Evidence for Two Catalytically Independent Clusters of Active Sites in a Functional Modular Polyketide Synthase†

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ABSTRACT: Modular polyketide synthases (PKSs), such as the 6-deoxyerythronolide B synthase (DEBS), catalyze the biosynthesis of structurally complex and medicinally important natural products. These large multifunctional enzymes are organized into "modules", where each module contains active sites homologous to those of higher eucaryotic fatty acid synthases (FASs). Like FASs, modular PKSs are known to be dimers. Here we provide functional evidence for the existence of two catalytically independent clusters of active sites within a modular PKS. In three bimodular derivatives of DEBS, the ketosynthase domain of module 1 (KS-1) or module 2 (KS-2) or the acyl carrier protein domain of module 2 (ACP-2) was inactivated via site-directed mutagenesis. As expected, the purified proteins were unable to catalyze polyketide synthesis (although the KS-1 mutant could convert a diketide thioester into the predicted triketide lactone). Remarkably however, the KS-1/KS-2 and the KS-2/ACP-2 mutant pairs could efficiently complement each other and catalyze polyketide formation. In contrast, the KS-1 and ACP-2 mutants did not complement each other. On the basis of these and other results, a model is proposed in which the individual modules of a PKS dimer form head-to-tail homodimers, thereby generating two equivalent and independent clusters of active sites for polyketide biosynthesis. Specifically, each subunit contributes half of the KS and ACP domains in each cluster. A similar complementation approach should also be useful in dissecting the organization of the remaining types of active sites within this family of multienzyme assemblies. Finally, blocked systems, such as the KS-1 mutant described here, present a new strategy for the noncompetitive conversion of unnatural substrates into polyketides by modular PKSs.

Modular polyketide synthases (PKSs) catalyze the biosynthesis of polyketides, a large family of structurally complex and medicinally important natural products (O'Hagan, 1991). Polyketide formation is analogous to fatty acid biosynthesis, in which successive decarboxylative condensations between coenzyme A (CoA) thioesters of carboxylic acids give rise to an extended carbon chain. However, in contrast to the fatty acid synthases (FASs) (Smith, 1994; Wakil, 1989), PKSs vary the choices of carboxylic acid monomers and catalyze varying extents of β-carbonyl reduction after each condensation. Additionally, they also control the stereochemistry of chiral carbon centers and the regiochemistry of cyclization(s) after chain synthesis. Together, this flexibility creates the potential for the controlled generation of molecular diversity.

The 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea* catalyzes the biosynthesis of 6-deoxyerythronolide B (6dEB) (**1**), the polyketide aglycone of the antibiotic erythromycin (Figure 1A) (Katz & Donadio, 1993). Genetic analysis of this modular PKS revealed three large proteins (each MW >300 000) that contain a repetitive series of catalytic centers homologous to FAS active sites: β-ketoacyl−acyl carrier protein synthase (KS), acyltransferase (AT), dehydratase (DH), enoyl reductase (ER), β-ketoreductase (KR), acyl carrier domain (ACP), and thioesterase (TE) (Cortes et al., 1990, Donadio et al., 1991). In modular PKSs, these active sites are organized into groups called "modules", where each module catalyzes one cycle of condensation and β-ketoreduction in 6dEB biosynthesis. Thus, modular PKSs are strikingly similar to avian and mammalian FASs with respect to the identity as well as linear arrangement of active sites within a module. However, two key differences exist between modular PKSs and higher eucaryotic FASs. First, whereas a FAS contains a full set of reductive domains (KR, DH, ER), each PKS module contains only the subset of reductive domains required for a particular condensation cycle. Second, whereas a single set of active sites is used iteratively in FASs, the active sites in a PKS module participate only once during the synthesis of a polyketide molecule.

Although the three-dimensional structure of a modular PKS remains unknown, gel filtration (Aparicio et al., 1994; Pieper et al., 1995a; Staunton et al., 1996) and sedimentation equilibrium experiments (Staunton et al., 1996) with the DEBS proteins have suggested that these complexes are dimeric. Recently, Staunton et al. showed that an elastase fragment containing module 5 from DEBS cross-links as a dimer in the presence of dibromopropanone. A similar proteolytic fragment containing module 6 without ACP-6 fails to cross-link, suggesting that the ACP domain is involved in the cross-linking reaction (Staunton et al., 1996).

