Demonstration of the catalytic roles and evidence for the physical association of type I fatty acid synthases and a polyketide synthase in the biosynthesis of aflatoxin B_1

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Background: Aflatoxin B_1 (compound **5**) is a potent environmental carcinogen produced by certain *Aspergillus* species. Its first stable biosynthetic precursor is the anthraquinone norsolorinic acid (compound **3**), which accumulates in the *Aspergillus* mutant strain NOR-1. Biochemical and genetic evidence suggest that this metabolite is synthesized *in vivo* by a specialized pair of fatty acid synthases (FAS-1 and FAS-2) and a separately transcribed polyketide synthase (PKS-A).

Results: The *N*-acetylcysteamine (NAC) thioester of hexanoic acid was shown to efficiently support the biosynthesis of norsolorinic acid (compound **3**) in the NOR-1 strain. In contrast, the mutants Dis-1 and Dis-2, which are derived from NOR-1 by insertional inactivation of *fas-1*, produced unexpectedly low amounts of norsolorinic acid in the presence of hexanoylNAC. Controls eliminated defects in the parent strain or enhancement of degradative β-oxidation activity as an explanation for the low level of production. Southern blots and restriction mapping of Dis-1 and Dis-2 suggested normal levels of expression of the PKS-A and FAS-2 proteins should be observed because the genes encoding these proteins are not physically altered by disruption of *fas-1*.

Conclusions: The impaired ability of Dis-1 and Dis-2, harboring modified FAS-1 enzymes, to carry out norsolorinic acid synthesis implies the need for FAS-1 (and possibly also FAS-2) to physically associate with the PKS before biosynthesis can begin. The failure of the unaffected PKS alone to be efficiently primed by hexanoylNAC, and the presumed requirement for at least one of the FAS proteins to bind and transfer the C_6 unit to the PKS, is in contrast to behavior widely believed to occur for type I PKSs.

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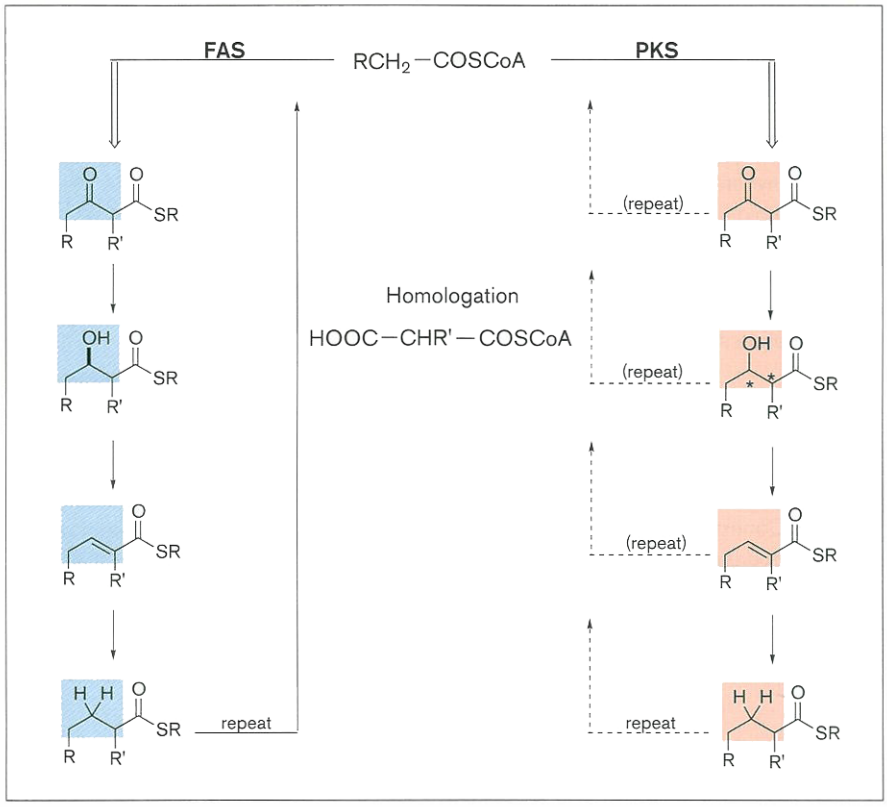
Introduction

Among natural products, the polyketides have achieved prominence in recent years not only for their important biological activities as, for example, antibiotics, immunosuppressants, antiparasitics and toxins, but also for the seminal advances that have been made at the genetic [1–3] and protein levels [4–9] in understanding the primary engines of their synthesis in nature, the polyketide synthases (PKSs). These large, macromolecular machines bear strong similarities to fatty acid synthases (FASs) but extend their synthetic power considerably [10]. The FASs typically homologate acetate by successive decarboxylative Claisen condensations with malonate followed by reduction and dehydration steps to yield the saturated hydrocarbon chain of a fatty acid (Fig. 1, R = R' = H). The PKSs use similar chemistry to achieve impressive synthetic versatility. They can also use low molecular weight primers other than acetate, and can catalyze homologation steps with malonate and with a variety of its α-alkylated derivatives. The reduction and dehydration steps carried out by PKSs may take place using a 'processive' elongation process in a manner similar to that of the FAS-catalyzed reactions; however, that series of steps may not be completed, yielding successive carboxyl-derived carbons at the oxidation state of a ketone, alcohol/double bond or methylene in the growing polyketide chain (Fig. 1; with both *R* and *S* configurations possible at the positions marked by a star).

