Spectroscopic investigations of intermediates in the reaction of cytochrome P450BM3–F87G with surrogate oxygen atom donors

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Abstract:

Rapid mixing of substrate-free ferric cytochrome $P450_{BM3}$–F87G with m-chloroperoxybenzoic acid (mCPBA) resulted in the sequential formation of two high-valent intermediates. The first was spectrally similar to compound I species reported previously for $P450_{CAM}$ and CYP 119 using mCPBA as an oxidant, and it featured a low intensity Soret absorption band characterized by shoulder at 370 nm. This is the first direct observation of a P450 compound I intermediate in a type II P450 enzyme. The second intermediate, which was much more stable at pH values below 7.0, was characterized by an intense Soret absorption peak at 406 nm, similar to that seen with $P450_{CAM}$ [T. Spolitak, J.H. Dawson, D.P. Ballou, J. Biol. Chem. 280 (2005) 20300–20309]. Double mixing experiments in which NADPH was added to the transient 406 nm-absorbing intermediate resulted in rapid regeneration of the resting ferric state, with the flavins of the flavoprotein domain in their reduced state. EPR results were consistent with this stable intermediate species being a cytochrome c peroxidase compound ES-like species containing a protein-based radical, likely localized on a nearby Trp or Tyr residue in the active site. Iodosobenzene, peracetic acid, and sodium m-periodate also generated the intermediate at 406 nm, but not the 370 nm intermediate, indicating a probable kinetic barrier to accumulating compound I in reactions with these oxidants. The P450 ES intermediate has not been previously reported using iodosobenzene or m-periodate as the oxygen donor.

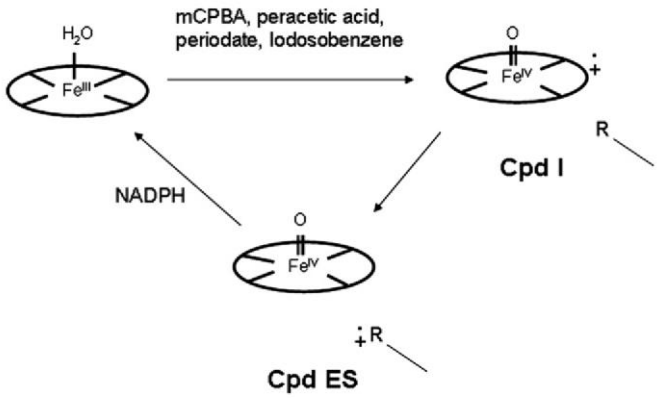
Keywords:

Cytochrome P450BM3; Stopped-flow; Compound I; Compound ES; Electron paramagnetic resonance; Iodosobenzene; m-Chloroperoxybenzoic acid

Article:

1. INTRODUCTION

There has been a great deal of interest in identifying reactive intermediates in the catalytic cycle of cytochrome P450 for the past quarter of a century. The application of new technologies to the direct observation of certain chemical species has contributed considerably to the characterization of these intermediates. For example, cryogenic methods have been used to study the properties of ferrous-oxy complexes of a variety of different P450 isoforms [1–3]. These low temperature studies have also facilitated the production and stabilization of several intermediates in the proposed P450 catalytic cycle beyond the second electron transfer, including a ferric-peroxo complex [4,5]. However, as information has accumulated, more questions have arisen as to the precise chemical nature of the ultimate hydroxylating species, as well as whether there is only one such oxidant in P450 catalysis. The consensus is that a ferryl [Fe^{IV}=O] porphyrin radical species, similar to the more stable compound I (Cpd I) from various peroxidases, is involved in the majority of oxidative reactions carried out



Scheme 1. Proposed reaction pathway for $P450_{BM3}$ reacting with various oxygen atom donors. Double mixing experiments showed that the proposed compound ES reacts with NADPH to produce the ferric enzyme.

by P450 enzymes (Scheme 1). While Cpd I has only been directly observed in two bacterial P450 enzymes [6–8], the argument is made that this species is probably universal among P450s. It is interesting that the only stopped-flow experiments in which clearly identifiable Cpd I could be directly observed required the use of the oxidant m-chloroperoxybenzoic acid (mCPBA) as the oxygen atom donor. No other oxidant has been successfully employed to directly form significant quantities of this species in solution. Stopped-flow studies of the reactions of rabbit liver microsomal P450s with peroxides, peracids [9], and iodosobenzene [10] have also been reported; however, no transient Cpd I-like intermediates were observed.

Schünemann et al., using EPR and Mössbauer techniques and peracetic acid as an oxygen donor, could not detect any Cpd I with $P450_{CAM}$, but they did identify a species containing a tyrosine radical and a Fe^{IV}=O [11,12]. This type of species, often called Compound ES (Cpd ES) [8], is well known in cytochrome c peroxidase (CcP). In CcP, Cpd ES has been shown by a variety of techniques to contain an Fe^{IV}=O prosthetic heme coupled to an active site Trp radical cation [13]. Recent reports on P450 [7,8] seemed to indicate that Cpd I was only observable when mCPBA was the oxidant, and that only Cpd ES was observable when peracetic acid was used [11,12,14]. Spolitak et al. recently addressed this discrepancy using detailed stopped-flow spectroscopic analysis of the reaction between substrate-free ferric $P450_{CAM}$ and either peracetic acid or mCPBA at several different pH values [8]. They concluded that a considerable fraction of the $P450_{CAM}$ formed a CcP Cpd ES-like intermediate below pH 7.0, whereas above pH 7.0, some Cpd I could be observed, albeit transiently. Cpd I decayed partially to a Cpd ES-like species as well as to heme degradation products due to further reactions of the higher oxidation states of the heme with the excess per- acid. Thus, for $P450_{CAM}$, the pH, as well as the oxidant, determined how much of each form of the oxidized intermediate was observed. Related intermediates containing oxidized amino acid residues have also been observed in a variety of heme containing enzymes including myoglobin and catalase-peroxidase [15,16].

