Purification and Characterization of Bimodular and Trimodular Derivatives 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. DEBS is a dimeric protein complex that consists of three large multidomain polypeptide chains, DEBS 1, DEBS 2, and DEBS 3. In turn, each polypeptide includes two modules, where one module is responsible for a single round of condensation and associated reduction reactions. A hybrid protein comprised of the first two modules of DEBS fused to a thioesterase domain (DEBS 1+TE) was purified to homogeneity in a fully active form (k_{cat} = 4.8 min $^{-1}$). Synthesis of the anticipated triketide lactone required the presence of (2*RS*)-methylmalonyl-CoA and NADPH. When available, propionyl-CoA is the preferred source of primer units. However, in its absence the enzyme can derive primer units via decarboxylation of a methylmalonyl extender. The two subunits of an engineered trimodular derivative of DEBS, DEBS 1 and module 3 of DEBS 2 linked to the TE domain (module 3+TE), were also individually purified and reconstituted to produce the expected tetraketide lactone *in vitro* (k_{cat} = 0.23 min^{-1}). The considerably lower specific activity of this trimodular PKS relative to its bimodular counterpart presumably reflects inefficient association between DEBS 1 and module 3+TE. As expected, module 3+TE could be efficiently cross-linked as a homodimer. In contrast, no cross-links were detectable between modules 2 and 3, even though biosynthesis of the tetraketide requires transient interactions to occur between these two modules. Since module 3 only contains the minimal set of active sites required in a module (a ketosynthase, an acyltransferase, and an acyl carrier protein domain) and is the first active unimodular protein to be purified to homogeneity, it represents an attractive target for future biophysical and structural studies.

Modular polyketide synthases (PKSs) catalyze the biosynthesis of polyketides, a large family of structurally complex and medicinally important natural products (Katz & Donadio, 1993; 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* (Cortes et al., 1990; Donadio et al., 1991) catalyzes the biosynthesis of 6-deoxyerythronolide B (6dEB) (**1**), the polyketide aglycon of the antibiotic erythromycin (Figure 1). Genetic analysis of this modular PKS revealed three large proteins (each MW > 300 kDa). Each protein contains two series of catalytic centers broadly 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). 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. Upstream modules are functionally independent of downstream ones, as illustrated by the activities of a series of mutants of DEBS, including DEBS 1 alone (Kao et al., 1994), DEBS 1+TE (Cortes et al., 1995; Kao et al., 1995), DEBS 1+module 3+TE (Kao et al., 1996a), and DEBS 1+2+module 5+TE (Kao et al., 1995) (Figure 2).

Recently, the development of a cell-free assay for DEBS 1+TE (Pieper et al., 1995; Wiesmann et al., 1995) and the complete DEBS system (Pieper et al., 1995) was reported. Active PKSs exist as dimers in which individual *modules* of a PKS dimer form head-to-tail homodimers, as indicated by chemical cross-linking studies (Staunton et al., 1996)[1] and the *in vitro* complementation patterns of various active site mutants (Kao et al., 1996b). Quantitative kinetic analysis of crude cell-free protein preparations revealed that, although DEBS 1+TE was fully active *in vitro*, the specific activity of the complete DEBS system was considerably lower than expected (Pieper et al., 1996).

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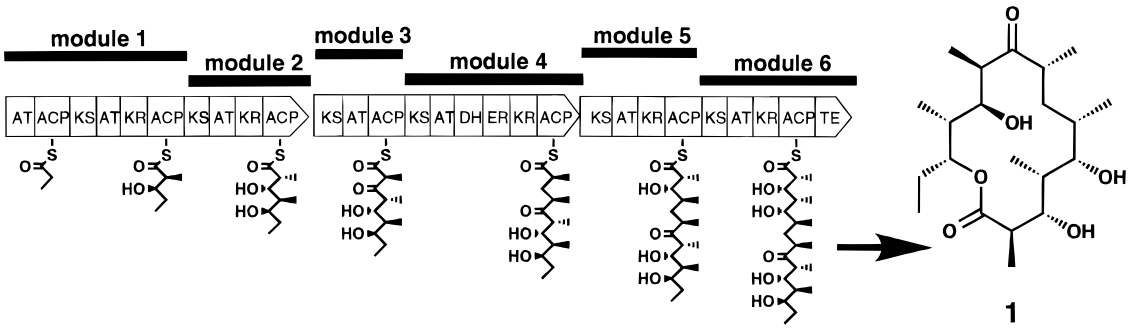

