

# A Self-Sufficient Peroxide-Driven Hydroxylation Biocatalyst\*\*

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Controlled, selective oxidations of unactivated C–H bonds are among the most desired transformations in catalysis.<sup>[1]</sup> Limitations to chemical oxidation catalysts include low turnover numbers, low or no regioselectivity, poor reaction specificity, their use of environmentally harmful components (for example, heavy metals or halogens), or their requirements for harsh, expensive reaction conditions. Many oxygenase enzymes catalyze the insertion of oxygen into unactivated C–H bonds<sup>[2,3]</sup> and are superior to synthetic catalysts with regard to selectivity and turnover rate.<sup>[4]</sup> Enzymes have found numerous industrial applications,<sup>[5]</sup> and the use of enzymes in industrial biocatalysis is expected to grow significantly.<sup>[6]</sup> One limitation to the application of oxygenases is their requirement for an expensive cofactor such as NAD(P)H, which precludes their use in vitro without addition of a coupled, cofactor-regenerating system.<sup>[7]</sup> The need for additional proteins to transfer electrons from the cofactor to the oxygenase presents a further complication. Here we describe a self-sufficient P450 BM-3 variant which utilizes hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to catalyze hydroxylation and epoxidation at high rates.

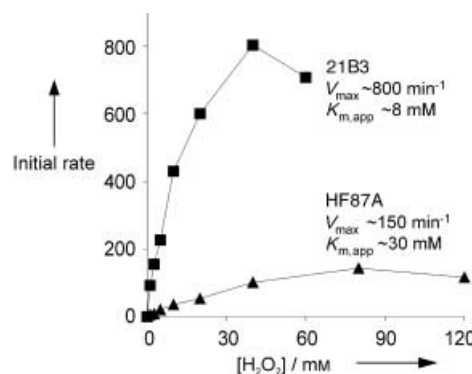
The cytochromes P450 are heme-containing oxygenases which collectively catalyze a variety of oxidations on a diverse array of substrates.<sup>[3,8]</sup> The natural P450 catalytic cycle utilizes two electrons from NAD(P)H to activate dioxygen, although P450s are also capable of utilizing peroxides as a source of oxygen through a peroxide “shunt” pathway. In principle, this “peroxygenase” activity offers an opportunity to employ cell-free P450 catalysis without requiring NAD(P)H regeneration, additional proteins, or dioxygen, and eliminates rate-limiting electron-transfer steps.<sup>[9]</sup> In practice, this non-natural pathway is too inefficient for any practical application.

The soluble P450 BM-3 enzyme catalyzes the hydroxylation of sub-terminal C–H bonds of medium chain (C<sub>12</sub>–C<sub>18</sub>) fatty acids and amides<sup>[10]</sup> with initial rates exceeding 3000 min<sup>−1</sup>.<sup>[11]</sup> The activities of P450 BM-3 and its corresponding F87A mutant in reactions driven by H<sub>2</sub>O<sub>2</sub> were recently characterized.<sup>[12,13]</sup> Whereas the wild-type enzyme is

inactivated after only a few turnovers, the F87A mutant supports low levels of peroxygenase activity (ca. 70 total turnovers). In a previous study we demonstrated that directed evolution could increase the rate of peroxide-driven naphthalene hydroxylation by cytochrome P450cam.<sup>[9]</sup> Since then, our efforts have focused on improving the peroxygenase activity of the more active P450 BM-3 as a step towards engineering a “biomimetic” hydroxylation catalyst.

The P450 BM-3 heme domain (BMP)<sup>[14]</sup> supports peroxygenase activity<sup>[12]</sup> and is more thermostable than the full-length P450 BM-3 protein (unpublished data). Removal of the reductase domain also results in an approximate fourfold increased molar expression (ca. 70 mg L<sup>−1</sup> in shake-flask cultures). We used BMP mutant F87A (HF87A) as the starting point (parent) to increase catalyst performance by sequential rounds of random mutagenesis and screening for H<sub>2</sub>O<sub>2</sub>-driven hydroxylation of 12-*p*-nitrophenoxycarboxylic acid (12-*p*NCA).<sup>[15]</sup> Experimental details and a description of the evolutionary path are available in the Supporting Information.

Fifth-generation mutant “21B3” is nearly 20-fold more active than HF87A in 10 mM H<sub>2</sub>O<sub>2</sub> using 12-*p*NCA as the substrate.<sup>[16]</sup> Figure 1 shows the activities of HF87A and 21B3



**Figure 1.** Initial rates ((mol pNP formed) (mol P450)<sup>−1</sup> min<sup>−1</sup>) of peroxide-driven 12-*p*NCA hydroxylation by P450 BMP variants HF87A and 21B3 in different H<sub>2</sub>O<sub>2</sub> concentrations. Reactions were performed at room temperature and contained 300 μM 12-*p*NCA, 6% DMSO, and purified enzyme (0.5 μM HF87A or 0.2 μM 21B3) in 100 mM Tris-HCl, pH 8.2. Tris = tris(hydroxymethyl)aminoethane.

