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Anabella Ivancich, Christa Jakopitsch, Markus Auer, Sun Un, and Christian Obinger

J. Am. Chem. Soc., **2003**, 125 (46), 14093-14102• DOI: 10.1021/ja035582+ • Publication Date (Web): 24 October 2003

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Published on Web 10/24/2003

Protein-Based Radicals in the Catalase-Peroxidase of *Synechocystis* PCC6803: A Multifrequency EPR Investigation of Wild-Type and Variants on the Environment of the Heme Active Site

Anabella Ivancich,*,† Christa Jakopitsch,‡ Markus Auer,‡ Sun Un,† and Christian Obinger‡

Contribution from the Service de Bioénergétique, URA 2096 CNRS, Département de Biologie Joliot-Curie, CEA Saclay, 91191 Gif-sur-Yvette, France, and Institute of Chemistry, BOKU, University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria

Received April 11, 2003; E-mail: ivancich@dsvidf.cea.fr

Abstract: Catalase-peroxidases are bifunctional heme enzymes with a high structural homology to peroxidases from prokaryotic origin and a catalatic activity comparable to monofunctional catalases. These unique features of catalase-peroxidases make them good systems to study and understand the role of alternative electron pathways both in catalases and peroxidases. In particular, it is of interest to study the poorly understood role of tyrosyl and tryptophanyl radicals as alternative cofactors in the catalytic cycle of catalases and peroxidases. In this work, we have used a powerful combination of multifrequency EPR spectroscopy, isotopic labeling of tryptophan and tyrosine residues, and site-directed mutagenesis to unequivocally identify the reactive intermediates formed by the wild-type *Synechocystis* PCC6803 catalase-peroxidase. Selected variants of the heme distal and proximal sides of the *Synechocystis* enzyme were investigated. Variants on the aromatic residues of the short stretch located relatively close to the heme and spanning the distal and proximal sides were also investigated. In the wild-type enzyme, the EPR signal of the catalases and peroxidases (typical) Compound I intermediate [Fe(IV)═O por$^{\bullet+}$] was observed. Two protein-based radical intermediates were also detected and identified as a Tyr$^{\bullet}$ and a Trp$^{\bullet}$. The site of Trp$^{\bullet}$ is proposed to be Trp 106, a residue belonging to the conserved short stretch in catalase-peroxidases and located at a 7−8 Å distance to the heme propionate groups. An extensive hydrogen-bonding network on the heme distal side, involving Trp122, His123, Arg119, seven structural waters, the heme 6-propionate group, and Trp106, is proposed to have a key role on the formation of the tryptophanyl radical. We used high-field EPR spectroscopy (95−285 GHz) to resolve the *g*-anisotropy of the protein-based radicals in *Synechocystis* catalase-peroxidase. The broad g_x component of the HF EPR spectrum of the Tyr$^{\bullet}$ in *Synechocystis* catalase-peroxidase was consistent with a distributed electropositive protein environment to the tyrosyl radical.

Introduction

Catalase-peroxidases constitute a family of heme enzymes that arose from gene duplication of an ancestral hydroperoxidase.[1] These enzymes are responsible for the disproportionation of hydrogen peroxide in the cell. Consequently, they are directly involved in the regulation of cellular oxidative stress. Comparisons of amino acid sequence showed a low homology of catalase-peroxidases with respect to catalases[2] but a high homology to peroxidases of prokaryotic origin.[3] In particular, the structural-based sequence alignment of the *Haloarcula marismortui* catalase-peroxidase shows a high homology to cytochrome *c* peroxidase (CcP) and ascorbate peroxidase.[4]

The cumbersome name given to this family of heme enzymes originates from their double function. From the activity point of view, catalase-peroxidases are typical peroxidases with broad substrate specificity.[5,6] In addition, they show an unusual feature for peroxidases, that is a high turnover rate of hydrogen peroxide disproportionation, comparable to monofunctional catalases. The different specificity toward hydrogen peroxide has been correlated to structural differences between catalases and peroxidases in relation to the (substrate) accessibility to the heme active

* Author to whom correspondence should be addressed: Centre d'Etudes de Saclay. DBJC/Service de Bioénergétique, Bat. 532, 91191 Gif-sur-Yvette, France.
† CEA Saclay.
‡ University of Natural Resources and Applied Life Sciences.

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site (see, for example, ref 7). The recently solved crystallographic structures of the catalase-peroxidases from *H. marismortui*[4] and *Burkholderia pseudomallei*[8] showed that the N-terminal domain contains the heme active site[9] and superimposes well with cytochrome *c* peroxidase, the differences being at the level of amino acid insertions located on the surface of the domain. In particular, a short stretch of residues (residues 74 to 83 in *H. marismortui* numbering[4]) that is highly conserved in all catalase-peroxidases[1] does not exist in ascorbate peroxidase and is partially present in CcP, although with substantially different amino acid residues in the latter. This short stretch is close to the heme propionates and makes interactions to residues placed both on the distal and the proximal heme pocket. The crystal structures of *H. marismortui* and *B. pseudomallei* catalase-peroxidase confirmed that the active site consists of an iron-protoporphyrin IX prosthetic group, the iron being pentacoordinated (via a histidine residue) in the resting state. Moreover, the catalytically essential residues on the proximal and distal heme sides of peroxidases are highly conserved (also from the point of view of spatial arrangement) in *H. marismortui* and *B. pseudomallei* catalase-peroxidases. The electrostatic environment of the triad (His-Asp-Trp) of the proximal side is very similar to that of CcP.[4,8]

A common property of peroxidases and catalases is the formation of a highly oxidizing intermediate, so-called Compound I, that catalyzes the oxidation of various substrates in peroxidases or that reacts with hydrogen peroxide in the case of catalases (for a review, see ref 10). This intermediate originates from the two-electron oxidation of the native enzyme by hydrogen peroxide that generates the oxoferryl moiety (Fe(IV)═O) and the concomitant formation of a porphyrin π-cation radical (por$^{\bullet+}$) or a protein-based radical species. A tryptophanyl radical, exchange-coupled with the oxoferryl moiety [Fe(IV)═O Trp$^{\bullet+}$] and located on the proximal-side residue (Trp 191) , was identified as the relevant intermediate for the substrate oxidation in cytochrome *c* peroxidase.[11] A tryptophanyl radical, located close to the enzyme surface, has been identified as the oxidation site for veratryl alcohol in lignin peroxidase.[12,13] The formation of an oxoferryl-tyrosyl radical intermediate [(Fe(IV)═O Tyr$^{\bullet}$] by means of an intramolecular electron transfer between the porphyrin cofactor and a tyrosine residue has been observed in catalases,[14] peroxidases,[15,16] and prosthaglandin H synthase.[17,18] The formation of a tyrosyl (or tryptophanyl) radical intermediate on a specific Tyr (Trp) residue strongly suggests that tyrosine (or tryptophan) residues can play the role of alternative oxidation sites both in catalases and peroxidases.