Cytochrome $P450_{BM3}$ has been heralded as a bacterial model enzyme for microsomal P450 enzymes because it contains both a flavin-containing redox partner (NADPH cytochrome P450 reductase) and the heme P450 domains as part of the same protein [17]. The reductase domain is similar to the mammalian P450 reductase [17]. Unfortunately, the BM3 enzyme is a fatty acid hydroxylase, and binds poorly to aromatic oxygen atom donors that have been used to generate Cpd I-like intermediates in the other bacterial isoforms. Recent studies by Jung et al., involving freeze-quench EPR analysis of intermediates of cytochrome $P450_{BM3}$ generated using peracetic acid, suggested the formation of protein radical intermediates similar to those observed in $P450_{CAM}$; however, no Cpd I was observed in these studies [14]. A mutant form of $P450_{BM3}$, BM3–F87G, has recently been shown to favorably bind certain aromatic substrates, and was used in a previous stopped- flow investigation of the reaction of P450 with aromatic aldehydes and hydrogen peroxide [18,19]. Those studies showed that adducts to the heme resulted from the reactions. The mutant enzyme has a larger, more

accommodating active site pocket that is well suited for binding relatively small aromatic oxene donor molecules such as mCPBA or iodosobenzene.

In the current study, we have examined the reaction of substrate-free ferric cytochrome $P450_{BM3}$–F87G with several different oxygen atom donors, including mCPBA, per- acetic acid, sodium m-periodate, and iodosobenzene, using stopped-flow spectrophotometric and freeze-quench EPR techniques, to directly observe intermediates that may play a role in the P450 catalytic function.

2. EXPERIMENTAL

2.1. Materials

Cytochrome $P450_{BM3}$ and the F87G mutant were produced in a recombinant bacterial expression system reported previously [18]. All reagents used in the experiments were purchased from Sigma, Aldrich, or Fisher Chemical suppliers. The reagents, mCPBA, peracetic acid, and sodium periodate, were purchased from Fisher. mCPBA was obtained as 70% pure reagent. Iodosobenzene (IOB) was purchased from TCI America chemical Corp.

2.2. Stopped-flow experiments

Experiments were carried out using either a Hi-Tech SF61DX double mixing stopped-flow system with diode array detection or an Applied Photophysics MVSX-18 single mixing system with diode array detector. The experiments involving $P450_{BM3}$–F87G with the oxygen atom donors mCPBA, peracetic acid, or iodosobenzene were performed by mixing P450 with oxidant (30–500 μM) in 100 mM phosphate buffer at the specified pH. Temperature was controlled using a water bath connected to the stopped-flow system. Due to the instability of mCPBA in aqueous solution, a stock solution in ethanol was prepared and stored on ice, and fresh aqueous mCPBA solutions were prepared every 15 min. Reactions carried out using sodium m-periodate were performed in the same way, except a 40 mM aqueous stock solution of the salt was prepared and used over the course of the day.

2.3. HPLC analysis of heme

Procedures for extracting and analyzing the heme cofactor from BM3–F87G are described elsewhere in the literature [18].

2.4. EPR analysis

All samples for EPR analysis were prepared by combining enzyme samples with the specified oxidant at 12 °C in an EPR tube and quenching the reaction by immersion in liquid nitrogen. Reaction times between 5 and 100 s were used in generating EPR samples with a final enzyme concentration of 165 μM in 100 mM phosphate buffer. The concentrations of mCPBA and iodosobenzene were between 0.50 and 2.0 mM. EPR spectra were recorded on a Bruker Instruments EMX 6/1 EPR spectrometer with microwave power between 0.005 and 20 mW, using a frequency of 9.48 GHz and a modulation amplitude of 5 gauss. Spectra were recorded at 15 K and the temperature was controlled with an Oxford Instruments ESR 900 liquid helium cryostat. Sixteen scans of 42 s each were summed for each spectrum.

3. RESULTS

3.1. Stopped-flow studies of the reaction of cytochrome $P450_{BM3}$–F87G with mCPBA

Addition of mCPBA (100 μM after mixing) to substrate- free ferric cytochrome $P450_{BM3}$–F87G at pH 7.4 (~20:1 ratio) resulted in the rapid appearance of a shoulder in the absorption spectrum at 370 nm and a decrease in the intensity of the Soret absorption peak with a slight red shift to 422 nm (Fig. 1). These spectral characteristics are indicative of the Cpd I shown previously [6–8]. Under these conditions, an additional intermediate with a λ_{max} of ~406 nm was formed in a subsequent phase of the reaction (not shown). However, the excess of mCPBA used in this reaction resulted in rapid bleaching of the heme, so that full spectral characterization of this second intermediate was not possible. When only a ~6-fold excess of oxidant was used,

the 370 nm shoulder, although present, was much less prominent, while the 406 nm absorbing species (B in Fig. 2a) was much more stable and ultimately decayed back to a species resembling the ferric form of BM3–F87G over the course of 5 min, with less bleaching (C in Fig. 2a) than seen with ~20:1 mCPBA:P450. The 406 nm species is spec-

