

Intermediates in the reaction of substrate-free cytochrome P450_{cam} with peroxy acetic acid

V. Schünemann^a, C. Jung^{b,*}, A.X. Trautwein^{a,1}, D. Mandon^c, R. Weiss^c

^aInstitute of Physics, Medical University, 23538 Lübeck, Germany

^bMax-Delbrück-Center for Molecular Medicine, 13092 Berlin, Germany

^cLaboratoire de Cristallogénie, UMR 7513, Université Louis Pasteur, 67070 Strasbourg, France

Received 21 June 2000; accepted 13 July 2000

Edited by Richard Cogdell

Abstract Freeze-quenched intermediates of substrate-free cytochrome ⁵⁷Fe-P450_{cam} in reaction with peroxy acetic acid as oxidizing agent have been characterized by EPR and Mössbauer spectroscopy. After 8 ms of reaction time the reaction mixture consists of ~90% of ferric low-spin iron with *g*-factors and hyperfine parameters of the starting material; the remaining ~10% are identified as a free radical (*S'* = 1/2) by its EPR and as an iron(IV) (*S* = 1) species by its Mössbauer signature. After 5 min of reaction time the intermediates have disappeared and the Mössbauer and EPR-spectra exhibit 100% of the starting material. We note that the spin-Hamiltonian analysis of the spectra of the 8 ms reactant clearly reveals that the two paramagnetic species, e.g. the ferryl (iron(IV)) species and the radical, are not exchanged coupled. This led to the conclusion that under the conditions used, peroxy acetic acid oxidized a tyrosine residue (probably Tyr-96) into a tyrosine radical (Tyr[•]-96), and the iron(III) center of substrate-free P450_{cam} to iron(IV). © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Cytochrome P450_{cam}; Peroxy acetic acid; Mössbauer spectrum

1. Introduction

The enzyme super-family cytochrome P450 plays an important role in many physiological processes as for example biotransformation of xenobiotics and synthesis of steroid hormones. Cytochromes P450 are found in all organisms where they catalyze a whole variety of reactions, such as aliphatic and aromatic hydroxylations, epoxidations, heteroatom oxidation, and *N*- and *O*-dealkylation, by transfer of an active oxygen from its heme unit to the substrates [1]. The common feature of the cytochrome P450 family is a substrate binding site next to the enzymatically active heme, which is a protoporphyrin IX prosthetic group with a cysteinate residue (Cys-357 in P450_{cam}) coordinately bound to the iron [2].

The proposed enzymatic cycle [1,3] starts with the resting

low-spin ferric state (*S* = 1/2) which converts to the ferric high-spin (*S* = 5/2) state on substrate binding. After reduction to the ferrous high-spin state, molecular oxygen binds to form the diamagnetic oxy-P450 state. Transfer of the second electron originating from NAD(P)H via redox proteins (reductase, iron-sulfur protein for some P450s) initiates further catalytic steps which are thought to end with a high-valent oxo-iron intermediate similar to the high-valent oxo-iron intermediates of the peroxidase enzymes, called compounds I [4]. This intermediate, sometimes represented as (FeO)³⁺, is thought to be the most likely candidate for the reactive oxygen intermediate of P450 that hydroxylates unactivated hydrocarbons [5]. It is this intermediate which has attracted the attention of many researchers for more than three decades [6]. Compound I intermediates of several peroxidases (horseradish peroxidase (HRP) [7,49], ascorbate peroxidase (APX) [8], *Micrococcus lysodeikticus* Catalase (MLC) [9] and chloroperoxidase (CPO) [10]) have been prepared by addition of either hydrogen peroxide or peroxy acids and have been characterized by a variety of spectroscopic methods like UV-vis, EPR and Mössbauer spectroscopy. In these enzymatic intermediates one oxidizing equivalent has been found to reside on the oxoferryl (Fe^{IV}=O) unit (*d*⁴, *S* = 1) whereas the other is probably more or less delocalized over the heme and the axial ligands of iron [11]. Many synthetic compound I analogues in which the second oxidizing equivalent resides on the porphyrin ring have been synthesized and characterized [6,12–15].

However, in the P450 catalytic cycle, little is known about intermediates beyond oxy-P450. Results in support of a compound I like intermediate in the 'shunt' pathway have been reported recently by Egawa et al. [16]. Following earlier work by Wagner et al. [17], rapid-scan-stopped flow absorption spectroscopy was used to study the reaction of substrate-free ferric P450_{cam} with *m*-chloroperbenzoic acid (*m*-CPBA). The authors of these studies reported preliminary evidence for the formation of a species with an absorption spectrum closely resembling that of CPO compound I (CPO-I): a well characterized cysteinate ligated peroxidase compound I. These results are in favor of a P450 active oxygen intermediate resembling CPO-I. Density functional calculations used recently to characterize this putative active oxygen intermediate of the P450 cytochromes [18] indicate that there are two parallel unpaired spins on the Fe=O moiety and an antiparallel unpaired spin distributed between the a_{2u}(π) orbital of the porphyrin ring and the π-orbital of ligand sulfur atom. The unpaired spin density on the sulfur ligand is described as being due of mixing of the a_{2u} porphyrin π-orbital and the S π orbitals in the highest occupied molecular orbital. Full geom-

*Corresponding author. Fax: (49)-30-9406 3329.
E-mail: cjung@mdc_berlin.de

¹ Also corresponding author. Fax: (49)-451-500 4214; E-mail: trautwein@physik.mu-luebeck.de

