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Cytochrome P450 Compound I

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The cytochromes P450 (P450s) are ubiquitous heme-containing enzymes that effect a vast range of oxidation reactions in nature.[1] By analogy to intermediates formed in other heme-containing enzymes,[2] the putative P450 oxidants are usually thought to be iron(IV)−oxo porphyrin radical cation species termed Compounds I.[1] The active oxidants in P450s have been sought since the initial reports of the enzymes in the 1960s,[3] and P450 Compound I has been the Holy Grail of those studies. Transients with millisecond lifetimes can be produced in low conversions by chemical oxidations of P450s, and these short-lived intermediates have been assigned as Compound I species.[4] Ironically, typical Compound I analogues in models[5] do not display reactivities consistent with those required for the oxidants in P450s, which can hydroxylate an unactivated C−H bond so rapidly that no oxidant accumulates to detectable levels.

As an alternative to two-electron, oxo-transfer chemical oxidations of iron(III) porphyrins, photochemical oxidations of iron(IV)−oxo neutral porphyrins (so-called Compound II species) can give Compound I species in enzymes and in models.[6] Formation of P450 Compound I species via photooxidation reactions of P450 Compound II transients can be expected if the latter species can be produced cleanly. Herein we report formation of a P450 Compound II species by peroxynitrite oxidation of the resting enzyme and subsequent conversion of this species to the P450 Compound I transient by photooxidation in laser flash photolysis (LFP) experiments.

Peroxynitrite[7] oxidizes some heme-containing enzymes, including $P450_{BM3}$ and $P450_{nor}$, to Compound II species.[8] We found that CYP119,[4b,9,10] a P450 enzyme from the thermophile *Sulfolobus solfactaricus* that we expressed in *Escherichia coli* cells, was oxidized to a Compound II species by peroxynitrite in a pH 7.4 solution. In the resting state, CYP119 displays a typical UV−visible spectrum with a Soret band absorbance at 417 nm. Reaction of CYP119 with peroxynitrite shifted the Soret band absorbance from 417 to 429 nm (Figure 1). The spectrum for CYP119 treated with peroxynitrite is quite similar to that for the known Compound II derivative of the heme-thiolate enzyme chloroperoxidase (CPO) (Figure 1), which was reported to give the Compound II derivative by reaction with peroxynitrite.[8c] The similarities in the spectra in Figure 1 and in the photochemical reactivities discussed below support the assignment of a Compound II transient from peroxynitrite oxidation of CYP119. The oxidations of CPO and CYP119 might involve two-electron, oxo-transfer reactions to give formal iron(V)−oxo species followed by rapid reductions of the iron−oxo species to the Compound II derivatives,[8a,b] or they might involve formation of transient peroxynitrito complexes that cleave homolytically to give Compound II derivatives directly in a net one-electron oxo-transfer reaction.[8f]

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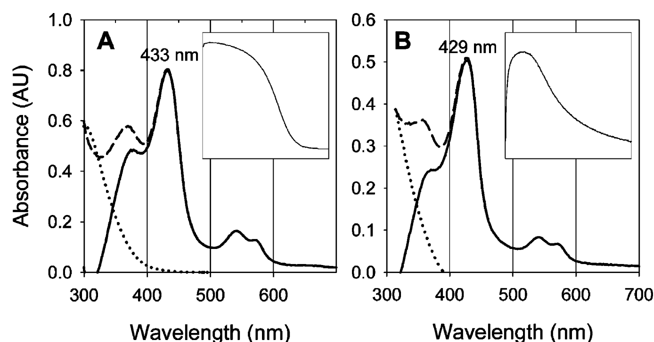

Figure 1. Spectra of Compound II species from CPO (A) and CYP119 (B). The dashed lines are the observed spectra, the dotted lines are background absorbances from peroxynitrite, which is decaying with time, and the solid lines are the spectra with the absorbance of peroxynitrite subtracted. The insets show kinetic traces at λ_{max} for the Compound II species for 0.4 s (A) and 40 s (B) after mixing the enzyme and peroxynitrite solutions.

The Compound II species from CYP119 was more stable than that from CPO (insets in Figure 1), and its rate of decay to resting enzyme was dependent on the intensity of the spectrometer's analyzing beam. With low-intensity monochromatic light, the Compound II species was stable for several seconds (Figure 1B).

Irradiation of the CYP119 Compound II species with ca. 5 mJ of 355 nm laser light (third harmonic of a Nd:YAG laser) in LFP studies gave a new intermediate in about 5% conversion with 95% of the Compound II derivative unaffected. A difference spectrum is shown in Figure 2. The enzyme concentrations and detection methods varied for different regions (Supporting Information), but bleaching of the Compound II signals (negative peaks) and formation of two new signals (positive peaks) at 400−410 nm and 600−700 nm are apparent. These new signals are assigned to the CYP119 Compound I species based on the photoreactivity of the Compound II species and the spectral features discussed below.

The photochemical behavior of CYP119 Compound II is similar to that of CPO Compound II, which gave the known CPO Compound I species[11] upon 355 nm irradiation (see Supporting Information). Photooxidations of three other Compound II species with 355 nm light to give Compound I species were previously reported,[6] and the method appears to be general.