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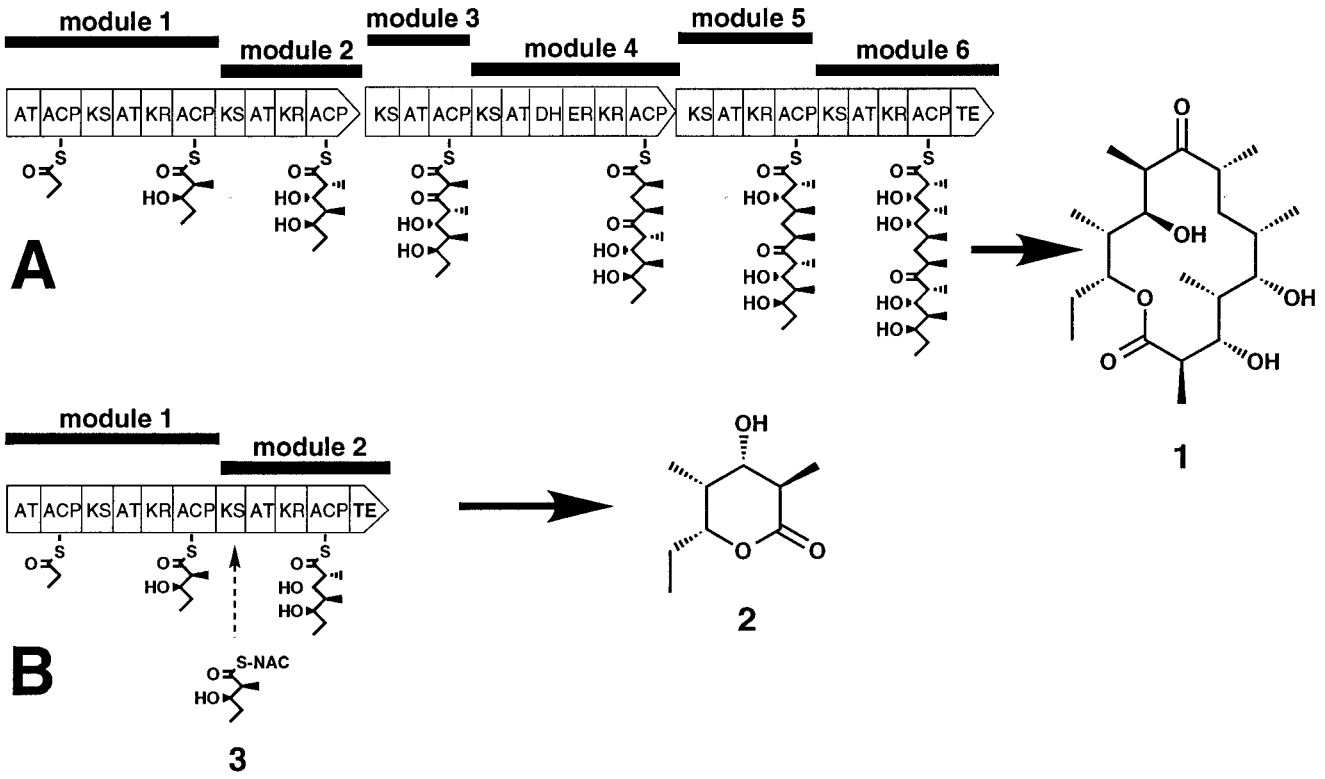

FIGURE 1: Genetic organization of (A) DEBS and (B) DEBS1 + TE. DEBS, which catalyzes the biosynthesis of 6dEB (**1**), consists of the polypeptides DEBS1, DEBS2, and DEBS3 (each MW >300 000) that each possess two modules. DEBS1 + TE consists of DEBS1 fused to the thioesterase domain from DEBS3 and catalyzes the *in vivo* and *in vitro* synthesis of triketide **2**. The same product can also be generated by DEBS1 + TE from the diketide analog **3** in the presence of methylmalonyl-CoA and NADPH. See text for details.

