Mechanistic Analysis of a Type II Polyketide Synthase. Role of Conserved Residues in the β-Ketoacyl Synthase−Chain Length Factor Heterodimer†

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ABSTRACT: Type II polyketide synthases (PKSs) are a family of multienzyme systems that catalyze the biosynthesis of polyfunctional aromatic natural products such as actinorhodin, frenolicin, tetracenomycin, and doxorubicin. A central component in each of these systems is the β-ketoacyl synthase−chain length factor (KS−CLF) heterodimer. In the presence of an acyl carrier protein (ACP) and a malonyl-CoA:ACP malonyl transferase (MAT), this enzyme synthesizes a polyketide chain of defined length from malonyl-CoA. We have investigated the role of the actinorhodin KS−CLF in priming, elongation, and termination of its octaketide product by subjecting the wild-type enzyme and selected mutants to assays that probe key steps in the overall catalytic cycle. Under conditions reflecting steady-state turnover of the PKS, a unique acyl-ACP intermediate is detected that carries a long, possibly full-length, acyl chain. This species cannot be synthesized by the C169S, H309A, K341A, and H346A mutants of the KS, all of which are blocked in early steps in the PKS catalytic cycle. These four residues are universally conserved in all known KSs. Malonyl-ACP alone is sufficient for kinetically and stoichiometrically efficient synthesis of polyketides by the wild-type KS−CLF, but not by heterodimers that carry the mutations listed above. Among these mutants, C169S is an efficient decarboxylase of malonyl-ACP, but the H309A, K341A, and H346A mutants are unable to catalyze decarboxylation. Transfer of label from [^{14}C]malonyl-ACP to the nucleophile at position 169 in the KS can be detected for the wild-type enzyme and for the C169S and K341A mutants, but not for the H309A mutant and only very weakly for the H346A mutant. A model is proposed for decarboxylative priming and extension of a polyketide chain by the KS, where C169 and H346 form a catalytic dyad for acyl chain attachment, H309 positions the malonyl-ACP in the active site and supports carbanion formation by interacting with the thioester carbonyl, and K341 enhances the rate of malonyl-ACP decarboxylation via electrostatic interaction. Our data also suggest that the ACP and the KS dissociate after each C−C bond forming event, and that the newly extended acyl chain is transferred back from the ACP pantetheine to the KS cysteine before dissociation can occur. Chain termination is most likely the rate-limiting step in polyketide biosynthesis. Within the *act* CLF, neither the universally conserved S145 residue nor Q171, which aligns with the active site cysteine of the ketosynthase, is essential for PKS activity. The results described here provide a basis for a better understanding of the catalytic cycle of type II PKSs and fatty acid synthases.

Polyketide synthases (PKSs)[1] are a large family of multifunctional enzymes responsible for the biosynthesis of a variety of structurally complex natural products in microorganisms and plants. Like the related fatty acid synthases (FASs), they include a set of homologous active sites embedded in architecturally diverse protein assemblies. One such example is the family of PKSs that catalyze the biosynthesis of polyfunctional aromatic natural products such as actinorhodin, frenolicin, tetracenomycin, and doxorubicin (for a review, see refs *1*−*4*). These enzymes, which are related to type II FASs which are present in bacteria and plants, include a heterodimeric ketosynthase (KS)−chain length factor (CLF) complex, an acyl carrier protein (ACP) bearing a pantetheinyl arm, and a malonyl-CoA:ACP malonyltransferase (MAT).[2] A model for chain initiation, elongation, and termination has been proposed, and is outlined in Figure 1.

A central component in polyketide biosynthesis is the KS−CLF heterodimer. The ketosynthase is homologous to condensing enzymes from all known PKSs and FASs, and

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[1] Abbreviations: CoA, coenzyme A; PKS, polyketide synthase; FAS, fatty acid synthase; KS, ketosynthase; CLF, chain length factor; ACP, acyl carrier protein; MAT, malonyl-CoA:ACP transacylase; *act*, actinorhodin; *fren*, frenolicin; TLC, thin-layer chromatography.

[2] There is some debate regarding the role of the MAT in polyketide synthesis (*5*, *6*); however, several laboratories have established that at physiologically relevant concentrations of individual proteins, the MAT-dependent pathway for malonyl-S-ACP formation is dominant (*7*−*10*).

