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Minireview

The mystery of cytochrome P450 Compound I A mini-review dedicated to Klaus Ruckpaul

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ABSTRACT

The cytochrome P450 protein-bound porphyrin complex with the iron-coordinated active oxygen atom is called Compound I, which is presumably the intermediate species which hydroxylates inert carbon–hydrogen bonds of substrates. In this mini-review, the milestones in discovering Compound I of cytochrome P450 are summarized. It will be discussed what was known and suggested in the years before 1984, the year when Klaus Ruckpaul's first book about cytochrome P450 appeared, and compared with recent approaches and studies to catch and characterize this intermediate oxygen species in the reaction cycle of cytochrome P450. Although many studies have been undertaken before and after 1984 to characterize Compound I, its electronic structure and physicochemical properties are still a mystery. The conclusion from this review is that the knowledge about Compound I has significantly increased; however, we still ask the same questions. There is a need for improved experimental approaches, detection techniques, and theoretical simulations for future studies of cytochrome P450 Compound I. This mini-review is dedicated to Klaus Ruckpaul on the occasion of his 80th birthday.

1. Introduction

Cytochromes P450 (P450) represent a large superfamily of heme thiolate proteins which catalyze the conversion of a variety of chemically diverse compounds by insertion of an activated oxygen atom into an inert C-H bond [1]. The activated oxygen is formed at the so-called sixth iron coordination position of the protoporphyrin IX iron complex, which is the catalytic center surrounded by the protein of the enzyme and with the substrate located close to the oxygen (Fig. 1). The porphyrin complex is held in the protein matrix by coordinating the H-bonded, negatively charged sulfur atom of a cysteine to the iron and by salt links, diverse van der Waals contacts and H-bonded water molecules in the heme pocket [2]. The protein-bound porphyrin complex with the coordinated active oxygen is called Compound I, which is presumably the intermediate species which hydroxylates the substrate. When reading these last sentences, it might seem that the nature and reactivity of Compound I have been well characterized and the mechanism of its formation and of substrate conversion is completely discovered and understood. However, the large number of experiments and theoretical studies, reported over more than 45 years, has not lead to a final characterization of Compound I in P450.

It was during my PhD work in the 1980s that I heard from "Compound I" for the first time when I worked in the group of Klaus Ruckpaul in Berlin. He initiated the writing of a series of publications about P450 in the journal "Die Pharmazie," volume 33, issues 6 and 7 (1978) where my part was to summarize thermodynamic aspects and ideas about the nature of the active oxygen species [3]. This series of publications was written with a view of the second Scientific Conference on "Cytochrome P-450: Structural and Functional Aspects" held in Eberswalde close to Berlin (July 9–13, 1978) which was organized by Klaus Ruckpaul [4,5] and taken as basis for the book in 1984 about P450 edited by Klaus Ruckpaul and Horst Rein [6]. Ruckpaul and Rein continued in reviewing the progress in P450 research by functioning as editors of the book series "Frontiers in Biotransformation" starting with volume 1 in 1989 [7].

What was known about Compound I of P450 at that time when Ruckpaul's first book [6] appeared and what new discoveries have been made in the last 26 years? The number of publications about Compound I of P450 is too big to consider completely in this mini-review. Therefore, I will highlight only selected studies which I would qualify as key experiments or milestones for discovering the secrets of P450 Compound I. Comprehensive reviews appeared in the past [8–13] and very recently [14,15]. The conclusion from this review is that

Abbreviations: P450cam, cytochrome P450cam from *Pseudomonas putida*, CYP101; Pdx, putidaredoxin; BMP, the heme protein domain of the monooxygenase P450BM-3 from *Bacillus megaterium*, CYP102; HRP, horseradish peroxidase; CPO, chloroperoxidase from *Caldariomyces fumago*; CcP, cytochrome c peroxidase; JRP, Japanese radish peroxidase; NADP(H), nicotinamide adenine (di)nucleotide phosphate, reduced form; mCPBA, meta-chloroperoxybenzoic acid; PA, peroxy acetic acid; EPR, electron spin paramagnetic resonance; EXAFS, Extended X-ray absorption spectroscopy; ENDOR, electron-nuclear double resonance; DFT, density functional theory

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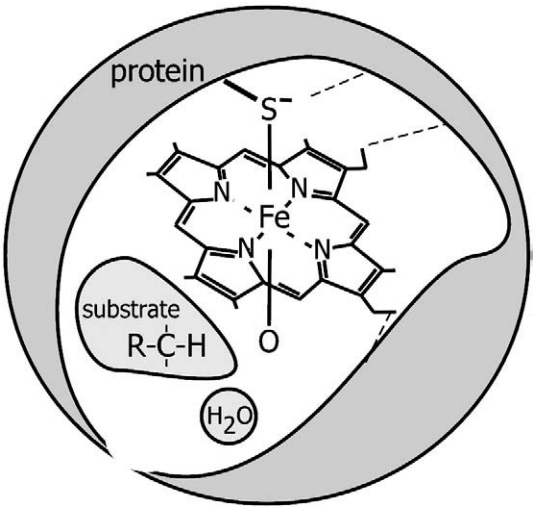

Fig. 1. Sketch of the structure of Compound I in cytochrome P450.

Compound I remains still a mystery and much efforts are needed to catch Compound I under experimental conditions which allow characterizing it with different spectroscopic techniques applied at the same sample.

2. Mechanisms of substrate hydroxylation and how the reactive oxygen species in P450 became assigned to Compound I—proposals in the 1980s

Ruckpaul [5,6], Estabrook [16], Omura [17] and many others have described how P450 was discovered and which key experiments led to describe the main steps in the P450 catalyzed substrate conversions (Fig. 2). The main steps include (i) substrate binding to the Fe^{3+} complex of P450; (ii) first reduction to build up the Fe^{2+} state; (iii) binding of O_2 to the Fe^{2+} heme, (iv) delivery of the second electron; (v) cleavage of the O-O bond of the iron-bound dioxygen; (vi) insertion of an oxygen atom into the substrate; and (vii) release of the hydroxylation product. Steps (iv), (v) and (vi) are certainly the most complicated ones to understand. Fig. 3 highlights the most important suggestions about these last steps discussed in the 1980s which were summarized in Ruckpaul's book [18].

2.1. Suggestion 1: The substrate carbon–hydrogen bond should be attacked by an electrophilic oxygen species

Ullrich and coworkers [19] made extensive studies on model systems. The systems "trifluoroperoxy acid" (Fig. 3-1) or "N-oxide + light" revealed the same reaction characteristics as the P450 catalyzed

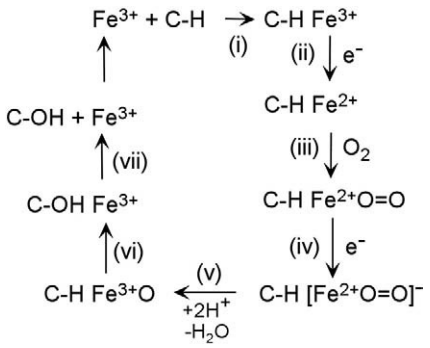

Fig. 2. Main steps of the reaction cycle of cytochrome P450; the oxidized heme complex is abbreviated by Fe^{3+} and the reduced heme complex by Fe^{2+}; C-H and C-OH represent the C-H bond of the substrate and the hydroxylated C-H bond, respectively.

hydroxylation, such as selective hydroxylation of aliphatic carbons and the hydroxylation of aromatic compounds, epoxide formation and migration of the substituents known as "NIH-shift." Because these models are classified as electrophilic species, the similarity to the P450 catalyzed reactions led suggest, that the active oxygen species in P450 should attack the substrate as an electrophilic agent (oxene species).

