Stereochemistry of Catalysis by the Ketoreductase Activity in the First Extension Module of the Erythromycin Polyketide Synthase[†]

Lars H. Østergaard,[‡] Laurenz Kellenberger,[§] Jesús Cortés,[∥] Marc P. Roddis,[⊥] Matthew Deacon,[‡] James Staunton,[#] and Peter F. Leadlay*[,△]

Cambridge Centre for Molecular Recognition and Departments of Biochemistry and Chemistry, University of Cambridge, Cambridge, U.K.

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ABSTRACT: Multiple ketoreductase activities play a crucial role in establishing the stereochemistry of the products of modular polyketide synthases (PKSs), but there has been little systematic scrutiny of catalysis by individual ketoreductases. To allow this, a diketide synthase, consisting of the loading module, first extension module, and the chain-terminating thioesterase of the erythromycin-producing PKS of *Saccharopolyspora erythraea*, has been expressed and purified. The DNA encoding the ketoreductase-1 domain in this construct is flanked by unique restriction sites so that another ketoreductase domain can be readily substituted. The purified recombinant diketide synthase catalyzes, at a very low rate ($k_{cat} \approx 2.5 \times 10^{-3}$ s^{-1}), the specific production of the diketide (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid. The activity of the ketoreductase domain in this model synthase was analyzed using as a model substrate (±)-2-methyl-3-oxopentanoic acid *N*-acetylcysteaminyl (NAC) ester for which k_{cat}/K_m was 21.7 M^{-1} s^{-1}. The NAC thioester of (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid was the major product and was strongly preferred over other stereoisomers as a substrate in the reverse reaction. The bicyclic ketone (9*RS*)-*trans*-1-decalone, a known substrate for ketoreductase in fatty acid synthase, was found also to be an effective substrate for the ketoreductase of the diketide synthase. Only the (9*R*)-*trans*-1-decalone was reduced, selectively and reversibly, to the (1*S*,9*R*)-*trans*-decalol. The stereochemical course of reduction and oxidation is exactly as found previously for the ketoreductase of animal fatty acid synthase, an additional indication of the close similarity of these enzymes.

Modular type I polyketide synthases (PKSs),[1] for example, the 6-deoxyerythronolide B synthase (DEBS) responsible for synthesis of the aglycone core of the macrolide antibiotic erythromycin A, are giant, multifunctional enzymes that catalyze the biosynthesis of structurally complex and clinically important polyketide products (*1*, *2*). Polyketide chain formation is akin to fatty acid biosynthesis, in which a starter unit and successive extender units, derived from the CoA esters of simple carboxylic acids, are condensed together to create a chain, without the intermediates being released into solution. In contrast to fatty acid synthases (FASs) (*3*, *4*) modular PKSs utilize a wider variety of starter and extender units, and the β-keto functionality created after each condensation step is not necessarily fully reduced before the next cycle of chain extension, leading to a far greater potential chemical and stereochemical diversity in the products. Nevertheless, each modular PKS normally produces only one major polyketide product.

The sequencing of the genes for DEBS (and for many other such PKSs) has revealed that each cycle of chain extension is catalyzed by a different set or "module" of active sites. Each module contains a ketosynthase (KS) domain which catalyzes the formation of a carbon−carbon bond. The acyltransferase (AT) domain recruits the chain extension unit, normally from either malonyl-CoA or methylmalonyl-CoA. The acyl carrier protein (ACP) cooperates in the carbon−carbon bond formation to form a β-ketoester and then carries the growing chain to various optional domains involved in processing the keto group. Depending on the module, there may be additional activities present: a β-ketoacyl reductase (KR) domain, which catalyzes reduction of the initially formed β-ketoester to a β-hydroxyester; a dehydratase (DH)

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* Address correspondence to this author at the Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge CB2 1GA, U.K. Tel: +44-1223-333656. Fax: +44-1223-766091. E-mail: p.f.leadlay@bioc.cam.ac.uk.

[‡] Department of Biochemistry. Present address: Novozymes, DK-2880 Bagsvaerd, Denmark.

[§] Department of Biochemistry. Present address: Basilea Pharmaceutica Ltd., Postfach 3255, CH-4002 Basel, Switzerland.

[∥] Department of Biochemistry. Present address: GlaxoSmithKline Research & Development, Medicines Research Centre, Gunnels Wood Road, Stevenage SG1 2NY, U.K.

[⊥] Department of Chemistry. Present address: Department of Medicinal Chemistry, Uppsala University, SE-751 23 Uppsala, Sweden.

[#] CCMR and Department of Chemistry.

[△] CCMR and Department of Biochemistry.

[1] Abbreviations: PKS, polyketide synthase; DEBS, 6-deoxyerythronolide B synthase; DEBS 1-TE, 6-deoxyerythronolide B synthase 1-thioesterase; DEBS 1-TE (ΔKR2), 6-deoxyerythronolide B synthase 1-thioesterase from which the ketoreductase domain of module 2 has been deleted; DKS, diketide synthase; AT, acyltransferase; ACP, acyl carrier protein; KS, β-ketoacyl synthase; KR, β -ketoacyl reductase; TE, thioesterase; FAS, fatty acid synthase; SDS, sodium dodecyl sulfate; NAC, *N*-acetylcysteamine.

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domain which dehydrates the β-hydroxy ester; and an enoyl reductase (ER) domain which reduces the double bond. For most modules, there is a convincing correlation between the predicted constituent domains and the chemical structure of the corresponding chain extension unit in the growing polyketide chain.

