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Review

Freeze-quenched iron-oxo intermediates in cytochromes P450

Christiane Jung [a,*,1], Volker Schünemann [b], Friedhelm Lendzian [c]

[a] *Max-Delbrück-Center for Molecular Medicine, 13125 Berlin, Germany*
[b] *Technical University of Kaiserslautern, Department of Physics, 67663 Kaiserslautern, Germany*
[c] *Max-Volmer Laboratory for Biophysical Chemistry, Technical University Berlin, PC 14, 10623 Berlin, Germany*

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Abstract

Since the discovery of cytochromes P450 and their assignment to heme proteins a reactive iron-oxo intermediate as the hydroxylating species has been discussed. It is believed that the electronic structure of this intermediate corresponds to an iron(IV)–porphyrin-π-cation radical system (Compound I). To trap this intermediate the reaction of P450 with oxidants (shunt pathway) has been used. The common approaches are stopped-flow experiments with UV–visible spectroscopic detection or rapid-mixing/freeze-quench studies with EPR and Mössbauer spectroscopic characterization of the trapped intermediate. Surprisingly, the two approaches seem to give conflicting results. While the stopped-flow data indicate the formation of a porphyrin-π-cation radical, no such species is seen by EPR spectroscopy, although the Mössbauer data indicate iron(IV) for P450cam (CYP101) and P450BMP (CYP102). Instead, radicals on tyrosine and tryptophan residues are observed. These findings are reviewed and discussed with respect to intramolecular electron transfer from aromatic amino acids to a presumably transiently formed porphyrin-π-cation radical.
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Oxygen—"the elixir of life," first recognized in the late 16th century by Michael Sendivogius, a Polish alchemist, philosopher, and medical doctor, and about 150 years later again discovered independently by the Swedish pharmacist Carl Wilhelm Scheele and the English theologian, philosopher, and researcher Joseph Priestley—is a fascinating element. In binding with itself to form molecular oxygen it reveals a significant multiface behavior. Fifty years ago it was Osamu Hayaishi who showed for the first time that an enzyme (pyrocatechase) is involved in catalyzing the incorporation of two atoms of molecular oxygen into an organic compound and he termed such enzymes oxygenases [1]. Around that time many in vivo studies on drug/steroid metabolism were performed [2], culminating a few years later in the discovery of the responsible monooxygenase enzyme cytochrome P450 [3,4]. Since these enzymes have been assigned to a heme protein [5,6] the existence of a highly reactive iron-oxo intermediate is discussed. It is amazing how this discussion has continued and even increased over the past 50 years. The reason is probably that this species is so short-lived and therefore very difficult to analyze. Attempts to trap this intermediate resulted in controversial findings. Suggestions about the chemical and electronic structure came from metal porphyrin studies and from the peroxidase field [7,8], where this intermediate is called Compound I (cpd I). In particular, the analogy to chloroperoxidase from *Caldariomyces fumago* (CPO) suggested that cpd I in P450 should be characterized by an iron(IV) and a porphyrin-π-cation radical.

Seven years ago we entered into the field with the vision of proving this suggestion by a rapid-mixing/freeze-quench technique and of characterizing the putative cpd I of P450cam by EPR and Mössbauer spectroscopy. To our surprise, these studies revealed that instead of the porphy-

* Corresponding author. Fax: +41 41 8104508.
E-mail address: christiane_jung@hotmail.com (C. Jung).
[1] Present address: KKS Ultraschall AG, Frauholzring 29, CH-6422 Steinen, Switzerland.

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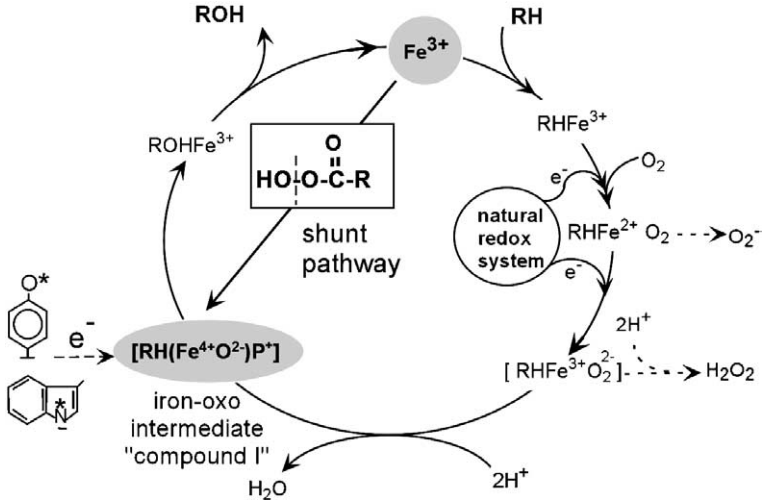