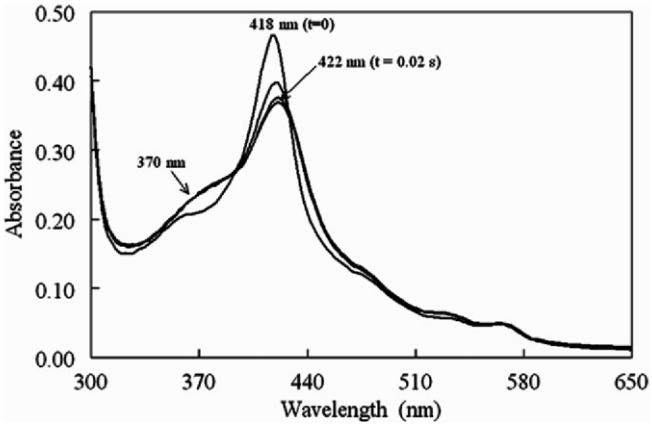


Fig. 1. UV–vis absorption spectra recorded in the stopped-flow spectrophotometer at 0.000, 0.002, 0.010 and 0.020 s after mixing equal volumes of ferric $P450_{BM3}$–F87G (9 μM) with 200 μM mCPBA at 10 °C in 100 mM potassium phosphate buffer, pH 7.4.

trally very similar to Cpd ES shown with $P450_{CAM}$ [8]. Representative kinetic traces taken at 370, 406 and 418 nm are shown in Fig. 2b and c. Rate constants for the different phases of this reaction were measured at 370 nm for phase 1, 406 nm for phase 2, and 418 nm for phase 3 as a function of mCPBA concentration. The average measured rate constants for each phase are reported in Table 1. As indicated by these data, only the initial phase of the reaction showed a significant dependence on the concentration of the oxidant used. The rate of conversion of the 370 nm absorbing intermediate to the one absorbing at 406 nm at pH 7.4 was nearly independent of the concentration of mCPBA, consistent with a sequential mechanism in which Cpd I initially formed converts to Cpd ES.

At pH 6.8, addition of mCPBA to the ferric BM3–F87G enzyme resulted in a rapid decrease and slight red shift of the Soret absorption peak, followed by an increase at 406 nm, with little or no appearance of the shoulder at 370 nm (Fig. 3). A clean isosbestic point at 412 nm observed after 10 ms was due to the conversion of the first intermediate to the species absorbing maximally at 406 nm. Along with the shift of the Soret peak to 406 nm, the absorption peaks at 535 and 568 nm decreased and a new feature at 623 nm appeared, as shown in Fig. 3. These results are consistent with formation of Cpd I followed by coversion to a Cpd ES-like species. The lower pH of 6.8 was more favorable for observing the Cpd ES-like species, similarly to the analogous reaction with $P450_{CAM}$ [8].

3.2. Double mixing stopped-flow analysis of the reaction between ferric $P450_{BM3}$–F87G and mCPBA

In a series of experiments involving double-mixing stopped-flow procedures, ferric BM3–F87G (8.0 μM final concentration) was initially combined with mCPBA (7:1) at pH 6.8 and aged for 5 s. Analysis of this reaction in the single mix mode revealed that formation of the 406 nm absorbing intermediate was maximal at 5 s.