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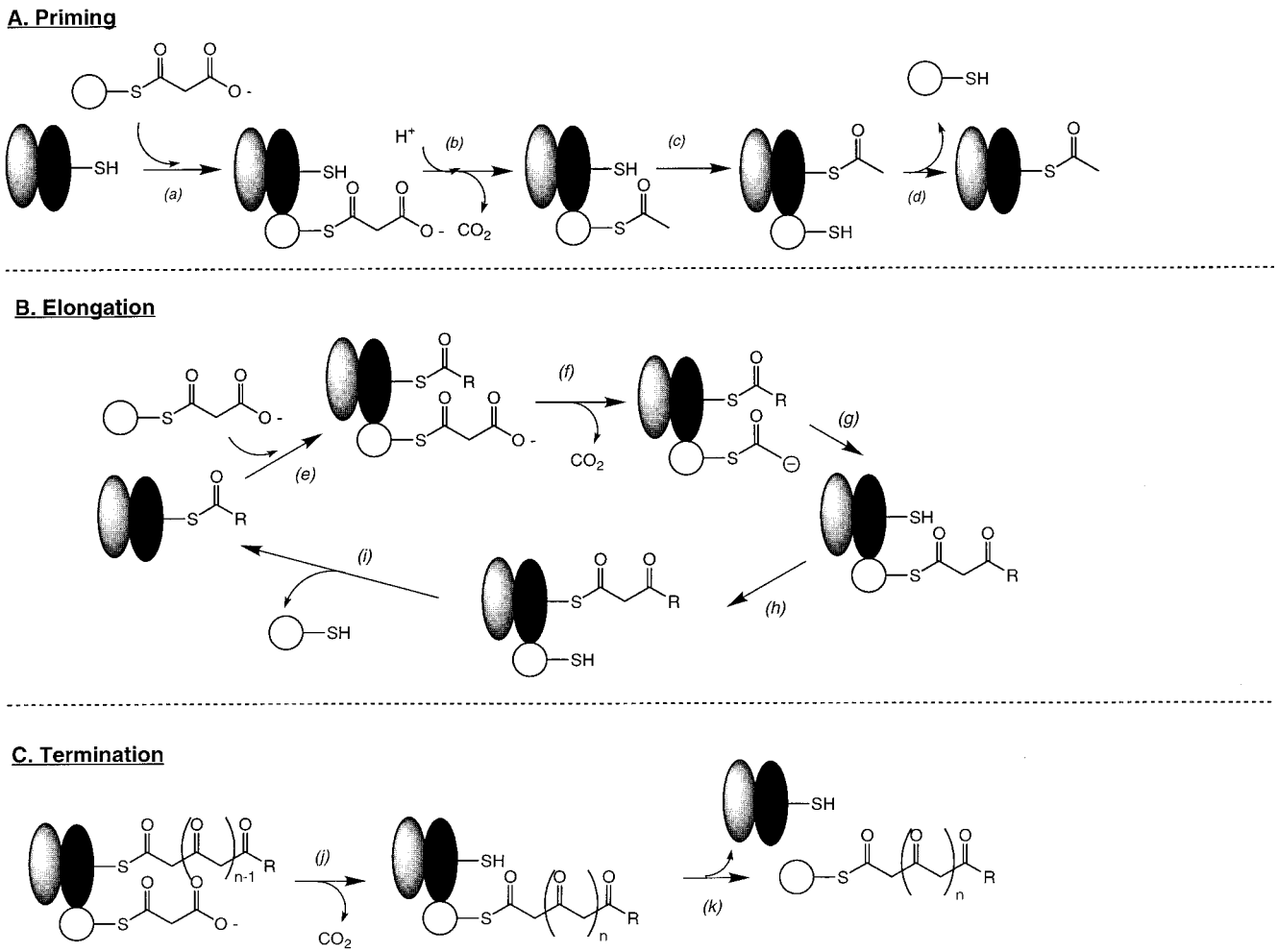

FIGURE 1: Proposed pathways for priming (A), chain elongation (B), and termination (C) reactions in the catalytic cycle of a bacterial aromatic PKS. The ketosynthase (KS)−chain length factor (CLF) heterodimers are shown as shaded ovals, with the active site cysteine thiol of the KS drawn explicitly. The acyl carrier protein (ACP) is shown as a white circle with the terminal thiol of its pantetheine arm explicitly drawn.

possesses an active site cysteine [C169 in the case of the actinorhodin (*act*) KS], to which the growing acyl chain is attached via a thioester linkage. The importance of the cysteine residue has been underscored in the case of the *act* and the tetracenomycin (*tcm*) PKS, where C → A, C → S, and C → Q mutants were found to abolish polyketide biosynthesis in vivo (*11*, *12*). The chain length factor also exhibits end-to-end sequence similarity with respect to ketosynthases, but lacks the active site cysteine residue. It has been implicated in the control of polyketide chain length control, but its mechanism remains unclear. Polyketide synthesis is initiated when a malonyl-ACP encounters an unoccupied KS−CLF heterodimer (Figure 1). Decarboxylation of the malonyl group, followed by transfer of the resulting acetyl group to the active site cysteine of the KS, leads to priming of the synthase. The acetyl primer unit then undergoes a defined number of extension cycles with malonyl-extender units, until a full-length poly-β-ketoacyl chain is synthesized. The chain is then released with concomitant (often regiocontrolled) cyclization.

A number of important questions remain unanswered regarding the mechanistic basis for this complex, multistep catalytic process. First, what is the precise nature of the interactions between the KS−CLF and the ACP? Although stable complexes of the KS−CLF and the holoACP have never been isolated, it is generally assumed that the polypeptide portion of the ACP docks against the KS−CLF during polyketide synthesis. Evidence for this comes from the observation that malonyl-CoA (which has a phosphopantetheine arm but lacks the protein moiety) cannot support polyketide synthesis in the absence of the ACP, and that the apoACP is a competitive inhibitor of polyketide synthesis (*8*). However, it remains unknown whether the same KS−CLF−ACP ternary complex chaperones the growth of a full-length polyketide chain through the entire catalytic cycle, or whether the ternary complex dissociates after each condensation reaction. If the latter is true, then one could ask whether the growing acyl chain remains bound to the ACP as the complex dissociates, or whether the chain is returned to the KS (where it must reside in preparation for the next round of condensation) before the ternary complex dissociates. Second, as shown in Figure 1A, chain initiation is believed to occur via decarboxylation of a malonyl group. What residues within the KS−CLF are responsible for catalyzing this decarboxylative priming reaction? Similarly, how is the decarboxylative condensation reaction (steps f and g, Figure 1B) catalyzed, and what residues within the KS−CLF are responsible for catalysis? Early studies on the yeast fatty acid synthase showed that the active site cysteine of the ketosynthase was not required for decarboxylation,

since malonyl-CoA decarboxylase activity of the synthase was stimulated by incubation with iodoacetamide (*13*). In a theoretical study (*14*), Dewar and Dieter have argued that a universally conserved lysine residue near the C-terminal end of the ketosynthase plays an important role in decarboxylation. However, alternative roles for this lysine residue have also been proposed (*15*, *16*), and the catalytic basis for the decarboxylation reaction remains a mystery. Finally, how does chain growth terminate?