The fact that catalase-peroxidases are considered as ancestors of hydroperoxidases renders this family of heme enzymes an extremely appealing system to understand better alternative electron pathways in catalases and peroxidases, involving tyrosines and tryptophans as oxidation sites. The catalytic intermediates formed by catalase-peroxidases have been studied by using steady-state and stopped-flow UV−vis absorption spectroscopy in the catalase-peroxidases from *Escherichia coli*,[19] *Mycobacterium tuberculosis*,[20] and cyanobacteria (*Synechocystis* PCC6803[21,22] and *Anacystis nidulas*[23]). Mutation of the distal-side histidine and arginine residues had an effect similar to those previously observed in peroxidases.[19,22] Mutation of the distal tryptophan (W122F in *Synechocystis* PCC6803) that is part of the catalytically essential triad Arg-Trp-His reduced dramatically the catalase activity of catalase-peroxidases.[19,22] Characterization of the electronic nature of the intermediates by conventional EPR spectroscopy has been reported on the *E. coli*[19] and the *M. tuberculosis*[20] enzymes. The observed 9-GHz EPR signal of the Compound I intermediate in *E. coli* catalase-peroxidase was attributed to an oxoferryl-porphyrin radical[19] [Fe(IV)═O por$^{\bullet+}$]. In the case the *M. tuberculosis* enzyme, the EPR signal has been recently reassigned to a tyrosyl radical.[24]

In this work, we have investigated the radical species formed in wild-type and selected variants of *Synechocystis* PCC6803 catalase-peroxidase. To understand better the reaction cycle of the enzyme, the first step consists of the accurate identification of the electronic nature of the different radical intermediates. This is an important issue for understanding the relationship between the different intermediates, the substrate specificity, and the alternative binding sites in this enzyme. Because of the overwhelming absorption bands of the heme in the electronic absorption spectra of the oxoferryl-porphyrin radical intermediate, it is not possible to distinguish the formation of protein-based radical(s). A key approach presented in this work is the use of multifrequency EPR spectroscopy combined with isotopic labeling of tyrosine and tryptophan residues, on the wild-type and variants of catalase-peroxidase from *Synechocystis* PCC6803. We were able to identify three different radical species: a porphyrin radical exchange-coupled to the oxoferryl moiety (the so-called Compound I) and two protein-based radicals (tryptophanyl and tyrosyl radicals). Selected tryptophan mutations were designed and characterized by multifrequency EPR spectroscopy combined with deuterium-labeling experiments. We propose Trp 106 as the radical site. On the basis of the structural changes induced by the distal side mutations and that were inferred from the EPR spectral changes on the ferric enzymes, the existence of an extensive hydrogen-bonding network on the heme distal side (including Trp 122, His 123,

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Arg 119, seven structural water molecules, the heme 6-propionate group) and extending to Trp 106 is proposed. Such a hydrogen-bonding network appears to have an unprecedented importance in relation to the formation of the tryptophanyl radical.

Materials and Methods

Materials. The perdeuterated tryptophan (DL-Trp d_8), perdeuterated tyrosine (DL-4-hydroxyphenyl-d_4-alanine-2,3,3-d_3) and the partially deuterated tyrosine (DL-4-hydroxyphenylalanine-3,3-d_2) were purchased from CDN Isotopes. l-4-Hydroxyphenyl-3,5-d_2-alanine was obtained from Isotech Inc. The isotopic purity was 98% in all cases.

Sample Preparation. Mutagenesis. Oligonucleotide site-directed mutagenesis was performed using PCR-mediated introduction of silent mutations as described previously.[22] A pET-3a expression vector that contained the cloned catalase-peroxidase gene from the cyanobacterium *Synechocystis* PCC6803[21] was used as the template for PCR. The deletion of the short stretch (residues 103 to 113 in *Synechocystis* numbering) was done via PCR with overlapping primers. All constructs were sequenced to verify DNA changes using thermal cycle sequencing. Expression and purification from nondeuterated *Synechocystis* wild-type KatG and variants were described previously.[21,22]

Isotope labeling. *E. coli* DL39W cells, auxotroph for tyrosine and tryptophan, were obtained from the *E. coli* Genetic Stock Center at Yale University. The *E. coli* cells were treated with the λDE3 Lysogenization Kit (Novagen) to obtain DL39W(DE3) cells and a pET-3a vector containing the sequences of wild-type or mutated *katG*s was transformed into those cells. To produce specifically labeled KatG on tyrosines and/or tryptophans, the *E. coli* DL39W(DE3) cells were grown in a defined M9 medium as previously described.[25] Cells were grown to an $OD_{600} = 1$ and expression was induced by isopropyl β-thiogalactoside (IPTG). At the time of induction, hemin (40 mg/L) was added to the media. Twenty hours after induction the cells were harvested. Isolation and purification of the specifically deuterated KatGs was done as described previosly for the nondeuterated samples.[21,22]

EPR Samples. Native enzyme in 0.1 M TRIS/maleate buffer was used for the pH titration experiments on the native (ferric) state. Typically, the Compound I samples were prepared by mixing manually 2.0 mM native enzyme (0.1 M TRIS/maleate buffer, pH 8.0) with a 10-fold excess of buffered peroxyacetic acid solution (final pH 4.5), 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 10 s for the wild-type enzyme and 5 s for the variants. The peroxyacetic acid concentration and the mixing time used in these experiments were those providing the higher yield on the narrow (protein-based) radical signal. No detectable differences in the relative contribution of the tyrosyl and tryptophanyl radicals to the high-field EPR spectrum were observed for the wild type enzyme in samples prepared with mixing times of 5 or 10 s. The use of lower excess of peroxyacetic acid induced the formation of the same EPR signal except that the yield was lower and scaled inversely with the conversion of the ferric iron signal.

EPR Spectroscopy. Conventional 9-GHz EPR measurements were performed using a Bruker ER 300 spectrometer with a standard TE_{102} cavity equipped with a liquid helium cryostat (Oxford Instrument) and a microwave frequency counter (Hewlett-Packard 5350B). The home-built high-field EPR spectrometer (95−285 GHz) has been described elsewhere.[26] The absolute error in g-values was 1×10^{-4}. The relative error in g-values between any two points of a given spectrum was 5×10^{-5}.

Results

The 9-GHz EPR Spectrum of the Ferric *Synechocystis* Catalase-Peroxidase: Wild-Type and Variants. EPR signals with two main resonances at $g^{\text{eff}}_{\perp} \approx 6$ and $g^{\text{eff}}_{||} \approx 2$ are characteristic of pentacoordinated heme iron enzymes ($S = 5/2$) in the high spin state. Differences in the coordination environment of the heme iron in catalases and peroxidases can be monitored by the extent of the rhombic distortion on the EPR signal, reflected by the difference between the g_x and g_y components of the **g**-tensor. Figure 1A (bottom) shows the 9-GHz EPR spectrum of the ferric form of *Synechocystis* catalase-peroxidase recorded at 4 K. Not surprisingly, this spectrum is very similar to the previously reported low-temperature EPR spectrum of the *M. tuberculosis* enzyme.[27] The complex pattern in Figure 1A (bottom) could be interpreted as the contributions from at least two different Fe(III) high-spin species represented by an axial signal with effective g-values of $g_{A_\perp} = 5.93$ and $g_{A_{||}} = 1.99$ and a rhombically distorted signal with $g_{B_x} = 6.57$, $g_{B_y} = 5.10$ and $g_{B_z} = 1.97$. Expansion of the $g \approx 6$ region of the EPR spectrum of the ferric enzyme is shown in the inset, with the axial and rhombic signals labeled as A and B, respectively. The pH variation in the relative intensity of the signals between pH 5.6 and 8.3 constitutes further support to the assignment to these signals (Figure 1A, inset). It is noteworthy that the catalase-peroxidase variants on the distal and proximal heme sides as well only one of the Trp mutants belonging to the short stretch (W106A) showed no pH dependence of the predominantly axial signal (see Supplementary Information). Simulations of the EPR spectrum of the *M. tuberculosis* enzyme allowed Rusnak and co-workers to propose a contribution of a third rhombic signal.[27] The shoulders to the axial signal in the EPR spectrum of *Synechocystis* catalase-peroxidase with approximate g^{eff}-values of $g_x = 6.05$ and $g_y = 5.73$ (see arrows in Figure 1A, inset) are consistent with such a signal. Multiple heme conformations were previously reported for the W175G variant in cytochrome *c* peroxidase (CcP).[28] The W175G variant (in the presence of 20 mM imidazole at pH 6.0) showed three ferric signals with different rhombicity and a pH-dependent relative intensity.[28]