Abbreviations: P450_{cam}, cytochrome P450_{cam} (CYP101) from *Pseudomonas putida* which catalyzes the hydroxylation of 1R-camphor; DTT, DL-dithiothreitol; DEAE, diethylaminoethyl

etry optimization of the lowest quartet and doublet states led to the finding of an antiferromagnetic ground state. However, in CPO I, resonance Raman investigations indicate that unpaired spin density on the porphyrin ring is predominantly of the $a_{1u}(\pi)$ type with, either a small mixing of the $a_{2u}(\pi)$ type orbital or, alternatively, a delocalization of the spin density on the thiolate ligand [19]. Very recently Schlichting et al. [20] have shown that the ferrous dioxygen adduct of P450_{cam} can be converted by irradiation with X-rays into an intermediate that would be consistent with an oxoferryl species. These studies show that an iron-oxo species can really exist in P450. However, the electron and spin distribution within this complex cannot be deduced from the structure.

The characterization of such active oxygen intermediates which hydroxylate unactivated hydrocarbons, occurring in both pathways of the reaction cycle of P450, remains a very challenging area of research. A clear answer about the spin distribution and iron-oxidation state in these intermediates can only be obtained with EPR and Mössbauer spectroscopy. Mössbauer studies of the stable ferric, as well as the ferrous forms of P450 with and without oxygen ligation have been performed already in the seventies [21].

In this communication, we present a complementary Mössbauer and EPR study on reaction intermediates obtained by reacting, in a freeze-quench-quench apparatus, ^{57}Fe - and ^{56}Fe -labeled P450_{cam} with peroxy acetic acid as the oxidizing agent. Peroxy acetic acid has been used to characterize compound I in CPO [10] and in HRP [22] and might be, therefore, an appropriate agent for the comparison of these studies with our own data on P450_{cam}. Our results suggest that the reaction intermediate obtained after 8 ms contains an iron(IV) center and a tyrosyl radical, with clear evidence that both are not exchange-coupled.

2. Materials and methods

2.1. Isolation and ^{57}Fe enrichment of cytochrome P450

Cytochrome P450_{cam} (CYP101) from *Pseudomonas putida*, expressed in *Escherichia coli* strain TB1 was isolated and purified to a Q -value (absorbance ratio $A_{392\text{ nm}}/A_{280\text{ nm}}$) of 1.32 as previously described [23]. The procedure for the preparation of the ^{57}Fe -derivative, by the ^{56}Fe -heme replacement, followed the protocol published by Wagner et al. [24]. In the first step apo-P450 was produced by heme extraction. 9 ml of camphor-bound P450_{cam} (577 μM) were dialyzed against 1 l of 100 μM camphor solution in bi-distilled water (three changes) within 32 h. 27 ml of the dialyzed P450 solution were mixed with 27 ml of 0.2 M DL-histidine. The pH was adjusted to 2.6 by stepwise addition of 0.1 N HCl at 4°C. The resulting 90 ml solution were mixed in a separation funnel with 90 ml of ice-cold butanone. The aqueous phase contains the apo-P450 and was separated from the organic phase in which the heme is dissolved. The aqueous solution with a final volume of 135 ml was dialyzed with three changes against 2 l of 0.01% NaHCO_3 , which was purged with nitrogen gas to remove dioxygen. During this dialysis the protein completely precipitated. The precipitated protein was resolubilized within 90 min by a procedure which includes a dialysis against 1.8 l of 0.1 M histidine, pH 8, 40% (v/v) glycerol within 10 h with subsequent incubation of the protein-containing dialysis hose in 1.5 l of 20 mM DTT in water at room temperature. Finally the protein was dialyzed three times against 1 l of 0.1 M histidine, pH 8, 20% (v/v) glycerol. DTT and camphor were added to the dialyzed apo-P450 solution yielding a final concentration of 20 mM and 500 μM , respectively. ^{57}Fe -heme reconstitution was performed at 20°C. 3.3 mg of ^{57}Fe -heme were dissolved in 200 μl of 0.1 N KOH and then diluted 10 times with water. The ^{57}Fe -heme solution was drop-wise added to apo-P450_{cam} under nitrogen gas atmosphere while stirring the solution. Reaction time was 45 h. The heme incorporation was checked each 3 h by taking a small aliquot

from the mixture for recording a CO-difference spectrum [25]. The solution of ^{57}Fe -heme-reconstituted P450_{cam} was concentrated by ultrafiltration and immediately loaded on a Sephadex G25 column equilibrated with 50 mM potassium phosphate buffer, pH 7.15, 100 μM camphor, to remove excess of heme.

The eluted protein was loaded on a DEAE cellulose anion exchange column equilibrated with 20 mM potassium phosphate buffer, pH 7.06, washed with the same buffer and eluted with the gradient 50 mM to 210 mM KCl. The DEAE cellulose chromatography was repeated until a Q -value of 1.1 was reached. After concentration with Centricon30 we got a 1 mM substrate-free ^{57}Fe -P450_{cam}. The reconstituted protein shows the same spectral characteristics like the starting material, i.e. the starting Soret low-spin spectrum, the typical Soret high-spin spectrum [26] (with the capacity of the protein to completely bind camphor), and the Soret band of the CO complex at 446 nm.

