Tyrosine Radical Formation in the Reaction of Wild Type and Mutant Cytochrome P450cam with Peroxy Acids

A MULTIFREQUENCY EPR STUDY OF INTERMEDIATES ON THE MILLESECOND TIME SCALE*

We report a multifrequency (9.6-, 94-, 190-, and 285-GHz) EPR study of a freeze-quenched intermediate obtained from reaction of substrate-free cytochrome P450cam (CYP101) and its Y96F and Y96F/Y75F mutants with peroxy acids. It is generally assumed that in such a shunt reaction an intermediate [Fe(IV)=O, porphyrin-\(\pi\)-cation radical] is formed, which should be identical to the species in the natural reaction cycle. However, for the wild type as well as for the mutant proteins, a porphyrin-\(\pi\)-cation radical is not detectable within 8 ms. Instead, EPR signals corresponding to tyrosine radicals are obtained for the wild type and the Y96F mutant. Replacement of both Tyr-96 and Tyr-75 by phenylalanine leads to the disappearance of the tyrosine EPR signals. EPR studies at 285 GHz on freeze-quenched wild type and Y96F samples reveal \(g\) tensor components for the radical (stretched \(g_{xx}\) values from 2.0078 to 2.0064, \(g_{yy} = 2.0043\), and \(g_{zz} = 2.0022\)), which are fingerprints for tyrosine radicals in a heterogeneous polar environment. The measurements at 94 GHz using a fundamental mode microwave resonator setup confirm the 285-GHz study. From the simulation of the hyperfine structure in the 94-GHz EPR spectra the signals have been assigned to Tyr-96 in the wild type and to Tyr-75 in the Y96F mutant. We suggest that a transiently formed Fe(IV)=O porphyrin-\(\pi\)-cation radical intermediate in P450cam is reduced by intramolecular electron transfer from these tyrosines within 8 ms.

The superfamily of cytochrome P450 enzymes are heme-containing monoxygenases in various organisms that catalyze a number of reactions, such as aliphatic and aromatic hydroxylations, epoxidations, heteroatom oxidation, and N- and O-dealkylation (1), by transfer of an activated oxygen atom from its heme iron-binding site to substrates. Cytochrome P450 (P450) plays a major role in biotransformation of xenobiotics and in the biosynthesis of various endogenous compounds in animals and humans (2). Understanding the reaction mechanism of this class of enzymes is therefore of considerable medical and pharmacological interest. The camphor-hydroxylating cytochrome P450cam\(^1\) (CYP101) from the soil bacterium \(Pseudomonas putida\) is the best studied P450 so far and is regarded to be a model protein for a large number of other P450s. It is assumed that during the P450 reaction cycle an iron-oxo intermediate, compound I (cpd I), is formed. In analogy to the heme cofactor in chloroperoxidase (CPO) from \(Caldariomyces fumago\), which has the same proximal cysteine iron ligand as P450, a Fe(IV)=O porphyrin-\(\pi\)-cation radical system has been suggested for cpd I in P450cam.

The Fe(IV)=O porphyrin-\(\pi\)-cation radical, as the electronic structure of cpd I in CPO, has been deduced from UV-visible spectroscopy (rapid-scan stopped-flow studies) that showed characteristic bands at \(\sim 370\) and \(\sim 690\) nm, from resonance Raman studies that revealed the Fe-O stretch vibration at \(\sim 790\) cm\(^{-1}\) (3) and from EPR and Mössbauer measurements of freeze-quenched samples obtained by reaction with peracetic acid (4). These experiments showed that the iron is Fe(IV) and that the radical couples antiferromagnetically with the iron, indicating a porphyrin radical.

For P450, artificial oxidants have been used in the shunt pathway to support substrate conversion and to characterize the iron-oxo intermediate by electronic absorption spectroscopy (5–7). Egawa et al. (6) performed stopped-flow studies for the reaction of P450cam with \(m\)-chloroperbenzoic acid (mCPBA) and found UV-visible spectra similar to cpd I of CPO. Recently, a transient absorption spectrum was observed in stopped-flow studies on CYP119, a cytochrome P450 from the thermophilic bacterium \(Sulfolobus solfataricus\), which reacted with mCPBA (8). The intermediate is formed to \(\sim 3\%\) under mCPBA-limiting conditions and revealed an absorption spectrum similar to cpd I of CPO. Thus, it could be expected that freeze-quenching the intermediate and analyzing the electronic structure by EPR and Mössbauer spectroscopies in a manner analogous to the studies on CPO would prove the nature of the Fe(IV)=O por-

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1 The abbreviations used are: P450cam, cytochrome P450cam; cpd I, compound I, PA, peracetic acid; mCPBA, \(m\)-chloroperbenzoic acid; CPO, chloroperoxidase; RNR, ribonucleotide reductase; hf, hyperfine; W, watts; mT, millitesla.
phosphor-π-cation radical for P450cam. However, as will be shown, our study reveals in contrast to CPO, no EPR signal of a phosphor-π-cation radical in P450cam, although Fe(IV) has been detected using 9.6-GHz EPR spectroscopy, which we have tentatively assigned to a tyrosine radical and have proposed Tyr-96 to be the radical site (9). Davydov et al. (10) reduced the dioxygen complex via γ-irradiation at 77 and 6 K in both wild type and D251N and T252A mutants of P450cam. They detected various intermediate states within the reaction cycle via EPR and electron nuclear double resonance spectroscopies by stepwise annealing of the reduced oxygen complex, increasing the temperature to ~220 K. Although the formation of a hydroxylation product of camphor was observed, the proposed cpd I intermediate could not be identified. In addition, mixed flow transient resonance Raman studies with a reconstituted P450cam system under catalytic conditions revealed an O-O stretch vibration signal very similar to the simple dioxygen complex, but no Fe-O characteristic Raman signal at ~790 cm\(^{-1}\) was observed (11). Transient state x-ray crystallographic studies by Schlichting et al. (12) revealed an electronic density near the heme iron in one of the two P450cam molecules located in the unit cell after reductive desolvation of the dioxygen complex via x-ray irradiation, which is interpreted to reflect the (Fe-O) species. Thus, the nature of cpd I in P450 has to date not been clarified. Theoretical studies propose several different electron and spin distributions for cpd I in P450 (13–15). From our first 9.6-GHz EPR and Mössbauer studies (9), we suggested that an intramolecular electron transfer from a tyrosine to the heme may quench the porphyrin-π-cation radical. In order to prove this hypothesis and to unequivocally identify and assign the radical observed, we performed a detailed multifrequency EPR study (9.6, 94, 190, and 285 GHz) of the intermediate of wild type, Y96F mutant, and Y96F/Y75F double mutant P450cam in the time regime up to 40 ms, which is presented in this work. These studies show that the radical observed in wild type P450cam is indeed a tyrosine radical and originates from intramolecular electron transfer to a presumably transiently formed phosphor-π-cation radical. It also strongly suggests that intramolecular electron transfer from tyrosines near the heme can generally occur in P450 as a possible competitive reaction to the decay of cpd I via substrate hydroxylation. This may be of functional importance for the whole P450 superfamily.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pUS200 plasmid carrying the wild type P450cam gene from *P. putida* was used as described (16). The plasmid carrying the Y96F mutant gene was described previously (17). The Y96F/Y75F double mutant was obtained by site-directed mutagenesis using double-stranded DNA templates made with the QuickChange\(^{TM}\) site-directed mutagenesis kit from Stratagene according to the instructions of the manufacturer. The 5.5-kb plasmid pUS200 with the wild type insert (16) was used as DNA template. In a first step, the mutagenic oligonucleotide primer pair, 5'-GAA GCC GCC GAA GCT TTC GAC TTC ATT CCC ACC-3' and 5'-GGT GGG GGT GAA GTC GGC TCC GCC GGG TTC-3', was used to generate a nicked double-stranded plasmid DNA carrying the Y96F mutation by PCR. Parental DNA was digested using the restriction enzyme DpnI. The nicked plasmid DNA was transformed into *Escherichia coli* strain XLI-Blue, which circularized the nicked DNA. In a second step, the plasmid having the Y96F mutation was used as template, together with the mutagenic primer pair, 5'-CTG ATC CGT GAG GCC GTT GAA GAT TGC CAC CAC-3' and 5'-GGT GGG GGT GAA GTC GGC TCC GCC GGG TTC-3', to generate a mutated plasmid carrying the Y75F/Y96F double mutation. Amplified plasmid DNA from PCR and subsequent DpnI digestion was also transformed into *E. coli* strain XLI-Blue. The correctness of mutation was checked by sequencing the plasmid.