The 9-GHz EPR Spectra of the Radical Species in *Synechocystis* Catalase-Peroxidase. Figure 1A (top) shows the 9-GHz EPR spectrum of the radical species obtained after the reaction of *Synechocystis* catalase-peroxidase with peroxyacetic acid. A narrow signal at $g \approx 2$, indicative of an isolated organic radical, was observed. The presence of a low proportion of the ferric signal arising from the native enzyme was attributed to either nonreactive enzyme or most probably to native enzyme that cycled back. A much broader (about 2000 G) and axial signal was also observed (Figure 1B). This broad signal with effective g-values of 2.35 and 2.00 for $g_\perp$ and $g_{||}$, respectively, was consistent with an oxoferryl-porphyrin radical intermediate such as those previously reported for catalases, peroxidases, and heme model complexes (see ref 16 and references therein). To facilitate the comparison, the [Fe(IV)═O por$^{\bullet+}$] intermediate spectrum of turnip peroxidase (Figure 1B, dashed trace) is overlapped to the broad EPR signal in catalase-peroxidase (Figure 1B, solid trace). The spectrum of the intermediate formed by the R119A variant is also shown for comparisons (Figure 1B, bottom). In this variant, the narrow-radical EPR signal as well as an EPR signal characteristic of a low-spin ferric

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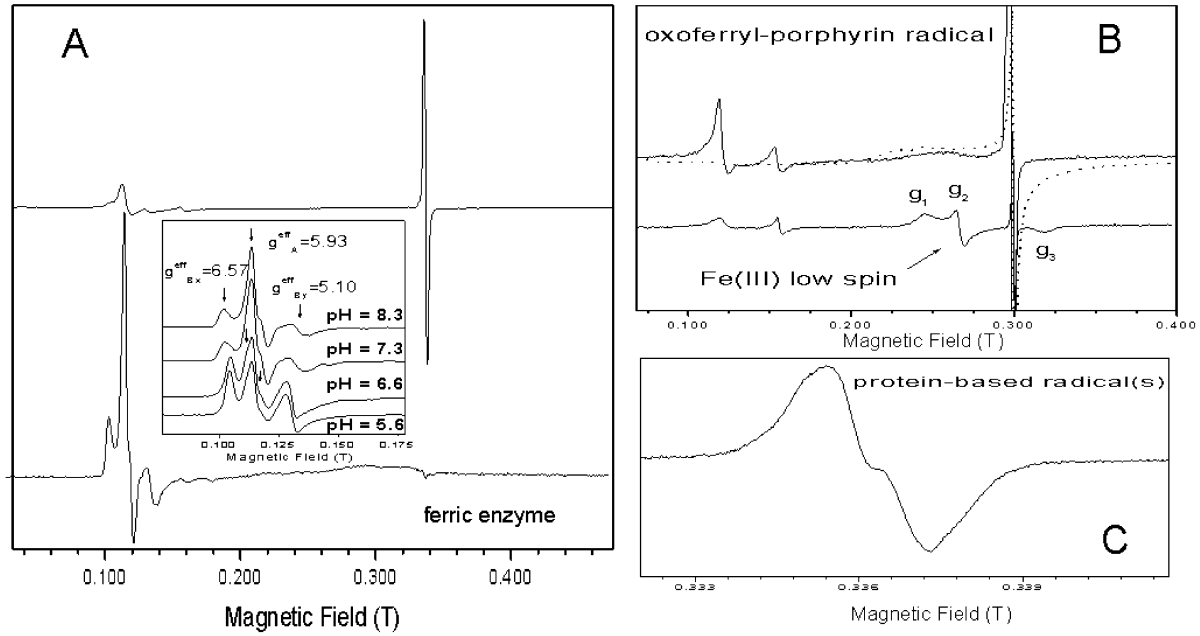

Figure 1. (A) The 9-GHz EPR spectra of wild-type *Synechocystis* PCC6803 catalase-peroxidase in the resting state (bottom) and after mixing with peroxyacetic acid (top). Spectra were recorded at 4 K, 5 G modulation amplitude, 1 mW microwave power, 100 kHz modulation frequency. Inset: the pH dependence ($5.6 \leq$ pH ≤ 8.3) of the Fe(III) high spin signals. (B) The broad radical spectrum of wild-type *Synechocystis* PCC6803 catalase-peroxidase (top). The [(Fe(IV)═O) por$^{\bullet+}$] intermediate of turnip peroxidase (dashed trace) and the narrow radical species of the *Synechocystis* R119A variant (bottom) are also shown. (C) The protein-based radical(s) spectrum of *Synechocystis* PCC6803 catalase-peroxidase recorded at 60 K, 0.05 mW microwave power and 0.5 G modulation amplitude.

iron species with effective g-values of $g_1 = 2.48$, $g_2 = 2.27$, and $g_3 = 1.88$ could be detected (Figure 1B, bottom). Signals with similar g-values to the latter, characteristic of weak-field ligands, were previously reported for other peroxidases[29,16] and cytochrome $P450_{cam}$[30] and were attributed to a water (or hydroxy) molecule acting as a sixth ligand to the heme. The clear spectral differences between the oxoferryl-porphyrinyl radical signal in wild-type catalase-peroxidase and the low-spin hexacoordinated heme signal in the R119A variant (see Figure 1B) allowed us to rule out the possibility that the broad feature observed in the wild-type enzyme spectrum originated from low-spin hexacoordinated heme. Moreover, the low spin hexacoordinated heme contribution was estimated to be less than 5% in the wild-type catalase-peroxidase samples (Figure 1A). A very low proportion of another hexacoordinated low-spin signal with effective g_{max} value of 2.9 was observed in most of the variants, with the exception of the W106A and the del[103−113] variants. Such g_{max} values are typical for strong ligand-field donors[32] and may arise from the rearrangement of the distal side residues (see, for example, ref 33). Since the H123Q variant also showed the contribution of this low-spin signal, it is tempting to discard the possibility of a bis-histidine heme iron complex.

Figure 1C shows the narrow organic radical spectrum (9-GHz EPR) recorded in nonsaturating conditions, i.e., $T = 60$ K and microwave power of 50 μW. The overall width of the spectrum is about 75 G and the peak-to-trough width is 19 G with an effective isotropic g-value of 2.004. Temperature dependence studies between 4 and 60 K showed no changes in the width of the EPR spectrum, unlike the tryptophanyl radical intermediate in cytochrome *c* peroxidase (see Figure 2). Thus, the EPR spectrum recorded at 60 K represents all the detectable radical signal. Spin quantitation of this signal resulted in 0.7 spin/heme. Similar spectra were reported for protein-based radical species formed in other enzymes. Nevertheless, the criteria of similarity in shape and relaxation behavior of the signal obtained with conventional EPR measurements is not sufficient to assign the protein-based radical species. Accord-

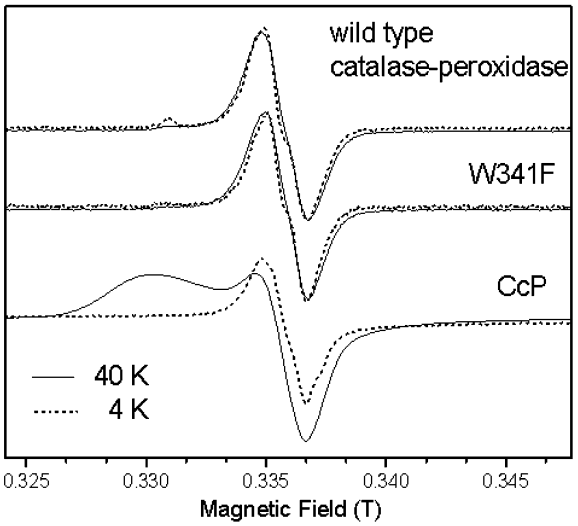

Figure 2. The 9-GHz EPR spectra of protein-based radicals in wild-type and the W341F variant of *Synechocystis* PCC6803 catalase-peroxidase. The exchange-coupled tryptophanyl radical intermediate of cytochrome *c* peroxidase (CcP) is plotted for comparisons. The spectra recorded at 40 K were scaled to the intensity of the 4 K spectra.