The methyl centers at C-2 and the hydroxyl centers at C-3 generated in many newly added chain extension unit can have either *R* or *S* configuration. Understanding how this stereochemistry is controlled is clearly central to any attempt to manipulate PKS catalysis to create novel polyketide products. In vitro studies with an engineered and purified model PKS, DEBS 1-TE, have already revealed crucial elements of the molecular basis of this control. This mutant PKS was created by relocating the chain-terminating thioesterase to the C-terminus of bimodular DEBS1 to effect chain release at the triketide stage giving a δ-lactone (*5*). Although the two methyl centers in this product have opposite configurations, work with DEBS 1-TE has shown that the acyltransferase (AT) domain is not responsible for determining the stereochemical outcome of chain extension, as had been proposed on the basis of classical in vivo experiments (*6*) and reproposed on the basis of the existence of a separate AT domain for each cycle of chain extension within DEBS (*7*, *8*). (2*S*)-Methylmalonyl-CoA acts as substrate for both modules 1 and 2 (*9*, *10*). There is an additional obligatory epimerization step in module 1 to produce the substrate for ketoreduction (*11*). The stereochemical outcome therefore depends on the interplay between the properties of individual KS and KR domains, with the ACP possibly also playing a role.

In a fully reduced extension unit the stereochemistry at a branching alkyl center is determined by the stereochemical outcome of the reaction catalyzed by the enoylreductase (ER) domain. Similarly, for an extension unit that preserves its hydroxyl group, it is obvious that the chirality at the hydroxyl group must be determined by the direction of hydride addition to the keto group (*re* or *si* face) within the active site of the KR domain. Individual KR domains have already been successfully swapped between modules of different PKSs, giving rise in some cases to an altered stereochemistry of the hydroxyl group in the product (*12*; L. Kellenberger, unpublished data), but the underlying reasons for the success or failure of such engineering attempts are not yet understood. On the basis of these limited examples, it has been suggested (*12*) that a heterologous KR in its new context in a hybrid PKS imposes the stereochemical course of its normal reaction on the new substrate. However, other evidence suggests that the true situation is more complex. The protein engineering may introduce unfavorable protein−protein contacts in the chimeric enzyme, but even if it does not, the intrinsic specificity of the transplanted KR domain may either be dominated by, or given free rein by, the action of the KS in the new host module (*13*, *14*).

The KS domain in the first extension module of DEBS is capable of catalyzing the epimerization of the initially formed (2*R*) isomer of the keto ester to the (2*S*) isomer (*11*), and the KR domain selects only the (2*S*) isomer from the mixture. In a chimeric diketide synthase which combined KS1 with the reductive enzymes corresponding to module 2 (*13*), a diketide was produced in which the stereochemistry was altered at both C-2 and C-3; this supports the idea that both enantiomeric keto esters are formed by KS1 and that the KR domain (in this case KR2) selects between them. Further, KR1 was shown to select only the (2*S*) enantiomer from a racemic mixture of the *N*-acetylcysteamine derivative of the keto ester diketide, and it produces the single product corresponding to the stereochemistry of reduction in module 1 (*15*).

In contrast, when the same model substrate is reduced in vitro by purified DEBS3 (which contains two KR domains, KR5 and KR6, producing identical stereochemical outcomes during normal chain extension), equal amounts of all four possible stereoisomers of the product alcohol are obtained (*16*). In a related experiment in vivo, the normal specificity of *both* KR5 and KR6 domains is apparently subverted when the KS5 domain is replaced by KS1, which contains an epimerization activity. These considerations therefore also apply when entire modules are swapped between natural PKSs. The alcohol stereochemistry is not controlled solely by the ketoreductase domain, as previously proposed (*12*), and an anti product may be formed by a KR domain which normally forms a syn product (cf. ref *14*).

The assay of individual activities of the fatty acid synthase multienzyme, using appropriate model substrates, has given important insight into the effects of mutation on individual steps in catalysis (*17*). So far, procedures have been developed for the AT (*9*, *18*), KR (*15*, *18*), and thioesterase (TE) (*19*−*21*) domains of the modular PKS. We describe here the development of an alternative experimental approach for investigating KR stereocontrol, consisting of a purified diketide synthase containing only one ketoreductase domain, and we show how the stereochemical course of reduction can be monitored using both specific substrate analogues and also *trans*-1-decalone, a potentially general substrate analogue which is a known model substrate for the ketoreductase activity of fatty acid synthase (*22*−*24*).

MATERIALS AND METHODS

Growth of Cells, Preparation of the Extract, and Purification of DEBS 1-TE (ΔKR2). Engineering of a strain of *Saccharopolyspora erythraea* containing a genetically modified version of the DEBS 1-TE (*5*), from which the ketoreductase domain of module 2 has been specifically deleted [DEBS 1-TE (Δ*K*R2)], will be described elsewhere (L. Østergaard, L. Kellenberger, J. Cortés, J. Staunton, and P. F. Leadlay, manuscript in preparation). The purification of the modified DEBS 1-TE from the recombinant *S. erythraea* was carried out essentially as described previously (*18*, *25*).