FIGURE 1: Modular organization of 6-deoxyerythronolide B synthase (DEBS) and genetically engineered truncated DEBS enzymes and their biosynthetic products. Each of the modules accounts for one polyketide chain extension and partial or complete β-ketoreduction cycle. The active sites denote acyltransferases (AT), acyl carrier proteins (ACP), β-ketoacyl-ACP transferases, β-ketoreductases, a dehydratase (DH), an enoylreductase (ER), and a thioesterase (TE). Full DEBS consists of three enzymes, DEBS 1, DEBS 2, and DEBS 3, and synthesizes 6-deoxyerythronolide B (**1**).

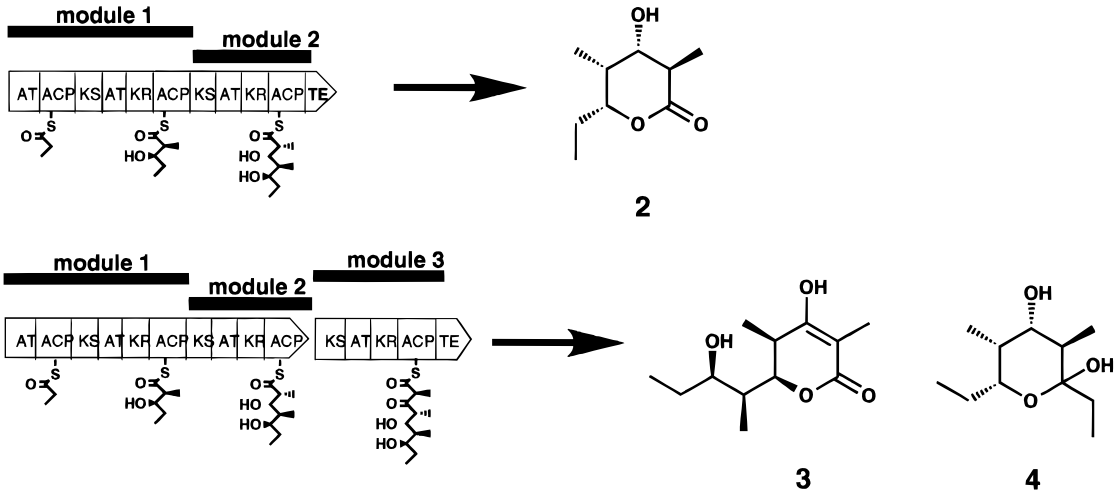

FIGURE 2: Bimodular and trimodular derivatives of DEBS. DEBS 1+TE is a fusion protein of the first DEBS enzyme with the thioesterase originally located at the end of DEBS 3 and generates a triketide C_9-lactone (**2**) enzymatically. DEBS 1 associated with a fusion protein of module 3 and thioesterase (module 3+TE) catalyzes the synthesis of two tetraketides, the six-membered lactone CK13a (**3**) and the hemiketal CK13b (**4**).

Here we report the development of a generally applicable purification procedure for modular PKSs that works especially well for subunits containing a C-terminal TE domain. Our procedure differs significantly from one reported earlier (Aparicio et al., 1994), from which active DEBS 1+TE has been recovered but not kinetically characterized (Wiesmann et al., 1995). Application of this procedure to two different engineered forms of the erythromycin PKS led to the recovery of active multi-enzyme proteins. In at least one case (DEBS 1+TE), the *in vitro* activity of the pure protein approximates the inferred *in vivo* activity.

MATERIALS AND METHODS

Reagents and Chemicals. DL-[2-*methyl*-^{14}C]Methylmalonyl-CoA (56.4 mCi/mmol) was obtained from ARC, Inc. [1-^{14}C]Propionate (59 mCi/mmol), purchased from Moravek Biochemicals, was enzymatically converted into [1-^{14}C]propionyl-CoA as described previously (Pieper et al., 1996). The cross-linking reagents EGS [ethylene glycol bis(succinimidyl succinate)] and BMH [bis(maleimidohexane)] were from Pierce. 1,3-Dibromopropanone was from ICN Pharmaceuticals, Inc. Biogel A-5m (200−400 mesh) was obtained from Bio-Rad, Resource Q from Pharmacia Biotech, and the C-18 Ultrasphere IP 5 μm column from Beckman Instruments Inc.

