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Functional Analysis of MycCI and MycG, Cytochrome P450 Enzymes Involved in Biosynthesis of Mycinamicin Macrolide Antibiotics

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SUMMARY

Macrolides are a class of valuable antibiotics that include a macrolactone ring, at least one appended sugar unit, and, in most cases, additional hydroxyl or epoxide groups installed by cytochrome P450 enzymes. These functional groups contribute to structural diversification and serve to improve the bioactivity profiles of natural products. Here, we have characterized in vitro two P450 enzymes from the mycinamicin biosynthetic pathway of *Micromonospora griseorubida*. First, MycCI was characterized as the C21 methyl hydroxylase of mycinamicin VIII, the earliest macrolide form in the postpolyketide synthase tailoring pathway. Moreover, we established that optimal activity of MycCI depends on the native ferredoxin MycCII. Second, MycG P450 catalyzes consecutive hydroxylation and epoxidation reactions with mycinamicin IV as initial substrate. These reactions require prior dimethylation of 6-deoxyallose to mycinose for effective conversion by the dual function MycG enzyme.

INTRODUCTION

The cytochrome P450 enzymes (P450s) form a very large family of oxidative hemeproteins that are responsible for diverse oxidative transformations across most life forms (Coon, 2005; Guengerich, 2001). These reactions typically involve modification of physiologic and xenobiotic compounds and include the biosynthesis of various bioactive compounds (e.g., steroids, antibiotics, and signaling molecules). Recent bacterial genome sequencing efforts have uncovered an unexpected large number of genes encoding P450 enzymes. For example, the model actinomycete *Streptomyces coelicolor* A3(2) that produces actinorhodin and undecylprodigiosin revealed the presence of 18 different P450 genes (Bentley et al., 2002), whereas *Streptomyces avermitilis* MA-4680, an avermectin producer, contains 33 P450s (Ikeda et al., 2003), and *Saccharopolyspora erythraea* NRRL 23338, the erythromycin-producing bacterium, encodes 36 P450s (Oliynyk et al., 2007). In secondary metabolic pathways, it is typical that P450 genes are integrated within the biosynthetic cluster, where their products catalyze regio- and stereospecific oxidation of precursors leading to structural diversity as well as improved bioactivities of these molecules (Lamb et al., 2003; Rix et al., 2002). Thus, cytochrome P450 enzymes EryF (Andersen and Hutchinson, 1992) and EryK (Stassi et al., 1993) that are encoded within the erythromycin biosynthetic gene cluster are involved in the biosynthesis of erythromycin A. Specifically, EryF hydroxylates the macrolactone precursor 6-deoxyerythronolide B, whereas EryK is a macrolide hydroxylase resulting in formation of erythromycin D. As prototypic P450 hydroxylases involved in secondary metabolism, EryF and EryK exhibit strict substrate specificity. In contrast, PikC cytochrome P450 involved in the methymycin/neomethymycin and pikromycin biosynthetic pathway of *Streptomyces venezuelae* has broader substrate tolerance (Xue et al., 1998). PikC catalyzes the final hydroxylation step toward the 12-membered ring macrolide YC-17 and the 14-membered ring macrolide narbomycin to produce methymycin/neomethymycin and pikromycin as major products.

Mycinamicins, a series of macrolide antibiotics produced by the rare actinomycete *Micromonospora griseorubida*, have shown impressive activities against a spectrum of Gram-positive strains, especially some antibiotic-resistant human pathogens (Kinoshita et al., 1988; Satoi et al., 1980; Suzuki et al., 1990). Stucturally, the major mycinamicin products of wild-type strain *M. griseorubida* A11725, including mycinamicin I (M-I), II (M-II), IV (M-IV), and V (M-V) (Figure 1), consist of a 16-membered ring polyketide macrolactone substituted with 6-deoxyhexose sugars desosamine and mycinose. Partial characterization of the biosynthetic pathway for mycinamicins has been obtained through analysis of blocked mutants and corresponding bioconversion studies (Kinoshita et al., 1991a; Suzuki et al., 1990). More recently, the nucleotide sequence of the complete mycinamicin biosynthetic gene cluster has been reported (Anzai et al., 2003),

