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Letters

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Stereospecificity of Enoylreductase Domains from Modular Polyketide Synthases

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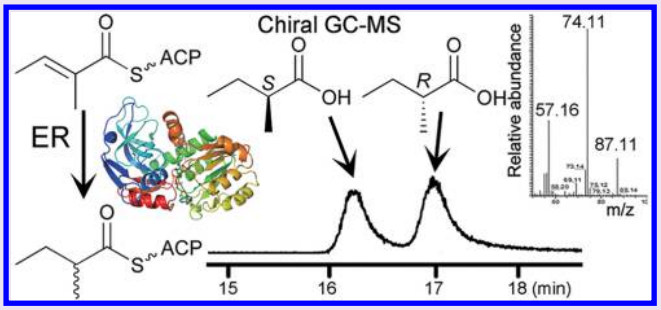
Supporting Information

ABSTRACT: An enoylreductase (ER) domain of a polyketide synthase module recruiting a methylmalonate extender unit sets the C2 methyl branch to either the *S* or *R* configuration during processing of a polyketide intermediate carried by an acyl carrier protein (ACP) domain. In the present study, pantetheine- and ACP-bound *trans*-2-methylcrotonyl substrate surrogates were used to scrutinize the stereospecificity of the ER domains. The pantetheine-bound thioester was reduced to mixtures of both 2*R* and 2*S* products, whereas the expected 2*S* epimer was almost exclusively generated when the cognate ACP-bound substrate surrogate was utilized. The analogous incubation of an ER with the substrate surrogate carried by a noncognate ACP significantly increased the generation of the unexpected 2*R* epimer, highlighting the dependence of stereospecificity on proper protein−protein interactions between ER and ACP domains. The ER mutant assays revealed the involvement of the conserved tyrosine and lysine in stereocontrol. Taken together, these results expand the current understanding of the ER stereochemistry and help in the engineering of modular PKSs.



Polyketides are a large group of natural products with diverse bioactivities, including antibacterial, antifungal, antitumor, and immunosuppressive properties.[1,2] Many polyketides are assembled by modular polyketide synthases (PKSs) from coenzyme A (CoA) activated carboxylic acids in a manner resembling fatty acid biosynthesis. However, a considerably wider variety of carboxylic acids can be utilized by modular PKSs to generate enormous chemical diversity. A ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) constitute a minimal module that is responsible for the elongation of a polyketide chain, whereas the combination of ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains in a module controls the final oxidation state of the extender unit newly added to the growing polyketide chain. The resulting polyketides are stereochemically dense due to the methyl-bearing chiral centers arising from the incorporation of methylmalonate extender units and the hydroxyl-bearing chiral centers introduced by KRs.[3,4]

The past two decades have seen considerable progress in understanding the way that modular PKSs control the stereochemistry of methyl and hydroxyl substituents and in relating the stereochemical outcomes to the intrinsic specificity of the catalytic domains. An AT recruits exclusively the (2*S*)-isomer of methylmalonyl-CoA, while a KS catalyzes the decarboxylative condensation of an extender unit with the inversion of configuration at the C2 position to generate a (2*R*)-2-methyl-3-ketoacyl-ACP intermediate.[5,6] Significant efforts have been made to determine the stereochemistry of KR-catalyzed reactions, since they determine the configuration of both C2 methyl and C3 hydroxyl of polyketide chains.[7] *In vivo* studies demonstrate that swapping KR domains between different modular PKSs results in the generation of polyketides with altered stereochemistry, suggesting that stereospecificity is an intrinsic property of KR domains and is transferable.[8] Structural analysis reveals the precise domain boundaries and facilitates the dissection of a complete module into discrete active domains.[9] In combination with the development of robust methods to assign the stereochemistry of the reduced products, the catalytic activities of individual KRs have been investigated intensively *in vitro*.[10] Divergent sequence motifs are correlated to the stereospecificity of KRs and used to predict the absolute configuration of polyketide products.[11,12] Alteration of the residues in these motifs can switch the stereochemical outcome of KRs at least *in vitro*.[13]

By contrast, the stereospecificity of ER domains of the modular PKSs is not well-studied. Analogous to their counterparts in fatty acid synthases (FASs), ERs of modular PKSs belong to the medium chain NAD(P)H-dependent

