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Stereochemistry of Reductions Catalyzed by Methyl-Epimerizing Ketoreductase Domains of Polyketide Synthases

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Supporting Information

ABSTRACT: Ketoreductase (KR) domains from modular polyketide synthases (PKSs) catalyze the reduction of 2-methyl-3-ketoacyl acyl carrier protein (ACP) substrates and in certain cases epimerization of the 2-methyl group as well. The structural and mechanistic basis of epimerization is poorly understood, and only a small number of such KRs been studied. In this work, we studied three recombinant KR domains with putative epimerase activity: NysKR1 from module 1 of the nystatin PKS, whose stereospecificity can be predicted from both the protein sequence and the product structure; RifKR7 from module 7 of the rifamycin PKS, whose stereospecificity cannot be predicted from the protein sequence; and RifKR10 from module 10 of the rifamycin PKS, whose specificity is unclear from both the sequence and the structure. Each KR was individually incubated with NADPH and (2*R*)- or (2*RS*)-2-methyl-3-ketopentanoyl-ACP generated enzymatically in situ or via chemoenzymatic synthesis, respectively. Chiral GC−MS analysis revealed that each KR stereospecifically produced the corresponding (2*S*,3*S*)-2-methyl-3-hydroxypentanoyl-ACP in which the 2-methyl substituent had undergone KR-catalyzed epimerization. Thus, our results have led to the identification of a prototypical set of KR domains that generate (2*S*,3*S*)-2-methyl-3-hydroxyacyl products in the course of polyketide biosynthesis.

Polyketides, which are produced by *Streptomyces* and other Actinomycetes and display a wealth of useful antibiotic, antitumor, and immunosuppressive activities, are notable for their intricate structures and stereochemical complexity. The majority of such polyketides are biosynthesized under the control of modular polyketide synthases (PKSs), which are multi-subunit megaenzymes in which each protein module is responsible for a single round of polyketide chain elongation and β-carbon modification under the control of proteins closely related in structure and mechanism to the well-characterized enzymes of fatty acid biosynthesis.[1] Each module contains a core set of three protein subunits: an acyl carrier protein (ACP) domain, to which the growing polyketide chain is covalently tethered by a characteristic 18 Å pantetheinyl prosthetic group; an acyltransferase, which is responsible for the loading of specific chain extender units, most often methylmalonyl- or malonyl-CoA, onto the terminal thiol of the ACP pantetheinate group; and a ketosynthase domain, which acquires the growing polyketide chain from the immediately upsteam module and then catalyzes a decarboxylative condensation with the ACP-bound methylmalonyl or malonyl unit to generate a chain-extended (2*R*)-2-methyl-3-ketoacyl-ACP or 3-ketoacyl-ACP polyketide stereospecifically. Individual modules can also harbor a specific subset of β-carbon-processing enzymes, including a ketoreductase (KR) domain, a dehydratase (DH) domain, and/or an enoylreductase (ER) domain. The resultant extended and modified ACP-bound polyketide intermediate is then passed to the immediately downstream KS domain for another round of chain elongation and β-carbon modification until the full-length, mature polyketide is finally released, often by cyclization to a macrolactone catalyzed by a dedicated thioesterase (TE) domain located at the C-terminus of the module furthest downstream.

Although each PKS domain has been shown to have a high level of substrate stereoselectivity and to act in a completely stereospecific manner,[1] the ultimate stereochemistry of the vast majority of both hydroxyl- and methyl-bearing stereocenters in the resultant complex polyketides is controlled primarily by the KR domains of the PKS modules. For example, we have shown that in the biosynthesis of 6-deoxyerythronolide B (**1**, 6-dEB), the parent macrolide aglycone of the erythromycin antibiotics, the configurations of the vicinal hydroxyl and methyl substituents at C-13/C-12, C-11/C-10, C-5/C-4, and C-3/C-2 are set by the EryKR1, EryKR2, EryKR5, and EryKR6 domains of modules 1, 2, 5, and 6 of 6-dEB synthase, respectively (Figure 1).[2,3]

Understanding the structural and mechanistic basis for the stereochemical control of KR-catalyzed reactions is therefore a major challenge to the detailed biochemical understanding and rational engineering of PKS enzymology. The stereochemistry

