Absorption spectra of cytochrome $P450_{CAM}$ in the reaction with peroxy acids

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The reaction of Fe(III) cytochrome $P450_{CAM}$ with *m*-chloroperbenzoic acid was studied by rapid scanning absorption spectroscopy. Native low-spin enzyme produced spectra characteristic of two reaction phases that were marked by time intervals with isosbestic positions. The high-spin enzyme substrate complex yielded a series of Soret-region spectra whose properties were dependent on peracid concentration. The simplest model describing the results was a sequence of at least two spectral intermediates, that were not entirely homologous with data measured in reactions with microsomal $P450_{LM2}$. Comparisons with related heme protein states indicate higher Fe(IV) oxidation levels provide a plausible interpretation of the $P450_{CAM}$ spectra.

Cytochrome P450 *Camphor monoxygenase* *Peroxygenase reaction* *Rapid scan absorption spectra*

1. INTRODUCTION

The P450 cytochromes are a substrate-diverse class of *b*-type heme monoxygenases [1,2], whose structural and functional properties are well represented by the bacterial cytochrome $P450_{CAM}$ model system [3,4]. Cytochrome $P450_{CAM}$ catalyzes a two-electron activation of O_2 resulting in the 5-*exo*-hydroxylation of camphor. In the overall rate-limiting step of this process, the transfer of a second reducing equivalent to a ternary Fe(II)–enzyme–substrate–O_2 complex leads to product formation and regeneration of native Fe(III) enzyme. The absence of physical evidence attributable to an activated heme–oxygen complex [5] has left unresolved the basic mechanism of O_2 cleavage and hydrocarbon oxygenation in the P450 monoxygenase cycle. Oxidized Fe(III) $P450_{CAM}$ will also catalyze an ROOH-dependent hydroxylation of camphor without the requirement of O_2 or the NADH redox protein components [6]. This P450 peroxygenase type shunt offers an alternative in vitro pathway that may provide insight into relevant states of the monoxygenase cycle [7]. Here, we have investigated by rapid scanning techniques the transient absorption spectra of $P450_{CAM}$ in reaction with peroxy acids. The results provide the first complete (360–720 nm) spectral comparisons of $P450_{CAM}$ with related heme proteins, including microsomal $P450_{LM2}$ [8,9], which represents a second P450 subclass characterized by a different mode of monoxygenase redox transfer [10].

2. EXPERIMENTAL

Cytochrome $P450_{CAM}$ was isolated [11] from *P. putida* strain PpG 786 (ATCC 29607) and crystallized [12,13] from $(NH_4)_2SO_4$ solutions as the enzyme substrate complex. The camphor-free form was prepared by gel filtration [13] of chromatographically purified enzyme [11]. Concentrations were determined spectrophotometrically at 417 nm for native enzyme, E = 115 $mM^{-1}.cm^{-1}$, and at 391 nm for the enzyme–substrate complex, E = 102 $mM^{-1}.cm^{-1}$ [11].

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m-Chloroperbenzoic acid (mCPB) was synthesized from the corresponding acid [14] and purified by solvent extraction [8] to >97% active oxygen. Peracetic acid (PA) was obtained as a 40% aqueous solution (FMC) and pretreated with catalase to remove H_2O_2 [15]. Both peracids were

