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Initial Characterization of a Type I Fatty Acid Synthase and Polyketide Synthase Multienzyme Complex NorS in the Biosynthesis of Aflatoxin B_1

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Summary

The biosynthesis of the potent environmental carcinogen aflatoxin B_1 is initiated by norsolorinic acid synthase (NorS), a complex of an iterative type I polyketide synthase and a specialized yeast-like pair of fatty acid synthases. NorS has been partially purified from *Aspergillus parasiticus*, has been found to have a mass of $\sim 1.4 \times 10^6$ Da, and carries out the synthesis of norsolorinic acid in the presence of acetylCoA, malonylCoA, and NADPH where hexanoylCoA is not a free intermediate. The *N*-acetylcysteamine thioester of hexanoic acid can substitute for the catalytic functions of HexA/B to initiate norsolorinic acid synthesis by the complex in the presence of only malonylCoA. An $\alpha_2\beta_2\gamma_2$ stoichiometry is proposed for NorS in keeping with its estimated mass and the observed dimeric or higher-order quarternary structures of PKS and FAS enzymes.

Introduction

Mold contamination of staple foods, especially carbohydrate-rich grains, has afflicted humankind from the earliest times. Prominant among mycotoxins borne by such infestations is aflatoxin B_1 (**2**, AFB1), a potent environmental carcinogen produced by a limited number of *Aspergillus* strains, but of wide distribution. The occurence of this relatively stable, lipophilic metabolite in the diet is associated with cancers of the liver and kidney—sites of oxidative activation, DNA intercalation, and covalent reaction [1, 2]. A direct link has been forged between the ability of aflatoxin to cause DNA damage at a mutational "hot spot" in the p53 tumor suppressor gene, giving rise to transversions G249T or C owing to faulty repair at this locus [3, 4]. Either base alteration results in an Arg→Ser change in the translated protein, which is now incompetent in its regulatory role. Thus, clonal expansion of this defect is maintained and constitutes one of the approximately 50% of human cancers correlated to mutations of the p53 gene [5–7].

Knowledge of the biochemistry and genetics of natural toxin production offers an important avenue for their control. Aflatoxin is a polyketide natural product and the result of at least 15 enzymic steps from norsolorinic acid (**1**, Figure 1), the first known intermediate in the biosynthetic pathway [8, 9]. Versicolorin A (**3**) and sterigmatocystin (**4**) are known intermediates in this process. While impressive advances have been made during the last decade in the understanding of the two general types of bacterial polyketide synthases (PKSs), the particulate and modular enzymes [10–12], remarkably little is known about the iterative eukaryotic PKSs of fungi [13]. The reasons for this are several, but historically these large proteins have proved extremely difficult to isolate or even to detect catalytically, and only a handful of gene sequences are available.

The best-characterized fungal PKS is 6-methylsalicylic acid synthase (6-MSAS) from *Penicillium patulum* [14–17]. Its encoding gene has been sequenced [18, 19], and the protein exists as a tetramer of identical subunits (α_4) but is functional as a dimer of molecular mass ~450 kDa. The CoA esters of acetate and malonate and one equivalent of NADPH are taken up by the enzyme to give a covalent intermediate thought to be **5** (assuming processive loss of water) prior to intramolecular aldol condensation, dehydration, and tautomerization to yield 6-methylsalicylic acid (**6**, Figure 2). Recently, evidence has been gathered that the aflatoxin biosynthetic pathway is initiated in an unusual manner whereby a specialized pair of yeast-like fatty acid synthases (HexA and HexB, Figure 2) [20, 21] extends acetylCoA and malonylCoA to a six-carbon acyl intermediate [22, 23]. Transfer of this "starter unit" to a fungal PKS (PkaA) and further elaboration with malonate can be visualized to give an intermediate formally thought of as **7**. Self-condensation by a Claisen reaction, as opposed to the aldol condensation proposed for 6-MSAS, should give anthrone **8**, which by spontaneous or enzyme-catalyzed oxidation would afford norsolorinic acid (**1**, Figure 2) [24]. When HexB was mutated by insertional inactivation of is encoding gene, metabolite production ceased as expected, but, unexpectedly, synthesis was only very poorly restored by the addition of a hexanoyl donor under conditions where incorporation should have been highly efficient. This observation and a series of control experiments provided circumstantial evidence to suggest that the intact HexA/B proteins might require intimate physical association with PksA in a well-defined complex to efficiently synthesize norsolorinic acid. The efficiency of the transfer of the C_6-starter, however, is perturbed by insertional inactivation of HexB [25]. In a broadly useful technical advance, we have developed a simple method for generating active, stabilized cell-free systems using diafiltration as the key preparative step [26]. The power of this method has been exemplified with *A. parasiticus*, where it was demonstrated that norsolorinic acid (**1**) could be converted through ≥15 enzymic transformations to AFB1 (**2**) in the presence of molecular oxygen, NADPH, *S*-adenosylmethionine (SAM), and FAD [26]. The enhanced stabilization of enzyme activities afforded by this method has been applied in the present work to the recalcitrant problem of fungal type I PKS enzymes to examine the proposed coordinate activities of HexA/HexB and PksA in this organism. Further evidence has been accumulated that hexanoylCoA is not a free intermediate in this process, partial purification of these three proteins has been