These results are consistent with a well-established model for the assembly of higher eucaryotic FASs as head-to-tail homodimers (summarized below). The DEBS cross-linking data also corroborate available evidence from deletion mutagenesis of DEBS, which indicates that modules are independent in structure and function (Cortes et al., 1995; Kao et al., 1994, 1995, 1996). [See, for example, the engineered bimodular PKS, DEBS1 + TE, which catalyzes the synthesis of the cyclized DEBS triketide intermediate (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (**2**) (Figure 1B) (Cortes et al., 1995; Kao et al., 1995).]

The current model for head-to-tail assembly of higher eucaryotic FASs, leading to the creation of two equivalent and independent active sites for fatty acid biosynthesis, crucially draws from not just structural but also functional evidence. Structural support for this model comes from cross-linking experiments demonstrating the proximity of the KS and ACP domains from opposite subunits (Stoops et al., 1983; Stoops & Wakil, 1981, 1982) and from fluorescence energy transfer measurements of the >56 Å distance between the two thioesterase domains (Yuan & Hammes, 1985). Functional evidence for the existence of two catalytic centers in a FAS complex is based on measurements of the number of fatty acid molecules produced per dimer in single-turnover experiments (Singh et al., 1984) and on active site complementation studies using chemically inactivated protein preparations, which conclusively demonstrated that each catalytic center is composed of a KS and ACP domain from opposite subunits (Wang et al., 1984). Here, we show that some, but not all, pairs of active site mutations in modular PKSs can complement each other. Our results directly reinforce the close structural and functional parallel between FASs and modular PKSs that was first recognized on the basis of sequence comparisons between these two classes of multifunctional proteins (Cortes et al., 1990; Donadio et al., 1991).

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Four recombinant strains of *Streptomyces coelicolor* were used in this study. CH999/pCK12, whose construction was described earlier, expresses the gene encoding DEBS1 + TE (Figure 1B) (Kao et al., 1995). CH999/pCK16, CH999/pCK18, and CH999/pCK19 express null mutants of the DEBS1 + TE gene in which the KS-1, KS-2, or ACP-2 domain was inactivated by site-directed mutagenesis; these mutants are designated $KS1^0$, $KS2^0$, and $ACP2^0$, respectively (Figure 2A). In $KS1^0$ and $KS2^0$, the mutations C729A and C2200A, respectively, replace the KS catalytic cysteine with alanine and generate a diagnostic *Nhe*I restriction site at the mutation site. In $ACP2^0$, the mutations S3419A and A3421Q replace the ACP-2 active site serine with alanine and the nonconserved A3421 with glutamine. The sequence encoding S3419A, L3420, and A3421Q is "GCCCTGCAG", so that a diagnostic *Pst*I restriction site is present at the mutation site. In all cases mutations were engineered via standard polymerase chain reaction (PCR) mutagenesis procedures.

Formation of Hybrid PKS Dimers. Crude preparations of DEBS1 + TE, $KS1^0$, $KS2^0$, and $ACP2^0$ enzymes were obtained as described previously (Pieper et al., 1996). DEBS protein concentrations were determined via the Bradford method (Bradford, 1976) and densitometry scanning using Adobe Photoshop 2.5.1 and NIH Image 1.5.2 software. The proteins were purified to 50−90%, either individually or as