Strains and Culture Conditions. Streptomyces coelicolor CH999/pCK12 (Kao et al., 1995) and CH999/pCK13 (Kao et al., 1996a) produce DEBS 1+TE and DEBS 1/module 3+TE, respectively (Figure 2); 400 mL of SMM liquid medium (Strauch et al., 1991) in a 2 L flask was inoculated with spores derived from one confluent lawn grown on a 9 cm petri plate. Cultivation for approximately 60 h yielded mycelial pellets of typically 8 g/L. Mycelia were harvested via centrifugation and directly suspended in disruption buffer.

Purification of the DEBS Enzymes. Mycelia from 1.2 L of culture were disrupted using a French press at 1300 psi. Crude extracts were processed as described earlier (Pieper et al., 1996). Approximately 3 mL of the ammonium sulfate fraction saturated to 45% (either from CH999/pCK12 or from CH999/pCK13) was loaded on a size exclusion chromatography column (Biogel A, 150 mL, 3 cm × 75 cm). Using buffer A (100 mM sodium phosphate, pH 7.1, 2 mM DTT, 2 mM EDTA, 1 mM benzamidine, and 10% glycerol), the

[1] Staunton et al. (1996) have reported that the ACP and KS domains of complementary chains of homodimeric modules (e.g., module 5) can be cross-linked by 1,3-dibromopropanone, indicating that these pairs of domains are within 3−5 Å of each other at the condensing enzyme active site. Although the "double helical" model they have proposed groups the successive modules "head-to-head" along the helical axis, the topological organization of individual module homodimeric pairs is necessarily head-to-tail, consistent with the well-accepted organization of vertebrate fatty acid synthases. For an alternate model of PKS organization which is completely consistent with the available data, see Kao et al. (1996b).

Table 1: Purification of DEBS 1 + TE

purification step for DEBS 1+TE	volume (mL)	total protein (mg)	total PKS act. (nmol of TKL/min)	sp act. (nmol of TKL min^{-1} mg^{-1})	purification (*x*-fold)	recovery (%)
crude extract (disruption)	17.5	196	29	0.15	1	91
supernatant (PEI-precipitation)	18.2	204	31.9	0.16	1.1	100
45% $(NH_4)_2SO_4$-saturated solution	2.8	104	23.3	0.22	1.5	73
gel filtration (BioGel A)	50	17	10.9	0.64	4.3	34
anion exchange (Resource Q)	1	1.5	14.7	9.8	65	46
sucrose gradient centrifugation	2	0.5	2.5	5	33	7.8

DEBS proteins were eluted using a flow rate of 0.43 mL/min in a volume range of typically 30−50 mL. Pooled fractions were applied on an anion exchange chromatography column (Resource Q; 6 mL column). A gradient from 0 to 0.22 M NaCl in buffer A was run for at 1 mL/min for 20 min, followed by a shallow gradient from 0.22 to 0.28 M NaCl at 1 mL/min for 45 min. Peak fractions (DEBS >70% of protein) released from the matrix at 0.25−0.27 M NaCl were pooled and concentrated on Centriprep 30 membranes (Amicon) to a protein concentration of 1−1.5 mg/mL in buffer B (150 mM sodium phosphate, pH 7.1, 2 mM DTT, 2 mM EDTA, and 20% glycerol). For sucrose gradient centrifugation, DEBS was equilibrated in buffer A (1 mg/mL) and centrifuged in a gradient of 10−40% sucrose in buffer A (Beckman SW41 rotor, 30 000 rpm, 22 h). Fractions containing pure DEBS proteins were concentrated as described above.