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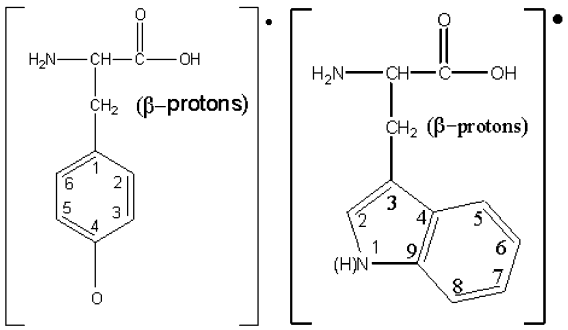

Figure 3. Molecular structure and numbering scheme used in the text for tyrosyl and tryptophanyl radicals.

ingly, we have used selectively deuterated tyrosines and tryptophans in order to unequivocally asses the chemical nature of the protein-based radical(s) in *Synechocystis* catalase-peroxidase. Figure 3 shows the numbering scheme for tyrosyl and tryptophanyl radicals used all through the text. Figure 4 (left panel) shows the 9-GHz EPR spectra of the narrow radical intermediate(s) in wild-type catalase-peroxidase containing perdeuterated tryptophans (A) or specifically deuterated tyrosines (B, C, D). Perdeuterated tryptophans and tyrosines as well as partially deuterated tyrosines (on the 3,5 phenol-ring protons (B) or the β-methylene protons (C), see scheme in Figure 3) were taken in by the *E. coli* DL39W cells auxotrophic for tryptophan and tyrosine residues (see Materials and Methods section). Changes in the EPR radical signal of the Tyr-deuterated or the Trp-deuterated enzymes were expected to be observed provided that a tyrosine or a tryptophan residue were the radical site. The expected change in the unresolved Gaussian line width (ΔH) of the radical EPR spectrum in the deuterium-labeled samples as compared to the fully protonated spectrum is given by $\Delta H(^1\mathrm{H}) = [\gamma(^1\mathrm{H})/\gamma(^2\mathrm{H})]\ \sqrt{3/8}\ \Delta H(^2\mathrm{H})$, where γ denotes the gyromagnetic ratio of protons and deuteriums.

The changes observed on the EPR spectra of the protein-based radicals in catalase-peroxidase containing partially and fully deuterated tyrosine residues (Figure 4B, 4C, 4D) were the same as those previously reported for deuterium-labeled tyrosyl radicals generated by photolysis in frozen solution as well as those of Tyr_D and Tyr_Z radicals in Photosystem II.[34,35] Specifically, upon deuteration of the phenol-ring protons at positions 3,5 a doublet spectrum was observed (Figure 4B, solid trace). Deuteration of the β-methylene protons removed the splitting and the resulting narrower spectrum had a peak-to-trough width of 10 G (Figure 4C). Finally, the perdeuterated-Tyr showed a single line spectrum with a peak-to-trough width of 8 G (Figure 4D). As expected, the EPR spectrum of the fully perdeuterated sample (perdeuterated on both Tyr and Trp residues, Figure 4E) was even narrower (peak-to-trough of 6 G). The changes in the EPR spectrum induced by using perdeuterated-Trp (Figure 4A, solid trace) clearly showed that a tryptophanyl radical also contributed to the fully protonated EPR signal (Figure 4A, dashed trace) and that such contribution was mainly visible on the wings of the spectrum. This can be demonstrated by subtracting the EPR spectrum of the fully protonated sample (spectrum A, dashed trace) and that of the perdeuterated-tryptophan sample (spectrum B, solid trace). The resulting spectrum (Figure 4, inset) is that of the (protonated) tryptophanyl radical with the contribution of the perdeuterated-tryptophanyl radical as the narrow feature in the middle of the spectrum.[37] Another way of obtaining the tryptophanyl radical spectrum is by subtraction of the EPR signals of the perdeuterated-tyrosine sample (spectrum D) and the fully perdeuterated (both on tyrosine and tryptophan) sample (spectrum E).[37] Both spectra resulting from the subtractions (Figure 4, inset) showed the contribution of the tryptophanyl radical spectrum as shoulders to the tyrosyl radical spectrum (see arrows in Figure 4, inset).

The 9-GHz EPR Spectra of the Protein-Based Radical Species in Selected Variants of *Synechocystis* Catalase-Peroxidase. A series of variants were studied by EPR in order to correlate the effect of such mutations and the nature of the protein-based radical species formed by the enzyme. The deuterium-labeling approach used for the wild-type enzyme (see above) was absolutely necessary for studying each of the *Synechocystis* catalase-peroxidase variants since, as we have just demonstrated the EPR signal in catalase-peroxidase is a superposition of two protein-based radicals, a tyrosyl radical and a tryptophanyl radical. Such species cannot be easily distinguished by 9-GHz EPR measurements.[38] Three sets of catalase-peroxidase variants were characterized by EPR in this work: (a) the heme distal-side mutations on Trp122, His123, and Arg119; (b) the heme proximal-side mutant on Trp341; and (c) the short stretch mutations on Trp105, Trp106, Trp110, Tyr113, and del[103−113] (deletion of residues 103 to 113).

Figure 4 (right panel) shows the 9-GHz EPR spectra of the protein radical intermediates formed by selected variants of catalase-peroxidase. For each variant, the EPR spectrum of the nondeuterated sample is plotted together with the EPR spectra of the Trp-perdeuterated (dashed trace) and the Tyr-perdeuterated (dotted trace) samples. The EPR spectra of the proximal tryptophan mutant (W341F) was identical to wild-type, with no temperature dependence of the width of the EPR signal (see Figure 2). More importantly, the spectral changes on the EPR signal due to the perdeuteration of tryptophan residues on this mutant were also the same as for the wild-type enzyme (Figure 4, left panel). This result clearly indicated that the tryptophanyl radical was formed in the W341F variant despite the mutation. By contrast, the EPR spectra of all three distal-side mutants (W122F, H123Q, and R119A) and the short-stretch tryptophan mutations at position 106 (W106A and W106Y) were clearly narrower than the wild-type spectrum. In addition, the deuteration experiments on these variants showed spectral changes on the EPR signal exclusively for the perdeuterated-Tyr samples (Figure 4, right panel). Accordingly, the absence of detectable spectral changes for the perdeuterated-Trp samples showed that in all four variants (W122F, H123Q, R119A, and W106A) only the tyrosyl radical was formed.