Chemicals: (1R)-camphor (Sigma), DL-histidine (Sigma), glycerol (Riedel-de-Häen), DL-dithiothreitol (DTT) (Sigma), 2-butanone (Sigma), Sephadex G25 (medium) (Pharmacia Biotech), DEAE cellulose (Sigma).

2.2. Rapid freeze-quench experiments

Rapid freeze-quench experiments [27] were performed with a System 1000 apparatus from Update Instruments by mixing a 0.93 mM cytochrome P450_{cam} and a 4.8 mM peroxy acetic acid solution in 100 mM potassium phosphate buffer, pH 7. The peroxy acetic acid stock solution was prepared from a ~40% aqueous solution (Fluka) diluted 1000 times in 100 mM potassium phosphate buffer pH 7. Residual hydrogen peroxide always present in the peroxy acetic acid stock was removed by trace amount of diluted bovine liver catalase. A Delrin cup with 4 mm inner diameter and a volume of 50 μl was attached to a quartz tube, which was connected to a funnel. The funnel was completely immersed into an isopentane bath at $T = -100^\circ\text{C}$. The reaction mixtures were quenched by spraying into the cold isopentane, and the so-obtained crystals were packed at the bottom of the Delrin cup using a packing rod made of Teflon. This procedure made it possible to record Mössbauer as well as EPR spectra from the same sample.

2.3. EPR spectroscopy

EPR spectra were recorded with a conventional X-Band spectrometer (Bruker 200D SRC) equipped with a He-flow cryostat (ESR 910, Oxford Instruments) in the temperature range 5–140 K. The spectra were simulated according to the procedure given by Beinert and Albracht [28] using orientation-dependent Gaussian line shapes. The microwave power at half saturation $P_{1/2}$ has been obtained from the saturation curve which is described by [29,30]

$$I \propto \sqrt{P}/(1 + P/P_{1/2})^{b/2} \quad (1)$$

where I is the EPR amplitude and P is the microwave power. The inhomogeneity parameter b has been taken as 1, which indicates the presence of an inhomogeneous broadening.

2.4. Mössbauer spectroscopy

Mössbauer spectra were recorded using a conventional spectrometer in the constant-acceleration mode. Isomer shifts are given relative to $\alpha\text{-Fe}$ at room temperature. The spectra obtained at 20 mT were measured in a He-bath cryostat (Oxford MD 306) equipped with a pair of permanent magnets. Typical measuring time was 3 weeks per spectrum. The magnetically split spectra were simulated within the spin-Hamiltonian formalism [31].

3. Results and discussion

Fig. 1a shows the Mössbauer spectrum of the starting material ^{57}Fe -P450_{cam} taken at 4.2 K in a field of 20 mT perpendicular to the γ -beam. This Mössbauer spectrum looks almost identical to that reported by Sharock et al. [21] and therefore has been analyzed by the spin-Hamiltonian formalism using their parameter set. The simulation fits the experimental spectrum well which additionally shows that the ^{57}Fe insertion has been successful.

The Mössbauer spectrum of a freeze-quenched sample ob-

tained after 8 ms reaction time is shown in Fig. 1b. 13% of the experimental area is due to a doublet with isomer shift $\delta=0.13$ mm/s and quadrupole splitting $\Delta E_Q=1.94$ mm/s. The small value of the isomer shift is indicative of the presence of an iron(IV) ($S=1$) center, if compared to the values reported for various compounds I and II and their analogues (Table 1). In fact, within the experimental error of ± 0.01 mm s^{-1} it is identical to that of the high-valent heme iron in CPO-I which, like in P450, has a cysteine sulfur as axial ligand. The observation of a quadrupole doublet under the present experimental conditions (4.2 K, 20 mT $\perp \gamma$), instead of a magnetic hyperfine pattern as found in other compound I species, in-

