Purification and in Vitro Reconstitution of the Essential Protein Components of an Aromatic Polyketide Synthase†

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ABSTRACT: A minimal set of proteins which catalyze the synthesis of aromatic polyketides from malonyl CoA has been purified and partially characterized. Plasmid-encoded actinorhodin (*act*) ketosynthase/chain-length factor (KS/CLF) complex was purified from *Streptomyces coelicolor* CH999/pSEK38, and assayed with purified aromatic PKS holo-ACPs which were overproduced and purified from *Escherichia coli* and phosphopantetheinylated in vitro using purified *E. coli* holo-ACP synthase. When highly purified preparations of KS/CLF, and holo-ACP failed to catalyze polyketide biosynthesis, a fourth protein was sought and purified from the *S. coelicolor* CH999 host on the basis of its ability to complement KS, CLF, and holo-ACP in polyketide synthesis. N-terminal sequencing identified this protein as the fatty acid synthase (*fabD*) malonyl CoA:ACP transacylase (MAT), recruited from primary metabolism. A $\alpha_2\beta_2$ structure was shown for the *act* KS/CLF complex, and three malonyl-enzyme biosynthetic intermediates were identified, defining an escorted path followed by malonyl groups en route from CoA to polyketide.

Aromatic polyketide synthases (type II PKSs[1]) are a family of enzymes that catalyze the biosynthesis of a structurally diverse and pharmaceutically important class of natural products known as bacterial aromatic polyketides (*1*−*5*). While many of these molecules have been exploited for their antibiotic (e.g. the tetracyclines), antineoplastic (e.g. doxorubicin), and other properties, the molecular mechanism of their biosynthesis is not well understood. Advances in the genetic manipulation of *Streptomyces* species enabled the identification and cloning of entire biosynthetic gene clusters which contain genes encoding polyketide synthases (cf. *6*−*9*). Subsequently, combinatorial expression of these genes resulted in the assignment of specific catalytic functions to the individual genes and an ability to produce novel, engineered polyketides (*4, 10, 11*). In an effort to understand the catalytic and molecular recognition properties of these pathways, crude cell-free systems for aromatic polyketide biosynthesis have been developed (*12, 13*); however, separation of the numerous protein components involved in these pathways during purification, along with the proposed requirement of host-encoded factors (*14, 15*), has made the in vitro reconstitution of aromatic polyketide biosynthesis difficult.

Streptomyces coelicolor CH999 is a recombinant strain which contains a defined chromosomal deletion of the entire actinorhodin (*act*) gene cluster (*10*). Plasmid-based expression of a minimal set of three proteins derived from the *act* polyketide synthase gene clusters, a ketosynthase (KS), chain-length factor (CLF), and acyl carrier protein (ACP) restore the strain's ability to produce polyketides (*16*). The KS and CLF are cotranscribed proteins which share primary sequence homology with each other as well as with the KS proteins involved in bacterial fatty acid synthesis; however, active site residues that play catalytic roles in fatty acid synthesis are conserved only in the KS (*17*). The CLF plays a poorly understood role in determining polyketide chain length (*10, 16, 18*). The ACP undergoes covalent posttranslational modification with a CoASH-derived 4′-phosphopantetheine prosthetic group, which is added to the protein through the action of a holo-ACP synthase (ACPS) (*19*−*21*). Natural PKS pathways also include "downstream" enzymes such as ketoreductases, cyclases, and methylases which tailor the polyketide backbone into the final, bioactive product. In this work, we have purified the *act* KS, CLF, and holo-ACP proteins and shown that their ability to catalyze the polymerization of malonyl CoA-derived building blocks requires the activity of a malonyl CoA:ACP transacylase (MAT) that also has a proposed role in *Streptomyces* primary fatty acid metabolism. No malonyltransferase or homologous protein is encoded within the *act* (*6*) or other (*7, 9, 22*) aromatic PKS gene clusters, suggesting that this enzyme is a functional link between primary and secondary metabolism.