The High-Field (285 GHz) EPR Spectra of the Protein-Based Radicals in *Synechocystis* Catalase-Peroxidase: Wild-

(34) Barry, B. A.; Babcock, G. T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *84*, 7099−7103.
(35) Tommos, C.; Tang, X.-S.; Warncke, K.; Hoganson, C. W.; Styring, S.; McCracken, J.; Diner, B. A.; Babcock, G. T. *J. Am. Chem. Soc.* **1995**, *117*, 10325−10335.
(36) Ivancich, A.; Jouve, H. M.; Gaillard, J. *J. Am. Chem. Soc.* **1996**, *118*, 12852−12853.
(37) To clarify this point, it is possible to consider the situation on an equation basis: S^1_{calc} = spectrum A(dashed) − spectrum A(solid) = [Tyr•(H) + Trp•(H)] − [Tyr•(H) + Trp•(D)], S^1_{calc}= Trp•(H) − Trp•(D), S^2_{calc} = spectrum D − spectrum E = [Tyr•(D) + Trp•(H)] − [Tyr•(D) + Trp•(D)] = S^1_{calc}, with Tyr•(H) and Tyr•(D) use to designate the fully protonated and perdeuterated tyrosyl radicals (the same for the Trp•).
(38) Bleifus, G.; Kolberg, M.; Potsch, S.; Hofbauer, W.; Lubitz, W.; Graslund, A.; Lassman, G.; Lendzian, F. *Biochemistry* **2001**, *40*, 15362−15368.

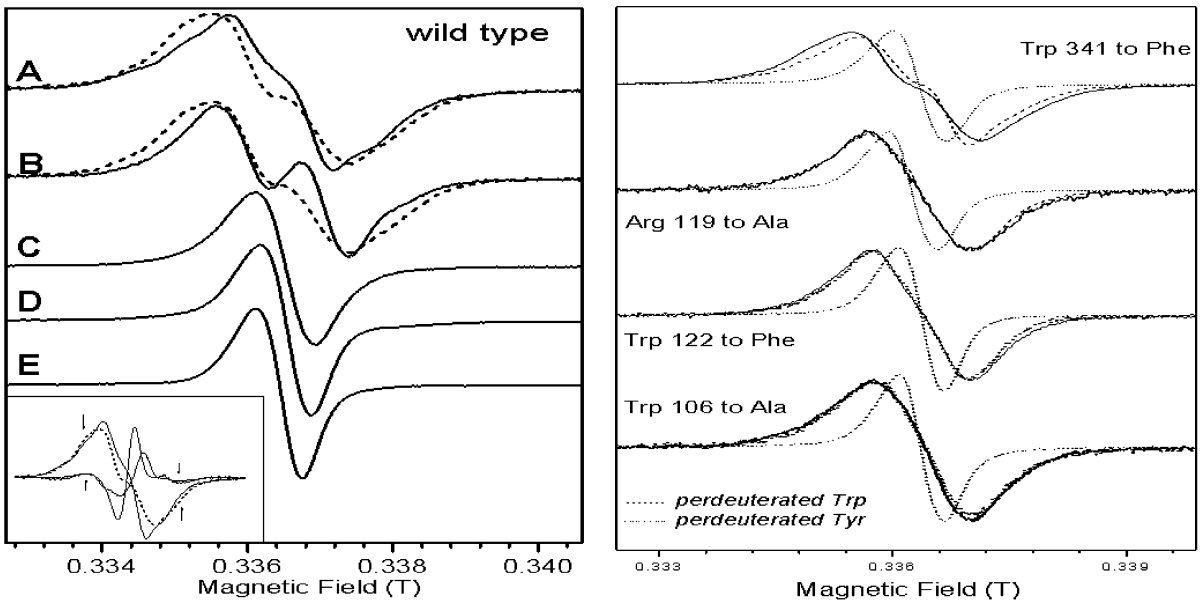

Figure 4. (Left) The 9-GHz EPR spectra of the protein-based radicals in wild-type *Synechocystis* catalase-peroxidase obtained for samples containing perdeuterated tryptophan (A), partially deuterated tyrosines on ring-protons 3,5 (B) and β-methylene protons (C), perdeuterated tyrosine (D), and perdeuterated Tyr and Trp (E). The fully protonated spectrum is also shown (dashed trace). Experimental conditions are the same as those in Figure 1C. Inset: the spectra of tryptophanyl radical obtained by the arithmetic difference of the experimental spectra.[37] (Right) The 9-GHz EPR spectra of the protein-based radicals in selected variants of *Synechocystis* catalase-peroxidase (solid trace). For each variant, the EPR spectra of the perdeuterated Trp (dashed trace) and the perdeuterated Tyr (dotted trace) samples are superimposed to the nondeuterated spectrum. Experimental conditions are the same as those in Figure 1C.

Type and Variants. High-field EPR spectroscopy was applied in order to obtain complementary information on the protein-based radicals formed by *Synechocystis* catalase-peroxidase. Enhanced resolution of the *g*-anisotropy can be obtained by using higher magnetic fields (see ref 26 and references therein). The 285-GHz (10 T) EPR spectrum of the radical species formed in wild-type catalase-peroxidase is shown in Figure 5 (top). As expected from the deuterium-labeling experiments (see above), the complex spectrum could not be explained by only one protein-based radical species. The three main components of the high-field spectrum, with observed *g*-values of 2.0064(4), 2.0040(5), and 2.0020(8) were consistent with those expected for the intrinsic *g*-values of tyrosyl radicals previously reported for other enzymes (see ref 39 and references therein) and for the in vitro tyrosyl radical generated by γ-irradiation of Tyr·HCl crystals.[15] Comparisons of the catalase-peroxidase HF EPR spectrum to that of a tyrosyl radical (dotted spectrum in Figure 5) clearly showed the presence of another signal in the g_y-g_z region of the catalase-peroxidase tyrosyl radical spectrum, contributing with extra intensity to the g_z component and with a break at $g = 2.0026$ (dotted ellipse in Figure 5, top). The *g*-anisotropy, $\Delta g = g_z - g_x$, of this other species ($\Delta g \approx 0.0012$) was totally consistent with that expected for an isolated tryptophanyl radical.[38] Unlike the tryptophanyl radical intermediate of cytochrome *c* peroxidase,[15] no other component to the signal was detected at lower fields in the 285-GHz spectrum (data no shown). The broad g_x component of the Tyr$^\bullet$ HF EPR spectrum of catalase-peroxidase was centered at a fairly low g_x-value, like the tyrosyl radical in the W191G CcP variant (Figure 5, top). The latter was interpreted as due to an electropositive and distributed microenvironment to the tyrosyl radical.[15] The width of the g_x component of the catalase-peroxidase Tyr$^\bullet$ spectrum was the same for the pH range of 5.5 to 8.5, like the case of the bovine catalase Tyr$^\bullet$.[39] This width was also independent of the frequency of measurement when plotted in a *g*-values scale (see 190-GHz EPR spectrum in the Supporting Information). Consequently, the broad g_x component

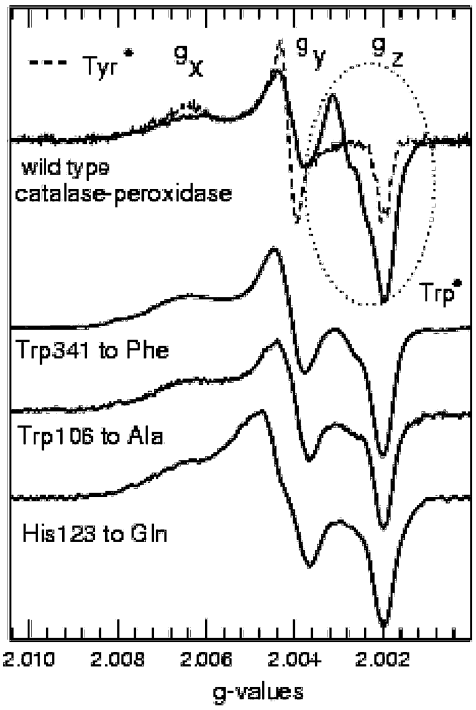

Figure 5. High-field (10 T, 285 GHz) EPR spectra of the protein-based radicals in wild-type (top, solid trace) and selected variants of *Synechocystis* PCC6803 catalase-peroxidase. The dashed ellipse indicates the spectral region for the tryptophanyl radical signal. The tyrosyl radical intermediate of the W191G variant from cytochrome *c* peroxidase is plotted for comparisons (dotted trace). Spectra were recorded at 4 K (20 K for CcP), using a frequency modulation of 30 kHz and a field modulation of 5 G.