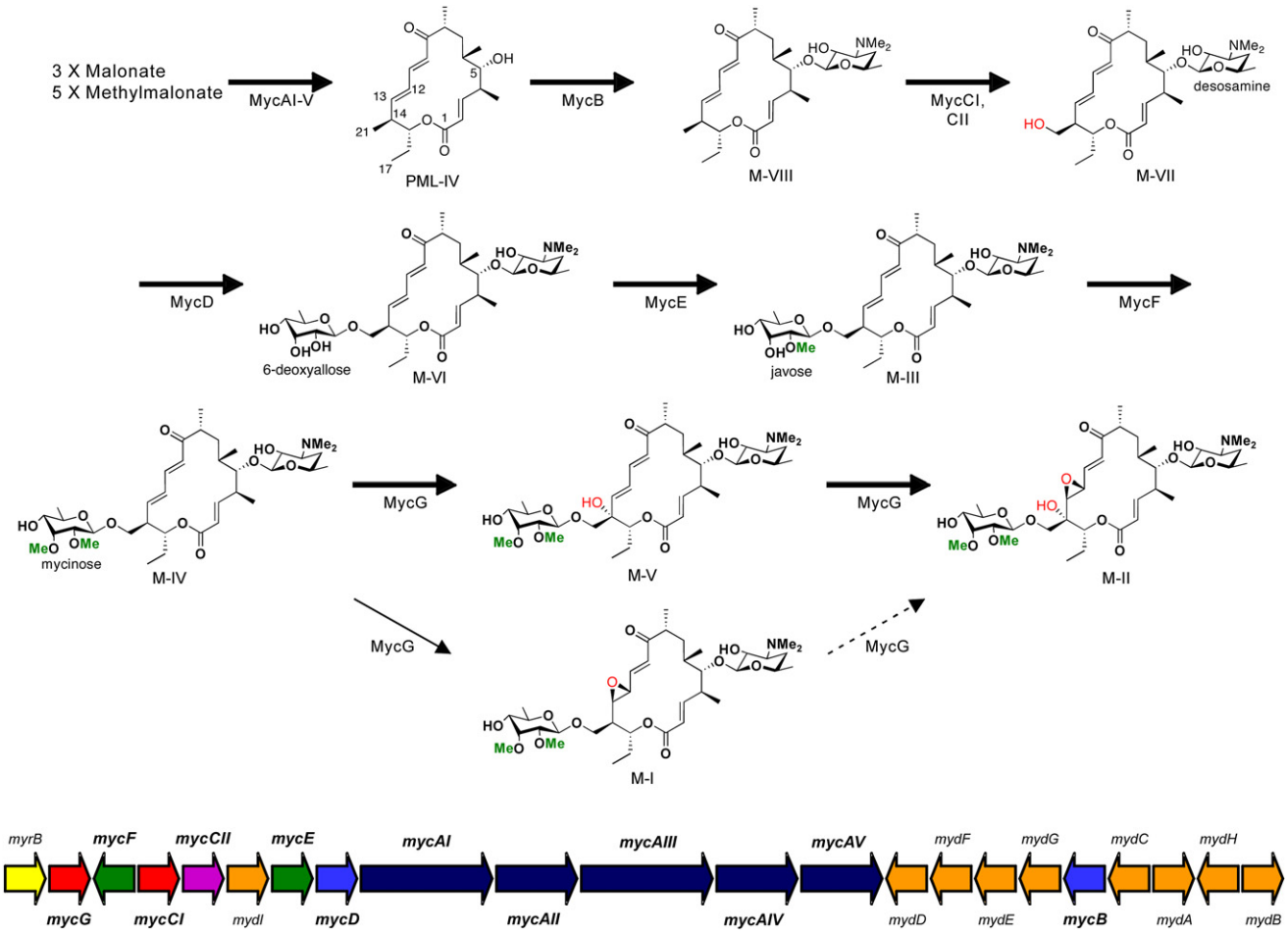

Figure 1. Mycinamicin Post-PKS Biosynthetic Pathway and Organization of the Mycinamicin Biosynthetic Gene Cluster

Methyl groups introduced by *O*-methyltransferases and oxidation steps catalyzed by cytochrome P450 enzymes are highlighted in green and red, respectively. Bold arrows represent the main pathway, whereas thin arrows indicate a shunt pathway. The dashed arrow indicates low-level conversion, as reported elsewhere (Inouye et al., 1994). Color codes in the *myc* gene cluster are as follows: red, cytochrome P450 genes; purple, ferredoxin gene; indigo blue, polyketide synthetase genes; blue, glycosyltransferase genes; green, *O*-methyltransferase genes; orange, deoxysugar biosynthetic genes; and yellow, self-resistance gene (rRNA methyltransferase gene).

wherein two putative P450 genes *mycCI* and *mycG* were identified (Figure 1).

Analysis of the 5′ region of the *myc* gene cluster upstream from the PKS locus revealed that *mycCI* is located adjacent to *mycCII*, which encodes a putative ferredoxin (Anzai et al., 2003). Since the deduced amino acid sequences of *mycCI* and *mycCII* show high sequence similarities to TylHI and TylHII (Figure 2), respectively, that are likely responsible for hydroxylation at the C23 methyl group of tylactone (Baltz and Seno, 1981), the function of MycCI and MycCII was accordingly proposed to mediate hydroxylation at the analogous C21 methyl group of protomycinolide IV (PML-IV) (Anzai et al., 2003) (Figure 1). On the basis of genetic complementation analysis of a targeted mutant strain of *M. griseorubida, mycG* was presumed to encode a P450 enzyme that catalyzes both hydroxylation and epoxidation at C14 and C12/13 on the macrolactone ring of mycinamicin (Inouye et al., 1994; Suzuki et al., 1990). In the current study, *mycCI* and *mycG* genes were overexpressed in *Escherichia coli*, and the functions of purified MycCI and MycG proteins were determined in vitro using natural substrates derived from semi-synthesis or isolated from wild-type or engineered strains of *M. griseorubida* that accumulate key mycinamycin intermediates. Moreover, specific roles have been proposed for both the desosamine and mycinose sugar residues in the oxidative cascade leading to M-II, the final product in the pathway.

RESULTS

Protein Sequence Analysis of MycCI and MycG

Comparison of the deduced amino acid sequences of MycCI and MycG showed relatively low sequence identity (33%). In the phylogenetic tree (Figure 2) of selected bacterial macrolide biosynthetic P450 enzymes, they were clustered in distinct branches, suggesting these two unrelated P450 genes may have been integrated into the mycinamicin biosynthetic gene cluster sequentially from different ancestors, as opposed to being derived from divergent evolution following duplication of a parental gene. Specifically, MycG, as the first biosynthetic monooxygenase

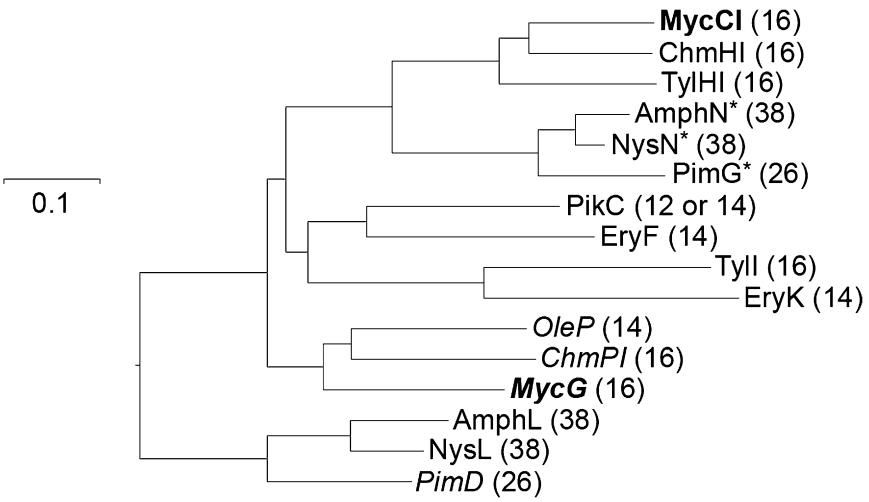

Figure 2. Phylogenetic Tree of Macrolide Biosynthetic P450 Mono-Oxygenases

The selected cytochrome P450s include OleP (accession number AAA92553) [oleandomycin pathway], ChmHI and ChmPI (accession numbers AAS79447 and AAS79453) [chalcomycin pathway], MycCI and MycG [mycinamicin pathway] (accession numbers BAC57023 and BAA03672), AmphL and AmphN (accession numbers AAK73504 and AAK73509) [amphotericin pathway], NysL and NysN (accession numbers AAF71769 and AAF71771) [nystatin pathway], PimD and PimG (accession numbers CAC20932 and CAC20928) [pimaricin pathway], TylHI and TylI (accession numbers AAD41818 and AAA21341) [tylosin pathway], EryF and EryK (accession numbers AAA26496 and YP-001102980) [erythromycin pathway], and PikC (accession numbers AAC68886) [pikromycin pathway]. The numbers in parentheses indicate the macrolactone ring size of the corresponding P450 substrate. Unless otherwise specified, all selected P450 enzymes are hydroxylases. The epoxidases are noted in italics. The enzymes marked with an asterisk are presumed to mediate carboxylic acid formation. MycCI and MycG are highlighted in bold.

characterized with an ability to catalyze both hydroxylation and epoxidation steps, is clustered with two other epoxidases—OleP (Rodriguez et al., 1995) and ChmPI (Ward et al., 2004)—whereas MycCI is closely related to ChmHI (Ward et al., 2004) and TylHI (Baltz and Seno, 1981), both of which are responsible for methyl group hydroxylations of 16-membered ring macrolides. Interestingly, we revealed that the clustering of macrolide biosynthetic P450s correlated with not only the functionalities (i.e., hydroxylases, epoxidases, or ones responsible for carboxylic acid formation) but also the substrate macrolactone ring size, suggesting that selection based on product structure derived from the upstream PKS biosynthetic system might be among the significant factors directing evolution of secondary metabolic P450s.