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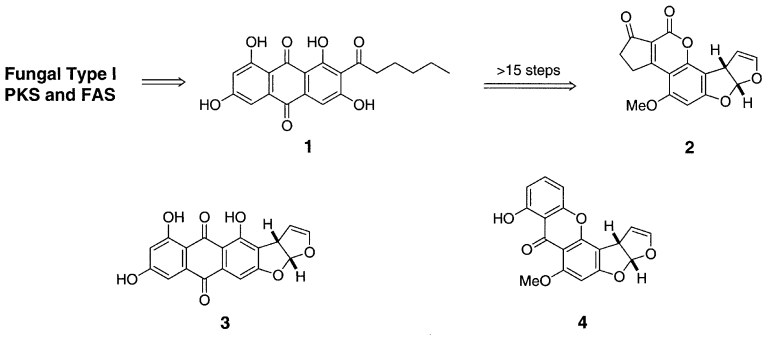

Figure 1. Aflatoxin Biosynthetic Pathway Is Initiated by a Type I PKS and FAS Pair

Type I PKS and FAS enzymes give norsolorinic acid (**1**), which is converted in ~15 steps via versicolorin A (**3**) and sterigmatocystin (**4**) to aflatoxin B_1 (**2**).

achieved in which a catalytically active complex of approximately 1.4×10^6 Da has been characterized, and three high-molecular-weight proteins have been identified by PAGE analysis.

Results

Preparation of the Cell-free Extract

Preparation of the cell-free extract to support the PksA and HexA/B activities was a slight modification of the method described previously for the cell-free conversion of norsolorinic acid (**1**) to aflatoxin B_1 (**2**) [26]. To achieve effective stabilization of the FAS and the PKS enzymes, the glycerol content of the buffer was raised as well as the ionic strength. Protease inhibitors were also eliminated as a precaution against the possible inactivation of active site thiols. The extraction buffer thus contained 100 mM potassium phosphate (pH 7.5), 50% glycerol, 1 mM EDTA, and 2 mM DTT. Following incubation and centrifugation, the supernatant was filtered to remove remaining cell debris and dialyzed with an Amicon RA 2000 apparatus equipped with a 100,000 molecular weight cut-off membrane by the gradual addition of buffer and maintaining a constant volume. The diafiltration step is the key element of the protocol and efficiently removes background metabolites and low-molecular-weight components that have been found detrimental to the enzyme activities [26].

Demonstration of Catalytic Activities

The catalytic activities of the HexA/B subunits and PksA were examined in *A. parasiticus* NOR-1 cell-free extracts (Figure 3; NOR-1 is a "leaky" classical mutant that accumulates norsolorinic acid [**1**]). To insure adequate sensitivity in the enzyme assays, [2-^{14}C]malonylCoA was utilized as a radioactive marker. Detection of aflatoxin pathway intermediates was achieved by two methods: either autoradiography of thin-layer chromatograms or HPLC and scintillation counting of collected fractions. Conversion of [2-^{14}C]malonylCoA by the FAS components was, as expected, dependent upon added NADPH. Without this cofactor, anthrone/anthraquinone synthesis could not take place in the presence or absence of the primer, acetylCoA (Figure 3, lanes 1 and 2). However, conversions to norsolorinic acid were readily observed when extracts were incubated in the presence of hexanoylCoA and [2-^{14}C]malonylCoA but in the absence of reductant, giving an unambiguous demonstration of the PKS activity (Figure 3, lane 3) and the ability to circumvent the catalytic functions of HexA and HexB. In con-

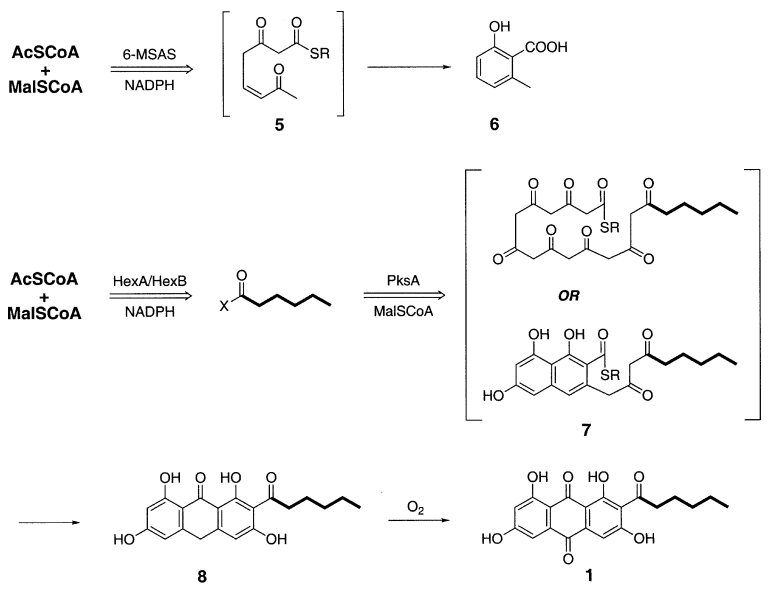

Figure 2. Proposed Iterative PKS Steps to 6-Methylsalicylic Acid and Norsolorinic Acid

Proposed iterative polyketide synthase steps to (top) 6-methylsalicylic acid (**6**) and (bottom) norsolorinic acid (**1**). PksA is primed by a hexanoyl starter generated by the HexA/B pair of yeast-like FAS subunits.