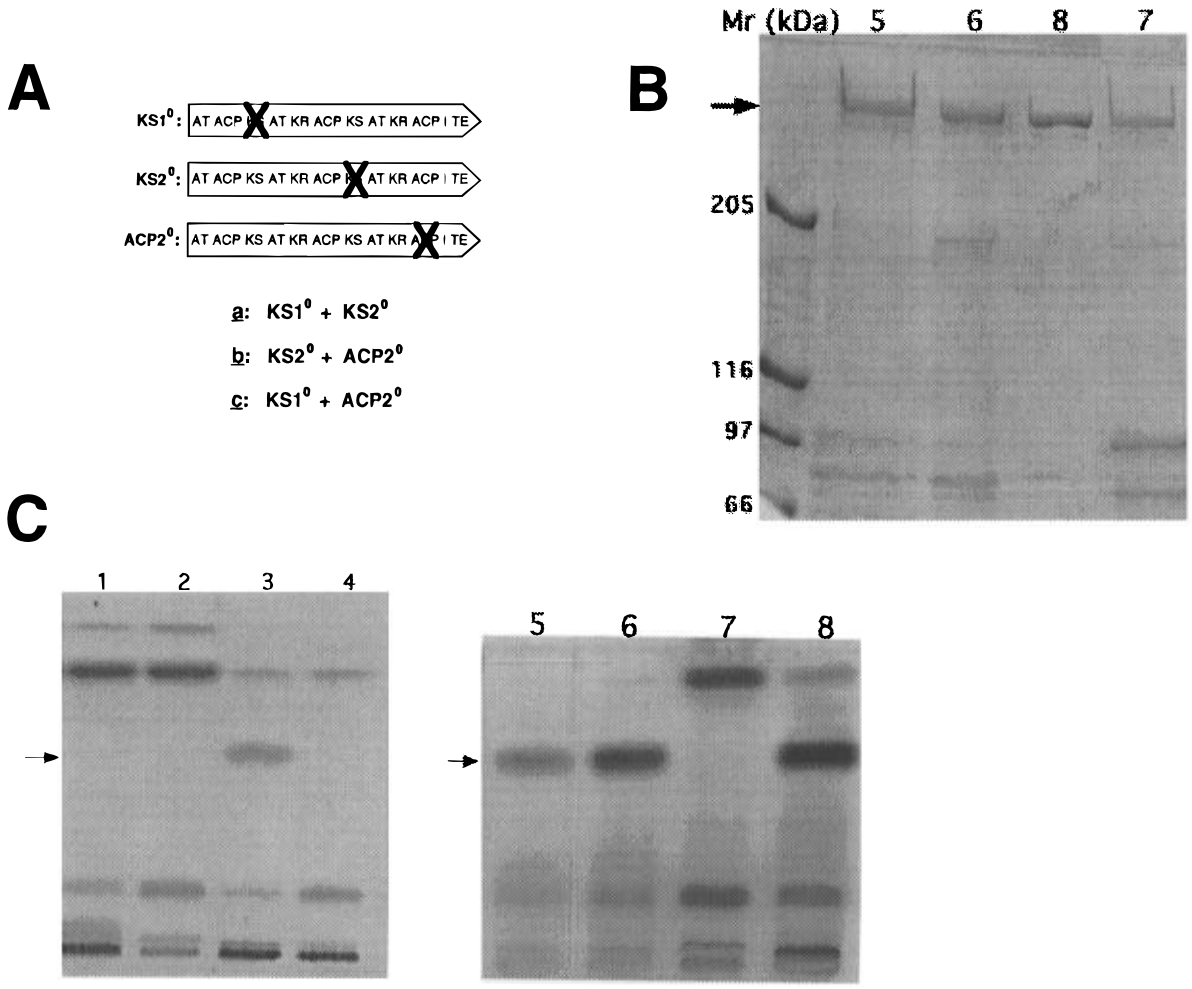

FIGURE 2: Complementation of KS and ACP domains in DEBS1 + TE hybrids. (A) Null mutants of DEBS1 + TE. The catalytic residue of the KS-1, KS-2, or ACP-2 domain is replaced by alanine in mutants $KS1^0$ (C729A), $KS2^0$ (C2200A), and $ACP2^0$ (S3419A), respectively. The three mutant hybrids tested for complementation are denoted **a**, **b**, and **c**. (B) Coomassie Blue-stained SDS−PAGE (5% acrylamide) of purified DEBS1 + TE and mutant hybrids. Lane numbers correspond to lane numbers in panel C (below). Lane 5: hybrid **b**. Lane 6: hybrid **a**. Lane 7: hybrid **c**. Lane 8: DEBS1 + TE. The arrow indicates the band for the 400 kDa DEBS1 + TE wild-type and mutant proteins. (C) TLC autoradiograph of ^{14}C-labeled triketide **2**. **2** is indicated by the arrow. [^{14}C]Methylmalonyl-CoA (200 μM, 5600 μCi/mmol) was the radiolabeled substrate from lanes 1−4, and [^{14}C]propionyl-CoA (250 μM, 5900 μCi/mmol) was the radiolabeled substrate for lanes 5−8. Final protein concentrations were 150−250 μg/mL, and concentrations of the unlabeled substrates (propionyl-CoA, methylmalonyl-CoA, NADPH) are as described previously, unless otherwise stated (Pieper et al., 1996). In lanes 3 and 4, 1 mM diketide analog **3** was used instead of propionyl-CoA. Lane 1: $KS1^0$. Lane 2: $KS2^0$. Lane 3: $KS1^0$ + diketide **3**. Lane 4: $KS2^0$ + diketide **3**. Lane 5: hybrid **b**. Lane 6: hybrid **a**. Lane 7: hybrid **c**. Lane 8: DEBS1 + TE. The intact activity of module 2 in $KS1^0$ is demonstrated by the conversion of the diketide analog **3** into triketide **2** (lane 3).

