A Model of Structure and Catalysis for Ketoreductase Domains in Modular Polyketide Synthases

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ABSTRACT: A putative catalytic triad consisting of tyrosine, serine, and lysine residues was identified in the ketoreductase (KR) domains of modular polyketide synthases (PKSs) based on homology modeling to the short chain dehydrogenase/reductase (SDR) superfamily of enzymes. This was tested by constructing point mutations for each of these three amino acid residues in the KR domain of module 6 of the 6-deoxyerythronolide B synthase (DEBS) and determining the effect on ketoreduction. Experiments conducted in vitro with the truncated DEBS Module 6+TE (M6+TE) enzyme purified from *Escherichia coli* indicated that any of three mutations, Tyr → Phe, Ser → Ala, and Lys → Glu, abolish KR activity in formation of the triketide lactone product from a diketide substrate. The same mutations were also introduced in module 6 of the full DEBS gene set and expressed in *Streptomyces lividans* for in vivo analysis. In this case, the Tyr → Phe mutation appeared to completely eliminate KR6 activity, leading to the 3-keto derivative of 6-deoxyerythronolide B, whereas the other two mutations, Ser → Ala and Lys → Glu, result in a mixture of both reduced and unreduced compounds at the C-3 position. The results support a model analogous to SDRs in which the conserved tyrosine serves as a proton donating catalytic residue. In contrast to deletion of the entire KR6 domain of DEBS, which causes a loss in substrate specificity of the adjacent acyltransferase (AT) domain in module 6, these mutations do not affect the AT6 specificity and offer a potentially superior approach to KR inactivation for engineered biosynthesis of novel polyketides. The homology modeling studies also led to identification of amino acid residues predictive of the stereochemical nature of KR domains. Finally, a method is described for the rapid purification of engineered PKS modules that consists of a biotin recognition sequence C-terminal to the thioesterase domain and adsorption of the biotinylated module from crude extracts to immobilized streptavidin. Immoblized M6+TE obtained by this method was over 95% pure and as catalytically effective as M6+TE in solution.

Polyketide synthases (PKSs)[1] catalyze the formation of diverse carbon chains and rings that form the core of many important natural products with therapeutic and agricultural uses (*1*). Polyketides are assembled through stepwise condensations between acyl-CoA precursors. The modular PKSs are large multifunctional, multisubunit homodimeric complexes that contain the active site domains required for each round of chain elongation (a module) grouped together (Figure 1). Most modules also contain a set of active site domains that modify the β-carbonyl resulting from each condensation to either a hydroxyl, alkene, or alkane functionality before the next condensation occurs. The ability to manipulate individual domains within a module or entire module(s) to alter the substrate specificity or β-carbon modification pattern of a PKS has spawned the application of genetic engineering approaches to generating novel compounds in support of drug development efforts.

Many techniques are currently available for manipulating modular PKSs and have been reviewed (*2*, *3*). One approach to altering β-carbon functionality is through inactivation of an existing domain so that it is bypassed. For example, an enoylreductase, dehydratase, or ketoreductase (KR) domain may be inactivated to introduce a double bond, hydroxyl, or ketone group, respectively. Deletion of a KR domain has been performed in several modules of the 6-deoxyerythronolide B synthase (DEBS) to effect hydroxyl to ketone changes (*4*, *5*). The deletion of such a large portion of the enzyme, however, may have deleterious or undesirable consequences on either protein folding, stability, or activity that cannot be predicted a priori. Therefore, a better understanding of the molecular mechanisms underlying each domain activity could lead to more effective methods of domain inactivation or engineering.

Here we propose a model for the KR domains of modular PKSs based on homology modeling to the enzyme superfamily of short chain dehydrogenases/reductases (SDRs). SDRs catalyze a wide variety of $NAD(P)^+$/NAD(P)H-dependent ketoreduction, dehydrogenenation, dehalogenation,

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[1] Abbreviations: KR, ketoreductase; SDR, short chain dehydrogenase/reductase; DEBS, 6-deoxyerythronolide B synthase; PKSs, polyketide synthases; FASs, fatty acid synthases; 6-dEB, 6-deoxyerythronolideB; AT, acyltransferase; ACP, acyl carrier protein; M6+TE, DEBS Module 6+TE; DH, dehydratase; TE, thioesterase; KS, ketosynthase; ER, enoylreductase.

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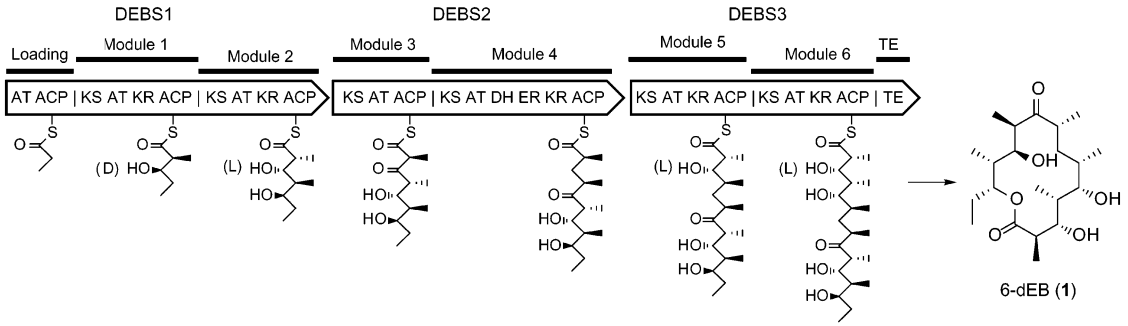