Heterologous Expression of MycCI, MycCII, and MycG

The *mycCI*, *mycCII*, and *mycG* genes were overexpressed in *E. coli* BL21(DE3), and the resulting proteins were purified (see Supplemental Data available online). MycCI was overexpressed as either a C- or N-terminal 6× His tagged protein. After purification using Ni-NTA agarose chromatography, the individual polypeptides showed molecular weights of approximately 44 and 45 kDa, corresponding to the estimated masses of C- and N-terminal 6× His tagged MycCI. The wild-type MycCII (MycCII-wt, m.w. ~8 kDa) and N-terminal 6× His-tagged ferredoxin MycCII (MycCII-NH, m.w. ~10 kDa) were purified to homogeneity (see Experimental Procedures). MycG expression was placed under the control of a T7 promoter including a 6× His-tag introduced at its amino terminus. Similarly, one-step purification was performed using a Ni-NTA agarose column to obtain homogeneous protein with expected molecular weight of 46 kDa. Subsequently, the CO-bound reduced difference spectra confirmed the identity of both MycCI and MycG as cytochrome P450 enzymes (see Supplemental Data).

Synthesis of Mycinamicin VIII (M-VIII) from Protomycinolide IV (PML-IV)

Because of the limited quantity (<0.1 mg) of totally available M-VIII isolated from *M. griseorubida* fermentation culture, the presumed substrate of MycCI, we developed an effective chemical glycosylation strategy to obtain this important intermediate by coupling desosamine as sugar donor to the readily available PML-IV aglycone precursor (Hayashi et al., 1981). Glycosylation with desosamine has previously employed either thioglycosides or glycosyl fluorides, with a methyl carbonate as the C2 protecting group (Martin et al., 1997; Matsumoto et al., 1988; Suzuki et al., 1988; Toshima et al., 1995; Woodward et al., 1981a, 1981b, 1981c). After examining several combinations of protecting groups and anomeric leaving groups, we found that the C2 acetoxy-protected glycosyl fluoride of desosamine was conveniently obtained, easily purified, stable to storage, and effective in the transfer of desosamine to a range of aglycones. In this procedure (Figure 3), erythromycin hydrate was hydrolyzed under acidic conditions to obtain a crude sample of desosamine hydrochloride that was converted to the bis(acetate) of desosamine in 88% isolated yield, as reported elsewhere (Chen et al., 2002). Further treatment of this material with HF·pyridine afforded the C2 acetoxy glycosyl fluoride of desosamine **1**, which serves as a stable and convenient precursor for chemical glycosylations. Direct treatment of PML-IV (Figure 1) with the glycosyl fluoride and $BF_3 \cdot Et_2O$ in CH_2Cl_2 afforded C2 acetoxy mycinamycin VIII as a 2:1 mixture of β and α anomers in 39% (not optimized) isolated yield. Higher yielding desosamine glycosylations with other mycinamicins have been reported (Matsumoto et al., 1988); however, these alternative procedures with different glycosyl donors were not explored for comparison. Purification by preparative HPLC (SiO_2) afforded the pure β-anomer (see Supplemental Data), which was deprotected with K_2CO_3 in methanol to afford semisynthetic M-VIII in 89% isolated yield. The ^1H NMR spectrum of this synthetic material was coincident with previously reported data (Kinoshita et al., 1989).

Functional Analysis of MycCI In Vitro

To establish the role of each mycinamicin P450, we first tested whether MycCI is capable of hydroxylating the predicted substrate M-VIII using a typical heterologous spinach ferredoxin and ferredoxin reductase system (Xue et al., 1998). As expected,

