Efficient Synthesis of Aromatic Polyketides *in Vitro* by the Actinorhodin Polyketide Synthase

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Polyketide synthases (PKSs) are a large family of enzymes that catalyze the biosynthesis of numerous structurally diverse and medicinally important natural products known as polyketides.[1] These enzymes direct the polymerization of activated carboxylic acids in a manner analogous to fatty acid biosynthesis. Unlike fatty acid biosynthesis, the β-carbonyl of a growing polyketide chain may be left unreduced or converted to hydroxyl, enoyl, or methylene functionalities. The level of ketoreduction, as well as the initial cyclization pattern of the full-length polyketide chain, is determined by the PKS.

Bacterial aromatic PKSs,[2] such as the actinorhodin (*act*) PKS, are composed of several monofunctional (and possibly bifunctional) proteins (Figure 1). Together, these proteins control the chain length,[3,4] regiospecificity of ketoreduction,[3,5,6] and regiospecificity of the initial cyclization(s)[7−10] of the nascent polyketide backbone. A subset of three proteins, the ketosynthase/putative acyltransferase (KS/AT), chain length factor (CLF), and acyl carrier protein (ACP), is essential for polyketide synthesis and comprises the "minimal" PKS.[7] The mechanisms by which bacterial aromatic PKSs synthesize a highly labile poly-β-ketone intermediate of precise chain length, and guide it toward a regiospecifically reduced and cyclized product, represent an exciting challenge in multifunctional enzymology and biomolecular engineering. Toward this end, mutagenesis and heterologous expression of recombinant bacterial aromatic PKSs have provided some insight into the functions and molecular recognition features of the different protein components of the PKS and have laid the groundwork for the combinatorial biosynthesis of "unnatural" natural products.[11] However, in the absence of fully active cell-free systems to facilitate purification, kinetic, physicochemical, and ultimately structural investigations, the mechanisms of catalysis, molecular recognition, and assembly of these remarkable multifunctional enzymes remain poorly understood. In this regard, the seminal work of Shen and Hutchinson led to the development of the first cell-free system for aromatic polyketide biosynthesis.[12]

Building on recent advances in the development of fully active cell-free systems for modular PKSs,[13−15] we report here the efficient conversion of acetyl and malonyl coenzyme A (CoA) into aromatic polyketides by cell-free preparations of the *act* minimal PKS. We also show that in the presence of the *act* ketoreductase (KR), aromatase (ARO), and cyclase (CYC) the minimal PKS can synthesize 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC, **3**), the primary *in vivo* product of the complete *act* PKS.

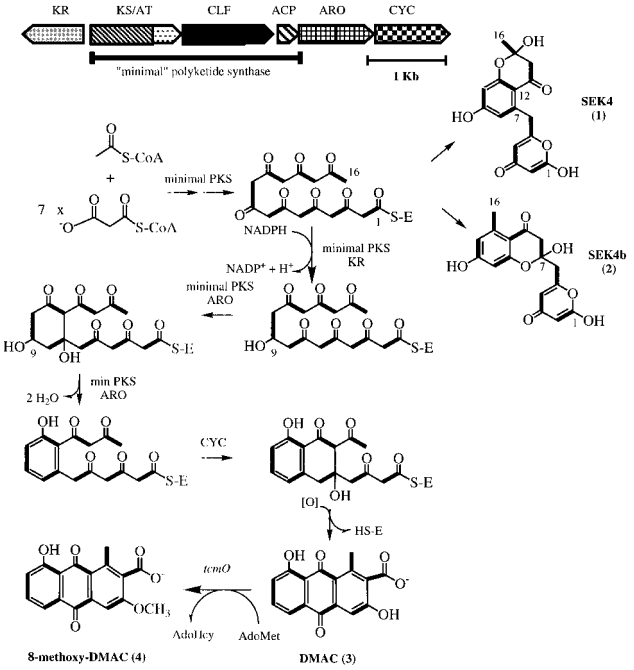

Figure 1. Polyketide biosynthesis by gene products of the *act* PKS cluster. *S. coelicolor* CH999/pSEK24 contains genes encoding the three proteins of the "minimal" PKS: an acyl carrier protein (ACP), a ketosynthase/acyltransferase (KS/AT), and a chain-length determining factor (CLF). The strain produces two 16-carbon polyketides, SEK4 (**1**) and SEK4b (**2**). In the presence of the *act* ketoreductase (KR), aromatase (ARO), and cyclase (CYC) (as in *S. coelicolor* CH999/pSEK38) the octaketide intermediate is converted into DMAC (**3**). DMAC (**3**) can be converted into 8-methoxy DMAC both *in vivo* and *in vitro* through the S-adenosylmethionine (Adomet)-dependent action of the *tcmO* methyltransferase.

