# Erythromycin Biosynthesis: Kinetic Studies on a Fully Active Modular Polyketide Synthase Using Natural and Unnatural Substrates[†]

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ABSTRACT: 6-Deoxyerythronolide B synthase (DEBS) is a modular polyketide synthase (PKS) that catalyzes the biosynthesis of the parent macrolide of erythromycin. On the basis of a recently developed cell-free assay (Pieper et al., 1995a) we report the results of steady-state kinetic studies on a modular PKS. A truncated form of DEBS (DEBS 1+TE), in which DEBS 1 is fused to the thioesterase domain from the C-terminal end of DEBS 3, was used for most of these studies. The overall $k_{cat}$ for (2*S*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid $\delta$-lactone ($C_9$-lactone) synthesis is 3.4 $min^{-1}$, indicating that the enzyme is at least as active *in vitro* as *in vivo*. The apparent $K_m$ for (2*S*)-methylmalonyl-CoA consumption by DEBS 1+TE is 24 $\mu$M. The catalytic activity of DEBS 1+TE is strongly dependent on the phosphate concentration in the reaction buffer in the range 0−250 mM, suggesting that hydrophobic interactions may be crucial to the assembly of DEBS monomers into a functional complex. Although DEBS 1+TE can convert acetyl-, propionyl-, or butyryl-CoA into the corresponding $C_8$-, $C_9$-, and $C_{10}$-lactones (Pieper et al., 1995b), it has a 32-fold preference for a propionate primer over an acetate primer and a 7.5-fold preference for a propionate primer over a butyrate primer. In the absence of any added primer unit, synthesis can be primed via decarboxylation of methylmalonyl-CoA; under these conditions the overall $k_{cat}$ for polyketide synthesis remains unchanged. Decarboxylation of methylmalonyl-CoA is negligible in the presence of saturating concentrations of propionyl-CoA but competes with the priming of the enzyme by acetyl-CoA or butyryl-CoA. The $k_{cat}$ for 6-deoxyerythronolide B synthesis by the complete DEBS is 0.5 $min^{-1}$. Under these assay conditions, the $C_9$-lactone is also produced as an abortive chain elongation product with a $k_{cat}$ of 0.23 $min^{-1}$, presumably due to inefficient assembly of the multimeric protein complex involving DEBS 1, 2, and 3. Together, these results provide the first comprehensive kinetic insights into a fully active modular PKS.

6-Deoxyerythronolide B synthase (DEBS) is a modular polyketide synthase (PKS) which catalyzes the biosynthesis of the polyketide precursor of the widely used broad spectrum antibiotic erythromycin. Sequence analysis has revealed that DEBS itself is composed of three large (ca. 3000 amino acids) multidomain proteins, designated DEBS 1, DEBS 2, and DEBS 3 (Cortes et al., 1990; Donadio et al., 1991). The domains within each protein appear to be clustered into two distinct functional units, called modules (Figure 1). Each module includes a full complement of active sites required for one of the six cycles of chain elongation and associated $\beta$-ketoreduction reactions that occur during the biosynthesis of 6-deoxyerythronolide B (6-dEB, **1**). The acyltransferase (AT) domain at the N-terminal end of DEBS 1 transfers the propionyl primer unit from coenzyme A (CoA) to the first module, whereas the thioesterase (TE) at the C-terminal end of DEBS 3 catalyzes the release of the polyketide chain via lactonization.

The above model for 6-dEB biosynthesis has been well substantiated by a number of studies. For example, by feeding *Saccharopolyspora erythraea*, the erythromycin producer, with $^{14}C$-labeled propionate, Corcoran and co-workers initially showed that the polyketide backbone of this natural product is derived from seven propionate units (Friedman et al., 1964). Using a variety of $^{13}C$, $^{18}O$, $^{2}H$, and multiply labeled substrates and advanced intermediate analogs, Cane and co-workers confirmed the propionate origin of erythromycin and demonstrated that 6-dEB biosynthesis occurs via a processive mechanism in which the final reduction state and stereochemistry of the growing polyketide chain is set immediately after each condensation reaction (Cane et al., 1983b, 1986; Cane & Yang, 1987). Cloning and sequencing of the genes encoding the DEBS proteins (Cortes et al., 1990; Donadio et al., 1991) facilitated their heterologous expression in *Streptomyces coelicolor*, leading to *in vivo* synthesis of 6-dEB (Kao et al., 1994a). Concurrent production of comparable quantities of 8,8a-deoxyoleandolide (**2**) by the recombinant strain first suggested that the enzyme possessed relaxed specificity for the primer unit. Knock-out mutagenesis of the ketoreductase (KR) domain of module 5 and the enoylreductase domain of module 4 of DEBS in *S. erythraea* resulted in *in vivo* synthesis of the expected oxo and anhydro analogs of erythromycin (Donadio et al., 1993, 1991). Expression of the gene encoding DEBS 1 alone in *S. coelicolor* CH999