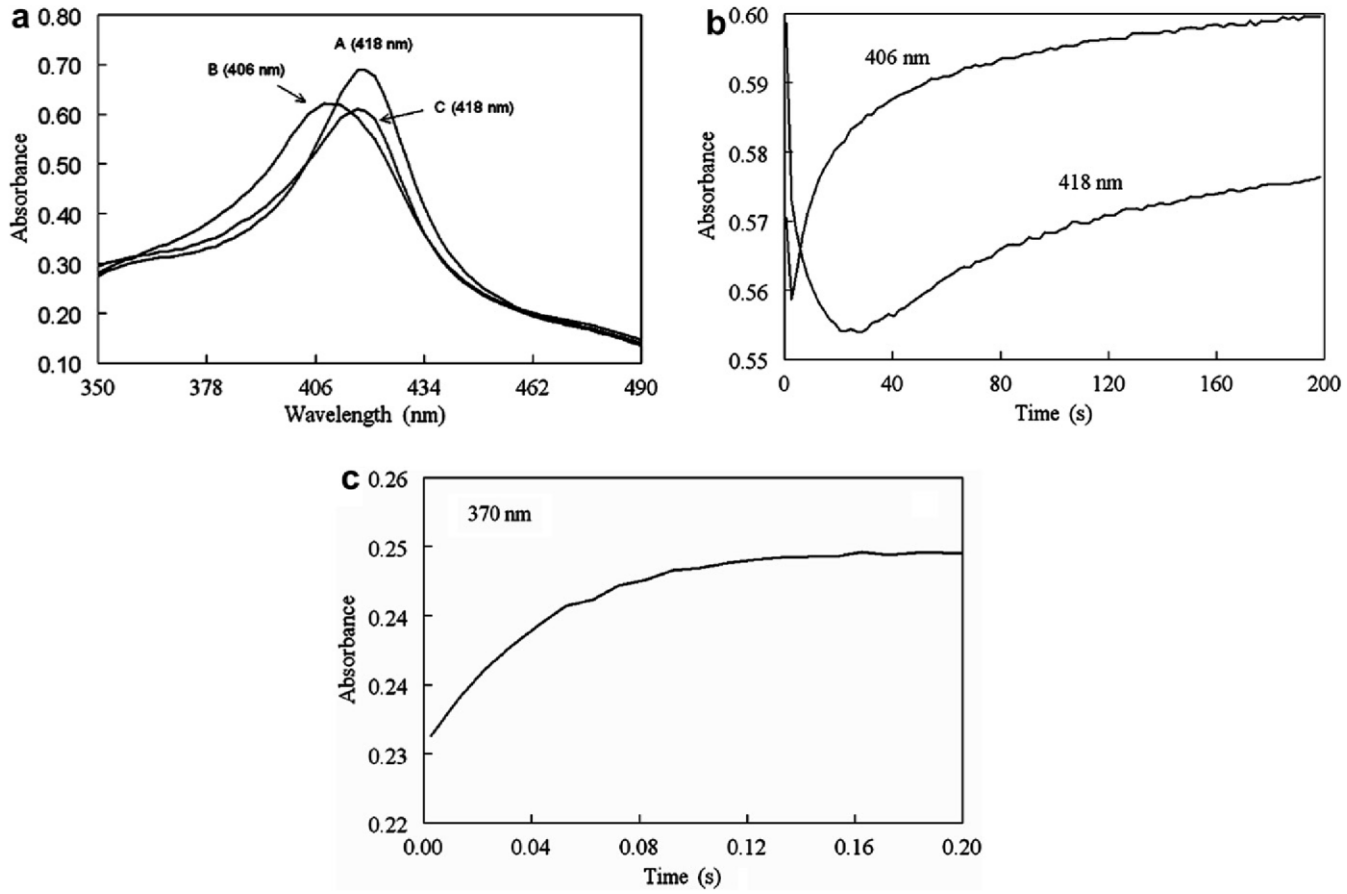


Fig. 2. (a) UV–vis absorption spectra recorded in the stopped-flow spectrophotometer at (A) 1.28 ms, (B) 1.0 s and (C) 20 s after mixing equal volumes of ferric $P450_{BM3}$–F87G (13 μM) with 80 μM mCPBA at 10 °C in 100 mM potassium phosphate buffer, pH 7.4. (b) Reprentative kinetic traces for the reaction of mCPBA with $P450_{BM3}$–F87G taken at 406 and 418 nm. (c) Kinetic trace for the reaction taken at 370 nm for the first 0.20 s.

Table 1
Dependence of the rate of each phase of the reaction of mCPBA with cytochrome $P450_{BM3}$–F87G

[*m*CPBA] (μM)	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})
35	17.5	0.190	0.015
53	22.0	0.125	0.013
63	30.0	0.122	0.017

Reactions were carried out at pH 7.4 in 100 mM phosphate buffer at 10 °C.

Following the 5 s aging time, NADPH (0.15 mM) was added in the second mix. At 15 °C, all of the 406 nm absorbing intermediate was converted back to ferric P450 within the mixing time (data not shown). Furthermore, excess NADPH was oxidized slowly by the resulting enzyme as indicated by loss of absorbance at 340 nm. This result implies that formation of the species absorbing at 406 nm did not prevent the enzyme from accepting electrons from NADPH. It should also be noted that BM3– F87G oxidized NADPH even in the absence of a substrate, presumably via production of either H_2O_2 or H_2O, indicating that initiation of the catalytic cycle did not require the presence of substrate. This type of uncoupling reaction in

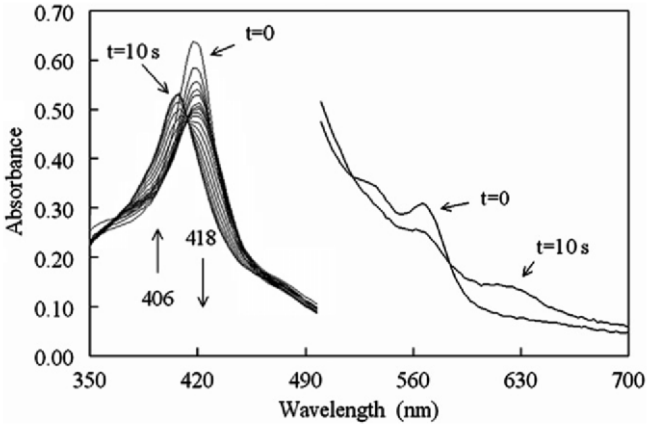


Fig. 3. UV–vis absorption spectral changes after mixing equal volumes of ferric $P450_{BM3}$–F87G (12.8 μM) and mCPBA (300 μM) at 15 °C in 100 mM potassium phosphate buffer, pH 6.8. Spectra were recorded in the stopped-flow spectrophotometer from 1.28 ms to 10 s after mixing. A reference spectrum was recorded under the same conditions, except mCPBA was not included. The region from 500 to 700 nm has been expanded by a factor of 4.

which a Cpd ES-like species becomes reduced back to the ferric heme with release of H_2O_2 or H_2O, may also occur with microsomal cytochrome P450 enzymes for which BM3 is often used as a model.

3.3. Reaction of P450BM3–F87G with other oxidants

In addition to mCPBA, iodosobenzene, sodium m-periodate, and peracetic acid were each utilized as oxidants for substrate-free ferric $P450_{BM3}$–F87G in stopped- flow experiments. In the reaction with iodosobenzene at pH 6.8, a direct conversion of the resting ferric enzyme to the 406 nm absorbing species was observed with a clean isosbestic point at 412 nm (Fig. 4) that remained until bleaching began after about 10.0 s (data not shown). As with mCPBA, a new broad spectral feature was observed in the spectrum at 623 nm, with loss of the bands at 535 and 568 nm. However, in contrast to the reaction with mCPBA, no shoulder at 370 nm was observed in the reaction at pH 7.4 with iodosobenzene; moreover, there was no red-shift in the Soret absorption peak prior to formation of the 406 nm peak, in contrast to that seen with mCPBA (Fig. 1). These results indicate that Cpd I does not accumulate in the reactions with iodosylbenzene, but that considerable Cpd ES-like species did. Presumably, the reaction with iodosobenzene to form the Cpd I was rate determining so that essentially no species with a shoulder at 370 nm accumulated before formation of the 406 nm absorbing species. Over a 5 min period, the 406 nm absorbing intermediate disappeared as the heme was slowly bleached.