Cell-free extracts were prepared[13] from *Streptomyces coelicolor* CH999/pSEK24[2] (which expresses genes for the *act* minimal PKS) by precipitation with 70% saturated $(NH_4)_2SO_4$. Polyketide synthesis in these extracts was detected using exogenous acetyl CoA and [^{14}C]malonyl CoA as substrates, followed by thin-layer chromatography (TLC)/autoradiography of the ethyl acetate-extractable products. Two ^{14}C-labeled compounds produced in these reactions were absent in reactions containing extracts of *S. coelicolor* CH999 lacking genes for the *act* minimal PKS. These products were detected when either [^{14}C]acetyl CoA or [^{14}C]malonyl CoA was used and comigrated with authentic samples of SEK4 (**1**) and SEK4b (**2**) purified from stationary phase cultures of *S. coelicolor* CH999/pSEK24[3,16] on TLC.[17] Additional evidence to confirm the structures of these products came *via* HPLC, UV−vis spectroscopy, and ^{13}C NMR spectroscopy. HPLC showed that the two unique radiolabeled products coeluted with authentic

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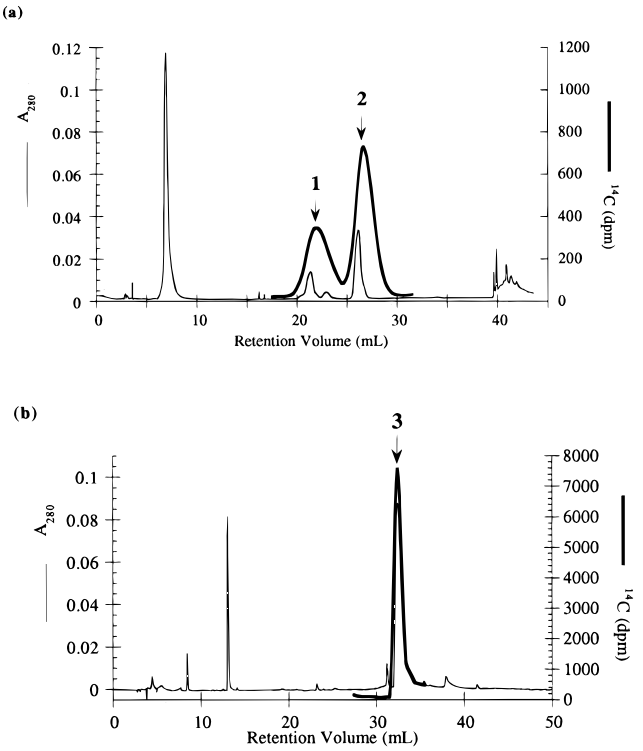

Figure 2. HPLC analysis of products formed by cell-free preparations of the *act* PKS. **(a)** *In vitro* synthesis of SEK4 (**1**) and SEK4b (**2**) by the *act* minimal PKS. Reaction mixtures (250 μL) contained 0.5 mM acetyl CoA, 0.5 mM [2-^{14}C]malonyl CoA (1 Ci/mol) and 6−9 mg/mL of *S. coelicolor* CH999/pSEK24 protein extract in 150 mM NaH_2PO_4, pH 7.3, 2 mM dithiothreitol, and 2 mM EDTA. Reactions were terminated after 30 min at 23 °C by addition of NaH_2PO_4 to saturation (~0.15 g) followed by extraction with 3 × 500 μL of ethyl acetate. The extract was dried *in vacuo* and redissolved in 15 μL of methanol. HPLC was performed using a Beckman Ultrasphere IP (4.6 mm × 25 cm) column and a mobile phase containing 5 mM NaH_2PO_4, pH 7.1, and 5 mM tetrabutylammonium sulfate with a gradient of 12−28% acetonitrile over 45 mL. Retention volumes for SEK 4 (**1**) and SEK4b (**2**) are indicated by arrows. **(b)** Synthesis of DMAC (**3**) in *S. coelicolor* CH999/pSEK38 extracts; reaction conditions were as described above, with the addition of 2 mM NADPH. DMAC (**3**) produced in these reactions was extracted from TLC plates and coinjected with an authentic sample. The mobile phase contained 1% acetic acid and a gradient of 0−100% acetonitrile over 60 mL; the retention volume for authentic **3** is indicated.

samples of **1** and **2** (Figure 2a) and had UV−vis spectra that were indistinguishable from those of the authentic compounds. For ^{13}C NMR experiments, [2-^{13}C]malonyl CoA was synthesized enzymatically using succinyl CoA transferase[18] and incubated with a cell-free preparation of the *act* minimal PKS. Sixteen enhanced singlet peaks, corresponding to C2, C4, C6, C8, C10, C12, C14, and C16 of **1** and **2**, were observed in the in the ^{13}C NMR spectrum of the extracted products.[19]

Based on radiochemical yield,[20] 40% of the [^{14}C]malonyl CoA added to reaction mixtures was converted into an approximately equimolar mixture of **1** and **2**. Typically, *ca*. 17 nmol of each polyketide product was synthesized per mL of reaction mixture containing *ca*. 7 mg of total protein. Thus, in the ^{13}C NMR experiment, over 0.5 μmol each of [$^{13}C_8$]**1** and [$^{13}C_8$]**2** was produced in a 30 mL reaction mixture.