MATERIALS AND METHODS

Materials. Cell-free protein extracts of *S. coelicolor* CH999/pSEK38 were prepared by precipitation with 70%

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[1] Abbreviations: PKS, polyketide synthase; KS, ketosynthase; CLF, chain-length factor; ACP, acyl carrier protein; MAT, malonyl CoA:ACP transacylase; ACPS, holo-ACP synthase; CoASH, coenzyme A; *act*, actinorhodin; *fren*, frenolicin; *gra*, granaticin; *otc*, oxytetracycline; *tcm*, tetracenomycin; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.

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$(NH_4)_2SO_4$ as previously described (*13*). pSEK38 encodes the *act* KS/AT, CLF, ACP, ARO, and CYC; the CLF has an N-terminal fusion with the 8-residue FLAG peptide (Kodak/IBI). [^{14}C]Malonyl CoA (56 Ci/mol) and [^{14}C]acetyl CoA (56 Ci/mol) were obtained from Moravek Biochemicals (Brea, CA). CoASH, malonyl CoA, acetyl CoA, and commonly available chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and used without further purification.

Assay of Polyketide Synthase Activity. PKS activity was measured by an endpoint assay that has been previously described (*13*). Protein concentrations were estimated from Coomassie blue-stained SDS-PAGE. Reaction mixtures (100−200 μL) contained, when included, ∼1 μM KS/CLF, ∼10 μM holo-ACP, ∼1 μM MAT, ∼1 mM [^{14}C]malonyl CoA (0.5 Ci/mol), 100 mM NaH_2PO_4, pH 7.3, 2 mM DTT, 2 mM EDTA. In some cases, chromatography fractions were assayed that contained up to 1 M $(NH_4)_2SO_4$ or 20% glycerol without deleterious effects. Reactions were incubated at room temperature for 2 h prior to quenching with ∼0.1 g of NaH_2PO_4 and extracting with 2 × 0.5 mL of ethyl acetate. Ethyl acetate extracts were dried in vacuo, resuspended in 10−15 μL of methanol and subjected to thin-layer chromatography on silica gel (Baker Si250F, methanol:acetic acid: ethyl acetate 5:1:94) and visualized using electronic autoradiography (Instantimager, Packard). In some cases, reactions were also analyzed by HPLC as previously described (*13*) or by atmospheric pressure chemical ionization mass spectrometry of an extract obtained from a 2 mL reaction.

Preparation and Purification of holo-ACP. The *fren* holo-ACP was prepared enzymatically from apo-ACP and *E. coli* holo-ACP synthase (ACPS), both of which were overexpressed and purified from *E. coli* as previously described (*20*, *21*). Holo-ACP prepared in this manner was used for routine complementation assays without further purification. In cases where highly purified holo-ACP was required, enzymatically synthesized holo-ACP was bound to Q-Sepharose (5 mL HiTrap, Pharmacia) in 20 mM Tris pH 6.0, 1 mM DTT, 1 mM EDTA at 1 mL/min, and eluted with a 40 mL gradient to the same buffer containing 1 M NaCl. Peak holo-ACP-containing fractions were subjected to two cycles of desalting and rechromatography in order to remove residual ACPS which apparently binds tightly to ACPs.

Purification of the act Ketosynthase/Chain-Length Factor Complex. All purification procedures were performed at 4 °C. Crude protein extract from ∼1.5 L of stationary phase *S. coelicolor* CH999/pSEK38 was desalted into 100 mM NaH_2PO_4, pH 7.3/NaOH, 2 mM DTT, 2 mM EDTA, 1 M $(NH_4)_2SO_4$ (buffer A) using Sephadex G-25 and bound to a Phenyl Sepharose 6 FF (high sub) column (HR 10/30, Pharmacia) at 2 mL/min. The column was washed with 30 mL of buffer A and developed with a 150 mL linear gradient to buffer A lacking $(NH_4)_2SO_4$. Fractions which supported polyketide synthesis when supplemented with holo-ACP eluted at the end of this gradient. Fractions containing ∼40% KS/CLF as estimated from SDS-PAGE were pooled, made 10% in glycerol, and frozen in liquid nitrogen prior to further purification.