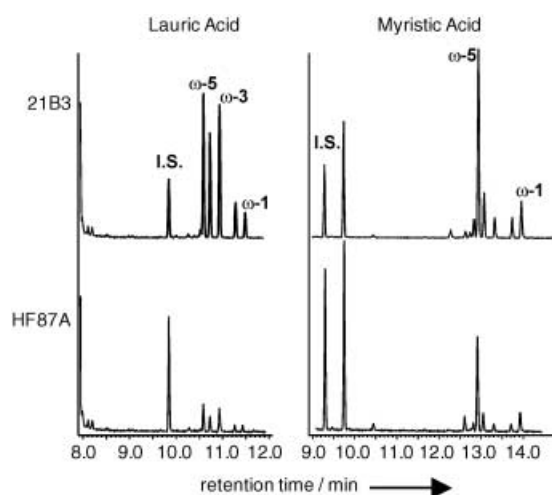
at different peroxide concentrations. Activities are reported as initial rates of *p*-nitrophenolate (pNP) formation at room temperature.<sup>[17]</sup> The peroxygenase reaction follows apparent Michaelis–Menten kinetics. Whereas the apparent *K<sub>m</sub>* value for H<sub>2</sub>O<sub>2</sub> in HF87A is about 30 mM, in 21B3 the apparent *K<sub>m</sub>* value is reduced to about 8 mM. Total turnover numbers (TON) of approximately 1000 are achieved.

Improved peroxygenase activity also extends to fatty acid substrates, with product distributions that are the same as those from HF87A, as demonstrated in Figure 2 for reactions of HF87A and 21B3 with lauric (dodecanoic) acid and myristic (tetradecanoic) acid. No products other than those shown were identified from these reactions. Using 10 mM H<sub>2</sub>O<sub>2</sub>, the initial rate of lauric acid hydroxylation, estimated from the amount of product formed in the first minute of

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[\*\*] This work was supported by the Biotechnology Research and Development Corporation (Peoria, IL) and an NSF Graduate Research Fellowship awarded to P.C.C. The authors thank Ulrich Schwaneberg for guidance and Denis Shcherbakov for laboratory assistance.

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.



**Figure 2.** Chromatograms of the trimethylsilyl-derivatized hydroxylated products from  $\text{H}_2\text{O}_2$ -driven reactions of P450 BMP variants HF87A and 21B3 with lauric acid and myristic acid. The reactions contained purified enzyme (1–4  $\mu\text{M}$ ) and 1–2 mM substrate in 500  $\mu\text{L}$  100 mM Tris-HCl, pH 8.2. The reactions were initiated by the addition of 5 mM or 10 mM  $\text{H}_2\text{O}_2$ , carried out at room temperature, and stopped by the addition of 7.5  $\mu\text{L}$  6 M HCl, as described.<sup>[12]</sup> I.S. is the internal standard (10-hydroxydecanoic acid), which was added to all samples in an equal amount (30 nmol) at the end of each reaction so that products could be quantified relative to the area of the I.S. peak.

reaction (see ref. [12] or Supporting Information), is about 50 mol (mol P450) $^{-1}$  min $^{-1}$  for 21B3. The TON for lauric acid in 5 mM  $\text{H}_2\text{O}_2$  is about 280. Similar results are observed with decanoic (capric) acid.

Oxidation of styrene to styrene oxide is also significantly improved with 21B3. (Relaxed substrate specificity was expected since specificity was not among the selection criteria during screening.) The initial rate of styrene oxide production by 21B3 is 55 mol (mol P450) $^{-1}$  min $^{-1}$  in 10 mM  $\text{H}_2\text{O}_2$  (estimated from the amount of product formed in the first minute of reaction), with a TON of about 240 in 5 mM  $\text{H}_2\text{O}_2$ . In contrast, the initial rate for HF87A in 10 mM  $\text{H}_2\text{O}_2$  is approximately 2.4 mol (mol P450) $^{-1}$  min $^{-1}$ , with a TON of only about 10. For comparison, the NADPH-driven styrene oxidation activity of wild-type BM-3 (for which styrene is a poor substrate) is about 30 mol (mol P450) $^{-1}$  min $^{-1}$ . Table 1

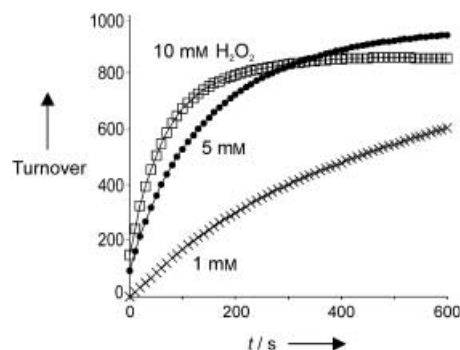
**Table 1:** Summary of peroxxygenase activities of P450 BMP variants HF87A and 21B3.