Measurement of Catalytic Activities and Kinetic Constants of DEBS 1+TE and DEBS 1/Module 3+TE. Assays for the synthesis of triketide (**2**) and tetraketide (**3**) lactones *in vitro* were performed in a 100 μL volume as described earlier (Pieper et al., 1996). [1-^{14}C]Propionyl-CoA (4.8 mCi/mmol, 0.25 mM), (2*RS*)-methylmalonyl-CoA (0.6 mM), and NADPH (0.8 mM) were added to enzyme preparations. Assay mixtures also contained 10% glycerol. Time courses were followed for 1 h with DEBS 1+TE and for 4 h with the combination of DEBS 1 and module 3+TE. Extraction and separation of the tetraketide lactones via TLC and the calculation of the amount of ^{14}C-labeled product were carried out as previously described for the triketide lactone (Pieper et al., 1996). Apparent k_{cat} values were calculated from the radioactivity incorporated into the relevant polyketide, as measured on a PhosphoImager (Molecular Dynamics). Protein concentrations were measured by the Bradford method and via densitometric scanning.

Chemical Cross-Linking. Module 3+TE of approximately 90% purity from a Resource Q chromatography step was equilibrated in buffer B without addition of DTT. The three cross-linking reagents used were dissolved in DMSO to give a final concentration of 2 mM in the enzyme solutions (100 μL volume). The reactions were quenched with 10 mM DTT in the case of bis(maleimidohexane) (BMH) and 1,3-dibromopropanone after 5, 10, and 15 min. The incubation of ethylene glycol bis(succinimidyl succinate) (EGS) with DEBS was quenched with 50 mM Tris, pH 7.6. The protein samples were denatured and electrophoretically separated using 3.5% acrylamide−SDS gels. The molecular masses of cross-linked protein species were estimated with DEBS 1+TE (410 kDa) and a solution of cross-linked phosphorylase B (97−582 kDa; Sigma) as markers.

*Identification of the Tetraketide Lactone CK13a (**3**) and the Tetraketide Hemiketal CK13b (**4**) by Autoradiography.* Portions of the ethyl acetate extract of an *in vitro* incubation of DEBS 1/module 3+TE with [1-^{14}C]propionyl-CoA, (2*RS*)-methylmalonyl-CoA, and NADPH were mixed with either 1 mg of authentic **3** or 6 mg of authentic **4**. The sample containing carrier **3** was purified by preparative TLC (SiO_2, 6% MeOH/$CHCl_3$) and the product visualized by UV and analysis in a BioRad PhosphoImager. The UV-active, radioactive band, R_f 0.2, corresponding to **3**, was eluted and analyzed by TLC/PhosphoImaging in six different solvent systems. In all six cases, the single UV-active component, the radioactive spot, and an authentic sample of **3** were completely coincident (6% MeOH/$CHCl_3$, R_f 0.23; 60% EtOAc/hexanes, R_f 0.14; 100% EtOAc, R_f 0.40; 60% acetone/hexanes, R_f 0.43; 20% MeOH/CH_2Cl_2, R_f 0.67; 50% EtOAc/hexanes, 0.12). When the sample containing authentic **4** as carrier was subjected to preparative TLC (50% EtOAc/hexanes), the radioactivity (45 100 dpm) comigrated with the authentic material, as determined by PhosphoImaging. The purified product was converted to the mixture of anomeric methyl ketals and then to the corresponding 3,5-dinitrobenzoate esters **5a** and **5e**, by the previously described procedure (Kao et al., 1996a). Analysis by PhosphoImaging and vanillin staining indicated that the radioactive components co-chromatographed with the authentic axial (**5a**) and equatorial (**5e**) anomers which were eluted from the plates and shown to have essentially similar specific activities of 3190 and 3090 dpm/mg.

*Identification of the Tetraketide Lactone CK13a (**3**) via HPLC.* The tetraketide lactone CK13a (**3**), generated *in vitro* by DEBS 1/module 3+TE and extracted with ethyl acetate, was chromatographed on a C-18 reverse-phase HPLC column (1 mL/min flow rate, 0−75% acetonitrile gradient in aqueous buffer containing 5 mM tributylammonium sulfate and 5 mM sodium phosphate, pH 7.1; 50 min). An authentic biosynthetic sample of the tetraketide lactone (Kao et al., 1996a) was used as a reference. UV spectra of the eluting peaks were monitored using an on-line multiwavelength detector. The peak corresponding to the tetraketide lactone appears at 25.2 min.