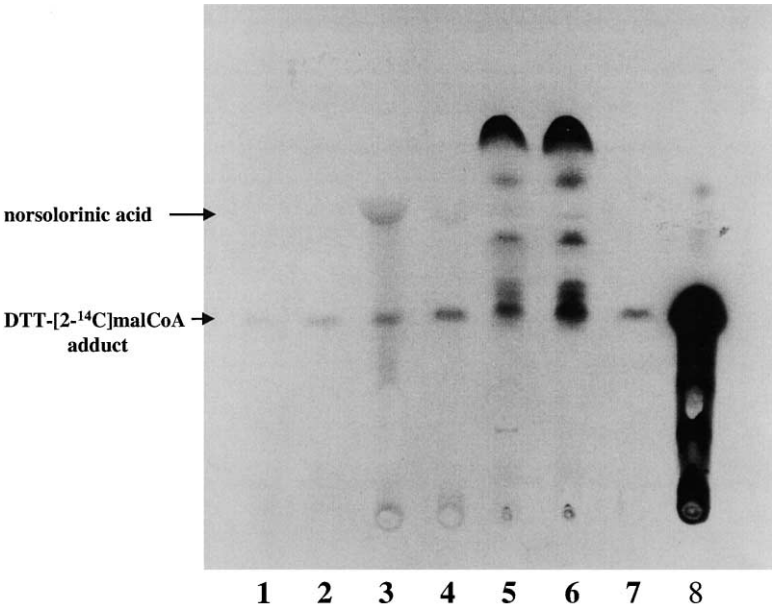

Figure 3. Autoradiogram of PKS and FAS Activities in the Diafiltered Cell-free Extract

Autoradiogram of metabolites extracted into ethyl acetate from diafiltered CFE incubated in the presence of: lane 1, [2-^{14}C]malonylCoA; lane 2, [2-^{14}C]malonylCoA + acetylCoA; lane 3, [2-^{14}C]malonylCoA + hexanoylCoA; lane 4, as in lane 3 + cerulenin; lane 5, [2-^{14}C]malonylCoA + hexanoylCoA + NADPH + SAM + FAD; lane 6, [2-^{14}C]malonylCoA + acetylCoA + NADPH + SAM + FAD; lane 7, as in lane 6 + cerulenin; lane 8, boiled diafiltered CFE + [2-^{14}C]malonylCoA.

trast, incubation in the presence of added cofactors (NADPH, SAM, and FAD) and either acetylCoA or hexanoylCoA with [2-^{14}C]malonylCoA gave rise to the formation of norsolorinic acid as well as other later intermediates of the biosynthetic pathway (Figure 3, lanes 5 and 6). The PKS/FAS activities were also found to be readily inactivated by the β-ketoacyl synthase-modifying reagent cerulenin (Figure 3, lanes 4 and 7), in keeping with the inactivation of other polyketide and fatty acid synthases [27–29]. The compound that appeared when the boiled extract (control) was incubated in the presence of [2-^{14}C]malonylCoA corresponded to transthioesterification of the malonyl unit with DTT, forming a [2-^{14}C]malonyl-DTT adduct (Figure 3, lane 8).

Partial Purification of the FAS and PKS Components

With a cell-free extract in hand capable of supporting both the HexA/B and PksA activities, purification of this triad of enzymes was undertaken. An intial attempt using gel filtration through Sephadex G-150 gave disappointingly little purification. Resort to affinity methods was considered and thought to confer particular advantages in the present case. These proteins are large, proteolytically vulnerable, and present at very low concentrations. The resolving power in principle achievable by this method was its first attraction [30]. Second, the central biosynthetic role of the hexanoyl primer suggested this as an easily synthesized affinity ligand. Thus, hexanoic acid was coupled to EAH Sepharose with a water-soluble carbodiimide to give the stable amide linkage (Figure 4). Chromatography was performed by gravity to insure that the proteins had sufficient time to bind to the modified resin. After washing the column with buffer to remove nonspecifically bound proteins, the proteins of interest were eluted with buffer containing 500 mM NaCl and dialyzed (enzyme activities diminish in the presence of high salt), concentrated, and loaded onto a column of Sephadex G-150. Both the HexA/B and PksA proteins should elute in the void volume. The protein fractions were assayed first by Bradford analysis, then directly assayed for HexA/B and PksA activities by incubation with radioactive [2-^{14}C]malonylCoA and either unlabeled hexanoylCoA or acetylCoA. NADPH was added to the assay mixtures to differentiate the FAS activity. Both sets of activities were observed in early eluting fractions, and SDS-PAGE gel analyses revealed three bands of comparable intensity in the high-molecular-weight range, suggesting approximately equivalent amounts of each. Western blot of these proteins with a polyclonal 6-MSAS antibody weakly recognized only one of the high-molecular-weight bands corresponding to that of intermediate relative molecular mass (data not shown).

Further purification of the FAS and PKS subunits was achieved by loading the proteins eluted from the hexanoyl-EAH Sepharose 4B column (following dialysis) onto a second affinity column with bound acetoacetyl groups obtained by treatment of the resin with diketene (Figure 4). The desired proteins were again eluted in high-salt buffer (500 mM NaCl) and subsequently dialyzed, concentrated, and loaded onto a Sepharose 6B gel filtration column to fractionate proteins of high molecular weight. As before, the fractions were assayed for HexA/B and PksA activities and analyzed by SDS-PAGE. Both catalytic activities were again observed, and examination of the SDS-PAGE gel revealed the three protein bands that coeluted in these early fractions (Figure 4). The correlation of full catalytic activity with fractions containing three proteins of approximately the correct relative mass suggested that HexA/B and the PksA functioned as a complex, which we denote norsolorinic acid synthase (NorS). Calibration of the Sepharose 6B column with known standards enabled estimation of the mass of the native complex at ~Mr 1.4×10^6 Da. The translated masses of HexA, HexB, and PksA are 181.3, 210.5, and 230.7 KDa, respectively [20, 21, 31]. The two highest-mass bands run very close together, and it would appear that HexB corresponds to an anomalously