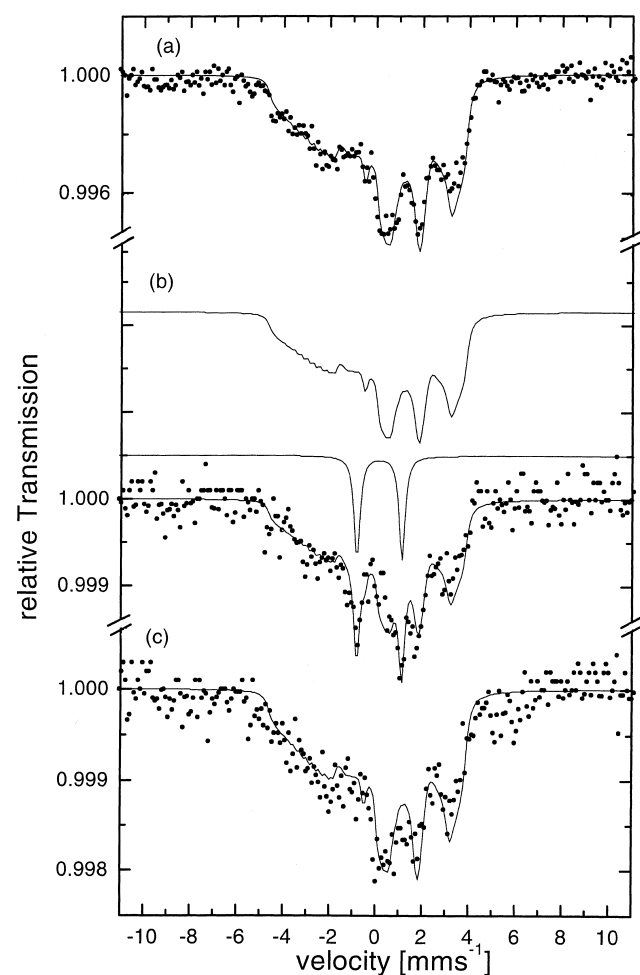


Fig. 1. a: Mössbauer spectra of substrate-free cytochrome P450_{cam} taken at 4.2 K in a field of 20 mT perpendicular to the γ -beam. The solid line is a spin-Hamiltonian simulation taking the parameters reported by Sharrock et al. [21]: $\delta=0.38$ mm s^{-1} , $\Delta E_Q=+2.85$ mm s^{-1} , $\eta=-1.8$, $\Gamma=0.30$ mm s^{-1} , $g=(1.91, 2.26, 2.45)$, $A/g_N\mu_N=(-45, 10, 19)$ T. b: Mössbauer spectrum of P450_{cam} after addition of peroxy acetic acid and 8 ms reaction time taken at 4.2 K in a field of 20 mT perpendicular to the γ -beam. The solid line is a spin-Hamiltonian simulation assuming two components: a doublet with $\delta=0.13 \pm 0.01$ mm s^{-1} and $\Delta E_Q=1.94 \pm 0.01$ mm s^{-1} characteristic for ferryl Fe^{IV} ($S=1$) ($13 \pm 2\%$ relative contribution) and the ferric starting material (calculated with the parameters given in (a); $87 \pm 2\%$ relative contribution). c: Mössbauer spectrum of P450_{cam} after addition of peroxy acetic acid and 5 min reaction time taken at 4.2 K in a field of 20 mT perpendicular to the γ -beam. The solid line is a spin-Hamiltonian simulation with the parameters used for the ferric starting material, see (a).

Table 1

Mössbauer parameters of high-valent intermediates of peroxidases, Met-myoglobin treated with H_2O_2 and two tetramesitylporphyrin (TMP) model systems

	δ (mm/s)	ΔE_Q (mm/s)	Reference(s)
P450 _{cam}	0.13 ± 0.01	1.94 ± 0.01	this work
JRP ^a Cpd I	0.10	1.33	[43]
HRP Cpd I	0.08	1.25	[7,49]
CPO Cpd I	0.14	1.02	[10]
CCP ^b Cpd ES	0.05	1.55	[44]
JRP Cpd II	0.03	1.59	[45]
HRP Cpd II	0.03	1.61	[7,49]
Mb ^c (H_2O_2)	0.09	1.43	[46]
$[Fe^{IV}=O(TMP)]^+$	0.08	1.62	[12]
$Fe^{IV}=O(TMP)$	0.04	2.3	[47]

^aJapanese radish peroxidase.

^bCytochrome *c* peroxidase compound ES.

^cMyoglobin complexed with H_2O_2 .

dicates that the ferryl iron is not taking part in a spin-coupled (ferromagnetic or antiferromagnetic) system.

After 200 ms reaction time the Mössbauer spectrum obtained at 4.2 K looks very similar to that of the 8 ms quenched sample (data not shown). 12% of the spectral area represents the high-valent ferryl species. After 5 min reaction time the Mössbauer pattern of the starting compound is restored (Fig. 1c) and the doublet, which we have attributed to a ferryl species has disappeared. This finding implies that the observed quadrupole doublet indeed represents a reaction intermediate.

The EPR spectra of the isotopically enriched starting material ^{57}Fe -P450_{cam} as well as the naturally abundant material ^{56}Fe -P450_{cam} are shown in Fig. 2a,b. The rhombic EPR signal around $g \sim 2$ is characteristic for the ferric low-spin state of cytochrome P450. The enzyme exhibits a g -tensor of $\bar{g}=(1.91, 2.26, 2.45)$ with the z -component being attributed to the heme normal [32].

After 8 ms reaction time a signal at $g=2$ evolves in both ^{57}Fe -P450_{cam} and ^{56}Fe -P450_{cam} (Fig. 2c,d) (accounting for 15 and 8% of the EPR absorption area, respectively), which is characteristic for a radical. After 200 ms reaction time the radical signal is observable with 30 and 16% for ^{57}Fe -P450_{cam} and ^{56}Fe -P450_{cam}, respectively (data not shown), while after 5 min it has almost disappeared. The radical signal, as shown by the EPR spectrum obtained at 140 K (Fig. 3), exhibits hyperfine splitting which is very similar to that reported for the tyrosyl radical of prostaglandin H synthase [33]. The hyperfine splitting of the latter is caused by the electronic interactions of the methylene hydrogens $H_{\beta 1}$ and $H_{\beta 2}$ of carbon C_7 with the π -orbital system of the phenoxyl ring. Similar spectra have also been observed for the tyrosyl radical Tyr[•]-107 of the double mutant W107Y/Y122F of *E. coli* ribonucleotide reductase (RNR) [34]. The hyperfine splitting is reported to be very sensitive to the angles of the β -methylene group in relation to the plane of the aromatic ring of the tyrosine [35].