Construction of a Strain of S. erythraea Expressing Diketide Synthase. The plasmid pDKS is derived from the integrative plasmid vector pCJR24 (*26*). It contains a truncated DEBS-derived gene encoding the loading module, the first extension module up to the beginning of the ACP1 domain (position 5780, numbering from the ATG start codon of the ORF), and the hybrid ACP-TE as found in DEBS 1-TE (*5*) of the erythromycin PKS. It was constructed starting from pE1, a pCJR24-based plasmid containing a modified DEBS 1-TE gene, in which a region of the ketoreductase domain KR2 in module 2, extending from an engineered *Avr*II site (position 8656) to an engineered *Hpa*I site (position 10121), has been replaced by the corresponding region from

module 1 of the erythromycin PKS. The plasmid pE1 was digested with *Nsp*V (which cuts within the DNA encoding KR1), and the resulting fragment was religated to give pDKS, from which most of module 2 has been extruded. Plasmid pDKS was used to transform protoplasts of *S. erythraea* JC2 (*27*). Plasmid-containing colonies were selected on solid medium containing thiostrepton (25 mg/L).

Analysis of the (2S,3R)-3-Hydroxy-2-methylpentanoic Acid Product. *S. erythraea* JC2/pDKS was grown in tryptone soy broth (TSB, Gibco) at 30 °C. The cultures were harvested after 4−5 days. After centrifugation, the supernatants were acidified to pH 2.5, saturated with NaCl, and extracted three times with an equal volume of ethyl acetate. The combined organic extracts were dried over sodium sulfate and evaporated. The residue was dissolved in acetone and treated with diazomethane. GC/MS analysis was carried out with chemical ionization (methane or ammonia as carrier gas) on a Finnegan/MAT GCQ instrument, using an Anachem chiral MEGA DAcTBuSilBETACDX-OV 1701 column (inner diameter 0.25 mm, length 25 m). The temperature was increased linearly from 50 to 100 °C over 12.5 min, held for 10 min, then increased linearly to 210 °C over 5.5 min, and finally held at 210 °C for 8 min.

Expression and Purification of the Diketide Synthase. *S. erythraea* JC2/pDKS, grown on tap water medium agar plates (*26*), was used to inoculate 500 mL of TSB medium containing 5 mg/L thiostrepton, in a 2 L baffled flask shaken at 200 rpm. After 4−5 days at 30 °C, the culture was used to inoculate 15 L of TSB medium in a 20 L fermentor (Applikon BioPilot) stirred at 200 rpm and aerated at the rate of 20 L/min. After 20 h at 30 °C, the cells (50−60 g wet weight/L) were harvested by centrifugation (16000*g*) and stored at −80 °C until required. The diketide synthase was isolated using the following procedure, in which all manipulations were carried out at 4 °C: About 30 g of mycelial pellet was resuspended in 50 mL of buffer A [50 mM Tris-HCl (pH 7.5 at 25 °C) containing 20% (w/v) glycerol, 1 mM EDTA, and 1 mM DTT] to which had been added 1 mM benzamidine, 1 μM pepstatin A, 20 μg/mL trypsin inhibitor, and 1 mM 1,10-phenanthroline. The cells were ruptured by passing them twice through a French pressure cell (1000 psi). The lysate was clarified by centrifugation (40000*g*), and the resulting supernatant (70 mL) was loaded onto a 50 mL Q-Sepharose Fast Flow column (2.6 cm i.d.) at 0.5 mL/min. The column was washed with 1.5 column volumes of buffer A, and proteins were eluted by a linear salt gradient (0−250 mM NaCl) over 2 column volumes, held at 250 mM for another 2 column volumes, and then again increased linearly to 500 mM NaCl over 5 column volumes. The majority of diketide synthase protein eluted in the region of 325−415 mM NaCl. These fractions were pooled, solid ammonium sulfate was added to a final concentration of 0.2 M, and the solution was applied to a 20 mL phenyl-Sepharose HP column (internal diameter 2.6 cm) at 2.5 mL/min. The column was washed with buffer A containing 0.2 M $(NH_4)_2SO_4$ until the absorbance of the eluate at 280 nm no longer diminished, and proteins were eluted using a gradient of decreasing ammonium sulfate, from 0.2 M to 0 over 2 column volumes. The diketide synthase eluted about halfway through this gradient. Early fractions containing the bulk of the diketide synthase were pooled and concentrated to 4−5 mL using filter membranes (Amicon) with a nominal molecular mass cutoff at 100 kDa. The concentrated protein was loaded onto a 500 mL Sephacryl S-400 column (internal diameter 2.6 cm) at 0.75 mL/min. The diketide synthase was eluted using buffer A containing 250 mM NaCl, in a peak emerging after about 250 mL. Fractions containing purified diketide synthase were pooled, concentrated, and rapidly frozen in liquid nitrogen before storage at −80 °C.

Limited Proteolysis of Diketide Synthase. Purified synthase was incubated with trypsin (Sigma) at an enzyme/substrate ratio of either 1/800 (w/w) or 1/8000 (w/w) in 50 mM Tris-HCl buffer (pH 7.5), 20% (w/v) glycerol, 1 mM EDTA, and 1 mM DTT (buffer A). The total reaction volume was 30 μL using a concentration of 2 μg of synthase/μL. Reactions were carried out at 30 °C for 10, 30, and 60 min, respectively. The reaction products were separated by SDS−PAGE using 7% and 10% polyacrylamide gels, and 2 mM thioglycolic acid was included in the running buffer. Protein bands were then transferred to ProBlott membranes for N-terminal sequencing. The wet electroblotting was performed for 1 h at 100 V and 350 mA in 25 mM Tris base and 192 mM glycine, containing 20% (v/v) methanol and 0.02% (w/v) SDS. Proteins were visualized with Coomassie Brilliant Blue R-250. Individual bands were subjected to N-terminal amino acid sequencing.