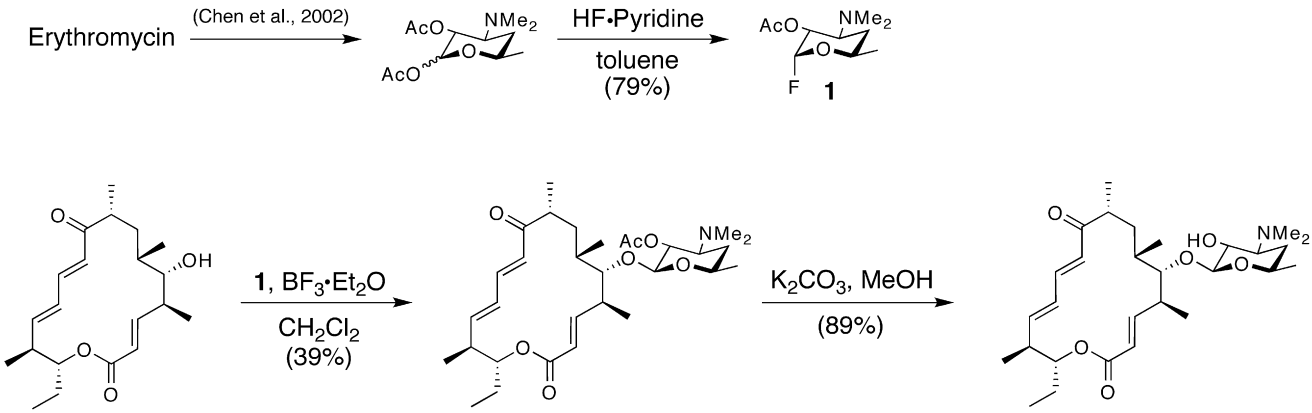

Figure 3. Synthetic Scheme for M-VIII

M-VIII was converted to the corresponding C21 hydroxymethyl product M-VII (Figure 4), albeit inefficiently. Notably, the C- and N-terminal His-tagged MycCI showed similar activities (data not shown), indicating that the location of His-tag has no significant impact on MycCI activity. We selected the C-terminal His-tagged MycCI to perform biochemical studies and refer to this form as MycCI for convenience. The low conversion ratio of M-VIII (11.0 ± 0.8%) might be due to the higher activation energy required to functionalize the primary C-H bond. Alternatively, the fact that a specific ferredoxin gene *mycCII* is clustered with *mycCI* suggests that MycCI might possess higher ferredoxin specificity than MycG. Thus, the capability of MycCII (together with spinach ferredoxin reductase, since the native ferredoxin reductase for MycCI remains unknown) to reconstitute the in vitro M-VIII hydroxylation activity of MycCI was assessed and compared to spinach ferredoxin. Thus, when partnered by MycCII-wt and MycCII-NH, MycCI converted 28.6 ± 2.0% and 37.4 ± 1.4% of M-VIII to M-VII, respectively, suggesting MycCII mediates the electron transfer from spinach ferredoxin reductase to MycCI more efficiently than does spinach ferredoxin (Figure 4). However, it is currently unclear why the activity of the His-tagged MycCII is higher than that of MycCII-wt. Considering that spinach ferredoxin and ferredoxin reductase are natural partners, we reason that the higher activity of MycCII could derive from a more favorable interaction with the MycCI P450, compared with the spinach ferredoxin reductase. Therefore, the binding affinities of different ferredoxins toward MycCI were compared with one another. As expected, the most active form (MycCII-NH) exhibited the lowest dissociation constant (K_d) of 7.0 ± 0.1 μM. In contrast, the binding affinity of spinach ferredoxin to MycCI (K_d = 148.9 ± 10.2 μM) is much lower than that of either MycCII-wt or MycCII-NH. Interestingly, we found MycCII is unable to support in vitro activity of MycG (see Supplemental Data), the other P450 enzyme involved in mycinamicin biosynthesis. These results strongly suggest MycCII has evolved to selectively serve MycCI catalysis.

In addition, similar MycCI reactions were assessed with the PML-IV, M-VI, M-III, M-IV, and M-V (Figure 1), but no products were observed, and starting materials were quantitatively recovered (data not shown). These results establish that MycCI catalyzes the first oxidation step, converting the C21 methyl group to the corresponding hydroxymethyl that is subsequently functionalized with 6-deoxyallose, followed by two methylation steps to mycinose. Moreover, the substrate requires desosamine modification at the C5 hydroxyl group in order to be accepted by MycCI. This is analogous to PikC, shown through cocrystal structure analysis with natural 12- and 14-membered ring macrolides to involve desosamine-mediated substrate anchoring (Sherman et al., 2006).

Functional Analysis of MycG In Vitro

Previous genetic studies on the mycinamicin biosynthetic pathway identified a gene fragment that complemented a M-II nonproducing mutant of *M. griseorubida* (Inouye et al., 1994). DNA sequence analysis revealed an open reading frame whose translated product showed high-level amino acid sequence similarity to cytochrome P450 enzymes. The mycinamicin biosynthetic intermediate isolated from the M-II nonproducing *M. griseorubida* strain lacked both C14 hydroxyl and C12-C13 epoxide functionalities, and the subcloned fragment bearing the putative P450 gene was able to restore production of M-II (Inouye et al., 1994). These data provided strong but indirect evidence that the *mycG* gene product is capable of catalyzing both oxidation steps at adjacent positions on the macrolactone ring (Figure 1).

To confirm the dual function of MycG, our in vitro analysis was initiated by testing directly the ability of MycG to convert the putative substrate M-IV. As shown in Figure 5A, the LC trace of the reaction extract (2) at 280 nm showed two peaks **a** and **b**. The corresponding mass spectra indicate M-V ($[M+H]^+$ = 712.35) and M-II ($[M+H]^+$ = 728.35) co-eluted as peak **a**, and peak **b** includes M-IV ($[M+H]^+$ = 696.35) and M-I ($[M+H]^+$ = 712.35). This LC-MC analysis clearly demonstrates the dual function of MycG as hydroxylase and epoxidase. However, despite efforts to optimize reverse-phase HPLC conditions, we were unable to separate M-IV and M-V from their epoxidized products.

To determine the precursor of M-II, M-V and M-I were incubated with MycG in separate reactions. When M-V was used as substrate, it was almost completely converted into M-II. Since M-II (and M-I) lacks strong absorbance at 280 nm (Satoi et al., 1980), only a minor peak **c** was observed in the LC trace, although substantial M-II was formed on the basis of mass spectral analysis (Figure 5B; notably, the M-II product was visible at 240 nm). However, M-I was not converted into M-II in vitro (see Supplemental Data), indicating that M-II can only be generated

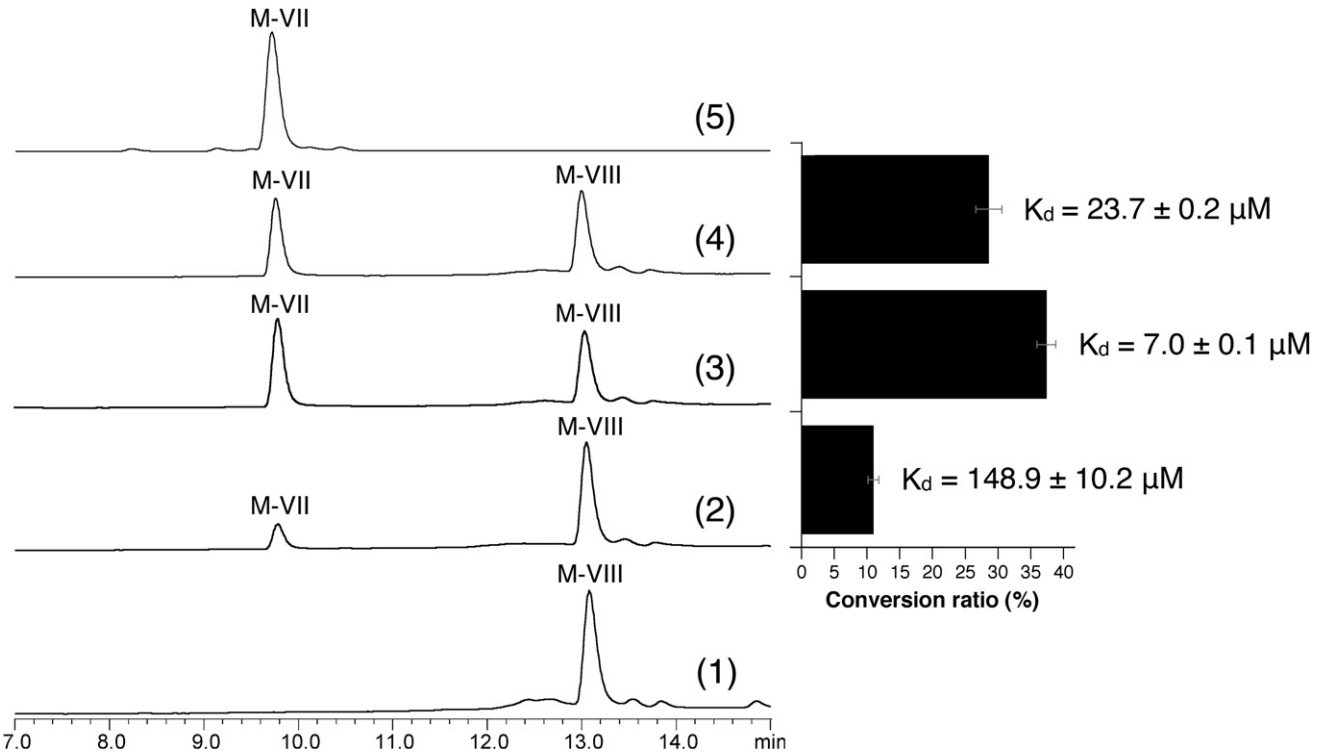

Figure 4. In Vitro M-VIII Conversions Catalyzed by MycCI

All LC traces were analyzed at 280 nm: (1) negative control, M-VIII + boiled MycCI; (2) M-VIII + MycCI + spinach ferredoxin; (3) M-VIII + MycCI + MycCII-NH; (4) M-VIII + MycCI + MycCII-wt; and (5) M-VII standard. The right diagram shows M-VIII conversion ratios calculated by using Equation 1-$AUC_{unreacted\ M\text{-}VIII}/AUC_{total\ M\text{-}VIII}$ (AUC: area under curve) based on the corresponding LC-traces in parallel. Error bars are shown since the conversion ratios (mean ± s.d.) are calculated from two independent experiments. In addition, dissociation constants (K_d) with standard errors of the spinach and MycCII ferredoxins toward MycCI are shown.