The saturation behavior of the EPR amplitude according to Eq. 1 is similar to that observed for the stabilized tyrosyl radical from RNR, i.e.: $P_{1/2}=6$ μ W at $T=5$ K for the ^{56}Fe -P450 sample (Fig. 4) is close to $P_{1/2}=4$ μ W at $T=4$ K for the stable tyrosyl radical of RNR from *E. coli* [36]. The temperature dependence of the $P_{1/2}$ -values of tyrosyl signals is shown in Fig. 5. It is obvious that the $P_{1/2}$ -values of the stabilized tyrosyl signal are enhanced with respect to those

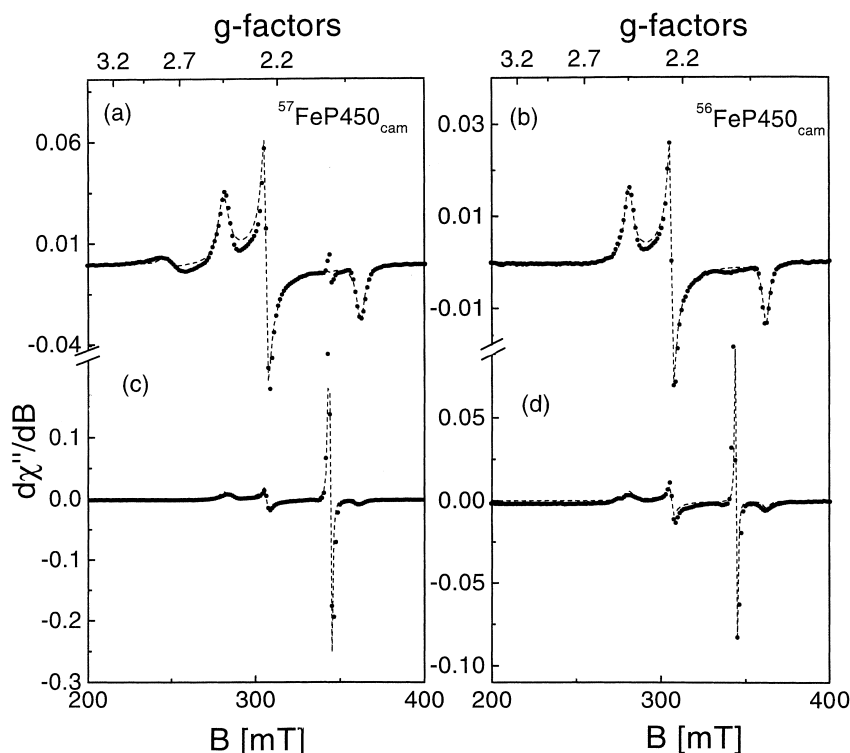


Fig. 2. EPR spectra of ^{57}Fe -P450_{cam} (a) and ^{56}Fe -P450_{cam} (b). The dashed lines are simulations yielding $\bar{g} = (2.45, 2.25, 1.9)$. An impurity of ^{57}Fe -P450_{cam} with $\bar{g} = (2.80, 2.75, 2.0)$ accounts for 3.5% of the total EPR signal. EPR spectra of ^{57}Fe -P450_{cam} (c) and ^{56}Fe -P450_{cam} (d) after addition of peroxy acetic acid and 8 ms reaction time. The top lines are simulations assuming two components: the ferric starting material with $\bar{g} = (2.45, 2.25, 1.9)$ and a radical with $g = 2.0$. The radical signal accounts for 15% in the ^{57}Fe -P450_{cam} sample and for 8% in the ^{56}Fe -P450_{cam} sample. The experimental spectra are recorded at 20 K with 9.6456 GHz microwave frequency at 80 μW microwave power, modulation amplitude 0.5 mT, modulation frequency 100 kHz.

of free tyrosyl [37], and semiquinone [38] radicals, the $P_{1/2}$ -values of which are also shown in Fig. 5. The enhancement of $P_{1/2}$ values is due to the magnetic interaction between the radical and a paramagnetic center causing the relaxation process to be more effective. This behavior is observed in several forms of RNR [36,37,39]. In fact, Tyr-96 in P450_{cam} is located ~ 9.4 Å from the heme iron and only ~ 7.4 Å from the nearest porphyrin *meso*-carbon atom [2]. Tyr-75, with a distance from the tyrosine oxygen atom to the iron of ~ 11.8 Å is farther away. Therefore we regard Tyr-96 as a potential candidate for the observed radical signal.

In summary we note that both the Mössbauer and EPR pattern of the freeze-quenched oxidation products of substrate-free cytochrome P450_{cam} with peroxy acetic acid are not characteristic for a compound I like exchange-coupled oxoferryl porphyrin π -cation radical system. Instead, our investigation reveals a ferryl heme iron ($S = 1$) and an organic radical ($S' = 1/2$) located at an amino acid of the protein. The two paramagnetic centers ($S = 1$ and $S' = 1/2$) are too far apart to cause, via exchange interaction, a measurable effect in the magnetic hyperfine (Mössbauer) pattern; however, they are close enough to significantly influence the (EPR) relaxation behavior of the radical spin.