(39) Ivancich, A.; Mattioli, T. A.; Un, S. *J. Am. Chem. Soc.* **1999**, *121*, 5743−5753.

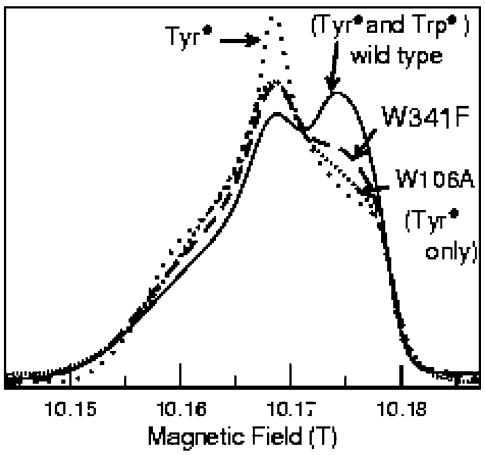

Figure 6. High-field EPR spectra of wild-type *Synechocystis* catalase-peroxidase, the W106A and W341F variants, and the tyrosyl radical of the cytochrome *c* peroxidase W191G variant all plotted in the absorption mode. All spectra were recorded at 4 K and with 10 G field modulation. Each spectrum was recorded at slightly different microwave frequencies; for comparison, all the spectra are aligned to a nominal field and were normalized so that the double integral was unity.

of the tyrosyl radical in *Synechocystis* catalase-peroxidase was clearly not due to hyperfine couplings.

Representative HF EPR spectra of the catalase-peroxidase variants investigated in this work are also shown in Figure 5. The HF EPR spectra of the short stretch mutations at position 106 (W106A and W106Y, represented by the W106A spectrum in Figure 5) and the distal side variants (W122F, H123Q, and R119A, represented by the H123Q spectrum[40] in Figure 5) exhibited only the tyrosyl radical signal. As expected, these results were consistent with the 9-GHz EPR studies on the deuterium-labeled samples (see Figure 4) in which the EPR spectra of the Trp-deuterated samples were identical to those of the fully protonated samples. The more isotropic radical signal with a break at $g = 2.0026$ (dotted ellipse) was absent in the HF EPR spectrum of the W106A and H123Q variants. By contrast, and as expected from the changes in the 9-GHz EPR spectrum of the W341F mutation with deuterated Trp residues (Figure 4), the signal attributed to the typtophanyl radical was detected in the HF EPR spectrum of the W341F mutant. In addition, the Trp contributed to a lower extent in the W341F variant as than in wild type. This effect can be better observed in the absorption-mode HF EPR spectra of the wild-type and variants of catalase-peroxidase, plotted in Figure 6.

Discussion

Structural Aspects of the Heme Active Site in *Synechocystis* Catalase-Peroxidase. The low-temperature (4 K) EPR spectrum of the ferric enzyme showed that the heme site of the wild-type *Synechocystis* catalase-peroxidase exists in a mixture of coordination environments, with at least two clearly distinct forms. Previous EPR measurements on the recombinant *M. tuberculosis* enzyme showed a similar spectrum, although the relative contribution of the different forms appeared to be variable.[5,27,41,42] In all cases, there has been agreement on the attribution of the rhombic and axial EPR signals to penta- and hexa-coordinated Fe(III) high-spin species, respectively. A recent analysis of the heterogeneity of the ferric EPR signals in the *M. tuberculosis* enzyme proposed that the presence of the axial signal was due to storage of the enzyme at low temperatures.[42] In our case, the mixture of different EPR signals in *Synechocystis* catalase-peroxidase was observed even for the freshly purified enzyme with no specific relationship between the different signals and the reactivity toward peroxyacetic acid.

The presence of a hydrogen-bonding network involving the catalytically essential distal-side residues (Trp122, His123, and Arg119) and connecting the distal and proximal sides has been previously proposed on the basis of room-temperature resonance Raman experiments on the *Synechocystis* catalase-peroxidase.[31] Since the *g*-values are very sensitive to small changes in the geometry of the heme site, the pH-dependent ferric EPR spectrum was used as a marker for possible structural changes induced by the mutations studied in this work. The absence of pH-induced changes on the ferric EPR spectra of the variants on the distal and proximal heme side was interpreted as due to structural changes on the heme environment.[43] An unexpected result was that among the mutations on the short stretch aromatic residues only Trp106 appears to also induce structural changes. A way to rationalize the effect of all these mutation is by the existence of a more extensive hydrogen-bonding network which links the catalytically essential distal-side residues (Trp122, His123, and Arg119), seven structural water molecules (826, 1189, 622, 215, 146, 119, and 103 in *H. marismortui* 3D structure[4]), the heme 6-propionate and Trp106 (Figure 7). To substantiate this proposal we designed a mutation at position 106 with a residue being a potential hydrogen-bond donor, specifically a tyrosine residue. Unlike the alanine mutation, the W106Y variant restored the original hydrogen-bonding network as judged from the pH-dependent EPR signal of the resting (ferric) enzyme.

The Oxoferryl-Porphyrin Radical Intermediate in *Synechocystis* Catalase-Peroxidase. The EPR spectrum of the intermediate obtained by reaction of the wild-type catalase-peroxidase with peroxyacetic acid showed the contribution of three different radical species: an exchange-coupled porphyrin radical, a tyrosyl radical, and a tryptophanyl radical. We have definitively assigned the chemical nature of the latter two radicals by the deuterium-labeling experiments in this study. A similar situation was previously reported for the bovine liver catalase although only two radical species were observed, the exchange-coupled porphyrin radical and a tyrosyl radical.[14] For this monofunctional catalase, the [Fe(IV)═O por$^{\bullet+}$] intermediate was observed in the millisecond time range and only the tyrosyl

(40) The H123Q HF EPR spectrum exhibited unusually broad shape. The EPR spectrum recorded at 190 GHz was identical in shape to the 285-GHz spectrum when plotted as a function of *g*-values. This indicated that the shape was due to distribution in **g**-tensor. Further work is require to understand the nature of this spectrum.

(41) Lukat-Rodgers, G. S.; Wengenack, N. L.; Rusnak, F.; Rodgers, K. R. *Biochemistry* **2000**, *39*, 9984−9993.
(42) Chouchane, S.; Girotto, S.; Kapetanaki, S.; Schelvis, J. P. M.; Shengwei, Y.; Magliozzo, R. S. *J. Biol. Chem.* **2003**, *278*, 8154−8162.
(43) The mutation of the distal histidine to a glutamine (H123Q) did not have the same structural effects as those of the two other distal residues (Trp122 and Arg119). The ferric EPR spectrum of the H123Q variant showed an inversion on the pH-dependence of the signal as compared to the wild-type enzyme. The crystal structure of the comparable mutation (H52Q) on cytochrome *c* peroxidase shows that the glutamine residue can replace the distal histidine (Bateman, L.; Léger, C.; Goodin, D. B.; Armstrong, F. A. *J. Am. Chem. Soc.* **2001**, *123*, 9260−9263). Specifically, the amide-oxygen of the glutamine residue replaced the imidazole N-ϵ of the distal His while the glutamine amide-NH_2 group served to retain the H-bond to the Asn82. If one assumes that the mutation of the distal histidine (H123Q) to a glutamine in catalase-peroxidase results in the same arrangement as the glutamine in the comparable CcP variant, the decreased basicity of the glutamine carbonyl relative to imidazole is likely to contribute to the "inverted" pH effect observed on the EPR spectrum of the H123Q variant.