RESULTS

Purification and Activity Profile of DEBS 1+TE. From a 400 mL liquid culture of *S. coelicolor* CH999/pCK12, ca. 200 mg of total protein was obtained. DEBS 1+TE, which contains modules 1 and 2, was present in this protein solution at an approximate concentration of 1 μM. As reported previously (Pieper et al., 1996), the 45%-saturated ammonium sulfate fraction was found to be highly active with regard to triketide C_9-lactone (**2**) synthesis, with a reported $k_{cat} = 3.4$ min^{-1}. As summarized in Table 1, gel filtration of this ammonium sulfate precipitate fraction led to a 3-fold purification of DEBS 1+TE (Figure 4) with a recovery of about 50% of the applied enzyme activity. Further purification and concentration on Resource Q (anion exchange) were

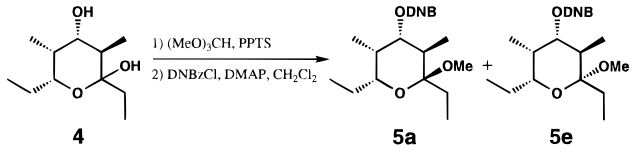

FIGURE 3: Conversion of hemiketal CK13b (**4**) to dinitrobenzoate esters of axial and equatorial methyl ketals **5a** and **5e**.

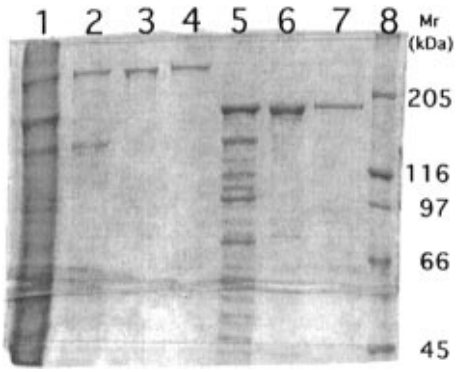

FIGURE 4: Steps of purification for DEBS 1+TE and module 3+TE analyzed by a 6% acrylamide−SDS gel. The gel was stained with Coomassie Blue; the molecular masses of standard proteins (lane 8) are indicated to the right of the gel in kDa. Lane 1: protein extract of CH999/pCK13 expressing DEBS 1 and module 3+TE, precipitate from extract saturated to 45% with ammonium sulfate. DEBS 1+TE (410 kDa) from the gel filtration step in lane **2**, from the anion exchange chromatography step in lane **3**, and from the sucrose gradient centrifugation step in lane **4**. Module 3+TE (190 kDa) in the same order of purification steps as DEBS 1+TE in lanes **5**−**7**. Details of purification procedures are described in the text.

found to be very effective. The activity was completely recovered, and a 15-fold purification factor was obtained in this step alone (Table 1). The resulting protein (approximately 90% pure; Figure 4) was concentrated to 1 mg/mL and purified to homogeneity via sucrose gradient centrifugation (Figure 4). The overall k_{cat} for synthesis of the triketide lactone using the protein obtained from the Resource Q column was calculated at 4.8 min^{-1}. Furthermore, the suspected ability of DEBS 1+TE to decarboxylate methylmalonyl-CoA (Pieper et al., 1996) was confirmed, since **2** could be synthesized using pure DEBS 1+TE in the absence of propionyl-CoA. Production of propionyl-CoA from methylmalonyl-CoA was undetectable in control incubations.

Cell-Free Biosynthesis of Tetraketides by DEBS 1/Module 3+TE. The trimodular DEBS system, which contains a fusion of the thioesterase domain of DEBS 3 to the ACP domain of module 3, has been analyzed recently with respect to its polyketide product profile *in vivo* (Kao et al., 1996a). The tetraketide products CK13a (**3**) and CK13b (**4**) were isolated in a ratio of 5:1, and characterized via NMR, mass spectroscopy, and stable isotope labeling techniques. In cell-free assays, protein preparations from CH999/pCK13 were incubated with all substrates including [^{14}C]propionyl-CoA. Radiolabeled products corresponding to CK13a (**3**) and CK13b (**4**) were detected via TLC autoradiography as well as by analysis of the corresponding pair of 3,5-dinitrobenzoate esters of the anomeric methyl ketals **5a** and **5e**, prepared as previously described (Kao et al., 1996a) from labeled **4** (Figure 3). The yields of the hemiketal CK13b (**4**) from the enzymatic incubation were considerably lower than those of CK13a (**3**). The identity of ^{14}C-labeled CK13a