2.2. Suggestion 2: The substrate hydroxylating oxygen species should be an iron–oxo complex

Rahimtula and O'Brian [20] and Gustafsson et al. [21] demonstrated that a system containing cumolhydroperoxide and the Fe^{3+}-P450 is able to hydroxylate aromatic compounds and steroids. In addition, the cumene-hydroperoxide dependent acetanilide hydroxylation revealed a 'NIH shift' comparable to the NADPH-dependent hydroxylation and studies with $H_2^{18}O$ demonstrated that the oxygen in the hydroxylation product stems from the peroxide and not from water [22]. Such peroxide-heme protein system is similar to the "horseradish peroxidase (HRP) + peroxide" system for which an iron–oxo species ("Fe-O") has been discussed. In addition, oxygen agents which do not show a peroxide structure such as iodosobenzene (Fig. 3-2), $NaIO_4$ and $NaClO_2$ were also be able to bind to P450 and to hydroxylate fatty acids and steroids [23,24]. These and a large number of other experiments in the 1970s and 1980s were the basis to suggest that an iron–oxo species such as in peroxidases should also be the active oxygen species in P450. However in contrast to peroxidases for which a substrate radical is formed as a first step [25], the P450 iron–oxo species should make an electrophilic attack on the inert C-H bond of the substrate to form a C-OH group.

2.3. Suggestion 3: The O-O bond should be cleaved heterolytically

To form an electrophilic iron–oxo species, the O-O bond in the dioxygen complex must be split heterolytically, that means the second electron delivered in step (iv) belongs to the released water molecule formed from the distal oxygen atom of the dioxygen ligand (Fig. 3-3). In consequence, the remaining oxygen atom at the iron cannot formally fill the electron octet and remains with an electron deficiency (oxene species), resulting in electrophilic properties.

2.4. Suggestion 4: The insertion of the oxygen atom into the C-H bond should occur via a "concerted" or an "attachment-rearrangement" mechanism

Such an electrophilic oxygen species (oxene) should hydroxylate aromatics and double bonds via an intermediate oxene-"π-complex" which rearranges to an OH-"σ-complex" (Fig. 3-4a)—a mechanism known in organic chemistry [26]. For aliphatic substrates, two types of reactions were discussed: (i) a "concerted" reaction where the oxene species attacks rather the C-H bond than the H or C atoms alone (Fig. 3-4b). This requires a slight polarization of the $C^{\delta+}$-$H^{\delta-}$ bond where electron deficient orbitals of the oxene species interact with the hydrogen atom while electron filled orbitals couple with the carbon atom followed by electron density rearrangement—a so-called electronic "push-pull" mechanism also discussed in organic chemistry [26]; (ii) an "attachment-rearrangement" mechanism where the oxene species approaches first the hydrogen atom followed by an rearrangement of the C-H···O to the C-OH form (Fig. 3-4b). Finally for both mechanisms, the iron-bound alcohol (Fig. 2) is formed which releases the substrate alcohol as product and recovers the Fe^{3+}-P450. For these mechanisms, the formation of an intermediate substrate radical is excluded and the retention of configuration during hydroxylation should be expected to occur as observed in many experiments.

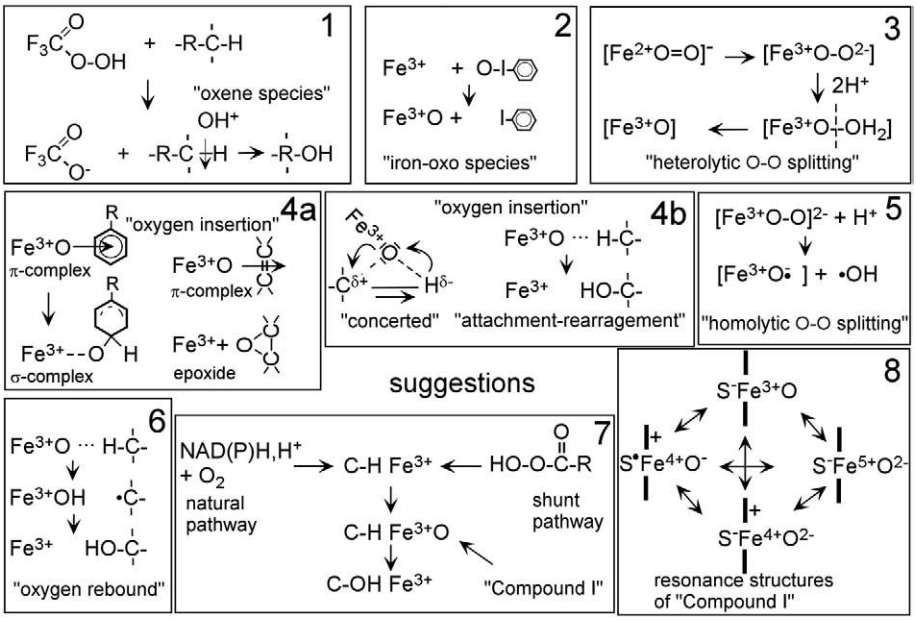

Fig. 3. Sketches for the suggestions 1–8 for the Compound I formation or reaction in the cytochrome P450 systems. In part-figures 1–7 Fe stands for the porphyrin complex in P450. The substrate and product are symbolized by R-C-H and R-C-OH. In part-figure 8 the proximal sulfur ligand of P450 is indicated by S and the heme is given as bars.

2.5. Suggestion 5: The O-O bond should also be able to be cleaved homolytically

In contrast to the previously discussed heterolytic splitting (Fig. 3-3), the studies using peroxyphenylacetic acid performed by White et al. [27] suggested a homolytic O-O bond cleavage (Fig. 3-5) which would also allow a radical attack on the substrate C-H bond.

2.6. Suggestion 6: The hydroxylation of the C-H bond should occur via a transiently formed substrate radical

Groves, Coon, White and coworkers suggested from different studies on the regio-selectivity, non-rearrangement and large internal kinetic hydrogen/deuterium isotope effect on the hydroxylation, i.e. norbornane, that the iron-oxygen species abstracts the hydrogen atom from the substrate C-H bond. This forms a transient substrate carbon radical which quickly recombines with the iron-bound OH-radical (two-step abstraction-recombination mechanism [28] which has been later on discussed as "oxygen rebound" mechanism [29]) (Fig. 3-6). Such a radical mechanism would require that the intermediate oxygen species should carry unpaired electrons or have some kind of "radical" character**.** The proximal sulfur ligand might be involved to realize a radical-like property [27,30].

2.7. Suggestion 7: The natural reaction pathway and the shunt pathway in P450 should run via the same oxygen intermediate which is called Compound I

The discussed mechanisms for the O-O bond cleavage and substrate hydroxylation have been derived from studies where oxygen and the redox equivalents were provided from two principally different sources—(i) from molecular oxygen along the natural enzymatic reaction cycle with NADP(H) as electron source and flavin-proteins (and an iron–sulfur protein in some cases) as redox partners for P450 and (ii) by addition of external oxygen agents, i.e. peroxy acids, which bring with them the two redox equivalents so that NADP(H) is no longer needed (Fig. 3-7). The latter one has been termed the "shunt pathway." This pathway (ii) has been extensively used to model steps in the natural pathway (i) and it is automatically assumed that both pathways run via the same intermediate—the iron–oxo species. Because the shunt pathway resembles formally the pathway known for peroxidases it is also assumed that the iron–oxo intermediate in the peroxidase pathway – the so-called Compound I [25,31] – is also valid for P450 and the name "Compound I of P450" was born (Fig. 3-7). However, it should be noted that the mode of reaction of this Compound I with substrates is different for peroxidases and P450. In a typical peroxidase reaction, the substrate reduces Compound I by transferring a hydrogen atom to the heme group (Compound II formation). This implies substrate radical formation followed by a further electron supply from the substrate as hydrogen atom to reform the resting Fe^{3+} state [25].