In this report, we seek to provide some answers to these questions. A major boost to this study came from the recent X-ray crystallographic analysis of the KAS II ketosynthase homodimer that catalyzes chain elongation during fatty acid biosynthesis in *Escherichia coli*, and is closely related in sequence and function to the KS−CLF heterodimers in bacterial aromatic PKSs (*17*, *18*). The crystal structure of KAS II facilitated identification of candidate residues for mutagenesis, as we sought answers to the above questions.

MATERIALS AND METHODS

Materials. Silica gel plates Si250F for thin-layer chromatography were from J. T. Baker. [^{14}C]Malonyl-coenzyme A (50 μCi/mL, 52 mCi/mmol) was obtained from Movarek Biochemicals. All other chemicals were from Sigma Chemical Co. and of the highest available grade.

Protein Expression and Purification. Wild-type and mutant *act* KS−CLF complexes were purified from strains of *Streptomyces coelicolor* CH999 (*19*) carrying appropriate derivatives of plasmid pSEK38, as described previously (*7*, *8*, *20*). *fren* apoACP, *fren* holoACP, and *S. coelicolor* MAT (*fab*D gene product) were purified as described previously (*8*). *Sfp* phosphopantetheinyl transferase (*21*, *22*) was expressed in *E. coli* and purified by a freezing−thawing method as described for ACP (*21*, *23*). Further purification was achieved with a Resource Q (Pharmacia) column using a gradient from buffer A [20 mM Tris (pH 6), 2 mM EDTA, 2 mM DTT, and 15% glycerol] to buffer B (A and 1 M NaCl). *Sfp* eluted at the very beginning of the gradient.

Construction of KS and CLF Mutants. Mutations were engineered using a T7 DNA polymerase-based double-stranded, site-directed mutagenesis method (Chameleon, Stratagene). They were cloned into plasmid pSEK38 (*20*), which carries the *act* KS and CLF genes, to yield pJDS91 (KS H309A), pJDS52 (KS K341A), pJDS61 (KS H346A), pJDS35 (CLF S145A), and pJDS45 (CLF Q171C/G173S double mutant). All mutants were confirmed via DNA sequencing.

PKS Activity Assays. Polyketide production was monitored in vitro by an assay based on [^{14}C]malonyl-CoA incorporation into polyketide products (*7*, *8*). Reactions were performed in a volume of 100 μL containing 100 mM $NaHPO_4$ (pH 7.3), 2 mM EDTA, 2 mM DTT, and proteins as described in the text. Unless stated otherwise, [^{14}C]malonyl-CoA (35.45 mM, 0.71 mCi/mmol) was added to a final concentration of 3.5 mM to start each reaction. Enzyme reactions were stopped by adding solid NaH_2PO_4. The products of individual reaction mixtures were extracted with ethyl acetate, applied to a TLC, and quantified using an Instant Imager 2024 (Packard) (*7*).

Preparation of Radiolabeled Proteins. To generate radiolabeled forms of holo-, acetyl-, or malonyl-ACP, 2.5 mL reaction mixtures [100 mM $NaHPO_4$ (pH 7.3), 2 mM EDTA, 2 mM DTT, and 10 mM $MgCl_2$] containing 40 μM purified *fren* apoACP, 5 μM purified Sfp, and the appropriate CoA analogue [0.5 μCi of [^{14}C]malonyl-CoA (50 μCi/mL, 56 mCi/mmol), 0.5 μCi of [^{14}C]acetyl-CoA (50 μCi/mL, 54 mCi/mmol), or 11.25 μCi of [^{3}H]CoASH (28.85 μCi/mL, 262 mCi/mmol)] were incubated for 1 h at 37 °C. After the buffer had been changed to 100 mM $NaHPO_4$ (pH 7.3), 2 mM EDTA, and 2 mM DTT using PD10 columns (Pharmacia), the ACP species was purified in each case by chromatography on a Hi Trap Q (Pharmacia) column as described previously (*8*).

To detect a radiolabeled PKS component, the appropriate set of proteins was incubated with [^{14}C]malonyl-CoA (50 μCi/mL, 56 mCi/mmol) as described in the text. For reaction volumes larger than 20 μL, proteins were precipitated with acetone and resuspended in 10−20 μL of 100 mM $NaHPO_4$ (pH 7.3), 2 mM EDTA, and 2 mM DTT. Enzymes were separated by SDS−PAGE, and the radioactive species were detected by X-ray film exposure of dried gels. To detect tritiated proteins, gels were soaked in 1 M sodium salicylate for 15 min prior to drying.

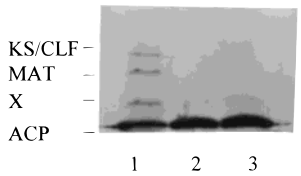

FIGURE 2: Detection of an ACP-bound acyl intermediate. Each lane contains proteins from an assay mixture containing wild-type KS−CLF, MAT, holoACP, and malonyl-CoA: lane 1, 1 nmol of ACP and [^{14}C]malonyl-CoA; lane 2, 1 nmol of [^{3}H]ACP and malonyl-CoA; and lane 3, 2 nmol of [^{3}H]ACP and malonyl-CoA. In all cases, the intense band at the bottom corresponds to radiolabeled malonyl-ACP. For details, see the text.

