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Engineering and Analysis of a Self-Sufficient Biosynthetic Cytochrome P450 PikC Fused to the RhFRED Reductase Domain

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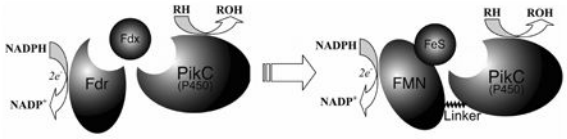
Abstract



Cytochrome P450 enzymes mediate important oxidative processes in biological systems including regio- and stereo-specific hydroxylation and epoxidation reactions. The inherent requirement of these biomolecules for separate redox partner(s) significantly limits their application in biotechnology. To address this challenge, naturally occurring and/or bio-engineered self-sufficient P450 systems with covalently fused redox partners have been utilized to harness their catalytic power. In this study, we describe the first *in vitro* characterization of a bacterial biosynthetic cytochrome P450 PikC fused to a heterologous reductase domain RhFRED that demonstrates single-component self-sufficiency. This novel fusion system not only produces a more active and effective biocatalyst, but also suggests a general design for a universal reductase to generate diverse self-sufficient fusions for functional identification or industrial applications of biosynthetic P450s.

Cytochrome P450 enzymes (P450s) are highly attractive biocatalysts due to their ability to catalyze a variety of regio- and stereo-specific oxidation reactions of complex organic compounds. These reactions occur under mild conditions by taking advantage of the two-electron activated dioxygen that is often challenging in organic synthesis.[1] To activate molecular oxygen, redox partners are required to sequentially transfer two reducing equivalents from NAD(P)H to P450.[2] Classically, there are two major redox partner systems, including an FAD containing reductase with a small iron-sulfur (Fe_2S_2) redoxin for most bacterial and mitochondrial P450s (Class I), and a single FAD/FMN containing flavoprotein for eukaryotic microsomal P450s (Class II).[3] The inherent requirement of cytochome P450s for separate protein partner(s) significantly limits their application in biotechnology.

The discovery of the first self-sufficient $P450_{BM3}$, which is naturally fused to a eukaryotic-like reductase represents an effective solution to this limitation.[4] The fusion nature of this enzyme dramatically improves electron transfer efficiency and coupling with the oxidative process, enabling it to be the most efficient P450 enzyme characterized to date.[5] Based upon the self-sufficiency of this naturally fused enzyme, a number of engineered proteins of diverse

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eukaryotic P450s bearing a reductase domain from $P450_{BM3}$ have been generated with *in vitro* activities.[6] This provides ready access to the great catalytic versatility of the membrane-bound eukaryotic P450s. In contrast, the biosynthetic P450s (Class I) lack such a universal reductase that can be used to engineer diverse self-sufficient P450s for either functional identification or potential industrial application.

Recently, a new class of self-sufficient cytochrome P450s exemplified by $P450_{RhF}$ from *Rhodococcus* sp. NCIMB 9784 was discovered to be naturally fused to a novel FMN/Fe_2S_2 containing reductase partner.[7] Although the physiological function of $P450_{RhF}$ remains unknown, its reductase domain (RhFRED), which is similar to the phthalate family of dioxygenase reductases, is capable of transferring electrons from NADPH to the heme domain of the monooxygenase, supporting 7-ethoxycoumarin dealkylation activity.[8] Moreover, recent reports from Misawa *et al*. demonstrated that this reductase domain could be used to reconstitute the catalytic activities of various Class I P450s *in vivo* through expression of corresponding genes fused to RhFRED in *Escherichia coli* cells.[9] This suggests that RhFRED might be developed into a generally effective redox partner for biosynthetic bacterial P450s. However, the lack of corresponding *in vitro* data could not unambiguously exclude *in trans* involvement of additional cellular redox partners.

