$M for R439N. The dissociation constants of these variants decreased substantially compared with the wild-type enzyme (15.8 $\mu$M).

**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 Tyr249 (11) or Trp122 (10), exchange of either Met275 or Arg439 allowed us to monitor the enzyme oxidation at 407 nm by using
hydrogen peroxide. This fits well with the decreased overall catalytic activity of these variants. In the R439N and R439A variants, the reaction of the ferric enzyme with a small excess of hydrogen peroxide or peroxyacetic acid was monophasic. Compound I formed by peroxyacetic 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 (Fig. 4A). The inset to Fig. 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 s 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 behavior (data not shown). The plot of the pseudo-first-order rate constants versus hydrogen peroxide concentrations (5–20 μM) was linear (Fig. 4B), and the kₘₐₓ 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 peroxyacetic 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 II).

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 peroxyacetic acid was used in both arginine variants (Fig. 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, 542, and 575 nm (bold line in Fig. 4C). The very rapid formation of this new intermediate prohibited 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 (Fig. 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, 430, and 526 nm (Fig. 4E). With peroxyacetic acid, the latter reaction was not observed, and compound I was stable.

Fig. 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, 542, and 575 nm (gray line), very similar to: (i) the spectral features of the first intermediate observed upon mixing of the Arg⁴⁳⁹ variants with excess H₂O₂ (see above) or (ii) to the recently observed intermediate that 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 behavior 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, 542, and 576 nm (11) when ferric Y249F was incubated with a 10-fold (or higher) excess of hydrogen peroxide.

The reaction of 5 μM M275I with 250 μM peroxyacetic acid is shown in Fig. 5B. A conventional compound I was observed within 200 ms and remained relatively stable. The spectrum of the intermediate monitored after 10 s (Fig. 5B, gray spectrum) was slightly red-shifted compared with conventional compound

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**TABLE II**

| Compound I formation | Compound I reduction | Compound I reduction |
|----------------------|----------------------|----------------------|
| Hydrogen peroxide    | 12.0                 | 10.5                 |
| Peroxyacetic acid    | 3.9                  | 30.8                 |
| Cyanide binding      | 24.8                 | 31.5                 |
| Ascorbate            | 0.7                  | 1.6                  |
| o-Dianisidine        | 271                  | n.d.                 |
| Anilinyl             | 1.4                  | 1.1                  |
| Ascorbate            | n.d.                 | 2.2                  |
| o-Dianisidine        | n.d.                 | 727                  |
| Anilinyl             | n.d.                 | 3.5                  |

a: Not detectable.
b: Conditions: 50 mM phosphate buffer, pH 7, and 15 °C. Compound I was formed with peroxyacetic acid.
c: Conditions: 50 mM phosphate buffer, pH 7, and 15 °C. Compound I was formed with hydrogen peroxide.
The plots of the pseudo-first order rate constants of formation of conventional compound I versus different concentrations of hydrogen peroxide (Fig. 5C) and peroxoacetic acid (Fig. 5D) were linear, and the calculated rate constants for compound I formation were $(1.2 \pm 0.1) \times 10^6$ M$^{-1}$ s$^{-1}$ and $(3.1 \pm 0.1) \times 10^5$ M$^{-1}$ s$^{-1}$, respectively (Table II). Thus, the peroxoacetic acid-mediated reaction was similar with both Arg439 and Met275 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 Figs. 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 $1.1 \times 10^5$ 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 enzyme 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; gray 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, 12, 19, 47 ms. After 47 ms compound I was reached. The reaction showed clear isosbestic points at 395 and 520 nm. Conditions as in A. E, decay of 5 μM compound I (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, 5, 7, and 10 s. Isosbestic points were at 360, 430, and 526 nm.
a biphasic behavior with a fast initial phase followed by a slower second phase. Fig. 6 shows the spectral changes after adding 2 mM ascorbate to a preformed compound I of R439N (2 \mu M). Compound I is in 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 s) is in gray. The inset of Fig. 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 II 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 (Fig. 6B) and plotting the pseudo-first order rate constants versus substrate concentration (Fig. 6C). In a typical experiment 4 \mu M ferric enzyme were mixed with either 200 \mu M 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 II). The hierarchy in reactivity toward the three investigated electron donors (ascorbate, \( \text{H}_2\text{O}_2 \), and peroxoacetic acid) was not changed compared with wild-type KatG, but the apparent bimolecular rate constants were significantly increased (Table II).