RESULTS

Detection of an Advanced ACP-Bound Biosynthetic Intermediate. Synthesis of SEK4 and SEK4b by the *act* minimal PKS requires a KS−CLF heterodimer, a MAT, and a holoACP (*7*). Earlier studies demonstrated that incubation of the minimal PKS with [^{14}C]malonyl-CoA resulted in the labeling of the KS, MAT, and holoACP proteins. In those studies, the reaction mixture was quenched with 5% TCA (*7*) and proteins were analyzed via SDS−PAGE, Coomassie blue staining, and autoradiography. By varying the quenching and analysis conditions, we were able to detect a new radiolabeled intermediate as follows. Reactions (100 μL) were stopped by adding 200 μL of H_2O and 1 mL of acetone. Precipitated proteins were analyzed as described in Materials and Methods. Under these conditions, as shown in Figure 2, a fourth strong radiolabeled protein could be reproducibly detected via autoradiography between the bands corresponding to the ACP and MAT. We suspected that the new species corresponds to an acylated form of the ACP. To confirm this hypothesis, the above experiment was repeated with unlabeled malonyl-CoA and ^{3}H-labeled holoACP. Three independent 100 μL assays were set up, each containing 100 pmol of KS−CLF, 10 pmol of MAT, and (a) 1 nmol of holoACP and 0.25 μCi of [^{14}C]malonyl-CoA (control reaction, lane 1), (b) 1 nmol of [^{3}H]holoACP and 4.5 nmol of malonyl-CoA (lane 2), and (c) 2 nmol of [^{3}H]holoACP and

4.5 nmol of malonyl-CoA (lane 3). As shown in Figure 2, the new band was weakly but detectably labeled in lanes 2 and 3, and was therefore confirmed to be an ACP derivative. This new species is neither acetyl-ACP nor malonyl-ACP, since authentic samples of these species (generated as described in Materials and Methods) have different mobilities on SDS−PAGE (data not shown). Moreover, the weak labeling of this species by tritiated pantetheine is consistent with the observation that only a weak band could be detected at the corresponding position in the gel by Coomassie blue staining (data not shown). Since the newly detected form of ACP has considerably higher specific radioactivity than malonyl-ACP [the expected (lowest) band in Figure 2] or the KS or the MAT, one can conclude that the acyl chain attached to the ACP in this newly detected species is an advanced polyketide intermediate, possibly even a full-length chain. Neither ^{14}C and ^{3}H scintillation counting nor mass spectrometric analysis of this acyl-ACP species was feasible due to the low amount of protein that could be recovered from an SDS−polyacrylamide gel. As described below, this acyl-ACP species could not be synthesized by KS mutants that are unable to catalyze polyketide biosynthesis.

Interaction between the KS−CLF and the ACP during Polyketide Chain Growth. Although physical complexes between a KS dimer and an ACP have not been isolated in type II fatty acid synthases or PKSs, recently it has been shown that the apoACP is a competitive inhibitor of polyketide biosynthesis (*8*). This result suggested that the protein moiety of the ACP must dock with the KS−CLF heterodimer during chain elongation. Two models were proposed for this interaction. In one model, a KS−CLF heterodimer and an ACP associate into a ternary complex that remains intact throughout the catalytic cycle, comprising seven C−C bond forming events and associated transfer reactions. An alternative model involves repeated assembly and disassembly of the ternary complex after every condensation step during polyketide chain growth. To discriminate between these models, we tested the ability of KS−CLF to synthesize polyketides in the presence of malonyl-ACP but not the MAT. Malonyl-ACP was prepared as described in Materials and Methods by incubating apoACP with malonyl-CoA and the *sfp* phosphopantetheinyl transferase. Sfp transfers acyl-phosphopantetheinyl groups from acyl-CoA to apoACP (*22*). The resulting [^{14}C]malonyl-ACP was purified as described in Materials and Methods, and used to synthesize SEK4 and SEK4b solely in the presence of 0.8 μM KS−CLF. All reactions were started by adding KS−CLF and the mixtures incubated for 15 min before the reactions were quenched. As shown in Figure 3, the yield of polyketide increases linearly with increasing amounts of malonyl-ACP in the reaction mixture. From the slope of the line in Figure 3, one can calculate that synthesis of 1 pmol of octaketide product requires 7 pmol of malonyl-ACP. Within experimental error, this stoichiometric ratio is consistent with theoretical predictions for octaketide synthesis. Moreover, from the initial rate of polyketide synthesis in this assay, a turnover number of 0.23 min^{-1} can be estimated, which is similar to the reported k_{cat} of the minimal PKS (0.4 min^{-1}) (*8*). If stable complexes were to form between the KS−CLF and the holoACP during the course of the catalytic cycle, one would not expect malonyl-ACP to be an effective substrate for polyketide synthesis. Therefore, we conclude

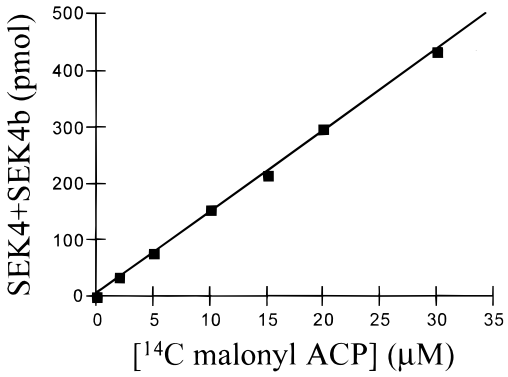

FIGURE 3: Conversion of malonyl-ACP into polyketides by the wild-type KS−CLF. The amount of SEK4 and SEK4b synthesized by KS−CLF is plotted as a function of increasing amounts of malonyl-ACP in the reaction mixture. For details, see the text.

that the catalytic cycle for SEK4 or SEK4b formation involves repeated assembly and disassembly of the KS−CLF−ACP ternary complex, as shown in Figure 1B. Moreover, for polyketide synthesis to occur as shown in Figure 3, the newly extended chain must be transferred back from the ACP to the KS−CLF heterodimer prior to dissociation of the ternary complex (step i in Figure 1B).

