Functional Orientation of the Acyltransferase Domain in a Module of the Erythromycin 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 multienzymes are organized into a series of functional units known as modules. Each dimeric module contains two catalytically independent clusters of active sites homologous to those of vertebrate fatty acid synthases. Earlier studies have shown that modules consist of head-to-tail homodimers in which ketosynthase (KS) and acyl carrier protein (ACP) domains are contributed by opposite subunits to form a catalytic center. Here, we probe the functional topology of the acyltransferase (AT) domain which transfers the methylmalonyl moiety of methylmalonyl-CoA onto the phosphopantetheine arm of the ACP domain. Using a bimodular derivative of DEBS, the AT domain of module 2 (AT2) was inactivated by site-directed mutagenesis. Heterodimeric protein pairs were generated in vitro between the inactivated AT2 (AT2°) polypeptide and an inactive KS1 (KS1°) or KS2 (KS2°) protein. Both of these hybrid proteins supported polyketide synthesis, suggesting that AT2 can perform its function from either subunit. The apparent catalytic rate constants for each of the two hybrid protein pairs, KS1°/AT2° and KS2°/AT2°, were identical, indicating that no significant kinetic preference exists for a particular AT2-ACP2 combination. These results suggest that the AT domain can be shared between the two clusters of active sites within the same dimeric module. Such a novel structural organization might provide a functional advantage for the efficient biosynthesis of polyketides.

The widely used broad spectrum antibiotic erythromycin A is a representative of a large class of pharmaceutically active polyketide natural products (*1*). The polyketide aglycone of the antibiotic erythromycin, 6-deoxyerythronolide B, is synthesized by a modular polyketide synthase (PKS)[1] known as 6-deoxyerythronolide B synthase (DEBS) (*2*, *3*). DEBS is a hexameric protein complex ($\alpha_2\beta_2\gamma_2$) consisting of three large homodimeric multidomain polypeptide chains, DEBS1, DEBS2, and DEBS3. Each polypeptide includes two modules, where each module is responsible for a single round of condensation and associated reduction reactions. This remarkable modular organization of the biosynthetic machinery appears to be a feature of a broad class of complex polyketides such as rapamycin (*4*), oleandomycin (*5*), and avermectin (*6*). These modular PKSs have a one-to-one correspondence between the organization of the active sites and the structure of the derived polyketide product. This modular organization, combined with the observed structural diversity among polyketides, raises the possibility that these multienzyme systems might possess vast combinatorial potential. Indeed, several studies have already shown that genes can be swapped between the erythromycin and rapamycin clusters to generate novel products with rationally predictable structures (*7*−*9*). The construction of large-scale combinatorial libraries depends critically on the ability to carry out genetic manipulations with a high frequency of success. Although several such manipulations at the gene level have produced novel polyketides, other attempts have failed. To exploit the catalytic versatility of modular polyketide synthases, it is therefore necessary to gain fundamental mechanistic insights into the function of these complex catalysts and to obtain a deeper understanding of the structural flexibility and catalytic organization of the DEBS proteins.

The heterologous expression of 6-deoxyerythronolide B synthase (*10*) and the cell-free synthesis of 6-dEB from the expressed protein containing at least 28 active sites (*11*) has provided an important step in understanding the modular organization and mechanistic principles of PKSs in general. DEBS contains a repetitive series of catalytic centers homologous to fatty acid synthase (FAS) active sites: β-ketoacyl-acyl carrier protein synthase (KS), acyltransferase

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[1] Abbreviations: PKS, polyketide synthase; DEBS, 6-deoxyerythronolide B synthase; KS, ketosynthase; KS1°, inactivated KS from module 1; KS2°, inactivated KS from module 2; AT, acyltransferase; AT2°, inactivated AT from module 2; KR, ketoreductase; ACP, acyl carrier protein; 6-dEB, 6-deoxyerythronolide; DH, dehydratase; ER, enoylreductase; TE, thioesterase; CoA, coenzyme A; FAS, fatty acid synthase; DTT, dithiothreitol; NADPH, nicotinamide adenine dinucleotide reduced; EDTA, ethylenediaminetetraacetic acid.