When M275I compound I (formed by mixing 5 \mu M M275I with 50 \mu M hydrogen peroxide; bold spectrum in Fig. 7A) was allowed to react with 500 \mu M ascorbate, a redox intermediate was formed within 1 s with absorbance maxima at 414, 542, and 575 nm (Fig. 7A). This transient intermediate (bold spectrum in Fig. 7B) was reduced back to the ferric enzyme. Both transitions exhibited clear isosbestic points at 400 nm (compare with compound I of M275I, Fig. 7B). The spectral features of the observed intermediate were very similar to those obtained when compound I of M275I formed by hydrogen peroxide decayed in the absence of a one electron donor (Fig. 7E). However, this transition was slow compared with compound I reduction mediated by ascorbate. Fig. 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 biphastic, 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 Fig. 7C). Fitting the first exponential phase and plotting the pseudo-first order rate constants versus ascorbate concentrations yielded apparent bimolecular rate constants of $2.2 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ (compound I preformed with hydrogen peroxide, Fig. 7D) and $1.6 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ (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 $10^2 \text{M}^{-1} \text{s}^{-1}$. 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, 542, and 575 nm (Fig. 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 s, but it was transformed to ferric M275I when adding ascorbate (Fig. 7F). The kinetics of this transition was very similar to that described in Fig. 7C, underscoring 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 (4 K) 9-GHz EPR signal of the wild-type Synechocystis catalase-peroxidase in the native state (13). The two main resonances observed at $g_{xx} = 1.99$, $g_{yy} = 5.93$, and $g_{zz} = 6.57$, and $g_{eff} = 5.10$, and $g_{eff} = 1.69$ are characteristic of pentacoordinated heme iron enzymes ($S = 5/2$) in the high-spin state. The EPR spectrum of the wild-type enzyme (Fig. 8, top) was assigned to two predominant Fe(III) high-spin species, represented by the superposition of an axial signal (effective $g$ values of $g_{xx} = 5.93$ and $g_{yy} = 1.99$) and a rhombically distorted ($g_{xx} = 5.93$, $g_{yy} = 5.10$, and $g_{eff} = 1.97$) signal (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 (Fig. 8, top). In the case of 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).

Fig. 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 Fig. 8, middle) and (b) the inversion of the pH dependence on the M275I ferric signals as compared with the wild-type enzyme (see Fig. 8, top). Similar distinct differences were previously reported for the proximal tryptophan (W341F) and the distal histidine (H123Q) variants, respectively (13). The M275I and R439AN 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 Fig. 1 in Ivancich et al., Ref. 13).

The EPR Characterization of Radical Intermediates—EPR spectroscopy was further used to precisely characterize the electronic structure of the radical intermediates formed by the Tyr$^{249}$, Met$^{275}$, and Arg$^{439}$ 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 [Fe(IV) = O por$^-$], typical for monofunctional catalases and peroxidases, was partially trapped in the time course of 10 s (the mixing was performed at 0 °C), as well as a tyrosyl (Tyr$^*$) and a tryptophanyl (Trp$^*$) radical species. The 9-GHz EPR spectra of the M275I, R439A, and R439N variants after reaction with peroxoacetic acid, showed the broad axial signal (total width of $\sim 2000 G$) 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 Figs. 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. Fig. 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 R439AN variants with a 10-fold excess of peroxoacetic acid (Fig. 9, middle) were identical to that observed for the wild-type enzyme (Fig. 9, top). 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 Fig. 4 in Ivancich et al., Ref. 13).