2.8. Suggestion 8: The electronic character of Compound I should correspond to a Fe^{4+}–oxo porphyrin–π-cation radical system

The structural and electronic properties of Compound I in peroxidases have been elucidated very early in the 1970s. Mössbauer studies [32,33], magnetic susceptibility [34] and EPR measurements indicated that the iron in Compound I exists in the Fe^{4+} state and that a free radical is present which should be located near the iron center in an aromatic amino acid residue of the protein [35] or in the porphyrin [31]. Dolphin et al**.** [36] suggested from UV-visible spectroscopic measurements that there should be a porphyrin–π-cation radical in Compound I of horseradish peroxidase (HRP) as well as of catalase. Schulz et al. [37] concluded from Mössbauer and EPR studies on HRP Compound I that there should be a spin coupling between the $S = 1$ at the Fe^{4+} and $S = 1/2$ at the porphyrin–π-cation radical. Furthermore, Roberts et al. [38] performed ENDOR studies on HRP Compound I and could also detect the porphyrin–π-cation radical.

The strongest input however came from studies on chloroperoxidase from *Caldariomyces fumago* (CPO) which possesses a negatively charged sulfur atom of a cysteine as proximal iron ligand analogous to the ligand in P450 [39]. Palcic et al. [40] observed an absorption spectrum for Compound I of CPO obtained from the reaction with meta-chloroperoxybenzoic acid (mCPBA) which resembled that of model porphyrin–π-cation complexes [41].

Complementary EPR and Mössbauer studies on freeze-quenched samples of CPO reacted with peroxyacetic acid [42,43] led to the conclusion that Compound I has one oxidation equivalent residing on

the Fe^{4+}=O unit (S=1), and one on the porphyrin (S=1/2), constituting an anti-ferromagnetically coupled Fe^{4+}–oxo porphyrin–π-cation radical system and it was assumed that this is also valid for P450 (Fig. 3-8). In addition, several electronic resonance structures for Compound I have also been discussed [30]. However, only quantum chemical methods can give information about electron and spin density distributions over the iron–porphyrin complex.

Quantum chemical calculations have been performed for a Compound I model using the Iterative Extended Hückel method previously [44]. These calculations showed that Compound I has three energetically high-lying molecular orbitals which can be filled each only with a single electron. Two of these orbitals are nearly degenerate, mainly localized (~38%) at the iron and belong to the $3d_{xz}$ and $3d_{yz}$ iron orbitals. These orbitals are mainly mixed with the oxygen $2p_x$ and $2p_y$ (50%) orbitals. Somewhat lower in energy is a singly occupied porphyrin orbital of a_{2u}-symmetry which reflects the porphyrin–π-cation radical. If this cation radical is reduced by supplying an electron the so-called Compound II is obtained which still maintains the iron–oxo unity [25].

Until 1984, a lot of knowledge about the structure and mechanism of the reactive oxygen species in heme proteins had been accumulated which was the basis for an assignment of the suggested reactive oxygen species of P450.

In the preface of the book from 1984 about P450 Ruckpaul and Rein wrote [6]: "The second reason which the authors would like to point out is the devotion to a more comprehensive approach with respect to regulation and control, respectively, of the enzyme system. These topics are mainly concerned with the physicochemical aspects of the enzymatic mechanism in order to elucidate the functional adaptation of the system in the organism. This analysis includes data from other heme proteins as well as from the *Pseudomonas putida* P-450, as being the best studied cytochrome."

Indeed, in the years after this statement we became the witness of an explosion of studies using different approaches to uncover the secrets of the reactive intermediate oxygen species of P450.

3. Hunting Compound I of P450—combined new and old approaches and suggestions

There is a general consensus in the P450 community to believe that Compound I, resulting from the heterolytic splitting of the O-O bond, does really exist and represents an iron–oxo species with iron in the Fe^{4+} state and the porphyrin with the cation radical in the π-system as described above for Suggestion 8. How could one prove this structural property?

The only way is to compare the spectroscopic properties and parameters of this presumed Compound I species with well studied reference samples for which the spectroscopic assignment to Fe^{4+} and the porphyrin–π-cation radical is unambiguously demonstrated within the same molecule or under comparable experimental conditions (Table 1). Indeed, such reference samples are available.

3.1. Structural properties of reference samples

Protein-free porphyrin complexes, HRP, cytochrome c peroxidase (CcP), catalase and CPO, the latter one as the closest relation to P450, have been extensively characterized by different techniques as mentioned above. These studies show (Table 1) that (i) the iron–oxo character is proved by the crystal structure showing electron density corresponding to a single oxygen atom bound to the iron in a distance of around 1.65–1.87 Å [45–47]; (ii) the longer Fe-O bond distance corresponds to the protonated state of the oxygen as EXAFS studies on Compound I and Compound II species indicate [11]; (iii) the Fe-O bond has a characteristic Raman band between 760 and 840 cm^{-1} [11,41]. The Fe=O stretch vibration frequency for six-coordinated porphyrin model complexes with non-radical character of the porphyrin (Compound II) appears between 818 cm^{-1} and 841 cm^{-1} [48]. If the porphyrin is a π-cation radical (Compound I) this frequency shifts to lower values at about 763 cm^{-1}; (iv) the Fe^{4+} state shows a doublet signal in the ^{57}Fe-Mössbauer spectrum with a low isomer shift between δ~0.03 and 0.14 mm/s [41]; (v) the porphyrin–π-cation radical in model complexes has a characteristic UV-visible spectrum with the Soret band between 390 and 406 nm, and a very weak band between 670 and 690 nm [41] which appear in CPO at about 370 nm and 690 nm, respectively [40]; and (vi) the iron–porphyrin radical system reveals a characteristic EPR spectrum depending on the ratio of the spin exchange interaction energy *J* and the zero-field splitting parameter *D* for the iron-spin/porphyrin-spin coupling [43]. Recent ENDOR studies on the Compound I of CPO showed that the radical is mainly located in the porphyrin π-system with some admixture of maximal 0.23 spin density at the sulfur ligand [49] which is suggested to be also the case in P450.

So, everything should be clear for characterization of P450 Compound I if one would be able to catch it. However, all experimental efforts undertaken so far revealed problems to trap the P450 species because it is extremely short-lived.

Fig. 4 summarizes the different approaches which have been applied to hunt the P450 species. One can differentiate between those approaches running via the iron–dioxygen complex in any kind and those approaches which exclude the formation of the dioxygen complex.

3.2. The natural P450 pathway

For the natural reaction pathway of P450, which runs via the iron–dioxygen complex no intermediate could be observed which has a life time long enough for a structural characterization. One should note here that the presence of the redox partner proteins such as i.e. putidaredoxin (Pdx) and putida redoxin reductase (Pdr) in case of P450cam (CYP101) may interfere with spectral properties of the P450 heme protein making a spectral characterization complicated anyway. The last species along the P450 reaction cycle which could be characterized by UV-visible spectroscopy in stopped-flow experiments, where reduced P450cam, reduced Pdx and O_2 were mixed, was a perturbed dioxygen complex [50]. A reconstituted P450cam system under catalytic conditions was studied using mixed-flow resonance Raman spectroscopy. Results from these studies 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 [51]. Sjodin et al. [52] measured the resonance Raman spectra of the oxygen complex of the wild-type and D251N mutant of P450cam in the presence of reduced Pdx in order to find the Compound I Fe=O stretch vibration. However, also in these studies no stretch vibration for Fe=O was found.