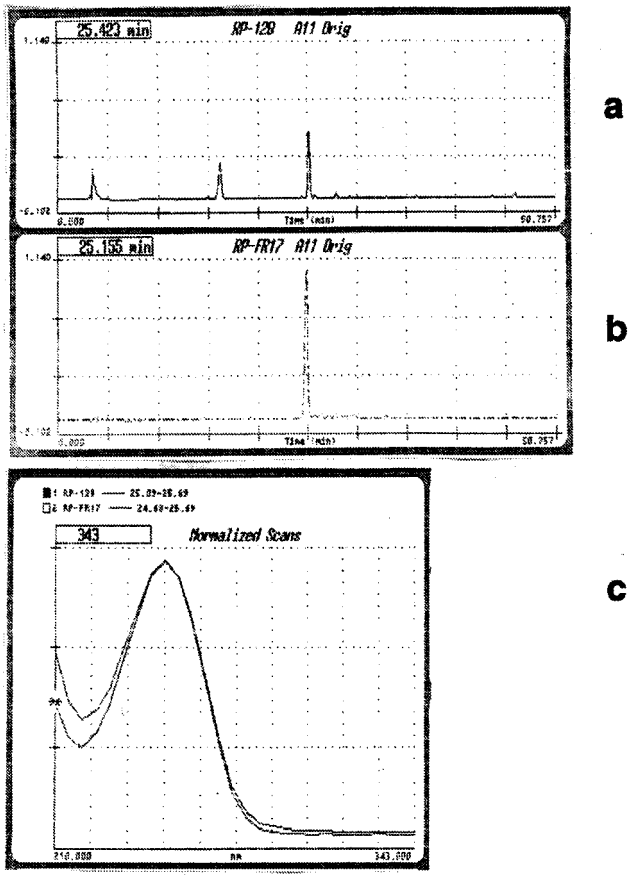

FIGURE 5: Comparison of reverse-phase HPLC elution profile and UV spectra of the tetraketide lactone CK13a from *in vivo* and *in vitro* sources. (a) HPLC trace of the cell-free assay with DEBS 1 and module 3+TE; (b) authentic sample of the tetraketide isolated from mycelium of the recombinant strain CH999/pCK13. In (a), the peak eluting at 25 min (43% of solvent B) is both UV-active (254 nm) and radioactive due to the incorporation of a propionyl moiety from the substrate [^{14}C]propionyl-CoA. (c) Superimposed UV scans from 210 to 343 nm of both peaks.

(**3**) was further verified via C-18 reverse-phase HPLC chromatography. Both the mobility (Figure 5a) and the UV spectrum (Figure 5b) of the cell-free product were identical to those of an authentic reference sample of **3**.

Purification and Kinetic Characterization of DEBS 1 and Module 3+TE. DEBS 1 and module 3+TE were individually purified from crude extracts of CH999/pCK13 using the procedure developed for DEBS 1+TE. Size-exclusion chromatography of the 45%-saturated ammonium sulfate fraction containing DEBS 1 and module 3+TE revealed that, whereas module 3+TE eluted as a sharp peak (analogous to DEBS 1+TE and DEBS 3, both of which carry a thioesterase domain at the C-terminus), DEBS 1 elutes over a broader fraction range. Furthermore, the two proteins elute in distinct fractions, suggesting the absence of tight association between modules 2 and 3. Analogous to DEBS 1+TE, anion exchange chromatography resulted in purification of module 3+TE to 90% purity (Figure 4), whereas DEBS 1 eluted over a broader range and was purified to 75% purity. Again, module 3+TE could be purified to homogeneity by sucrose gradient centrifugation on a 1 mg scale (Figure 4).

In anticipation of the relatively weak association between the two proteins, module 3+TE was present in a 10-fold excess stoichiometric ratio over DEBS 1 during the measurement of the overall rate constant for tetraketide synthesis.