FIGURE 1: Modular organization of DEBS and 6-dEB (**1**). The erythromycin PKS, DEBS, consists of six modules encoded on three separate polypeptide subunits (DEBS 1, 2, and 3). Each module in a modular PKS contains domains for one round of polyketide chain elongation (KS, AT, ACP) and various degrees of β-keto modification (KR, DH, ER). DEBS1 also contains a bidomain loading module, and DEBS3 contains a TE domain for cyclization and release of the macrolactone. DEBS assembles 6-dEB from a propionyl-CoA loading unit and six methylmalonyl-CoA extender units. In this work the stereoconfiguration produced by KR domains (relative to the polyketide backbone) in modules 2, 5, and 6 is referred to as the L configuration and that produced by the KR domain in module 1 is referred to as the D configuration.

dehydration, isomerization, and other reactions (*6*). They are characterized by a Rossmann fold for cofactor binding and a triad of conserved Tyr, Lys, and Ser residues located in the active site. Crystallographic and mutational studies of SDRs have led to proposed models in which these residues participate in catalysis through hydrogen bonding and proton transfer (*7*, *8*). The putative corresponding residues in KR domains of modular PKSs were identified, and the effect of replacing these residues with those predicted to attenuate or eliminate ketoreductase activity were evaluated in vitro and in vivo in model PKS systems. Our results implicate these amino acids in catalysis and provide a highly functional approach to inactivation of KR domains for polyketide analogue engineering. The discovery of the relationship between modular PKS KRs and the SDR family will be a useful tool for developing additional mechanistic hypotheses to guide future KR engineering approaches. This is illustrated with an example of how the product stereospecificity of a KR domain can be predicted from its primary sequence by key amino acid residues.

MATERIALS AND METHODS

Strains, Culture Conditions, and DNA Manipulation. Growth, culture conditions, and DNA manipulations were essentially as described (*9*) unless noted otherwise. *S. lividans* K4-114 (*10*) containing pBoost (*11*) was used as the host for in vivo production of polyketides. Strain BAP1 (*12*) containing pBIRAcm (Avidity, Denver, CO), a plasmid encoding *Escherichia coli* biotin ligase, was used for expression of PKS proteins in *E. coli* for purification.

Construction of KR Mutants. Site-directed mutations were introduced in the KR domain of module 6 from DEBS using the Altered-Sites mutagenesis kit (Promega). A *Not*I-*Xho*I DNA fragment from the plasmid pRSG54 (*13*) was cloned into a pAlter-1 (Promega) derivative carrying a modified multiple cloning site. The mutations were introduced using the oligonucleotides 5′-CCGGTCACCTGCGCCGCGCAG (for K2664Q), 5′-CCCGCTCCGGCCGAGAACAGG (for S2686A), and 5′-GGCCGCGGAGGCGCCGAG (for Y2699F) following protocols provided by the manufacturer. The amino acid number corresponds to its position in DEBS3, encoded by *eryAIII* (*4*). All mutations were verified by DNA sequencing. For construction of *S. lividans* expression plasmids, the mutation-containing fragments were introduced into the full DEBS expression plasmid pKOS011-77 (*11*) through standard cloning procedures. The final expression plasmids were pKOS198-15 (K2664Q), pKOS198-16 (S2686A), and pKOS198-17 (Y2699F). pKOS011-13 is the DEBS expression plasmid containing the ΔKR6 mutation previously described (*5*).

For expression of DEBS Module 6+TE (M6+TE) in *E. coli* for purification, a biotinylation recognition sequence, LNDIFEAQKIEWH (AviTag, Avidity), was engineered at the C-terminal end, immediately upstream of the 6-His tag by introducing the DNA sequence 5′-CCTGAACGACATCT-TCGAGGCTCAGAAAATCGAATGGCAC-3′ between the *Eco*RI and *Hin*dIII sites of pRSG54 (*13*). An 11 amino acid peptide encoding a Flag epitope tag was engineered at the N-terminus of M6+TE by introducing the DNA sequence 5′-AATAATTTTGTTTAACTTTAAGAAGGAGATAT-AATGCATGACTACAAGGACGACGACGACGACAAG between the *Xba*I and *Nde*I sites of the above plasmid. The resulting plasmid, pKOS196-123, encodes M6+TE with a N-terminal Flag tag and C-terminal biotin and His tags. Plasmids expressing M6+TE KR mutant proteins were made by moving the three mutation-containing *Xho*I-*Not*I fragments from above to pKOS196-123. A ΔKR6 expression plasmid was made similarly by using a *Not*I-*Eco*RI fragment from pKOS21-106 (*14*). The resulting plasmids were pKOS159-56 (K2664Q), pKOS159-57 (S2686A), pKOS159-58 (Y2699F), and pKOS159-59 (ΔKR6).

Expression and Analysis of KR Mutants in S. lividans. *S. lividans* strains containing the DEBS KR mutant plasmids were grown and analyzed by LC−MS for polyketide production as previously described (*11*). Purified standards of 6-deoxyerythronolide B (**1**, 6-dEB) and 3-deoxy-3-oxo-6-dEB (**2**) (*5*) were used to verify the identity of these compounds. Production levels were determined by LC−MS−MS using an Applied Biosystems API-3000 triple-quadrupole MS equipped with a Turbo-ionspray and interfaced to an Agilent 1100 HPLC system. The polyketides were detected using multiple reaction monitoring in positive ion mode. Fragmentation of precursor ions was achieved using nitrogen as the collision gas with a collision energy of 13 eV for both compounds. The mass transitions monitored were *m*/*z* 387 $[M + H]^+$ to 239 for 6-dEB and *m*/*z* 367 $[M + H - H_2O]^+$ to 251 for 3-deoxy-3-oxo-6-dEB. Analyte quantification was done by measuring peak areas using a linear equation for 3-deoxy-3-oxo-6-dEB (R^2 = 0.9999) and a polynomial equation for 6-dEB (R^2 = 0.9992).