Activity of KS Mutants. The cysteine residue corresponding to C169 of the *act* KS is universally conserved among all ketosynthases belonging to the FAS/PKS superfamily of enzymes, and has been shown to be the site of attachment of the growing polyketide chain (*15*−*18*). Consistent with this notion, earlier studies in vivo have confirmed that alteration of C169 in the *act* KS, as well as the corresponding C173 in the *tcm* KS, inactivates the PKS (*11*, *12*). To evaluate the properties of a KS in which the active site thiol is replaced with a hydroxyl, a C169S mutant was constructed as described in Materials and Methods. As expected (*12*), the resulting KS was unable to produce detectable quantities of polyketide in vivo, when coexpressed with the *act* CLF and an ACP. Moreover, the KS−CLF complex purified from this strain was unable to produce detectable quantities of polyketide in the presence of the *fren* ACP, the MAT from *S. coelicolor*, and [^{14}C]malonyl-CoA. The inability of the mutant to catalyze chain elongation was also underscored by the lack of appearance of the advanced ACP-bound intermediate described above in the case of the wild-type enzyme (Figure 2).

Amino acid sequence comparison has also led to the identification of a lysine residue and two histidine residues (K341, H309, and H346 in the *act* KS) as being universally conserved among all known prokaryotic and eukaryotic ketosynthases. The potential importance of these residues has also been underscored by the proximity of these residues to the active site cysteine in the only KS that has been crystallographically characterized to date (*17*, *18*). Furthermore, mutagenesis of the *tcm* KS has led to the identification of the histidine corresponding to H346 (*act* numbering) as being essential for polyketide biosynthesis (*12*). To dissect the roles of these residues, each was independently replaced with an alanine in the *act* KS. None of the resulting PKS mutants produced detectable quantities (<1 mg/L) of any known polyketide in vivo. The wild-type *act* PKS produces polyketides in quantities of >100 mg/L (*19*). Each mutant KS−CLF heterodimer was purified to >90% purity using

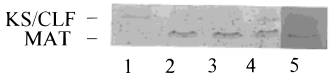

FIGURE 4: Labeling of the wild-type and mutant KS proteins. Reaction mixtures containing holoACP, MAT, and KS−CLF were incubated with [^{14}C]malonyl-CoA. Labeled proteins were detected by autoradiography of a 10% SDS−polyacrylamide gel: lane 1, wild type; lane 2, H309A; lane 3, H346A; lane 4, K341A; and lane 5, C169S. Equal amounts of each protein are loaded in all lanes. In all cases, the upper band corresponds to the KS whereas the lower band corresponds to the MAT.

the protocol described for the wild-type protein (*7*, *8*). In all cases, incubation of 0.8 μM KS−CLF with 20 μM ACP, 100 nM MAT, and [^{14}C]malonyl-CoA led to no detectable formation of SEK4 or SEK4b over the course of 1 h. Moreover, as with the C169S mutant, none of these three mutants yielded the advanced ACP-bound intermediate observed for the wild-type PKS in Figure 2.

Labeling of KS Mutants. Since the C169S, H309A, H346A, and K341A mutants were judged to be inactive by in vivo and in vitro criteria, we wished to determine which step(s) in the overall catalytic cycle (Figure 1) is blocked in each case. Earlier studies have shown that acyl-KS intermediates can be detected by transfer of a radiolabeled acetyl or malonyl group from the appropriate acyl-ACP to the active site cysteine of the KS (*7*, *23*). [The malonyl group is presumably decarboxylated prior to transfer, since the acetyl primer unit in aromatic polyketide biosynthesis is derived from malonyl-CoA (*7*; see also Figure 1).] To determine whether these mutant KS proteins could be labeled with [^{14}C]-malonyl groups, 100 μL reaction mixtures containing 100 pmol of KS−CLF, 2 nmol of holoACP, 10 pmol of MAT, and 0.25 μCi of [^{14}C]malonyl-CoA were incubated for 15 min at room temperature and analyzed as described in Materials and Methods. Figure 4 shows an autoradiograph of a 10% SDS−polyacrylamide gel. Both the C169S and K341A mutants can be labeled in this assay at levels comparable to those of the wild-type KS. In contrast, neither H309A nor H346A is labeled. Prolonged exposure of the gel to an X-ray film resulted in detection of very weakly labeled H346A, but not H309A, protein (data not shown).

Decarboxylase Activity of KS Mutants. To assess the ability of the KS mutants described above to decarboxylate malonyl-ACP, we took advantage of the fact that malonyl-ACP is a substrate for the wild-type KS−CLF in the absence of the MAT (Figure 3). Thus, preincubation of a defective KS−CLF with malonyl-ACP, followed by addition of wild-type KS−CLF to the reaction mixture, should result in polyketide synthesis only if the mutant enzyme has substantially reduced decarboxylase activity. To validate this assay, 500 pmol of [^{14}C]malonyl-ACP (prepared as described in Materials and Methods) was preincubated with either 50 pmol (10:1 malonyl-ACP:KS ratio) or 250 pmol (2:1 malonyl-ACP:KS ratio) of the C169S KS−CLF heterodimer for 30 min at room temperature in a reaction volume of 100 μL. The wild-type KS−CLF (50 pmol) was then added to each tube, and the reaction was allowed to proceed for an additional 30 min at room temperature. Utilization of the unmodified malonyl-ACP was assessed by quantifying the amount of SEK4 and SEK4b formation. As summarized in Table 1, no polyketide product could be detected at either malonyl-ACP:KS ratio, suggesting that the decarboxylase activity of the C169S mutant is comparable to that of the wild-type enzyme. This observation is consistent with earlier studies on the yeast and vertebrate fatty acid synthases, where the active site cysteine was found to be dispensable for the decarboxylase activity of the KS (*13*, *15*, *16*). In contrast, the preincubation of malonyl-ACP with either KS H309A, KS H346A, or KS K341A under the above conditions did not lead to detectable depletion of the malonyl-ACP pool at either malonyl-ACP:KS ratio (Table 1). We therefore conclude that H309A, K341A, and H346A are impaired with respect to decarboxylase activity.