Fig. 1. The heme-based reaction cycle for cytochrome P450, indicating the shunt pathway and the iron-oxo intermediate that is involved in the tyrosine and tryptophan radical formation as side reaction. The uncoupling H_2O_2 and O_2^- reactions are also indicated.

rin radical, a tyrosine radical was formed, although iron(IV) is seen when this species is produced in the reaction of ferric P450cam with external oxidants within the so-called shunt pathway [9] (Fig. 1). In the present paper, we review the main results of these studies on cytochromes P450 and make the comparison to CPO and nitric oxide synthase (NOS) which both form together with P450 the class of thiolate heme proteins. It will be shown that in the light of analogous studies on many other heme proteins performed by several laboratories in the recent years the existence of intermediate protein radicals in P450 is not as surprising as originally thought and might be a general phenomenon in heme-based oxygenases.

The cytochrome P450 reaction cycle

The reaction cycle of P450 (Fig. 1) [10] starts with binding the substrate to the heme protein. The next step is the reduction of the heme iron by electron transfer from NAD(P)H via the redox proteins (flavin protein, iron–sulfur protein). In a subsequent step the reduced heme iron binds molecular oxygen. The dioxygen complex accepts a second electron with synchronous or subsequent attachment of a proton to the oxygen ligand. This intermediate dioxygen species quickly decomposes by splitting the O—O bond and by releasing a water molecule. Thus a highly reactive iron-oxo species (cpd I) is formed, which is assumed to hydroxylate the substrate. This iron-oxo intermediate can also be produced by reaction of the heme enzyme with external oxidants such as peracids, peroxides, and iodosobenzene (shunt pathway), for which substrate conversion was been observed already in the early 70th [11]. This strongly suggests that the intermediate formed with external oxidants may be identical with that formed in the natural reaction cycle via the dioxygen complex and may justify producing cpd I by the shunt pathway for further characterization.

Chloroperoxidase as reference protein

So far, there are two main approaches to identifying the intermediate: (i) the stopped-flow technique taking the heme UV–visible spectrum for detection and (ii) the rapid-mixing/freeze-quench technique using EPR and Mössbauer spectroscopy for characterization of the trapped intermediate. For both approaches CPO is taken as a reference. When CPO is mixed in stopped-flow experiments with an oxidant such as H_2O_2, peroxyacetic acid (PA), or *meta*-chloroperbenzoic acid (mCPBA), a green-colored cpd I is formed, which shows a UV–visible absorption spectrum after several milliseconds, characterized by a broad Soret band around 370 nm and a weak but significant long-wavelength band at ~680 nm [12,13]. This spectrum is characteristic of a porphyrin-π-cation radical, very well known from iron porphyrin model complexes [8]. EPR (9.6-GHz) and Mössbauer spectroscopic studies on CPO samples obtained by rapid-mixing with PA and freeze-quenching [14] identified cpd I as an Fe(IV)=O (spin $S = 1$) and porphyrin-π-cation radical (spin $S' = 1/2$), where both spins couple antiferromagnetically. The EPR spectrum of cpd I in CPO produced with peracids has *g*-values $g_{\|} = 2$ and $g_{\perp} = 1.75$. The Mössbauer study gives an isomer shift of $\delta = 0.14\ \text{mm s}^{-1}$ and a quadrupole splitting of $\Delta E_Q = 1.02\ \text{mm s}^{-1}$. The Fe=O stretch vibration of CPO cpd I lies at $790\ \text{cm}^{-1}$, as determined by resonance Raman spectroscopy [15]. Similar values have been found for porphyrin iron-oxo species [16]. One-electron reduction of cpd I reduces the porphyrin radical but leaves the ferryl state of the Fe(IV)=O moiety unchanged. This species is called Compound II (cpd II). The Fe=O distance of CPO

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