Pooled fractions from phenyl sepharose chromatography were diluted 2-fold with 100 mM mM NaH_2PO_4, pH 7.3/NaOH, 2 mM DTT, 2 mM EDTA, 20% (v/v) glycerol (Buffer B) and bound to a Resource Q column (6 mL, Pharmacia) at 1 mL/min. The column was washed with 5 mL of buffer B and developed with a 42 mL linear gradient to buffer B containing 0.5 M NaCl. KS/CLF-containing fractions eluted near 400 mM NaCl and were identified by 12.5% SDS-PAGE where they run as a ∼50 kDa doublet, slightly larger than the 42 kDa predicted from their deduced amino acid sequences. The doublet was electroblotted onto a PVDF membrane (Immobilon P, Millipore) and submitted for N-terminal amino acid sequencing at the University of California, San Francisco, Biomolecular Resource Center.

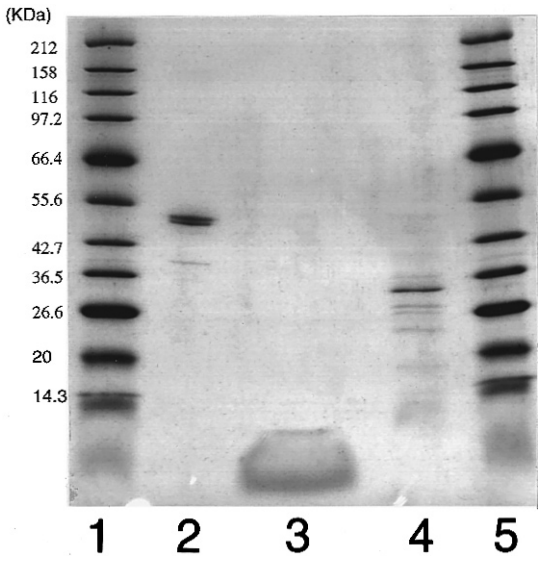

FIGURE 1: 12.5% SDS PAGE of purified PKS proteins. Lane 1, molecular weight standards; lane 2, *act* ketosynthase and chain-length factor (KS and CLF); lane 3, *fren* holo-acyl carrier protein (ACP); lane 4, *fabD* malonyl CoA:ACP transacylase (MAT); lane 5, molecular weight standards.

Purification of the fabD Malonyl CoA:ACP Transacylase. Crude extract from ∼3 L of stationary phase *S. coelicolor* CH999 was desalted into buffer A using Sephadex G-25 and bound to a Phenyl Sepharose 6 FF (high sub) column (AK 26/40, Pharmacia) at 2 mL/min. The column was washed with 100 mL of buffer A, followed by a 1225 mL linear gradient to buffer A lacking $(NH_4)_2SO_4$. Fractions which complemented purified KS/CLF and holo-ACP in polyketide synthesis eluted near the end of the gradient and were further purified by gel filtration chromatography (Superdex 200pg AK 26/60, Pharmacia) in 20 mM Tris, pH 8.0/NaOH, 2 mM DTT, 2 mM EDTA, 15% glycerol (Buffer C). Fractions from four separate gel filtration chromatography runs which complemented purified KS/CLF and holo-ACP in polyketide synthesis were pooled and bound to a Resource Q column (6 mL, Pharmacia) equilibrated with buffer C. The column was washed with 5 mL of buffer C, followed by a 42 mL linear gradient to buffer C containing 0.5 M NaCl. Fractions complementing purified KS/CLF and holo-ACP had in common a dominant 32 kDa protein. N-terminal amino acid sequencing was performed on the sample shown in Figure 1, which was ∼ 90% enriched in this protein.

Gel Filtration Chromatography. The molecular weights of the KS/CLF complex and the MAT were estimated using Superdex 200pg (AK 26/60, Pharmacia) with Buffer B at 0.5 mL/min (*23*). Elution volumes determined by absorbance at 280 nm were verified by SDS-PAGE and PKS activity assays of relevant fractions.

Detection of Covalent Intermediates. Reaction mixtures (20 μL) contained 250 μM [^{14}C]malonyl CoA (56 Ci/mol) and when included, ~1 μM KS/CLF, ~25 μM holo-ACP, and/or ~1 μM MAT. Following incubation at room temperature for 5−10 min, reactions were quenched with 5 μL of acidic, nonreducing loading buffer (0.1% bromophenol blue, 1% SDS, 40% glycerol, 5% trichloroacetic acid), and loaded directly onto 12.5% SDS-PAGE. Following staining, dried gels were subjected to electronic autoradiography.