Fig. 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 peroxoacetic 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 Fig. 4 in Ref. 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 peroxoacetic acid. The 9 GHz EPR spectra of this variant (Fig. 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 agreed well 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 catalytic activity in the Y249F, M275I, and R439AN variants (Table I) 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 (Fig. 9, dotted trace) were narrower than those obtained by using peroxoacetic acid (Fig. 9, solid traces). Deuterium label-
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 Fig. 9, except for the sample concentration.

Discussion

Structural Changes Induced by the Mutations Related to the Cross-linked 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 (Trp122, Tyr249, and Met275 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 similar 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 (Trp122) being part of this unusual adduct (Fig. 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 (Met275), has been postulated (6). In addition, the structure of B. pseudomallei KatG showed that a conserved arginine (Arg426, corresponding to Arg439 in Synechocystis) exists in two orientations (6); in the predominant form (Fig. 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 underscore 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 Trp122 or Tyr249 prevents the formation of the covalent adduct, whereas exchange of Met275 still allowed bond formation between Trp122 and Tyr249 (7). In this work, we have demonstrated that both Arg439 variants do not affect the linkage between Trp122, Tyr249, and Met275 existing in the wild-type enzymes in solution. Moreover, the EPR spectra of the Arg439 variant in frozen solution further confirmed that no H-bond was formed between Arg439 and the amino acids Tyr249 and Met275, because 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 Arg439 variant should have been detected if Arg439 is a H-bond donor to Tyr249 and Met275.

As observed form 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 Arg439 variants showed that the only difference with the wild-type enzyme is the contribution of a higher proportion (about 20% as compared with 5% in the wild-type KatG) of the 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 Trp122 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 Tyr249, which (as shown by our previous RR
studies) weakens the heme binding, most probably as a result of a readjustment of the KatG-typical (Tyr^{249} containing) LL1 loop at the heme edge (18), but only slightly affects the binding of fluoride to the iron atom as well as its H-bond interaction with Trp^{122} (18). The EPR studies in this work specifically showed that the effect of the mutation on Tyr^{249} is less dramatic than that induced by the Trp^{122} variant (13). It can be explained by a milder repositioning of the distal Trp induced by the disruption of the covalent link between Trp^{122} and Tyr^{249}, 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 Trp^{122} and Tyr^{249} does not significantly perturb the relative orientation of the Trp^{122} and the heme. However, exchange of Met^{275} 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 inverse compared with the wild-type enzyme (Fig. 8). A similar dramatic effect was observed when the histidine (His^{123}) was exchanged to a glutamine (13). The exchange of Met^{275} still allowed the formation of an adduct involving Trp^{122} and Tyr^{249} (7) that would undergo a dramatic repositioning induced by the absence of the covalent link between Met^{275} and the partially formed adduct. Mutation of Met^{275} still allows fluoride binding to some extent (18). Similarly, the kinetics of cyanide binding to ferric M275I and both Arg^{439} variants closely resembles 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 Cross-linked 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 cross-link 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 Figs. 4 and 7), make it impossible to monitor the formation of protein-based radical intermediates in catalase-peroxidases 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 would be underneath the α and β heme bands at ~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 to 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 discerned 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 Fig. 6 in Ref. 11, and Figs. 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 compared with 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. Because the behavior of the ferric heme EPR spectrum of the Y249F variant (Fig. 8) was the same as that of the W341F variant (13), the relatively lower yield of Trp’ in the Y249F variant can be attributed to a mild repositioning of the distal tryptophan residue (Trp^{122}) induced by the absence of the covalent link between Trp^{122} and Tyr^{249} in this variant (7), without disruption of the extensive hydrogen-bond network. A different situation was observed when exchanging the Met^{275} 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 (Fig. 8). Most probably, the exchange of the Met^{275} by an isoleucine induced a substantial repositioning of the remaining Trp^{122}–Tyr^{249} 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 peroxoacetic 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 compared with those of the R439A, R439N, and Y249F variants (see Table II), 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 cross-linked tyrosine residue in *Mycobacterium 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 compared with the wild-type *M. tuberculosis* enzyme was observed by Magliozzo and co-workers (see Fig. 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 cross-linked tyrosine residue is not the site for the Tyr’ formation either in *Synechocystis* or 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, 542, and 575 nm attributed to a compound III-type species. Since this intermediate has been proposed based on the changes in the
of Trp<sup>122</sup>, Tyr<sup>249</sup>, or Met<sup>275</sup> mainly affect <i>k</i><sub>2</sub>. But this scenario appears to be much too simplified since many of our findings would remain unexplained: (i) Wild-type compound I produced with peroxyacetic acid has the typical spectral features of an oxoferryl porphyrin π-cation radical species but reacts extremely slow with H<sub>2</sub>O<sub>2</sub> as demonstrated by the sequential-stopped flow technique (10); (ii) in wild-type Synecocystis catalase-peroxidase, the chemical nature of the intermediate referred to 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 (Figs. 4 and 5) and compound II (Figs. 6 and 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 the case of catalase-peroxidases the standard reduction potential of the ferric/ferrous couple of <i>M. tuberculosis</i> 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 <i>Syneecocystis</i> 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. O<sub>2</sub> production) it is not a thermodynamic requirement since $E^\circ$ (O<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>) = 280 mV (28).

