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

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Abstract

Ketoreductase (KR) domains from modular polyketide synthases catalyze reduction of 2-methyl-3-ketoacyl-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. We have studied three recombinant KR domains with putative epimerase activity – NysKR1 from module 1 of the nystatin PKS whose stereospecificity can be predicted from both protein sequence and product structure, RifKR7 from module 7 of the rifamycin PKS whose stereospecificity cannot be predicted from protein sequence, and RifKR10 from module 10 of the rifamycin PKS whose specificity is unclear from both sequence and 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 chemo-enzymatic 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 which 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), multisubunit mega-enzymes in which each protein module is responsible for a single round of polyketide chain elongation and β -carbon modification, under 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 that is responsible for the loading of specific chain extender units, most often methylmalonyl- or malonyl-CoA, onto the terminal thiol of ACP pantetheinate group, and a ketosynthase domain that acquires the growing polyketide chain from the immediately upstream module and then catalyzes a decarboxylative condensation with the ACP-bound methylmalonyl or malonyl unit to generate stereospecifically a chain-extended (2*R*)-2-methyl-3-ketoacyl-ACP or 3-ketoacyl-

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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>.

ACP polyketide. Individual modules can also harbor a specific subset of β -carbon-processing enzymes, including a ketodeuctase (KR) domain, a dehydratase (DH) domain, and/or an enoylreductase (ER) domain. The resultant extended and modified ACP-bound polyketide intermediate is then passed on 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 furthest downstream module.

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 individual ketoreductase (KR) domains of each PKS module. 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 ketoreductase domains of modules 1, 2, 5, and 6 of the 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 of the reductions catalyzed by KR domains is an intrinsic property that is independent of either modular context or substrate structure, including 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 the tylactone synthase) with NADPH and racemic ($\pm$)-(2*RS*)-2-methyl-3-ketopentanoyl-*N*-acetylcysteamine (SNAC) thioester 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 diastereo-specific 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 have reported that the intrinsic stereospecificity of recombinant KR domains can be fully preserved during *in vitro* experiments if the 2-methyl-3-ketoacyl substrates are covalently tethered to an ACP domain.^{2,6} Thus incubation of a recombinant ketosynthase-acyl transferase [KS][AT] didomain and an ACP derived from the same PKS module with an acyl-*N*-acetylcysteamine (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 resultant ACP-bound 2-methyl-3-hydroxyacyl thioester product can then be hydrolyzed and derivatized, followed by chiral GC-MS analysis, including direct comparison with synthetic standards of known structure and stereochemistry.^{2,6} In this manner, we have established that EryKR2, EryKR5, and EryKR6 each generate exclusively (2*R*,3*S*)-2-methyl-3-hydroxy-pentanoate (**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 (2*R*,3*R*)-**2b**.^{2b,6} Finally both EryKR1 and PicKR1 (from

the picromycin synthase) catalyzed both reduction and *epimerization* of the initially generated (2*R*)-2-methyl-3-ketoacyl-ACP intermediate to give exclusively the (2*S*,3*R*)-**2c**.^{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}

Although we have already established the stereospecificity of a variety of KR domains that generate 3 of the 4 possible diastereomers of 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 to the elucidation of the biochemical basis for KR-catalyzed methyl group epimerization as well as for 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 the 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 correlation with conserved sequence motifs of 5 other KR domains whose stereospecificity has 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 Y⁹⁸⁹RVTW motif at the N-terminal boundary of the NysKR1 structural subdomain up to R1472 at the conserved RLHGL boundary of the NysACP1 domain (Supporting Information, Figures S1 and S2).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 that was generated *in situ* by the decarboxylative condensation of propionyl-SNAC and methylmalonyl-CoA, using the previously described combination of Ery[KS6][ACP6] 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 previously described,^{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, based on 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 reveal that RifKR7 lacks the conserved LDD motif that has been correlated with the predicted formation of a (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 harbors instead a pair of Phe and Ser residues at the corresponding amino acid positions, thereby obscuring sequence-based prediction of the intrinsic reductase stereospecificity and possible epimerase activity of RifKR7. The requisite recombinant RifKR7 protein was expressed from a synthetic gene that encoded the region spanning from A1186, located 4 residues upstream of the conserved YRVDW motif at the N-terminal boundary of the KR7 structural subdomain, to A1610, 3-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 above using the coupled Ery[KS6][AT6] plus EryACP6 system with RifKR7 plus NADPH gave predominantly (80%) (2*S*,3*S*)-**2d**, 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 His⁶-tag had been