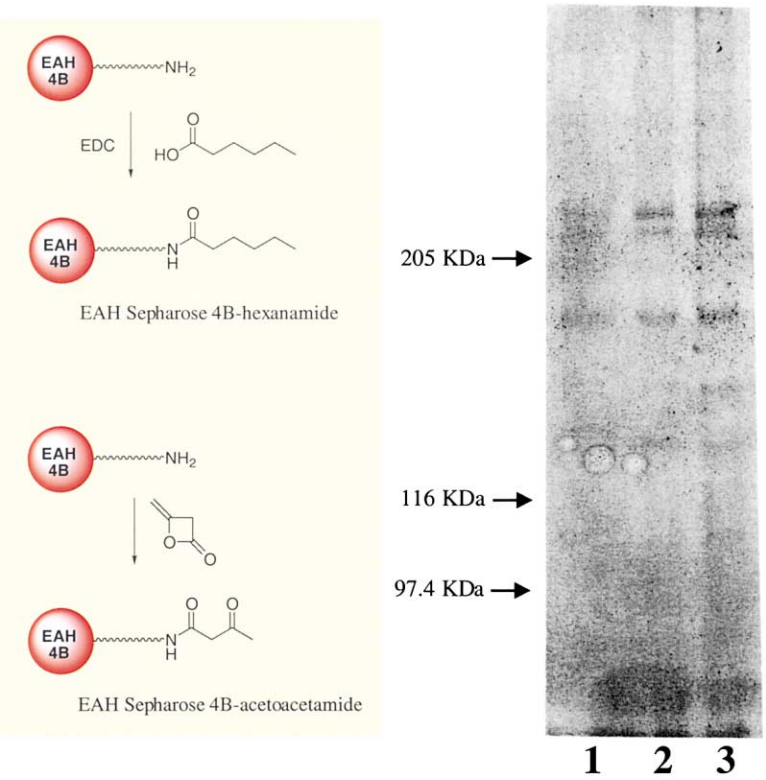

Figure 4. Partial Purification of the PksA-HexA/B Complex

Silver-stained SDS-PAGE gel of catalytically active fractions containing NorS after EAH Sepharose 4B-hexanamide, EAH Sepharose 4B-acetoacetamide affinity chromatography steps, and size fractionation on Sepharose 6B: lane 1, fractions 40/41; lane 2, fractions 42/43; lane 3, fractions 44/45.

high relative mass (Figure 4), making the reasonable assumption that PksA crossreacts with the polyclonal 6-MSAS antibody and is the central band. The extent of posttranslational modification of these proteins, however, apart from pantethienylation, is not known, or a simple structural artifact affecting its mobility in the gel may account for this apparent discrepancy.

HexanoylCoA Is Not a Free Intermediate

A functional, isolable complex of $\sim$1.4 $\times$ 10^6 Da containing all three proteins, PksA, HexA, and HexB, was consistent with the proposal based previously on the greatly diminished norsolorinic acid-synthesizing ability of HexB-disrupted mutants grown in the presence of a hexanoyl donor [25]. It can be proposed as a further consequence of this stable complex that the C_6-primer synthesized by HexA/B could be transferred directly (channeled) to the PKS to initiate anthrone/anthraquinone formation. A process of this kind could be visualized to occur, for example, by transthioesterification from the ACP of HexB to the ACP or the KS of PksA, as illustrated in Figure 5. The result of such a direct transfer would be that the C_6-intermediate would not be released as a free species; for example, as its CoA ester, which is the mode of product discharge from yeast FAS. While negative in outcome, both in vitro and in vivo experiments were carried out to establish whether the C_6-starter unit is simply released into solution or is directly transferred to the PKS, requiring at the very least transient association of the HexA/B and PksA proteins.

In Vitro Experiment

Utilizing the previously described methods, a cell-free extract was prepared with the polyketide synthase disruption mutant PKS-A [32]. This strain would be expected to accumulate hexanoylCoA if it were released into solution as a free intermediate, in accord with the normal course of fatty acid synthesis. Control experiments with the crude extract (prior to diafiltration) demonstrated the efficient conversion of norsolorinic acid (**1**)→versicolorin A (**3**) and sterigmatocystin (**4**)→AFB1 (**2**, Figure 1), which is consistent with the genotype of this strain. PKS-A was constructed from the Wh-1 mutant strain, which harbors a genetic block in the *ver-1* gene and accumulates the anthraquinone versicolorin A (**3**). As controls confirmed the proper expression of later pathway enzymes, the extract was subjected to diafiltration (100,000 MW cut-off membrane) and subsequently incubated with acetylCoA, [2-^{14}C]malonylCoA, and NADPH. The protein suspension was then extracted with ethyl acetate and briefly centrifuged. A control experiment demonstrated that [1-^{14}C]hexanoylCoA dissolved in water does not extract into ethyl acetate. The organic extracts were decanted and the protein precipitate was discarded. The aqueous suspension was then loaded onto an AG-MP1 column (a strongly anionic macroporous resin), washed with distilled water, and the bound components were eluted with 700 mM LiCl. Most of the LiCl was removed from the solution by filtration/dialysis. The desalted solution was then lyophilized to dryness, resuspended in distilled water, and analyzed by HPLC. HexanoylCoA was not detected.. Based upon the amount of added substrate, [2-^{14}C]malonylCoA, the method should have detected as little as a $\sim$0.2% overall conversion to hexanoylCoA.