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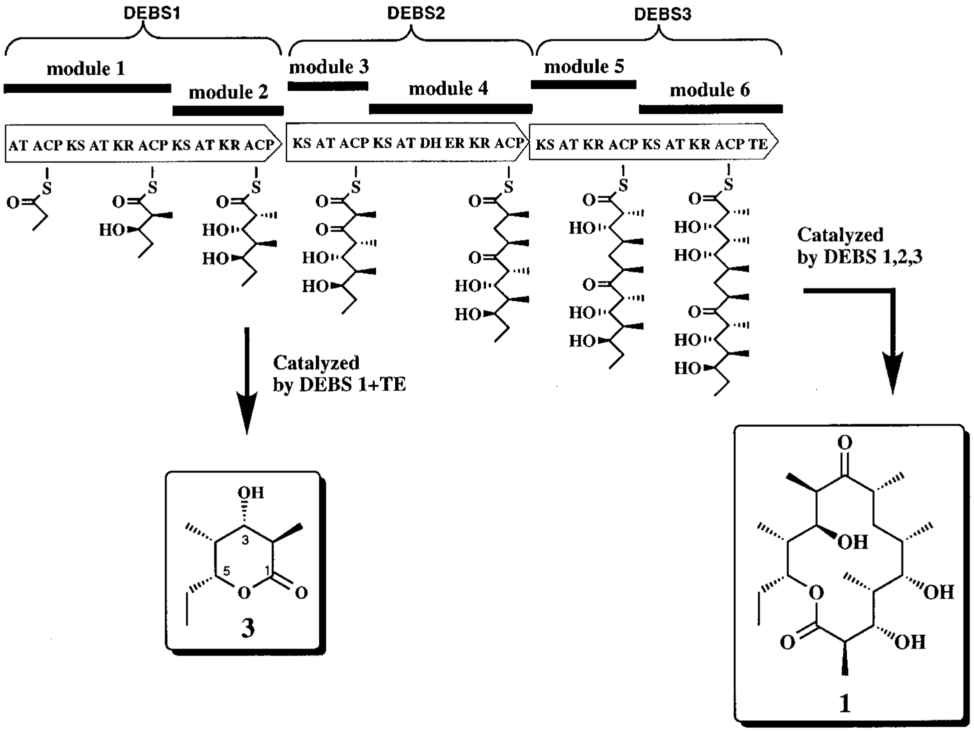

FIGURE 1: Model for the modular organization of 6-deoxyerythronolide B synthase (DEBS) and the biosynthesis of 6-deoxyerythronolide B (**1**) and (2*S*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid $\delta$-lactone (**3**) by DEBS and DEBS 1+TE, respectively. Each of the six modules accounts for one polyketide chain extension and one partial or complete $\beta$-ketoreduction cycle. Complete DEBS comprises DEBS 1, DEBS 2, and DEBS 3, whereas DEBS 1+TE consists of the first two modules fused to the thioesterase domain of DEBS 3. The active sites are designated as follows: acyltransferase (AT), $\beta$-ketoacyl-ACP transferase (KS), acyl carrier protein (ACP), $\beta$-ketoreductase (KR), dehydratase (DH), enoylreductase (ER), and thioesterase (TE). For further details see Donadio et al. (1991).

yielded small amounts of (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxy-*n*-heptanoic acid $\delta$-lactone (**3**) (Kao et al., 1994b). In contrast, fusion of the TE domain from DEBS 3 to the C-terminal end of DEBS 1 (designated DEBS 1+TE) yielded substantially enhanced levels of **3**, as well as its $C_8$ analog derived from an acetate primer unit (**4**) (Brown et al., 1995; Cortes et al., 1995; Kao et al., 1995), arguing in favor of a catalytic role for the TE domain in chain release (Figure 1). Finally, repositioning of the TE domain from the C-terminal end of module 6 to the C-terminal end of module 5 led to the synthesis of the expected 12-membered macrolactone (8*R*,9*S*)-8,9-dihydro-8-methyl-9-hydroxy-10-deoxymethynolide, suggesting that the TE plays a role in recognition of the terminal hydroxyl of the completed chain during lactonization (Kao et al., 1995).

Notwithstanding the above advances, our understanding of the mechanistic basis for chain transfer and molecular recognition within this remarkable multienzyme assembly remains rudimentary at best. In large measure this has been due to the absence of methods for monitoring the complete activity of DEBS *in vitro*. Studies on partially active DEBS proteins have shed light on the stereochemical control of methyl-branched centers in 6-dEB. Thus, (2*S*)-methylmalonyl-CoA was shown to be the substrate for all six extender unit AT domains of DEBS, suggesting that epimerization of three methyl groups must occur at each of two stereogenic centers corresponding to C-8 and C-12 of 6-dEB, either before or after the relevant condensation reactions (Marsden et al., 1994). More recently, cell-free activity of the complete DEBS 1/DEBS 2/DEBS 3 assembly (Pieper et al., 1995a) and the DEBS 1+TE mutant (Pieper et al., 1995a,b; Wiesmann et al., 1995) has been reported. In some cases the enzymatic activity was high enough to allow detection of the polyketide product by $^{13}$C NMR spectroscopy (Pieper et al., 1995a,b). The availability of cell-free systems has facilitated the direct probing of the unusual molecular recognition features of these multienzyme assemblies. For example, DEBS 1+TE was shown to tolerate a relatively broad range of primer units (including butyrate, which is converted into **5**; Figure 2) (Pieper et al., 1995b; Wiesmann et al., 1995). It was also observed that the oxidation level and substitution pattern of a potential primer are more significant determinants for its recognition than chain length itself (Pieper et al., 1995b). Lastly, in the absence of NADPH, DEBS 1+TE produced the unreduced triketide, 6-ethyl-2-hydroxy-3,5-dimethyl-2*H*-pyran-2-one (**6**; Figure 2), suggesting that the enzyme was capable of processing unreduced polyketoacyl intermediates (Pieper et al., 1995b).