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dehydrogenase/reductase (MDR) superfamily.[14] In a module that recruits methylmalonyl-CoA, the ER reduces the carbon−carbon double bond of the 2-enoyl intermediate and simultaneously sets the methyl branch at the C2 position in either *R* or *S* configuration (Figure 1). *In vivo* functional assays

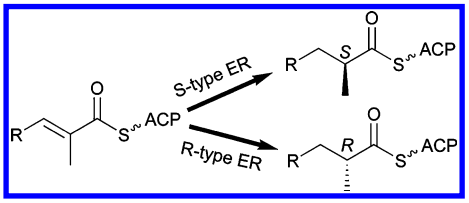

Figure 1. The α-methyl substituent set to either *S* or *R* configuration in the reduction of the double bond by the ER domain of a modular polyketide synthase.

within the context of a complete module reveal a correlation between a unique residue in the ER active site and chirality of the generated methyl branch.[15] In an ER producing an *S*-configured methyl branch, this residue is systematically conserved as tyrosine, while in the ER domains producing an *R*-configured methyl branch, valine (occasionally alanine or phenylalanine) is observed at this position. A single mutation of tyrosine to valine in the ER from module 4 of erythromycin PKS (EryER4) switches the configuration of the corresponding methyl branch from *S* to *R*. We have solved a KR-ER didomain structure from the second module of spinosyn PKS.[16] This didomain structure defines the precise boundary of ER domains and facilitates the expression of SpnER2 as a discrete active domain. The inspection of the structure reveals that a lysine and an aspartic acid are situated near the nicotinamide ring of the NADPH cofactor beside the fingerprint tyrosine of *S*-type ERs. However, no single mutation alone abolishes the *in vitro* activity of SpnER2 completely, consistent with the *in vivo* mutational study on the ER domain from module 13 of rapamycin PKS (RapER13), which also fails to reveal a single residue that is essential for activity.[17]

In this study, we report the *in vitro* characterization of the stereospecificity of ER domains from the modular PKSs. The stereochemical control of isolated KRs has been extensively assayed, and it revealed the potential of several KRs as catalysts for the stereospecific reduction of ketones in organic synthesis. But, to date, SpnER2 is the only isolated ER domain of the modular PKS characterized *in vitro*.[16] To investigate the *in vitro* stereospecificity of discrete ER domains, two additional ERs from the fourth modules of erythromycin and pikromycin PKSs were also expressed in *Escherichia coli* BL21 (DE3). The boundaries of ER domains were chosen using sequence alignment with SpnER2, of which two β-strands (β1 and β9) in the substrate-binding subdomain were defined as the N-terminus and C-terminus, respectively. A high level of overall sequence identity was observed between SpnER2 and EryER4 (54%), SpnER2 and PikER4 (65%), and EryER4 and PikER4 (56%). All these ER domains were expressed in a soluble form with average yields of more than 5 mg/L. SpnER2 has been previously shown to reduce *trans*-α,β-unsaturated crotonyl-pantetheine. Although there is no methyl branch on α-carbon of the natural substrate *trans*-(5*S*)-hydroxyhept-2-enoyl-SpnACP2 thioester,[18] SpnER2 could indeed reduce this substrate carrying an α-methyl branch (Figure 2). The high-performance liquid chromatography (HPLC) analysis of the reaction mixtures showed the generation of a reduced product, which was verified to be 2-methylbutyryl-pantetheine by the mass spectrum (HR-ESI-MS *m*/*z* 385.1767 observed; calculated for $C_{16}H_{30}N_2NaO_5S$ 385.1768 $[M + Na]^+$). As shown in Figure 2B, EryER4 and PikER4 could also reduce the *trans*-2-methylcrotonyl-pantetheine substrate surrogate (Figure 2B). N-acetyl cysteamine (NAC) and coenzyme A (CoA) derived thioesters were also synthesized, whereas the reduced product was not observed when they were incubated with these ERs in the same reaction condition (Figure S1). It has been reported that an ER domain from a highly reducing iterative fungal PKS also prefers pantetheine-bound substrate to the corresponding NAC thioesters.[19]

