New Reactions and Products Resulting from Alternative Interactions between the P450 Enzyme and Redox Partners

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Supporting Information

ABSTRACT: Cytochrome P450 enzymes are capable of catalyzing a great variety of synthetically useful reactions such as selective C–H functionalization. Surrogate redox partners are widely used for reconstitution of P450 activity based on the assumption that the choice of these auxiliary proteins or their mode of action does not affect the type and selectivity of reactions catalyzed by P450s. Herein, we present an exceptional example to challenge this postulate. MycG, a multifunctional biosynthetic P450 monooxygenase responsible for hydroxylation and epoxidation of 16-membered ring macrolide mycinamicins, is shown to catalyze the unnatural N-demethylation(s) of a range of mycinamicin substrates when partnered with the free Rhodococcus reductase domain RHFRED or the engineered Rhodococcus-spinach hybrid reductase RHFRED-Fdx. By contrast, MycG fused with the RHFRED or RHFRED-Fdx reductase domain mediates only physiological oxidations. This finding highlights the larger potential role of variant redox partner protein–protein interactions in modulating the catalytic activity of P450 enzymes.

INTRODUCTION

The superfamily of cytochrome P450 enzymes (CYPs) is one of the most versatile biocatalyst systems in nature.1–3 CYPs are capable of catalyzing more than 20 distinct types of reactions including regio- and stereoselective oxidation of unactivated C–H bonds (hydroxylation and epoxidation), dealkylation, C–C bond cleavage, aromatic coupling, and others.4,5 Recently, more novel activities such as decarboxylation,6 nitrations,7 farnesene synthase activity,8 and carbene transfer9 have been reported, reflecting the ability to further diversify the function of these versatile enzymes.

Catalytic activities of the vast majority of CYPs require one or more redox partner proteins to sequentially deliver two electrons from NAD(P)H to the heme iron reactive center for dioxygen activation.1,2 Classical redox partner systems,10,11 including (1) a two-component system for most bacterial and mitochondrial CYPs comprising a small iron–sulfur reductin and an FAD-containing reductin reductase and (2) a single FAD/FMN-containing CYP reductase that serves in eukaryotic microsomal P450s as a separate partner protein or in a small number of bacterial CYPs (e.g., P450<sub>2E1</sub>) as a fused functional domain. Interestingly, nonclassical redox systems have been discovered continuously,10,12 among which the FMN/Fe<sub>2S<sub>2</sub></sub>-containing reductase domain termed “RHFRED” that is naturally fused with the P450 enzyme from Rhodococcus sp. NCIMB 978413 has been extensively studied in our laboratories.14–16

For practical purposes due to the difficulty of obtaining native partners, one or more surrogate redox partners, acting either in isolation or as artificially fused protein complexes, are often employed in functional characterization or synthetic applications of CYPs. The choice of surrogate partners or their mode of action is not necessarily expected to affect the type and selectivity of reactions catalyzed by P450s. Although alternative redox partners may influence catalytic efficiency and/or product distribution,17–22 the chemical identity of products themselves is apparently determined by the P450 enzyme. Our previous experience constructing and characterizing various self-sufficient biosynthetic P450 enzymes (whose activity is independent of isolated redox partners) fused with the RhFRED reductase domain14–16 also supports this “postulate”.

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Notably, previous work revealed that cytochrome \( \text{b}_5 \) is able to modulate the bifunctional role of human CYP17A1 as a 17\( \alpha \)-hydroxylase or a 17,20-lyase in steroid biosynthesis.\(^{23}\) This well-studied example demonstrates that the catalytic type of a P450 enzyme can be altered by a third-party protein effector via alternative interactions among the P450 enzyme, P450 reductase, effector protein, and distinct substrates.\(^{24−27}\) However, there have been no reports for a redoxin protein or a P450 reductase acting as the specific effector by itself to endow the serving P450 enzyme with new catalytic activity.

In this work, novel demethylated mycinamicin products were generated by P450 MycG supported by a stand-alone form of the RhFRED reductase domain, as opposed to RhFRED fused to MycG, thus challenging the generally accepted postulate. Further engineering of RhFRED led to didemethylated mycinamicin macrolides, demonstrating a novel route for structural diversification of natural products. Mechanistically, our results suggest that variant protein–protein interactions with the redox partner may serve as a modulator of P450 function by dramatically affecting the substrate binding mode in the P450 active site.