3-deoxy-3-oxo-6-dEB (**2**): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.21 (1H, ddd, J = 1, 5, 10 Hz, H13), 4.05 (1H, t, J = 3.5 Hz, H5), 3.95 (1H, q, J = 7 Hz, H2), 3.57 (1H, dd, J = 10, 2 Hz, H11), 2.86 (1H, dq, J = 7, 3.5 Hz, H4), 2.81 (1H, dq, J = 7, 2 Hz, H10), 2.70 (1H, ddq, J = 4, 7, 12 Hz, H8), 1.84 (1H, unresolved, H6), 1.81 (1H, unresolved, H12), 1.80 (1H, unresolved, H14a), 1.61 (1H, unresolved, H7a), 1.53 (1H, unresolved, H14b), 1.27 (1H, dd, J = 8, 14 Hz, H7b), 1.33 (3H, d, J = 7 Hz, Me2), 1.29 (3H, d, J = 7 Hz, Me4), 1.08 (3H, d, J = 7 Hz, Me6), 1.03 (3H, d, J = 7 Hz, Me8), 1.02 (3H, d, J = 7 Hz, Me10), 0.95 (3H, d, J = 7 Hz, Me12), 0.93 (3H, t, J = 7 Hz, H15). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 215.4 (C-9), 207.6 (C-3), 171.9 (C-1), 77.0 (C-13), 71.3 (C-5), 70.6 (C-11), 50.5 (C-2), 47.4 (C-4), 42.2 (C-10), 41.7 (C-12), 40.0 (C-8), 36.5 (C-7), 35.2 (C-6), 25.2 (C-14), 17.8 (Me-6), 14.5 (Me-8), 13.4 (Me-2), 12.2 (Me-4), 10.4 (C-15), 9.2 (Me-12), 7.1 (Me-10).

Expression and Purification of DEBS Module 6+TE KR Mutants in E. coli. DEBS M6+TE expression plasmids were used with pBIRAcm to cotransform *E. coli* BAP1 electrocompetent cells. A 500 μL aliquot from an overnight culture grown in LB medium (plus 100 μg/mL carbenicillin and 25 μg/mL chloramphenicol) was used to inoculate 500 mL of LB in a 2-L flask. Cultures were grown at 37 °C to an OD_{600} of 0.4 at which point the temperature was equilibrated to 22 °C, and IPTG (1 mM) and biotin (50 μM) were added. Cells were grown at 22 °C for ~18 h post induction, harvested by centrifugation, and washed 3× with 30 mL of lysis buffer (300 mM NaCl, 250 mM $NaPO_4$ (pH 7.1), 30% glycerol, 2 mM EDTA (pH 8.0), and 2 mM DTT) to remove excess biotin. Cells were resuspended in 30 mL of lysis buffer and disrupted by sonication. Cellular debris was removed by centrifuging the suspension for 45 min at 47 000*g*. Biotinylated protein was captured by passing the supernatant over a 1-mL Ultralink Neutravidin Plus (Pierce) bead column preequilibrated with lysis buffer. The column was washed with 10 mL of lysis buffer and then with 10 mL of assay buffer (100 mM $NaPO_4$ (pH 7.1), 20% glycerol, 1 mM EDTA, 2.5 mM DTT). Beads were resuspended in 1 mL of assay buffer and recovered from the column. Protein bound to the Neutravidin bead was used immediately for enzyme assays. Prior to SDS−PAGE analysis, the protein−bead complex was denatured by boiling in 1 volume Tricine sample buffer (Bio-Rad) for 10 min. Protein concentration was determined by SDS−PAGE using bovine serum albumin standards. Protein bands were stained with Sypro Red (Amersham Biosciences) fluorescent dye and quantified with a Typhoon (Amersham Biosciences) fluorescence imager.

In Vitro Assays with Purified DEBS Module 6+TE KR Mutants. Kinetic assays were performed in triplicate using 0.4−0.8 μM M6+TE protein, 0.5 mM [2-^{14}C]-methylmalonyl Coenzyme A (ARC), 4 mM NADPH, and varying diketide (equimolar mixture of (2*S*, 3*R*) and (2*R*, 3*S*)-2-methyl-3-hydroxy-hexanoic acid, *N*-propionylcysteamine thioester) concentrations (0.5−20 mM) in 50 μL of assay buffer. Reactions were incubated at 30 °C and terminated at 2, 5, or 10 min with addition of 200 μL of ethyl acetate and vortexing. The organic fraction was collected, and the aqueous phase was extracted with an additional 2 × 200 μL of ethyl acetate. The ethyl acetate fractions were pooled, concentrated, and analyzed by silca gel TLC (1:1 ethyl acetate/hexanes). Triketide lactone product on the TLC was quantified with a Typhoon imager in phoshporimaging mode using [2-^{14}C]-methylmalonyl Coenzyme A standards.