A convenient and sensitive protocol to resolve the two isomers of 2-methylbutyric acid by the chiral gas chromatographic/mass spectrometric (GC/MS) method has been developed previously and used to determine the *in vitro* stereospecificity of the loading AT from avermectin PKS toward the racemic 2-methylbutyryl-NAC thioester.[20] The direct comparison of the experimentally measured retention

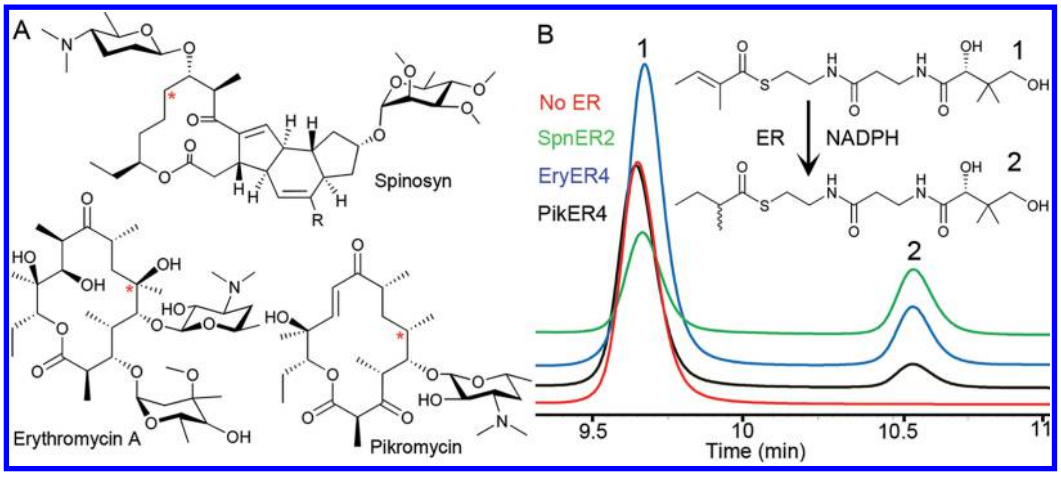

Figure 2. ER-catalyzed reduction of *trans*-2-methylcrotonyl-pantetheine. (A) Structure of spinosyn, erythromycin, and pikromycin. The methyl substituents in which the stereochemistry is controlled by EryER4 and PikER4 are labeled by a red asterisk. SpnER2 catalyzes the reduction of the polyketide intermediate without the α-methyl branch in the biosynthesis of spinosyn. (B) All three tested ERs can reduce the pantetheine-bound substrate surrogate.

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times obtained using a β-DEX 120 capillary column, as well as the corresponding mass spectra with those of the authentic standards, could enable the determination of the absolute configuration and the relative yield of each 2-methylbutyric acid isomer (Figure 3). To establish the stereochemical course of

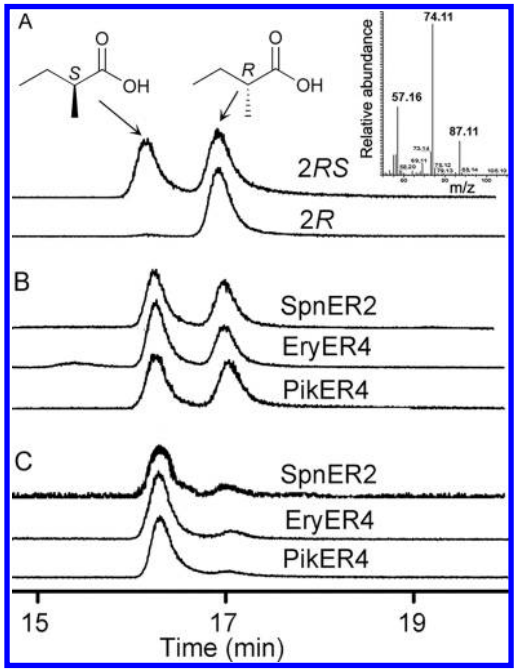

Figure 3. Stereospecificity of ER domains. (A) The two isomers of 2-methylbutyric acid can be resolved by chiral gas chromatography using a β-DEX 120 capillary column and show identical fragmentation patterns in the mass spectra. (B) Both 2*S* and 2*R* products were produced in the reduction reactions of *trans*-2-methylcrotonyl-pantetheine catalyzed by SpnER2, EryER4, and PikER4. (C) Each ER almost exclusively reduced its cognate *trans*-2-methylcrotonyl-ACP to the expected 2*S* product.