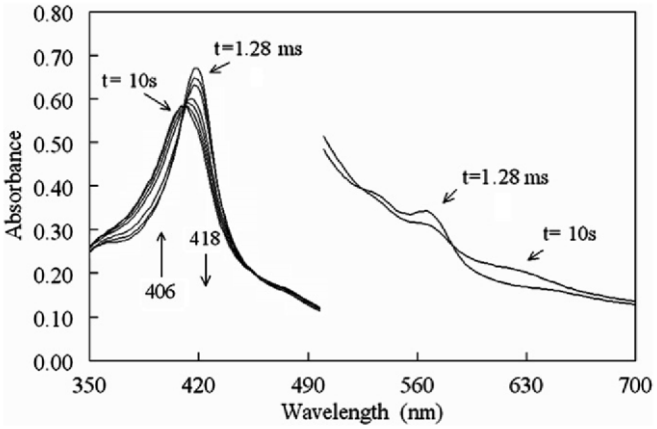


Fig. 4. UV–vis absorption stopped-flow spectral data recorded after mixing equal volumes of ferric $P450_{BM3}$–F87G (12.8 μM.) with iodoso-benzene (300 μM) at 15 °C in 100 mM potassium phosphate buffer, pH 6.8. Spectra were recorded from 1.28 ms to 10 s after mixing. The region from 500 to 700 nm has been expanded by a factor of 4.

Reaction of ferric BM3–F87G with sodium m-periodate at pH 6.8 also resulted in the formation of the 406 nm absorbing intermediate, again with an isosbestic point at 412 nm, and, as with iodosobenzene, no Cpd I-like species with a shoulder at 370 nm could be observed under any conditions. Interestingly, in this reaction the Soret absorption peak decreased slightly over the first 100 ms and over the next 50 ms returned to its original absorbance (Fig. 5a). Over the same time period, the region of the spectrum between 350 and 400 nm steadily increased in absorbance. We do not know the chemical nature of this initial reaction. Starting at 0.15 s and continuing for 10 s, a monophasic shift of the Soret peak from 418 to 406 nm was observed (Fig. 5b), again consistent with formation of a Cpd ES-like species. This figure also shows the new broad peak at 625 nm associated with this intermediate, analogous to that seen with the other oxidants.

Finally, peracetic acid was rapidly mixed with ferric BM3–F87G at pH 6.8 (Fig. 6) and at 7.4 (data not shown); only the 406 nm absorbing intermediate could be detected, even at concentrations in excess of 20 mM peracetic acid. As with the other oxidants, the appearance of the 406 nm Soret absorption peak was accompanied by a decrease in the 535 and 568 nm bands, and formation of a new broader

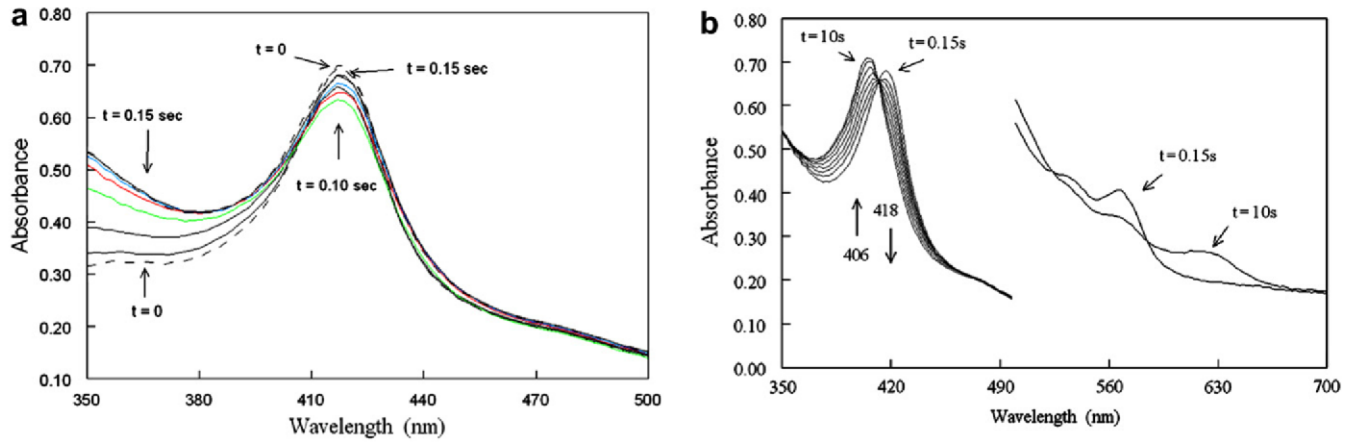


Fig. 5. (a) UV–vis absorption spectral changes recorded in the first 0.15 s after mixing equal volumes of ferric $P450_{BM3}$–F87G (12.8 μM) and sodium *m*-periodate (20 mM) at 15 °C in 100 mM potassium phosphate buffer, pH 6.8. The Soret absorption peak decreases for the first 0.1 s and then increases again. The dashed line spectra were recorded at $t = 0$ and $t = 0.15$ s. (b) Changes in the UV–vis absorption spectrum of ferric $P450_{BM3}$–F87G after mixing equal volumes of the P450 (12.8 μM) and sodium *m*-periodate (20 mM) at 15 °C in 100 mM potassium phosphate buffer, pH 6.8 over the time period from 0.15 to 10.0 s. The region from 500 to 700 nm has been expanded by a factor of 4.