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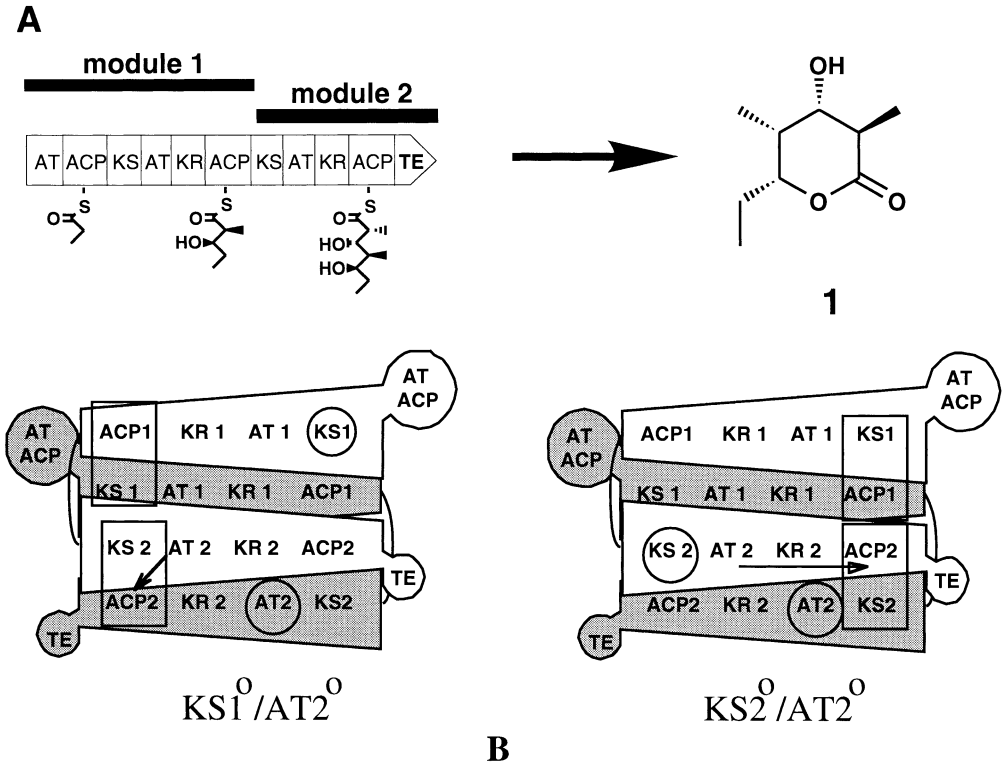

FIGURE 1: (A) Modular organization of DEBS1 + TE, which catalyzes the synthesis of triketide lactone (**1**), is an engineered hybrid protein in which DEBS1 is fused to the thioesterase (TE) domain from DEBS3. (B) Model of the heterodimeric mutants of DEBS1 + TE, depicting the strategy used for mapping the functional orientation of the AT and ACP domains. The shaded subunit contains the inactivated AT2 domain (AT2°). The other unshaded subunit contributes inactivated KS domains of either module 1 (KS1°) or module 2 (KS2°). The inactivated domains are circled and the boxed regions are active catalytic centers that are functional in these heterodimers. The two heterodimeric proteins probe the properties of ACP domains present on different subunts in relation to the AT2° mutation. The arrows shows the AT2-ACP2 pair that cooperate to produce triketide lactone in the respective mutants.

(AT), dehydratase (DH), enoyl reductase (ER), β-ketoreductase (KR), acyl carrier protein domain (ACP), and thioesterase (TE). Although the sequential arrangement of the domains in the linear polypeptide chain is established, there is very little understanding of their spatial interactions in the folded dimeric state. Studies with a series of truncated mutants of DEBS (*10*, *12*−*14*) have demonstrated that upstream modules are functionally independent of downstream modules and suggested that each protein module exists as a structurally independent folding unit.

According to our current working model, each module is a head-to-tail dimer possessing two equivalent clusters of active sites (*15*), consistent with the well-accepted organization of the vertebrate FAS (*16*−*18*). Evidence for this model comes from two types of studies: first, chemical cross-linking with dibromopropanone of KS and ACP domains from the opposite subunits (*19*), and second, in vitro complementation analysis in which the pair of inactive dimers carrying mutations in different functional domains were reconstituted to a catalytically active heterodimer (*15*). Here, we use a similar active site complementation strategy to determine the functional topology of the acyltransferase (AT) domain. Our studies show that the AT domain can remarkably perform its function from either subunit. The overall kinetic rate constant for the synthesis of (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid δ-lactone (referred to as triketide lactone **1** henceforth as shown in Figure 1A,) catalyzed by DEBS1+TE is similar irrespective of whether the ACP2 domain accepts methylmalonyl-CoA from the AT2 domain present on the same subunit or from the AT2 of the paired, equivalent subunit. These results provide evidence of a novel mechanism by which these multienzyme complexes might perform their function.