Size Exclusion Chromatography. The molecular weight of the intact active protein was determined by size exclusion chromatography of the purified enzyme. A Superose 6 (3.2/30) column was loaded with 10 μL of a protein solution (10 g/L) and then subjected to isocratic elution using buffer A containing 250 mM NaCl on the Smart micro-FPLC system (Pharmacia). The elution volume on this column was determined for a number of proteins, including DEBS 1-TE, of known relative molecular mass.

Analytical Ultracentrifugation. The molecular weight and state of aggregation of the diketide synthase was analyzed by equilibrium centrifugation in an Optima XLi analytical centrifuge (Beckman) essentially as described previously for the DEBS 1-TE protein (*28*). Three loading concentrations were used of 0.5, 0.4, and 0.3 mg/mL in a 50 mM Tris-HCl buffer, containing 100 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA, at a pH of 7.5 and a temperature of 5 °C. The samples were centrifuged at 3000 rpm for 28 h, after which time the sample had reached equilibrium. The data were fitted globally using the Beckman Origin 4.1 analysis program which uses a nonlinear least-squares method for fitting the data (Beckman Coulter). The partial specific volume of the protein was calculated from the amino acid composition, and the densities of the buffer were calculated using the SEDNTERP 1.02 program (*29*).

Analysis of the Overall Reaction. The course of the overall reaction was followed by incorporation of radiolabel from methylmalonyl-CoA into the product. Incubations were performed in the presence of 300 μM propionyl-CoA, 300 μM (2*RS*)-methylmalonyl-CoA, 1 mM NADPH, and 24.7 μM (2*RS*)-[*methyl*-^{14}C]methylmalonyl-CoA (56.4 mCi/mmol) in 400 mM potassium phosphate (pH 7.5) at 30 °C. The reaction was started by addition of protein to a final concentration of 0.25 mg/mL. Equal portions of the reaction mixture were removed and loaded directly onto TLC plates at 1, 2, 3, 6, 11, and 23 h. The radiolabeled products were separated using as the mobile phase methanol containing

0.1% (v/v) HCl. The plates were counted for 24 h using a PhosphorImager (Molecular Dynamics Storm 840). The counts were converted into disintegrations per minute using a standard with known radioactivity. The results of assays without radiolabel were analyzed by GC/MS as described above.

Analysis of Ketoreductase Activity. All experiments were carried out at 25 ± 0.1 °C in 400 mM potassium phosphate buffer (pH 7.5). The continuous NADPH-linked assay, used for determination of the steady-state kinetic parameters, was conducted on a Shimadzu 2100PC spectrophotometer. The course of the reaction was followed by monitoring the change in absorbance at 340 nm (A_{340} = 6220 M^{-1} cm^{-1}) typically for 1 min in a cuvette with a 1 cm path length. The concentrations of enzyme and of substrate were adjusted to ensure that less than 1% of the substrate was used during this interval. The concentration of cofactor used in the ketoreductase assay was 10-fold higher than the value of the K_m for NADP(H) determined at saturating substrate levels. The observed initial rate data were converted into moles of NADP(H) utilized by 1 mol of enzyme per second. The obtained values were fitted to the Michaelis−Menten equation using nonlinear least-squares regression. The quoted errors in K_m are the standard deviations from least-squares fitting, whereas the quoted errors in k_{cat} are standard deviations from multiple assays at saturating substrate concentration ($10K_m$).

Synthesis of Decalols. The (±)-*trans*-*cis*-1-decalols [i.e., a racemic mixture of (1*R*,9*S*)- and (1*S*,9*R*)-*trans*-decalols] were synthesized as previously described (*30*). (±)-*trans*-*trans*-1-Decalol [i.e., a racemic mixture of (1*S*,9*S*)- and (1*R*,9*R*)-*trans*-decalols] was also synthesized as previously described (*31*). Fatty acid synthase was purified, essentially as described (*32*), from fatty rat liver (kindly provided by Professor M. Schweizer). The rat FAS was used to reduce *trans*-1-decalone selectively to (1*S*,9*R*)-*trans*-1-decalol essentially as described by Dutler and colleagues (*23*, *24*). The reaction mixture was extracted three times with equal volumes of diethyl ether, the combined extracts were dried over $MgSO_4$, the ether was evaporated, and the residue was dissolved in acetone.

Analysis of the (1S,9R)-trans-Decalol Product. Purified diketide synthase enzyme was used to reduce *trans*-1-decalone. The reaction mixture contained NADPH (10 mM), glucose 6-phosphate (7.5 mM), $MgCl_2$ (4 mM), glucose-6-phosphate dehydrogenase (0.1 mg), diketide synthase (0.5 mg), and *trans*-1-decalone (10 mM) in 100 mM potassium phosphate buffer (pH 7.5) at 30 °C in a total volume of 3 mL. The course of the reaction was readily monitored by the change in absorbance at 340 nm. The product mixture was extracted as described above for the products of the fatty acid synthase, and GC/MS analysis was carried out with chemical ionization (methane or ammonia as carrier gas) on a Finnegan/MAT GCQ instrument, using an Anachem chiral MEGA DAcTBuSilBETACDX-OV 1701 column (inner diameter 0.25 mm, length 25 m). The following temperature program was used for separations: the temperature was increased linearly from 50 to 100 °C over 12.5 min, held for 10 min, then increased linearly to 210 °C over 5.5 min, and finally held at 210 °C for 8 min.