The spectral features of the new transient also are consistent with those expected for the Compound I derivative. The Soret band has λ_{max} in the range of 400−410 nm, and this absorbance apparently is broader, less intense, and blue-shifted from that of the Soret band of Compound II. The Q-band of the new transient is weak and has λ_{max} in the range of 640−670 nm, which is strongly red-shifted from the Q-band of Compound II. The spectral features in both

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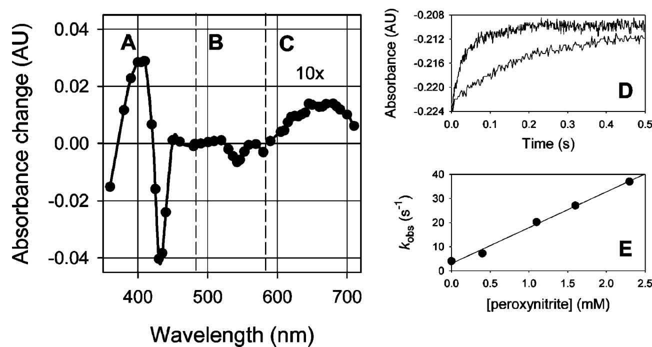

Figure 2. (A−C) Difference spectra from 355 nm LFP of CYP119 Compound II. In the experiments, the P450 enzyme in a pH 7.0 solution was mixed with a basic peroxynitrite solution to give a pH 7.4 mixture, and the laser was fired after a delay of 4 s, delivering approximately 5 mJ of 355 nm light to the reaction cell in 7 ns. The spectra show the change in absorbance in a 1 cm path cell. Signals with negative absorbance change are from net bleaching, and those with positive absorbance change are from net increases in absorbing species. The methods for the spectral regions are as described in detail in Supporting Information. (A) 1×10^{-5} M CYP119 with PM tube detection, (B) 2×10^{-5} M CYP119 with photodiode array detection, (C) 2×10^{-5} M CYP119 with PM tube detection shown at 10× observed AU change. (D) Kinetic traces at 430 nm for reaction of CYP119 Compound I at 20 °C in the presence of 2.3 mM (top) and 0.0 mM (bottom) peroxynitrite. (E) Observed pseudo-first-order rate constants for reactions of CYP119 Compound I with peroxynitrite at 20 °C; the slope of the line gives a second-order rate constant of $(1.5 \pm 0.2) \times 10^4$ M^{-1} s^{-1}.

the Soret and Q-band regions for CYP119 Compound I are similar to those of known Compound I species in enzymes and models.[12]

Generation of CYP119 Compound I in LFP experiments permits kinetic studies of very fast reactions, but surprisingly, the Compound I derivative appeared to be relatively stable. In the absence of peroxynitrite, it decayed at 20 °C with a lifetime of ca. 200 ms. In the presence of peroxynitrite, the Compound I derivative reacted at 20 °C with a second-order rate constant of $(1.5 \pm 0.2) \times 10^4$ M^{-1} s^{-1} (2σ) (Figure 2E), a smaller rate constant than that for reaction of myeloperoxidase Compound I with peroxynitrite.[8f] In the presence of laurate, a known substrate for CYP119 under turnover conditions,[10] the decay of the Compound I derivative was essentially unchanged even when the laurate concentration was greater than necessary for saturation of the resting enzyme. Styrene, a poor CYP119 substrate,[10] similarly had no significant effect on the decay of Compound I.

The apparent lack of reactivity of CYP119 Compound I is striking. In low-temperature studies of the $P450_{cam}$ hydroxylation of camphor, no transient oxidant was detected,[13] indicating that the oxidation of the unactivated C−H bond in camphor has a rate constant exceeding 1000 s^{-1} at ambient temperature, but we observed no significant reaction of the CYP119 Compound I derivative with the known substrate laurate. Much more kinetic characterization of Compounds I will be required to understand the P450 oxidants. It is possible that laurate has limited access to the active site of the CYP119 Compound I derivative, but it also is possible that Compound I species are not the predominant oxidants in P450 enzymes.[14] The first-formed oxidant in P450 enzymes could be an iron(V)−oxo species,[15] a high-energy isomer of Compound I,[16] that effects oxidations before it relaxes to the more stable Compound I species by internal electron transfer.

The photochemical production of CYP119 Compound I from the Compound II derivative is similar to photooxidation reactions of CPO Compound II and other iron(IV)−oxo neutral porphyrin species.[6] One should anticipate that the technique will be general for most P450 Compound II derivatives, and that formation of P450 Compound II derivatives by peroxynitrite oxidations of the resting enzymes, while not universally successful,[17] should work well with many of the thousands of known P450s. One expects that a wide range of studies of P450 Compound I derivatives will be possible using the methods we have developed.