MATERIALS AND METHODS

Reagents and Chemicals. DL-2-[*methyl*-^{14}C]Methylmalonyl-coenzyme A (CoA) (56.4 mCi/mmol) was purchased from ARC Incorporation (St. Louis, MO). [1-^{14}C]Propionyl-CoA (54 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA). All other chemicals used were of the highest purity commercially available grade.

Strains and Culture Conditions. Four recombinant strains of *Streptomyces coelicolor* were used in this study. Construction of CH999/pCK12 expressing DEBS1 + TE (*12*) and CH999/pCK16 and CH999/pCK18 (*15*) expressing null mutants of DEBS1+TE gene in which domains KS1 and KS2, respectively, have been inactivated and have been described earlier. The null mutants are designated as KS1° and KS2°. CH999/pLJ1, expressing the DEBS1 + TE gene with an inactive AT2 domain (referred to as AT2°), was obtained by mutating the catalytic serine to alanine (S2649A) by standard polymerase chain reaction mutagenesis procedures.

Construction of the Mutant Containing Inactive AT2. The crystal structure of the *Escherichia coli* malonyl-CoA:acyl carrier protein transacylase had revealed a serine residue, predicted to be the catalytically essential nucleophile, which is located at the elbow between a β-strand and a α-helix, typical of α/β hydrolases (*20*). This serine residue is strictly

conserved in the sequences of 40 other known acyltransferases. The relevant serine residue in the AT2 domain of DEBS1 + TE was mutated to alanine by PCR-mediated mutagenesis. The resulting mutant DEBS1 + TE construct (AT2°), containing an inactive AT2 domain, did not produce any triketide lactone (**2**) when incubated with the normal polyketide chain-elongation substrates.

Purification of the DEBS Enzymes and Formation of Heterodimers. Homogeneous preparations of DEBS were obtained by a modifying the reported purification protocol (*21*). Mycelia from 1.2 L of culture were disrupted using a French press at 1300 psi. The lysate was centrifuged for 1 h at 25000*g*, and the supernatant was collected. Nucleic acids were precipitated with polyethylenimine (0.15%) and removed via centrifugation (20 min at 25000*g*). The protein solution was made 45% (w/v) saturated with ammonium sulfate and precipitated overnight. After centrifugation (30 min, 25000*g*), the pellet containing the DEBS proteins was redissolved in 100 mM sodium phosphate, pH 7.1, 4 mM DTT, 2 mM EDTA, and 20% glycerol. Approximately 25% of the protein sample was loaded on a size exclusion chromatography column. Fractions containing DEBS were pooled and applied on an anion exchange column (Resource Q; 6 mL column, Pharmacia). Samples containing DEBS were further purified on a Superdex 200 pg 26/60 (Pharmacia) gel filtration column. Heterodimeric proteins were generated by combining two mutant DEBS1 + TE proteins in equal amounts and subjecting the mixture to anion-exchange (Resource Q) chromatography, as previously described (*15*). This last step results in an exchange of subunits so as to generate a statistical mix of mutant hybrids containing each of the original inactive homodimeric pairs (25% each) as well as the heterodimeric pair (50%). For the sake of simplicity, all studies were carried out with mutants of the bimodular DEBS1 + TE which ordinarily produces triketide lactone (**2**).

Assay Conditions. A typical assay mixture contained 150−250 μg/mL of protein and saturating concentrations of each substrate (propionyl-CoA, methylmalonyl-CoA, NADPH) as described earlier (*22*). Polyketide products were extracted with ethyl acetate and separated via thin-layer chromatography (TLC). The product was confirmed by simultaneously running triketide lactone on TLC plate.

Kinetic Measurements. Time courses for the formation of triketide lactone were performed for 3 h. The reaction was quenched by adding ethyl acetate after 40, 80, 110, 150, and 180 min. The products were separated on TLC, and the apparent k_{cat} was calculated from the radioactivity incorporated into the triketide lactone. Quantitative measurements were performed on an Packard InstantImager.