**Protein Expression**—*E. coli* strain TB1 was used as expression host for the wild type as well as for the mutant proteins. Cells were grown in standard LB medium in a 221 fermentor as described previously (18) or in flasks.

**Protein Purification**—The purification of the expressed wild type and mutant proteins followed the same protocol (18). The final purification step of the proteins was performed with fast protein liquid chromatography-anion exchange chromatography to get an absorbance ratio \( Q = 392 \text{ nm}/280 \text{ nm} \) of > 1.4 for 100% high spin state content. The camphor-bound wild type protein has 100% high spin content, whereas the mutant proteins show a high spin content of ~52% determined with the fit program described earlier (19), which also allows us to recalculate the absorbance corresponding to 100% needed to estimate the \( Q \) value. Camphor was removed by dialysis against 50 mM Tris buffer, pH 7.4, 5% (v/v) glycerol, Sephades G-25 gel chromatography and final dialysis against 100 mM potassium phosphate buffer, pH 7. For comparison, mCPBA was also used as oxidant with a wild type P450cam/mCPBA ratio of 1:1.5, analogous to the previous study on chloroperoxidase (22).

For the 9.6-GHz EPR study, a quartz tube (4 mm outer diameter, 3 mm inner diameter) was connected to a funnel. The funnel was completely immersed into an isopentane bath at \( T = -110^\circ C \). The reaction mixtures were quenched by spraying into the cold fluid isopentane, and the frozen material so obtained was packed at the bottom of the quartz tube using a packing rod made of Teflon.

For the 190- and 285-GHz EPR measurements, a Teflon cup with 7 mm inner diameter and a volume of 200 \( \mu l \) was attached to a quartz tube, which was connected to a funnel. The preparation procedure was identical to that used for 9.6-GHz EPR.

For the preparation of the samples for the 94-GHz EPR instrument, we have constructed the setup shown in Fig. 1. It allowed collection of freeze-quenched material in a fragile quartz sample tube (0.87 mm outer diameter, 0.70 mm inner diameter) at \( T = -110^\circ C \) by stabilizing the tube with an additional brass holder. The high heat capacity of the brass holder served to prevent rapid warming of the sample during preparation and transfer of the small sample. The brass sample holder was connected to the glass funnel in which the freeze-quenched material was collected. A channel of 1-mm diameter connected the supported quartz tube with the glass funnel. Thus it was possible to transfer fine sample grains into the small quartz tube and to collect them at the bottom of the tube. By carefully packing these grains with a stainless steel rod (0.5 mm diameter) in fluid isopentane (\( T = -110^\circ C \)), it was possible to obtain a filling height of 1–2 mm, corresponding to 1–2 \( \mu l \) sample volume, which was sufficient for the 94-GHz EPR instrument (see below). All manipulations were performed in the cold isopentane bath. After completion of the packing procedure, the quartz tube was immediately transferred into liquid nitrogen.

**EPR Spectroscopy at 9.6 GHz**—EPR spectra were recorded with a conventional 9.6-GHz spectrometer (Bruker 200D SRC), equipped with a helium-flow cryostat (ESR 910, Oxford Instruments) at temperatures of 20 and 140 K. EPR spectra were simulated using effective \( g \) values and angular-dependent Gaussian line shapes according to the procedure described by Beirnert and Albracht (23). Thus the radical signals in the high- and low-\( g \) EPR spectra, which showed no resolved hyperfine structure, were simulated by using an isotropic \( g \) value and Gaussian line shape. Spin quantification was performed by double integration of simulated absorption-derivative subspectra. Differences in \( g \) values were taken into account using the Aasa \( g \) factor (24) for corrections. We also performed double integration of the total experimental EPR signal and compared it to the theoretical signal. These results were about the same as those obtained by the above method with an absolute experimental error of ±5%.

**High Frequency EPR Spectroscopy at 94 GHz**—Continuous wave high frequency EPR measurements at 94 GHz were performed on a
Connection to glass funnel

tube support

W-band quartz tube ∅ 0.87 mm

Fig. 1. Setup for preparing freeze-quenched 94-GHz EPR samples. A, the quartz 94-GHz EPR sample tube, with an outer diameter of 0.87 mm, is protected both thermally and mechanically by the tube support made of brass. The upper brass piece can be connected to the glass funnel (B). The reaction mixture is sprayed into the glass funnel, which is located in the cold isopentane bath (see text), and packed by a stainless steel rod (diameter 0.5 mm) into the 94-GHz EPR quartz tube.

Bruker Elexsys 680 spectrometer equipped with a fundamental mode microwave resonator. Spectra were recorded at $T = 80$ K. Low microwave power, typically only a few microwatts, was used in order to avoid saturation effects. For determination of precise $g$ tensor components, the microwave frequency was measured by a frequency counter, which was integrated in the spectrometer. The magnetic field was calibrated with a $g$ standard (lithium in LiF, $g = 2.002293(2)$, Ref. 25) at two different frequencies (typically 93.8 and 94.2 GHz). All spectra were recorded in the “persistent mode” of the superconducting magnet, using the room temperature coils for the field sweep to ensure high linearity and stability of the field (maximum sweep width 800 G). The modulation amplitude was kept at 0.2 mT in order to avoid modulation broadening. The design of the 94-GHz EPR microwave resonator ensures high sensitivity and a small active sample dimension (0.7 mm diameter and ~1.5 mm height). This leads to a high homogeneity of the field over the active sample volume (better than 5 ppm) and an instrumental limit for the absolute $g$ resolution obtained for the narrow signal of the $g$ tensor standard of ±0.000005 (lithium in LiF, approximate line width 0.01 mT).

High Frequency EPR Spectroscopy at 190 and 285 GHz—Continuous wave high frequency EPR measurements at 190 and 285 GHz were performed at the Grenoble High Magnetic Field Laboratory with a setup described in detail elsewhere (26). The spectrometer works in the transmission mode without a microwave resonator. This enables EPR spectra to be recorded at several frequencies with the same experimental setup and without removing the sample. For these measurements a Gunn oscillator with a fundamental frequency of 95 GHz was used together with a frequency doubler or tripler (Radiometer Physics, Meckenheim, Germany), thereby extending the EPR frequency to 285 GHz. The lower sensitivity resulting from the absence of a microwave resonator is partly compensated by a larger sample volume, typically 0.3 ml. The field homogeneity on the sample volume is about 50 ppm, which may mask some of the hyperfine structure of the radical. The absolute $g$ values were obtained by using the $g$ value, as determined from the 94-GHz EPR spectrum of the same freeze-quenched material.