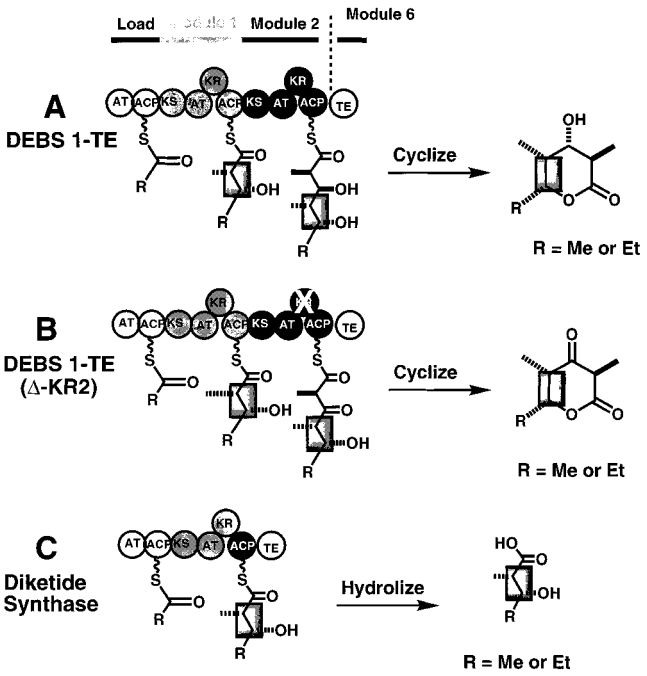

FIGURE 1: The erythromycin PKS consists of three giant multifunctional proteins, DEBS 1−3. The DEBS proteins are arranged in modules, which catalyze the stereospecific condensation of an extender unit onto the growing chain, and specify the level of reduction of the β-carbon of the resulting intermediate. (A) DEBS 1-TE is a truncated version of DEBS comprising the loading module and the first two extension modules, to which is attached part of the ACP from module 6 and the pendant thioesterase (TE) domain. (B) DEBS 1-TE (ΔKR) is a derivative of DEBS 1-TE from which the KR domain of module 2 has been deleted. (C) The diketide synthase studied here has the domain organization of DEBS 1-TE from which the ACP domain of module 1 and the KS, AT, and KR domains of module 2 have been deleted. The gene for this recombinant diketide synthase contains unique restriction sites engineered 5′ (*Bst*BI) and 3′ (*Hpa*I) of the region encoding the ketoreductase domain of module 1 (KR1).

RESULTS AND DISCUSSION

Expression and Purification of a Diketide Synthase. The minimal modular polyketide synthase gene used in these experiments contains the loading module of DEBS and the first extension module up to and including the end of the ketoreductase domain of DEBS module 1 (Figure 1). The DNA for the KR1 domain is flanked by unique *Bst*BI and *Hpa*I restriction sites to allow future substitution of alternative KR domains. At its C-terminal end the KR1 domain is connected directly to the ACP2/ACP6-TE6 didomain successfully used in previous experiments with DEBS 1-TE (*5*). This chimeric PKS was expressed in *S. erythraea*, and the diketide product was extracted from the broth, methylated, and subjected to GC/MS analysis. Under the conditions used, the methyl esters of the four stereoisomers of 2-methyl-3-hydroxypentanoic acid have different retention times. By comparison with synthetic standards of known configuration, it was shown that the product isolated from the broth of *S. erythraea* JC2/pDKS was (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid, as expected (Figure 1).

The diketide synthase was purified to homogeneity from this strain. Approximately 1−2 mg of purified diketide synthase was obtained from 30 g of cells (wet weight). In contrast, a diketide synthase we previously constructed, with an extension module composed of the DEBS KS1 domain