equal mixtures of hybrids **a** (=$KS1^0$ + $KS2^0$), **b** (=$KS2^0$ + $ACP2^0$), or **c** (=$KS1^0$ + $ACP2^0$) (Figure 2A,B) as described elsewhere (R. Pieper, D. E. Cane, and C. Khosla, manuscript in preparation). An anion-exchange chromatography step on a Resource Q column (Pharmacia) served as a device to "scramble" (dissociate and reassociate) the two different mutants in hybrids **a**, **b**, and **c**, giving rise to all possible PKS dimer combinations. (For a description of all possible dimer conbinations, see the results below on the measured k_{cat} of the $KS1^0$ + $KS2^0$ hybrid **a**.)

Assay Conditions. To assay for catalytic activity, the final DEBS protein concentration was adjusted to 150−250 μg/mL and incubated for 3 h with saturating concentrations of substrates (propionyl-CoA, methylmalonyl-CoA, and NADPH, unless otherwise stated) as described previously (Pieper et al., 1996). Polyketide products were extracted with ethyl acetate, separated via thin-layer chromatography (TLC), and quantified on a PhosphorImager (Molecular Dynamics). Kinetic measurements of triketide formation with purified DEBS1 + TE and hybrid **a** were conducted as described previously (Pieper et al., 1996).

RESULTS

Due to the simplicity of the system, the well-characterized nature of the protein, and the high yields of polyketide product generated *in vitro*, the bimodular PKS, DEBS1 + TE, was used as a model system to examine the complementation of enzymatic domains in modular PKSs. In the presence of propionyl-CoA, (2*RS*)-methylmalonyl-CoA, and NADPH, this PKS catalyzes the synthesis of the triketide **2** *in vivo* and *in vitro* (Figure 1B and Figure 2C, lane 8) (Kao et al., 1995; Pieper et al., 1995a). The same product can also be generated by the enzyme at comparable rates in the absence of propionyl-CoA, presumably due to the ability of one or more KS domains to decarboxylate methylmalonyl-CoA (or its enzyme-bound version) (Pieper et al., 1996). Finally, **2** can also be synthesized by DEBS1 + TE upon incubation with (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-NAC