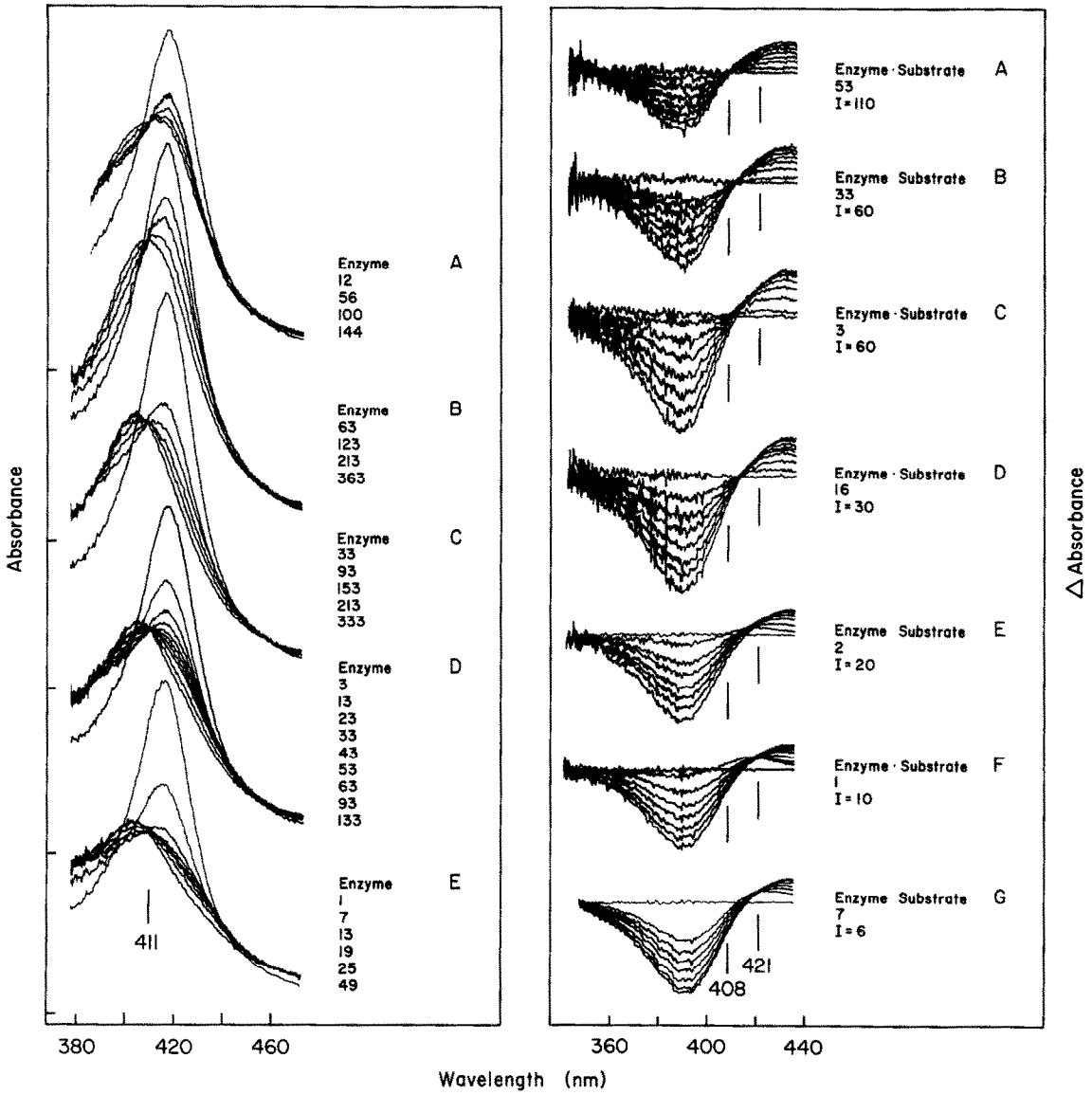

Fig.1. Soret absorption spectra of Fe(III) $P450_{CAM}$ reacted with mCPB. (Left) Native low-spin enzyme. Initial conditions after mixing: 50 mM KP_i, pH 7.0 (μ = 0.11); P450/mCPB (mM) was – (A) 0.0040/0.025; (B) 0.0047/0.013; (C) 0.0047/0.024; (D) 0.004/0.10; (E) 0.0047/0.25. Enzyme spectrum recorded after mixing with H_2O. Decreasing 420 nm absorbance corresponds to increasing reaction time. Reaction times in ms are given in the figure; temp., 20.0 ± 0.2°C. (Right) High-spin enzyme–substrate complex. Initial conditions after mixing: 50 mM KP_i, pH 7.0 (μ = 0.11) plus 0.2 mM D–(+) camphor; 0.0093 mM P450; mCPB was (mM) – (A) 1.94; (B) 1.45; (C) 0.96; (D) 0.48; (E) 0.36; (F) 0.24; (G) 0.12. Enzyme–substrate difference spectrum produced by computer subtraction of two absolute spectra measured after mixing with H_2O. Decreasing 390 nm absorbance corresponds to increasing reaction time. Initial reaction time and subsequent time intervals I in ms are given in the figure; temp., 20.0 ± 0.2°C.

dissolved in H_2O and standardized spectrophotometrically at 353 nm in the HRP catalyzed triiodide assay, using $E = 25.5\ mM^{-1}.cm^{-1}$ [16]. All solutions in this study were prepared aerobically with multidistilled H_2O [17].

Normal absorption spectra were determined with a Cary 219 spectrophotometer. Rapid reaction spectra were measured on a Union Giken RA-601 stopped-flow spectrophotometer [18]. Transient changes in light intensity after mixing were detected by a multi-channel photodiode array at 95 nm/ms and stored in a Sord M223 computer. Ratio–log intensities and spectral subtractions were computed and displayed on an X–Y plotter. Absorption spectra (360–720 nm) were constructed from 15 nm overlaps of separate 95 nm intervals. Maxima and isosbestic wavelengths were reproducible to within a ± 1 nm limit and were calibrated from absorption maxima of the unreacted states [11].