## RESULTS

MycG is the multifunctional P450 monoxygenase involved in the biosynthetic pathway of 16-membered ring macrolide mycinamicins in the rare actinomycete *Micromonospora griseorubida*.\(^{19,28,29}\) Physiologically, it catalyzes the C14 hydroxylation, and the C12=C13 epoxidation of mycinamicin IV (M-IV) leading to mycinamicin V (M-V) and mycinamicin I (M-I), respectively. M-V is subsequently epoxidized by MycG, giving rise to the major final product mycinamicin II (M-II), while M-I cannot be further modified by MycG, thus
representing the other terminal product in the pathway (Figure 1A). All diglycosylated mycinamicins display strong antibiotic activity against Gram-positive bacteria and mycoplasma,30 and M-II has been developed into a veterinary anti-infective agent.31 Detection of formaldehyde in the reaction by Purpald reagent37 provides additional evidence for oxidative demethylation (see Supporting Information). To confirm the site of demethylation, the 1H NMR of enzymatically prepared dMe-M-IV was analyzed using M-IV as reference (Figures 1C, S2 and S3). Instead, a new singlet peak was observed at 2.35 ppm singlet peak of M-IV that corresponds to the N-dimethyl group disappeared in the spectrum of dMe-M-IV. Instead, a new singlet peak was observed with half the integrated area at 2.72 ppm, indicating the −NHMe group resulted from the MycG-catalyzed demethylation. This observation is intriguing because a heretofore unknown dMe-M-IV product was obtained following separation of the previously fused protein domains, MycG and RhFRED. Next, we measured the oxidation and demethylation activities of MycG under a variety of molar ratios (1:1, 1:10, and 1:100) between MycG and RhFRED. In all cases, both oxidative and demethylated products were detected (Figure S4), indicating the ability of MycG to catalyze demethylation is not the consequence of a stoichiometric effect of RhFRED. A time course of the reaction (Figure S5) showed proportional accumulation of oxidative and demethylated mycinamicin products.

Table 1. Quantitative Measurements of M-IV Conversions Mediated by Different MycG Catalytic Systems (2 h reactions)

| MycG + RhFRED | MycG + RhFRED-Fdx | MycG-RhFRED | MycG-RhFRED-Fdx |
|---------------|-----------------|-------------|----------------|
| 22.1 ± 1.1%   | 5.8 ± 0.8%      | 54.1 ± 2.0% | 5.0 ± 0.4%     |
| 27.9 ± 1.4%   | 34.5 ± 1.5%     | –           | –              |
| 18.5%         | 24.0%           | –           | –              |
| 0.036 ± 0.008 | 0.036 ± 0.007   | 0.052 ± 0.012 | 0.017 ± 0.004 |

aNumbers of relative AUC280 nm (areas under curve at 280 nm) were calculated from the integrated area of certain peaks on HPLC traces. Coupling efficiencies were calculated as the percentage of NADPH used for product formation over the total NADPH consumption.

To investigate if the free form of the RhFRED domain can support the catalytic function of MycG, the ability of this two-component system to convert mycinamicin IV (M-IV) was tested. Surprisingly, in addition to the expected oxidative products, M-I, M-II, and M-V (coeluted with M-II), a new product formed with 18.5% isolated yield (Table 1) with the one phase exponential decay curve, the rate constants (k) for the four studied MycG catalytic systems were determined (Table 1 and Figure 2A). In comparison, isolated RhFRED and RhFRED-Fdx served MycG with close efficiency despite distinct product profiles. The M-IV oxidation mediated by MycG-RhFRED and MycG-RhFRED-Fdx demonstrated the apparent reaction velocity at 500 μM of M-IV being 26.0 ± 6.0 μM min⁻¹ and 8.5 ± 2.0 μM min⁻¹ (k × [S]), respectively. The steady-state Michaelis–Mentken kinetics of MycG could not be ascertained due to multiple reactions occurring simultaneously. It is well-known that the use of an unnatural redox partner in a CYP catalyzed reaction likely uncouples the electron generation upon NAD(P)H consumption and P450 product formation.40-42 Separation of the fused P450 and reductase domains could have a similar effect.43 The uncoupling process generates reactive oxygen species (ROS) such as superoxide anions (O₂⁻) and H₂O₂, which could direct the P450 catalytic route into the “peroxide shunt pathway.”1,2 To examine whether the demethylation of mycinamicins is a consequence of the peroxigenase activity by undergoing this shunt pathway, we first determined the NADPH coupling efficiency of the four catalytic systems using M-IV as substrate. As shown in Table 1, the coupling efficiencies for P450-reductase fusions (18.7% for MycG-RhFRED and 21.3% for MycG-RhFRED-Fdx) were lower than those of corresponding reactions using separated reductases (29.4% for MycG + RhFRED and 24.2% for MycG + RhFRED-Fdx). These data indicate that separation of...
reductase and P450 domains did not result in greater uncoupling in these P450 catalytic systems. Moreover, catalase, superoxide dismutase (SOD), ascorbic acid, and the combination of these three ROS scavengers were added into the MycG/RhFRED/M-IV reaction to remove ROS possibly generated from the uncoupling process. Although these ROS scavengers lowered the M-IV conversion (except for ascorbate) as well as the ratio of demethylated product to oxidized products (except for SOD) in the majority of reactions (Figure 2B), these effects were not dose dependent, and the N-demethylation activity remained even at the highest concentration of catalase, SOD, and ascorbate (Figure S6).