Table 1: Decarboxylase Activity of Wild-Type and Mutant Ketosynthases[a]

	decarboxylase activity at a 10:1 ACP:KS−CLF ratio	decarboxylase activity at a 2:1 ACP:KS−CLF ratio
wild type	+	+
C169S	+	+
H309A	−	−
K341A	−	−
H346A	−	−

[a] For assay details, see the text.

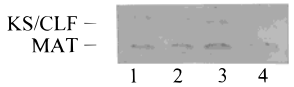

FIGURE 5: Preacetylation of wild-type KS and the K341 mutant. The KS was preacetylated with unlabeled acetyl groups as described in the text, and subsequently chased with [^{14}C]malonyl-CoA in the presence of MAT and ACP. The autoradiograph of the resulting 10% SDS−polyacrylamide gel is shown: lane 1, wild-type KS control (no preacetylation); lane 2, preacetylated wild-type KS; lane 3, K341A control (no preacetylation); and lane 4, preacetylated K341A mutant. For details, see the text.

Inhibition of KS K341A Labeling by Preincubation with Acetyl-ACP. As described above, the K341A mutant of the KS lacks decarboxylase as well as PKS activity, but can catalyze transfer of the radiolabel from [^{14}C]malonyl-ACP to the KS. This suggests that, in contrast to the normal priming mechanism of the PKS (Figure 1A), the intact malonyl group is transferred from the ACP to the active site of the KS by this mutant. To test whether the inability of the K341A mutant to catalyze chain extension was the result of mispriming the KS with a malonyl primer instead of an acetyl primer, we directly primed both the wild-type and the K341A heterodimer with unlabeled acetyl units, followed by a chase with [^{14}C]malonyl-ACP. Acetyl-KS was generated by preincubating 120 pmol of KS−CLF with excess acetyl-ACP, prepared as described in Materials and Methods. [Earlier studies with the wild-type enzyme had confirmed that acetyl-KS thus prepared was a viable intermediate in polyketide biosynthesis (*23*).] Following this preincubation step, 12 pmol of MAT, 1.5 nmol of holoACP, and 0.375 μCi of [^{14}C]malonyl-CoA were added to 120 pmol of preacetylated KS−CLF. Control reactions, in which the preacetylation step was omitted, were performed in parallel. Figure 5 shows an autoradiograph of the 10% SDS−polyacrylamide gel on which the assay mixtures were applied. As expected, both the wild-type KS and the K341A mutant were labeled in the control reactions (lanes 1 and 3). Likewise, the preacetylated wild-type KS was also labeled with [^{14}C]malonyl extender units (lane 2), consistent with its ability to turn over in the presence of holoACP, MAT, and [^{14}C]malonyl-CoA. However, the preacetylated K341A mutant could not be labeled by [^{14}C]malonyl-ACP (lane 4).

This result confirms that the inability of the K341A mutant to catalyze chain elongation is due to its lack of decarboxylase activity, rather than simply being the result of mispriming with a malonyl group.

Activity of CLF Mutants. Although the chain length factor (CLF) component of the KS−CLF heterodimer is known to be essential for PKS activity (*11*), and is an important determinant of chain length specificity (*19*, *24*), its mechanism remains obscure. The problem is further complicated by the fact that, although the KS and the CLF are homologous, the active site cysteine of the ketosynthase is replaced with a conserved glutamine (or glutamic acid) residue (Q171 in the *act* CLF). As a step toward understanding the precise role of the CLF, we attempted to identify residues that are crucial to its activity via site-directed mutagenesis. Two mutants of the *act* CLF were generated.

A double mutant Q171C/G173S was designed to mimic the conserved active site of a ketosynthase in the background of a CLF. To test whether the CLF can be converted into a KS by the simple presence of an active site CXS motif, the mutant gene was coexpressed with both the wild-type *act* KS (in pJDS45) and the C169S null mutant of the *act* KS (in pJDS46). When transformed into *S. coelicolor* CH999, pJDS45 produced octaketides in quantities similar to that of the wild-type control, CH999/pSEK38. In contrast, CH999/pJDS46 yielded a null phenotype analogous to the control strain that only carried the C169S mutation in the *act* KS. These results suggest that CLF is not simply a dysfunctional ketosynthase, nor is the conserved glutamine in this protein essential for its activity.

A second mutation, S145A, was independently engineered in the *act* CLF to test the function of a universally conserved serine residue. Again, coexpression of this mutant CLF gene with wild-type *act* KS and ACP genes yielded a recombinant strain that produced normal quantities of the expected polyketides in vivo. We therefore conclude that S145 is also not essential for CLF activity.

DISCUSSION

KS−CLF and ACP Form Dynamic Ternary Complexes As They Chaperone Polyketide Chain Growth. Earlier work in this and other laboratories has shown that a minimal PKS consisting of the KS−CLF, the ACP, and the MAT is sufficient for polyketide synthesis in vitro (*7*, *9*). More recently, we have subjected the reconstituted *act* minimal PKS to kinetic analysis (*8*). Our results indicate that optimal activity of the minimal PKS requires a stoichiometric ACP:KS−CLF:MAT ratio of approximately 200:10:1. We also showed that the catalytically inactive apo form of ACP competitively inhibits polyketide synthesis, suggesting a role for protein−protein interactions between the ACP and the KS−CLF or the MAT or both. However, we could not distinguish between two alternative models for type II PKS activity: (a) a "static" model in which a single KS−CLF−ACP ternary complex catalyzes seven rounds of condensation to generate an octaketide and (b) a "dynamic" model, where the ternary complex dissociates following every round of condensation. The fact that KS−CLF can stoichiometrically convert malonyl-ACP into polyketide products (Figure 3) at rate constants approaching the k_{cat} of the entire minimal PKS suggests that the latter model is valid for type II PKS systems (step i in Figure 1B). If the former model were correct, polyketide synthesis in this assay would be undetectable, or at least significantly attenuated. Moreover, this result throws light on yet another unanswered question regarding type II PKSs. An essential feature of the elongation cycle is that the newly extended polyketide chain must be returned by the ACP to the KS after every round of condensation (step h in Figure 1B). For efficient synthesis of full-length chains to occur in the presence of malonyl-ACP alone, this step must precede the dissociation of the ternary complex (step i in Figure 1B). Perhaps the bulk of the polyketide chain remains bound to the KS−CLF dimer with only the acyl−thioester linkage undergoing exchange between the KS and the ACP. This would be consistent with the observation that the KASII ketosynthase possesses an extended hydrophobic pocket located near the dimer interface (*17*), and highlights the central difference between an iterative and a modular PKS (although no modular PKS structures are available as yet to confirm this hypothesis). In the former case, the chain presumably remains bound to the KS throughout its biosynthesis, whereas in the latter case, the chain is transferred from one KS to the next; therefore, its association with the KS must be weaker.