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Supporting Information Available: Experimental details, kinetic traces for formation of Compounds II, and spectra from LFP irradiation of CPO Compound II. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Ortiz de Montellano, P. R., Ed. *Cytochrome P450 Structure, Mechanism, and Biochemistry*, 3rd ed.; Kluwer: New York, 2005. (b) Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H. *Chem. Rev.* **1996**, *96*, 2841. (c) Denisov, I. G.; Makris, T. M.; Sligar, S. G.; Schlichting, I. *Chem. Rev.* **2005**, *105*, 2253.
(2) Dawson, J. H. *Science* **1988**, *240*, 433. (b) Hiner, A. N. P.; Raven, E. L.; Thorneley, R. N. F.; García-Cánovas, F.; Rodríguez-López, J. N. *J. Inorg. Biochem.* **2002**, *91*, 27.
(3) (a) Omura, T.; Sato, R.; Cooper, D. Y.; Rosenthal, O.; Estabrook, R. W. *Fed. Proc.* **1965**, *24*, 1181. (b) Lu, A. Y. H.; Coon, M. J. *J. Biol. Chem.* **1968**, *243*, 1331. (c) Katagiri, M.; Ganjuli, B. N.; Gunsalus, I. C. *J. Biol. Chem.* **1968**, *243*, 3543.
(4) (a) Egawa, T.; Shimada, H.; Ishimura, Y. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 1464. (b) Kellner, D. G.; Hung, S.-C.; Weiss, K. E.; Sligar, S. G. *J. Biol. Chem.* **2002**, *277*, 9641. (c) Spolitak, T.; Dawson, J. H.; Ballou, D. P. *J. Biol. Chem.* **2005**, *280*, 20300.
(5) Pan, Z.; Zhang, R.; Newcomb, M. *J. Inorg. Biochem.* **2006**. In press.
(6) Zhang, R.; Chandrasena, R. E. P.; Martinez, E., II; Horner, J. H.; Newcomb, M. *Org. Lett.* **2005**, *7*, 1193.
(7) Uppu, R. M.; Pryor, W. A. *Anal. Biochem.* **1996**, *236*, 242.
(8) (a) Floris, R.; Piersma, S. R.; Yang, G.; Jones, P.; Wever, R. *Eur. J. Biochem.* **1993**, *215*, 767. (b) Mehl, M.; Daiber, A.; Herold, S.; Shoun, H.; Ullrich, V. *Nitric Oxide Biol. Chem.* **1999**, *3*, 142. (c) Daiber, A.; Herold, S.; Schöneich, C.; Namgaladze, D.; Peterson, J. A.; Ullrich, V. *Eur. J. Biochem.* **2000**, *267*, 6729. (d) Exner, S.; Herold, S. *Chem. Res. Toxicol.* **2000**, *13*, 287. (e) Boccini, F.; Herold, S. *Biochemistry* **2004**, *28*, 16393. (f) Furtmüller, P. G.; Jantachko, W.; Zederbauer, M.; Schwanninger, M.; Jakopitsch, C.; Herold, S.; Koppenol, W. H.; Obinger, C. *Biochem. Biophys. Res. Commun.* **2005**, *337*, 944.
(9) (a) Wright, R. L.; Harris, K.; Solow, B.; White, R. H.; Kennelly, P. J. *FEBS Lett.* **1996**, *384*, 235. (b) McLean, M. A.; Maves, S. A.; Weiss, K. E.; Krepich, S.; Sligar, S. G. *Biochem. Biophys. Res. Commun.* **1998**, *252*, 166. (c) Tschirret-Guth, R. A.; Koo, L. S.; Hoa, G. H. B.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **2001**, *123*, 3412.
(10) Puchkaev, A. V.; Ortiz de Montellano, P. R. *Arch. Biochem. Biophys.* **2005**, *434*, 169.
(11) Palcic, M. M.; Rutter, R.; Araiso, T.; Hager, L. P.; Dunford, H. B. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1123.
(12) Watanabe, Y.; Fujii, H. In *Metal-Oxo and Metal-Peroxo Species in Catalytic Oxidations*; Meunier, B., Ed.; Springer-Verlag: Berlin, 2000; pp 61−89.
(13) (a) Davydov, R.; Makris, T. M.; Kofman, V.; Werst, D. E.; Sligar, S. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **2001**, *123*, 1403. (b) Denisov, I. G.; Makris, T. M.; Sligar, S. G. *J. Biol. Chem.* **2001**, *276*, 11648.
(14) (a) Watanabe, Y. *J. Biol. Inorg. Chem.* **2001**, *6*, 846. (b) Newcomb, M.; Chandrasena, R. E. P. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 394.
(15) (a) Harischandra, D. N.; Zhang, R.; Newcomb, M. *J. Am. Chem. Soc.* **2005**, *127*, 13776. (b) Pan, Z.; Zhang, R.; Newcomb, M. Submitted for publication.
(16) (a) Ogliaro, F.; de Visser, S. P.; Groves, J. T.; Shaik, S. *Angew. Chem., Int. Ed.* **2001**, *40*, 2874. (b) Dey, A.; Ghosh, A. *J. Am. Chem. Soc.* **2002**, *124*, 3206.
(17) Daiber, A.; Schöneich, C.; Schmidt, P.; Jung, C.; Ullrich, V. *J. Inorg. Biochem.* **2000**, *81*, 213.

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