RESULTS

Purification of the act KS/CLF Complex. Using our previously reported assay for cell-free polyketide synthesis (*13*), we fractionated crude extracts of *S. coelicolor* CH999/pSEK38 and assayed the fractions for their ability to catalyze polyketide synthesis from [^{14}C]malonyl CoA. The process was complicated by the requirement of several proteins for PKS activity, and separation of these proteins resulted in a loss of activity. Following phenyl sepharose chromatography, ~40% enriched preparations of KS/CLF were obtained that catalyzed polyketide synthesis when supplied with purified holo-ACPs derived from the *fren*, *gra*, *otc*, or *tcm* PKS gene clusters (*21*). Further fractionation of this preparation using anion exchange chromatography resulted in copurification of a nearly homogeneous (~95% pure) mixture of KS and CLF which was unable to catalyze polyketide biosynthesis when supplied with purified holo-ACPs and malonyl CoA (Figure 1). Approximately 10 mg of KS/CLF were obtained from each liter of *S. coelicolor* CH999/pSEK38 culture processed. Quantitative N-terminal amino acid sequencing of the KS/CLF doublet obtained from SDS-PAGE unambiguously identified the KS and CLF proteins and indicated their copurification as an equimolar mixture.

Isolation and Purification of the fabD MAT. The integrity of the KS, CLF, and holo-ACP proteins following purification was verified by the observation that they were reactivated upon addition of a crude extract from the *S. coelicolor* CH999 host strain which lacks genes encoding these proteins and has no PKS activity of its own. The extract's ability to complement KS, CLF, and holo-ACP survived desalting but was lost during heating, implicating a protein as the essential factor. We therefore sought and purified the responsible protein from *S. coelicolor* CH999 by assaying for its ability to complement purified KS, CLF, and holo-ACP in polyketide synthesis. A preparation was obtained following phenyl sepharose, gel filtration, and anion exchange chromatography that could complement purified KS, CLF, and holo-ACP and was highly enriched (>85%) in a ~32-kDa protein (Figure 1). N-terminal sequencing of the first 16 residues identified the protein as the *fabD* malonyl CoA:ACP transacylase (MAT). Approximately 1 mg of MAT was obtained from each liter of *S. coelicolor* CH999 processed.

Assay of Purified PKS Components. Figure 2 shows a thin layer chromatographic assay of the PKS activity of purified PKS components. Polyketide synthase activity required purified KS/CLF, holo-ACP, and MAT in order to catalyze polyketide synthesis from malonyl CoA, and omission of any of these components resulted in a loss of activity. Inclusion of acetyl CoA was not required to obtain PKS activity. Activity was obtained when holo-ACPs from the

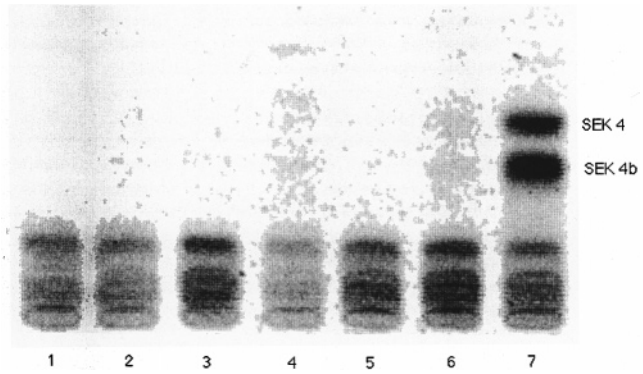

FIGURE 2: Thin-layer chromatography assay of the PKS activity of purified PKS proteins. Lane 1, *fren* holo-ACP only; lane 2, *act* KS/CLF only; lane 3, *fabD* MAT only; lane 4, holo-ACP + KS/CLF; lane 5, holo-ACP + MAT; lane 6, MAT + KS/CLF; lane 7, holo-ACP + MAT + KS/CLF.