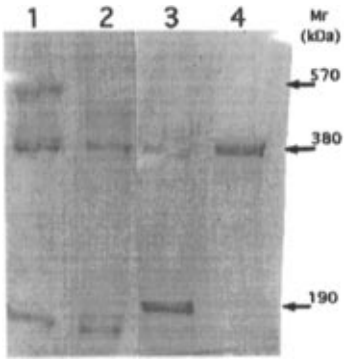

FIGURE 6: Chemical cross-linking of module 3+TE. Module 3+TE (0.6 mg/mL) was incubated with 2 mM BMH [bis(maleimidohexane)] (lane 1) and 1,3-dibromopropanone (lane 2) for 5 min, respectively. The reaction was quenched with 10 mM DTT, and the samples were electrophoretically run in a 3.5% acrylamide−SDS gel. Lane 3: module 3+TE. Lane 4: DEBS 1. The molecular masses of the module 3+TE (190 kDa), DEBS 1 (380 kDa), and trimeric cross-linked species (570 kDa) are indicated to the right of the gel. Cross-linked phosphorylase B (97−582 kDa) multimers were used to calculate the high molecular mass species.

The k_{cat} for the synthesis of **3** was measured at 0.23 min^{-1}. (In these assays, the appearance of **4** was approximately an order of magnitude slower.) Thus, the turnover rate for a PKS containing two distinct subunits was found to be considerably lower than the rate for triketide lactone synthesis by DEBS 1+TE. Interestingly, the rate of appearance of **2** (which can also be generated by DEBS 1 alone in small quantities) was increased in the presence of module 3+TE. The possible significance of this observation is discussed below.

Covalent Cross-Linking of Module 3+TE with Bifunctional Reagents. Earlier, bifunctional cross-linking reagents such as 1,3-dibromopropanone have been shown to cross-link proteolytically generated modules as homodimers (Staunton et al., 1996). Here we confirm these observations with recombinant module 3+TE, and extend them by demonstrating the applicability of alternative cross-linking agents as well. EGS, which specifically reacts with the amino groups of lysine residues, cross-linked module 3+TE to generate one dimeric species and two trimeric species (data not shown). Likewise, BMH, which reacts with the sulfhydryl groups of cysteine residues, cross-linked module 3+TE similarly into two dimeric species of approximately 380 kDa and one trimeric species of approximately 570 kDa (lane 1, Figure 6). As the incubation time was increased, one of the dimer bands disappeared, and the trimeric band intensified (data not shown). Both EGS and BMH have a spacer arm of ca. 16 Å. In contrast, 1,3-dibromopropanone, which has a spacer arm of ca. 5 Å, cross-linked module 3+TE into only one dimeric species (lane 2, Figure 6). In contrast to the reaction with BMH and EGS, the rate of appearance of the dibromopropanone cross-linked species was significantly retarded by preincubation with methylmalonyl-CoA, suggesting that dibromopropanone exclusively cross-links the active sites of the ACP and the KS. No cross-linking was observed between DEBS 1 and module 3+TE using the above-mentioned cross-linkers.

DISCUSSION

Since its discovery, the erythromycin PKS has been regarded as a fundamentally interesting and medicinally important challenge in multifunctional enzymology. The recent development of cell-free systems for assaying this multienzyme assembly and its mutants has opened the door to direct structural and mechanistic investigations of their remarkable properties. Here we describe a generally applicable three-step method for the purification of modular PKS subunits, especially those with C-terminal TE domains. The development of this procedure was guided by our earlier observation (Pieper et al., 1995) that the chromatographic behavior of DEBS 1 and DEBS 2 differed from that of DEBS 3 (whose N-terminus harbors the thioesterase) on a gel filtration column.