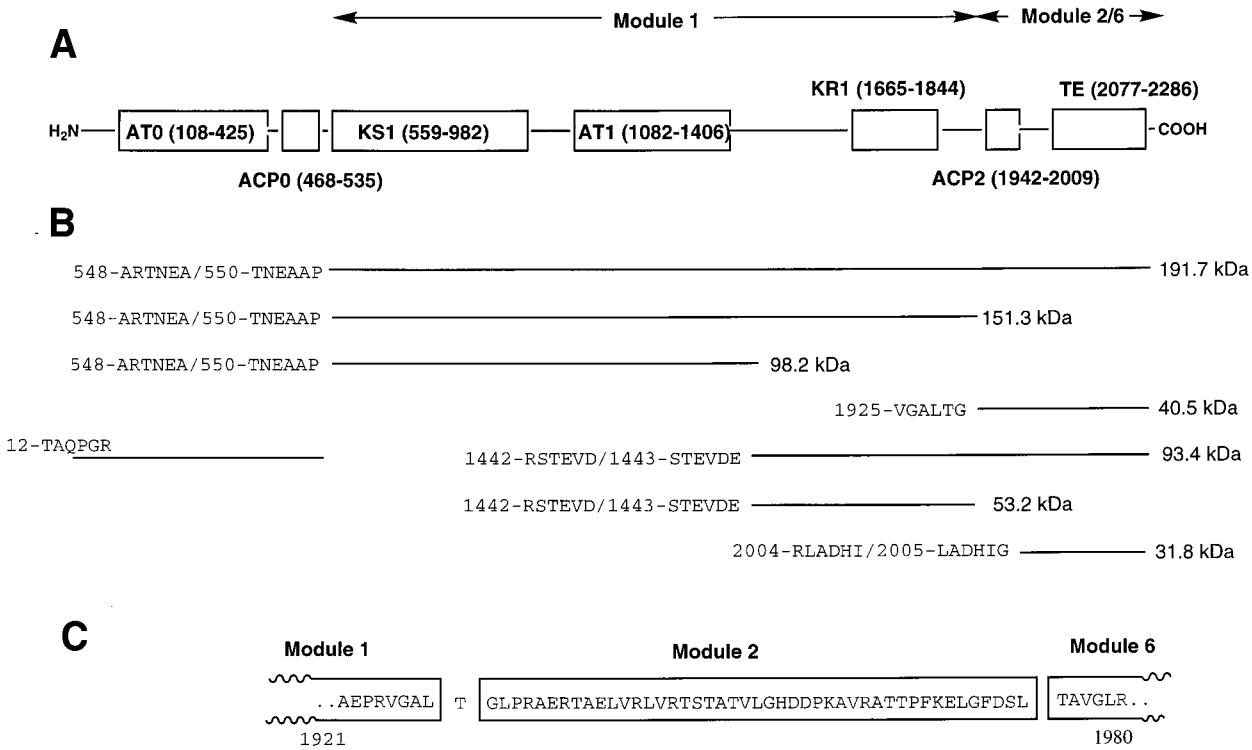

FIGURE 2: Domain organization of the diketide synthase (A) as predicted from sequence comparisons with other polyketide synthases (*35*) and (B) as revealed by limited proteolysis using trypsin. The N-terminal sequence of each fragment is indicated along with its estimated molecular weight and its alignment with the primary structure of the diketide synthase. (C) Amino acid sequence of the diketide synthase in the region of the splice points between modules 1 and 2 and between module 2 and module 6-TE.

and the AT, KR, and ACP domains of DEBS module 2, has so far proved to be refractory to purification (L. Østergaard, unpublished data) although it is satisfactorily active in vivo (*13*).

Size Exclusion Chromatography, Analytical Ultracentrifugation, and Limited Proteolysis. Homodimeric fatty acid synthase active site mutants involving single residue changes have been found to undergo more ready subunit dissociation (*17*), with concomitant loss of activity. In the chimeric diketide synthase, at the junction of the KR1 and the ACP2 domains, the genetic manipulation involved the introduction of an additional threonine residue at the site of fusion, in the linker region (Figure 2). Although this change in the linker region is not likely to perturb the structure, the quaternary structure of the recombinant diketide synthase was checked using analytical size exclusion chromatography on a Smart micro-FPLC system (Pharmacia) fitted with a Superose 6 column (3.2/30) and eluted with buffer A containing 250 mM NaCl. The relative molecular weight of the protein was estimated to be 610000, which argues in favor of a dimer of two identical subunits, each of relative molecular weight 241000, rather than a monomer. The diketide synthase therefore appears to be homodimeric, as are native DEBS multienzymes (*25*, *28*, *33*), but to secure a more reliable estimate of molecular weight, analytical ultracentrifugation measurements at equilibrium were carried out as described in the Materials and Methods section. The weight average molecular weight determined by this means was 455000, which is in good agreement with the value anticipated if the diketide synthase is correctly associated into a homodimeric structure.

Purified diketide synthase was also subjected to limited proteolysis with trypsin, and the pattern of proteolysis at different times was compared with that of native DEBS multienzymes (*28*, *33*). The proteolytic fragments were separated by SDS−PAGE, providing an estimate of the size of each polypeptide fragment, and their respective N-termini were sequenced. The deduced fragmentation pattern after tryptic digestion is shown schematically in Figure 2. The most labile sites in the diketide synthase were found, as expected from previous work (*28*, *33*), to lie within the linker regions between the loading module and the extension module, between the ACP and thioesterase domains, and in the regions flanking the ketoreductase domain. No proteolytic cleavage sites were found that differed from those found in a comparable native DEBS multienzyme, confirming the structural integrity of the diketide synthase.

Analysis of Diketide Production in Vitro. The overall reaction catalyzed on the purified diketide synthase was studied by incubation in the presence of methylmalonyl-CoA and propionyl-CoA substrates and NADPH. The product was analyzed either by chiral GC/MS or (when radiolabeled substrate was used) by thin-layer chromatography followed by autoradiography. It was identified as (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid (data not shown). Analysis of the time course of diketide production, using radiolabeled methylmalonyl-CoA at saturating levels of both substrates and cofactor, showed that the overall rate of diketide production (k_{cat}) was only about 9 h^{-1}, about 50-fold lower than for triketide production by DEBS 1-TE (*18*). Model studies (*21*) with the four stereoisomers of 2-methyl-3-hydroxypentanoic acid derivatized as their NAC thioesters show that all four compounds can be released by the TE, so the detailed reasons for this low turnover remain to be established.

Reduction and Oxidation of Specific Substrate Analogues by the Ketoreductase. The ketoreductase activity of the diketide synthase was studied using (±)-2-methyl-3-oxopentanoic acid, as its *N*-acetylcysteamine (NAC) thioester, as a specific substrate analogue. The NAC thioester mimics the natural substrate when it is bound to the ACP domain via the phosphopantetheine prosthetic group. The rate of hydrolysis of this analogue by the diketide synthase, a potential side reaction, was found to be negligible under the conditions used. The kinetic parameters obtained for reduction of the specific substrate analogue (±)-2-methyl-3-oxopentanoic acid NAC thioester at a saturating concentration of NADPH (0.25 mM) were $k_{cat} = 0.27 \pm 0.01$ s^{-1} and $K_m = 13 \pm 2$ mM, giving a value for the specificity constant $k_{cat}/K_m = 21.7 \pm 5.0$ M^{-1} s^{-1}.