Here we report on the key overall steady-state kinetic parameters for DEBS 1+TE and the complete DEBS system. Using this quantitative assay, insights have been obtained into the primer unit specificity as well as interesting mechanistic and protein chemical aspects of these multifunctional enzymes.

## MATERIALS AND METHODS

*Reagents and Chemicals.* DL-2-[*methyl*-$^{14}$C]Methylmalonyl-CoA (56.4 mCi/mmol) was purchased from ARC, Inc. [1-$^{14}$C]Acetyl-CoA (54 mCi/mmol) was from Moravek Biochemicals. [1-$^{14}$C]Butyryl-CoA (4 mCi/mmol) and [1-$^{14}$C]-propionate (51.0 mCi/mmol) were from NEN DuPont. [1-$^{14}$C]Propionyl-CoA was synthesized using a slightly altered version of a published procedure (Patel & Walt, 1986). Acetyl-CoA synthetase (5 units/mL; Sigma) was incubated with [1-$^{14}$C]propionate (100 $\mu$Ci/mL), coenzyme

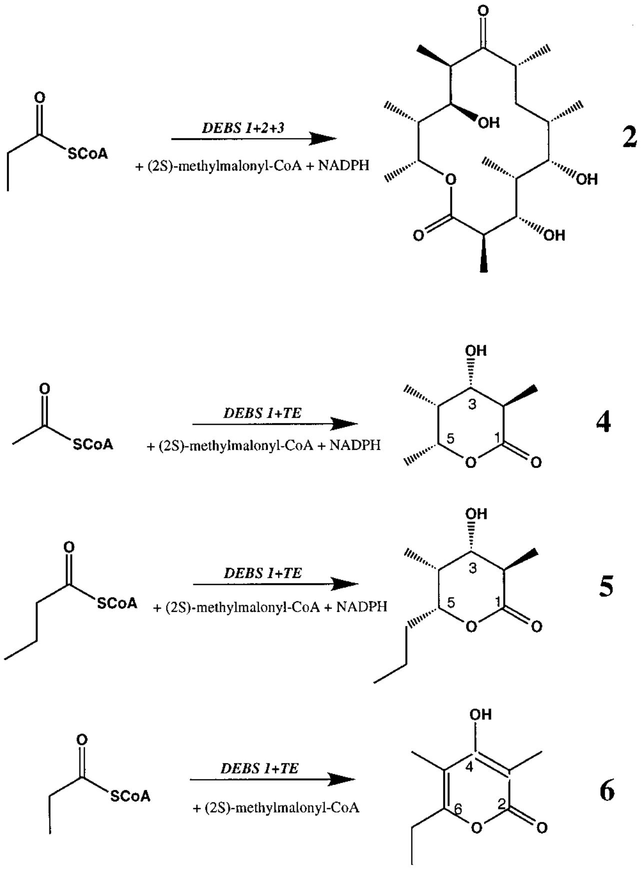

FIGURE 2: Structures of some polyketides derived from DEBS and/or its mutants. For further details, see text.

A (2.5 mM), ATP (5 mM), and $MgCl_2$ (5 mM) in 100 mM sodium phosphate buffer (pH 7.4) for 12 h at 20 °C. [1-$^{14}$C]-Propionyl-CoA was purified by $C_{18}$ reverse-phase HPLC using 20% methanol in 50 mM potassium phosphate (pH 5.0) as the eluant.

*Strains and Culture Conditions.* Two recombinant strains of *S. coelicolor*, CH999/pCK7 (Kao et al., 1994a) and CH999/pCK12 (Kao et al., 1995), were used in this study. The former produces the complete DEBS comprising DEBS 1, DEBS 2, and DEBS 3, whereas the latter produces DEBS 1+TE. Spores were inoculated in SMM medium (Strauch et al., 1991) and grown at 30 °C for approximately 60 h. Mycelia (typically 8 g/L wet weight) were harvested via centrifugation at 3000*g* and washed two times with water and two times with 0.5 M sodium chloride.

*Preparation of the DEBS Enzymes.* Mycelial pellets were resuspended in buffer A [250 mM sodium phosphate (pH 7.1), 0.3 M sodium chloride, 2 mM DTT, 1 mM benzamidine, 2 mM EDTA, 3 mg/L leupeptin, 3 mg/L pepstatin, and 30% glycerol] and disrupted by sonication (five bursts of 30 s, Branson sonifier) at 0 °C. The lysate was centrifuged for 1.5 h at 192000*g*, and the supernatant was collected. Nucleic acids were precipitated with poly(ethylenimine) (0.2%) and removed via centrifugation (20 min at 23100*g*). The protein solution was saturated with ammonium sulfate to 50% and precipitated overnight. After centrifugation (30 min, 31100*g*), the pellet containing the DEBS proteins was redissolved in buffer B [250, 100, or 50 mM sodium phosphate (pH 7.1), 2 mM DTT, 2 mM EDTA, and 10% glycerol] and desalted on a Sephadex G25 M column using buffer B (250 mM unless otherwise mentioned).