3. RESULTS

Consecutive Soret absorption spectra of both the native enzyme and the enzyme–substrate complex reacted with mCPB are shown in fig.1. The spectra describe reaction sequences that ultimately produce a bleaching of absorption (not shown),

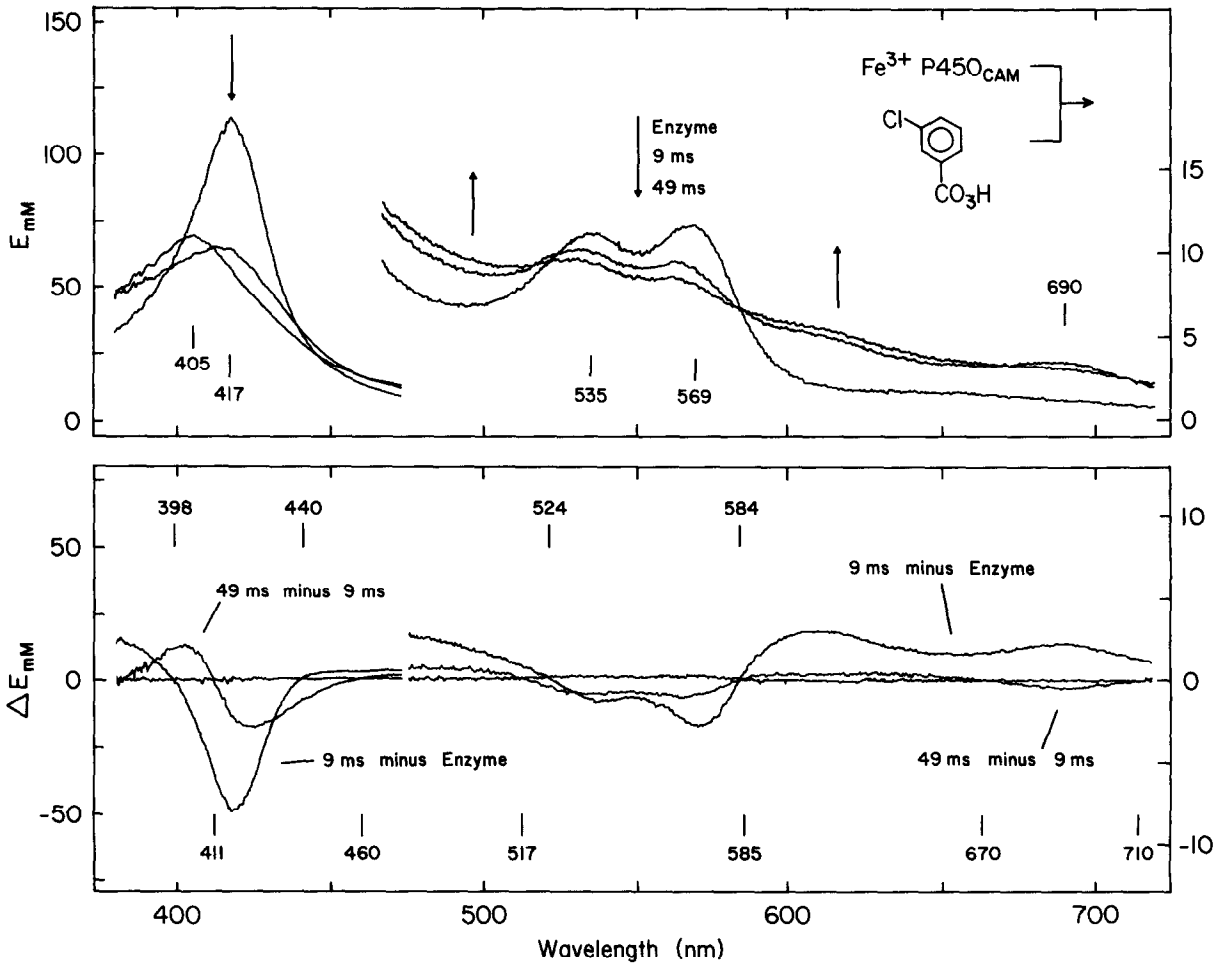

Fig.2. Absorption spectra of native low-spin $P450_{CAM}$ enzyme reacted with mCPB. Initial conditions after mixing: 0.012 mM P450 (Soret region) or 0.018 mM P450 (visible region), 0.26 mM mCPB. All other conditions are given in fig.1 (left).

which is consistent with heme destruction. Prior to this decay process, the data in fig.1 illustrate spectral transformations of the Fe(III) proteins that are marked by isosbestic positions over selected time intervals.