Therefore other approaches, in particular the shunt pathway, has been applied to try catching Compound I.

3.3. The shunt pathway

The shunt pathway, which excludes iron–dioxygen complex formation, has been run in stopped-flow and freeze-quench experiments. Although both techniques have been applied earlier in the 1980s, only recent developments allow extracting detailed information. Modern rapid-scan stopped-flow spectrometers have a shorter dead time of less than 2 ms and are furnished with software for single-value decomposition of large sets of spectra at different times. Egawa et al. [53] performed rapid-scan stopped-flow studies combined with single-value decomposition analysis on the reaction of substrate-free ferric P450cam with mCPBA and calculated one single-component spectrum which revealed a blue-shifted Soret band at ~367 nm and a very weak band at around 694 nm. Such spectrum resembles spectra for porphyrin–π-cation radicals [41] (Table 1). However, this spectrum is strongly overlaid by the low-spin spectrum of the ferric

Table 1
Parameters for Compound I, Compound II and Compound ES of selected heme proteins and porphyrin model complexes and assignment to structural properties.

Species[a]	Assignment[a]	UV-visible spectroscopy		Reference
		Soret (nm)	Visible bands (nm)	
$[Fe^{IV}=O(TMP^{\bullet})]^{+}$	π-radical	406	674	Tab. 8 in [41][b]
$[Fe^{IV}=O(TCMPP^{\bullet})]^{+}$	π-radical	392	673	Tab. 8 in [41][b]
Various porphyrin model complexes	π-radical	391–406	673–687	[41][b]
HRP Compound I	π-radical	400	577, 622, 651	[25]
Catalase Compound I	π-radical	405	660	[36], [40]
CPO Compound I	π-radical	367	610, 666	[40]
P450cam, wt Compound I	π-radical	367	694	[53]
P450cam, Y97F; Y75F; Y96F-Y75F mutants, Compound I	π-radical	367	–	[55], [56]
P450 119	π-radical	370	608, 690	[54]
$[Fe^{IV}=O(TMP)]$	–	414	545	Tab. 1 in [41][b]
HRP Compound II	–	420	527, 554	[25]
Catalase Compound II	–	429	536, 568	[53]
CPO Compound II	–	436	542, 572	[94]
P450cam, wt Compound II	–	–	–	[57]
P450cam, Y97F; Y75F; Y96F-Y75F mutants, Compound II	–	420	–	[57]
P450cam, wt, Compound ES	–	406	~535	[55], [56]
P450cam, Y97F; Y75F; Y96F-Y75F mutants, Compound ES	–	406–407	~535	[55], [56]

Species[a]	Assignment[a]	Mössbauer spectroscopy		Reference
		δ (mm/s)	ΔEQ (mm/s)	
$[Fe^{IV}=O(TMP^{\bullet})]^{+}$	π-radical, Fe^{4+}	0.08	1.62	[95]
Various porphyrin model complexes	π-radical, Fe^{4+}	0.02–0.08	1.28–1.62	Tab. 14 in [41] [b]
JRP Compound I	π-radical, Fe^{4+}	0.10	1.33	[33]
HRP Compound I	π-radical, Fe^{4+}	0.08	1.25	[37], [99]
CPO Compound I	π-radical, Fe^{4+}	0.14	1.02	[96]
JRP Compound II	Fe^{4+}	0.03	1.59	[98]
HRP Compound II	Fe^{4+}	0.03	1.61	[37], [99]
CPO Compound II	Fe^{4+}–OH (70%)	0.10	2.06	[104]
	$Fe^{4+}=O$ (30%)	0.11	1.94	
CcP Compound ES	Fe^{4+}	0.05	1.55	[97]
P450cam, wt, Compound ES or II	Fe^{4+}	0.13	1.94	[57]
P450cam, wt, Compound II	Fe^{4+}-OH	0.14	2.06	[63]
P450BM3, heme domain, Compound ES or II	Fe^{4+}	0.13	1.94	[62]
P450BM3, heme domain, Compound II	Fe^{4+}-OH	0.13	2.16	[63]

Species[a]	Assignment[a]	Resonance Raman spectroscopy		Reference
		ν(Fe-O) (cm^{-1})	Fe-O distance (Å)	
Various porphyrin model complexes (Compound I analogs)	$Fe^{4+}=O$, π-radical	801–835		[48]
Various porphyrin model complexes (Compound II analogs)	$Fe^{4+}=O$	818–852	1.64–1.66	Tab. 4 in [41] [b]; [100], [48]
HRP Compound I	$Fe^{4+}=O$, π-radical (?)	790	1.64–1.67	[101]
CcP Compound I (ES)	$Fe^{4+}=O$	753; 767	1.67	[63]
CPO Compound II	Fe^{4+}–OH	565	1.82	[106]
CPO Compound I	$Fe^{4+}=O$, π-radical	790	1.65	[102], [103]
HRP (-A) Compound II	$Fe^{4+}=O$	789		[100]
HRP Compound II, high pH	$Fe^{4+}=O$	787		[62]
HRP Compound II, low pH	$Fe^{4+}=O$	776		[62]
Catalase Compound II, high pH	$Fe^{4+}=O$	786		[100]
Catalase Compound II, low pH	$Fe^{4+}=O$	775		[62]

Species[a]	Assignment[a]	EPR spectroscopy		Reference
		g value	J/D[c]	
CPO Compound I	$Fe^{4+}=O$, π-radical	$g_{\perp} \sim 1.73$, $g_{\parallel} \sim 2$	1.02	[43]
CPO Compound I	$Fe^{4+}=O$, π-radical	$g_{\perp} \sim 1.75$, $g_{\parallel} \sim 2$	0.97	[61]
Catalase Compound I	$Fe^{4+}=O$, π-radical	$g_{\perp} \sim 3.32$, $g_{\parallel} \sim 2$	0.4	[105]
HRP Compound I	$Fe^{4+}=O$, π-radical	$g_{eff.} \sim 2$	~0.1	[99]

[a] TMP = tetramesityl porphyrin; TCMPP = tetrakis-(2-chloro-6-methylphenyl) porphyrin; JRP = Japanese radish peroxidase; CcP = Cytochrome c peroxidase; HRP = horseradish peroxidase; CPO = chloroperoxidase; π-radical = porphyrin–π-cation radical.
[b] Review articles are primarily cited.
[c] J = exchange interaction energy; D = zero-field splitting parameter.

P450 and is hampered from the loss of total heme absorption due to heme destruction induced by the peroxy acid. Analogous results have been obtained for CYP119 for which the calculated spectrum with the Soret band at 370 nm and the weak band at 690 nm counts for only 3% of the total enzyme concentration [54]. Spolitak et al. [55–57] performed a number of stopped-flow experiments with P450cam reacting with peracids. These studies revealed a single-component spectrum with a Soret band at 370 nm; but the 690 nm absorption band is not convincingly demonstrated. This spectrum was assigned to Compound I. It converts rapidly to another spectrum with the Soret band at 406 nm which is assigned to a Fe^{4+} species where the porphyrin–π-cation radical should be reduced by i.e. tyrosines from the protein (called Compound ES) [56]. Interestingly, this spectral behaviour was observed for the wild-type P450cam as well as for its mutants Y96F, Y75F and Y96F-Y75F. Later on, Compound ES was interpreted as Compound II in which the oxygen at the iron should be