In Vivo Experiment

To examine with greater sensitivity the possible release of hexanoylCoA from the mutant blocked in PksA, we repeated the experiment but instead employed an in vivo

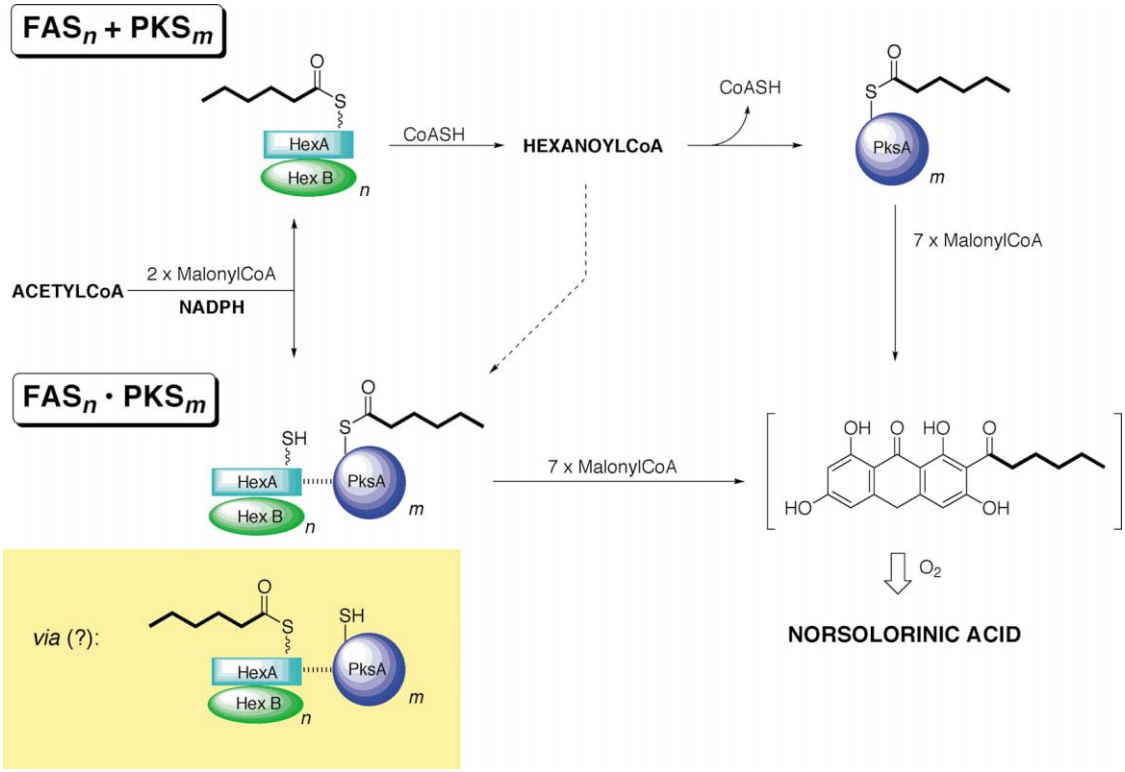

Figure 5. Nature of the Hexanoyl Priming of PksA

The transfer of the hexanoyl primer can occur either (top) by release of free hexanoylCoA from HexA/B and intramolecular transfer to PksA to initiate polyketide synthesis or (bottom) by intracomplex transfer (channeling) within NorS by transthioesterification to the PksA ACP or KS.

approach. Labeled acetate, as its *N*-acetylcysteamine thioester, [1-^{14}C]acetylNAC, was prepared as previously described [22] and supplied to whole-cell cultures of the PKS-A mutant. The disruptant was initially cultured on Adye and Mateles medium [33] for 48 hr and resuspended in a replacement medium, where four flasks received 10 mg of [1-^{14}C]acetylNAC, and two flasks were supplied with either 5 mg of norsolorinic acid (**1**) or 5 mg of sterigmatocystin (**4**) to serve as controls. The cells were grown for an additional 65 hr and harvested by vacuum filtration. Cultures that received the aflatoxin pathway intermediates were flash frozen in liquid nitrogen and steeped in acetone overnight. Following this treatment, the organic layer was decanted, concentrated in vacuo, and resuspended in a small volume of ethyl acetate. Analysis by TLC gave the expected results, as norsolorinic acid (**1**) was very efficiently converted to versicolorin A (**3**) and sterigmatocystiin (**4**) to AFB1 (data not shown). Cultures that received radiolabeled acetylNAC were flash frozen in liquid nitrogen, pulverized in an Osterizer blender, and suspended in chilled, deionized distilled water. The mixture was incubated at 4°C with stirring for 2 hr and then centrifuged. To remove the unreacted [1-^{14}C]acetylNAC, the aqueous extract was diafiltered with an Amicon stirred cell fitted with a 500 MW cut-off filter. The aqueous mixture was extracted once with ethyl acetate and lyophilized to dryness. The lyophilite was then resuspended in distilled water and analyzed by HPLC as above. Again, no detection was made of released hexanoylCoA (Figure 6). This method should have allowed detection of ~0.001% conversion to hexanoylCoA.