proteolytically removed gave a 90:10 mixture of (2*S*,3*S*)-**2d** and (2*S*,3*R*)-**2c** (Scheme 2B, 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 is 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 the 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 NysR1 and other (2*S*,3*S*)-2-methyl-3-hydroxyacyl-ACP-specific reductases (Figure 3). Although the desired recombinant RifKR10 protein, spanning from G2877, 3 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 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 exclusively methyl (2*S*,3*S*)-2-methyl-3-hydroxypentanoate (**2d**) (Scheme 2B, Table S3, Figures S9 and S10). Thus all three KR domains examined, NysKR1, RifKR7, and RifKR10, catalyze 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 of (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10 (Scheme 1B). Thus synthetic (2*RS*)-2-methyl-3-ketopentanoyl-CoA,¹⁶ was used to covalently modify the active site Ser residues of recombinant *apo*-EryACP6 and of *apo*-RifACP10-NusA, under control of the surfactin phosphopantetheinyl transferase Sfp.¹⁷ RifKR7 plus NADPH was then incubated with the resultant (2*RS*)-2-methyl-3-ketopentanoyl-EryACP6 or (2*RS*)-2-methyl-3-ketopentanoyl-RifACP10, respectively, followed by PicTE-catalyzed hydrolysis and subsequent methylation to yield in each case exclusively (2*S*,3*S*)-**2d**, as established by chiral GC-MS analysis (Scheme 1B, Table S3, 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, Figures S11 and S12).

These results expand the range of KR domains with experimentally validated stereochemistry to methylepimerizing ketoreductases that generate (2*S*,3*S*)-2-methyl-3-hydroxyacyl-ACP products, diastereomeric to those generated by the previously studied methylepimerizing 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 parent polyketide. Investigations to reveal the stereospecificity of additional KR domains of cryptic stereochemistry that are paired with DH domains and to elucidate the biochemical mechanism of epimerization are in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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11. NysKR1, RifKR7 and RifKR10 were each expressed using synthetic genes optimized for expression in *Escherichia coli* that were inserted into the NdeI and XhoI restrictions sites of pET28 and expressed as N-terminal His₆-tagged proteins in *E. coli* BL21(DE3). The domain boundaries were based on the structures of EryKR1, TylKR1, and AmpKR2 (Cf. ref 8). Each of the recombinant His₆-tag proteins was purified by immobilized metal ion affinity chromatography on Ni-NTA and, as necessary, gel filtration chromatography, then characterized for purity and

molecular wt (M_r) by SDS-PAGE (Figure S7). The N-terminal His₆-tag could also be removed from His₆-RifKR7 by digestion with HRV 3C protease.

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18. Control incubations in the presence of RifKR7 or RifKR10 but without added Sfp established that negligible 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 RifKR7 whether the RifKR7 or 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 (ref. 5) for the reduction of the corresponding racemic SNAC thioesters.

Figure 1.

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

Figure 2.
Postulated reductions carried out by NysKR1, RifKR7, and RifKR10 in the biosynthesis of
A. Nystatin and B. Rifamycin.

AmpKR1	HTAAVIELAAL	SSTAGM W GSGV H AAYVAGN
NysKR1	HAAAAIELSAL	SSTAGM W GSGV H AAYVAGN
ConKR5	HAAGTGLLVPL	SSISGV W GSGD H GAYAAAN
AmpKR11	HTAATIELHTL	SSTAGM W GSGA H AAYVAGN
ConKR11	HAAAYIQLASL	SSVAGV W GSSN H AAYTAGN
MyxKR2	HAAALLTESNL	SSTSTL W GASGLAHYAAGN
RifKR10	HTAGVPDAGVI	SSVSAV F MGAG S GSYAAAN
RifKR7	HTAGVFDAGVT	SSASSI F MGAG S GGYAAAN

Figure 3.

Sequence alignments of KR Domains Known or Predicted to Generate (2*S*,3*S*)-2-Methyl-3-hydroxyacyl-ACP Products. (Boxed region indicates corresponding site of the conserved LDD motif in (3*R*)-hydroxyl-specific KR domains.)

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.