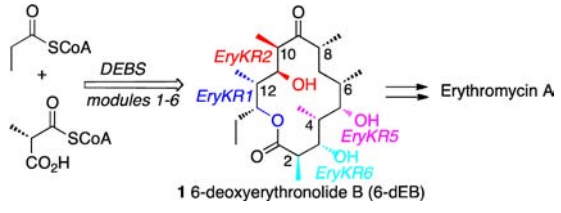

Figure 1. Biosynthesis of 6-deoxyerythronolide B (**1**, 6-dEB) by 6-dEB synthase, showing the stereochemistry of the methyl and hydroxyl centers controlled by the designated ketoreductase (EryKR) domains.

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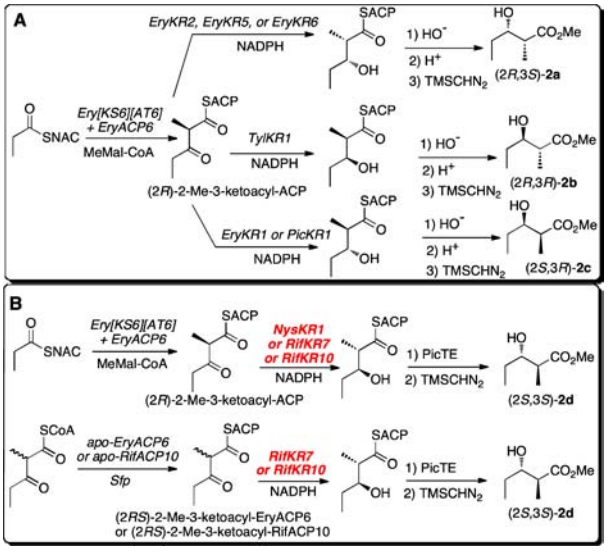
of the reductions catalyzed by KR domains is an intrinsic property that is independent of either the modular context or substrate structure, including the substitution pattern and chain length, as first demonstrated by engineered replacement of native KR domains by alternative KR domains from heterologous PKS modules.[4] A major advance in the understanding of KR stereospecificity was the discovery that incubation of recombinant EryKR1 or TylKR1 (from module 1 of tylactone synthase) with NADPH and racemic (±)-(2*RS*)-2-methyl-3-ketopentanoyl-SNAC thioester (SNAC = *N*-acetylcysteamine) resulted in the exclusive formation to the corresponding (2*S*,3*R*)- and (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-SNAC products, respectively, thereby demonstrating that KR domains are capable of diastereospecific reductions that fix not only the target hydroxyl configuration but that of the vicinal methyl group as well.[5] On the other hand, these experiments could not distinguish whether the KR domains simply select the correct diastereomer of the substrate or if they also can catalyze epimerization of the methyl substituent itself. Unfortunately, attempts to extend these findings to other KR domains using SNAC thioester substrates resulted in a complete loss of stereochemical control.

We previously reported that the intrinsic stereospecificity of recombinant KR domains can be fully preserved during in vitro experiments if the 2-methyl-3-ketoacyl substrate is covalently tethered to an ACP domain.[2,6] Thus, incubation of a recombinant ketosynthase−acyltransferase ([KS][AT]) didomain and an ACP derived from the same PKS module with an acyl-SNAC thioester and a methylmalonyl-CoA extender unit generates exclusively the corresponding (2*R*)-2-methyl-3-ketoacyl-ACP intermediate, which can then be reduced in situ by the KR domain of interest in the presence of NADPH cofactor (Scheme 1A). The hydrolysis and derivatization of the resultant ACP-bound 2-methyl-3-hydroxyacyl thioester product can then be followed by chiral GC−MS analysis, including direct comparison with synthetic standards of known structure and stereochemistry.[2,6] In this manner, we established that EryKR2, EryKR5, and EryKR6 all exclusively generate (2*R*,3*S*)-2-methyl-3-hydroxypentanoate [(2*R*,3*S*)-**2a**], corresponding precisely to the configuration of the corresponding vicinal hydroxyl and methyl substituents of 6-dEB (Figure 1),[2] while TylKR1 gives the corresponding (*R*,*R*)-configured isomer (2*R*,3*R*)-**2b**.[2b,6] Finally, both EryKR1 and PicKR1 (from picromycin synthase) catalyze both reduction *and epimerization* of the initially generated (2*R*)-2-methyl-3-ketoacyl-ACP intermediate to give (2*S*,3*R*)-**2c** exclusively.[2b] Kinetic analysis confirmed that epimerization of the methyl group is catalyzed exclusively by the KR domain itself prior to diastereoselective reduction of the transient (2*S*)-2-methyl-3-ketoacyl-ACP intermediate.[2b]