Herein, we describe the first *in vitro* characterization of a single component bacterial biosynthetic cytochrome P450 fused to RhFRED that demonstrates high catalytic efficiency. The PikC cytochrome P450 in this study is involved in the pikromycin biosynthetic pathway of *Streptomyces venezuelae*.[10] PikC catalyzes the final hydroxylation step toward both the 12-membered ring macrolactone YC-17 (**1**) and the 14-membered ring macrolactone narbomycin (**4**) to produce methymycin/neomethymycin (**2/3**) and pikromycin (**5**) as major products (Scheme 1).[11] Recently, we elucidated the structural basis for the remarkable substrate flexibility by analyzing ligand-free and substrate-bound structures of PikC.[12] However, since the native redox partner of PikC remains unknown, its *in vitro* activity has depended on expensive spinach ferredoxin reductase (Fdr) and ferredoxin (Fdx) (Scheme 2A), as are many other biosynthetic P450s.[13] To investigate an alternative electron transfer pathway mimicking the fusion organization in $P450_{RhF}$ (Scheme 2B), the *pikC* gene was linked to the RhFRED gene including the native 16 amino acid linker sequence. The hybrid gene was cloned into pET28b(+), and overexpressed in *E. coli* BL21 (DE3) to generate N-terminal His_6-tagged PikC-RhFRED. After Ni-NTA chromatography, the purified red-colored recombinant P450 displayed (upon reduction) the signature peak at 450 nm in the CO-difference spectrum. Interestingly, gel filtration chromatography indicated that PikC-RhFRED predominantly dimerizes in storage buffer solution containing 0.2 mM dithioerythritol (DTE). In contrast, wild type (wt) PikC was shown to be monomeric under the same conditions. It was thus unclear whether the inter-monomer electron transfer could occur in the dimeric PikC-RhFRED as in $P450_{BM3}$.[14]

We next tested the ability of PikC-RhFRED to hydroxylate **1** and **4** *in vitro* when provided electron donor NADPH. We were gratified to observe that this chimeric protein showed significantly improved catalytic activity compared to wt PikC in the presence of exogenous redox partners (spinach Fdr and Fdx), producing higher yields of **2/3** and **5** under identical reaction conditions (Figure 1). This result unambiguously confirms that PikC-RhFRED is a self-sufficient P450 enzyme. Interestingly, we also constructed the pET21b(+)-*pikC*-RhFRED and obtained the purified C-terminal His_6-tagged PikC-RhFRED. This protein showed a similar CO-difference spectrum as its N-terminal His_6-tagged counterpart (Data not shown). However, it lacks catalytic activity, which is consistent with a similar C-terminal His_6-tagged form of original $P450_{RhF}$.[8] This provides additional evidence for the importance of the C terminus of RhFRED for electron transfer.

As mentioned above, one benefit of the fusion arrangement is that the covalent linkage presumably stabilizes the interaction between the P450 and redox partner, thus enhancing electron transfer efficiency. As such, one would expect this to improve the catalytic activity in terms of k_{cat}, whereas the substrate specificity would not be changed significantly.[15] To test whether this also applies to PikC-RhFRED, we first determined the substrate binding affinity of **1** and **4** toward both PikC and PikC-RhFRED. As expected, **1** and **4** binds to PikC-RhFRED with K_d values of 92.6 ± 0.5 μM and 215.0 ± 4.2 μM, respectively, which are similar to 112.9 ± 1.9 μM (**1**) and 288.3 ± 7.1 (**4**) toward wt PikC. This indicates that attachment of the heterologous reductase domain has no significant impact on substrate binding to PikC. Subsequently, we compared the kinetic parameters of PikC-RhFRED with those of the PikC-Fdr-Fdx three component system. As previously reported,[11,16] substrate inhibition was observed in all cases when substrate concentration was greater than 250 μM. Moreover, the solubility limitation (less than 500 μM) of macrolides in aqueous solution prevented us from deducing the K_i value. Therefore, we determined the apparent specificity constants (k_{cat}/K_m) by fitting the low-concentration data to the linear region of the Michaelis-Menten curve. By directly monitoring the substrate consumption by HPLC, the k_{cat}/K_m values of PikC-RhFRED were determined to be 0.96 and 1.20 $\mu M^{-1}\cdot min^{-1}$ for **1** and **4**, respectively. In contrast, the specificity constants of wt PikC partnered by Fdr and Fdx were 0.24 $\mu M^{-1}\cdot min^{-1}$ for **1** and 0.31 $\mu M^{-1}\cdot min^{-1}$ for **4**. It is evident that the fusion enhanced the catalytic activity approximately 4 fold for both **1** and **4**. Notably, the kinetic parameters of wt PikC differ significantly from those previously determined indirectly, using a NADPH depletion assay,[11,16] suggesting the stoichiometric ratio between NADPH and substrate hydroxylation could not be 1:1. The presumed de-coupling between electron transfer and hydroxylation might account for this difference.