Simulation of High Frequency EPR Spectra—The EPR spectra were analyzed using a software for simulating and fitting EPR spectra for $S = 1/2$ systems with anisotropic $g$ and $h$ tensors described previously (27). Thus the spectra are simulated by computing the resonant field position correct to second order at the given microwave frequency, dependent on the orientation of the $g$ and $h$ tensors with respect to the external magnetic field (28, 29). No restriction for the relative orientation of the principal axes of the different tensors is applied (27).

RESULTS

EPR Spectroscopy at 9.6 GHz

Wild Type P450cam—The 9.6-GHz EPR spectrum obtained from substrate-free $^{57}$Fe-P450cam, which reacted with PA within 40 ms, is shown in Fig. 2A. The spectrum does not show any signal due to a magnetically coupled cpd I species. Instead, the spectrum of the starting material is superimposed by a strong radical signal that accounts for 40 ± 5% of the total observable EPR intensity in the low spin Fe(III) EPR spectral range. EPR spectra of substrate-free $^{57}$Fe-P450cam obtained after 8-ms reaction time have been already published previously (9) and resemble qualitatively the spectrum in Fig. 2A except that the corresponding radical yields are 15 ± 5% (3).

It should be noted that the radical signal obtained at 20 K has been simulated by means of an isotropic $g$ value of $g = 2$. In order to resolve hyperfine structure of the radical, a spectrum of the $g ~ 2$ region has been taken at 140 K (inset of Fig. 2A). The native ferric form of the heme cannot be observed at this temperature, and the hyperfine structure of the radical observed at 140 K is expected not to be influenced by line broadening due to dipole interaction with nearby paramagnetic centers. The hyperfine structure of the radical observed after 40-ms reaction time presented here is the same as that observed for the 8-ms reaction time (9) and is independent of whether $^{57}$Fe or $^{56}$Fe is in the heme.

The hyperfine pattern of the radical resembles that of tyrosine radical intermediates of turnip isoperoxidase-7 and bovine liver catalase (31) and is well reproduced by the simulation shown in the inset of Fig. 2A. The simulation has been calculated using the parameters obtained for Y96* by the analysis of the high frequency data presented below (see Table II and “Discussion”). The consistent analysis of the EPR data obtained

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Footnote: The reported relative yields are based on the integration of the EPR spectra at 20 K. Because quantification of freeze-quenched EPR samples is generally unreliable due to packing effects, we have performed a quantification of the ferric low spin signal by Mössbauer spectroscopy and obtained a concentration of 405 μM after 8-ms and 330 μM after 40-ms reaction time. By using these values and the relative radical yields of 15 ± 5 and 40 ± 5%, we obtain 71 ± 24 μM for the radical concentration after 8-ms and 220 ± 28 μM after 40-ms reaction time, respectively.
Experimental parameters: $T = 20 \, \text{K}$; microwave power $= 80 \, \mu\text{W}$; microwave frequency $9.64 \, \text{GHz}$; modulation amplitude $0.5 \, \text{mT}$; modulation frequency $100 \, \text{kHz}$; and a field sweep of $0.25 \, \text{mT/s}$.

A very similar spectrum is also observed both for the 8- and 40-ms reaction time when mCPBA is used as an oxidant (spectra not shown). In this case, the radical accumulates for $39 \pm 5\%$ of the total spin density for 40 ms, respectively.

The microwave power saturation behavior revealed faster spin relaxation as compared with a radical in a pure diamagnetic environment as we have reported previously (9). For example, the wild type radical exhibits a $P_{\nu}$ value of 1 mW at 70 K. This is in the same order of magnitude as $P_{\nu}$ values reported for e.g. tyrosine radicals from RNR M. tuberculosis (e.g. 0.7 mW at 77 K), but it is much larger as that reported for a free semiquinone radical (0.07 mW at 90 K) (9). This indicates weak magnetic coupling to a paramagnetic species located at a moderate distance. Therefore, the radical has already been tentatively assigned to Tyr-96 (9), which is $\sim 7.4 \, \text{Å}$ away from the heme ring and $\sim 9.6 \, \text{Å}$ from the heme iron (32, 33).

However, a second tyrosine, Tyr-75, is located at a similar distance ($\sim 7.6 \, \text{Å}$) from the heme ring edge and may also be a candidate for the radical. The presence of a tryptophan radical also cannot be ruled out on the basis of the 9.6-GHz EPR data, because tyrosine and tryptophan radicals can exhibit very similar EPR spectra at 9.6 GHz (34). It should also be noted that when amino acid radicals are even closer to the heme (3-4 Å), for example the tryptophan radical in cytochrome c peroxidase, they exhibit a completely different EPR spectrum, which is governed by a strong exchange interaction between the radical and the heme iron (35).

**Y96F Mutant of P450cam**—Fig. 2B shows the 9.6-GHz EPR spectrum of a P450cam Y96F mutant sample that was reacted with PA for 8 ms. Again, a strong radical signal at $g = 2.0$ and the low spin ferric heme EPR signature are observed, identical to the unreacted P450cam Y96F mutant with $g$ factors of 2.45, 2.26, and 1.91. The heme iron $g$ factors of the mutant are almost identical to the $g$ factors of the wild type protein (2.45, 2.25, and 1.91 (9)). The relative EPR intensity of the radical is $22 \pm 5\%$ which is comparable with the radical yield observed for the wild type after 8 ms of reaction time (9).

The hyperfine structure of the mutant radical signal obtained at 140 K, shown in the inset of Fig. 2B, however, is significantly different from that formed during the reaction with the wild type enzyme after 40 (inset of Fig. 2A) and 8 ms (9). In contrast to the wild type, the mutant radical spectrum shows a large doublet splitting similar to the tyrosine radical observed for Y122* of E. coli RNR (36).

Again, the hyperfine structure of the radical is well reproduced by a simulation using the $g$ tensor and ring proton hyperfine tensors obtained from the 94- and 285-GHz EPR at all frequencies presented in this study allowed a reliable determination of $g$ and hyperfine parameters that are required to identify and assign the radical. In particular the $g$ tensor values cannot be obtained from an analysis of the 9.6-GHz EPR data alone.

The inset shows a spectrum of the $g = 2$ region obtained at $T = 140 \, \text{K}$; $p = 200 \, \mu\text{W}$; modulation amplitude 0.05 mT; modulation frequency 100 kHz; and a field sweep of 0.25 mT/s; dotted trace, simulation calculated with parameters obtained from high field EPR (see text and Table II). $C$, substrate-free Y96F/Y75F double mutant of cytochrome P450cam (8-ms reaction time). The dotted line is a simulation assuming two components with Gaussian line shape: the signal of the start material (with $g$ values of 1.91, 2.25, and 2.44 and line widths of 6.0, 4.0, and 8.4 mT; relative contribution 78% $g = 2$ and isotropic line width of 3.0 mT; relative contribution 22% $g = 1.91$).