Soret spectra of the reaction between mCPB and native low-spin enzyme (fig.1, left) are biphasic. The first phase is characterized by a progressive decrease in intensity at the 417 nm Soret maximum. The second is characterized by a spectral transformation marked by a 411 nm isosbestic wavelength which results in a blue-shifted Soret maximum at 405 nm. Absorption spectra over the 380–720 nm region in fig.2 are given to represent the essential features of this reaction. Since the reaction phases are not well-resolved, the spectra at 9 and 49 ms are only intended to indicate qualitatively the properties of the two intermediate states. The visible spectrum corresponding to the initial phase (9 ms) is characterized by an increased absorption above 600 nm with a weak maximum at 690 nm. In contrast, the spectrum of the later phase (49 ms) is marked by a decrease of the 690 nm band.

Reaction of mCPB with the high-spin enzyme–camphor complex produced spectra (fig.1, right) characterized by a red-shift from the 391 nm Soret maximum of the unreacted state. The spectral changes are dependent on [mCPB], as seen at the limits in fig.1. At the lowest [mCPB], a spectral transformation of the enzyme–substrate complex is described by an isosbestic wavelength at 408 nm. At the highest peracid concentration, the spectral transformation is now characterized by a 421 nm isosbestic point that is not coincident with the enzyme–substrate spectrum (i.e., the baseline in the difference mode).

Preliminary studies in the Soret region of native enzyme reacted with PA showed spectral changes in the early phase that are similar to the initial transformation with mCPB (fig.2, at 9 ms). However, the later and dominant phase of reaction is characterized by a bleaching process that masks any detailed analysis of the spectra.

4. DISCUSSION

The simplest scheme describing the reactions of Fe(III) $P450_{CAM}$ with mCPB requires at least two spectral intermediates of differing stability and time constant. Spectra of low-spin native enzyme in fig.2 provide clear evidence of at least two different types of intermediates. Spectra of the high-spin enzyme–substrate complex are also consistent with at least two transient species, as indicated in fig.1 by the loss of a transformation that maintains an isosbestic wavelength with the initial state.

The results of the native low-spin enzyme are comparable to the microsomal $P450_{LM2}$ system [8] in the number of transient species detected and in the type of changes in the Soret region during the initial phase of reaction. However, whereas the $P450_{CAM}$ transformation from the first to second species (fig.2) is characterized by a Soret blue-shift, the opposite is true for the analogous conversion of complex C to D in $P450_{LM2}$ [8]. These results may reflect different pathways in turnover and/or heme destruction between crystallized $P450_{CAM}$ and lipid-solubilized $P450_{LM2}$.

The well-documented reactions of ROOH with related *b*-type heme proteins (e.g., HRP [19,20], CCP [21,22] and CPO [23] and catalase [18]) are characterized by intermediates corresponding to two oxidizing equivalents above the native Fe(III) proteins. In general these states are formally described by an Fe(IV) heme–oxygen compound, with a second oxidizing equivalent localized as a free radical on either the porphyrin or a protein residue. Direct spectral comparisons between these intermediates and $P450_{CAM}$ are viewed with caution since there are distinct spectroscopic differences in the initial Fe(III) states. However, spectral trends are apparent in this study and are similar to these related systems. Intermediates that are represented by the porphyrin radical formalism [18–20,23] are characterized by a Soret intensity nearly half that of the initial Fe(III) state and a broad visible spectrum with a weak absorption in the 650–700 nm region. Spectral parameters of this type are similar to the 9 ms reaction spectrum of native enzyme and suggest a related electronic structure in $P450_{CAM}$. When the radical species is either localized on a protein residue [21,22] or absent [19], the Fe(IV) heme–oxygen compound is described by a red-shift of the Soret band from the initial Fe(III) state and resolved α/β structure in the 530–560 nm region. The visible region of the 49 ms reaction spectrum with native enzyme indicates a weak correspondence to this type of intermediate, although the blue-shifted Soret max-

imum is anomalous in this comparison. A strong red-shift of the Soret band from the initial Fe(III) state is, however, apparent in the reaction with the enzyme–substrate complex. Allowing for the noted differences, an assignment of higher Fe(IV) oxidation states to intermediates of the $P450_{CAM}$ peroxygenase-shunt remains as a plausible interpretation of the spectra here.

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