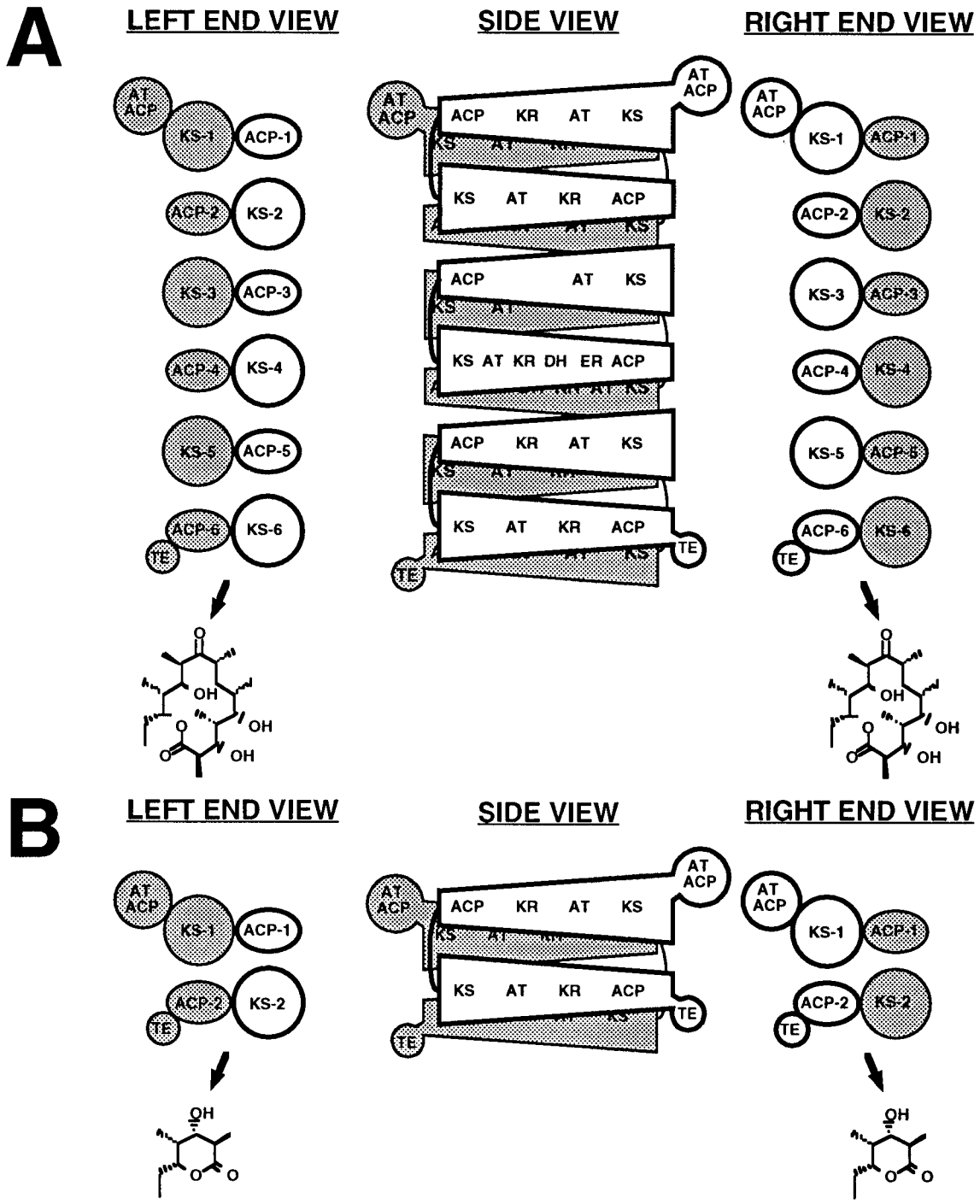

FIGURE 3: Proposed organization of KS and ACP domains in dimeric (A) DEBS and (B) DEBS + TE. The two identical subunits are shaded and unshaded. Side view: Each module forms a head-to-tail homodimer (=shaded + unshaded module), analogous to higher eucaryotic FASs. End views: Two equivalent catalytic centers are present at opposite ends of the PKS complex. Each subunit contributes half of the KS and ACP domains to each catalytic center, and KS-*n* and ACP-*n* from opposite subunits interact during the synthesis of a polyketide molecule.

thioester (**3**) (an analog of the enzyme-bound diketide intermediate), methylmalonyl-CoA, and NADPH (Figure 1B) (Pieper et al., 1995a).