*Multifrequency EPR on Freeze-quenched P450 Intermediates* (86)
Multifrequency EPR on Freeze-quenched P450 Intermediates

Wild Type P450cam—Fig. 3 shows EPR spectra obtained at 190 and 285 GHz of substrate-free $^{56}$Fe-P450cam, which was reacted with PA in 5-fold excess to P450 and freeze-quenched after 40 ms. The absence of a clear hyperfine structure on these spectra is not due to the large modulation amplitude used (2.2 mT), because similar spectra are obtained for much lower modulation amplitudes. At 190 GHz (Fig. 3A) the $g_x$ component on the high field side is clearly resolved, and at 285 GHz (Fig. 3B) the $g_x$ component on the low field side also exhibits good resolution. However, the $g_y$ range is significantly broadened, indicating a distribution of $g_y$ values. This broadening is not due to hyperfine interaction, because it has a different width (on a field scale) at 190 and 285 GHz. A similar distribution of $g_y$ values has been observed earlier for tyrosine radicals in several enzymes, i.e. in turnip peroxidase (31). The distribution indicates two maxima at $g_y$ values of 2.0078 and 2.0064. The values for $g_x$ and $g_z$ obtained from the simulation shown in Fig. 3B are 2.0043 and 2.0022, respectively. These $g_y$ values are fingerprints for tyrosine radicals in a polar hydrogen bonding heterogeneous environment (see Table I and “Discussion” below).

$^{56}$Fe Mutant of P450cam—Fig. 3C shows the EPR spectrum of the P450 Y96F mutant obtained at 285 GHz. The simulation (upper trace) yields $g_x$ = 2.0022 (1), $g_y$ = 2.0042 (1), and a bimodal distribution of the $g_z$ values around $g_z$ = 2.0080 and around $g_z$ = 2.0067, which is much more pronounced than for the wild type (Fig. 3B). Again, these $g_z$ values are characteristic of a tyrosine radical (Table I). The maximum of the $g_z$ distribution at $g_z$ = 2.0080 is characteristic of a tyrosine radical in a weak polar environment, and the maximum at $g_z$ = 2.0067 is characteristic for a strong hydrogen-bonding environment (see below) (31).

Y96F/Y75F Double Mutant of P450cam—EPR measurements at 190 and 285 GHz have not been performed with the double mutant because of the small radical yield.

EPR Spectroscopy at 94 GHz

Wild Type P450cam—Fig. 4 (trace b) shows the 94-GHz EPR spectrum of substrate-free $^{57}$Fe-P450cam, which reacted with PA for 40 ms and stems from the same freeze-quenched material as used for 9.6-GHz spectrum in Fig. 2A. The $g_x$ and $g_y$ components are clearly separated in the spectrum and both show a weak polar environment (see Table I and “Discussion” below). The maximum of the $g_z$ distribution of the $g_z$ values was modeled by summing up four simulated spectra with $g_z$ values of 2.0081, 2.0079 (weight 1), 2.0066, and 2.0068 (weight 0.5), with some minor contribution of a very low value of 2.0062 (weight 0.3). The same distribution of $g_z$ values was also used in the simulation of the 94-GHz spectrum (Fig. 4, trace d).

Fig. 3. High frequency EPR spectra of substrate-free wild type and Y96F mutant cytochrome P450cam freeze-quenched with peracetic acid. P450 wild type or mutant (1 mM) reacted with peracetic acid (5 mM). The buffer was 100 mM potassium phosphate, pH 7. Spectra were obtained in transmission geometry. Experimental parameters: A, wild type $^{56}$Fe-P450cam, 40-ms reaction time, $T = 5$ K; modulation amplitude 2.2 mT, modulation frequency 0.8 kHz and microwave frequency 190 GHz, field sweep 0.5 mT/s; B, wild type $^{56}$Fe-P450cam, 40-ms reaction time, $T = 5$ K; modulation amplitude 2.4 mT, modulation frequency 0.8 kHz and microwave frequency 285 GHz, field sweep 0.5 mT/s. Exp, experimental spectrum; Sim, simulated spectrum after field calibration using $g_x$ = 2.0022 (from 94-GHz EPR simulations) yielding the $g_x$ value and a distribution of $g_y$ values around two maxima at $g_x$ = 2.0064 and $g_x$ = 2.0078 (Table I). Single component line width used in the simulation: 4 mT. C, $^{56}$Fe-P450cam; 8-ms reaction time, obtained in transmission geometry (lower trace). Experimental parameters: $T = 5$ K; modulation amplitude 2.4 mT, modulation frequency 0.8 kHz, and microwave frequency 285 GHz, field sweep 0.5 mT/s. Upper trace, simulated spectrum after field calibration using $g_x$ = 2.0022 (from 94-GHz EPR simulations). The observed bimodal distribution of the $g_z$ values was modeled by summing up four simulated spectra using $g_z$ values of 2.0081, 2.0079 (weight 1), 2.0066, and 2.0068 (weight 0.5), with some minor contribution of a very low value of 2.0062 (weight 0.3). The same distribution of $g_z$ values was also used in the simulation of the 94-GHz spectrum (Fig. 4, trace d).
errors in the last digit. A bimodal distribution of $g_x$ values was seen in the 94-GHz spectrum which probably originates from the different oxidant residues (acetic acid or m-chlorobenzoic acid) which may still stay in the heme pocket after O–O bond cleavage.

Y96F Mutant of P450cam—Fig. 4 (trace c) shows the 94-GHz EPR spectrum of the substrate-free $^{56}\text{Fe}$-P450cam Y96F mutant, reacted with PA for 8 ms. The simulation of the spectrum (Fig. 4, trace d) yields $g_x$ values of $g_x = 2.00219 \pm 0.00004$ and $g_y = 2.0043 \pm 0.0001$. In the simulations the bimodal distribution of $g_x$ values was modeled by summing up several simulated spectra, using $g_x$ values of 2.0081, 2.0079, 2.0066, and 2.0068, with some minor contribution of a very low value of 2.0062 but the same $g_x$ and $g_y$ values for all spectra (Fig. 4 legend). The same distribution of $g_x$ values was used in the simulation of the 285-GHz spectrum (Fig. 3C, upper trace) where the two higher and the two lower $g_x$ values are merged into the mean values at 2.0080 and 2.0067, respectively.

The most obvious difference to the 94-GHz EPR spectrum of the wild type (Fig. 4, trace b) is the large isotropic doublet splitting, which is clearly resolved on the $g_x$ component and leads also to a changed hyperfine pattern on the $g_y$ component. The simulation yields an isotropic value of 1.55 ± 0.05 mT for this hyperfine coupling, which is assigned to one of the $\beta$-CH$_2$ protons of the side chain. The hyperfine splitting values of the ring protons used in the simulations were the same as for the spectrum of the wild type P450cam (Table II).