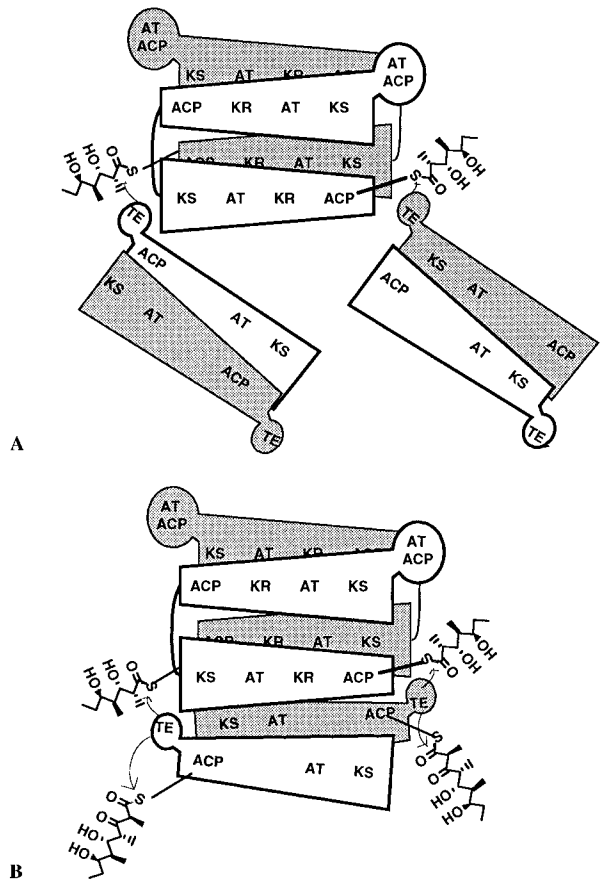

FIGURE 7: Models for premature release of the triketide in the trimodular derivative of DEBS. (A) Nonproductive association of DEBS 1 and module 3+TE, such that TE can release the growing chain. (B) Productive association of the trimodular system where the TE domain has considerable flexibility, such that it can release the polyketide chains attached to the final as well as the penultimate ACP domain.

The above procedure has been applied to an engineered bimodular derivative (DEBS 1+TE; Figure 2) as well as a trimodular derivative (DEBS 1/module 3+TE; Figure 2) of the erythromycin PKS with good overall yield. In both cases, the anion-exchange step results in a substantial purification (>10-fold) of individual subunits. Purified DEBS 1+TE is shown to possess high catalytic competence; its apparent k_{cat} of 4.8 min^{-1} would permit the steady-state synthesis of approximately 20 mg of triketide lactone **2** per day from the amount of protein that can be recovered from a 1 L culture. This number compares very well with the *in vivo* yield of **2** reported earlier (Kao et al., 1995). Although the reconstituted trimodular PKS is ca. 20-fold less active *in vitro*, we believe that the reduction in catalytic competence is primarily due to inefficient association of DEBS 1 and module 3+TE.

Regardless, module 3+TE represents the first active single-module protein to be purified from a modular PKS. Furthermore, since this module is one of the smallest known modules (it lacks both DH or ER domains and has a nonfunctional KR domain), it is likely to be an attractive target for future structural and biophysical studies aimed at understanding the properties of the three essential domains within each module (a KS, AT, and ACP).

Earlier we have shown that DEBS 1 is capable of synthesizing the triketide lactone **2**, albeit at a substantially lower level *in vivo* than DEBS 1+TE (Kao et al., 1994, 1995). Consistent with this observation, small amounts of **2** are synthesized *in vitro* by purified DEBS 1 alone. Curiously, this rate of synthesis increases with increasing concentrations of module 3+TE in the assay mixture. On one hand, this could be explained by the nonproductive association of DEBS 1 and module 3+TE such that, although the triketide cannot be extended further, the ACP2 from DEBS 1 can interact with the TE from module 3+TE (Figure 7A). This mechanism would imply that ACP and TE domains can associate *in trans* in a general sense, and is therefore testable by coexpression of DEBS 1 and the TE domain as separate proteins. An alternative mechanism would involve the productive association of DEBS 1 and module 3+TE into a trimodular head-to-tail assembly (Kao et al., 1996b); here the TE domain can competitively reach out to the growing chains attached to either ACP2 or ACP3 (Figure 7B). This mechanism would eliminate the possibility for the TE to hydrolyze growing chains attached to all but the final and the penultimate ACP in a modular PKS.

Finally, the cross-linking results reported here confirm the dimeric nature of individual modules, as demonstrated recently by gel filtration (Aparicio et al., 1994; Pieper et al., 1995), cross-linking of monomers of a proteolytically generated module (Staunton et al., 1996), and mutagenesis (Kao et al., 1996b). Additionally, they also point to the existence of other functional groups (thiols and amines) which are proximally located within a dimeric module. Further protein chemical analysis of these cross-linked complexes could lead to a better understanding of the relative geometries of individual subunits within a head-to-tail dimer.

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