Complementation of KS and ACP Domains in DEBS1 + TE Hybrids. As shown in Figure 2C (lanes 1 and 2), neither $KS1^0$ nor $KS2^0$ was able to catalyze polyketide synthesis by itself in the presence of propionyl-CoA, methylmalonyl-CoA, and NADPH; $ACP2^0$ was similarly nonfunctional (not shown). However, $KS1^0$ appears to be completely competent at converting the synthetic diketide analog **3** into the corresponding triketide product **2**, while $KS2^0$ is inactive in this assay (Figure 2C, lanes 3 and 4). This suggests that the lesion in module 1 does not adversely affect the properties of module 2 in $KS1^0$. [A more detailed study of the enzymatic and *in vivo* biotransformation properties of this mutant will be published elsewhere (J. R. Jacobsen, G. Luo, H. Huang, M. McPherson, D. E. Cane, and C. Khosla, manuscript in preparation).] In Figure 2C, lanes 5−7, the reaction products of PKS hybrids **b**, **a**, and **c** are shown, respectively. (See Figure 2A for hybrid descriptions.) Triketide synthesis by hybrids **a** and **b** is indicative of active site complementation of the KS-1 and KS-2 mutants and the

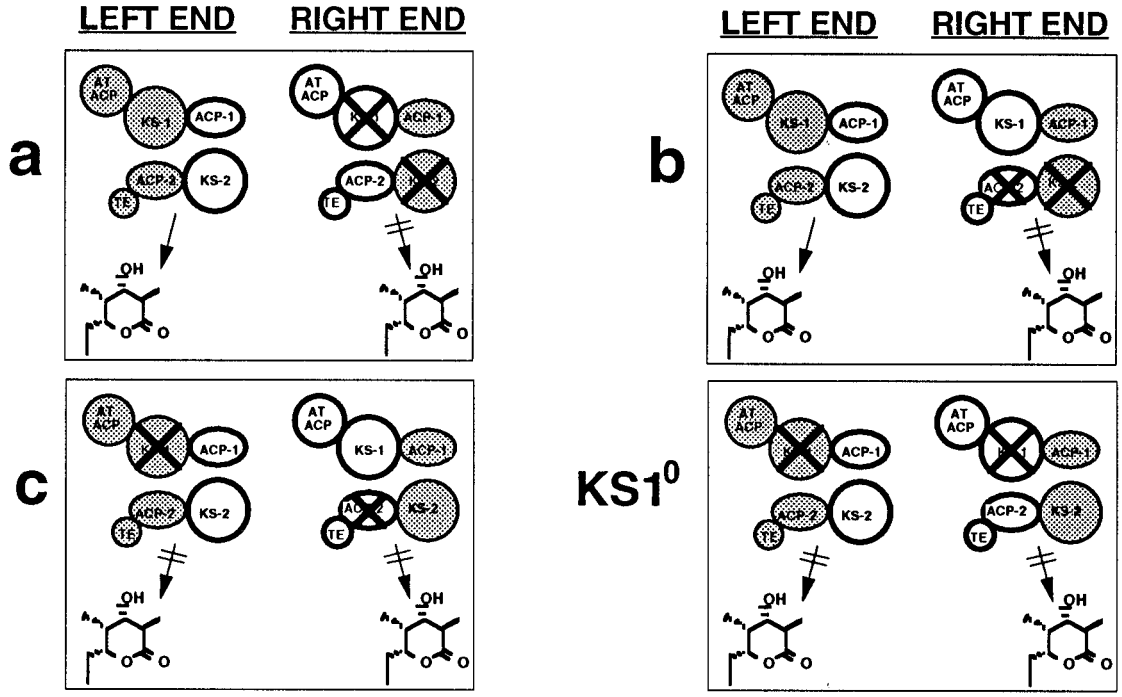

FIGURE 4: Localization of mutations in DEBS1 + TE hybrids **a**, **b**, and **c** (end views only of mutant heterodimers). Active site complementation in hybrids **a** and **b** and lack of complementation in hybrid **c** are depicted. The null mutant $KS1^0$ lacks activity when propionyl-CoA, methylmalonyl-CoA, and NADPH are the substrates; however, $KS1^0$ will convert diketide **3**, methylmalonyl-CoA, and NADPH to triketide; $KS2^0$ and $ACP2^0$ are similarly nonfunctional (not shown).