ER-catalyzed reduction, the reactions were quenched after 48 h of incubation by the addition of an aqueous base. Following acidification and extraction, the resulting 2-methylbutyric acid was subjected to chiral GC/MS analysis to assign the stereochemistry of the C2 methyl branch. SpnER2 contains a tyrosine at the corresponding position of the active site, suggesting its function as an *S*-type ER. However, the expected 2*S* isomer only constituted ~60% of all reduced products (Figure 3B). Due to the absence of a C2 methyl branch in the natural substrate, SpnER2 may not be exposed to the corresponding evolutionary pressure. We sought to determine the stereochemical outcome of the reactions catalyzed by the recombinant EryER4 and PikER4, which incorporated *S*-configured methyl substituents into erythromycin and pikromycin, respectively (Figure 2A), consistent with the conserved tyrosine at their active sites. Intriguingly, incubation of *trans*-2-methylcrotonyl-pantetheine with EryER4 and PikER4 also generated both 2*S* and 2*R* products (Figure 3B). More than 60% of the substrate still remained after the reactions were incubated for 48 h (Figure S2). We determined the *R*/*S* ratios at 12, 24, 36, and 48 h. Modest fluctuations were revealed, suggesting that the *R*- and *S*-isomers were likely produced at similar rates.

The effects of ACPs on the stereospecificity of ERs are assayed, since all intermediates are tethered to ACPs during the processing of polyketide intermediates. The *trans*-2-methyl-crotonyl-ACPs were enzymatically synthesized from the corresponding CoA thioester by the promiscuous *Sfp* phosphopantetheinyl transferase from *Bacillus subtilis*.[21] The reduced product was treated with trypsin protease before base-catalyzed hydrolysis to increase the release of 2-methylbutyric acid from the phosphopantetheine arm of the ACP domain. It has been reported previously that the ACP-bound thioesters are more stable compared with their corresponding NAC thioesters. A hypothesis that an ACP stabilizes the phosphopantetheinate-tethered substrate by accommodating it in a cavity has been proposed.[10] Notably, all three ER domains retained their intrinsic stereospecificity toward the cognate ACP-bound substrate surrogates and predominantly yielded the expected (2*S*)-methylbutyryl-ACP (Figure 3C). Analogous incubations were performed using ERs and noncognate ACPs (Table S1). EryER4 and PikER4 reduced the SpnACP2-bound substrate to the expected 2*S* product, whereas a significant amount of unexpected (2*R*)-methylbutyryl-ACPs was produced by the paired SpnER2 + EryACP4, SpnER2 + PikACP4, EryER4 + PikACP4, and PikER4 + EryACP4, highlighting the dependence of ER stereospecificity on proper protein−protein interactions between ER and ACP domains. Conversely, stereochemistry of the reduced 2-methyl-3-hydroxyacyl-ACP was directly correlated with the specificity of the relevant KR domain, independent of which the ACP was used to generate the initial 2-methyl-3-ketoacyl-ACP intermediate.[22] Crystal structures of an AT−ACP complex from the vicenistatin PKS and a curacin β-branching enzyme in complex with ACP indicate that the ACP surface of αII, loop II, and αIII interacts with partner enzymes.[23,24] The SpnACP2 surface for ER interaction is likely more promiscuous compared with EryACP4 and PikACP4. Pairwise sequence alignments of three ACPs suggested a high degree of sequence identity (SpnACP2-EryACP4 51%, SpnER2-PikACP4 54%, and EryACP4-PikACP4 58%) but revealed two conserved aspartate residues on the tentative contact surface of EryACP4 and PikACP4. The distinctive surface shape and charge distribution of ACPs may contribute to their specificity against ERs (Figure S3). A structure of the ER-ACP complex will improve our understanding of the basis of ACP recognition for ER stereochemistry.