**FIG. 4.** 94-GHz EPR spectra of substrate-free wild type and mutant P450cam freeze-quenched intermediate. P450 wild type or mutants (1 mM) reacted with peracetic acid (5 mM). The buffer was 100 mM potassium phosphate, pH 7. Amplitudes of spectra a, b, and c are scaled to the same size for better comparison. Spectra b and f are shown on the same scale and hence represent approximately the relative signal intensities. Trace a, best simulation for the experimental EPR spectrum of wild type $^{56}\text{Fe}$-P450cam (trace b) freeze-quenched with PA after 40-ms reaction time; $g_x = 2.00219$, $g_y = 2.0043$, and $g_z = 2.0044$, the $g_z$ distribution was modeled by the sum of four simulated spectra using $g_z$ values between 2.0078 and 2.0062; hyperfine tensors from two ring protons and one $\beta$-proton ($A_{\alpha\beta}(H_{9004}) = 1.1$ mT) of the side chain (see Table II). Single component line width, 0.6 mT. Trace c, experimental spectrum of wild type cytochrome $^{57}\text{Fe}$-P450cam, freeze-quenched with mCPBA after 40-ms reaction time. Trace d, best simulation of the experimental spectrum of substrate-free Y96F mutant $^{56}\text{Fe}$-P450cam freeze-quenched with PA after 8-ms reaction time (trace e) yielding $g_x = 2.00219$ (5), $g_y = 2.0043$ (1); the numbers in parentheses are estimated errors in the last digit. A bimodal distribution of $g_x$ values (2.0081, 2.00079, 2.0068, and 2.0066) was used. The two mean values (2.0080 and 2.0067) are resolved clearly in the 285-GHz spectrum (Fig. 3C). Obtained hyperfine tensor values from two ring protons and one $\beta$-proton ($A_{\alpha\beta}(H_{9004}) = 1.55$ (5) mT) of the side chain (see Table II). Single component line width, 0.6 mT. Trace f, experimental spectrum of substrate-free Y96F/Y75F double mutant $^{56}\text{Fe}$-P450cam freeze-quenched with PA after 8-ms reaction time. Experimental parameters for all spectra: $T = 80$ K, microwave power = 5 $\mu$W, modulation amplitude 0.2 mT, modulation frequency 10 kHz, microwave frequency 94,000 GHz, field calibrated with lithium/LiF to an accuracy of 5 ppm, each spectral trace obtained by numerical summation of 20–22 single scans (80 s each) corresponding to a total accumulation time of 30 min.

resolved hyperfine structure, in particular the $g_x$ value. The simulation of the spectrum (Fig. 4, trace a) yields $g_x$ values of $g_x = 2.00219 \pm 0.00004$ and $g_x = 2.0043 \pm 0.0001$. The $g_x$ component is broadened and without structure. The spectrum was simulated using the same distribution of $g_x$ values between 2.0078 and 2.0062 as for the simulation of the 285-GHz EPR spectra and a superposition of two very close $g_x$ values of 2.0044 and 2.0042, which were not resolved within the single component line width of the 285-GHz simulations.

Furthermore, this simulation reveals the hyperfine tensor principal component $A_e = -0.7$ mT from two ring protons (positions 3 and 5, Fig. 5). For the other tensor components of these protons, values of $A_e = -0.9$ mT and $A_e = -0.35$ mT were used, as they are known from other tyrosine radicals (Table II). This assumption is justified because the hyperfine values for these ring protons are similar for the majority of protein tyrosine radicals observed so far (34, 37–41). The largest hyperfine coupling in tyrosine radicals, which is almost isotropic, results from one of the $\beta$-protons of the tyrosine side chain. The simulation (trace a) yields a value of 1.1 ± 0.1 mT for this $\beta$-proton (Table II). When P450cam was reacted for 40 ms with the other oxidant, mCPBA, a 94-GHz EPR spectrum (Fig. 4, trace c) similar to that for PA as oxidant (trace b) was obtained analogous to the 9.6-GHz spectra discussed above. Only a slight difference for the distribution of the $g_x$ values was seen in the 94-GHz spectrum which probably originates from the different oxidant residues (acetic acid or m-chlorobenzoic acid) which may still stay in the heme pocket after O–O bond cleavage.

**DISCUSSION**

Comparison of Wild Type and Y96F P450cam

The Radical EPR Signals Reflect Tyrosine Radicals—The $g$ tensor components obtained from an analysis of the high frequency EPR data clearly show that the radicals, both in the wild type and the Y96F mutant, are tyrosine radicals. The deviation $\Delta g_{x,y} = g_{x,y} - g_e$ from the free electron $g$ value 2.002319 results from spin-orbit coupling and increases when significant spin densities occur at heavier nuclei such as oxygen, which exhibits a larger spin-orbit coupling constant as compared with carbon or nitrogen (42). $\Delta g_e$ values are $-0.0015$ for tyrosophan radicals (34), having spin density only on carbon and nitrogen nuclei, whereas for radicals with spin density on oxygen nuclei, such as tyrosines and quinones, $\Delta g_e$ values range from 0.004 up to 0.007 (34, 43–45) (Table I). Therefore, $\Delta g_x$ and $\Delta g_y$ are indicative of the type of radical. Tyrosine radicals have long been detected in plant photosystem II (46,
47) and in aerobic RNR (36, 48). They were also found to be transient intermediates in a variety of enzymes, such as peroxidases (31), cytochrome c oxidase (49), and in DNA photolyase, where, depending on the organism, tryptophan or tyrosine radicals have been observed (50). The reported $g_z$ values of tyrosine radicals range from 2.0091 for a non-polar environment in RNR of *E. coli* (34, 38, 51) to 2.0076 for a hydrogen-bonded situation in RNR of mouse and yeast (34, 38, 39) and in plant photosystem II (37, 43). Recently, for a tyrosine radical in turnip peroxidase, a significantly lower value of 2.0066 was reported, probably indicating strong hydrogen bonding (31). A similar low $g_z$ value has been reported for tyrosine radicals in $\gamma$-irradiated crystals (52) (Table I).

Comparison of the $g$ tensor components obtained for the freeze-quenched transient radical in substrate-free wild type and mutant Y96F P450cam with those of other tyrosine radicals shows that they are typical of a tyrosine radical in a polar environment, which is probably hydrogen-bonded at the phenoxy oxygen (Table I). Tryptophan radicals were shown to have much smaller $g_x$ and $g_y$ values (34) which excludes tryptophans as the radical site in P450cam. Beyond this identification of the nature of the observed radical, the $g$ and hyperfine values obtained contain information about the geometry and the environment of the tyrosine radical, which allows the assignment to specific tyrosines in the structure of the protein (see below).

### Table I

| Tyrosine radical/organism | $g_x$ | $g_y$ | $g_z$ | Ref. |
|---------------------------|-------|-------|-------|------|
| Y96$^*$ in P450cam, wt$^*$ | 2.0078–2.0064 | 2.0044–2.0042 | 2.00219 | This work |
| Y75$^*$ in mutant Y96F$^*$ | 2.0080–2.0067 | 2.0043 | 2.00219 | This work |
| Y122$^*$ in RNR *E. coli* | 2.00912 | 2.00457 | 2.00225 | 51 |
| Y122$^*$ in RNR *E. coli* | 2.0091 | 2.0043 | 2.0022 | 38 |
| Y122$^*$ in RNR *E. coli* | 2.0091 | 2.0046 | 2.0021 | 34 |
| Y177$^*$ in RNR mouse$^*$ | 2.0076 | 2.0044 | 2.0021 | 34 |
| Y177$^*$ in RNR mouse$^*$ | 2.0076 | 2.0043 | 2.0022 | 38 |
| Tyr$^*$ in RNR herpes simplex (HSV 1)$^*$ | 2.0076 | 2.0043 | 2.0022 | 38 |
| Y$_{10}$ in PS II from spinach$^*$ | 2.00756 | 2.00432 | 2.0015 | 43 |
| Y$_{10}$ in PS II from *S. elongates*$^*$ | 2.00767 | 2.00438 | 2.0019 | 37 |
| Y$^*$ in wild type cytochrome c peroxidase, cpd I | 2.00644 | 2.00436 | 2.00232 | 76 |
| Y$^*$ in bovine liver catalase | 2.00777 | 2.0046 | 2.00232 | 77 |
| Y$^*$ in turnip isoperoxidase | 2.0066 | 2.0043 | 2.0020 | 31 |
| Y$^*$ in $\gamma$-irradiated tyrosine crystals$^*$ | 2.0067 | 2.0045 | 2.0023 | 52 |
| Y$^*$ in RNR, yeast$^*$ | 2.00770 | 2.00435 | 2.00229 | 39 |

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$^*$ A distribution of $g_z$ values, around two maxima (2.0078 and 2.0064 in wild type (wt)); 2.0080 and 2.0067 in Y96F) was obtained from EPR simulations (see “Results”). The estimated error of the $g_x$, $g_y$, and $g_z$ values from simulations of the 94-GHz EPR spectra is ±0.0001 and ±0.00005, respectively.