KS-2 and ACP-2 mutants in the DEBS1 + TE system, whereas the null activity of hybrid **c** indicates that the KS-1 and ACP-2 mutants are unable to complement each other.

Relative k_{cat} of the $KS1^0$ + $KS2^0$ Hybrid. The specific activity of $KS1^0$ + $KS2^0$ hybrid **a** relative to purified DEBS1 + TE was measured. Under saturating concentrations of propionyl-CoA, methylmalonyl-CoA, and NADPH, the k_{cat} for hybrid **a** was found to be 1.7 ± 0.5 min^{-1} averaged over two experiments. This k_{cat} is approximately 25% of the measured k_{cat} for the purified DEBS1 + TE, 6.9 min^{-1} (R. Pieper, D. E. Cane, and C. Khosla, manuscript in preparation). In the model described below, since one-half of an equimolar mixture of $KS1^0$ and $KS2^0$ proteins would be comprised of two inactive homodimers ($KS1^0$ + $KS1^0$ and $KS2^0$ + $KS2^0$) and the other half comprised of the 50% active hybrid dimer ($KS1^0$ + $KS2^0$), the maximum theoretical specific activity obtainable from the hybrid mixture **a** (or **b**) would be 25% that of wild-type DEBS1 + TE.

DISCUSSION

The studies described here demonstrate that (1) a dimeric modular PKS possesses two equivalent clusters of active sites for polyketide biosynthesis and (2) each PKS subunit contributes half of the KS and ACP domains to each catalytic center. In particular, our experiments with DEBS1 + TE show that one protein subunit contributes KS-1 and ACP-2 and the other subunit contributes KS-2 and (by inference) ACP-1 to a catalytic center. On the basis of these and other results, a model delineating the functional interactions of the KS and ACP domains is shown in Figure 3A for DEBS and Figure 3B for DEBS1 + TE. In this model, each module forms a head-to-tail homodimer, allowing KS-*n* and ACP-*n* from opposite subunits to interact in a manner analogous to higher eucaryotic FASs. Two independent and equivalent catalytic centers for polyketide synthesis exist at each end of the PKS complex (Figure 3, end views). As shown in Figure 4, the model completely explains the data presented here: The mutations in hybrids **a** and **b** are confined to the active sites at only one end of the PKS complex, leaving the other end of the dimer intact and fully competent to catalyze polyketide formation. On the other hand, the mutations in hybrid **c** occur at both ends of the PKS complex, inactivating both catalytic centers and completely abolishing polyketide synthesis. This model, as well as our results, is consistent with the observations of Staunton et al. for interactions within a module dimer and for the transfer of polyketide intermediates between adjacent modules (Staunton et al., 1996). Together, these results reinforce the close structural and functional parallel between FASs and modular PKSs that was first observed on the basis of sequence comparisons between these two classes of multifunctional proteins (Cortes et al., 1990; Donadio et al., 1991). The analytical strategy presented here could also be used to map the patterns by which other catalytic domains, such as acyltransferases, ketoreductases, dehydratases, and enoylreductases, are contributed by the PKS subunits to the two functionally independent but covalently interconnected catalytic clusters.

Finally, the ability of the $KS1^0$ mutant to efficiently process exogenously fed diketide analogs presents a new strategy for *in vitro* and *in vivo* generation of novel polyketides. Although modular PKSs are known to recognize and process a variety of nonnatural primer units (Pieper et al., 1995a,b; Wiesmann et al., 1995), the process is relatively inefficient due to the ability of the PKS to decarboxylate methylmalonyl extender units and use the resulting propionyl groups as preferred primers (Pieper et al., 1996). As shown in Figure 2C (lane 1), this decarboxylative priming process does not take place in the $KS1^0$ mutant. Thus, such a blocked mutant could serve as an

invaluable resource for future studies on the molecular recognition properties of these remarkable enzymes, as well as for the generation of novel polyketides via mutasynthetic approaches.

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