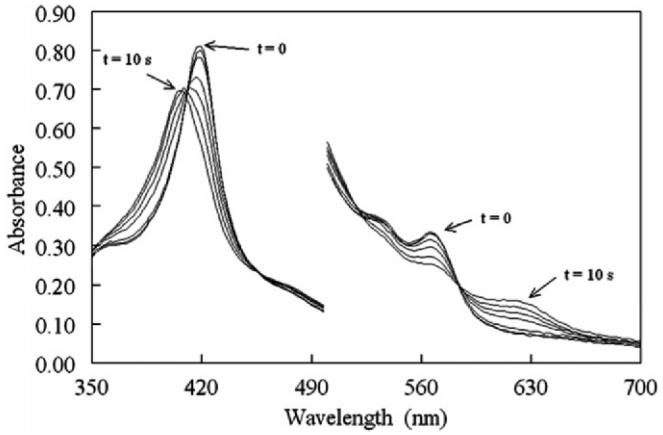


Fig. 6. UV–vis absorption spectral changes after mixing equal volumes of ferric $P450_{BM3}$–F87G (14.4 μM) and 10.0 mM peracetic acid at 15 °C in 100 mM potassium phosphate buffer, pH 6.8. Spectra were recorded from 1.28 ms to 10 s after mixing the reactants. A reference spectrum was recorded under the same conditions ($t = 0$), except that peracetic acid was not included. The region from 500 to 700 nm has been multiplied by a factor of 4.

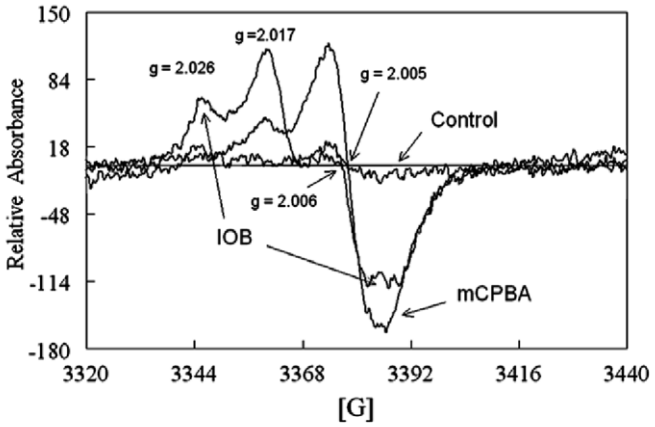


Fig. 7. EPR spectra of the intermediates formed during the reaction of ferric $P450_{BM3}$–F87G with either *m*-chloroperoxybenzoic acid (mCPBA) or iodosobenzene (IOB). Final enzyme concentration was 0.165 mM; mCPBA was 0.50 mM, and iodosobenzene was 2.0 mM. Reactions were carried out at 15 °C in 100 mM potassium phosphate buffer, pH 6.8, for approximately 5 s and quenched by immersion in liquid nitrogen. Spectra were recorded at a microwave power of 5 μW. The control consists of enzyme with no oxidant added.

band at 623 nm. Kinetic analysis of the data revealed a lag phase during the first 100 ms, with only a slight decrease in absorbance at 418 nm. This lag phase was followed by a direct conversion to the 406 nm absorbing intermediate.

3.4. Electron paramagnetic resonance analysis of intermediates in the reaction of $P450_{BM3}$–F98G with oxidants

The reactions of ferric BM3–F87G with either mCPBA or iodosobenzene at pH 6.8 were carried out for 1–5 s, after which the reaction mixtures were rapidly frozen by immersing the EPR tubes into liquid nitrogen. The estimated time for freezing the sample was 4 ± 2 s. Spectra of mixtures with various P450:mCPBA ratios were recorded at 10 K. Increasing the concentration of mCPBA resulted in a more prominent signal at g = 2, with a decrease in the low-spin ferric heme signal with spectral components at g = 2.41, 2.25, and 1.92. The region around g = 2 was analyzed at microwave powers ranging from 5 μW to 20 mW for reactions with both mCPBA and with iodosobenzene. The spectrum obtained from the reaction with mCPBA appeared to show two different radical species, one at g = 2.005 with an isotropic line shape, and the other a multiple-lined signal represented by distinct components at g = 2.006, 2.017, and 2.026 (Fig. 7). The microwave power saturation behavior of the two signals differed noticeably, with the features at g = 2.006, 2.017, and g = 2.026 more easily saturated. The spectrum obtained from the reaction with iodosobenzene showed similar signals, but they were much more easily distinguished by varying the microwave power. The signal at g = 2.005 was very difficult to saturate at high powers, while the multiple lined signal was the dominant feature at low power. At 5 μW power, the latter signal comprised three features (g = 2.026, 2.017, 2.006) separated by 14–14.5 gauss, suggestive of an amino acid radical. Additional preliminary EPR data were acquired using 165 μM BM3–F87G and 1.7 mM mCPBA. With this high mCPBA:P450 ratio, a broad low intensity feature in the EPR spectrum at g = 1.78 emerged along with a sharp g ~ 2 peak, and the ferric P450 signals at g = 2.41, 2.25 and 1.92 were weak (data not shown).

3.5. Analysis of heme products following reaction with oxidants

Studies by Kuo et al. showed that the reaction of mammalian ferric $P450_{2B4}$ from rabbit liver with mCPBA resulted in the formation of a covalently labeled adduct of the heme cofactor [20]. In order to ensure that such an adduct was not forming with the BM3–F87G enzyme, HPLC was performed on the heme cofactors. Following the reaction with each of the oxidants, samples of BM3– F87G were treated with isobutanone to extract the heme. The extract was dried under vacuum and the resulting residue was subjected to HPLC analysis to determine whether the heme had been modified during the reactions. HPLC analysis showed that the retention times and visible absorption spectra for hemes from each of the treated samples were identical to those of the native cofactor, indicating that no adducts analogous to those seen with $P450_{2B4}$ formed.