*Enzymatic Synthesis of Polyketides.* Assays involving the turnover of DEBS proteins were carried out as described recently (Pieper et al., 1995a). Unless otherwise mentioned, substrates were added at the following initial concentrations: acetyl-CoA (200 $\mu$M), propionyl-CoA (200 $\mu$M), butyryl-CoA (200 $\mu$M), (2*RS*)-methylmalonyl-CoA (600 $\mu$M), and NADPH (1 mM, replenished every 20 min). One hundred microliters of enzyme solution was typically used in each assay, and buffer B (final phosphate concentration 250 mM) was added to a final volume of 200 $\mu$L. Reactions were started by addition of methylmalonyl-CoA. When used, $^{14}$C-labeled substrates were diluted to the following final specific activities: (2*RS*)-methylmalonyl-CoA, 0.59 mCi/mmol; propionyl-CoA, 3.19 mCi/mmol; acetyl-CoA, 7.94 mCi/mmol. Control experiments confirmed that the enzyme preparations did not lose appreciable activity for up to 3 h (data not shown). The reaction mixtures were quenched and extracted as described before (Pieper et al., 1995a). Control experiments showed that more than 95% of the polyketide products were extractable from the aqueous into the organic phase.

*Kinetic Measurements.* $^{14}$C-Labeled polyketide products were separated on TLC plates (Si250F, Baker) using ethyl acetate/hexane (65:35) as the mobile phase. Plates were counted on a PhosphorImager (Molecular Dynamics) for 2−3 days. These counts were converted into dpm using a triketide $C_9$-lactone (**3**) standard of known radioactivity. The initial rates, *V*, were determined from the initial slopes of the time courses for lactone formation, with $V_{max}$ being determined using saturating concentrations of each variable substrate. From this value, the apparent $k_{cat}$ was calculated on the basis of knowledge of DEBS concentration in the assay mixture, determined via SDS−PAGE/densitometry as described below. The apparent $K_m$ for (2*S*)-methylmalonyl-CoA was measured by varying its concentration between 3 and 300 $\mu$M, using 200 $\mu$M [$^{14}$C]propionyl-CoA and 1 mM NADPH, as described above, and fitting the experimentally determined intial rates directly to the Michaelis−Menten equation.

*Densitometric Measurement of DEBS and DEBS 1+TE Concentrations.* Known quantities (200, 250, 400, and 600 $\mu$g/mL) of bovine serum albumin (BSA) were used as calibration standards. Serial dilutions of the DEBS protein(s) present in each batch of enzyme were resolved via SDS−PAGE in 5% acrylamide gels (data not shown). Densitometric scanning of Coomassie Blue stained proteins was performed using an ArtiScan Imager (Tamarack Telecom).

## RESULTS

Due to the simplicity of the system and the high yields of a variety of triketide lactones (Pieper et al., 1995a,b), DEBS 1+TE was used for most of the experiments that follow. However, the overall rate constant of the complete DEBS system is also reported. For each experiment, all data points were collected using the same batch of enzyme. Experiments were repeated using independent enzyme preparations (starting with separate cultivations), and average parameters are reported, where appropriate. Typical protein preparations contained DEBS 1+TE at a concentration of 0.9−1.2 $\mu$M (3−4% of the total protein) and the complete DEBS system at a concentration of 1.2 $\mu$M (7% of the total protein).

*Measurement of Kinetic Parameters for DEBS 1+TE.* The yield of the $C_9$-lactone (**3**) after an incubation of 3 h was

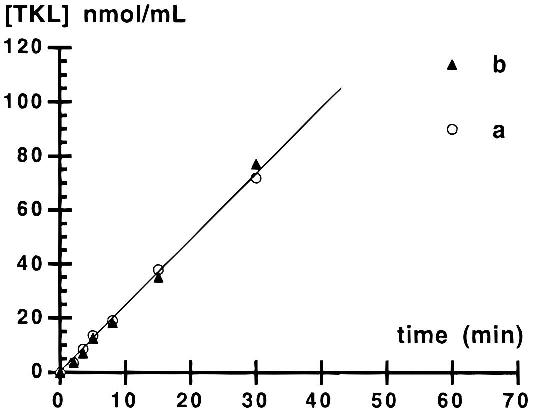

FIGURE 3: Time−course for triketide $C_9$-lactone (TKL) synthesis catalyzed by DEBS 1+TE. Incubation mixtures (200 $\mu$L) containing saturating concentrations of each substrate (see Materials and Methods) were quenched after incubation times of 2, 3.5, 5, 8, 15, 30, and 60 min. The products were extracted with ethyl acetate and separated via TLC. The amount of TKL synthesized was measured on a PhosphorImager as described in Materials and Methods. In (a) [$^{14}$C]propionyl-CoA and in (b) [$^{14}$C]methylmalonyl-CoA were used as the radiolabeled substrate. The $V_{\max}$ for product formation was determined from the slope of [TKL] versus time in the linear range of the data.