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$^\dagger$ Non-polar environment and non-hydrogen-bonded (see references and “Results”).

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$^\ddagger$ Polar environment and hydrogen-bonded (see references). A hydrogen bond to the tyrosine oxygen was confirmed for RNR of mouse (38) and yeast (39).

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$^a$ A distribution of $g_z$ values (2.0066, width 2$\Delta g_z$ = 0.0007) was observed; polar environment.

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$^b$ In Ref. 52 also $g$ tensor axes have been determined; $g_x$ is parallel to the C=O bond, and $g_z$ is parallel to the tyrosine molecular plane (see Fig. 5).

### Table II

| Proton hyperfine tensor principal values (mT) for tyrosine radicals in different enzymes |
|------------------------------------------|
| $\propto(H_1)$ | $\propto(H_2)$ | $\propto(H_3)$ |
| Y96$^*$ in P450cam (this work$^*$) | −0.90(5) | −0.35(5) | −0.70(5) | 1.11(1) | <0.3 | A.
| Y75$^*$ in mutant Y96F P450cam (this work$^*$) | −0.96(5) | −0.30(5) | −0.70(5) | 1.19(1) | <0.3 | A.
| Y$_{10}$ in spinach (41) | −0.91 | −0.29 | −0.71 | 1.02 | 0.22 | A.
| Y$_{10}$ in *S. elongates* (37) | −0.93 | −0.28 | −0.70 | 1.04 | <0.3 | A.
| Y172$^*$ in R2, *E. coli* (34) | −0.96 | −0.28 | −0.70 | 2.06 | <0.2 | A.
| Y122$^*$ in R2, *E. coli* (40) | −0.96 | −0.28 | −0.70 | 2.01 | <0.2 | A.
| Y122$^*$ RNR, *E. coli* (39) | −1.0 | −0.4 | −0.6 | 2.1 | 0.3 | A.

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$^a$ Values are from simulations of the 94-GHz EPR spectra (Fig. 4). Numbers in parentheses are estimated errors in the last digit. The hyperfine tensor principal axes $x$ and $y$ for the protons at positions 3 and 5 (Fig. 5) are rotated by approximately +20$^\circ$ (position 3) and −20$^\circ$ (position 5) with respect to the $g$ tensor $x$ and $y$ axes (see Table I and Refs. 34, 37, 39, and 40).

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$^b$ $\propto$ axes of hyperfine tensors of position 3 and 5 and $g$ tensor $z$ axis are colinear (see Table I and Refs. 34, 37, and 43).

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$^\dagger$ Isotropic values, $A_{iso}(H_\parallel)$, of the hyperfine components, $A_{1,2,3}(H_f)$, from simulations of 94-GHz EPR spectra (see Table I and Ref. 34). Other isotropic values were calculated from the $A_x$, $A_y$, and $A_z$ values measured by ENDOR (37, 41). Calculated hyperfine angle from $A_{iso}(H_f)$, for the radical in wild type P450cam: $\theta = 45.5 \pm 3^\circ$; and for mutant Y96F, $\theta = 25–27^\circ$ (94-GHz data) and $\theta = 19–23^\circ$ (9.6-GHz data, see text).

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$^\ddagger$ $\Delta g$ values were determined by ENDOR (40, 41) or represent maximum values as estimated from EPR simulations (this work and Refs. 34 and 37).
tyrosine radicals in wild type P450cam with \( g_s = 2.0078 \) is probably weakly hydrogen-bonded, whereas the fraction with \( g_s = 2.0064 \) is probably strongly hydrogen-bonded. According to the electrostatic model of Un et al. (55), such a low \( g_s \) value should correspond to a very short hydrogen bond. The x-ray structure shows that a cluster of 6 water molecules is located at distances between 4.6 and 7.8 Å from Tyr-96 in the wild type substrate-free P450cam (Fig. 5A, Protein Data Bank code 1phc (33)). Thus, it seems likely that this relatively loosely bound water cluster is redistributed inhomogeneously upon radical generation, which causes either a very strong or a weak hydrogen bond to the resulting radical Y96\(^*\). The low \( g_s \) value of 2.0064 may indicate interaction even with several water molecules. Thus, the environment likely depends on the actual geometry of this water cluster in each protein molecule, leading to the observation of a range of \( g_s \) values that is consistent with the environment of the tyrosine radical being heterogeneous, yet polar in nature.

In the 285-GHz EPR spectrum observed for the mutant Y96F, both maxima of the bimodal \( g_s \) distribution are shifted to higher values (2.0080 and 2.0067, as compared with 2.0078 and 2.0064 in the wild type), as it is evident from the comparison of Fig. 3, B and C. Tyr-75, which is the closest tyrosine to the heme in the Y96F mutant, and therefore likely to be the radical site, is far from the water cluster but is hosted in a polar cage formed by the Gln-322 side chain NH\(_2\) and CO groups, the guanidinium group of Arg-299, the peptide CO group of Asp-297, and the heme propionic acid group. The nitrogen atom of the Gln-322 NH\(_2\) side chain is in a hydrogen-bonding distance to the oxygen atom of Tyr-75 (2.8 Å) (Fig. 5). The lack of a water cluster near the radical site could explain the shifted \( g_s \) values for the tyrosine radical in Y96F P450cam. Apparently, the protein offers two different conformations also of the Tyr-75 site, corresponding to the \( g_s \) values of 2.0080 and 2.0067. However, the distribution around each of these two maxima is much narrower in the mutant than observed for the wild type, where the broader distribution could result from the structural heterogeneity of the water cluster near the Tyr-96 radical site. In addition to these possible hydrogen bonds and polar interactions, it should also be considered that the remaining acetic acid, after splitting the O–O bond in PA, may still stay in the heme pocket and interact with the tyrosine, in particular with Tyr-96, which is close to the water cluster in the wild type protein. Nevertheless, based on the \( g \) values alone, it cannot be excluded that the \( g_s \) distribution is a consequence of the presence of tyrosine radicals at two different positions in the wild type enzyme.

Assignment of the Radical Signals to Specific Tyrosines Based on the Hyperfine Structure—Two classes of protons give rise to the observed hyperfine couplings in tyrosine radicals, i.e. the ring protons (\( \alpha \)-protons) and the \( \beta \)-protons of the side chain. Large couplings arise from the two magnetically equivalent ring protons in the ortho position to the oxygen. Their magnitude depends solely on the \( \pi \) spin density at the adjacent carbon atom and is very similar for all observed tyrosine radicals (Table II). The hyperfine tensor is strongly anisotropic, with typical components \( A = -0.9 \text{ mT}, A_{\beta \text{iso}} = -0.7 \text{ mT} \). These values lead to observable splittings of the \( g_s \) and \( g_c \) components in the 94-GHz EPR spectra, whereas the small \( A_{\beta \text{iso}} \) component is not resolved on the \( g_c \) component. An additional large hyperfine coupling arises from one of the two \( \beta \)-protons of the side chain. This coupling exhibits only small anisotropy, and its isotropic value, \( A_{\beta \text{iso}}(H_p) \), does not only depend on the \( \pi \) spin density \( \rho_{\pi} \) of the adjacent \( \pi \)-carbon but is also strongly dependent on the geometry of the side chain, according to Equation 1,

\[
A_{\beta \text{iso}}(H_p) = \rho_{\pi}(B'(B' + B\cos^2\theta)) \tag{Eq. 1}
\]

where \( B' \) and \( B'' \) are empirical constants (\( B' \sim 0, B'' = 5.0–5.24 \text{ mT} \)) and \( \theta \) is the dihedral angle between the axis of the \( \pi \)-orbital (\( \rho_{\pi} \)) of the adjacent carbon atom and the projected \( C_{\beta}H_{\beta} \) bond (27, 34, 56, 57) (Fig. 5B). The dependence of \( A_{\beta \text{iso}}(H_p) \) on the side chain orientation with respect to the tyrosine ring is strong, because \( \cos^2\theta \) can vary between 1 and 0 for each of the two \( \beta \)-protons.