The tyrosine residue which is oxidized is probably Tyr-96, lying in the substrate binding cavity of P450_{cam} [2]. Several reaction schemes compatible with the formation of a tyrosine radical and an iron(IV) center, may be considered: (i) one possibility could correspond to the direct oxidation of Tyr-96 by a peroxy acetic acid molecule entering the substrate-free binding cavity of P450_{cam} accompanied by a formation of a

water molecule and an acetic acid radical $\text{CH}_3\text{-C}(\text{O})\text{O}^\bullet$. This radical oxidizes then iron(III) to iron(IV), either with substitution of the water molecule bound to iron in the resting state of P450_{cam} [48] by the resulting acetic acid anion or, alternatively in addition, by deprotonation of the axially bonded water molecule into a hydroxide anion. The acetic acid anion has been shown to bind to substrate-free ferric P450_{cam} [40]. (ii) another possibility could be that a peroxy acetic acid molecule oxidizes the ferric heme to a compound I like oxo-ferryl

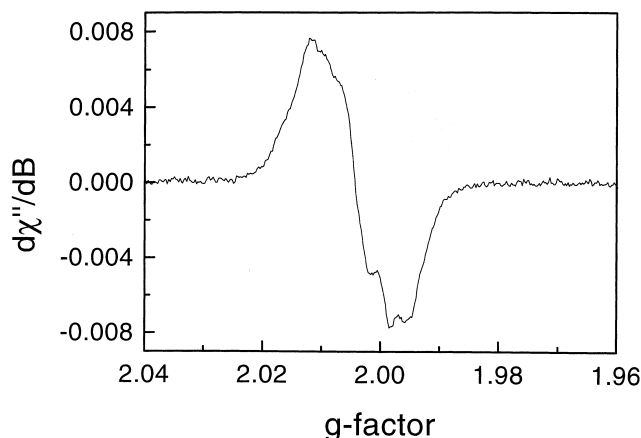


Fig. 3. EPR spectrum of ^{57}Fe -P450_{cam} taken at $T = 140$ K with a 9.6451 GHz microwave frequency at 200 μW microwave power, modulation amplitude 0.05 mT, modulation frequency 100 kHz.

porphyrin species by binding to the metal and by heterolytic cleavage of the peroxy acetic acid O–O bond. This reaction would then be followed by a migration of an electron from Tyr-96 to the porphyrin π -cation radical yielding the Tyr $^{\bullet}$ -96 radical and a transfer of a proton to pocket water. The iron center would in this case be a compound II like oxo-ferryl-porphyrin unit, *trans*-ligated to a cysteinate proximal ligand. Such a kind of intramolecular electron transfer between a porphyrin and a tyrosine has recently been discussed for the HRP mutant Phe172Tyr [41], in which the distance from the tyrosine–O to the heme iron is ~ 9.9 Å and to the nearest porphyrin *meso*-carbon ~ 6.7 Å, and for bovine liver catalase with a Tyr-369 oxygen–iron distance of ~ 13.8 Å and a Tyr-369 oxygen–porphyrin *meso*-carbon distance of ~ 12.2 Å [42].

The properties of the 8 ms intermediate presented here, namely a ferryl heme iron ($S=1$) and an organic radical ($S'=1/2$) located at an amino acid of the protein, are similar to those found for cytochrome *c* oxidase [44], for prostaglandin H synthase [38] and bovine liver catalase [42]. It is thus tempting to speculate that compound I of P450 may not be an exchange-coupled oxoferryl porphyrin π -cation radical system. However, more investigations, both theoretical and experimental, are needed to clarify this hypothesis.

Acknowledgements: Support to C.J. and to A.X.T. by the Deutsche Forschungsgemeinschaft (Ju 229/4-1, Sk 35/3-1 and Tr97/26-1) is gratefully acknowledged.

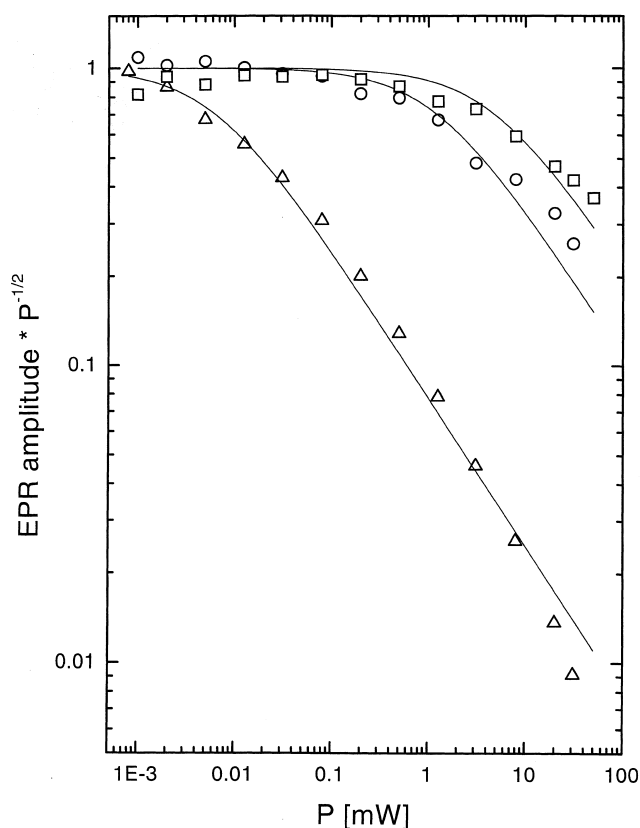


Fig. 4. EPR microwave power saturation curve of the tyrosyl radical of ^{56}Fe -P450_{cam} at $T=5$ K (Δ), $T=71$ K ($\circ$) and $T=156$ K ($\square$). The solid lines represent fits according to Eq. 1 yielding $P_{1/2}(T=5\text{ K})=6\pm 2\text{ }\mu\text{W}$, $P_{1/2}(T=71\text{ K})=1.2\pm 0.36\text{ mW}$ and $P_{1/2}(T=156\text{ K})=7\pm 2\text{ mW}$.