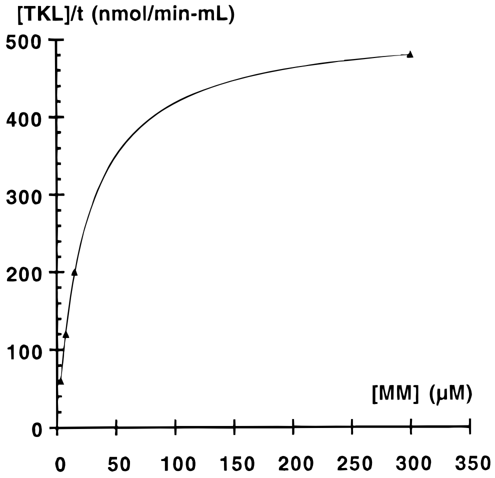

FIGURE 4: Apparent $K_m$ for (2*S*)-methylmalonyl-CoA for triketide $C_9$-lactone (TKL) synthesis by DEBS 1+TE. Initial rates (*V*) of TKL synthesis were plotted against (2*S*)-methylmalonyl-CoA concentrations. [$^{14}$C]Propionyl-CoA was the radiolabeled substrate.

typically about 50% under the standard assay conditions described in the Materials and Methods section. Under saturating concentrations of all three substrates (propionyl-CoA, methylmalonyl-CoA, and NADPH), the rate of formation and final yield of radiolabeled **3** were independent of whether methylmalonyl-CoA or propionyl-CoA was used as the radiolabeled substrate (Figure 3). This suggests that, in the presence of excess propionyl-CoA, the fraction of **3** that contains a primer unit derived via DEBS 1+TE-catalyzed decarboxylation of methylmalonyl-CoA is negligible (see below). From a time−course of formation of **3**, the overall $k_{cat}$ was determined to be 3.4 ±1.2 min$^{-1}$ (averaged over several protein preparations). By varying the concentration of (2*S*)-methylmalonyl-CoA, the apparent $K_m$ for this substrate was found to be 24 $\mu$M (Figure 4).

Earlier studies have shown that active DEBS 1+TE migrates as a dimeric complex on a gel-filtration column (Pieper et al., 1995a). To investigate the effect of enzyme dilution on its catalytic activity, a DEBS 1+TE preparation (12 mg/mL total protein, 1.4 $\mu$M DEBS 1 + TE) was diluted 3-, 6-, 12-, and 24-fold. As shown in Figure 5, activity varies linearly with dilution. Thus, if dimerization is essential to its activity, the dissociation constant for the DEBS 1+TE complex must be below 60 nM.

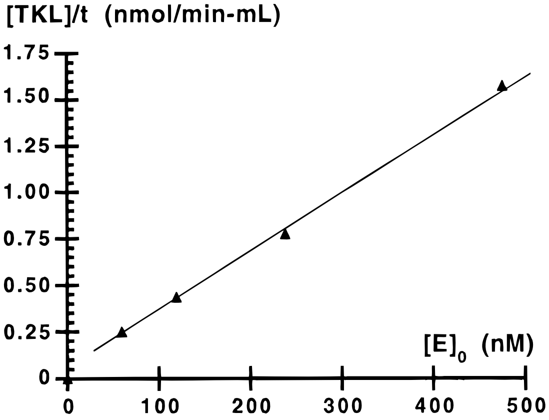

FIGURE 5: Dependence of DEBS 1+TE activity on enzyme concentration. A preparation containing 1.4 $\mu$M DEBS 1+TE was diluted 3-, 6-, 12-, and 24-fold. $V_{\max}$ was measured for each dilution, as described in the caption to Figure 3.

*Decarboxylation of Methylmalonyl-CoA Can Provide DEBS 1+TE with a Starter Unit.* When propionyl-CoA is excluded from the reaction mixture, DEBS 1+TE can synthesize **3** with a comparable overall $k_{cat}$ as reported above (data not shown). This phenomenon, which has also been reported in the case of FASs (Kresze et al., 1977), is presumably due to the ability of DEBS 1+TE to catalyze the decarboxylation of methylmalonyl-CoA. Curiously, in the absence of propionyl-CoA an initial lag phase is reproducibly observed in the time−course for synthesis of **3**. This lag phase is more pronounced at lower DEBS 1+TE concentrations. Thus, competition from DEBS-catalyzed decarboxylation of methylmalonyl-CoA under nonsaturating propionyl-CoA concentrations precluded an accurate determination of the apparent $K_m$ for propionyl-CoA.