from M-V as substrate. Epoxidation of M-IV prior to hydroxylation evidently results in termination of this post-PKS tailoring pathway (Figure 1).

To assess further enzyme selectivity and ability to catalyze alternative oxidative reactions against the mycinamicin aglycone and several early glycosylated intermediates (PML-4, M-VIII, M-VII, M-VI, and M-III), MycG-mediated reactions were performed. No products were observed when using PML-4, M-VIII, and M-VII (see Supplemental Data), but two new compounds appeared at low levels in LC-MS analysis of the M-VI and M-III reactions (Figure 5C). When M-III was used as a substrate, a minor new peak **d** was detected with *m/z* = 698.40, which is 16 Da higher than the molecular weight of M-III. Moreover, in the MycG reaction with M-VI, a new peak **e** (*m/z* = 684.25), presumed to be oxidized M-VI, was detected at an even lower conversion level. Both peak **d** and **e** were visible under 280 nm, and their retention times were faster than corresponding starting materials, strongly suggesting they are hydroxylated instead of epoxidized products. Moreover, the MS-MS analysis (data not shown) of **d** and **e** indicated that both hydroxylations occurred on the macrolactone ring, although we could not further determine the site of modification because of limited amounts of each compound. Interestingly, the C14 hydroxylated forms of M-III and M-VI have already been reported as minor components of the mycinamicin pathway designated as mycinamicin IX (M-IX) and mycinamicin XV (M-XV), respectively (Kinoshita et al., 1992). Accordingly, it is likely that **d** and **e** correspond to M-IX and M-XV, providing direct evidence for the in vivo origin of these two minor compounds. More importantly, we established that efficient catalysis by MycG requires mycinose, derived from dimethylation of the second sugar residue as C21 OH linked 6-deoxyallose.

Measurement of Substrate Dissociation Constants

To understand why mycinamicin P450 enzymes (especially MycG) behave differently toward compounds with subtle structural differences, we performed spectrophotometric substrate binding assays to determine dissociation constants (K_d). As shown in Table 1, M-VIII binds to MycCI with a K_d value of 28.1 ± 3.2 μM, which is significantly higher than 0.7 ± 0.1 μM, the K_d value of M-IV toward MycG. Evidently, the substrate binding to MycG is much tighter than that to MycCI, which is likely attributed to additional interactions of mycinose within the P450 substrate-binding pocket. Interestingly, the C14 hydroxylated M-V showed approximately 14 times lower binding affinity toward MycG than M-IV, suggesting that M-V is a less suitable substrate for MycG. However, this finding is inconsistent with the results from in vitro assays (described in Figure 4C above) in which M-V was converted to oxidized product more effectively than M-IV (kinetic analysis [see below] provided insights into this apparent paradox). In contrast to M-V, the epoxidized product M-I was not able to function as a substrate since we did not observe a Type-I difference spectra even at high substrate concentrations. This result is consistent with a lack of MycG activity toward M-I in vitro. It was particularly surprising that the final product

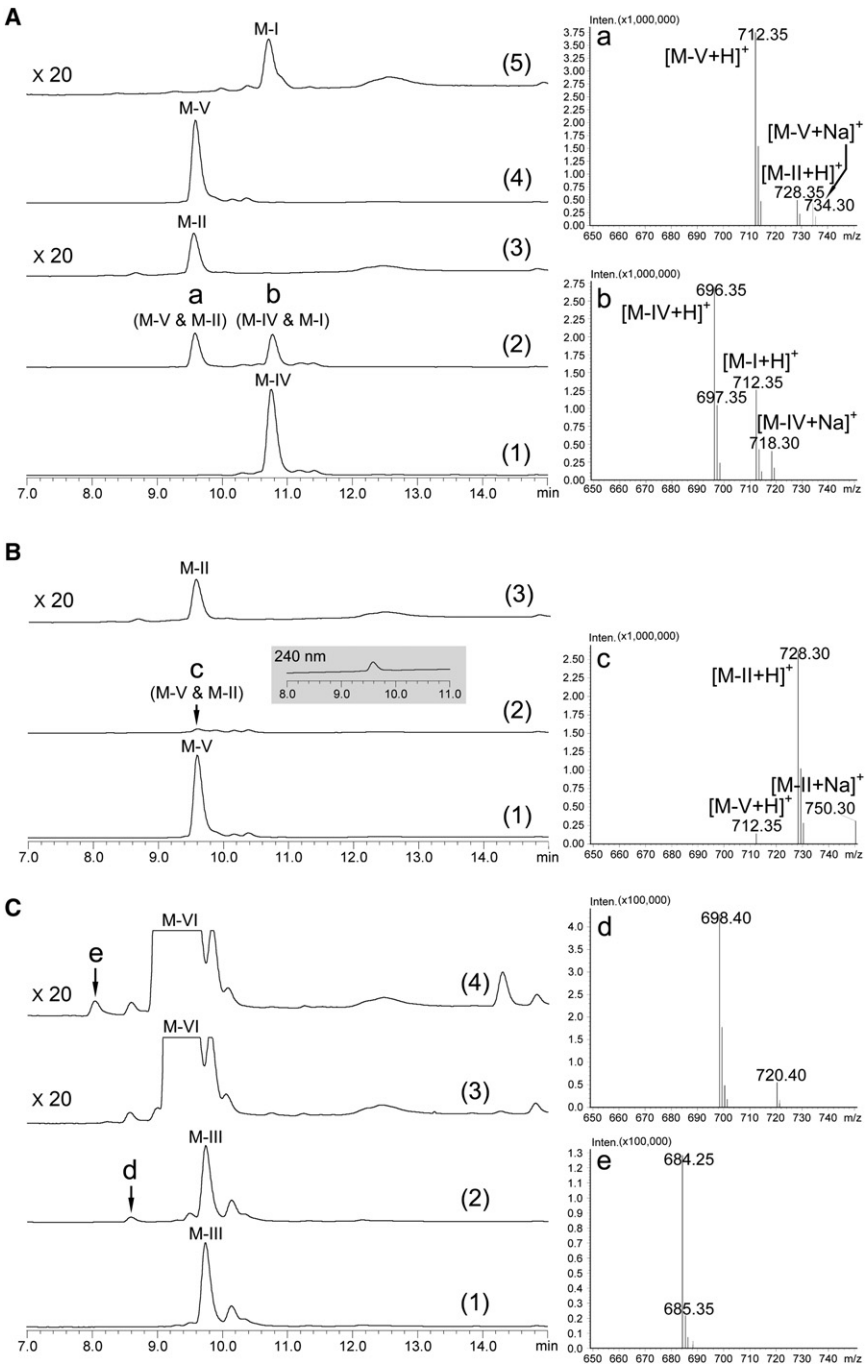

Figure 5. LC-MS Analysis of In Vitro Conversions Catalyzed by MycG

All LC traces were analyzed at 280 nm unless otherwise specified.