Fig. 5A shows the structure of the heme moiety, together with the two nearest tyrosines, Tyr-96 and Tyr-75, for the substrate-free P450cam (32). For both tyrosines the distance...
between their oxygen atom and the nearest carbon atom of the porphyrin ring is similar (Tyr-96, 7.4 Å; Tyr-75, 7.6 Å). Their side chain geometries, however, are significantly different, with dihedral angles for the two β-protons of \( \theta_1 = 41^\circ \), \( \theta_2 = -79^\circ \) for Tyr-96, and \( \theta_1 = 26^\circ \), \( \theta_2 = -94^\circ \) for Tyr-75 (Fig. 5B).

From the simulation of the 94-GHz EPR spectrum of the wild type protein (Fig. 4, trace a), an isotropic hyperfine value, \( A_{iso}(H_{11}) = 3.1 \pm 0.1 \) mT, is obtained for one of the β-protons. This value is also in agreement with the simulation of the 9.6-GHz EPR spectrum (Fig. 2). By using Equation 1 with \( \rho^{c1}_1 = 0.38 \) from (58), \( B^1 = 5.0 \) mT and \( A_{iso}(H_{11}) = 1.1 \pm 0.1 \) mT, a dihedral angle of \( \theta_2 = 40.5 \pm 3^\circ \) is calculated, which matches very well the 41° obtained for Tyr-96 from the crystal structure of substrate-free P450cam (Protein Data Bank code 1pht). For the Y96F mutant, on the other hand, the value \( A_{iso}(H_{11}) = 1.55 \pm 0.05 \) mT is obtained for the β-proton from the simulation of the 94-GHz EPR spectrum (Fig. 4, trace d). A slightly higher value of \( A_{iso}(H_{11}) = 1.65 \pm 0.05 \) mT is obtained from the simulation of the 9.6-GHz EPR spectrum (Fig. 2). The deduced dihedral angle \( \theta_2 \) for the β-proton corresponds to 23–27° and 19–23° for the 94-GHz and 9.6-GHz hyperfine values, respectively.4 The range of 19–27° thus obtained is consistent with the angle of 26° for Tyr-75 seen in the crystal structure.

The resolved doublet splitting resulting from the larger β-proton hf coupling is a distinct feature of the Y96F EPR spectra both at 94 GHz and at 9.6 GHz. Simulations of superimposed spectra show that such a feature of the Y96F EPR spectrum, which is obviously characteristic for the Tyr-75 radical, should be visible in the wild type spectrum if at least 15% of Tyr-75 radical would co-exist with the Tyr-96 radical. However, no indication of such a superposition of the Tyr-75 radical spectrum was observed in the EPR spectrum of wild type P450cam. Therefore, we conclude that there is none or only negligible yield of Tyr-75 radical in the wild type P450cam. If both tyrosine radicals would coexist, one would also expect a significantly larger percentage of radicals for the wild type compared with the Y96F mutant, which is not the case.

The estimated radical yields for the wild type (15 ± 5% (9)) and the Y96F mutant (22 ± 5% (this study)) in the 8-ms trace are comparable within the experimental error. All these observations are in agreement with the existence of only one radical site either located at Tyr-96 (wild type) or at Tyr-75 (Y96F mutant).

The above assignment assumes that no major reorientation of the side chain occurs upon radical formation. Such a reorientation has been discussed recently for prostaglandin H synthase (59). However, a reorientation of the tyrosine radical side chains in P450cam is not indicated. The radical yield in the wild type protein increases with reaction time, with a maximum of 29% (this study)) in the 8-ms trace of a much broader (180 mT) and weaker spectrum (63, 64). They are therefore also ruled out. For the two latter forms of cysteine-based radicals, the g anisotropy leads to resolved g components even in the 9.6-GHz EPR spectra (63, 64). They are therefore also excluded.

The g values given above were reported for the uncoupled “free” amino acid radicals. Spin-coupling between the radical spin and a paramagnetic (Fe(IV)=O) moiety with \( g = 2.003 \) is not consistent with the observed \( g \) value of 2.014 (60, 61), which is not consistent with the observed \( g \) value of 2.003. Thus, we exclude the presence of peroxyl radicals.

As a further possibility a cysteine-based radical may be discussed, which can occur as thyl radical (R-S*) or as persulphyl radical (R-S-S-R*). Thiyl radicals were reported to have an axial g tensor with \( g_z \) values between 2.18 and 2.3, dependent on hydrogen bonding, and \( g \) values of 2.008 (62, 63) and are therefore ruled out.

The formation of a tryptophan radical, at least one large hyperfine splitting would be expected from the \( ^3P^0 \) spin density (34). In addition, the formation of a tryptophan radical is not very likely because of the large distance of the tryptophan closest to the heme (Trp-42–N atom to heme iron (17.6 Å) and to the heme edge (14.4 Å)). Peroxyl radicals, which could in principle be formed by homolytic splitting of the O–O bond, have been reported to have \( g \) values of 2.014 (60, 61), which is not consistent with the observed \( g \) value of 2.003. Thus, we exclude the presence of peroxy radicals.

For a further possibility a cysteine-based radical may be discussed, which can occur as thyl radical (R-S*) or as persulphyl radical (R-S-S-R*). Thiyl radicals were reported to have an axial g tensor with \( g_z \) values between 2.18 and 2.3, dependent on hydrogen bonding, and \( g \) values of 2.008 (62, 63) and are therefore ruled out. For the two latter forms of cysteine-based radicals, the g anisotropy leads to resolved g components even in the 9.6-GHz EPR spectra (63, 64). They are therefore also excluded.

The \( g \) values given above were reported for the uncoupled “free” amino acid radicals. Spin-coupling between the radical spin and a paramagnetic (Fe(IV)=O) moiety with \( S = 1 \) is expected to change these values. In this case the radical could be an amino acid radical close to the heme, i.e. an iron-ligating cysteine, or even a Fe(IV)=O-porphyrin cation radical.

Interestingly, a narrow EPR signal was reported in a 9.6-GHz EPR and electron nuclear double resonance study of the cpd I-type complex in cytochrome c oxidase generated with hydrogen peroxide (65), which was described as a narrow, intense portion of a much broader (180 mT) and weaker spectrum (66). Due to the weak intensity of the double mutant signal, the shape of this EPR signal could not be well resolved, and the validation of the possible explanations mentioned requires further investigations.