*Phosphate Concentration Dependence of DEBS 1+TE Activity.* Initial experiments during the development of a cell-free assay for triketide $C_9$-lactone synthesis were performed using DEBS preparations in 50 mM Tris buffers. In contrast to a recently published report (Wiesmann et al., 1995), no $C_9$-lactone (**3**) production could be detected using this buffer (data not shown). The presence of phosphate in buffer solutions was found to be essential for catalytic activity. In order to study the effect of phosphate on DEBS 1+TE activity, 50, 100, and 250 mM sodium phosphate buffers were used. The highest $k_{cat}$ was observed in 250 mM phosphate (3.4 ± 1.2 min$^{-1}$). In 100 and 50 mM phosphate, the $k_{cat}$ was 2.6 and 0.7 min$^{-1}$, respectively. Notably, the experimental attainment of maximum activity required desalting the ammonium sulfate-precipitated enzyme in a buffer with the appropriate salt concentration; the activity of DEBS 1+TE prepared by desalting with low-salt buffers could be only partially recovered upon addition of sodium phosphate. The actual yield of DEBS 1+TE protein, as distinguished from its activity, was independent of phosphate concentration in the desalting buffer. DEBS 1+TE activity was not influenced by the nature of the monovalent cation (potassium or sodium) (data not shown).

*Rate Constants for DEBS 1+TE in the Absence of NADPH.* In the absence of NADPH, DEBS 1+TE converts

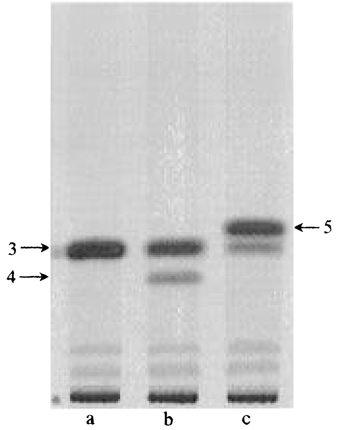

FIGURE 6: TLC autoradiograph of $^{14}$C-labeled $C_8$-, $C_9$-, and $C_{10}$-lactones **3**, **4**, and **5**. [$^{14}$C]Methylmalonyl-CoA ($^{14}$C-MM) was the radiolabeled substrate; concentrations of all substrates and DEBS 1+TE are described in Materials and Methods. Lanes: (a) $^{14}$C-MM + NADPH; (b) $^{14}$C-MM + NADPH + acetyl-CoA; (c) $^{14}$C-MM + NADPH + butyryl-CoA. Compounds: (**3**) $C_9$-lactone; (**4**) $C_8$-lactone; (**5**) $C_{10}$-lactone.

propionyl-CoA and methylmalonyl-CoA into the pyrone **6** (Pieper et al., 1995b). Using the same enzyme preparation, the $k_{cat}$ for pyrone formation was 7-fold reduced, compared to the $k_{cat}$ for triketide $C_9$-lactone synthesis. Under these reaction conditions, the final pyrone yield was 20%.

*Primer Unit Specificity of DEBS 1+TE.* Recent studies have shown that DEBS 1+TE can be acylated by $^{14}$C-labeled acetyl, propionyl, and butyryl residues with comparable efficiency (Pieper et al., 1995a,b). Furthermore, all three primer units could be converted into the corresponding triketide lactone products, **3**, **4**, and **5** (Pieper et al., 1995a,b; Wiesmann et al., 1995). Although these results were consistent with earlier *in vivo* experiments suggesting that DEBS possessed relatively broad specificity toward unnatural primer units (Brown et al., 1995; Cortes et al., 1995; Kao et al., 1994a, 1995), they failed to explain the exclusive formation of polyketides with propionate starters in *S.* erythraea, the erythromycin-producing organism. In the course of the current studies, we observed that both $C_8$- and $C_9$-lactones (1:2.8 ratio of **4** to **3**) can be detected when [$^{14}$C]-methylmalonyl CoA and unlabeled acetyl-CoA are used as substrates in the absence of propionyl-CoA (Figure 6). Similarly, both $C_{10}$- and $C_9$-lactone are synthesized (2.6:1 ratio **5** to **3**) when butyryl-CoA and [$^{14}$C]methylmalonyl-CoA are the substrates (Figure 6). Thus, propionyl primer units, generated *in situ* by decarboxylation of methylmalonyl-CoA, compete with varying efficiency with non-propionate primers. As shown above, however, when propionyl-CoA is used as substrate, the fraction of tripropionate lactone that contains a primer unit derived by decarboxylation of methylmalonyl-CoA is negligible. Together, these results suggested that DEBS had greater overall specificity toward propionyl primer units than toward alternative primers. These differences were further quantitated by competition experiments in which two competing substrates were present in equimolar ratios in the same reaction mixture (Figure 7). The ratio of the initial rates of appearance of the two lactone products is a measure of the relative apparent bimolecular rate constants, $k_{cat}/K_m$, for the overall reaction for each of the two substrates. When propionyl-CoA and acetyl-CoA are present in the same reaction mixture, this ratio was 32 (propionate:acetate) (Figure 7A); in the presence of propionyl- and butyryl-CoA, the ratio was found to be 7.5 (propionate:butyrate) (Figure 7B). Thus, although DEBS 1 + TE can incorporate a variety of primer units into its polyketide product, it has a significantly higher specificity toward its natural substrate, propionate, over alternative starter units.