Scheme 1. Stereochemistry of KR-Catalyzed Reduction of 2-Methyl-3-ketoacyl-ACP Intermediates: (A) Reductions Catalyzed by EryKR2, EryKR5, EryKR6, and TylKR1 and Epimerization/Reductions Catalyzed by EryKR1 and PicKR1; (B) Epimerization/Reductions Catalyzed by NysKR1, RifKR7, and RifKR10



Although that previous work established the stereospecificity of a variety of KR domains that generate three of the four possible diastereomeric 2-methyl-3-hydroxyacyl-ACP products, the generation of the remaining (2*S*,3*S*)-2-methyl-3-hydroxyacyl-ACP stereoisomers has not yet been systematically examined. An understanding of the formation of these diastereomers is essential for the elucidation of the biochemical basis for KR-catalyzed methyl group epimerization as well as for the correlation of KR structure and amino acid sequence motifs with intrinsic reductase stereochemistry and epimerase activity.[7]

The first potential epimerase/ketoreductase that we examined was NysKR1 from module 1 of nystatin synthase.[10] NysKR1 was expected to generate a (2*S*,3*S*)-2-methyl-3-hydroxyacyl-ACP product, as suggested by both the known structure and absolute configuration of the relevant centers in nystatin (Figure 2A) as well as a correlation with the conserved sequence motifs of five other KR domains whose stereospecificities have been inferred from the structure and stereochemistry of the derived polyketides (Figure 3).[8a] Recombinant NysKR1 was constructed with boundaries corresponding to the region encompassed by the conserved Y[989]RVTW motif at the N-terminal boundary of the NysKR1

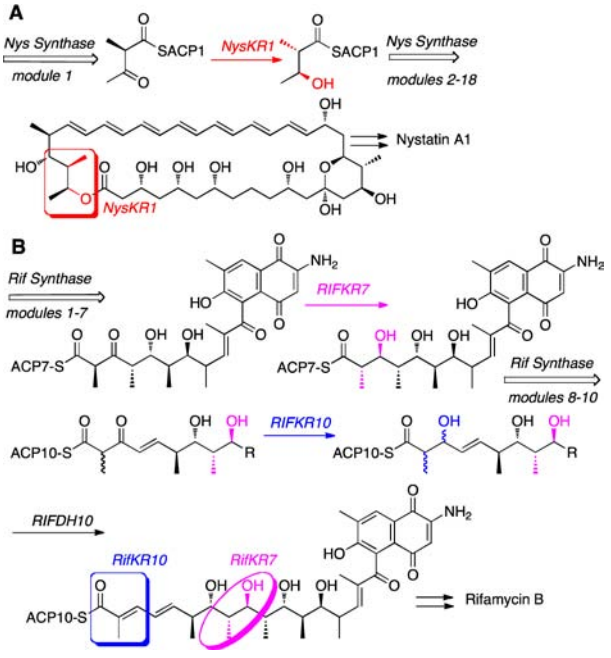


Figure 2. Postulated reductions carried out by (A) NysKR1 in nystatin biosynthesis and (B) RifKR7 and RifKR10 in rifamycin biosynthesis.