Finally, when RhFRED was fused to another prototype biosynthetic P450 EryF,[13] a more active self-sufficient biocatalyst was obtained once again (See supporting information). Together with previous *in vivo* work,[9] our studies demonstrate that further development of RhFRED as the basis for an efficient cost-effective redox partner for bacterial biosynthetic P450s is warranted. Further efforts to understand this unique reductase, especially the electron transfer process involving heterologous fusion systems are now in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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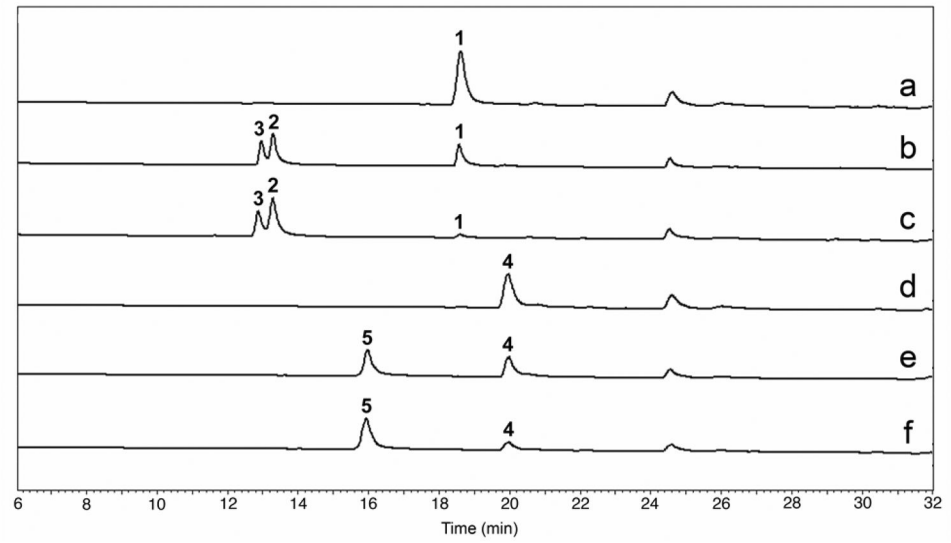
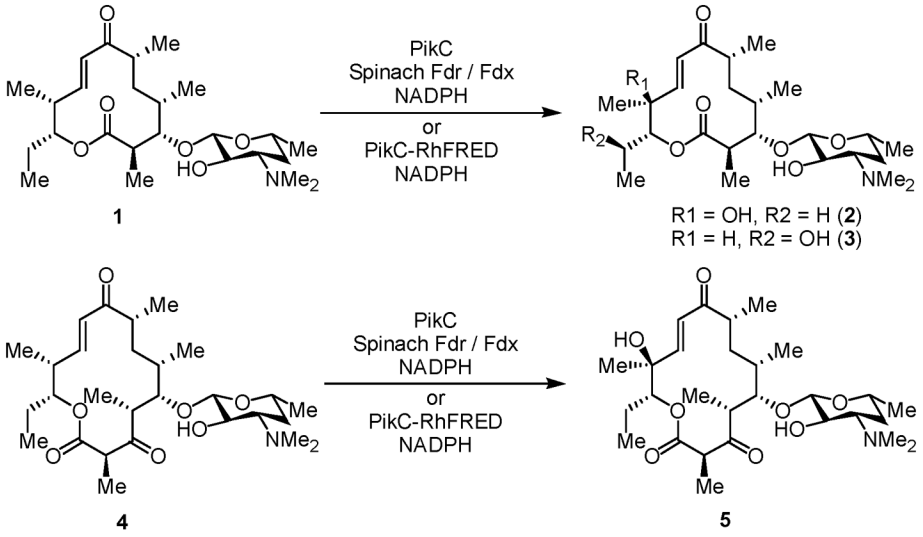
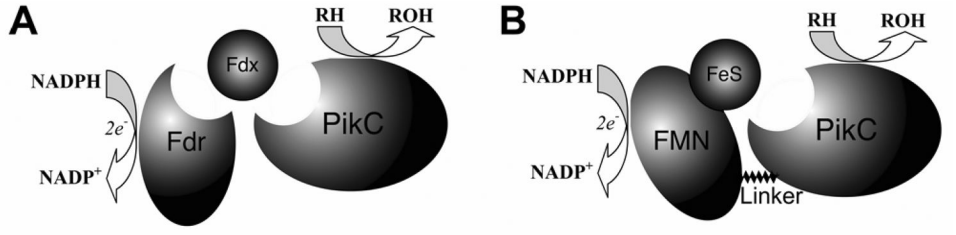


Figure 1.

HPLC analysis of reactions (1 h) catalyzed by wt PikC and fusion enzyme PikC-RhFRED. a) Negative control of **1** in absence of P450. b) **1** with wt PikC in presence of Fdr, Fdx, and NADPH. c) **1** with PikC-RhFRED in presence of only NADPH. d) Negative control of **4** in absence of P450. e) **4** with wt PikC in presence of Fdr, Fdx, and NADPH. f) **4** with PikC-RhFRED in presence of only NADPH.



Scheme 1.
Major physiological reactions catalyzed by PikC



Scheme 2.

Two redox partner systems (electron transfer pathways) used in this study for PikC. A, three components system; B, one component RhFRED system.