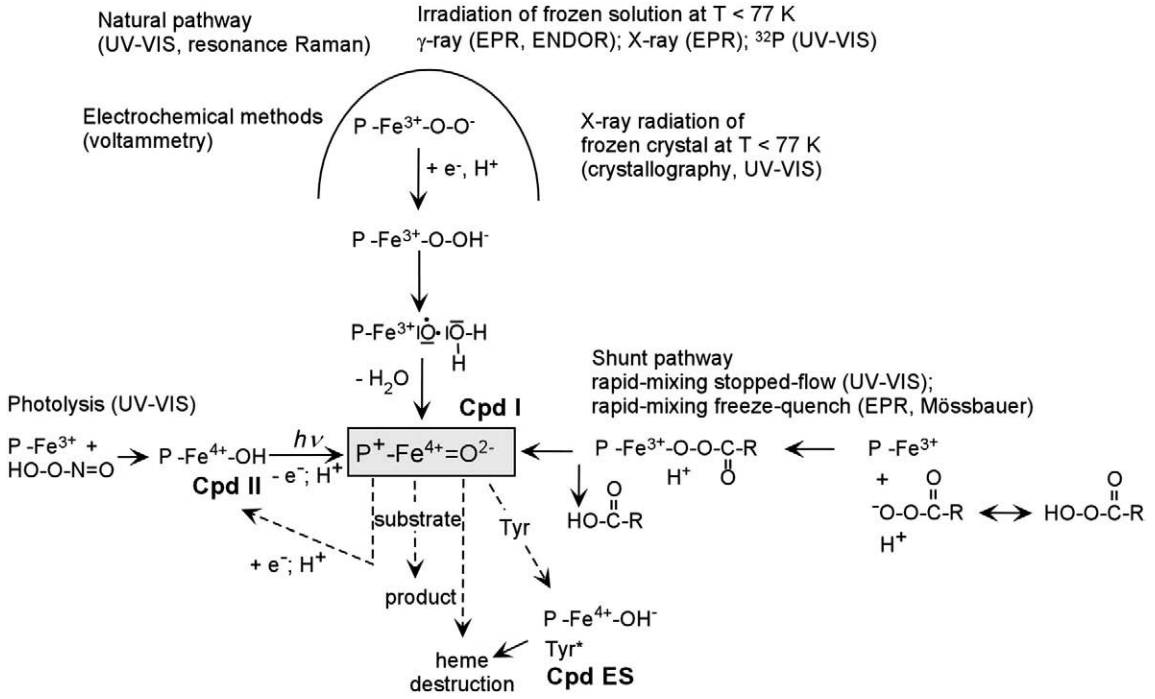

Fig. 4. Overview about the experimental approaches used to catch and characterize Compound I in cytochrome P450. P represents the porphyrin-π-system. Tyr and Tyr* indicate tyrosine and tyrosine radical, respectively. Cpd I, Cpd II and Cpd ES indicate Compound I, Compoud II and Compound ES, respectively. Dashed arrows indicate decay processes for Compound I.

protonated, while the unprotonated form of Compound II should absorb at 420 nm [56,57].

This last mentioned assignment of the species absorbing at 406 nm [55–57] (Compound ES) is based on the EPR characterization of an intermediate detected by Schünemann, Jung et al. in studies on substrate-free P450cam reacting with peroxy acetic acid and immediately freeze-quenching at −110° C after a reaction time of 8 ms [58]. These studies revealed a radical signal at $g \sim 2$. Although an undefined EPR signal at $g \sim 2$ has been reported already earlier and suggested to originate from a "non-heme protein function" [59] an unambiguous assignment to a tyrosine radical was possible only recently by developing an appropriate technique to collect freeze-quenched samples in the fragile EPR tubes needed for 94 GHz EPR instruments and by a detailed multifrequency EPR study (9.6, 94, 190, and 285 GHz) performed by Lendzian, Jung, Schünemann et al. [60]. In all P450cam samples such g-values were obtained, $g_x = 2.0078–2.0064$, $g_y = 2.0044$ and $g_z = 2.0022$, which are fingerprints for tyrosyl radicals. From the simulation of the 94 GHz EPR spectrum of the P450cam wild-type protein, an isotropic hyperfine value, $A_{iso}(H_{\beta 1}) = 1.1 \pm 0.1$ mT, was obtained for one of the tyrosine β-CH_2 protons. Using this value a dihedral angle of $\theta_1 = 40.5° \pm 3°$ between the axis of the π-orbital (p_z) of the adjacent carbon atom and the projected $C_\beta H_\beta$ bond in tyrosine was calculated, which matches very well the 41° obtained for Y96 from the crystal structure of substrate-free P450cam. However, in contrast to the results of the stopped-flow studies, an EPR signal corresponding to a porphyrin-π-radical, as seen in CPO [61], could not been detected for this wild-type P450cam species. In order to prove this spectroscopic assignment and to check whether amino acid radicals may be generated on other residues, it was straightforward to investigate the mutant Y96F, with a phenylalanine at residue 96. Indeed, mutant Y96F showed also a strong radical signal [62]. However, the EPR signature of this radical signal was changed and revealed a significantly larger hyperfine coupling of the side chain proton ($A_{iso}(H_{\beta 1}) = 1.55 \pm 0.05$ mT). The deduced dihedral angle θ_1 for the β-proton was in the range of 23–27°, which was consistent with the angle of 26° for Y75, seen in the crystal structure of the enzyme. Furthermore, both tyrosines Y96 and Y75 were replaced by phenylalanines in an attempt to detect the proposed porphyrin–π-cation radical. Even in this Y96F-Y75F double mutant, a porphyrin radical signal was not detected [61]. However, no further radical of an amino acid side chain was seen either. A signal at $g = 2.003$, with very low intensity and no resolved hyperfine structure, has been found in the 94-GHz spectrum only of the double mutant of P450cam which cannot been assigned so far.

In summary, neither in the wild-type nor in the mutants of P450cam, any indication for the supposed porphyrin–π-cation radical could be obtained from the EPR spectra.

Mössbauer studies by Schünemann, Jung et al. [58] revealed that the iron in this freeze-quenched ^{57}Fe-P450cam species shows a doublet signal with a low isomer shift of $\delta = 0.13$ mm/s and a large quadruple splitting ΔE_Q of 1.94 mm/s which counts for 13% of the total P450 concentration while 87% belongs to the ferric start material. The doublet indicates that indeed a Fe^{4+} state was formed [58] (Table 1). Analogous results were obtained in freeze-quench studies with P450BM3-heme domain (BMP) [62]. However, in addition to the tyrosine radical, a tryptophan radical has also been found for BMP. Integration of the spectra indicated that approximately 30% of the total radical signal intensity belongs to the presumed tryptophan and 70% to a tyrosine radical. In independent studies Behan et al. [63] confirmed the existence of a doublet signal in the Mössbauer spectra of P450cam and P450BM3 when freeze-quenched with peroxy acetic acid. Furthermore, density functional theoretical calculations (DFT) suggested that the large value for the quadrupole splitting ΔE_Q of 1.84–2.17 mm/s may be indicative for a protonated oxo ligand in the iron–oxo species [63].