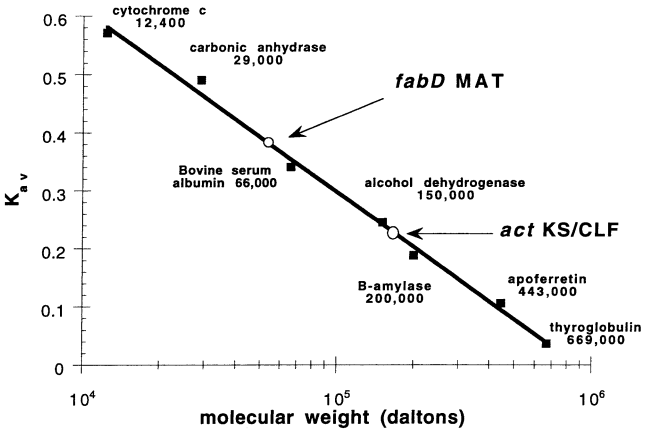

FIGURE 3: Estimation of KS/CLF and MAT complex molecular weights by gel filtration chromatography. $K_{av} = (V_t - V_e)/(V_t - V_o)$ (see ref 23). V_o was determined using blue dextran (~2×10^6 Da) and V_t was determined using tyrosine.

fren, *gra*, *tcm*, or *otc* PKS pathways were used; however, no activity was obtained with the corresponding apo-ACPs (*20*, *21*). SEK 4 and SEK 4b polyketide products were identified on the basis of their chromatographic properties on TLC and HPLC (data not shown), which were the same as for our previously reported in vitro synthesized samples of SEK 4 and SEK 4b whose structures were verified by ^{13}C NMR (*13*). In addition, mass spectroscopy of SEK 4 and SEK 4b synthesized using the purified system showed the expected molecular ion ($M + H^+$) of 319 Da as well as a dehydrated fragment at 301 Daltons (SEK4 and SEK4b are structural isomers).

Complex Molecular Weight Estimations. Gel filtration chromatography was used to characterize the KS/CLF complex and the MAT (Figure 3). The KS/CLF complex behaved as a ~168 kDa tetramer, eluting between alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Together with the N-terminal sequencing of the complex which indicated that the KS and CLF proteins copurify as an equimolar mixture, these data suggests an $\alpha_2\beta_2$ structure for the *act* KS/CLF complex. MAT activity eluted between carbonic anhydrase (29 kDa) and bovine serum albumin (66 kDa), midway between the elution volumes expected for its monomeric and dimeric forms. This is in accord with the crystal structure of the *E. coli fabD* MAT, which shows an elongated monomer (*24*).

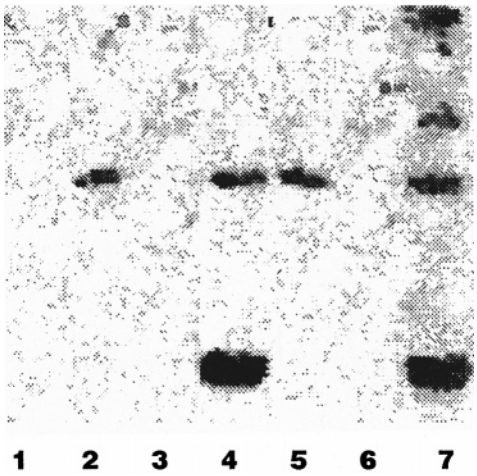

FIGURE 4: Autoradiogram of a 12.5% SDS-PAGE gel showing labeling of purified PKS proteins by [^{14}C]malonyl CoA. Lane 1, *fren* holo-ACP only; lane 2, *fabD* MAT only; lane 3, *act* KS/CLF only; lane 4, holo-ACP + MAT; lane 5, MAT + KS/CLF; lane 6, holo-ACP + KS/CLF; lane 7, holo-ACP + MAT + KS/CLF.