RESULTS

Strategy for Mapping Functional Orientation of AT and ACP Domains. Previous in vitro complementation experiments with inactive KS and ACP mutants showed that, whereas both the KS1°/KS2° and KS2°/ACP2° mutant pairs were catalytically active, as a consequence of the formation of self-complementing heterodimeric pairs, a heterodimer formed from the KS1° and ACP2° mutant proteins was inactive (*15*). The model then proposed had individual modules of a PKS dimer organized in a head-to-tail fashion thereby generating two equivalent and independent clusters of active sites for polyketide biosynthesis. The inability of the KS1°/ACP2° heterodimer to carry out synthesis of polyketides suggested that the transfer of growing polyketide chain between modules takes place within the same subunit. On the basis of these observations, we designed a strategy to study the functional orientation of an AT-ACP pair. The aim was to investigate whether AT2, the acyltransferase domain of module 2, transfers the methylmalonyl moiety of from methylmalonyl-CoA to the ACP2 domain of the same subunit or to the ACP2 domain on the paired subunit. As shown in Figure 1A, a heterodimer formed between the AT2° and the KS1° mutants would synthesize triketide lactone provided that the active AT2 domain perform its function with the ACP2 domain present on the opposite subunit. The circled domains in Figure 1A indicate inactive domains while the squared boxes enclose the functional catalytic center constituted by the respective KS and ACP domains. Similarly, a heterodimeric protein between the AT2° and KS2° mutants (Figure 1B) would synthesize polyketide only when the functional domains of AT2 and ACP2 are present on the same subunit.

Complementation of the AT2° Domain with KS1° and KS2° Domains. Figure 2 , panels A and B, shows the result of in vitro assays carried out with the AT2°/KS1° and AT2°/KS2° heterodimers, respectively. All the homodimeric mutants, AT2° (lanes 3 and 5), KS1° (lane 4) and KS2° (lane 6), were unable to catalyze polyketide synthesis in the presence of propionyl-CoA, methylmalonyl-CoA, and NADPH. Significantly, however, both of the heterodimeric proteins, AT2°/KS1° and AT2°/KS2°, produced triketide lactone (**1**) (lane 2 and lane 7, respectively). Triketide lactone synthesis by these hybrid proteins indicates that the AT2 domain can catalyze transfer of the methylmalonyl moiety of methylmalonyl-CoA to the ACP2 domain of either strand of the dimeric protein.

Kinetics of Heterodimeric Proteins. Initial velocity measurements were carried with each of the heterodimeric proteins AT2°/KS1° and AT2°/KS2° under saturating substrate concentrations. The derived k_{cat} values of 3.1 ± 0.6 and 3.2 ± 0.5 min^{-1}, respectively, were calculated from these initial velocity measurements assuming that the dissociation constants of the DEBS1 + TE mutant heterodimers are unchanged compared to the native DEBS1 + TE homodimer. The k_{cat} value of DEBS1 + TE was calculated to be 9.9 ± 0.7 min^{-1}.

DISCUSSION

The studies described here demonstrate that (1) a PKS containing an AT2° null mutation is inactive, suggesting that acyltransfer between neighboring modules does not occur; (2) ACP2 can accept the methylmalonyl extender unit from the AT2 domain present on either subunit of the dimeric PKS protein; and (3) there appears to be no obvious kinetic preference whether the AT2 domain interacts with the ACP2 from the same subunit or that from the paired subunit. These results thus suggest that, in a heterodimeric protein, the AT2° mutation is functionally "recessive". Thus, a single AT2 domain appears to be sufficient to support polyketide chain elongation at each of the two independent catalytic centers found in a dimeric module. Such sharing of a single AT