Substrate	Measurement	HF87A	21B3
12-pNCA <sup>[a]</sup>	initial rate <sup>[b]</sup>	23	430
12-pNCA <sup>[a]</sup>	TON <sup>[c]</sup>	90	980
lauric acid	initial rate	10	50
lauric acid	TON	70	280
styrene <sup>[d]</sup>	initial rate	2.4	54
styrene <sup>[d]</sup>	TON	10	240

[a] Activity on 12-pNCA was measured in the presence of 6% DMSO (see ref. [16]). [b] Initial rates were determined in 10 mM  $\text{H}_2\text{O}_2$  and are reported as (mol product) (mol P450) $^{-1}$  min $^{-1}$ . [c] Total enzyme turnovers. TON values were determined in 5 mM  $\text{H}_2\text{O}_2$ . [d] Styrene oxide is the only product.

summarizes the activities of 21B3 compared to HF87A for the various substrates tested.

The major limitation of this system is rapid enzyme inactivation as a result of peroxide-mediated heme degradation, as indicated by a decrease in the heme absorbance peak in the presence of  $\text{H}_2\text{O}_2$  (not shown). Figure 3 shows the time-



**Figure 3.** Time-courses of pNP formation for reactions catalyzed by P450 BMP variant 21B3 with 12-pNCA in 1, 5, and 10 mM  $\text{H}_2\text{O}_2$ . Increasing peroxide concentration increases the peroxxygenase activity as well as the rate of enzyme inactivation. The reactions were performed at room temperature and contained 250  $\mu\text{M}$  12-pNCA, 6.3% DMSO, and either 0.94  $\mu\text{M}$  F87A or 0.15  $\mu\text{M}$  21B3 in 100 mM Tris-HCl, pH 8.2. Turnover is expressed in (mol pNP formed) (mol P450) $^{-1}$ .

courses of pNP formation from reactions of 21B3 on 12-pNCA in 1 mM, 5 mM, and 10 mM  $\text{H}_2\text{O}_2$ . The enzyme is essentially inactive within 5 minutes in 10 mM  $\text{H}_2\text{O}_2$ . Inactivation appears to be turnover-dependent (similar TONs are reached from reactions in 1 mM, 5 mM, and 10 mM  $\text{H}_2\text{O}_2$ ), and the ratio of peroxxygenase turnovers to heme-degrading turnovers increased with each generation of evolution (half-lives in a given concentration of peroxide did not increase).

Sequence analysis of 21B3 reveals thirteen nucleotide substitutions relative to the HF87A sequence, nine of which result in amino acid substitutions. The BMP crystal structure<sup>[18]</sup> shows that the mutations are dispersed throughout the protein scaffold. No mutations appear in the active site, substrate binding channel, or F and G helices of the heme domain. There are also no mutations in any  $\beta$ -sheets of the  $\beta$ -sheet-rich region of the structure. Four mutations lie on the protein surface. A list of the amino acid substitutions and information on the positions of these mutations are available in the Supporting Information.

The fungal heme enzyme chloroperoxidase (CPO) has received much attention for its ability to catalyze the  $\text{H}_2\text{O}_2$ -driven, enantioselective epoxidation of alkenes.<sup>[19]</sup> CPO does not efficiently hydroxylate unactivated C–H bonds,<sup>[20]</sup> which is the hallmark of P450 chemistry. Furthermore, protein engineering on CPO has been extremely limited, as a result of its lack of functional expression in bacteria or yeast. The P450s SP $\alpha$  (CYP152B1) and BS $\beta$  (CYP152A1) are two recently discovered  $\text{H}_2\text{O}_2$ -driven fatty acid  $\alpha$ - and  $\beta$ -hydroxylases.<sup>[21–23]</sup> Their highly specialized catalytic mechanisms<sup>[24]</sup> limits the substrate range and therefore the applications of these P450s for biotransformations.

Cytochrome P450 BM-3 is a versatile hydroxylase whose “designability” has been demonstrated<sup>[25]</sup> and whose biotechnological relevance has been established.<sup>[26]</sup> Directed evolution allows us to engineer into this enzyme functions not required or permitted in its natural biological context. For example, a P450 BM-3 variant which efficiently hydroxylates alkanes was recently described.<sup>[27]</sup> An efficient H<sub>2</sub>O<sub>2</sub>-driven BMP variant allows us to exploit this powerful and versatile hydroxylase in a cell-free reaction system that requires neither NADPH nor reductase. This catalyst is easy to synthesize (high expression levels are achieved in *E. coli*), requires minimal preparation,<sup>[28]</sup> and is active under mild conditions (namely, 25 °C, 1 atm). The self-sufficiency of the H<sub>2</sub>O<sub>2</sub>-driven heme domain encourages further protein engineering to explore potential synthetic applications of this catalytic system.

Received: March 18, 2003 [Z51434]

**Keywords:** biocatalysis · cytochrome P450 · enzymes · oxidation · peroxxygenase

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