Synthesis of **1** and **2** was inhibited by either 100 μM *N*-ethyl maleimide or 100 μM cerulenin (data not shown), which are also known to inhibit fatty acid synthases,[21] modular PKSs,[13] and *tcm* minimal PKS.[12] Likewise, the *act* minimal PKS appears to be capable of deriving primer units *via* decarboxylation of (extender) malonyl units, a reaction that has been noted in the fatty acid synthase,[22] modular PKS,[15] and *tcm* PKS[12] systems. This was apparent from the results of radiolabeling experiments in which [^{14}C]**1** and [^{14}C]**2** were produced from [^{14}C]malonyl CoA in the absence of acetyl CoA (data not shown). Furthermore, the ^{13}C NMR spectrum of **1** and **2** produced in reactions containing [2-^{13}C]malonyl CoA and unlabeled acetyl CoA showed enriched singlets for C16 of both products.[23]

Finally, using protein extracts from *S. coelicolor* CH999/pSEK38,[24] a cell-free assay for the complete *act* PKS, including the minimal PKS, ketoreductase (KR), aromatase (ARO), and cyclase (CYC) activities, was developed (Figure 1). When *S. coelicolor* CH999/pSEK38 extract (7 mg/mL of total protein) was incubated with 0.5 mM acetyl CoA, 0.5 mM [2-^{14}C]malonyl CoA, and 2 mM NADPH, one of the major radiolabeled products observed *via* TLC/autoradiography comigrated with DMAC (**3**), the major *in vivo* product of the complete *act* PKS. HPLC analysis of this reaction also revealed a major ^{14}C-labeled peak that coeluted with an authentic sample of **3** (Figure 2b). A secondary enzymatic assay was used to confirm the identity of this product. Recently, the *tcmO* methyltransferase was shown to selectively catalyze the conversion of **3** into 8-methoxy DMAC (**4**), both *in vivo* and *in vitro*.[25] Upon incubation of the TLC-purified ^{14}C-labeled product with an extract from *S. coelicolor* CH999/pZP4[25,26] (which contains the *tcmO* methyltransferase) in the presence of 2 mM *S*-adenosyl methionine, a new product was formed which coeluted with authentic samples of **4** on HPLC (data not shown).

In conclusion, several aspects of the *act* minimal PKS have been reconstituted in cell-free systems. First, efficient conversion of malonyl CoA into **1** and **2** demonstrates the catalytic potency of the preparation. Second, enzymatic synthesis of equimolar amounts of SEK4 (**1**) and SEK4b (**2**) is in excellent quantitative agreement with the *in vivo* behavior of the *act* minimal PKS.[16] Finally, the ability of the *act* ketoreductase, aromatase, and cyclase to assist in the conversion of the nascent 16-carbon polyketide produced by the minimal PKS into DMAC (**3**) shows that the entire metabolic pathway consisting of at least six different proteins can be reconstituted *in vitro*. Together, these results provide an attractive starting point for kinetic, protein chemical, and structural studies of the *act* PKS as a paradigm for this interesting class of enzymes.

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(19) ^{13}C NMR (400 MHz, DMSO-d_6) **1**: δ (ppm) C2, 88.3; C4, 102.9; C6, 38.7; C8, 113.0; C10, 100.3; C12, 111.3; C14, 49.3; C16, 27.5. **2**: δ (ppm) C2, 89.1; C4, 104.0; C6, 44.1; C8, 47.5; C10, 111.8; C12, 101.6; C14, 112.7; C16, 22.5. All signals were within 0.6 ppm of reported values (refs 6 and 16) with the exceptions of the signal for C6 of SEK4 (**1**) (38.7 obs, 37.6 authentic reference). This deviation is likely to be due to the presence of the DMSO-d_6 heptet at 39.5 ppm.

(20) ^{14}C-labeled products were quantitated on TLC plates using a Phosphorimager 400S (Molecular Dynamics) with ImageQuant software. No correction was made for product lost during the extraction/TLC procedure. Quantitation of ^{14}C-containing HPLC fractions using Beckman LS3801 scintillation counter yielded comparable results.

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(23) However, we cannot exclude the possibility that this reaction is catalyzed by a different enzyme, distinct from the PKS.

(24) pSEK38 is an unpublished derivative of pRM21 (see ref 7) which includes the *act* KS/AT, CLF, ACP, ARO, and CYC; the CLF has an N-terminal fusion with the 8-residue FLAG peptide (Kodak/IBI). The polyketide products of *S. coelicolor* CH999/pSEK38 are qualitatively and quantitatively the same as those of *S. coelicolor* CH999/pRM21.

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(26) In additon to *tcmO*, pZP4 contains genes for the complete *tcm* PKS and does not produce **3** or **4**.