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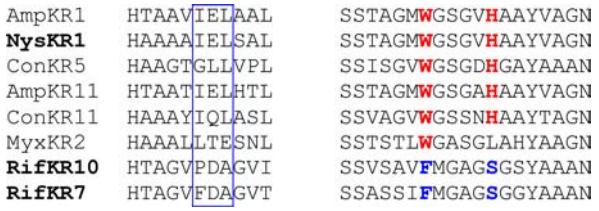

Figure 3. Sequence alignments of KR domains known or predicted to generate (2*S*,3*S*)-2-methyl-3-hydroxyacyl-ACP products. The boxed region indicates the corresponding sites of the conserved LDD motif in (3*R*)-hydroxyl-specific KR domains.

structural subdomain up to R1472 at the conserved RLHGL boundary of the NysACP1 domain (Figures S1 and S2 in the Supporting Information).[11] To establish the stereochemistry of the NysKR1-catalyzed epimerization/reduction, recombinant NysKR1 and NADPH were incubated with (2*R*)-2-methyl-3-ketopentanoyl-EryACP6 generated in situ by decarboxylative condensation of propionyl-SNAC and methylmalonyl-CoA using the previously described combination of Ery[KS6][AT6] and EryACP6 (Scheme 1B).[2,6] Hydrolytic release of the resultant 2-methyl-3-hydroxypentanoic acid using the picromycin synthase thioesterase domain (PicTE)[2,12] followed by chiral GC−MS analysis of the derived methyl ester as described previously[2b] established the exclusive formation of (2*S*,3*S*)-**2d** (Scheme 1B, Table S3, and Figures S9 and S10), as had been predicted both from the structure of nystatin (Figure 2A) and the sequence of NysKR1 (Figure 3).[8a,13]

We next turned to an examination of RifKR7 from module 7 of the rifamycin PKS.[14] RifKR7 was predicted to generate a (2*S*,3*S*)-2-methyl-3-hydroxyacyl intermediate on the basis of the established modular organization of the rifamycin PKS in combination with the known structure and absolute configuration of the relevant center in rifamycin (Figure 2B). On the other hand, although sequence alignments revealed that RifKR7 lacks the conserved LDD motif that has been correlated with the predicted formation of (3*R*)-hydroxyacyl products,[9] it also lacks the characteristic pair of conserved Trp and His residues present in NysKR1 and related domains that have been correlated with the formation of (2*S*,3*S*)-2-methyl-3-hydroxyacyl intermediates (Figure 3).[8a] RifKR7 instead harbors Phe and Ser residues at the corresponding amino acid positions, thereby obscuring sequence-based prediction of the intrinsic reductase stereospecficity and possible epimerase activity of RifKR7. The requisite recombinant RifKR7 protein was expressed from a synthetic gene encoding the region spanning from A1186, located four residues upstream of the conserved YRVDW motif at the N-terminal boundary of the KR7 structural subdomain, to A1610, located three residues downstream of the conserved RLAGL domain boundary of RifACP7 (Figures S3 and S4).[11] Incubation of (2*R*)-2-methyl-3-ketopentanoyl-EryACP6 generated in situ as described above with RifKR7 plus NADPH predominantly gave (2*S*,3*S*)-**2d** (80%) accompanied by minor amounts of (2*R*,3*S*)-**2a** (5%) and (2*S*,3*R*)-**2c** (15%), while treatment with RifKR7 from which the His_6 tag had been proteolytically removed gave a 90:10 mixture of (2*S*,3*S*)-**2d** and (2*S*,3*R*)-**2c** (Scheme 1B, Table S3, and Figures S9 and S10). RifKR7 is therefore stereospecific for generation of (3*S*)-hydroxyacyl-ACP groups and catalyzes epimerization of the 2-methyl-3-ketoacyl-ACP, consistent with the structure of the ultimately derived natural product rifamycin (Figure 2B).