The specific product of reduction was shown by analysis using chiral GC/MS to be the NAC thioester of (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid (Figure 1). Its retention time under the conditions used was 20.5 min, which exactly matches that of an authentic synthetic sample of the (2*S*,3*R*) isomer. Synthetic samples of the other isomers had retention times of 19.24 min (2*S*,3*S*), 19.52 min (2*R*,3*R*), and 20.18 min (2*R*,3*S*), respectively. The NAC thioester of (2*S*,3*R*)-2-methyl-3-hydroxypentanoic acid also served as a substrate for the reverse reaction, in the presence of NADP$^+$. Its low solubility prevented determination of the maximal velocity, but the specificity constant for this substrate was estimated from the slope at low substrate concentration of the curve fitted to the Michaelis−Menten equation, $k_{cat}/K_m = 3.3 \pm 0.1$ M^{-1} s^{-1}.

The diketide synthase also oxidized the NAC thioester of (2*R*,3*R*)-2-methyl-3-hydroxypentanoic acid, in which the methyl group at C-2 has the opposite configuration, although the estimated specificity constant k_{cat}/K_m was only 0.2 ± 0.05 M^{-1} s^{-1}. The NAC thioester of (2*S*,3*S*)-2-methyl-3-hydroxypentanoic acid, in which the configuration of the hydroxy group at C-3 is reversed, was an even poorer substrate, with k_{cat}/K_m less than 0.01 M^{-1} s^{-1}, and there was no detectable reaction with the NAC thioester of (2*R*,3*S*)-2-methyl-3-hydroxypentanoic acid, in which the configuration is reversed at both C-2 and C-3. These data provide the first demonstration of the reversibility of the KR-catalyzed reduction, and they confirm the view that the individual domains of an extension module can show a clear-cut stereospecificity for their substrates. The KR1 domain in particular has a distinct preference for those diketides with configurations matching the normal diketide intermediate at C-2 and C3, particularly discriminating between the diastereomers differing in the hydroxyl group configuration at C-3 (cf. ref *21*). The reversibility of the reaction allowed an estimate of the equilibrium constant to be made, using the Haldane relationship (*34*) as the ratio between the specificity constants for the forward and reverse reactions (54, using the diketide substrate analogues). On this estimate, the energetic balance between substrates and products of the ketoreductase-catalyzed reaction is relatively even, and the reaction is normally carried to completion by the continuous removal of the reduced products by the subsequent enzyme activities in the PKS. The reversibility of the KR-catalyzed reaction opens up the possibility that if the more rapidly produced isomer of the hydroxy thioester intermediate is a significantly poorer substrate for the ketosynthase domain of the succeeding module than is the slower formed isomer, then the configuration of the slower formed isomer might even be favored in the formation of the full-length polyketide chain.

Table 1: Kinetic Parameters for Reduction of *trans*-1-Decalone and for Oxidation of *trans*-1-Decalol by the KR Domain from Module 1 of DEBS, Housed either in the Diketide Synthase (DKS) or in a Version of DEBS 1-TE in Which the KR Domain of Module 2 Has Been Deleted (ΔKR2)

	k_{cat} (s^{-1})[e]	K_m (mM)[e]	k_{cat}/K_m (s^{-1} M^{-1})
DKS (±)-*trans*-1-decalone[b]	0.53 ± 0.02	1.2 ± 0.3	450 ± 150
DKS NADPH[a]	0.53 ± 0.01	0.011 ± 0.001	48920 ± 8000
ΔKR2 (±)-*trans*-1-decalone[b]	0.44 ± 0.05	1.12 ± 0.33	395 ± 230
ΔKR2 NADPH[a]	0.46 ± 0.16	0.011 ± 0.002	43490 ± 5000
DKS *trans*-*cis*-1-decalol[c]	0.50 ± 0.03	1.2 ± 0.3	417 ± 200
DKS NADP$^+$[d]	0.48 ± 0.01	0.10 ± 0.01	5870 ± 900

[a] Determined in the presence of 10 mM *trans*-1-decalone. [b] Determined in the presence of 0.25 mM NADPH. [c] Determined in the presence of 10 mM *trans*-1-decalol. [d] Determined in the presence of 0.25 mM NADP$^+$. [e] The values of k_{cat} and K_m must be considered apparent values because of the presence in each case of a nonreacting enantiomer of the substrate.