(A) MycG reaction using M-IV as substrate: (1) negative control, M-IV + boiled MycG; (2) M-IV + MycG. The right panel shows the mass spectra of peak **a** and **b**; (3) M-II standard amplified 20× because of its poor absorbance at 280 nm; (4) M-V standard; (5) M-I standard amplified 20×.

(B) MycG reaction using M-V as substrate: (1) negative control, M-V + boiled MycG; (2) M-V + MycG. The inset shows the 240 nm LC trace, under which M-II has better absorbance. The right panel shows the mass spectrum of peak **c**; (3) M-II standard amplified 20×.

(C) MycG reactions using M-III and M-VI as alternative substrates: (1) negative control, M-III + boiled MycG; (2) M-III + MycG; (3) negative control, M-VI + boiled MycG; (4) M-VI + MycG. LC traces (3) and (4) amplified 20 × compared to (1) and (2) to visualize the peak **e** in trace amount. The right panel shows the mass spectra of peak **d** and **e**.

M-II bound to MycG with a dissociation constant of 71.5 ± 8.4 μM, indicating the compromising effect of the epoxide moiety toward substrate binding could be overridden by an adjacent C14 hydroxyl group. The reason for this unusual finding will await substrate cocrystallographic analysis of MycG, which is currently in progress.

Steady-State Kinetic Analysis of MycCI and MycG

Assuming a 1:1 stoichiometric relationship between NADPH consumption and substrate oxidation, the steady-state kinetic parameters of MycCI were determined using purified synthetic M-VIII as substrate. When partnered with spinach ferredoxin, MycCI demonstrated a K_m of 34.5 ± 5.5 μM and a k_{cat} of 71.7 ± 3.2 min^{-1} for the C21 methyl group hydroxylation step. In contrast, with MycCII-NH coupled with MycCI, catalysis became more efficient with a decreased K_m of 5.8 ± 0.7 μM and an improved k_{cat} of 104.1 ± 1.8 min^{-1}. This finding suggests that MycCII not only induces better substrate binding of MycCI but also improves electron transfer efficiency as reflected by increased turnover number (k_{cat}).

Although MycG is capable of catalyzing three different reactions (M-IV to M-V; M-IV to M-I; and M-V to M-II), we limited our study to kinetic constants for M-V to M-II conversion (K_m = 16.2 ± 3.1 μM; k_{cat} = 415.7 ± 22.9 min^{-1}). This was because of the complexity of the analysis when M-IV was used as substrate, since multiple reactions (M-IV to M-V or M-I and M-V to M-II) did not allow fitting of data to the Michaelis-Menten equation. However, it is noteworthy that we observed the overall NADPH consumption rate at high M-IV concentration (e.g., 100 μM) to be considerably reduced, compared with low M-IV concentration (e.g., 10 μM), suggesting strong substrate inhibition. This could explain the above-mentioned contradiction between the conversion ratio and binding affinity. According to the in vitro analysis of MycG activity (Figures 5A and 5B), M-V appears to be a better substrate than M-IV on the basis of the conversion ratios. However, this result might be misleading since M-IV has better binding affinity toward MycG (Table 1). Thus, on the basis of in vitro reactions using substrate concentrations at

Table 1. Binding and Steady-State Kinetic Analysis of MycCI and MycG

Enzyme	Substrate	K_d (μM)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot min^{-1}$)
MycCI	M-VIII	28.1 ± 3.2	34.4 ± 5.5[a]	71.7 ± 3.2[a]	2.1[a]
			5.8 ± 0.7[b]	104.1 ± 1.8[b]	17.9[b]
MycG	M-IV	0.7 ± 0.1	ND[c]	ND[c]	ND[c]
	M-V	10.4 ± 0.2	16.2 ± 3.1[a]	415.7 ± 22.9[a]	25.7[a]
	M-I	NB[d]	–	–	–
	M-II	71.5 ± 8.4	–	–	–

–, No reaction.
[a] Kinetic parameters were determined when spinach ferredoxin was used.
[b] Kinetic constants were determined when MycCII-NH ferredoxin was used.
[c] ND: The kinetic parameters were not determined because multiple reactions occur at the same time.
[d] NB: No binding.

500 μM, we believe that the lower conversion of M-IV is a reflection of substrate inhibition.

DISCUSSION

Mycinamicins represent a large family of macrolide antibiotics with more than 20 members (Kinoshita et al., 1991b, 1992). The structural diversity is derived primarily from post-PKS tailoring modifications, including glycosylation, oxidation, and methylation steps. Therefore, this biosynthetic pathway represents an important system to explore the mechanism and significance of secondary metabolite diversification.

Here, we provide a detailed analysis of two cytochrome P450 enzymes, MycCI and MycG, and their role in late-stage chemical modifications. By gene cloning, protein expression, purification, and reconstitution assays with heterologous redox partners, we unambiguously confirmed their physiological role in vitro. To the best of our knowledge, the functional and kinetic analysis of MycCI represents the first enzymatic characterization of a biosynthetic monooxygenase responsible for methyl group hydroxylation. In addition, we established that MycCII is a specific ferredoxin, whose corresponding gene (*mycCII*) is immediately adjacent to *mycCI* and capable of effectively supporting MycCI activity. Compared with spinach ferredoxin, MycCII binds to MycCI more tightly, presumably leading to more efficient electron transfer. On the other hand, we established that C21 hydroxylation by MycCI depends on C5-linked desosamine, which is analogous to PikC (Xue et al., 1998), another cytochrome P450 involved in pikromycin biosynthesis. Recent cocrystallographic analysis of PikC (Sherman et al., 2006) and its natural substrates (YC-17 and narbomycin) revealed that desosamine acts as an indispensable anchor responsible for productive binding and proper positioning of substrate in the active site. Accordingly, we surmise that desosamine plays a similar role in hydroxylation of precursor macrolide M-VIII.