RESULTS

Homology Modeling of Ketoreductase Domains from Modular Polyketide Synthases. Amino acid alignments between several KR domains from modular PKSs, KR domains from type I animalian fatty acid synthases (FASs) of both vertebrates and invertebrates, and selected SDR members reveal good end-to-end sequence similarities (Figure 2). Among SDRs with solved crystal structures, the KR domains align well with the tropinone reductases (TR-I and TR-II) from the plant species *Datura stramonium* (Figure 3) that, like KRs from PKSs, contain highly homologous members that catalyze ketoreduction with opposite stereochemical outcomes (*15*) (see further details in Discussion section).

In addition to the Rossmann fold motif, the catalytic Tyr and Ser residues of SDRs are clearly conserved in KR domains of modular PKSs. An apparent divergence between KRs and SDRs, however, is the substitution of an Asn residue in the KRs at the position occupied by the catalytic Lys residue in SDRs. Substitution of this Lys with Arg has been observed in halohydrin dehalogenases (*16*), and therefore, the presence of an Asn is not entirely unusual. In the SDR family, a highly conserved Asn lies ~32 amino acids upstream of the conserved Ser. A specific role for this Asn residue has not been proposed; however, in the X-ray crystal structures of SDRs, the side chain of this Asn occupies space near the catalytic Lys residue (Figure 3). At the corresponding position in KR domains, a conserved Lys is present and, based on SDR crystal structures, could extend to the site occupied by the conserved Lys that lies downstream in SDR sequences. We therefore hypothesize that the Lys and Asn residues are switched in the linear sequence of KRs in relation to the general SDR configuration. This switched arrangement is also found in KR domains of the closely related animalian FASs (*17*) (Figure 2) and could represent either an evolutionary event or a mechanistic constraint imposed by the similar acylthioester substrates for the two enzymes. Implications in the overall mechanism are discussed below.

In Vivo Evaluation of Mutations in the KR6 Domain of DEBS. On the basis of the analysis above, it was predicted that mutation of the identified Tyr, Ser, or Lys residues in a PKS module would impair or eliminate KR activity in that module. This was tested in module 6 of DEBS, for which deletion of the entire KR domain was previously performed to generate the 3-keto derivative of 6-dEB, **2** (Figure 4) (*5*). Therefore, each mutant was expected to produce either 6-dEB (**1**) if the mutation had no effect on activity, 3-deoxy-3-oxo-6-dEB (**2**) if the domain was completely inactivated, or a mixture of both molecules if activity was only diminished. Three separate amino acid replacements, K2664Q, S2686A, and Y2699F, were constructed in the KR6 domain of DEBS3 and expressed together with the remaining subunits, DEBS1 and DEBS2, in *S. lividans* K4-114. The ΔKR6 mutant, in which the entire 447 amino acid KR region between the acyltransferase (AT) and the acyl carrier protein (ACP) of module 6 has been deleted and replaced with an 18 amino acid linker (*5*), was also included for comparative analysis.

Production profiles were examined initially by LC−MS analysis. Each of the strains produced a mixture of polyketides