These results, together with the small change of coupling efficiency upon reductase domain separation, suggest that dMe-M-IV is unlikely to be a product of the peroxide shunt pathway. However, the lowered demethylated product/oxidized product ratio implies superoxide species might still contribute to some of the observed demethylation. This could be supported by detection of a low level of H₂O₂ in MycG reactions (Figure S7) and the fact that addition of ascorbic acid improved the overall conversion of M-IV (Figure 2B) since this chemical scavenger is capable of protecting the enzyme and substrate/products from radical damage by superoxides.44

Next, we comparatively tested the activity of MycG, driven by each of the four different redox systems, against M-IV analogues M-I, II, III, and V, isolated from fermentation cultures of wild-type or mutant Micromonospora griseorubi-da.30,45 The two fused systems displayed the typical physiological set of the hydroxylation and/or epoxidation products (Figure 3). However, the reactions driven by free reductors gave rise to more diversity of demethylated products. Specifically, both RhFRED and RhFRED-Fdx efficiently supported the demethylation of M-I and M-III by MycG, generating the new products dMe-M-I and dMe-M-III, respectively. M-II and M-V were demethylated by MycG at a low level driven by RhFRED-Fdx, but not by RhFRED. Of particular interest, MycG partnered by the stand-alone form of RhFRED-Fdx was able to further remove the second N-methyl group from monodemethylated dMe-M-I and dMe-M-III, leading to the unique double-demethylated product d2Me-M-I and d2Me-M-III (Figure 3), respectively. Previously, in both the in vivo30,45 and in vitro reactions that employed the surrogate spinach Fdx/FdR system,19 M-I and M-II have been shown to be the two end products of the mycinamicin biosynthetic pathway, and M-III bearing the monomethoxy sugar javose is oxidized by MycG at a very low level. Here, the change of the redox partners and their interaction mode has led to seven novel demethylated products (Figures 1, 3, and S8), demonstrating a new route for structural diversification of natural products.

**DISCUSSION**

In this study we show for the first time the ability of the multifunctional P450 monooxygenase MycG to catalyze N-demethylation of various mycinamicin natural product molecules. This novel functionality of MycG occurs when the monooxygenase is partnered with either the free *Rhodococcus* reductase domain, RhFRED, or the *Rhodococcus*-spinach hybrid, RhFRED-Fdx. In contrast, no similar demethylation activity was observed with the corresponding fused reductase partner systems. The minor differences in the NADPH coupling efficiency upon P450-reductase domain separation (Table 1) and dose-independent effects of catalase, SOD, and the chemical radical scavenger ascorbate on both MycG activity and the demethylation/oxidation product distribution (Figure 2B) strongly suggest that the unnatural demethylation activity is primarily due to heme-iron-oxo catalysis, as opposed to the peroxide shunt pathway. Thus, this finding highlights a broader role of reductors as a third-party protein–protein interactions in modulating catalytic activity of the P450 enzyme. It is well-known that alternative or surrogate reductase protein(s) might not provide the optimal supporting activity toward mismatched P450 enzymes17,19,22 and could influence product distribution.14,16 The modulating effect of a third party protein, cytochrome b₅₅, on the hydroxylase/lyase bifunction of CYP17A1 supported by cytochrome P450 reductase23–26 has also been demonstrated. However, to the best of our knowledge, the entirely new catalytic activity of a P450 enzyme associated with an alternative reductase partner is unprecedented.