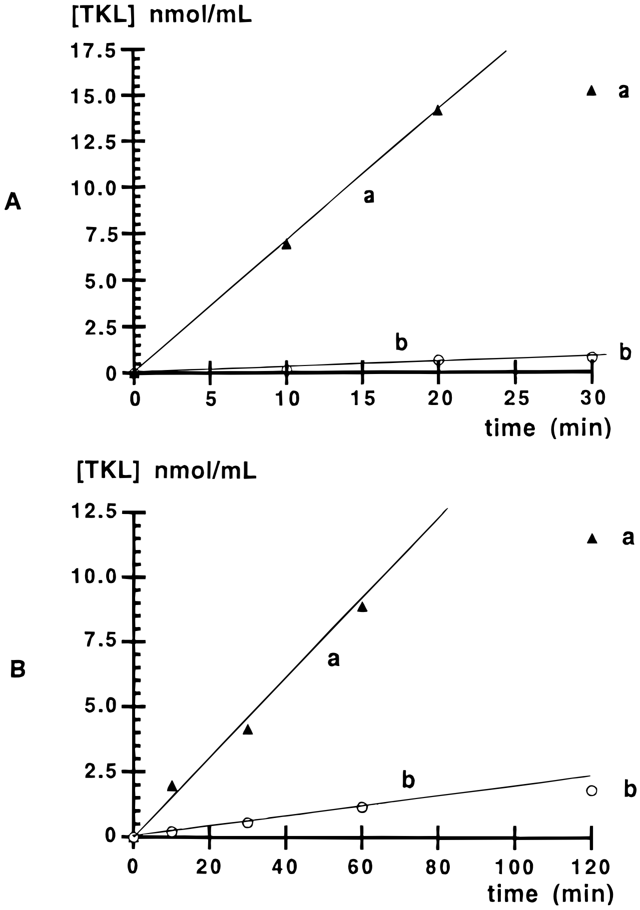

FIGURE 7: (A) Competition of acetyl-CoA and propionyl-CoA as primer substrates for DEBS 1+TE. DEBS 1+TE was incubated with [$^{14}$C]propionyl-CoA and [$^{14}$C]acetyl-CoA (both 200 $\mu$M), (2*S*)-methylmalonyl-CoA (300 $\mu$M), and NADPH (1 mM). The concentrations of (a) $C_9$- (**3**) and (b) $C_8$-lactone (**4**) synthesized in the same reaction are plotted versus reaction time. Rates were calculated from the linear range of the data. (B) Competition of propionyl-CoA and butyryl-CoA as primer substrates for DEBS 1+TE. DEBS 1+TE was incubated with [$^{14}$C]propionyl-CoA and [$^{14}$C]butyryl-CoA (both 200 $\mu$M), (2S)-methylmalonyl-CoA (300 $\mu$M), and NADPH (1 mM). The concentrations of (a) $C_9$- (**3**) and (b) $C_{10}$-lactone (**5**) synthesized in the same reaction are plotted versus reaction time. Rates were calculated from the linear range of the data.

*Rate Constant for Complete DEBS Activity.* In vitro the complete DEBS assembly synthesizes both 6-dEB (**1**) and the $C_9$-lactone (**3**). 6-dEB is produced with a $k_{cat}$ of 0.5 min$^{-1}$, whereas the $k_{cat}$ for formation of the side-product $C_9$-lactone is 0.23 min$^{-1}$. The $C_9$-lactone has not been detected in cultures of CH999/pCK7, which produce **1** and **2** as their primary products (Kao et al., 1994a).

## DISCUSSION

The development of an efficient cell-free assay for triketide lactone and 6-dEB synthesis has facilitated, for the first time, kinetic analysis of modular PKSs. The overall rate constants for the synthesis of the polyketide products of both DEBS 1+TE and the complete DEBS 1/DEBS 2/DEBS 3 assembly have been measured. Given the observed turnover rate of 3.4 $\text{min}^{-1}$, the amount of DEBS 1+TE obtained from 1 L of culture (ca. 1 $\mu$mol) would be expected to yield up to 35 mg/h triketide lactone, assuming constant activity. This is comparable to the observed yield of triketide lactone from cultures of *S. coelicolor* CH999/pCK12 (Kao et al., 1995). Thus, it is noteworthy that, under the above assay conditions, DEBS 1+TE is as active *in vitro* as it is *in vivo*.

In the case of fatty acid synthases, malonyl-CoA can be decarboxylated by the ketosynthase in the absence of acetyl-CoA (Kresze et al., 1977). Here we have shown that modular PKSs can also catalyze decarboxylation and that the product of this reaction can be used for polyketide biosynthesis. Interestingly, although decarboxylation of methylmalonyl-CoA does not occur at an appreciable level in the presence of adequately high concentrations of propionyl-CoA (but not other priming substrates; see below), under steady-state conditions decarboxylation of methylmalonyl-CoA is not rate-limiting for polyketide formation in the absence of propionyl-CoA.