Identification and Sequence of Covalent Intermediates. [^{14}C]Malonyl CoA labeling of purified holo-ACP, KS/CLF complex, MAT, and combinations of these proteins was used to investigate covalent intermediates formed during polyketide biosynthesis (Figure 4). When purified holo-ACP, KS/CLF complex, or MAT were incubated individually with [^{14}C]-malonyl CoA, only the MAT could be labeled. When [^{14}C]-malonyl CoA and MAT were incubated with holo-ACP, both the MAT and holo-ACP were labeled; however, when [^{14}C]-malonyl CoA and MAT were incubated with KS/CLF, only the MAT was labeled. When MAT, holo-ACP, and the KS/CLF complex were incubated together with [^{14}C]malonyl CoA, labeled forms of all three of the protein preparations were observed, and the polyketides SEK 4 and SEK 4b were formed. 10−20% gradient SDS-PAGE was used to obtain increased resolution between the KS and CLF proteins and showed that only the slower moving of the two proteins was labeled. Although both KS and CLF have anomalously low mobilities on SDS-PAGE, it is likely that the labeled protein is the KS, since its calculated molecular weight is ~5% larger than that of the CLF (45016 vs 42523 Da). In addition, the *tcm* KS (*tcm K*) and CLF (*tcm L*) proteins behave similarly on SDS-PAGE and have been identified by western blotting (*25*).

DISCUSSION

In vitro reconstitution has been used to define a minimal set of essential proteins required for the biosynthesis of bacterial aromatic polyketides from malonyl CoA. The *act* KS and CLF proteins and holo-ACPs derived from several PKS gene clusters were purified to homogeneity and found to require the *fabD* malonyl CoA:ACP transacylase (MAT) for PKS activity. Together, these four proteins, KS, CLF, holo-ACP, and MAT are necessary and sufficient for the synthesis of polyketide backbones from malonyl CoA.[2]

In a striking confirmation of the crosstalk between primary and secondary metabolism, the *fabD* MAT has been isolated as an essential component of PKS activity. This protein is encoded as part of the fatty acid synthase (FAS) gene cluster which is distant from the PKS gene cluster on *Streptomyces* chromosomes and catalyzes malonyl transfer from CoA to the FAS holo-ACP during bacterial fatty acid synthesis (*14*, *15*, *26*). This same protein was previously isolated from *Steptomyces* protein extracts based upon its ability to catalyze malonyl transfer from CoA to aromatic PKS holo-ACPs, suggesting a link between the fatty acid (primary) and polyketide (secondary) biosynthetic pathways (*14*, *15*). Isolation of the *fabD* MAT using a direct biochemical assay for its ability to complement the PKS activity of purified KS/CLF and holo-ACP provides definitive evidence for the dual role played by this protein. Utilization of the *fabD* MAT in both fatty acid and polyketide biosynthesis makes it a potential regulatory point for the control of primary and secondary metabolic flux.

While genetic studies and analogies to bacterial fatty acid synthesis have long implicated the KS and CLF proteins in polyketide biosynthesis, this work represents the first direct observation of their covalent involvement in the oligomerization of malonyl CoA in a purified system. The *act* KS and CLF proteins copurify under all conditions investigated, and the purified KS/CLF complex behaves as an $\alpha_2\beta_2$ heterotetramer during gel filtration chromatography. The *tcm* KS and CLF proteins also copurify using the same methods; however, these proteins behave as a heterodimer on gel filtration (Bao and Hutchinson, personal communication; Carreras and Khosla, data not shown).

[^{14}C]Malonyl CoA labeling studies were used to define an escorted path followed by malonyl groups en route from CoA to polyketide (Figure 5). The ability of purified MAT, to covalently bind [^{14}C]malonyl CoA in the absence of other PKS proteins, together with the inability of these other components to become labeled in the absence of MAT, indicates that malonyl-MAT is the first covalent intermediate of the pathway. The MAT-dependent labeling of PKS holo-ACPs is consistent with the previously reported activity of the MAT (*14*, *15*), and indicates that malonyl-ACP is the second covalent intermediate formed in the pathway. The absence of detectable labeling in a mixture of ACP and KS/CLF is in accord with mutagenesis experiments which indicate that the KS protein does not contain MAT activity (*25*). Labeling of the KS protein of the KS/CLF complex in the presence of MAT and holo-ACP indicates that labeling of the KS occurs via the malonyl-ACP intermediate and demonstrates a third covalent intermediate of the pathway. The labeled protein may actually represent several different lengths of growing polyketide chain attached to KS that are unresolved during electrophoresis. Finally, since intermediates must accumulate in significant quantities and be stable during SDS-PAGE in order to be detected in these studies, our failure to observe a labeled form of the CLF does not prove the lack of a covalent catalytic role for this PKS component.