Implications for the Catalytic Mechanism

Heme iron-oxo species are key intermediates in many heme proteins. In P450cam this species is also regarded to be the catalytically relevant oxygen species that attacks the substrate. So far, the electron and spin distribution of this species has not been identified experimentally for the native reaction cycle in P450cam. In analogy to CPO, it is generally assumed that in all P450s, a cpd I intermediate with the iron in the Fe(IV)=O state (\( d^5, S = 1 \)) and a porphyrin-\( \pi \)-cation radical (\( S' = 2^+ \))...
1/2), is formed. In CPO, these spins couple antiferromagnetically (4, 22).

Our studies, however, show that such a spin system is not observed in P450cam for reaction times of ~8 ms (9, 67), which is the shortest quench time we can achieve with our freeze-quench setup. In contrast to the expected porphyrin π-cation radical, a tyrosine radical, now clearly assigned to Tyr-96 in the wild type protein and to Tyr-75 in the Y96F mutant, is observed.

The appearance of the tyrosine radical is independent of whether peracetic acid or m-chloroperbenzoic acid is used as the oxidant, indicating that a specific effect of peracetic acid does not exist. The iron is in the Fe(IV) state, as already the oxidant, indicating that a specific effect of peracetic acid radical, a tyrosine radical, now clearly assigned to Tyr-96 in the experiments.

The generation of the tyrosine radicals is not related to oxidant-induced bleaching of the porphyrin ring of P450cam. We have determined recently the bleaching rate and found that in the first 8 ms only ~0.005% and within 200 ms only ~0.1% of P450 is bleached (67), indicating that P450 bleaching due to peroxy-acetic acid plays no role in the freeze-quench experiments.

The binding of camphor apparently suppresses the tyrosine radical formation because the access to the heme pocket for peracetic acid is blocked (67). This is also the case for the Y96F mutant (data not shown). If the iron coordination site is blocked by a ligand such as metyrapone then the tyrosine radical formation is also suppressed (67). This observation shows that first the oxidant has to bind to the heme iron before the tyrosine radical can be formed. However, for camphor-bound wild type P450cam, a weak tyrosine radical signal with a similar electronic absorption spectrum that is similar to that seen for the Fe(II)=O peroxynitron–cation radical of CPO (71). There are two differences between the stopped-flow and freeze-quench experiments. For the freeze-quench experiments, higher protein concentrations are needed. However, the oxidant to P450 molar ratio is similar to that used in stopped-flow studies. Thus, the concentration difference does not seem to be important. Unspecific binding sites of the oxidant to produce tyrosine radicals just due to higher concentrations of the protein and the oxidant can be excluded because such unspecific effects should also appear if the specific binding site (the heme iron) is blocked by camphor or metyrapone which was not observed (see above). The other difference is the time mode of spectroscopic monitoring of the intermediate. While the spectrum of the intermediate is directly measured after 6–8 ms in the stopped-flow experiment, the intermediate is trapped at ~110 °C first, and then some time for filling the EPR tube (~10 min) is needed and for freezing it to liquid nitrogen temperatures. We found that the yield of radical formation is independent of the time needed to fill the EPR tube (10–40 min), thus excluding the possibility that the electron transfer from the tyrosines to the porphyrin radical occurs only within this time range at ~110 °C.

The transient tyrosine radical may principally be formed as follows: (i) by homolytic splitting of the peroxo O−O bond, resulting in formation of a compound II-like Fe(IV)=O species
and an acetic (or m-chlorobenzoic) carboxyl radical, which may abstract a hydrogen atom from tyrosine to yield a tyrosine radical, or (ii) by heterolytic splitting of the peroxy O–O bond resulting in formation of a Fe(IV)=O species and a porphyrin-π-cation radical, which is rapidly reduced by electron transfer from Tyr-96 and by release of a proton, with the latter being taken up by the remaining acetic (or m-chlorobenzoic) carboxylate. The heterolytic splitting modes have been found for porphyrin complexes in reaction with peroxopyrroleacyclic acid (72). We suggest that the latter mechanism (ii) is more likely to occur, because a similar electron transfer has recently been observed for bovine liver catalase reacted with peracetic acid in a heterolytic O–O bond splitting mode, and it was shown that the porphyrin radical is formed first and then reduced by a tyrosine (73). There are recent reports by several laboratories demonstrating similar findings on other proteins (74). In the case of P450cam studied here, the intramolecular electron transfer from Tyr-96 in the wild type will likely proceed via Thr-101 to the propionic acid side group of the heme and from there to the porphyrin ring, as calculations using the pathway program HARLEM suggest (67). If this Tyr-96 is replaced by phenylalanine, then the electron transfer occurs from the next possible tyrosine, which is Tyr-75, via Gln-92 to the nearest methyl group of the heme, according to the pathway calculation using HARLEM.

In the P450cam double mutant Y96F/Y75F of this study, the electron transfer from the remaining tyrosines which are closest to the heme seems not to be efficient because of the long distance. Tyr-201 and Tyr-29 are located at distances to the heme iron (heme edge) of 16.1 (14.3 Å) and 16.6 Å (14.9 Å), respectively. In this case, where the electron transfer from tyrosines to the porphyrin is disrupted, an EPR spectrum is observed that is almost identical with that of the resting enzyme, except for a weak radical signal at g = 2.003 of yet unknown origin. If this weak signal in Y96F/Y75F does not reflect that of an exchange-coupled porphyrin-π-cation radical, then our results could mean that either the coupled Fe(IV)=O porphyrin-π-radical is simply not formed in P450 or that another reductive quench process exists which becomes important in this double mutant and leads to an EPR silent product.

The question arises as to whether the tyrosine radical plays any functional role for substrate conversion. For this particular P450 (P450cam), we consider it unlikely that in the native camphor-bound enzyme, the tyrosine radical itself would directly be involved in the hydroxylation, based upon the structure of the camphor-bound enzyme that shows the 5-carbon to be very close to the position where the ferryl oxygen would bind. However, such intramolecular electron transfer competes with the hydroxylation and may diminish the efficiency of hydroxylation, similarly to those for the well known uncoupling processes as follows: (i) at the dioxygen complex level (autoxidation, O2 formation); (ii) at the peroxo level (formation of H2O2); and (iii) at the level of the iron-oxo species (oxidase reaction, formation of the second water molecule) (2). For other cytochromes P450 which convert diverse substrates such a transient tyrosine radical may also function in abstraction of a hydrogen atom from a substrate before it is further converted. Analogous conclusions about tyrosine radical function have been discussed recently (74) for the catalase peroxidase from Mycobacterium tuberculosis.

In conclusion, the important finding in our studies is that a new intramolecular reaction has been detected that competes with the cpd I level with the oxygen insertion into the substrate. Such competitive electron transfer has never been considered previously for P450cam but may also occur in other P450s. Such potential competitive intramolecular electron transfer could also explain why it is so difficult to characterize the cpd I intermediate experimentally. To further study the reaction intermediate and to check whether a porphyrin-π-cation radical is really formed in P450, it is necessary to shorten the reaction time significantly. For our freeze-quequen setup we are limited to ~8 ms. Very recently, a new freeze-quequen device with a dead time of ~120 μs (reaction time shorter by a factor of 10) has been reported by Tanaka et al. (75) and applied to study the short lived intermediate in the reaction of horseradish peroxidase with hydrogen peroxide. The application of such device to P450 could give information whether the porphyrin-π-cation radical is formed in the time range of 100 μs to 8 ms or whether at least the tyrosine radical yield is diminished.
Tyrosine Radical Formation in the Reaction of Wild Type and Mutant Cytochrome P450cam with Peroxy Acids: A MULTIFREQUENCY EPR STUDY OF INTERMEDIATES ON THE MILLISECOND TIME SCALE
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