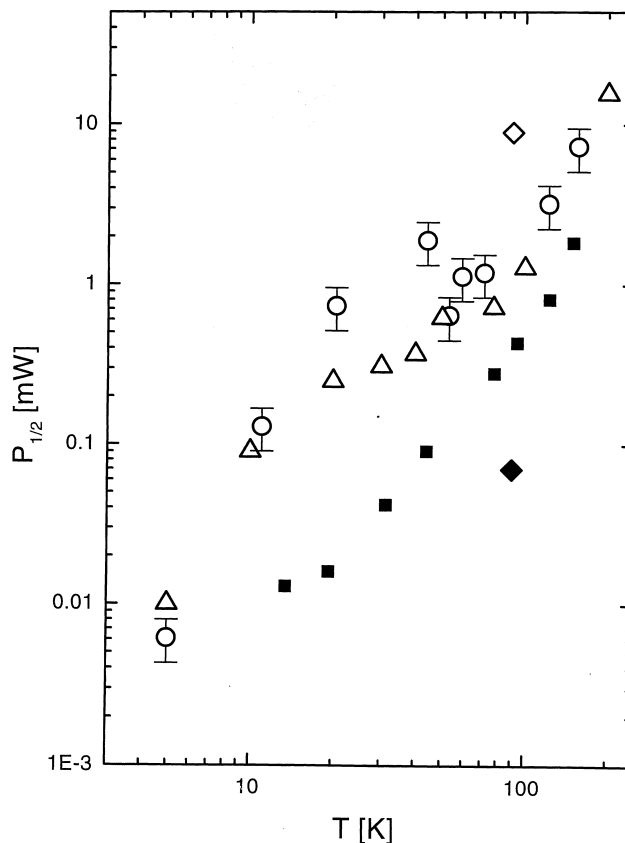


Fig. 5. Temperature dependent EPR microwave power saturation data of free tyrosyl radicals ($\blacksquare$, calculated from [37]); a free semiquinone radical ($\blacklozenge$, obtained from [38]). Compared to saturation data of tyrosyl radicals from RNR (*M. tuberculosis*) (Δ , [39]); from prostaglandin synthase ($\blacklozenge$, obtained from [38]) and from this study ($\circ$).

References

- [1] Lewis, D.F.V. (1996) Cytochromes P450 – Structure, Function and Mechanism, Taylor and Francis, London.
- [2] Poulos, T.L. and Howard, A.J. (1987) Biochemistry 26, 8165–8174.
- [3] Kaim, W. and Schwederski, B. (1995) Bioanorganische Chemie B.G., Teubner, Stuttgart.
- [4] Ortiz de Montellano, P.R., (1995) in: Oxygen Activation and Reactivity in Cytochrome P450: structure, Mechanism, and Biochemistry, 2nd edn. (Ortiz de Montellano, P.R., Ed.), pp. 245–308, Plenum, New York.
- [5] Sono, M., Roach, M.P., Coulter, E.D. and Dawson, J.H. (1996) Chem. Rev. 96, 2841–2887.
- [6] Debrunner, P.G. (1989) in: Iron Porphyrins Part III, Physical Bioinorganic Chemistry Series (Lever, A.B.P. and Gray, H.B., Eds.), pp. 139–234, VCH, New York.
- [7] Schulz, C., Rutter, R., Sage, J.T., Debrunner, P.G. and Hager, L.P. (1984) Biochemistry 23, 4743–4754.
- [8] Patterson, W.R., Poulos, T.L. and Goodin, D.B. (1995) Biochemistry 34, 4342–4345.
- [9] Benecky, M.J., Frew, J.E., Scowen, N., Jones, P. and Hoffman, B.M. (1993) Biochemistry 32, 11929–11933.
- [10] Rutter, R., Valentine, M., Hendrich, M.P., Hager, L.P. and Debrunner, P.G. (1983) Biochemistry 22, 4769–4774.
- [11] Weiss, R., Mandon, D., Wolter, T., Trautwein, A.X., Mütter, M., Bill, E., Gold, A., Jayaraj, K. and Terner, J. (1996) JBIC 1, 377–383.
- [12] Mandon, D., Weiss, R., Jayaraj, K., Gold, A., Terner, J., Bill, E. and Trautwein, A.X. (1992) Inorg. Chem. 31, 4404–4409.