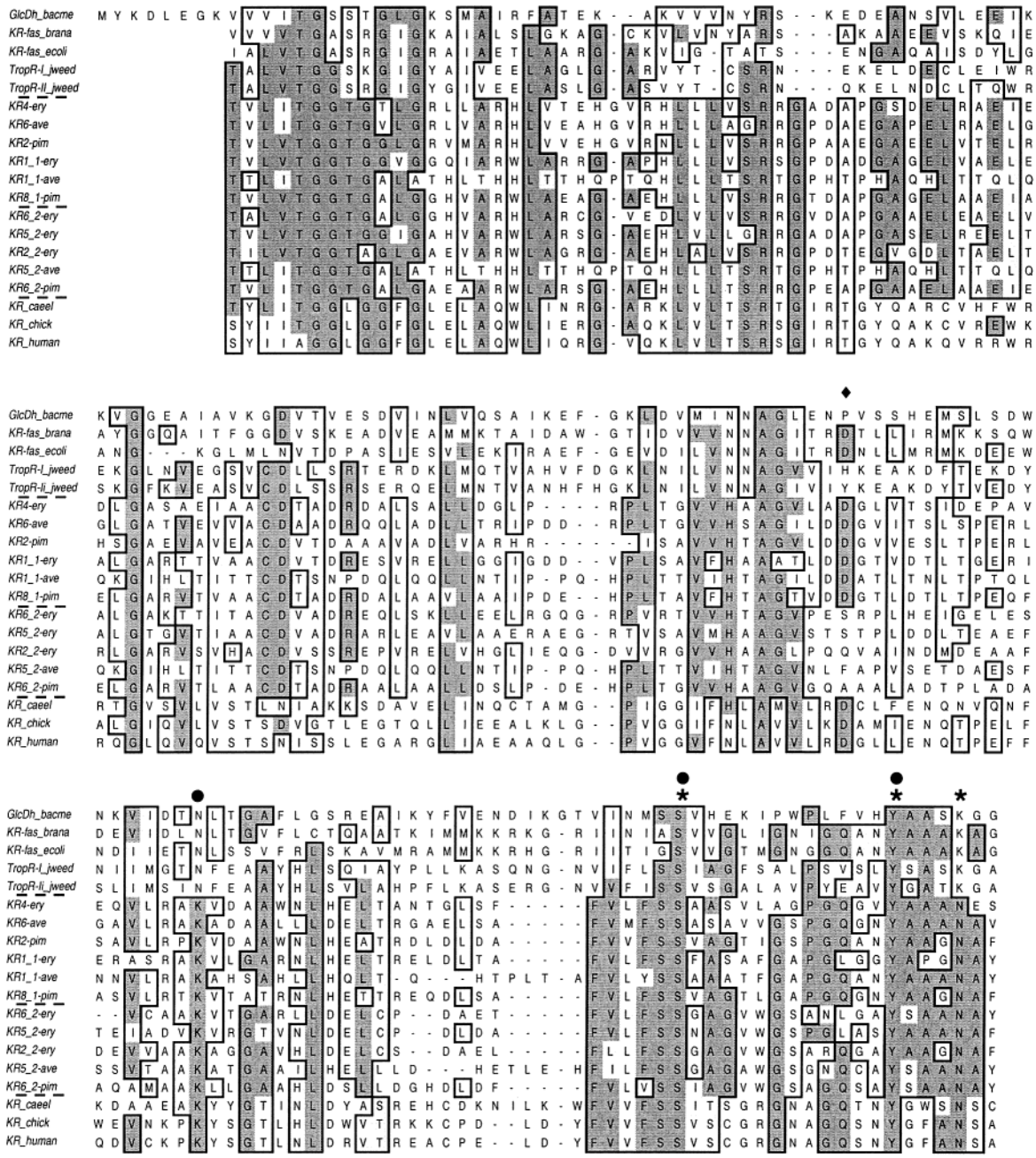

FIGURE 2: Alignment of SDRs (first 5), modular PKS KR domains (next 11), and animalian FAS KR domains (last 3). Partial sequences beginning from the Rossmann fold (solid bar) near the N-terminus through the SDR catalytic residues (asterisks) are shown. The amino acids mutated in this work are labeled with a circle. The Asp residue diagnostic of PKS KR specificity is labeled with a diamond. Those containing the Asp are either known (KR1_ery, KR1_ave, KR8_pim) or predicted to catalyze reduction with D configuration. Those without the Asp are known to catalyze ketoreduction with L configuration. The FAS KR domains, which all contain the Asp, catalyze reduction with D configuration. KR domains from PKSs include examples from DEBS (ery), the avermectin PKS (ave), and the pimaricin PKS (pim). GlcDh_bacme, glucose dehydrogenase from *Bacillus megaterium* (PDB: 1GCO); KR-fas_brana, β-keto ACP reductase from *Brassica napus* (PDB: 1EDO); KR-fas_ecoli, β-keto ACP reductase from *E. coli* (PDB: 1I01); TropR-I_jweed, tropinone reductase-I from jimsonweed (*Datura stramonium*) (PDB: 1AE1); TropR-II_jweed, tropinone reductase-II from jimsonweed (PDB: 2AE1); KR-caeel, KR domain from *Caenorhabditis elegans* FAS (GB: T21676); KR-chick, KR domain from chicken FAS (GB: P12276); KR-human, KR domain from human FAS (GB: P49327).

that included both **1** and **2** (Table 1). Since these two compounds were difficult to resolve completely by liquid chromatography, LC−MS−MS analysis using characterized standards was used to distinguish between these two molecules and estimate titers. As wild-type DEBS produces no detectable amount of **2**, it appears that all three of the point mutations significantly diminish ketoreductase activity in module 6, with the K2664Q and S2686A mutations to a lesser extent than the Y2699F mutation. Residual amounts of 6-dEB were unexpectedly produced by the ΔKR6 containing strain (Table 1) and may result from KR activity in another DEBS module or from an unknown enzyme in *S. lividans*. The level of 6-dEB produced by the Y2699F mutant was similar to ΔKR6 and therefore likely arises via the same mechanism. Although the mechanism(s) for 6-dEB formation in each of the point mutants cannot be determined from these

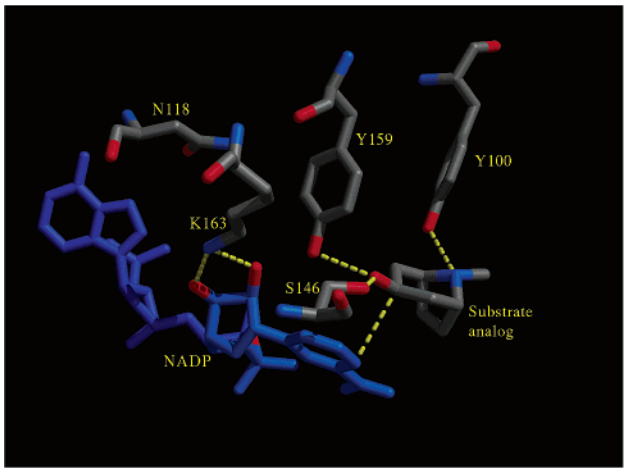

FIGURE 3: Active site arrangement of the tropinone reductase II from *Datura stramonium* with NADP and substrate analogue pseudotropine bound (PDB: 2AE2). Tyr159, Ser146, and Lys163 form the catalytic triad. Also shown is Asn118, which aligns with the putative catalytic Lys residue of the PKS KR domains and Tyr100, which aligns with the conserved Asp of the KR domains producing carbon centers with D configuration. See text for discussion.

experiments, we interpret the formation of significant amounts of 6-dEB from the K2664Q and S2686A mutations as an attenuation of the KR6 activity, while the formation of only residual 6-dEB in the Y2699F mutation, as in ΔKR6, suggests a complete inactivation of KR6 activity. Consistent with previous results (*5*), the DEBS ΔKR6 protein also produced an approximately equal amount of an additional polyketide, 2-desmethyl-3-deoxy-3-oxo-6-dEB (**3**) (Table 1, Figure 4), that results from misincorporation of an acetate unit rather than a propionate unit in module 6. On the basis of mass spectrometry profiles, **3** is not produced by any of the point mutations engineered here.