The apparent stereospecificity of RifKR10-catalyzed reduction was even more obscure a priori, since the relevant hydroxyl- and methyl-bearing centers are absent, having been replaced in both rifamycin and its biosynthetic intermediates by a double bond generated by coupled dehydration catalyzed by the paired DH10 domain of module 10 of the rifamycin PKS (Figure 2B).[14,15] Interestingly, RifKR10 exhibits the same anomalous pair of Phe and Ser residues found in RifKR7 in place of the normally conserved Trp and His residues characteristic of NysKR1 and other (2*S*,3*S*)-2-methyl-3-hydroxyacyl-ACP-specific reductases (Figure 3). Although the desired recombinant RifKR10 protein, spanning from G2877, three residues upstream of the conserved YRVDW motif, to R3323, at the conserved RLAGL motif at the N-terminal boundary of RifACP10 (Figures S5 and S6), was initially obtained only as insoluble inclusion bodies under a wide range of conditions, the corresponding protein could be expressed in soluble form as either a C-terminal glutathione S-transferase (GST) or NusA fusion protein.[11] Incubation of recombinant RifKR10−GST plus NADPH with (2*R*)-2-methyl-3-ketopentanoyl-EryACP6 generated in situ as described above, followed by the usual PicTE-catalyzed hydrolysis and chiral GC−MS analysis of the derived methyl ester, gave (2*S*,3*S*)-**2d** exclusively (Scheme 1B, Table S3, and Figures S9 and S10). Thus, all three of the KR domains examined, NysKR1, RifKR7, and RifKR10, catalyze the reduction of the (2*R*)-2-methyl-3-ketopentanoyl-EryACP6 substrate by a mechanism in which epimerization of the 2-methyl substituent is followed by stereospecific delivery of hydride from NADPH to the *si* face of the epimerized, enzyme-bound (2*S*)-2-methyl-3-ketoacyl-EryACP6 intermediate (Scheme 1B).

In order to evaluate any influence that the structure of the covalently bound ACP domain might exert on the observed substrate specificity and stereochemistry of the RifKR-catalyzed reductions, we carried out a complementary set of experiments using chemoenzymatically prepared samples of (2*RS*)-2-methyl-3-ketopentanoyl-EryACP6 and (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10 (Scheme 1B). Thus synthetic (2*RS*)-2-methyl-3-ketopentanoyl-CoA[16] was used for covalent modification of the active-site Ser residues of recombinant *apo*-EryACP6 and *apo*-RifACP10−NusA, respectively, under the control of the surfactin phosphopantetheinyl transferase Sfp.[17] RifKR7 plus NADPH was then incubated with the resultant (2*RS*)-2-methyl-3-ketopentanoyl-EryACP6 and (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10, respectively, and subsequent PicTE-catalyzed hydrolysis followed by methylation in both cases yielded (2*S*,3*S*)-**2d** exclusively, as established by chiral GC−MS analysis (Scheme 1B, Table S3, and Figures S11 and S12).[18] Similarly, incubation of RifKR10-NusA plus NADPH with (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10 produced exclusively (2*S*,3*S*)-**2d**. Both RifKR7 and RifKR10 therefore catalyze the diastereoselective reduction of only (2*S*)-2-methyl-3-ketopentanoyl-ACP regardless of the structure of the covalently attached ACP domain (Scheme 1B, Table S3, and Figures S11 and S12).

These results expand the range of KR domains with experimentally validated stereochemistry to methyl-epimerizing ketoreductases that generate (2*S*,3*S*)-2-methyl-3-hydroxyacyl-ACP products, which are diastereomeric to those generated by the previously studied methyl-epimerizing EryKR1 and PicKR1 domains. The experimental results are clear, direct, and unambiguous and do not depend on inferences based on either PKS sequence or the structure of the ultimately formed

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parent polyketide. Investigations to reveal the stereospecificities of additional KR domains of cryptic stereochemistry that are paired with DH domains and to elucidate the biochemical mechanism of epimerization are in progress.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, sequence alignments, PKS module and domain sequences, GC−MS data, and kinetic analysis. 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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(18) Control incubations in the presence of RifKR7 or RifKR10 but without added Sfp established that neglible 2-methyl-3-hydroxypentanoate was released by PicTE-catalyzed hydrolysis of unreacted CoA thioester at the end of the incubation period. These results with (2*RS*)-2-methyl-3-ketoacyl-ACP substrates alone cannot distinguish whether the RifKR7 and RifKR10 domains can mediate epimerization of the 2-methyl group or simply catalyze the diastereoselective reduction of the (2*S*)-2-methyl-3-ketopentanoyl-ACP component of the (2*RS*) substrate, similar to the inherent ambiguity associated with the earlier results of Siskos et al.[5]) for the reduction of the corresponding racemic SNAC thioesters.