In contrast, in the presence of alternative primer units such as acetyl-CoA and butyryl-CoA, decarboxylation of methylmalonyl-CoA and condensation between the unnatural primer and methylmalonyl-CoA are competing reactions. Presumably, this is due to the significantly greater specificity of DEBS 1+TE toward propionate over alternative primer units. Thus, the lower the specificity for the unnatural primer unit, the higher the yield of $C_9$-lactone **3** derived via decarboxylation of methylmalonyl-CoA. This result has potential physiological significance. As mentioned above, whereas recombinant *S. coelicolor* produces comparable amounts of acetate primer and propionate primer derived lactones, *S. erythraea* primarily produces propionate primer derived polyketides. We believe that this may be due to differences in intracellular levels of propionyl-CoA. Earlier we have reported that *S. coelicolor* produces propionate and acetate-derived polyketides in a ratio of ca. 3:1 (Kao et al., 1994a, 1995). These yields are in excellent agreement with our *in vitro* results reported here, suggesting that the intracellular concentration of free propionyl-CoA in *S. coelicolor* may be extremely low or negligible.[1] *S. erythraea*, on the other hand, appears to generate a substantial fraction of its intracellular pool of propionyl-CoA through the activity of a malonyl-CoA decarboxylase (Hsieh & Kolattukudy, 1994). The exclusive formation of significantly lower yields of **3** by cultures of *S. coelicolor* harboring DEBS 1 without a fused TE domain may reflect preferential utilization of the small pool of endogenous propionyl-CoA (Kao et al., 1994b). Interestingly, DEBS 1+TE appears to tolerate a butyryl primer better than an acetyl primer. Thus, the biosynthetic potential of modular PKSs may be substantially greater than is evident from the products observed *in vivo*. In view of the potential medicinal utility of derivatives of erythromycin that are modified at C-14 (Kirst, 1993), it should be worthwhile and relatively straightforward to explore the ability of DEBS to tolerate other functionalized primer units as well.

The 32-fold preference for propionate over acetate primers in DEBS 1+TE assumes greater significance in light of our recent finding that the AT domain at the N-terminal end of DEBS 1+TE (Figure 1) can be acylated with approximately equal efficiency by both acetate and propionate primer units (Pieper et al., 1995b). We speculate that the ability of DEBS 1+TE to discriminate between the two substrates is primarily due to the recognition features of an active site other than the loading AT (possibly one of the KS domains). This distinction cannot be made solely by comparison of steady-state parameters for the overall multistep transformation and will require further investigation.

The activity of both DEBS 1+TE and the complete DEBS system requires a high phosphate concentration, suggesting that hydrophobic interactions may play a significant role in the assembly of a functional enzyme complex. Consistent with this interpretation is the finding that DEBS proteins migrate as dimers (or higher order complexes) on gel-filtration columns (Caffrey et al., 1992; Pieper et al., 1995a). The importance of high phosphate concentrations for proper assembly of active homodimers has previously been reported for avian fatty acid synthase (Wang et al., 1984).

In the absence of NADPH, DEBS 1+TE catalyzes the formation of an unreduced aromatic polyketide, 6-ethyl-2-hydroxy-3,5-dimethyl-2*H*-pyran-2-one (**6**) (Pieper et al., 1995b), with an overall $k_{\text{cat}}$ one-seventh that for synthesis of **3** when NADPH is present. Formation of the pyrone requires that at least two catalytic domains, KS2 and TE, be able to tolerate substrates that are significantly altered in their oxidation state compared to the natural chain-elongation intermediates. The relatively modest difference in overall rates of formation of **3** and **6** is consistent with the previously reported tolerance of DEBS toward knock-out mutations in downstream reducing domains (Donadio et al., 1993, 1991).

Finally, the observed rate constant for formation of 6-dEB by the complete DEBS assembly is significantly lower than that for generation of triketide lactone **3** by DEBS 1+TE. This difference could be due to the greater complexity of the reaction sequence catalyzed by the complete DEBS complex. For example, it is not known how many chain-elongation intermediates can be loaded simultaneously onto either DEBS 1+TE or the complete set of DEBS proteins. It is likely, however, that under the reported assay conditions, the association of the three DEBS subunits has not yet been optimized. Consistent with this hypothesis is the fact that the $C_9$-lactone is generated as an abortive chain-elongation product.[2] Improving the assay conditions so as to optimize the yield of 6-dEB could yield important insights into the mechanisms of intermodular chain transfer within modular PKSs.

The modular organization of the DEBS PKS provides an elegant evolutionary solution to the problem of programming a complex sequence of biosynthetic reactions based on the repetitive utilization of a small repertoire of simple biochemical transformations. The kinetic studies reported here demonstrate that, superimposed on this purely organizational control, a level of structural selectivity exists, allowing kinetic discrimination in favor of natural substrates and intermediates. The fact that this selectivity is not absolute and that

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[1] The independent report that **4** can be formed *in vivo* in substantial preference to **3** by *S. coelicolor* harboring a DEBS 1+TE construct in an unspecified vector/promoter system may be due to higher endogenous levels of acetyl-CoA under the fermentation conditions used (Brown et al., 1995).

[2] Katz and Donadio (Donadio et al., 1992) have reported *in vivo* formation of **3** as a side product by the KR5 deletion mutant in *S. erythraea*.

downstream catalytic domains can tolerate a variety of substrate analogs not only has intriguing implications for the evolution of the more than 100 closely related naturally occurring macrolides (Cane et al., 1983a; Celmer, 1965) but offers exciting possibilities for the rational production of entirely new polyketide metabolites.

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