In less pure preparations of PKS holo-ACPs, we observed the apparent autocatalytic labeling of holo-ACP with [^{14}C]-malonyl CoA (data not shown). It is possible that this activity derives from residual ACPS used in the phosphopantetheinylation reaction, or contaminating *E. coli* MAT which has a high activity in the *E. coli* BL21 DE(3) extracts from which the apo-ACPs were purified (C. Carreras and

[2] The presence of added acetyl CoA was not required, and [^{14}C]-acetyl CoA incorporation seemed to occur only at lower malonyl CoA concentrations, suggesting that the enzyme prefers to prime polyketide synthesis by decarboxylating malonyl CoA.

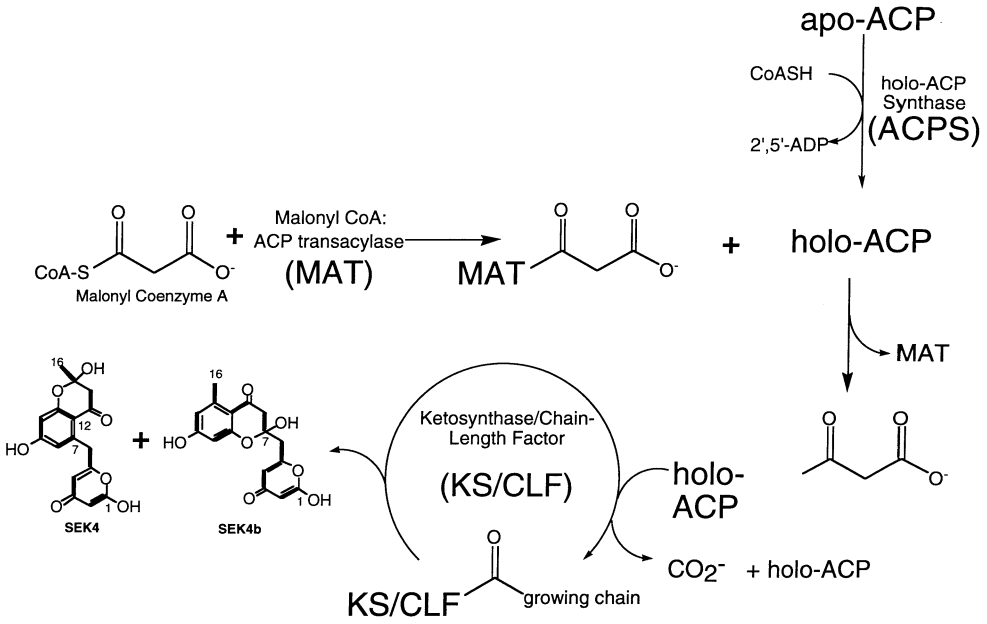

FIGURE 5: Escorted path followed by malonyl CoA-derived two carbon units en route from CoA to polyketide.

C. Khosla, unpublished results). Alternatively, it is possible that the apparent autocatalytic malonyl transfer is the result of a chemical thioester exchange reaction that is difficult to detect following the dilution that occurs upon repeated chromatography of the holo-ACP. In either case, the reaction is not biologically relevant since it cannot support polyketide synthesis in the absence of MAT.

The active, reconstituted system containing purified KS/CLF complex, holo-ACP, and MAT provides an attractive model system for mechanistic studies of this interesting class of enzymes, and preliminary experiments suggest these methods are suitable for the purification of KS and CLF proteins derived from the *tcm*, *fren*, *whiE* and other gene clusters (C. Carreras and C. Khosla, unpublished results). We are currently using the purified system to address issues such as the molecular basis of chain length determination, the number of each subunit that participates in the synthesis of an individual polyketide chain, and the interaction of minimal PKS proteins with downstream enzymes. Continued study of the basic catalytic and molecular recognition features of these enzyme systems is likely to enhance technologies aimed at the generation of engineered polyketides with novel biochemical and pharmaceutical properties.

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