In summary, the freeze-quench studies revealed that a Fe^{4+}-oxo porphyrin species and a protein radical were trapped. If a porphyrin-π-radical had been really formed initially in the reaction with the peroxy acids, as generally be assumed, then reduction of the radical by aromatic amino acids of the protein (Fig. 5) had to occur very fast, whereby the number of redox equivalents should maintain constant in the whole protein (Compound ES). In addition, secondary reactions with the peracids, increasing the number of redox equivalents in the whole protein by one (Compound II), may also be considered as stopped-flow

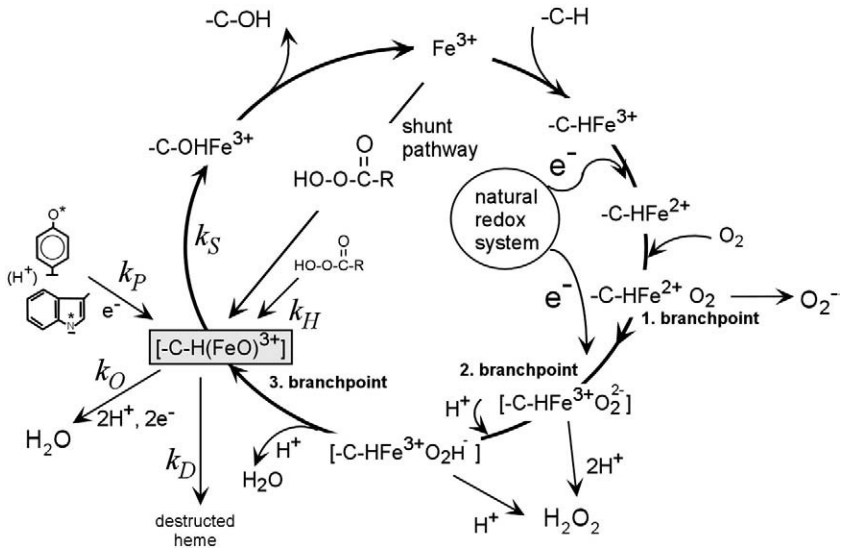

Fig. 5. Reaction cycle of cytochrome P450 indicating the formation and decay processes for Compound I ([-C-H(FeO)$^{3+}$]) at the third branchpoint. -C-H and -C-OH indicate the substrate and the hydroxylation product, respectively. *k* are the rate constants for the oxygen insertion step (k_S) and for the individual leakage processes such as oxidase reaction (k_O), protein radical formation (k_P), heme destruction (k_D), and reaction with a second molecule of the used oxygen species (k_H).

studies by Spolitak et al. [56,57] indicate. It is likely that both reactions and different decay processes occur because only at 8 ms (the shortest reaction time which could be realized in the freeze-quench studies) a concentration ratio of approximately 1:1 for the Fe^{4+} species and the tyrosine radical was observed [64]. At longer times (>40 ms) this ratio is lost. For example, an axial ferric high-spin species has been detected which is built up with time.

It should be mentioned here that the rapid-mixing stopped-flow as well as the freeze-quench experiments were performed in the absence of a substrate. When the experiments for P450cam were performed in the presence of the substrate camphor the amount of tyrosine radical is very low. But a porphyrin radical was also not observed. It turned out that the reason for the low yield of tyrosine radical formation is that the substrate camphor bound near the heme hinders the access of the iron in the heme pocket for the peracid molecule. The yield of tyrosine radical formation increases again if such substrates are used which bind more loosely in the heme pocket [65].

3.4. "Oxidation" approach using photolysis

For the previously discussed stopped-flow and freeze-quench experiments, the sequence of the species formation is building-up of the presumed Compound I at first, followed by the formation of Compound ES or Compound II at second. Obviously, the time for trapping and for spectroscopic detection is too long in these approaches to get high accumulation of Compound I. If Compound II is produced by reduction of the porphyrin radical, one might be able to reform Compound I from Compound II by re-oxidation of the porphyrin to obtain the porphyrin–π-cation radical. Re-oxidation may be performed by laser flash photolysis with light of a wavelength of 355 nm. Indeed, this approach has been successfully applied on porphyrin model complexes as recently reviewed [13,14,66]. Newcomb and coworkers have followed this idea in a number of recent studies on P450 CYP119 [67–69], P450 CYP102A1 heme domain [70] and P450CYP2B4 [71,72]. They prepared Compound II by reaction of ferric P450 with peroxynitrite which has been shown to produce Compound II in other heme proteins. In the following flashphotolysis with 355 nm laser light the porphyrin-π-system should be oxidized within 7 ns and the UV-visible spectrum with a minimum integration time of 1.2 ms was recorded with a diode array detector [68]. The spectral response after the laser flash was obtained as difference spectrum which was afterwards transformed to an absolute spectrum by adding it to a scaled absolute spectrum of Compound II. It is difficult to judge whether this sum-spectrum can be really assigned to 100% of the oxidation product - the presumed Compound I. Probably only a certain amount of Compound I is reflected by this spectrum. However, Behen et al. [73] claimed that the species produced in the reaction of P450BM3 (CYP102A1) with peroxynitrite should be in reality the P450-NO complex and not Compound II because of the identity of the UV-visible as well as Mössbauer spectra with the species produced by the reaction of ferric P450 with NO. In response on these studies, Newcomb et al. [74] demonstrated by X-ray absorption spectroscopic studies on CYP119 that the species produced by reaction with peroxynitrite exhibits a species which shows a Fe-O distance of 1.82 Å indicative for a Fe-OH bonding type. The CYP119–NO complex have also been measured in these studies showing that a Fe-N bond exists with a Fe-N distance also of 1.82 Å which is longer than usual Fe-NO distances. Importantly, the species obtained from Compound II by photolysis was able to induce a number of substrate conversions.

3.5. "Reduction" approach using irradiation techniques

It is well known that solvated electrons can be produced by high-energy irradiation of aqueous solutions, in particular in the presence of glycerol. Solvated electrons could be used to reduce the P450 heme iron. If this is performed in the presence of dioxygen one may expect that the P450 dioxygen complex and even its reduced dioxygen complex could be formed. Kobayashi et al. [75] performed pulse radiolysis studies on the dioxygen complex of 2,4-diacetyldeutero-cytochrome P450cam in the presence of camphor at 4° C and were able to construct point-wise an absorption difference spectrum in the range of 500–300 nm for the time of 10 μs after the radiolysis pulse. This spectrum, exhibiting an absorption maximum at 470 nm and a broad band at 370 nm, revealed some similarities with the spectrum of CPO Compound II. This P450 species decays at about 300 ms and is completely re-converted to the ferric form after 5 min. It was suggested that the species produced by radiolysis is rather Compound II due to homolytic O-O bond splitting than Compound I.

When performing such experiments at low temperatures there might be a chance to stabilize Compound I on the way of the decay of the reduced dioxygen complex. This approach has been used by Davydov, Hoffman and coworkers in a number of studies at cryogenic

temperatures using γ-irradiation of ferric P450cam in the presence of dioxygen and the substrate camphor. EPR and ENDOR spectroscopy were used to analyze the resulting species. A Compound I species could not be detected. Instead EPR signals of a ferric peroxo-complex ($g_\perp \sim 2.25$) and hydroperoxo-complex ($g_\perp \sim 2.3$) were found [76]. In the wild-type P450cam, a significant amount of the ferric peroxo-complex has been detected at 6 K, which converted to the ferric hydroperoxo-complex ($g_\perp \sim 2.3$) when heated to 77 K. The Thr252Ala mutant of P450cam behaved similar. However, in the Asp251Asn mutant the ferric peroxo species was already the primary intermediate at elevated temperature, indicating that proton transfer to the distal oxygen atom was delayed. Stepwise annealing the wild-type samples bound with the camphor substrate to temperatures above the glass transition temperature of the solvent/protein mixture at about 200 K converted the hydroperoxo intermediate directly via three intermediates in the sequence of $g_\perp \sim 2.6$; $g_\perp \sim 2.5$; and $g_\perp \sim 2.48$ to the hydroxy-camphor product complex and then to the ferric low-spin resting state ($g_\perp \sim 2.41$). An analogous annealing study performed by Nyman and Debrunner [77] gave similar results. Although the appearance of the 5-exo-hydroxy-camphor product complex suggests that the O-O bond was cleaved, the expected iron–oxo porphyrin intermediate was not detected.