- [13] Ochsenbein, Ph., Mandon, D., Fischer, J., Weiss, R., Austin, R., Jayaraj, K., Gold, A., Terner, J., Bill, E., Mütter, M. and Trautwein, A.X. (1993) *Angew. Chem.* 32, 1437–1439.
- [14] Jayaraj, K., Terner, J., Gold, A., Roberts, D.A., Austin, R.N., Mandon, D., Weiss, R., Bill, E., Mütter, M. and Trautwein, A.X. (1996) *Inorg. Chem.* 35, 1632–1640.
- [15] Ayougou, K., Mandon, D., Fischer, J., Weiss, R., Mütter, M., Bill, E., Schünemann, V., Trautwein, A.X., Terner, J., Jayaraj, K., Austin, R.N. and Gold, A. (1996) *Chem. Eur. J.* 2, 1159–1162.
- [16] Egawa, T., Shimada, H. and Ishimura, Y. (1994) *Biochem. Biophys. Res. Commun.* 201, 1464–1469.
- [17] Wagner, G.C., Palcic, M.M. and Dunford, H.B. (1983) *FEBS Lett.* 156, 244–248.
- [18] Loew, G.H. and Harris, D.L. (2000) *Chem. Rev.* 100, 407–419.
- [19] Terner, J., Gold, A., Weiss, R., Mandon, D. and Trautwein, A.X. (2000) *J. Porph. Phthal.*, in press.
- [20] Schlichting, I., Berendzen, J., Chu, K., Stock, A.M., Maves, S.A., Benson, D.E., Sweet, R.M., Ringe, D., Petsko, G.A. and Sligar, S.G. (2000) *Science* 287, 1615–1622.
- [21] Sharrock, M., Debrunner, P.G., Schulz, C., Lipscomb, J.D., Marshall, V. and Gunsalus, I.C. (1976) *Biochim. Biophys. Acta* 420, 8–26.
- [22] Ivancich, A., Jouve, H.M. and Gaillard, J. (1996) *J. Am. Chem. Soc.* 118, 12852–12853.
- [23] Jung, C., Hui Bon Hoa, G., Schröder, K.-L., Simon, M. and Doucet, J.P. (1992) *Biochemistry* 35, 12855–12862.
- [24] Wagner, G.C., Perez, M., Toscano Jr., W.A. and Gunsalus, I.C. (1981) *J. Biol. Chem.* 256, 6262–6265.
- [25] Omura, T. and Sato, R. (1964) *J. Biol. Chem.* 239, 2370–2378.
- [26] Jung, C., Ristau, O. and Rein, H. (1991) *Biochim. Biophys. Acta* 1076, 130–136.
- [27] Ballou, D.P. (1978) *Methods Enzymol.* 54, 85–93.
- [28] Beinert, H. and Albracht, S. (1982) *Biochim. Biophys. Acta* 683, 245–277.
- [29] Rupp, H., Rao, K.K., Hall, D.O. and Cammack, R. (1978) *Biochim. Biophys. Acta* 537, 255–269.
- [30] Sahlin, M., Gräslund, A. and Ehrenberg, A. (1986) *J. Magn. Reson.* 67, 135–137.
- [31] Trautwein, A.X., Bill, E., Bominaar, E.L. and Winkler, H. (1991) *Struct. Bond.* 78, 1–95.
- [32] Murray, R.I., Fisher, M.T., Debrunner, P.G. and Sligar, S.G. (1985) in: *Metalloproteins, Part I* (Harrison P.M., Ed.), pp. 157–206, Macmillan, London.
- [33] DeGray, J.A., Lassmann, G., Curtis, J.F., Kennedy, T.A., Marrett, L.J., Eling, T.E. and Mason, R.P. (1992) *J. Biol. Chem.* 267, 23538–23588.
- [34] Katterle, B., Sahlin, M., Schmidt, P.P., Pötsch, S., Logan, D.T., Gräslund, A. and Sjöberg, B.-M. (1997) *J. Biol. Chem.* 272, 10414–10421.
- [35] Barry, B.A., El-Deeb, M.K., Sandusky, P.O. and Babcock, G.T. (1990) *J. Biol. Chem.* 265, 20139–20143.
- [36] Persson, B.O., Karlsson, M., Climent, I., Ling, J., Loefer, J.A., Sahlin, M. and Sjöberg, B.-M. (1996) *JBIC* 1, 247–256.
- [37] Sahlin, M., Petersson, L., Gräslund, A., Ehrenberg, A., Sjöberg, B.-M. and Thelander, L. (1987) *Biochemistry* 26, 5541–5548.
- [38] Kartheim, R., Dietz, R., Nastainczyk, W. and Ruf, H.H. (1988) *Eur. J. Biochem.* 171, 313–320.
- [39] Liu, A., Pötsch, S., Davydov, A., Barra, A.-L., Rubin, H. and Gräslund, A. (1998) *Biochemistry* 37, 16369–16377.
- [40] Dawson, J.H., Anderson, L.A. and Sono, M. (1982) *J. Biol. Chem.* 257, 3606–3617.
- [41] Miller, V.P., Goodin, D.B., Friedman, A.E., Hartman, C. and Ortiz de Montellano, P.R. (1995) *J. Biol. Chem.* 270, 18413–18419.
- [42] Ivancich, A., Jouve, H.M., Sartor, B. and Gaillard, J. (1997) *Biochemistry* 36, 9356–9364.
- [43] Maeda, Y. and Morita, Y. (1967) *Biochem. Biophys. Res. Commun.* 29, 680–685.
- [44] Lang, G., Spartalian, K. and Yonetani, T. (1976) *Biochim. Biophys. Acta* 451, 250–258.
- [45] Harami, T., Maeda, Y., Morita, Y., Trautwein, A. and Gonser, U.J. (1977) *Chem. Phys.* 67, 1164–1169.
- [46] Schulz, C., Chiang, R. and Debrunner, P.G. (1979) *J. Phys. Colloq.* 40-C2, 534–536.
- [47] Groves, J.T. and Gilbert, J.A. (1986) *Inorg. Chem.* 25, 123–125.
- [48] Poulos, T.L., Finzel, B.C. and Howard, A.J. (1986) *Biochemistry* 25, 5314–5322.
- [49] Schulz, C., Devaney, P.W., Winkler, H., Debrunner, P.G., Doan, N., Chiang, R., Rutter, R. and Hager, L.P. (1979) *FEBS Lett.* 103, 102–105.