4. DISCUSSION

Substrate-free ferric cytochrome P450BM3–F87G reacted with mCPBA to form several different spectral intermediates over the course of 1 s. The initial intermediate formed at pH 7.4 had characteristics similar to those of Cpd I from both $P450_{CAM}$ and CYP119 [6–8]. This Cpd I-like species converted partially to a Cpd ES-like species, with a λ_{max} of 406 nm, but a large fraction of the heme was also bleached via secondary reactions with the mCPBA. At pH 6.8, the species with a λ_{max} of 406 nm was the predominant intermediate formed. Only a small amount of the Cpd I-like species could be detected by a very rapid increase at 370 nm (Fig. 2c). The Cpd ES-like species persisted for about 2 s before converting back mainly to ferric P450. Furthermore, at pH 7.4, the rate of formation of the Cpd I-like intermediate was dependent on the concentration of mCPBA used, whereas the rate of conversion of this intermediate to the species absorbing at 406 nm was independent of mCPBA concentration. This observation suggests that the intermediate at 406 nm results directly from the decomposition of the Cpd I-like intermediate. Spolitak et al. recently showed similar behavior with substrate-free ferric cytochrome P450CAM using mCPBA as an oxidant, and also demonstrated that peracetic acid could generate this intermediate that absorbed at ~406 nm [8]. They have tentatively assigned this species as a CcP Cpd ES-like intermediate in which a protein radical is formed by electron transfer to Cpd I. Freeze-Quench EPR and ENDOR experiments using wild-type and mutant forms of $P450_{CAM}$ suggested that the position of the protein radical was either Tyr96 or Tyr75, because mutation of both residues completely eliminated the Tyr EPR radical signal, whereas the single mutation at Tyr96 did not [11]. In contrast to $P450_{CAM}$, $P450_{BM3}$ has a Trp residue directly adjacent to the heme in the active site, as shown in Fig. 8 [21]. In fact, the environment surrounding Trp96 in BM3 is very similar to that of Trp 191 in CcP [22]. A propionate group from the heme cofactor in BM3 is within hydrogen bonding distance from the indole nitrogen of Trp (Fig. 8). The negative charge on the carboxylate can be expected to stabilize a

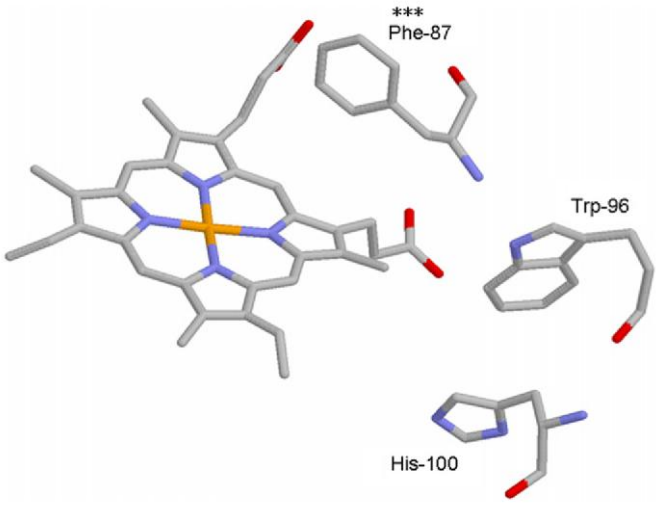


Fig. 8. Active site structure of the heme domain of the ferric $P450_{BM3}$ enzyme. The phenylalanine at position 87 was replaced with glycine in the F87G mutant, and the Trp at position 96 is the proposed site of the radical cation in the 406 nm intermediate species (PDB ID;2BMH [21]).

positive charge on a Trp radical cation in Cpd ES, and this is consistent with the observation that the Cpd ES-like species was clearly more stable in $P450_{BM3}$–F87G than in $P450_{CAM}$. A similar arrangement of a side chain carboxylate is believed to be involved in stabilization of the Trp radical in CcP where Cpd ES is quite stable [13].

The fact that $P450_{BM3}$ is a fusion protein containing both a heme domain and a flavoprotein domain, allowed us to introduce electrons specifically to the active site of the protein via the physiological electron donor to BM3, NADPH. Thus, using a double mixing stopped-flow protocol, we showed that addition of NADPH to the 406

nm absorbing intermediate resulted in rapid conversion of this species into ferric P450 with the FAD and FMN becoming reduced. Excess NADPH was oxidized slowly in this experiment as indicated by the loss of absorbance at 340 nm. These experiments showed that the intermediate species in question was clearly an oxidized form of the BM3 that could be rapidly reduced to give the ferric state of the enzyme in a peroxidase-like reaction. Furthermore, the resulting enzyme, following oxidation to this species and reduction back to the ferric form, did not appear to lose its ability to oxidize NADPH. This implies that although substrate was not present, some of the heme can be reduced in a rate-limiting step, as indicated by the predominant species observed being the ferric heme.

Prior studies with $P450_{CAM}$ showed that two different peracids (mCPBA and peracetic acid) were each effective in forming the Cpd ES-like intermediate; however, it has not previously been demonstrated that other oxidants have this capability. In the current study, we have shown that iodosobenzene also gives rise to this Cpd ES-like species, but without detectable formation of Cpd I. This is the case at both pH 6.8 and pH 7.4, in contrast to mCPBA, which leads to observable Cpd I formation at pH 7.4. The reaction of iodosobenzene with mammalian P450 isoforms was examined previously by stopped-flow techniques; however, the Cpd ES-like intermediate described in the current work was not detected [10]. We find that the inorganic salt, sodium m-periodate, is also capable of generating the Cpd ES-like intermediate. Although previous reports have indicated sodium m-periodate can support P450-catalyzed hydroxylation reactions, no direct evidence for formation of oxidized P450 intermediates has been reported using this oxidant [23]. In addition to the Cpd ES-like intermediate, a new spectral feature was observed with this oxidant that was absent using other oxidants. An increase in absorbance below 400 nm was observed within 0.15 s of mixing. Periodate salts are known to initiate tyrosine dimer formation in proteins, and these dimers are characterized by electronic absorbances between 300 and 350 nm [24]. Two tyrosine residues at the surface of the BM3 enzyme, Tyr278 and Tyr429, are located within 3.6 Å of one another, and represent a potential site for dimer formation. The concentration of periodate (10 mM final concentration) was much higher than any of the other oxidants used, which may explain why this feature was only observed with periodate. Overall, these results with other oxidants suggest that Cpd ES arises from Cpd I. However, the kinetics of formation of Cpd I with the iodosylbenzene and with periodate were rate determining so that no Cpd I accumulated. We can suggest that the hydroxylation reactions brought about by using these latter two oxidants with P450 are thereby likely to occur via the nascent Cpd I.