X-rays have also been used to liberate electrons from the solvent/protein matrix which reduce the heme iron and the subsequently formed dioxygen complex. Davydov et al. [78] used EPR spectroscopy at 77 K, analogously to the studies mentioned above, and observed signals for P450cam corresponding to a peroxo species similar to oxy-hemoglobin and oxy-myoglobin. In the light of these studies the observation of an iron–oxo species in the crystal structure of P450cam [79] appears to be surprising. Schlichting et al. [79] prepared the dioxygen complex of P450cam by diffusing dithionite into the crystal and applying a higher oxygen pressure. At cryogenic temperature, the second electron is provided by the solvent matrix which releases free electrons during exposure to long-wavelength X-ray radiation. It turned out that the dioxygen complex decays to an iron–oxo species with a Fe-O distance of 1.65 Å assigned to Compound I. It is argued that the restraints on the P450 flexibility imposed by the crystal lattice or the unusual source of electrons are the reason that an iron–oxo complex is produced while in other experiments such complex does not accumulate. Interestingly, 5-exo-hydroxy-camphor has been detected in the crystal structure after warming up the crystal for a short time and refreezing it again. Product formation has been afterwards confirmed by gas chromatography.

A clever experiment has been performed by Denisov et al. [80] who used the β-decay of the radioactive ^{32}P of the phosphate buffer at 77 K as ionization radiation to deliver electrons from the solvent matrix. However also in these experiments, the UV-visible spectra taken during the thermal annealing did not indicate the accumulation of a spectrum which could be assigned to Compound I.

3.6. Electron delivery using electrochemical methods

The P450 reaction cycle includes two electron transfer processes. Therefore, it is straightforward trying to deliver the electrons by electrochemical methods. This approach has been followed already very early in the P450 research and in a number of recent studies [81]. In almost all of these publications, the so-called first electron transfer step (Fe^{3+}/Fe^{2+} redox couple) is studied. The potential for this redox couple is in the range from −550 mV to −230 mV versus SCE (−282 mV to +38 mV versus NHE). The potential for the second electron delivery step (Fe^{4+}/Fe^{3+} redox couple) is expected to be at higher values. Recently, Udit et al. [82] reported the electrochemical generation of a high-valent state of P450 (termed as state "E") at a redox potential of $E_{1/2} = +830$ mV (versus Ag/AgCl) (1054 mV versus NHE) assigned to Fe^{4+} formation. Importantly, this state "E" is only obtained at high scan rates in the cyclic voltammogram (>30 V/s) and higher pH or at lower temperatures (4° C) with lower scan rates of ~1 V/s. From the pH dependence of the redox potential (-47 mV/pH) it was suggested that this reduction step follows a proton-coupled electron transfer mechanism suggesting that rather a Fe=OH (Compound II) species than the Fe=O (Compound I) species is formed. Only thioanisole oxidation is induced by this P450 intermediate. Obviously, the Fe=OH (Compound II) species is not powerful enough to attack inert C-H bonds. To produce Compound I electrochemically one needs to oxidize the porphyrin-π-system at still higher potentials in the range of 1350 mV (versus NHE) [83]. An alternative way to the electrochemically induced electron transfer has been proposed by using metal-diimine wires which can be bound to the protein or directly dipped into the access channel to the active site if it carries a substrate-like tail. On the other end of the wire, outside the channel, it carries a ruthenium dipyridyl complex as head group which can be used as photo-inducible reduction or oxidation agent [84]. Studies for the oxidation step using this approach revealed that rather a protonated iron–oxo species (Compound ES or Compound II) and a tyrosine radical should be produced [84].

3.7. Quantum chemical and QM/MM calculations

Results of extensive theoretical studies in characterizing the O-O bond cleavage mechanism, the electronic structure of Compound I and the mechanism of insertion of the oxygen atom into the substrate have been published over the years for P450 [85–90]. The DFT calculations by Shaik et al. revealed that Compound I may exist in two states [90]. Three odd electrons occupy the a_{2u} porphyrin–sulfur mixed orbital and the two π*-orbitals (mainly $3d_{xz}$ and $3d_{yz}$ orbitals of the iron) as analogously obtained by the early IEHT calculations [44]; however the admixture of sulfur orbital revealed to be more important. If the spin in a_{2u} couples anti-ferromagnetically with the spins in π*, the low-spin state (doublet) is formed which represents the ground state. Only 10–21 cm^{-1} energetically above, the high-spin state (quartet) exists with a ferromagnetic coupling between the spins. These calculations showed that both states behave similar in the first step of the substrate hydroxylation (the transient abstraction of the hydrogen atom from the substrate) but differ significantly in the radical rebound step. A comprehensive review about the status of the theoretical studies appeared recently [15].

3.8. Indirect chemical approaches

The large number of studies on the product analysis of the P450 catalyzed reaction with specially designed substrates may be classified as indirect approaches to characterize Compound I. Isotope-labeled substrates have been used already before 1984 to determine the k_H/k_D primary kinetic isotope effect [28] as noted above. Large isotope effects are indicative for a C-H scission process as rate-limiting step which may produce a carbon radical or carbon cation. If it is not the rate-limiting step, then intrinsic isotope effects can be determined from the product analysis of stereo-specifically isotope-labeled substrates. Values of up to 18 have been observed for the isotopic effect of P450 in some studies indicating that the presumed Compound I abstracts the hydrogen atom from the C-H-bond as assumed in the radical rebound mechanism (Fig. 3-6) [14]. Therefore, Compound I should not have bound a hydrogen atom at the iron–oxo unit before. Since 1991, the kinetics of the rebound step has been analyzed in a number of studies using so-called radical clock substrates. In case of an intermediate radical formation by abstraction of a hydrogen atom, these radical clock substrates undergo an immediate collapse with the iron-bound OH-radical to give an un-rearranged hydroxylation product or form another distinct product as result of a competitive rearrangement to another radical. From the known rate of the rearrangement step, the rate of the rebound step can be estimated. It turned out that the rate constants for the rebound

step lies in the range of 10^{10} to 10^{12} s^{-1} or even faster. The last value is already in the picoseconds time range of C-H vibrations and one might exclude the formation of a real radical in this case. A comprehensive review of these studies appeared recently [14] and will therefore not be discussed here in detail. Although all these studies give important evidence for the mechanism of the substrate hydroxylation itself, only little information about the structural properties of Compound I can be obtained.

It should be mentioned here that the hydroperoxo–ferric complex of P450, which is produced before the Compound I formation, has also been considered to function as an alternative electrophilic agent in some substrate conversions [91]. However, DFT calculations revealed that the hydroperoxo–ferric complex is a strongly basic species which should function more as a nucleophilic than as an electrophilic species [92]. On the other hand, thermodynamic analysis suggests that the hydroperoxo–ferric complex is isoenergetically converted to Compound I (Fe^{4+}–oxo prophyrin–π-cation radical) [83]. The activation barrier for i.e. epoxidation by the hydroperoxo–ferric complex has been shown however to be 20–40 kcal/mol higher compared to the Compound I reaction [92].