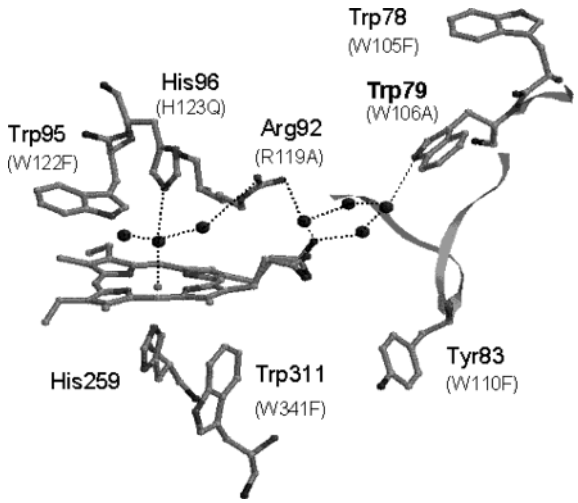

Figure 7. Structure of the proximal and distal sides of the heme cofactor, as well as the short stretch and structural water molecules (represented by the oxygen atom) in *H. marismortui* catalase-peroxidase[4] showing all the positions for which mutations of the *Synechocystis* enzyme (shown in parentheses) were characterized by EPR spectroscopy in this work. The proposed hydrogen-bonding network is indicated by the broken lines. The Tyr residue at position 83 in *H. marismortui* enzyme is a Trp (W110) in the *Synechocystis* enzyme.

radical intermediate signal was still present in the time scale of manual mixing (5 to 15 s, at 0° C).[14] In light of this observation, it is not surprising that for *Synechocystis* catalase-peroxidase only a small fraction of the [Fe(IV)═O por$^{\bullet+}$] species was trapped when using manual mixing.

The [Fe(IV)═O por$^{\bullet+}$] intermediate, so-called Compound I, has been well characterized for some peroxidases and catalases (see ref 16 and references therein). This intermediate is an exchange-coupled species resulting in a very broad (about 2000 G) EPR spectrum, with the $g_{||}$ component at $g \approx 2$ (see dotted spectrum in Figure 1B). Consequently, the narrow EPR spectra (less than 100 G) centered at $g \approx 2$ previously reported for the *E. coli*[19] and *M. tuberculosis*[20] catalase-peroxidase Compound I are not consistent with the oxoferryl-porphyrin radical species, unless they are only part of the expected broad spectrum. Recently, a comprehensive study on the *M. tuberculosis* radical intermediate led the authors to reassign the narrow EPR spectrum to a tyrosyl radical.[24] These authors also mentioned that they could not detect the oxoferryl-porphyrin radical signal in their EPR spectrum, although the stopped-flow optical measurements showed the formation of such species in the *M. tuberculosis* samples.[24] The fact that we were able to detect the EPR spectrum of the porphyrin radical intermediate in the *Synechocystis* catalase-peroxidase can be possibly explained by differences in the intramolecular electron-transfer rates, between the porphyrin radical and the protein-based radical, for the two enzymes.

The Tryptophanyl and Tyrosyl Radicals in *Synechocystis* Catalase-Peroxidase. The selective deuteration of tyrosine and tryptophan residues in the *Synechocystis* enzyme unequivocally showed that *both* Tyr$^{\bullet}$ and Trp$^{\bullet}$ signals were at the origin of the narrow radical EPR spectrum (Figure 4). In the W122F and H123Q variants for which only the tyrosyl radical was detected, the radical yield was estimated as 60% of that of the wild-type enzyme (from spin quantitation of the 9-GHz EPR spectra at 60 K). Accordingly, the 40% relative contribution of the tryptophanyl radical has to be considered as an upper limit since it is estimated under the assumption that the tyrosyl radical yield is the same for the WT and the mutants. It is not clear yet how the formation of both radicals is related (see pH effect on the relative contribution of the radicals in Supplementary Information). A lower limit of 10% is obtained if considering the relative contribution of the Trp$^{\bullet}$ signal in the wild-type high-field EPR spectrum shown in Figure 6.

Recently, deuterium-labeling experiments were reported for the *M. tuberculosis* enzyme including a time course rapid-mix freeze−quench EPR experiments from 50 ms to 10 s.[24] From the absence of changes on the radical spectrum (trapped in 200 ms) of the Trp-deuterated samples, it was concluded that only the tyrosyl radical was formed.[24] In the *M. tuberculosis* study, specific labeling of only the Trp indole-ring protons (at positions 2,4,5,6,7; see numbering scheme in Figure 3) was used. It has been previously reported that the isotropic ^{1}H-hyperfine couplings on the Trp indole ring are rather small as compared to those of the β-protons.[44,45] Specifically, the hyperfine couplings of the β_1 and β_2 protons in the Trp$^{\bullet}$ of *E. coli* ribonucleotide reductase (Y122F variant) were 13.6 and 28.3 G, respectively, while the largest hyperfine couplings of the indole protons were 6.3 and 1.5 G for $^1H_5$ and $^1H_7$, respectively.[44] Similar values were reported for the mouse RNR Trp$^{\bullet}$.[46] In both cases, minimal changes due to labeling could be observed in the 9-GHz EPR spectrum. Hence, taking into account the magnitude of the indole-ring hyperfine couplings of the Trp$^{\bullet}$, the quality of the 9-GHz EPR spectrum previously reported for *M. tuberculosis* enzyme and the fact that a Tyr$^{\bullet}$ signal is superposed to the Trp$^{\bullet}$ spectrum, it is not surprising that the authors[24] could not detect spectral differences in their Trp-deuteration experiments. Unfortunately, in the *M. tuberculosis* study, the EPR spectrum corresponding to the Trp-deuterated sample containing the radical trapped in 10 s was not shown.[24] This leaves an open question about the time scale of the formation of the radicals. It would be interesting to use perdeuterated trytophan and 10 s mixing time for the *M. tuberculosis* catalase-peroxidase in order to test the formation of the tryptophanyl radical in this enzyme. It is possible that catalase-peroxidases from different bacterial origins may form different radical species. This is known to be the case in other peroxidases and catalases.

The Tryptophanyl Radical Site. A tryptophanyl cation radical constitutes the active intermediate for the ferrous to ferric conversion of cyt *c* in cytochrome *c* peroxidase.[11] This radical has a broad EPR spectrum (more than 200 G at 9 GHz, see Figure 2) originating from the weak exchange interaction with the oxoferryl-iron moiety.[47,48] On the basis of the primary sequence homology and the structural similarities on the heme proximal side between cytochrome *c* peroxidase and the catalase-peroxidases from *H. marismortui* and *B. pseudomallei*, it has been proposed that the conserved proximal tryptophan residue at the equivalent position of the CcP Trp 191 could be the radical site in the bifunctional enzymes.[4,8] This proposal