Additional polyketides could be detected in lower but significant quantity by LC−MS in all of the strains. These compounds were not characterized but, based on mass spectrometry, appear to be related to 3-deoxy-3-oxo-6-dEB and are possibly derivatives that have prematurely hydrolyzed from the enzyme before TE-catalyzed lactone formation and have undergone various spontaneous cyclizations. This complex mixture of compounds, therefore, made it difficult to determine with certainty the relative efficiencies of the different mutations versus the KR deletion, and we therefore used in vitro experiments for better quantitative comparisons.

In Vitro Analysis of Mutations in the KR Domain of DEBS Module 6+TE. The truncated DEBS M6+TE enzyme system developed by Gokhale et al. (*13*) was employed to evaluate the activities of the different engineered mutations for production of corresponding keto derivatives. In the presence of synthetic diketide substrates and methylmalonyl-CoA, M6+TE will catalyze formation of triketide lactone products (Figure 5A). Although M6+TE is expressed as a C-terminal 6-His fusion protein, in our experience, purification of this and other PKS proteins expressed with His tags has not been reliable and in some cases impossible to purify by Ni^{2+} capture. Therefore, we wished to develop a simple and robust purification method for PKS modules and subunits to expedite the study of large numbers of engineered PKS proteins in parallel.

A single-step method for purification of PKS proteins from *E. coli* lysates was developed by engineering a biotin recognition sequence at the C-terminal end of M6+TE upstream of the 6-His site (see Materials and Methods for description). Coexpression of this construct with the *E. coli* biotin ligase (*bir*A) gene led to production of biotinylated M6+TE in *E. coli* that was easily purified by incubation with Neutravidin (Pierce) immobilized to polyacrylamide beads followed by a series of wash steps. Biotinylated M6+TE purified by this method was estimated to be >95% pure based on PAGE analysis. Additional biotinylated versions of DEBS Module 2+TE, Module 4+TE, and Module 5+TE (*13*) were also constructed and found to bind to the beads with similar efficiency. Assays performed in parallel with M6+TE bound to Neutravidin beads and free M6+TE purified by Ni^{2+} affinity chromatography did not indicate any differences in activity.

KR mutants of M6+TE were constructed, and the purified enzymes were analyzed using diketide substrate **4** in the presence of methylmalonyl-CoA (Figure 5A). In these experiments, both **4** and its 2*R*,3*S* isomer were present in equimolar concentration. It was reported previously that, with an analogous set of diketide enantiomers, k_{cat}/K_m is 20-fold lower for the 2*R*,3*S* isomer than for the 2*S*,3*R* isomer (*18*). Therefore, the contribution of the 2*R*,3*S* diketide to product formation was assumed to be negligible in our assays. Under conditions in which wild-type M6+TE produced predominantly the reduced triketide lactone, **5**, the Y2699F, K2664Q, S2686A, and ΔKR6 KR mutant enzymes produced >95% of the unreduced triketide lactone, **6**, as judged from radio-TLC analysis (Figure 5B). Thus, in the M6+TE system, all three amino acid substitutions appear to completely abolish KR activity. In the case of the Y2699F mutation, this is entirely consistent with the above in vivo experiments. For the K2664Q and S2686A mutations, which resulted in formation of both reduced and unreduced products in the complete DEBS system in vivo, it was expected that both **5** and **6** would be produced by these mutations in M6+TE, and therefore, somewhat surprising not to detect significant amounts of the reduced product, **5**. However, in considering that the M6+TE protein is an engineered truncated protein and must process an unnatural substrate, it is possible that the activity of KR6 in M6+TE is reduced relative to the natural complete DEBS system so that these two mutations have a more significant effect on ketoreduction. Michaelis−Menten constants were determined for the Y2699F and ΔKR6 KR enzymes under saturating methylmalonyl-CoA concentrations and revealed similar K_m values for the diketide substrate and ∼1.6-fold higher apparent k_{cat} for the Y2699F mutant as compared to the ΔKR6 mutant (Table 2).

DISCUSSION

Hypothetical Model for Catalysis. A model for KR structure and catalysis in modular PKSs can be proposed from the homology studies and mutagenesis data reported here. First, the ability to inactivate the KR6 domain of DEBS M6+TE and to either reduce or inactivate KR6 activity in the DEBS3 subunit by mutation of the conserved Tyr, Ser, and Lys residues is consistent with the hypothesis that these residues constitute a catalytic triad in the active sites of KRs (Figure 6). In SDR models, proton transfer occurs from the Tyr residue of the enzyme to the carbonyl oxygen of the

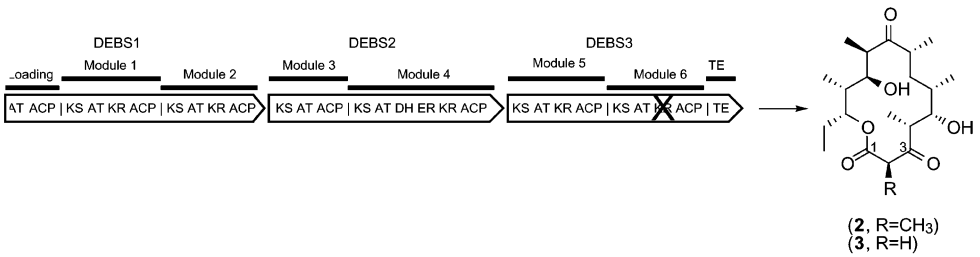

FIGURE 4: Compounds produced by inactivation or deletion of DEBS KR6. Lack of ketoreductase activity in module 6 leads to formation of 3-deoxy-3-oxo-6-dEB (**2**). Deletion of DEBS KR6 gives rise additionally to 2-desmethyl-3-deoxy-3-oxo-6-dEB (**3**) by affecting the AT specificity of module 6 (see text for details).