4. Conclusions

4.1. Apparently inconsistent results are obtained

Compound I, well characterized for CPO, HRP and other peroxidases to be a Fe^{4+}-oxo porphyrin–π-cation radical complex, turned out to be a strange species in P450. The different methods (Fig. 4), used to catch and characterize this intermediate, revealed apparently inconsistent results. In the natural pathway, Compound I could not be detected. For the shunt pathway a UV-visible spectrum resembling that of CPO Compound I could be obtained only using the rapid-mixing stopped-flow technique. However, this spectrum resulted from a single-value decomposition of the set of spectra at different times revealing a small contribution of only few percent. In contrast, rapid-mixing freeze-quench experiments gave no indication for Compound I; instead, the Fe^{4+} state, detected by Mössbauer spectroscopy, and the formation of radicals of tyrosines and tryptophanes, detected by EPR spectroscopy, were found indicating Compound ES or Compound II formation. Production of intermediates using γ- or X-ray radiation of frozen P450 solutions showed only the peroxo– and/or hydroperoxo–ferric complexes. In contrast, irradiation of a P450 crystal at cryogenic temperatures by X-rays resulted in the formation of an iron–oxo species. However, the Fe^{4+} oxidation state and the existence of a porphyrin–π-cation radical cannot be deduced from such studies. Spectral changes in the UV-visible spectra recorded after oxidation of a pre-formed Compound II by photolysis using near-UV light are not sufficiently clear for an unequivocal assignment to a porphyrin–π-cation radical. Electrochemical studies indicate that a second redox couple at high potentials indeed exists, but a structural assignment is only indirectly possible from a pH dependence indicating Compound II formation instead of Compound I formation. Theoretical studies suggest that Compound I should exist in two electronic states.

4.2. Too different experimental conditions must be used

One might ask whether one really catches the same species with the different techniques which all require different experimental conditions for their application (Table 2). For example, the P450 concentration lies in the range of 10–21 μM in stopped-flow experiments with UV-visible spectroscopic detection of the intermediate. Freeze-quench experiments with Mössbauer and EPR spectroscopic detection require P450 concentrations in the range of 0.5–2 mM. Both techniques base on bimolecular reaction kinetics as the initial step followed by several side reactions. The concentration ratio of P450 : peracid vary between 3 and 120 for the different experiments. In contrast, radiation and photolysis techniques follow completely different mechanisms and should produce the resulting P450 species in another electronic state at the initial event followed by relaxation processes. When completed at low temperatures, the produced species are in a meta-stable state and the annealing step is too slow to resolve short-live intermediates. Electrochemical methods require an attachment of the protein on the electrode surface. Even, if coupling agents are used, P450 should be involved in a heterogeneous reaction in contrast to homogenous reactions of P450 in solution which may restrict degrees of freedom for the reaction.

4.3. Too many players are in the game

In order to catch and to characterize Compound I, one needs to realize a steady-state concentration of Compound I (Cpd I) which fits in the time window of the experimental method used. With the steady-state condition $d[\text{Cpd I}]/dt = 0$ and assuming that the hydroperoxo-complex ("Compound 0"; Cpd 0) is always the species before Cpd I one may derive the condition for the concentration ratio $[\text{Cpd I}]/[\text{Cpd 0}] = k_2/(k_3 + k_{-2})$ [15]. k_2 and k_{-2} are the rate constants for the formation of Cpd I from Cpd 0 and for the reverse reaction,

Table 2
Experimental conditions used in experiments according to different approaches to catch and characterize P450 intermediates.

Approach	Concentration range for P450	Concentration range for oxygen agent[a]	Temperature	State
Natural pathway, UV-visible spectra	3–20 μM	O_2: ~200 μM (natural saturation in puffer at ambient conditions)	4–30° C	Solution
Shunt pathway, stopped-flow, UV-visible spectra	P450cam: ~10 μM	mCPBA: 30–300 μM PA: 600–1200 μM	4–25° C	Solution
	P450119: 21 μM	mCPBA: 1–20 μM	4° C	
Shunt pathway, freeze-quench EPR, Mössbauer spectra	P450cam, P450BM3: 0.5–2 mM	PA: 2.5–10 mM	~20° C, trapped at −110° C	Solution
γ-Irradiation EPR spectra	1 mM	O_2 bound to P450 = 1 : 1	−196° C	Frozen solution
X-ray radiation EPR spectra	0.6 mM	–	−196° C	Frozen solution
X-ray radiation crystallography	>5 mM	O_2: ~1 mM (solubility of O_2 in aqueous solutions, after O_2-pressure of 150 bar and pressure release),	−170° C to −190° C	Crystal
^{32}P-radioactive decay UV-visible spectra	~30 μM	O_2: ~200 μM (natural saturation in puffer at ambient conditions)	−196° C	Frozen solution
Electrochemistry voltammetry	6 μM as soaking solution; 2.0–2.5 ng/electrode film	O_2: ~200 μM (natural saturation in puffer at ambient conditions)	4–25° C	Heterogeneous reaction
Photolysis UV-visible spectra	5–10 μM	–	−10° C to −50° C	Cooled solution

[a] mCPBA = meta-chloroperoxybenzoic acid; PA = peroxy acetic acid.

respectively. k_3 represents the rate constant for the decay of Cpd I, which may include all possible reactions. In the ideal case that would be only the insertion of the oxygen into the substrate C-H bond. However, as discussed above and reviewed in [93] Compound I may be regarded as the third branch point in the reaction cycle of P450 (Fig. 5) leading to leakage of the cycle on the way to substrate conversion. Thus, k_3 appears to be the sum $k_3 = k_S + k_H + k_P + k_D + k_O + k_U$ of the rate constants for the oxygen insertion step (k_S) and for the individual leakage processes such as oxidase reaction (k_O), protein radical formation (k_P), heme destruction (k_D), reaction with a second molecule of the used oxygen species (k_H), i.e. in the shunt pathway, and other still unknown reactions (k_U). Assuming that $k_{-2} \ll k_3$ and $c_0 = [\text{Cpd I}] + [\text{Cpd 0}]$ (total P450 concentration) one may reform the above equation to $[\text{Cpd I}] = c_0/(k_3/k_2 + 1)$. Following, if i.e. k_3 is tenfold larger than k_2 one could accumulate only ~9% of the total P450 concentration as Compound I. Assuming further that the P450 concentration is 1 mM as used i.e. in freeze-quench experiments then the final protein concentration is 0.5 mM after mixing and the concentration of Compound I would be $0.09 \times 0.5 = 0.045$ mM = 45 μM; a concentration which is on the border of the detection limit of some spectroscopic techniques, i.e. Mössbauer. For a P450 concentration of ~20 μM, as used for UV-visible spectroscopy, one would get a concentration of $0.09 \times 0.20 = 1.8$ μM; a concentration which could still be detected by UV-visible spectroscopy using the Soret band as spectral signal.

So, this simple estimation shows that accumulation of Compound I depends on the relative rate constants for the various competing reactions which finally determine the chance to detect Compound I by the different spectroscopic technique. Simulation of the kinetics will probably help to design experiments to catch Compound I.

5. Outlook

Reviewing the studies discussed above, one might think that Compound I in P450 appears to be a mystery. Indeed, over more than 45 years the P450 community tries to uncover the secrets of Compound I formation and mechanism of its action in substrate conversion. The knowledge has significantly increased although we still ask the same questions—does Compound I as Fe^{4+}–oxo porphyrin–π-cation radical really exist and how would the oxygen atom be transferred to the C-H bond of the substrate. I remember discussions with Klaus Ruckpaul about oxygen species and their formation by heme proteins and model porphyrin complexes almost 40 years ago when I joined his group as a freshly graduated chemistry student which had a passion for theoretical chemistry. He encouraged me to write my first paper in the P450 field dealing with the estimation of thermodynamic parameters for the formation of active oxygen species and the P450 catalysis [3] but he always pointed out that theory must be proved by experiments. This statement is certainly true and still valid. Future studies on the P450 Compound I story need improved and new experimental approaches and detection techniques combined with theoretical simulations for the design of appropriate experiments. Klaus Ruckpaul has always encouraged young people to go in this way. I am very thankful to Klaus Ruckpaul that he gave me the opportunity to start my scientific career in such interesting scientific field.

Dear Klaus, congratulations on your 80th birthday!

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