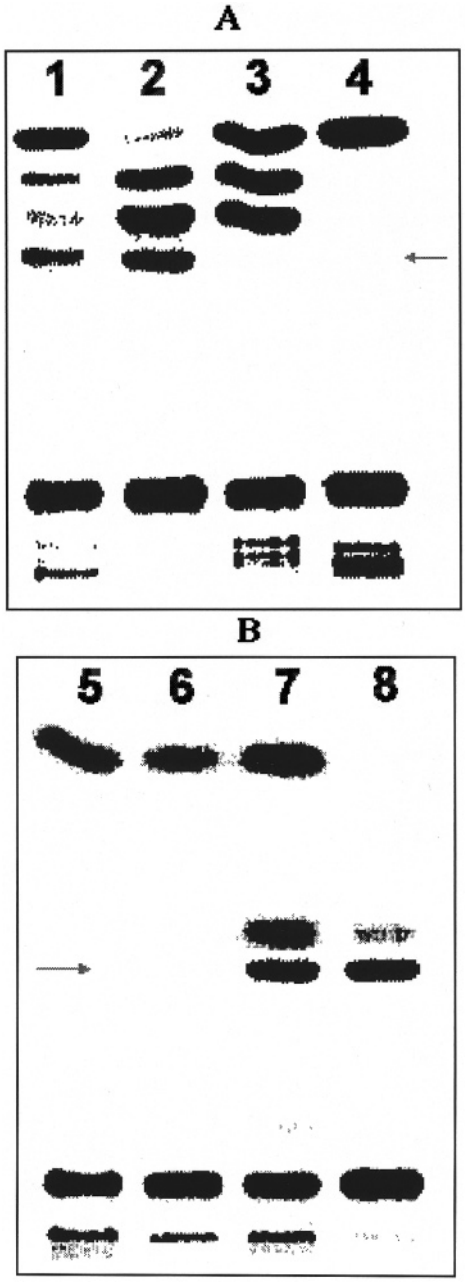

FIGURE 2: TLC autoradiographs of the in vitro assays carried out with heterodimeric proteins AT2°/KS1° (A) and AT2°/KS2° (B). [^{14}C]Propionyl-CoA was the radiolabeled substrate. Lane 1 and Lane 8 are standards, the band corresponding to triketide product is marked by an arrow. Lane 2: AT2°/KS1°; lane 3: AT2°; lane 4: KS1°; lane 5: AT2°; lane 6: KS2°; lane 7: AT2°/KS2°.

domain might provide a functional advantage in multidomain proteins.

Earlier work from our laboratory has demonstrated the active site complementation of the KS1° and KS2° mutant proteins and of the KS2° and ACP2° mutant proteins. These results together suggested that in a catalytically competent dimeric protein KS1 and ACP2 are contributed by one subunit and KS2 and ACP1 by the other subunit. In contrast, the inability of the KS1° and ACP2° mutants to complement one another established that a KS and an ACP domain within the protein subunit (on the same polypeptide strand) do not interact productively (*15*). In this report, we show that a mutant protein, AT2°, with a defective AT2 domain, can complement with both the KS1° and the KS2° mutant proteins. These findings, combined with the results of earlier studies, suggest that the AT2 domain can cooperate with ACP2 domains on either the same or the complementary module 2 subunit. The ability of the flexible ACP phosphopantetheine arm to acquire methylmalonyl chain extender units from the AT domain of either subunit might enhance the efficiency of polyketide synthesis. The similarity in the apparent k_{cat} values for the two hybrid proteins, AT2°/KS1° and AT2°/KS2°, probably indicates that no particular AT2-ACP2 pair has a kinetic preference sufficient to alter the overall steady-state rate constant for triketide lactone formation. The observed kinetic rate constant of the hybrid mutants are approximately 30% that of the DEBS1 + TE, a value somewhat higher than the predicted 25% of the wild-type catalytic rate. Such a small discrepancy is most likely due to the variation in protein preparations or minor experimental errors. The intrinsic rates of acyl transfer for each AT-ACP mutant pair have yet to be determined. In fact, these rates may well be masked by a slower catalytic event in the multistep polyketide chain elongation process.

The simplest explanation for these results is that each of the two AT2 domains could be effectively equidistant from the two corresponding ACP2 domains within the dimeric module 2. The flexible phosphopantetheine arm of the ACP could then accept the methylmalonyl substrate from either AT. Presumably, the relationship between the AT and ACP domains of module 2 is typical of all other DEBS modules as well. Thus, the KS and ACP domains from complementary subunits form a catalytic center, and each of these catalytic centers would be essentially equidistant from each of the individual AT domains within the same module. Our results are also consistent with recent findings of Smith and co-workers who showed that the DH domain of a FAS interacts with the ACP domain of the same polypeptide subunit (*18*), and are inconsistent with a simple side-by-side, fully extended model, that would predict that the DH domain should interact with the ACP domain from the opposite subunit. Interestingly, the DH and ACP domains in the same FAS subunit are separated by more than 600 amino acid residues. More recently the Smith group has also shown that the AT domain of the FAS can interact functionally with both KS-ACP pairs in the homodimer (see the preceding paper in this issue).

The studies presented here provide insight into the ways modular enzymes might catalyze polyketide synthesis. Ongoing studies with KR and TE domains are expected to delineate the functional status of these domains providing additional important mechanistic details and helping to flesh out a functional model for DEBS and modular polyketide synthases in general.

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