The current study has revealed that MycG is a more versatile enzyme than MycCI, providing the first example of a biosynthetic P450 involved physiologically in catalyzing both hydroxylation and epoxidation reactions. In addition, MycG represents the primary basis for structural diversification in the mycinamicin pathway since it is solely responsible for generating multiple products, including M-V, M-I, and M-II, as well as some minor components, including M-IX and M-XV. This work has also demonstrated that MycG function depends on the presence of both desosamine and the second sugar mycinose in the substrate. It is also noteworthy that both deoxysugars are essential for mycinamicin bioactivity (Kinoshita et al., 1989). Although the precise role of the second sugar for MycG substrate recognition remains obscure because of the lack of X-ray structural information, on the basis of the high binding affinity of M-IV toward MycG (K_d = 0.7 ± 0.1 μM), we predict that there might be a specific active site-binding pocket in the polypeptide to accommodate mycinose, thereby significantly improving substrate affinity. Moreover, of particular interest, the activity of MycG against diglycosylated substrates (M-VI, M-III, and M-IV) is related to the extent of methylation mediated by sequential *O*-methyltransfer by MycE and MycF. In the absence of methylether groups installed on the second sugar molecule, M-VI bearing 6-deoxyallose appears to be a very poor substrate for MycG. This situation can be improved by the first methylation to form javose in M-III, although its conversion is low (Figure 5C). Subsequently, the second methylation catalyzed by MycF imparts a pronounced effect upon MycG-mediated binding of M-IV. It appears likely that these stepwise methylations decrease the polarity of 6-deoxyallose in M-VI, thus enabling it to be accepted by a putative hydrophobic MycG-binding pocket.

Substrate inhibition occurs in about 20% of all known enzymes (Copeland, 2000). In biosynthetic P450 enzymes, this phenomenon has been observed in previous kinetic analysis of PikC (Li et al., 2007), EryK (Lambalot and Cane, 1995), and PimD (Mendes et al., 2005). In this study, M-IV inhibition of MycG was also detected indirectly even though the kinetic parameters were not obtained because of the complexity of the multiple-reaction system. Thus, the mechanism of this substrate inhibition cannot be further elucidated without generating detailed kinetic curves. However, we realize that this behavior of MycG might be physiologically significant for maintaining the chemical diversity within the mycinamicin pathway. In the event that M-IV inhibition does not occur, MycG would convert all M-IV into final products M-I and M-II, instead of a mixture of M-I, II, IV, and V as shown in Figure 5A and observed in the fermentation culture of *M. griseorubida strain* A11725 (Satoi et al., 1980). This would ultimately limit the spectrum of its metabolic output and perhaps compromise the ability of the microorganism to adapt to a variable and competitive environment.

SIGNIFICANCE

This report provides detailed information about the oxidative cascade that introduces structural diversity into the mycinamicin class of macrolide antibiotics. We have established in vitro that MycCI is a cytochrome P450 enzyme responsible for the C21 methyl hydroxylation of M-VIII, the starting substrate of the mycinamicin post-PKS tailoring pathway. In addition, we have demonstrated that the MycG mono oxygenase catalyzes sequential hydroxylation and epoxidation steps with M-IV or M-V as substrates. MycCI represents the first biosynthetic methyl group hydroxylase characterized in vitro. Its optimal activity depends on MycCII, a ferredoxin whose gene is encoded within the mycinamycin biosynthetic gene cluster. MycG is the first biosynthetic mono oxygenase to be characterized whose physiological function includes hydroxylation and epoxidation reactions. Moreover, the unprecedented requirement of the second deoxysugar with essential methylether modifications for activity of MycG reveals the important interplay between three typical posttailoring modifications in biosynthesis, including glycosylation, methylation, and oxidation. Through detailed functional analysis of these two P450 enzymes, we have unambiguously established the mycinamicin post-PKS oxidation pathway.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise specified, all chemical reagents were ordered from Sigma-Aldrich. DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Molecular cloning used New England Biolabs (Ipswich, MA) restriction enzymes, Stratagene (La Jolla, CA) Pfu Turbo DNA polymerase, Novagen (Madison, WI) pET vectors, Invitrogen (Carlsbad, CA) T4 DNA ligase, and Z-Competent *Escherichia coli* Transformation Buffer set from Zymo Research (Orange, CA). Protein purification used QIAGEN (Valencia, CA) Ni-NTA resin, Millipore (Billerica, MA) Amicon Ultra centrifugal filter, PD-10 desalting columns from GE Healthcare (Piscataway, NJ), and Thrombin restriction grade Kit from Novagen (Madison, WI). LB Broth was from EM Sciences (Gibbstown, NJ). All mycinamicins except M-VIII were obtained from the fermentation broth of *M. griseorubida* A11725. M-VIII was synthesized from PML-IV using the method described above.

DNA Manipulation, Cloning, and PCR

Using cosmid pMR01 (Anzai et al., 2003) as template, *mycCI*, *mycCII*, and *mycG* genes were amplified by PCR under standard conditions with primers as follows: forward, 5′-CAGCATATGGTGGTCTGGCCCATGGACCGCACCTG-3′ for *mycCI*, 5′-GTGCCATATGCGGATAGTCCTGGACGCCGAAC-3′ for *mycCII*, and 5′-CGGTCATATGACTTCAGCTGAACCTAGGGCGTATCC-3′ for *mycG* (the underlined bases represent the introduced *Nde*I site for further cloning); reverse, 5′-TCGT*AAGCTT*CCACTCGACCAGCAGCTCGTCGATG-3′ for cloning *mycCI* gene without a stop codon, 5′-TCC*AAGCTT*TCCGCATACCG CACCCCCATTCGTC-3′ for amplifying the *mycCI* gene retaining a stop codon, 5′-TGAC*AAGCTT*ACTCCTGTTGGCCCACCTGTCCCGTG-3′ for *mycCII*, and 5′-GGCA*AAGCTT*CTCCGACGAGATCGTCGAGATCGAC-3′ for *mycG* (the italic letters indicate a *Hind*III restriction site for later cloning). The gel purified cDNAs were rescued by double digestion of *Nde*I and *Hind*III. Then, the fragment containing the *mycCI* gene with stop codon removed was ligated into *Nde*I/*Hind*III treated pET21b to generate recombinant plasmid pET21b-*mycCI* for expression of C-terminal His-tagged MycCI. Genes retaining stop codons, including *mycCI*, *mycCII*, and *mycG*, were ligated into previously *Nde*I/*Hind*III-digested pET28b to generate recombinant plasmids for expression of N-terminal His-tagged proteins. After confirming the identity of inserted genes by DNA sequencing, the constructs were used to transform *E. coli* BL21(DE3) for protein overexpression.