Table 1: Polyketides and Titers Resulting from DEBS KR6 Mutations in *S. lividans*.

mutation	6-dEB (**1**) (mg/L)	3-deoxy-3-oxo-6-dEB (**2**) (mg/L)	2-desmethyl-3-deoxy-3-oxo-6-dEB (**3**) (mg/L)
ΔKR6	2	21	10−20
K2664Q	35	17	
S2686A	24	9	
Y2699F	<1	41	

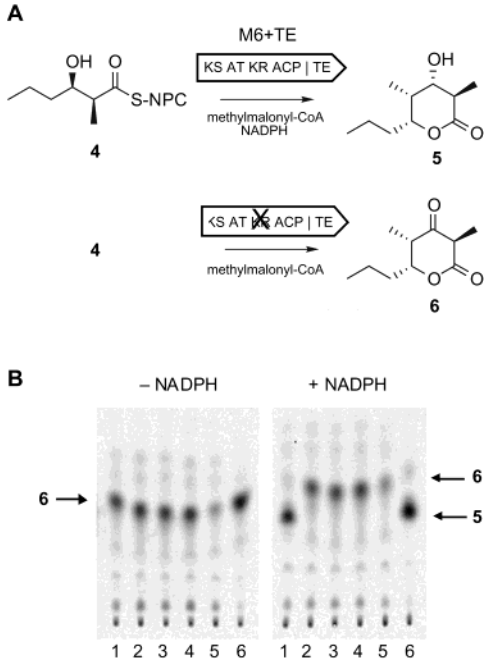

FIGURE 5: Production of triketide lactones by DEBS M6+TE. (A) Reactions catalyzed by purified M6+TE in the presence of diketide-*N*-propionyl cysteamine (NPC) thioester **4** and methylmalonyl-CoA. Inactivation of the KR domain or omission of the NADPH cofactor results in formation of triketide lactone **6**. (B) TLC analysis of triketide lactone formation by wild-type and KR mutants of M6+TE. Biotinylated proteins were expressed in *E. coli* and captured onto Neutravidin polyacrylamide beads. Assays were conducted with diketide **4** and ^{14}C-methylmalonyl-CoA, with or without NADPH. Lanes 1 and 6, wild-type M6+TE; 2, K2664Q; 3, S2686A; 4, Y2699F; and 5, ΔKR.

Table 2: Kinetic Constants Determined for Wild-type and Mutant DEBS M6+TE

mutation	K_m (mM)[a]	k_{cat} (min^{-1})
wild-type	7 ± 1	1.3 ± 0.3[b]
ΔKR6	8 ± 2	0.47 ± 0.04[c]
Y2699F	8 ± 2	0.77 ± 0.07[c]

[a] For diketide **4**. [b] For formation of triketide lactone **5**. [c] For formation of triketide lactone **6**.

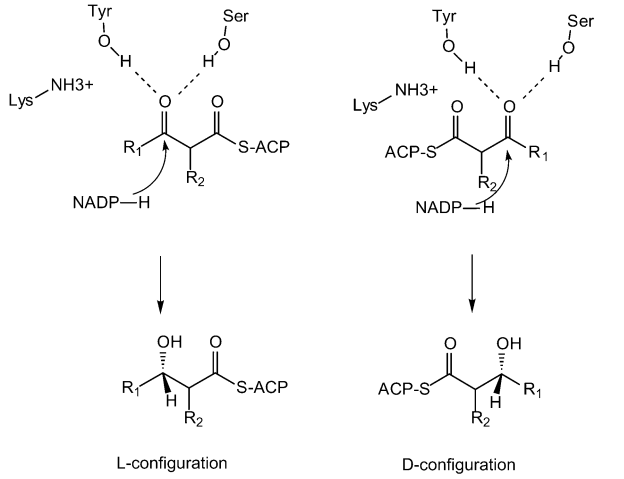

FIGURE 6: Proposed mechanism for stereospecific reduction by modular PKS KR domains based on SDR homology modeling. In the model proposed here, the substrate is rotated about the axis of the reduced carbonyl bond to orient the substrate differently in the two types of KRs. The relative positions of the catalytic residues and the NADPH are maintained for the two different stereochemical outcomes, and only a single active site geometry is required for each type of KR. McPherson et al. (*21*) have proposed a different mechanism in which the polyketide backbone is rotated about an axis perpendicular to the carbonyl bond, thereby reversing the direction of the carbonyl bond relative to the NADPH cofactor. In their model, the relative positions of the proton donating residue and NADPH cofactor must be changed, implying two different active site geometries for the two different KRs.

substrate (*7*, *15*, *19*). In our in vivo studies, mutagenesis of the Tyr resulted in the most dramatic affect on ketoreductase activity, suggesting that this critical residue is also the proton donating constituent in the KR domains of modular PKSs. The conserved Ser residue in SDRs is thought to help stabilize bound substrates or intermediates through hydrogen bond interactions (*8*). In at least two reported cases with SDRs, substitution of the Ser with Ala results in complete inactivation of the enzyme (*8*, *16*). In DEBS KR6, there is an additional Ser adjacent to the conserved Ser (Figure 2) that could possibly compensate in part for the lost function in the KR6 S2686A mutant. This adjacent Ser residue is present in a large number of KR domains, as well as in some SDRs, and a small number of active KRs contain only the adjacent Ser in sequence alignments. Since only the conserved Ser residue was examined here, further studies are necessary to determine any role of the adjacent Ser when present.