(44) Lendzian, F.; Sahlin, M.; MacMillan, F.; Bittl, R.; Fiege, R.; Pötsch, S.; Sjöberg, B.-M.; Gräslund, A.; Lubitz, W.; Lassman, G. *J. Am. Chem.* **1996**, *118*, 8111−8120.
(45) Himmo, F.; Eriksson, L. A. *J. Phys. Chem. B* **1997**, *101*, 9811−9819.
(46) Pöstch, S.; Lendzian, F.; Ingemarson, R.; Hörnberg, A.; Thelander, L.; Lubitz, W.; Lassman, G.; Gräslund, A. *J. Biol. Chem.* **1999**, *274*, 17696−17704.
(47) Housseman, A. L.; Doan, P.; Goodin, D. B.; Hoffman, B. M. *Biochemistry* **1993**, *32*, 4430−4443.
(48) Huyett, J. E.; Doan, P. E.; Gurbiel, R.; Houseman, A. L. P.; Sivaraja, M.; Goodin, D. B., Hoffman, B. M. *J. Am. Chem. Soc.* **1995**, *117*, 9033−9041.

motivated our detail characterization of the proximal Trp variant (W341F) of *Synechocystis* catalase-peroxidase, including selective proton perdeuteration on the Trp residues of the mutated enzyme. Such a study allowed us to demonstrate that the proximal tryptophan is unlikely to be the radical site. The EPR spectrum of the Trp-deuterated samples of the W341F variant showed the same spectral changes than the wild type enzyme (Figure 4). The only difference between the wild-type enzyme and the W341F variant was the lower relative contribution of the tryptophanyl radical in the W341F sample, as observed in the HF EPR spectrum (Figure 6). An explanation is that the indirect structural changes induced by the W341F variant with respect to the extensive hydrogen-bonding network of the distal side (see above) were sufficient to modify the kinetics of the tryptophanyl radical formation as compared to the wild type. In the *M. tuberculosis* catalase-peroxidase, the proximal tryptophan variant (W321F) was also reported to have the same 9-GHz EPR spectrum as the wild-type enzyme[49] although no deuteration experiments were reported.

Three other tryptophan variants (W105F, W106A, and W110F) of *Synechocystis* catalase-peroxidase were also investigated in this work. These tryptophan residues are components of the highly conserved short-stretch of catalase-peroxidases. Such a stretch is effectively a loop positioned close to the heme edge and spanning to the distal and the proximal sides of the heme (see Figure 7). The complete deletion of this short stretch in the *Synechocystis* enzyme (del[103−113] variant) induced a major structural change leading to the 90% conversion of the ferric heme iron to the hexacoordinated low-spin form, the consequence being a drastically reduced enzyme activity. In the case of the mutations at positions 105, 110, and 113 of the short stretch, the pH-dependence of their ferric heme EPR spectra (Supplementary Information) and the EPR spectra of the protein-based radicals allowed us to conclude that these mutations (W105F, W110F, Y113F) have no effect on the heme environment nor on the chemical nature of the protein-based radicals formed. By contrast, deuterium labeling experiments on the W106A variant showed no detectable Trp$^{\bullet}$ signal in the EPR spectrum (Figure 4). An unexpected result from the deuterium-labeling experiments on all three distal side variants (W122F, H123Q, and R119A) was that, as in the case of W106A, no Trp$^{\bullet}$ was observed. In principle, both Trp 106 and Trp 122 must be considered as candidates for the Trp$^{\bullet}$ radical site. Clearly, it is not possible to identify the site of the radical solely based on the EPR findings.[50]

However, by combining the EPR spectroscopy with site-directed mutagenesis and structural considerations we propose that Trp 106 is the actual site for the radical formation. Three residues on the distal heme pocket (His 123, Arg 119, and Trp 122) are known to be structurally important for hydrogen peroxide reduction and oxidation in *Synechocystis* catalase-peroxidase.[6] The tryptophanyl radical did not form in any of these distal site variants. This can be interpreted as an inhibition of the radical formation due to the structural role of these three residues, *irrespective of the amino acid type*. Consequently, we can discard Trp122 as being the radical site. On the other hand, the three short-stretch tryptophans (Trp 106, Trp 105, and Trp110) are reasonably close to each other and at comparable distances for electron transfer to the heme moiety (see Figure 7). The fact that only the variants at position 106 (W106A and W106Y) did not form the Trp$^{\bullet}$ strongly argues for a selectivity toward this position as compared to the other tryptophans. Trp 106 is the sole aromatic residue on the short stretch that is part of the extensive hydrogen-bonding network on the distal side (see Figure 7). An important finding was the fact that the W106Y mutant restores the network, but yet the tryptophanyl radical is not formed. Taken together these facts argue for Trp 106 being the site of Trp$^{\bullet}$ formation in the *Synechocystis* catalase-peroxidase, with the extensive hydrogen-bonding network of the distal side having a crucial role in the formation of the radical. Inspection of the known catalase-peroxidase crystal structures[4,8] shows that there exist a possible access channel from the surface to Trp 106 that may then act as an alternative substrate oxidation site. Further studies have to be carried out to substantiate this proposal. Finally, the fact that the Trp radical was formed when other tryptophans were mutated (W341F, W105F, and W110F) strongly argues against the common idea that the radical will form on any other of the 21 tryptophans of the catalase-peroxidase N-terminal domain. More work is required to understand the specific role of the Trp 106 in the catalytic cycle of the enzyme.

Conclusion. The *Synechocystis* PCC6803 catalase-peroxidase forms the typical Compound I intermediate [Fe(IV)═O por$^{\bullet+}$] of catalases and peroxidases as well as two subsequent protein-based radical intermediates. These radicals were unequivocally identified as a Tyr$^{\bullet}$ and a Trp$^{\bullet}$ by using a combination of multifrequency EPR spectroscopy, isotope labeling, and site-directed mutagenesis. Trp106, located on the distal side of the heme, is proposed as the tryptophanyl radical site. This residue belongs to the short stretch that is highly conserved in catalase-peroxidases but absent in ascorbate peroxidase. The existence of an extensive hydrogen-bonding network on the distal side of the heme, involving Trp122, His123, Arg119, seven structural waters, the heme 6-propionate group, *and* Trp106 (Figure 7) was inferred from the EPR studies on the ferric heme. This hydrogen-bonding network appears to be relevant for the tryptophanyl radical formation. Site-directed mutagenesis on the two other tryptophans of the short stretch (Trp105 and Trp110) and on the proximal Trp 341 showed that Trp 106 is rather unique. The existence of a possible access channel from the surface to Trp 106 argues for its catalytic role as alternative oxidation site. The ability to identify unambiguously the three different radical species in catalase-peroxidases opens up the possibility to clearly define their substrate specificity and their role in the complex catalytic cycle of the enzyme.

Acknowledgment. Dedicated to the memory of Frank M. Rusnak (1960−2002), for his excellent contribution to the investigations on the *M. tuberculosis* catalase-peroxidase, his enthusiasm, and his predisposition for stimulating discussions. We thank Dr. A. William Rutherford for his support of this work, Guillaume Voyard for technical developments on the high-field EPR spectrometer, and Drs. Giulietta Smulevich, Kenton Rodgers, Peter Loewen, and Ignacio Fita for stimulating discussions. Dr. Tanaka is acknowledged for having provided us with the crystal structure of *H. marismortui* prior to

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(50) The dihedral angles of the β-protons are similar, as judged from the crystallographic structure (ref 4). Hence, the β-protons hyperfine couplings would be of little help to decide between the Trp122 and Trp106.
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publication. This work was supported in part by the Austrian Science Fund (Project P15417) to C.J. and C.O. The COST Action D21 is acknowledged for supporting exchange among the laboratories.

Supporting Information Available: The pH dependence of the Fe(III) high-spin (9 GHz) EPR spectra of selected variants of the *Synechocystis* PCC6803 catalase-peroxidase. Comparison of the HF EPR spectra, recorded at 190 and 285 GHz, of the protein-based radicals formed in wild-type catalase-peroxidase at two different pH values. This material is available free of charge via the Internet at http://pubs.acs.org.

JA035582+