To further characterize the intermediates formed with BM3, we performed EPR analysis on samples that had been treated with mCPBA and rapidly frozen in liquid nitrogen after about 7 s. The stopped-flow data suggested that the ES-like intermediate in BM3 was considerably more stable than it was in $P450_{CAM}$. This might be expected, given the potential stabilization of the putative Trp radical cation in BM3 via the heme propionate. As a result of the increased stability, the Cpd ES-like intermediate could be trapped without the need for a rapid-quench system. EPR spectra were recorded at different P450:mCPBA ratios. Increasing the relative amount of mCPBA resulted in a more prominent radical signal in the spectrum, with the disappearance of the low-spin ferric heme signal. The radical signal was examined in more detail at microwave powers ranging from 5 μW to 20 mW. The saturation behavior of the radical signal and the observed hyperfine splitting at low power were consistent with the presence of a protein radical; however, different components of the EPR signal responded differently to changes in the microwave power, indicating the likely presence of multiple radical species. This became more apparent when iodosobenzene was used as an oxidant and the EPR spectrum was recorded. The three signals observed in the mCPBA reaction were all present, but their relative abundance was very different. The main species present in the iodosobenzene reaction was characterized by a multi- component EPR signal with g-values of 2.026, 2.017 and 2.006. Although these peaks were also present in the mCPBA reaction, they were minor compared to the main peak at g = 2.005. While we are unable to assign the observed radical to a specific amino acid residue in this intermediate, the behavior of the radical signal is consistent with either a Tyr or a Trp, or possibly a combination of both [25].

A possible explanation for this is that the two radical species originate on different amino acid side chains, and that the two oxidants react differentially with these residues. Assuming that at least one of the two surface tyrosine residues discussed earlier may be oxidized, this is certainly feasible. Alternatively, it is possible that the

two oxidants produce different ratios of the intermediates represented in Scheme 1. For example, the g = 2.005 signal that dominates the mCPBA reaction may represent a small amount of Cpd I (porphyrin n-cation radical), whereas the multi-component signal dominating the iodosobenzene spectrum would represent a protein radical, possibly Trp-96. Rutter et al. reported the 9.5 GHz EPR spectrum for compound I of chloroperoxidase (CPO) under several different conditions, and in each case the signal consisted of g ~ 2 and g = 1.73 components [26,27]. As with CPO, the preliminary EPR spectrum of $P450_{BM3}$–F87G, when mixed with mCPBA for ~1 s, using a 10:1 mCPBA:BM3 ratio, contained a sharp g ~ 2 component and a broad weak signal at g = 1.78 (data not shown). The experiment with BM3 was carried out at 10 K using 20 mW power, in contrast to the CPO experiment, where the microwave power was between 50 μW and 1 mW, and the temperature was between 3.6 and 30 K. Although the similarity of the CPO and BM3–F87G EPR spectra alone does not prove that Cpd I is present in the P450 experiments, the combination of the EPR and the stopped-flow data (discussed above), argue more strongly that a compound I-like intermediate and a compound ES-like intermediate both are produced using mCPBA as an oxidant, whereas with iodosobenzene, the Cpd ES-like intermediate accumulates almost exclusively.

The fact that this ES-like species can undergo rapid reduction by NADPH suggests that formation of Cpd ES may provide a viable protective or uncoupling mechanism for this enzyme. For example, when the enzyme reacts with NADPH and oxygen in the absence of substrate or in the presence of a non-optimal substrate, Cpd I is produced (a very reactive species). Instead of heme destruction, Cpd I converts to Cpd ES, which is less reactive than Cpd I. Subsequently, two additional electrons from NADPH pass through the reductase domain to reduce Cpd ES to the ferric state. The only products formed are water and $NADP^+$, rather than reactive oxygen species. This proposed sequence of events is outlined in Scheme 1. From the EPR and stopped-flow studies, we conclude that a Cpd ES-like complex in cytochrome $P450_{BM3}$ results from the oxidation of an active site aromatic amino acid by Cpd I formed in the reaction of this P450 isoform with alternate oxygen atom donors. The relevance of this intermediate to the catalytic function of this or certain microsomal enzymes remains to be determined.

In summary, the stopped-flow data presented herein provide the first direct observation of a Cpd-I-like intermediate in a class-II or III cytochrome P450 enzyme, BM3– F87G. Preliminary EPR data have also been obtained that are consistent with this interpretation that is based on analogous studies with chloroperoxidase [26,27]. Finally, a compound ES-like intermediate has been observed using not only peracid-type oxygen atom donors, but also iodosobenzene and sodium m-periodate. Cpd ES in BM3–F87G was shown to be easily reduced to the ferric state by NADPH. A protective biological role for P450 Cpd ES in avoiding the production of reactive oxygen species from P450 Cpd I is proposed. Similar results have been seen in parallel studies of cytochrome $P450_{CAM}$ [28].

5. ABBREVIATIONS

mCPBA	m-Chloroperoxybenzoic acid
Cpd I	compound I
Cpd ES	compound ES
CcP	cytochrome c peroxidase
BM3–F87G	cytochrome $P450_{BM3}$–F87G, BM3; cytochrome $P450_{BM3}$
EPR	electron paramagnetic resonance
ENDOR	electron nuclear double resonance

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