Role of Conserved Residues in the Active Site of the KS in Chain Priming and Extension. X-ray crystallographic analysis of the KASII ketosynthase from *E. coli* has shown that three universally conserved residues [H309, K341, and H346 (*act* KS numbering)] lie close to the active site cysteine (C169 in the *act* KS) on which the acyl chain is appended (*17*, *18*). Earlier studies on type I PKSs have led to proposals of the roles of some of these residues in chain elongation (*15*, *16*). A major advantage of a type II system in mechanistic studies of the KS is that the individual active sites (the KS, the ACP, and the MAT) can be individually prepared, covalently modified (if desired), and titrated into the assay mixture in arbitrary ratios. In this study, we exploit this feature of a type II system to gain a better understanding of the roles of C169, H309, K341, and H346 in the *act* KS.

The nucleophilicity of the C169 thiol and the electrophilicity of the corresponding thioester were probed by construction and analysis of a C169S mutant. Interestingly, we observed that the occupancy of the active site of this mutant with acyl chains is comparable to that of the wild-type enzyme under steady-state turnover conditions (Figure 4). However, within detectable limits, the mutant enzyme is unable to support polyketide synthesis. Therefore, either the rate of the ACP → KS acyl transfer (analogous to steps c and h in Figure 1), the ability of the resulting oxoester to be attacked by the carbanion (analogous to step g in Figure 1), or both steps have been impaired in the C169S mutant. The former possibility is consistent with a proposal by Smith and co-workers, who developed an inter-thiol transfer reaction from CoA (or pantetheine) to the KS of the rat fatty acid synthase, and used it to quantify the ability of the C → S mutant to catalyze inter-thiol transfer (*16*). Since the *act* KS cannot accept acyl chains directly from CoA thioesters in the absence of an ACP, we were unable to perform similar kinetic analysis on our C169S mutant. Importantly, however, this mutant retains its ability to decarboxylate malonyl-ACP (Table 1). This is also consistent with earlier studies on type I PKSs (*13*−*16*).