Discussion

Enormous progress has been made in the last decade in understanding both the genetic organization and biochemical function of bacterial polyketide synthases [11–13]. These advances encompass both the particulate enzymes, notably of *Streptomyces* species, and the giant modular proteins responsible for, e.g., erythromycin biosynthesis. In contrast, the eukaryotic polyketide syn-

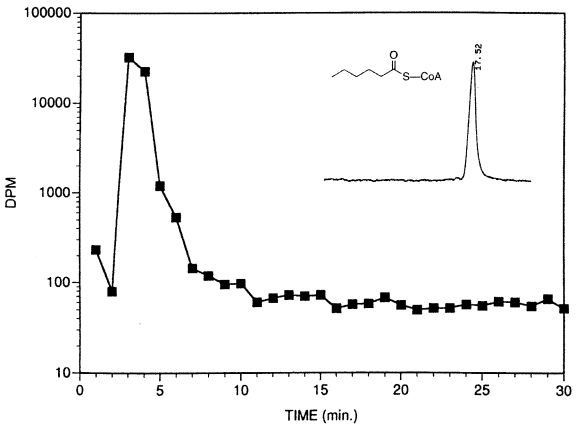

Figure 6. Attempted Detection of HexanoylCoA by HPLC

Radiometric HPLC analysis of an extract from suspended cells of PKS-A (disruption mutant of *pksA*) grown in the presence of [1-^{14}C]acetylCoA. No radioactivity was detected for hexanoylCoA (t_R = 17.5 min).

thases, despite their comparatively simple arrangement of domains, are poorly understood functionally [13]. A central question is that of "programming"—that is, how are chain length, sites of reduction, and order of cyclizations determined in an "iterative" system in which catalytic domains are used repeatedly, but in a fixed number and order, in the selective synthesis of their products?

While 6-methylsalicylic acid synthase (6-MSAS) is a notable exception, historically fungal PKSs have proved notoriously unstable [34]. Comparatively few of these type I enzymes have been detected even in cell-free systems, much less purified and their catalytic cycles characterized [13]. As a consequence, little biochemistry exists, and few gene sequences are known. The method of cell-free extract preparation described here involving diafiltration as its key step is a technical advance that has allowed the PKS and FAS enzymes initiating AFB1 biosynthesis to be stabilized, partially purified, and the first characterization carried out. Applications of this general method to other wild-type enzymes and heterologously expressed PKSs [35, 36] promise further advances in this area of polyketide research.

Evidence from whole-cell incorporation experiments suggested that the biosynthesis of norsolorinic acid (**1**), the earliest known precursor of AFB1 (**2**) biosynthesis [8], required a saturated, linear six-carbon starter unit [22]. When the gene cluster encoding the aflatoxin biosynthetic pathway was elucidated, a yeast-like pair of FAS enzymes correlated to a pair of genes very near *pksA* at one end of the cluster. Biochemical experiments [25] and gene disruptions [32] confirmed the suspected roles of HexA, HexB, and PksA in aflatoxin biosynthesis. A complex containing each of these proteins has been substantially purified by two affinity chromatography steps on resins bearing hexanoyl and acetoacetyl ligands. Size separation on a calibrated Sepharose 6B column afforded a single catalytically active fraction at Mr 1.4×10^6 Da. SDS-PAGE revealed three high-molecular-weight proteins with apparent masses around 200 KDa. Western blot analysis with an antibody raised to 6-MSAS reacted with the middle band on the gel, in keeping with the expected relative mass of the PKS involved in AFB1 biosynthesis (HexB appears larger than expected). On the basis of these observations, we propose a dimeric structure for this complex, $\alpha_2\beta_2\gamma_2$. The order of catalytic domains in HexA and HexB is identical to yeast FASs, which, it is noted, form an $\alpha_6\beta_6$ structure of molecular mass $>2.2 \times 10^6$ Da. The structural model proposed suggests that the specialized pair of FASs (HexA and HexB) synthesizes the C_6-starter unit, which is then transferred within the complex to PksA by transthioesterification. That this transfer is intramolecular without release of free hexanoylCoA could be implied from earlier studies [25], but was directly demonstrated in this study by in vivo and in vitro experiments designed to trap radiolabeled hexanoylCoA. No free C_6-primer could be found.

The restoration of catalytic activity upon removal of salt parallels previous observations with 6-hydroxymellein synthase isolated from carrot [34]. This protein is active as a homodimer, which could be dissociated at high salt to catalytically inactive monomer units. These similar findings suggest electrostatic interactions play a role in assembly of the functional complexes. Future experiments with recombinant proteins will enable more detailed examination of this assembly process as well as an evaluation of the catalytic behavior of the PKS alone and complexed with the FAS pair. For the moment, however, the proposed dimeric $\alpha_2\beta_2\gamma_2$ model is an intriguing variant on the emerging theme of functional homodimers seen in 6-hydroxymellein synthase [34], 6-MSAS [14], and the modular bacterial type I enzymes [13].