The mechanism of ER-catalyzed reduction assumes a direct transfer of a hydride from NADPH to the C3 position of the enoyl substrate followed by stereospecific protonation at the C2 position, which determines the configuration of the methyl substituent.[15] A recently proposed ene mechanism involves the formation of a transient covalent NADPH-substrate adduct ("C2-ene adduct") in the transfer of the hydride equivalent.[25] The C2-ene adduct is further converted to the consensus enolate intermediate, which accepts a proton to form the reduced product. In either case, a proper proton donor at the active site is indispensable and determines the configuration of the C2 methyl branch in the reductions catalyzed by ERs of modular PKSs. The structure of SpnER2 reveals that three conserved residues Y241, K422, and D444 are ~6 Å from the nicotinamide hydride (Figure 4). We sought to identify the effects of these residues on the stereospecificity of ERs. K422 and D444 were mutated to alanine to determine the contributions of the side chains, while Y241 was mutated to phenylalanine to abolish its potential function as a proton donor. The D444A of SpnER2 produced the 2*S* product exclusively, whereas Y241F and K422A mutants lost the

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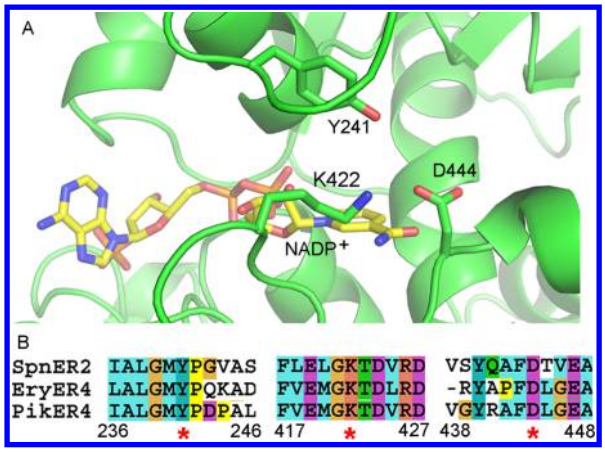

Figure 4. Active site of SpnER2. (A) The structure shows the SpnER2 active site. Conserved catalytic residues (Y241, K422, and D444) and $NADP^+$ coenzyme are shown as sticks. (B) Sequence alignment of SpnER2, EryER4, and PikER4. Residues are numbered according to SpnER2. The catalytic residues are labeled by a red asterisk.

stereospecificity toward the ACP-bound substrate thioester and produced a mixture of both 2*S* and 2*R* products (Table S2). Similar results were obtained for the corresponding mutants of EryER4 and PikER4, suggesting that the conserved tyrosine and lysine are involved in the stereocontrol of ER domains. An *in vivo* functional assay of EryER4 shows that the single mutation of tyrosine to valine switches the configuration of the resulting methyl branch from *S* to *R* completely.[15] Surprisingly, both *S*-type (~50%) and *R*-type (~50%) products were generated *in vitro* by the tyrosine to valine mutants of SpnER2, EryER4, and PikER4 (Table S2). In addition to the relatively shorter acyl chain compared with the native substrate, other catalytic domains in the PKS modules may also affect the stereospecificity of ERs. More sensitive experiments are necessary to clarify the exact role of each catalytic residue.

In summary, understanding the molecular basis for the stereospecificity of modular PKSs is crucial for production of novel polyketides by protein engineering. In this study, pantetheine and ACP bound *trans*-2-methylcrotonyl substrate surrogates were incubated with discrete ERs from different modular PKSs in the presence of NADPH. Following the release of 2-methylbutyric acid by base-catalyzed hydrolysis, the stereochemistry of the reduced product was assigned by chiral GC/MS. The dependence of ER stereospecificity on cognate ACP was revealed with this method. The effects of catalytic residues on the stereospecificity of ERs were investigated in combination with site-specific mutagenesis. Taken together, the results presented in this report expand the current understanding of ER stereochemistry and could facilitate the rational engineering of modular PKSs.

METHODS

See the Supporting Information for a detailed description of the experimental methods.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00982.

Supporting tables, supporting figures, and detailed methods that describe expression and purification of ERs; site-directed mutagenesis; synthesis of *trans*-2-methylcrotonyl-CoA; reduction of thioester substrate and *trans*2-methylcrotonyl-ACP; chiral GC/MS; and homology modeling (PDF)

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Notes

The authors declare no competing financial interest.

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