How the reversed order of the conserved Lys and Asn residues relates to mechanism in the KR family of modular

PKSs and animal FASs can also be postulated. In SDRs, the amino group of the conserved Lys residue has apparent dual function, providing hydrogen bond interactions with the nicotinamide ribosyl moiety of the cofactor and lowering the pK_a of the proton on the catalytic Tyr through ionic interactions (*8*, *20*). On the basis of the theoretical positions that the switched Lys and Asn residues would occupy in KR domains, it is possible that these functions could either be provided by the Lys residue or shared between the two. Again, further studies are necessary.

Comparison between Site-Directed Mutagenesis and KR Deletion Approaches. For engineering 3-keto derivatives of 6-dEB, the advantage of using the site-directed approach for KR inactivation is clear from the in vivo experiments with full DEBS. Whereas complete deletion of the KR6 leads to a large amount of the 2-desmethyl side products, this product is eliminated in the Y2699F mutant. The misincorporation of an acetate unit by the ΔKR protein could be caused by either the KR deletion itself, which aberrantly affects AT specificity in module 6, or result from the selectivity of the downstream thioesterase (TE) domain for different 3-keto intermediates. However, the absence of acetate incorporation in the point mutants demonstrates that TE selectivity is not the cause, and therefore, the structure of the AT domain has been affected by the KR deletion. The in vitro experiments conducted with DEBS M6+TE also indicate the engineered Y2699F enzyme has better catalytic properties than the ΔKR deletion since the apparent k_{cat} was ∼60% higher.

Modeling Provides Clues to KR Control of Stereospecificity. A model for stereochemical control of ketoreduction in KR domains can also be extrapolated from these homology studies and extensive alignments of KR domains (not shown). In crystal structures of SDRs, only the pro-S face of the cofactor is exposed for hydride transfer. Biochemical experiments have established that the KR1, KR2, KR5, and KR6 domains of DEBS also utilize the pro-S hydride of NADPH (*21*, *22*). Extension of the single SDR cofactor binding arrangement to all KRs then implicates two different substrate binding orientations as the control for ketoreduction stereochemistry in modular polyketide biosynthesis (Figure 6). This is exemplified by two homologous tropinone reductases belonging to the SDR family recently studied (*15*). The two enzymes contain very similar folds and active site geometries, but crystallographic and modeling studies suggest that the substrates' binding modes are related by a 180° rotation about the axis of the target carbonyl (*20*). In these enzymes, specific substrate binding residues were identified and subsequently used to interconvert the stereospecificities (*23*).

Alignment of over 200 KR domains from modular PKSs led to the identification of an Asp residue that occurs in all KRs that catalyze ketoreduction known to produce the D configuration, as in animalian FASs (e.g., that of DEBS KR1), whereas this residue was absent and varied in all KRs producing the L configuration (i.e., DEBS KR2, KR5, and KR6) (Figure 2). This position coincides with the His112/Tyr100 residue of the two tropinone reductases that were determined to contribute to stereochemical control (*23*). On the basis of the tropinone precedent, this diagnostic Asp may interact with the substrate in D configuration type KRs where it is present but would interfere with substrate binding in the L configuration type KRs. Nearly all known cases in which ketoreduction is followed by dehydratase (DH) activity result in formation of a trans double bond, and these KRs possess the conserved Asp. Although the stereospecificity of these KRs cannot be determined by the structure of the resulting compounds, we predict these to catalyze ketoreduction producing the D stereoconfiguration. This has been proven for the KR domain from module 4 of the rapamycin PKS (*24*). The KR10 domain from the rifamycin PKS (*25*) is one of the few known KRs followed by a DH that is predicted to produce the L stereoconfiguration. The structure of rifamycin contains a cis double bond where the KR-DH10 activity occurs (*25*). Therefore, the resulting stereochemistry of the ketoreduction may have implications in control of double bond geometry.

Finally, we have shown that by incorporating a biotin recognition sequence C-terminal to the TE domain of a PKS module, and causing biotinylation during protein expression, it is possible to very rapidly purify the module to >95% homogeneity from crude cell extracts by simple adsorption to immobilized strepavidin and subsequent washing. Moreover, immobilized M6+TE showed kinetic properties indistinguishable from the same protein in solution. This technology is being further refined to provide a general approach to high-throughput purification and in vitro characterization of individual PKS modules and multimodule subunits.

In summary, we have provided substantial evidence that the KR domains of modular PKSs and animalian FASs comprise a unique subfamily of reductases within the SDR superfamily of enzymes. Using this knowledge, mechanistic models have been proposed and were useful in identifying an alternative method for KR inactivation. The widespread occurrence and collective knowledge surrounding SDRs will certainly be an invaluable aid to future studies of KRs from modular PKSs. Last, the novel method for purification and characterization of PKS modules should be useful tool for applications or studies requiring robust and high throughput in vitro analysis of PKSs.

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