Significance

The organization of the aflatoxin biosynthetic genes in *A. parasiticus* in which a type I polyketide synthase *pksA* and a yeast-like pair of fatty acid synthase subunits *hexA* and *hexB* reside at one end of the gene cluster provides circumstantial evidence of their involvement in pathway initiation through the synthesis of norsolorinic acid. Whole-cell incorporation experiments with hexanoic acid and hexanoylNAC support this proposal, and disruption of *pksA* and *hexB* (formerly *fas-1A*) lead to loss of aflatoxin production. Here, we have demonstrated the presence of a $\sim 1.4 \times 10^6$ Da complex comprised of these three proteins which proved sufficiently robust to be partially purified from *A. parasiticus*. In the presence of acetylCoA, malonylCoA, and NADPH, the synthesis of the first anthraquinone precursor, norsolorinic acid, takes place but fails if reductant to drive fatty acid synthesis is left out of the reaction mixture. In the presence of hexanoylNAC, however, the catalytic functions of HexA/B can be circumvented, and added malonylCoA is all that is required to achieve the synthesis of norsolorinic acid. Fatty acid synthesis in yeast terminates with release of the product CoA ester. We searched by two experimental methods for the release of free hexanoylCoA by NorS but found none. We propose that the role of HexA/B is the synthesis of a hexanoyl primer which is transferred directly by transthioesterification to the PksA ACP or KS domain (channeling) within the complex to initiate polyketide synthesis. An $\alpha_2\beta_2\gamma_2$ composition for NorS is hypothesized in keeping with its observed mass and the dimeric or higher-order quarterary structures characteristic of polyketide and fatty acid synthases.

Experimental Procedures

Instrumentation and General Methods

Analytical HPLC separations were achieved with a Phenomenex C-18 column (250 × 4.60 mm) on a Varian 5020 liquid chromatograph with an attached ABI (model 1000s) diode array detector. Concentration of proteins to small volumes (≤1 ml) was conducted with a set of Amicon stirred cells (200 ml, 50 ml, or 10 ml) employing Millipore PTLK 100,000 MW cut-off membranes (Bedford, MA). Sephadex G-150 (superfine) and the Sepharose 6B resins were obtained from Sigma (St. Louis, MO), as were biotinylated and high-molecular-weight protein markers. The EAH Sepharose 4B resin (aminohexyl-linker arm) was purchased from Pharmacia Biotech (Piscataway, NJ). The Silver Stain Plus kit and the Enhanced Colloidal Gold Total Protein Detection kit were both obtained from BioRad (Hercules, CA). The MW markers utilized to calibrate the Sepharose 6B gel filtration column, blue dextran (2,000,000), thyroglobulin (669,000), β-amylase (200,000), and bovine serum albumin (66,000), were all obtained from Sigma.

Organisms

The *A. parasiticus* norsolorinic acid-accumulating mutant NOR-1 (ATCC 24690) was obtained from the American Type Culture Collection (Rockville, MD). The *A. parasiticus* polyketide synthase disruption mutant of PksA (PKS-A) was a generous gift from Professor J.E. Linz (Michigan State University).

Culture Techniques

Fungal strains were grown on coconut agar plates adjusted to pH 7.0 for 4–7 days [26]. Spores were suspended in a solution of 9 ml of Tween 80 (0.05% v/v), which was diluted with 9 ml of aqueous NaCl (0.85%). A portion of this suspension (5 ml) was used to inoculate 1.5 liters of growth medium.

Cell-free Extract Preparation

FAS/PKS Activities

A. parasiticus mutant NOR-1 was cultured for 108 hr in AM growth medium (1.5 l/4 l Erlenmeyer flask) as described for SU-1 [26]. The mycelia were harvested, flash frozen in liquid nitrogen, and the diafiltered cell-free extract was prepared as previously described with the following modifications. The powdered mycelia were suspended in 300 ml of buffer (100 mM potassium phosphate [pH 7.5], 50% glycerol, 2 mM dithiothreitol, and 1 mM EDTA). The suspension was stirred at 4°C for 2 hr and centrifuged for 20 min at 20,000 × g to give the crude cell-free extract. Filtration through four layers of cheesecloth followed by dialysis (against 4 liters of buffer supplemented with 30% glycerol, CFE buffer) with the Amicon RA 2000 (Millipore) apparatus equipped with a 100,000 molecular weight cut-off membrane produced the diafiltered cell-free extract in a volume of ~175 ml.

CFE Enzyme Assays

FAS Activity

To the NOR-1 cell-free extract (10 ml) was added 10 μl of [2-^{14}C]malonylCoA (25 μCi/1 ml), 1 μl of acetylCoA (1 mg/ml), and 80 μl of cofactor solution, unless stated otherwise. The cofactor solution included, per ml: 1 mg NADPH, 1 mg SAM, and 1 mg FAD in distilled water.

PKS Activity

To the NOR-1 cell-free extract (10 ml) was added 10 μl of [2-^{14}C]malonylCoA (25 μCi/ml) and 1 μl of hexanoylCoA (1 mg/ml).

TLC Analyses

For NOR and other intermediates of the aflatoxin pathway, a ternary mixture of 8:1.8:0.2; hexane:ethyl acetate:acetic acid was utilized. TLC plates were sprayed with three coats of En^{3}Hance spray (New England Nuclear) and wrapped in Saran Wrap for exposure to Kodak X-OMAT film.

HPLC Analyses

^{14}C-labeled norsolorinic acid (t_r = 13.5 min) was isolated by employing an isocratic gradient: 20% A: 80% B (A: 0.1% trifluoroacetic acid: water; B: acetonitrile), flow rate 1 ml/min monitored at λ 310 nm. Fractions were subjected to scintillation counting to assay for labeled NOR. Separation of aflatoxin and its intermediates (including NOR) was carried out as previously described [26]. Samples were analyzed at λ 310 nm.