Nevertheless, the classical compound I formed by mixing of wild-type KatG with peroxyacetic acid reacts only very slowly with H<sub>2</sub>O<sub>2</sub>. Because 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 toward H<sub>2</sub>O<sub>2</sub> 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 toward 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 toward H<sub>2</sub>O<sub>2</sub> is impaired and the conventional compound I can accumulate even in the presence of H<sub>2</sub>O<sub>2</sub>.

The electronic structure and the visible spectrum of this postulated catalatically reactive compound I is unknown as is its contribution to the observed spectral transitions in the Fig. 4, C and D as well as 5A. The spectrum of the intermediate, which was observed upon incubation of both Arg<sup>439</sup> variants with excess hydrogen peroxide (bands at 414, 542, and 572 nm; Fig. 4, C and D), was very similar to that of the hexacoordinated low-spin species obtained upon mixing of hydroxylamine with <i>Athromyces ramosus</i> peroxidase (29). This was interpreted as a structural model for compound 0, normally difficult to observe because its formation is followed by the rapid heterolytic cleavage of hydrogen peroxide.

However, this rapidly formed intermediate of both Arg<sup>439</sup> variants was also similar to the intermediate that was ob-
observed when the classical compound I of M275I decayed (414, 542, and 575 nm; Fig. 5A) and to the intermediate that was formed from Y249F compound I via compound II in the absence of one electron donors (11). In the case of both Arg439 variants the appearance of this intermediate was monitored spectroscopically before the classical compound I, whereas in the 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.

An interesting observation was 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 Fig. 5, A and B). 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 reoxid 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 o xoferryl-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 o xoferryl 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 (including both Arg439 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 because the endogenous donor is still unknown. If one electron donors and H2O2 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 of M275I. This is best underscored 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 than 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 (Fig. 6A), whereas compound II formed from M275I classical compound I (preformed with H2O2) upon addition of one electron donors has absorption bands at 414, 542, 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 H2O2, 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 II). Under the assumption that the protein radical compound II has spectral features similar to ferric KatG and taking 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 was a red-shift observed: in the 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 that showed a significantly increased overall peroxidase activity (Table I). In addition to R439A/N, it shows 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 Met275 and Arg439 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 Arg439 variants and that the pH-profile was affected in the 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 reoxid intermediates. The contribution to protein stability is mainly by anchoring a KatG-specific loop to the molecular surface via Tyr249 and by participation in the extensive hydrogen-bond network on the distal side of the heme, involving Trp122, His123, Arg119, several structural waters, the heme 6-propionate group, and Trp106; the latter being the site of the tryptophanyl radical (13). Disruption of this network by mutagenesis mainly affects H2O2 oxidation although not H2O2 reduction during compound I formation. Concomitantly, the tryptophanyl radical (on Trp106) 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 for understanding the role of enzyme pathways in peroxidases and catalases and the mechanism of catalase activity of a heme protein, which is not well understood even in the case of monofunctional catalases (28, 31, 32).

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