The novel catalytic activity demonstrated by MycG likely stems from the poor accessibility of the substrate binding site, as evidenced by the X-ray structure analysis of the MycG-substrate complexes.46 Thus, the bulky and conformationally restrained mycinamicin substrates were largely unable to penetrate the L-shaped binding site to adopt a catalytically productive binding mode. NMR modeling using paramagnetic...
restraints suggests that displacement of a number of the protein secondary structure elements takes place in order to accommodate mycinamicin substrates in an orientation conducive to the observed oxidation pattern. By analogy to P450cam and the human P450 3A4, reorganization of the MycG active site may be driven by binding of the redox partner. As has been recently demonstrated by elegant work from the Poulos laboratory, binding of the endogenous redox partner putidaredoxin stabilizes the “open” conformation of P450cam, facilitating substrate access to the active site. Consistent with the structurally resolved MycG-substrate complexes, mycinamics exhibiting pseudo two-fold symmetry enter the active site in one of the two possible orientations: “mycinose-in-desosamine-out” (Figure 4A) or “desosamine-in-mycinose-out” (Figure 4B). The desosamine-in-mycinose-out entry results in the catalytically nonproductive binding mode represented by the M-III costructure (PDB ID code 2YCA). The mycinose-in-desosamine-out entry in the absence of the redox partner resulted in both the M-IV and M-V substrates being stalled in the intermediate nonproductive binding pose in all the structurally resolved complexes. If substrate relocation to the catalytically competent mode is indeed initiated by specific interactions with the endogenous redox partner, the alternative surrogates may to some extent mimic this allosteric effect, either facilitating or hampering substrate progression to the catalytic site. Tethered to MycG, both RhFRED and RhFRED-Fdx afforded physiological product profiles, with the qualification that the product yield was low for MycG-RhFRED-Fdx. Alternatively, when used as stand-alone redox partners, both the RhFRED and RhFRED-Fdx domains produced demethylated (RhFRED) or didemethylated (RhFRED-Fdx) products of not only the native MycG substrates M-IV and M-V but also the M-III precursor, an otherwise poor substrate for MycG oxidation, which was efficiently demethylated (Figure 3).

To explain this phenomenon we speculate that while the electron transfer between MycG and the surrogate redox partners is retained, the allosteric effect facilitating substrate progression to the normal catalytically competent binding mode (“mycinose-in-desosamine-out”) is likely diminished, if not entirely lost. This might be due to intrinsically low affinity between the surrogate partners and MycG, particularly if concentration dependence in binary complex formation becomes a rate-limiting issue in the bimolecular reaction. Also, loss of the positioning effect of the linker following domain separation may be a factor in changing the interaction mode of free reductase and P450 domains. We conclude that in the absence of the allosteric influence of the redox partner, mycinamics are more inclined to be stalled in the “desosamine-in-mycinose-out” noncatalytic entry pose, increasing the probability of N-demethylation occurring in an otherwise atypical reaction site. Despite discovery of a new N-demethylation activity for monoxygenase MycG, caution should be exercised in interpreting reconstituted P450 activity generated in a surrogate redox system, particularly until the generality of this approach is
further investigated. However, an interesting hypothesis is suggested: P450 enzymes could be even more versatile in vivo than previously considered, because these biocatalysts might interact with a variety of redox partners to gain alternative activities. This may impart evolutionary advantages to the host organisms through detoxifying a more diverse range of xenobiotics or synthesizing more secondary metabolites to adapt to ever-changing environments. Clear indications that the P450-omes of microbes (e.g., *Streptomyces coelicolor*) are capable of interacting with a limited set of redox partners are consistent with a broader role for redoxin-mediated selectivity in these fascinating enzyme systems.

**ASSOCIATED CONTENT**

*Supporting Information*

Experimental methods, DNA sequences for the synthesized *fdx* gene and the gene encoding the RhFRED-Fdx hybrid protein, NMR spectra, the data for effects of catalase, superoxide dismutase, and ascorbate on MycG activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**
The authors declare no competing financial interest.

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