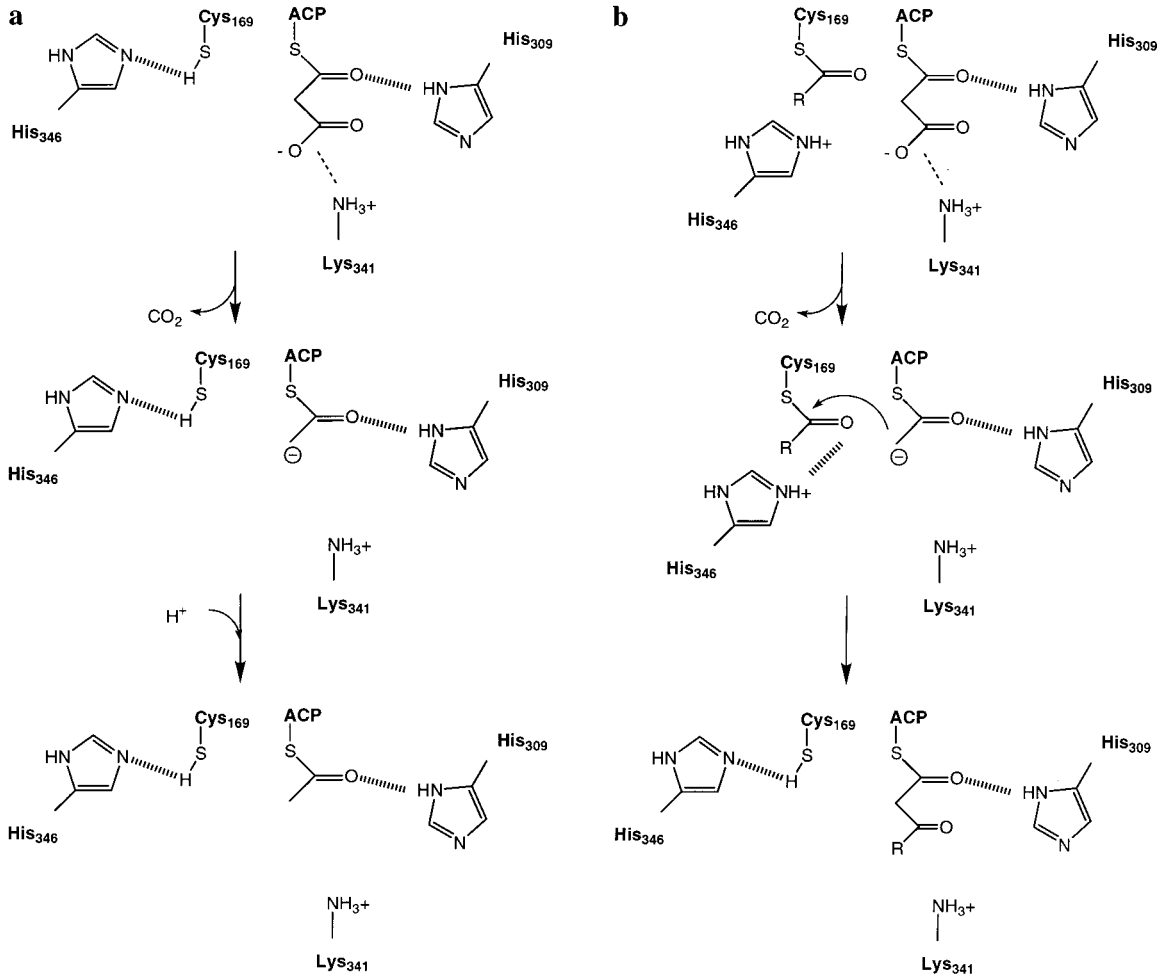

FIGURE 6: Model for the mechanism of the KS-catalyzed reactions. (a) Decarboxylative priming of the KS and (b) chain extension via C−C bond formation.

Both the H309A and H346A mutants of *act* KS exhibited no detectable PKS activity. (The detection limits of our turnover assay are ca. 5% of wild-type activity.) Moreover, neither mutant could be efficiently labeled by [^{14}C]malonyl-ACP (Figure 4), nor were they able to catalyze decarboxylase activity within detectable limits (Table 1). The inability of H309A and H346A to be labeled suggests that either the nucleophilicity of the C169 thiol is attenuated in these mutants or their ability to correctly position the malonyl-ACP substrate for the inter-thiol transfer reaction is deficient in these mutants (or both). X-ray crystallographic analysis of the KASII protein has revealed that the Nϵ atoms of the corresponding histidines are located 4.6 and 3.3 Å, respectively, from the Sγ atom of the active site cysteine (*18*). It has been suggested that H346 acts as a base abstracting the proton from the C169 thiol (*act* KS numbering), whereas H309 aids in binding and decarboxylation of malonyl-ACP, as well as in stabilizing the resulting carbanion. These proposals are consistent with our results; in addition, our results suggest that H346 must also play a role in catalyzing decarboxylation of the malonyl group. It is unclear whether the effect of H346 on decarboxylation is direct (i.e., through interaction with the malonyl group) or indirect (i.e., through the positioning of another functional group that is important for decarboxylase activity).

The K341A mutant of the *act* KS fails to catalyze polyketide formation or malonyl-ACP decarboxylation (Table 1), but can be labeled in the presence of [^{14}C]malonyl-CoA, holoACP, and MAT (Figure 4). This suggests that the malonyl group is transferred to C169 in this mutant without undergoing decarboxylation. We have ruled out the possibility that the inability of this mutant to catalyze C−C bond formation is the result of mispriming of the KS with malonyl groups (Figure 5). The lack of decarboxylase activity in the K341A mutant contrasts with the observation by Smith and co-workers that the corresponding mutant of the rat FAS catalyzes decarboxylation at levels comparable to that of the wild-type enzyme (*16*). We do not know if this discrepancy is the result of differences in assay protocols (our assay with a type II system tests for the ability of the KS to catalyze mutiple rounds of decarboxylation by using ACP:KS−CLF ratios of 2−10, whereas the Smith assay uses a type I system, where the ratio of KS:ACP is fixed at 1:1) or whether it represents a fundamental difference between diverse ketosynthases. However, the absence of decarboxylase activity in the K341A mutant is consistent with a proposal by Dewar and Dieter (*14*), who argue that electrostatic interactions between the ammonium cation and the malonyl anion have a strong influence on catalysis of decarboxylation, as long as the ions are far apart to inhibit proton transfer between them. Indeed, in the active site of the KASII, the amino group of this lysine is within hydrogen bonding distance of the peptide oxygen of the histidine corresponding to H309 (believed to assist in malonyl-ACP binding; see above).

Therefore, the crystal structure suggests that the two ions are likely to be in proximity.

A model for the mechanism of the KS-catalyzed reactions, which is consistent with the data presented above, is proposed in Figure 6. Confirmation of this model must await high-resolution X-ray crystallographic analysis of the *act* KS−CLF heterodimer and further mechanistic studies.

Chain Termination. As discussed above, the growing polyketide chain presumably remains bound in the binding pocket of KS−CLF as it is extended through multiple condensation cycles. During this process, it is incrementally extruded into the binding pocket. If so, then chain termination (Figure 1C) probably occurs when the size limit of the binding pocket has been reached, and further growth is no longer possible. The discovery of a single long-chain acyl-ACP species (Figure 2), together with the fact that it is not observed in the presence of any of the null KS mutants described in this study, suggests that the chain attached to this ACP species is a full-length product. If so, then chain extension is likely to be relatively rapid, and chain termination, which must involve release of the acyl chain by the KS−CLF heterodimer, is rate-limiting in polyketide synthesis. Recent studies involving gene shuffling of the KS−CLF genes from two PKSs with different chain length specificities have reinforced the notion that the CLF plays an important role in chain length control (*24*).

Chain Length Factor Mechanism. The mechanism of action of the CLF subunit of the KS−CLF heterodimer is unknown. In an effort to probe its function, we tested two hypotheses. First, we tested whether Q171 (whose position corresponds to the active site cysteine of the KS) and S145 (a universally conserved serine residue in all known CLFs) are essential for activity by replacing them with a cysteine and alanine, respectively. Neither mutation was found to have any detectable effect on polyketide biosynthesis. Second, we evaluated the possibility of grafting a KS-like active site in the CLF through the generation of a Q171C/G173S double mutant and coexpressing it with the C169S null mutant of the KS. The inability of the resulting KS−CLF to produce detectable quantities of polyketide suggests that the two subunits are not sufficiently similar to permit a simple exchange of their active sites. Thus, the precise mechanism by which the CLF participates in polyketide synthesis remains a mystery.

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