Partial Purification of the FAS and PKS Subunits

Following diafiltration, the NOR-1 cell-free extract (~175 ml) was loaded onto the hexanoyl EAH Sepharose 4B column (50 ml resin) and washed with 150 ml of CFE buffer (containing 30% glycerol). The bound proteins were eluted with 200 ml of CFE buffer supplemented with 500 mM NaCl. The solution was then dialyzed (Spectro-Por 12–14,000 MW cut-off) against 4 liters of CFE buffer and concentrated with an Amicon stirred cell (30KDa cut-off) to a volume of ~20 ml, and 2 ml of this solution was loaded onto a G-150 column. Alternatively, the dialyzed proteins were loaded onto the acetoacetyl EAH Sepharose 4B column. The unbound proteins were again washed from the column with CFE buffer (150 ml), and the specifically bound proteins were eluted with CFE buffer (200 ml) supplemented with 500 mM NaCl. The eluted proteins were dialyzed against 4 liters of buffer, concentrated, and loaded onto the Sepharose 6B column.

Preparation of Hexanoyl EAH Sepharose 4B Affinity Resin

EAH Sepharose 4B (50 ml, sedimented gel) was washed thoroughly with 500 mM NaCl. Hexanoic acid (696 mg, 10 times the concentration of spacer groups) in 100 ml of coupling solution (1:1; dioxane:water) (pH 4.5) was added to the gel, and water-soluble carbodiimide [EDC, 2.3 g (20 times the concentration of spacer groups)/20 ml doubly distilled H_2O adjusted to pH 4.5)] was added and gently shaken for 24 hr. The pH of the solution was maintained between 4.5–6.0 with 2 N NaOH. The gel slurry was washed sucessively with 0.1 M sodium acetate buffer, 500 mM NaCl (pH 4.0), 0.1 M Tris-HCl buffer, 500 mM (pH 8.0).

Preparation of Acetoacetyl EAH Sepharose 4B Affinity Resin

EAH Sepharose 4B resin (50 ml, sedimented gel) was washed as described above. Diketene (101 μl, 1 mmol) was dissolved in 100 ml of 1:1; dioxane:water (pH 7) with 10 mol% DMAP (7.3 mg) and reacted for 24 hr.

In Vitro HexCoA Experiment (PKS-A Mutant)

To 170 ml of diafiltered PKS-A extract was added acetylCoA (17 μl, 1 mg/ml), malonylCoA (170 μl, 25μCi/1 ml), and NADPH (1.36 ml, 1 mg/ml). Following incubation overnight at 30°C, the protein suspension was extracted with ethyl acetate and centrifuged at 5000 rpm, 4000 × g, for 30 min. The aqueous layer was then loaded onto a pre-equilibrated AG-MP-1 column (BioRad; 10 g of resin). Unbound components were removed by washing with 100 ml of distilled water, while anionic compounds bound to the column were eluted with 150 ml of a 700 mM LiCl. The LiCl was then removed using an Amicon stirred cell with a 500 MW cut-off membrane. While the LiCl flowed through the membrane, malonylCoA, acetylCoA, and any hexanoylCoA generated by the extract were retained (as demonstrated by control experiments (<2% loss). The sample was concentrated, diluted with water, and reconcentrated several times to remove LiCl. The aqueous solution remaining was lyophilized to dryness and resolubilized with 5 ml of doubly distilled water. The sample (500 μl) was analyzed by HPLC utilizing an isocratic gradient (70% 50 mM potassium phosphate [pH 5.3] and 30% methanol). Under these HPLC conditions, hexanoylCoA eluted at t_R = 17.5 min.

In Vivo HexanoylCoA Experiment

A. parasiticus PKS-A cells were cultured for 48 hr on AM growth medium [33] (four 1 l cultures/ 4 l flasks), vacuum filtered, and rinsed thoroughly with distilled water. The cells (10 g) were transferred into six 500 ml Erlenmeyer flasks, each containing 125 ml of replacement medium [22]. To four of the flasks was added [1-^{14}C]acetylNAC (10 mg, 7 mCi/mol) dissolved in 1.5 ml of dimethylformamide. To the remaining two flasks (control flasks) was added either 5 mg of norsolorinic acid (**1**) or 5 mg of *O*-methylsterigmatocystin [the methyl ether of sterigmatocystin (**4**)] in 1.5 ml of dimethylformamide. After incubation at 30°C, 175 rpm, for an additional 65 hr, the cells were vacuum filtered and washed thoroughly with distilled water. The mycelia from cultures incubated with [1-^{14}C]acetylNAC were consolidated, flash frozen with liquid nitrogen, and pulverized in an Osterizer blender with dry ice. The pulverized cells were then steeped in distilled water, stirred at 4°C for 2 hr, and centrifuged at 16,000 × g for 20 min. The supernatant was ultrafiltered using an Amicon stirred cell (200 ml, 500 MWT cut-off), and when the reaction mixture reached a volume of ~20 ml, chilled distilled water was added, and the ultrafiltration procedure was repeated. The reaction mixture was extracted with ethyl acetate (to remove unreacted [1-^{14}C]acetylNAC from the suspension) and centrifuged at 4080 × g for 10 min. The organic layer was discarded, and the aqueous layer was then flash frozen with liquid nitrogen and lyophilized to dryness. The lyophilized material was solubilized with 1 ml of distilled water, and 500 μl of this sample was analyzed by HPLC, utilizing the same separation conditions as described previously.

[1-^{14}C]acetylNAC was prepared from 250 μCi of [1-^{14}C]acetic acid and an additional 250 mg of unlabeled acetic acid as previously described [22].

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