Protein Overexpression and Purification

MycCI and MycG (P450s) overexpression and purification followed previously developed procedures with minor modifications (Li et al., 2007; Xue et al., 1998). The *E. coli* BL21 (DE3) transformants carrying certain plasmids were grown at 37°C in 1 l of LB broth containing thiamine (1 mM), 5% glycerol, 50 μg/ml selective antibiotics (ampicillin for pET21b and kanamycin for pET28b), and a rare salt solution (6750 μg/l $FeCl_3$, 500 μg/l $ZnCl_2$, $CoCl_2$, Na_2MoO_4, 250 μg/l $CaCl_2$, 465 μg/l $CuSO_4$, and 125 μg/l H_3BO_3) until OD_{600} reached 0.6–0.8. Then isopropyl β-D-thiogalactoside (IPTG, 0.1mM) and δ-aminolevulinic acid (1mM) were added, and the cells were cultured at 18°C overnight. After harvesting cells by centrifugation, 40 ml of lysis buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 10% glycerol, and 10 mM imidazole) was used to resuspend the cell pellet. Lysis was accomplished on a Model 500 Sonic Dismembrator (ThermoFisher Scientific). The insoluble material was separated by centrifugation (35,000 × g, 30 min at 4°C). The soluble fraction was collected and incubated for 1 h at 4°C after addition of 1ml Ni-NTA resin. The slurry was loaded onto an empty column, and the column was then washed with 40 to 80 ml of wash buffer (50 mM NaH_2PO_4 [pH8.0], 300 mM NaCl, 10% glycerol, and 20–30 mM imidazole). The elution buffer (50 mM NaH_2PO_4 [pH8.0], 300 mM NaCl, 10% glycerol, and 250 mM imidazole) was added onto the column, and eluted protein fraction was concentrated with Amicon Ultra 4, Ultracel −30K. Subsequent desalting was attained by buffer exchange into desalting buffer (50 mM NaH_2PO_4 [pH7.3], 1 mM EDTA, 0.2 mM dithioerythritol, and 10% glycerol) with a PD-10 column.

Overexpression and purification of N-terminal 6× His-tagged ferredoxin MycCII-NH are similar to above procedures with minor modifications as follows: 1) δ-aminolevulinic acid, the precursor for heme biosynthesis, was omitted in the culture broth; and 2) the protein obtained from elution buffer was concentrated using Amicon Ultra 4, Ultracel −5K because of the low molecular weight of MycCII (~10 kDa). To obtain MycCII-wt lacking a His-tag, 2.4 mg of MycCII-NH in desalting buffer was digested by 2 units of thrombin at 4°C overnight. The cleaved His-tag and residual thrombin were removed by use of Ni-NTA resin and a 30K size exclusion filter sequentially. The concentration of purified ferredoxins was determined by Coomassie protein assay using spinach ferredoxin as standard.

CO-Bound Reduced Difference Spectra

The identification of MycCI and MycG as active P450 enzymes was performed through getting the CO-bound reduced difference spectra using a UV-visible spectrophotometer 300 Bio (Cary). First, the P450 enzyme in desalting buffer was reduced by adding several milligrams of sodium dithionite ($Na_2S_2O_4$), and a spectrum was recorded from 350 to 600 nm. Then, after CO bubbling of the solution for 30–60 sec, the spectrum of CO-bound reduced P450 species was recorded using previous reduced spectrum as reference. This assay was also used to determine the functional P450 concentration using the extinction coefficient of 91,000 $M^{-1} \cdot cm^{-1}$ (Omura and Sato, 1964).

Functional Analysis of In Vitro Activities of MycCI and MycG

The standard conversion was accomplished by combining 1 μM of desalted MycCI or MycG, whose functional concentrations were determined using UV-visible absorption spectrum method (Omura and Sato, 1964), 0.5 mM mycinamicin biosynthetic intermediate, 3.5 μM spinach ferredoxin or MycCII ferredoxin, 0.1 U/ml spinach ferredoxin-$NADP^+$ reductase, and 0.5 mM NADPH in 100 μl of desalting buffer. The reaction with boiled P450 enzyme was used as a negative control. The reaction was performed for 2 h at 30°C and was terminated by extraction, using 3 × 200 μl of $CHCl_3$. The resulting organic extraction was dried and redissolved in 150 μl of methanol. The LC-MS analysis of reaction extract was performed on LCMS-2010 EV (Shimadzu) by using an XBridge™ C18 3.5 μm 150 mm reverse-phase HPLC column under following conditions: mobile phase, 20–100% solvent B over 18 min (A = deionized water + 0.1% formic acid, B = acetonitrile + 0.1% formic acid); flow rate: 0.2 ml/min; UV wavelength: 240 and 280 nm.

Spectral Substrate Binding Assay

Spectral substrate binding assay was performed on UV-visible spectrophotometer 300 Bio (Cary) at room temperature by titrating substrate DMSO solution (blank DMSO for reference group) into 1 ml of 0.5–1 μM P450 solution in 1 μl aliquots. The series of Type I difference spectra were used to deduce

ΔA ($A_{peak} - A_{trough}$). Then, the data from duplicated experiments were fit to Michaelis-Menten equation to obtain the dissociation constant K_d.

Spectral Ferredoxin Binding Assay

Spectral ferredoxin titrations were performed as described elsewhere (Coghlan and Vickery, 1991) using 0.7 μM MycCI and 10 μM M-VIII in desalting buffer at room temperature. Ferredoxin solutions in appropriate concentrations were used to titrate the P450 solution. Binding of ferredoxins to MycCI induced increased M-VIII binding, thus leading to a larger absorbance difference ΔA ($A_{peak} - A_{trough}$). The data from duplicated experiments were linearized by using a Hanes-Wolf plot to deduce dissociation constant K_d.

Steady-State Kinetics of MycCI and MycG

The standard reactions contain 0.6 μM of MycCI (when partnered with spinach ferredoxin), 0.1 μM of MycCI (when partnered with MycCII-NH), or 0.1 μM of MycG, 1.9 μM spinach or MycCII ferredoxins, 0.02 U/ml spinach ferredoxin-$NADP^+$ reductase, 5–200 μM M-VIII for MycCI and 2–160 μM M-V for MycG in 90 μl of desalting buffer. After preincubation in 96-well plate at room temperature for 5 min, the reactions with different substrate concentrations were initiated spontaneously by adding 10 μl of 2 mM NADPH with a multichannel pipette. The rate of NADPH consumption was monitored continuously over 2 min under 340 nm by SpectraMax M5 spectrophotometer (Molecular Devices). The initial velocities of NADPH consumption were deduced from the absorbance curves within the linear range (0–20 s). Then, assuming a 1:1 stoichiometric relationship between NADPH consumption and substrate oxidation, the initial velocities of hydroxylation reactions were calculated by using the millimolar absorption coefficient 6.22 $mM^{-1} \cdot cm^{-1}$ of NADPH at 340 nm (Greenbaum et al., 1972). Finally, the results from duplicated experiments were fit to Michaelis-Menten equation to obtain steady-state kinetic parameters.

SUPPLEMENTAL DATA

Supplemental data include five figures and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/9/950/DC1/.

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