Reduction of trans-1-Decalone and Oxidation of trans-1-Decalol Substrate Analogues by the Ketoreductase. (9*RS*)-*trans*-1-Decalone has been successfully used as a surrogate substrate for the ketoreductase activity of fatty acid synthase (*22*−*24*). The kinetic data for reduction by the diketide synthase of *trans*-1-decalone are summarized in Table 1. The kinetic data for NADPH were obtained by varying the concentration of NADPH while keeping the concentration of *trans*-1-decalone constant at 10 mM. Reduction of (9*RS*)-*trans*-1-decalone was shown by GC/MS analysis to result in production of (1*S*,9*R*)-*trans*-1-decalol. A chemically synthesized racemic mixture of (1*S*,9*R*)- and (1*R*,9*S*)-*trans*-1-decalols gave baseline separation of the enantiomers with retention times of 13.31 and 13.41 min for the (1*S*,9*R*) and (1*R*,9*S*) isomers, respectively. The identity of the peak assigned to the (1*S*,9*R*) enantiomer was revealed by comparison with the GC/MS analysis of an authentic sample of this product, obtained through the reduction of (9*RS*)-*trans*-1-decalone catalyzed by rat fatty acid synthase (*24*). A peak initially present with a retention time of 13.47 min and corresponding to (9*R*)-*trans*-1-decalone clearly diminished as a result of ketoreduction. The mass spectra of the decalol compounds showed the expected *m*/*z* at 154 (M^+) and 136 ($M^+ - H_2O$), while the decalones showed the expected *m*/*z* of 170 ($M + NH_4^+$) and 152 (M^+). Other stereoisomers of *trans*-1-decalol comprised <1% of the amounts of the (1*S*,9*R*) enantiomer. For comparison, the relative rates of formation of *trans*-1-decalol products catalyzed by fatty acid synthase under the conditions used are (1*S*,9*R*) isomer, 100; (1*S*,9*S*) isomer, 1.5; (1*R*,9*S*) isomer, 0.5; and (1*R*,9*R*) isomer, <0.05; respectively (*24*).

The ability of the ketoreductase domain to catalyze oxidation of decalol substrates was demonstrated by the reduction of NADP$^+$ in the presence of a racemic mixture of (1*S*,9*R*)- and (1*R*,9*S*)-*trans*-1-decalols (Figure 3). The kinetic data for these conversions are also presented in Table 1. The K_m value for NADP$^+$ was determined by varying the concentration of cofactor while keeping the concentration

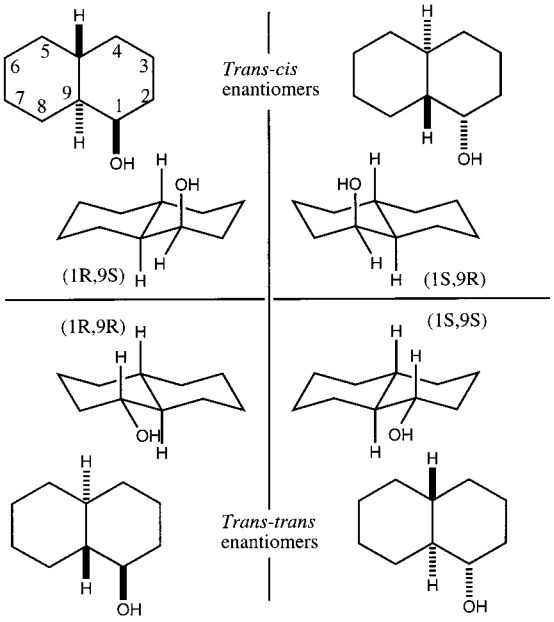

FIGURE 3: Structures of the four stereoisomers of *trans*-1-decalol shown in two alternative types of chemical representation. The (1*S*,9*R*) isomer is the specific product of the diketide synthase KR domain acting on (9*RS*)-*trans*-1-decalone.

of racemic 1-decalol constant at 10 mM. A racemic mixture of (1*R*,9*R*)- and (1*S*,9*S*)-*trans*-1-decalols (Figure 3) was not accepted as a substrate for oxidation by the ketoreductase. From these data it appears that the hydride ion is preferably (and reversibly) added to the equatorial position in *trans*-1-decalone and to the *re* side of the carbonyl group. This is exactly the outcome of the analogous reaction catalyzed by fatty acid synthase acting on *trans*-1-decalone (*23*, *24*). Given the very high amino acid sequence similarity between the KR domains of fatty acid synthase and of modular PKSs (*35*) and the similarity of the reactions that they catalyze, they are very likely to adopt the same NADPH-binding fold and to bind the coenzyme in the syn form from which the C-4 *pro-S* hydrogen is specifically transferred (*36*). In confirmation of this, both fatty acid synthase (*24*) and DEBS 1 (*37*) have been shown to transfer the *pro-S* hydrogen to their substrates (B-side dehydrogenases). The stereoselectivity observed here toward the *trans*-1-decalone and the corresponding decalol similarly underlines the very close relationship between the structure and function of the KR active sites in the two types of synthase.

As a further check that the protein context of the KR1 domain housed in the diketide synthase is close to that in the native PKS, kinetic analysis was carried out using *trans*-1-decalone as a substrate for purified DEBS 1-TE (ΔKR2), a mutant of DEBS 1-TE from which the ketoreductase domain of module 2 has been specifically deleted. The data from this analysis are presented in Table 1, from which it is clear that the kinetic constants derived for KR1 housed in this enzyme do not differ, within the error of the experiments, from those determined for the same domain in the diketide synthase.

The limiting rate constant for the reaction with *trans*-1-decalone is of the same order of magnitude as those observed for FAS utilizing (9*S*)-*trans*-1-decalone as substrate but 2 orders of magnitude lower than that for (9*R*)-*trans*-1-decalone (*24*). Nevertheless, the rigid alicyclic ketone is still a very good substrate for the ketoreductase (Table 1), and the ability to use this surrogate substrate in an NADPH-linked assay for modular PKSs should be particularly valuable in further mechanistic studies on these enzymes. It may also prove more appropriate than the ketoester diketide for probing the stereoselectivity of substrate binding and stereospecificity of hydride addition in KR domains of other isolated PKS modules.

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