Aflatoxin B_1 (compound **5**, Fig. 2) is a potent environmental carcinogen produced by certain *Aspergillus* species, which are vigorously growing fungi that infect, among other things, grains and nuts. The mycotoxin survives food processing to become an important contaminant of foodstuffs worldwide [11,12]. It has been directly linked to carcinomas of the liver and kidney [13,14] as a result of interaction of its metabolically activated form with DNA, notably the p53 gene in humans.

The biosynthesis of aflatoxin is unusually long and complex [15–18], but is known to begin with norsolorinic acid (compound **3**, Fig. 2) — a metabolite that is clearly of polyketide origin [19]. This tetrahydroxyanthraquinone is thought to be derived from the corresponding

Figure 1

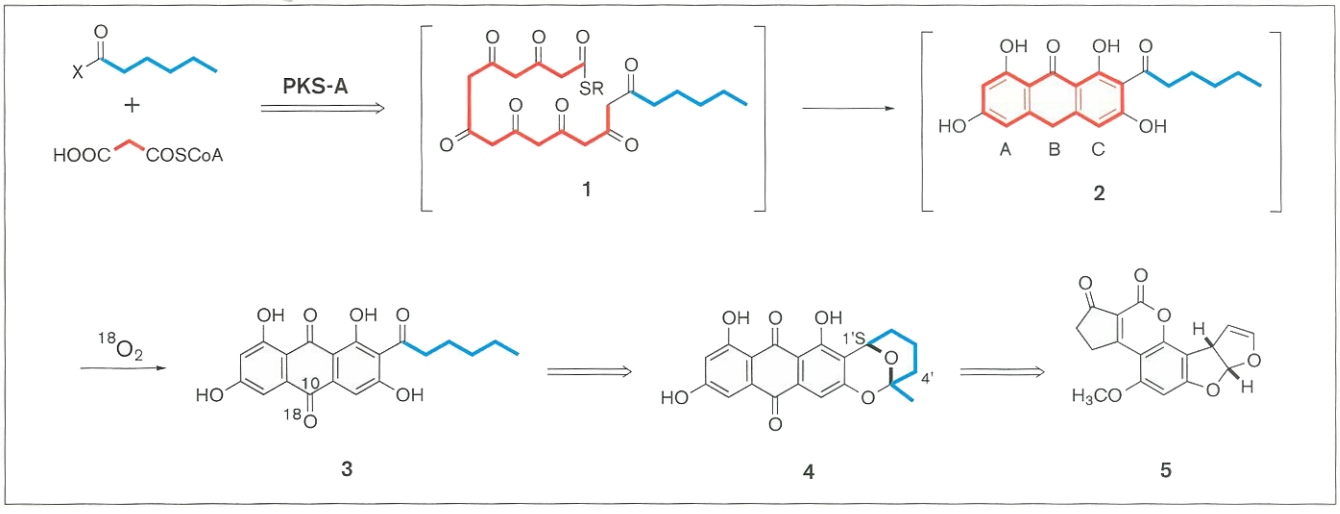


Comparison of fatty acid synthase (FAS) homologation of acetylCoA with malonylCoA (and their substituted derivatives with different groups at R and R') to a generic polyketide synthase (PKS) that shows a varying extent of reduction/dehydration/reduction at the thioacyl β-carbon prior to further homologation (highlighted in color). Both *R* and *S* configurations are possible at the positions marked (*).

anthrone **2** based on experiments with $^{18}O_2$ in which C-10 is uniquely labeled [20] (Fig. 2). The mechanism of formation of compound **2** has been investigated by extensive ^{13}C-incorporation studies with C_4, C_6 and C_8 acids and their corresponding *N*-acetylcysteamine (NAC) thioesters [21–23], which were prepared as potential surrogates of the hypothetical CoA or acyl carrier protein (ACP) thioester intermediates. These studies, as well as ^{18}O labeling experiments [21] and stereochemical correlations [24], point decisively to either of two mechanistic possibilities in the formation of compound **2** [25]. The first would involve a polyfunctional enzyme that acts as a FAS to homologate acetate, generating a six carbon unit, before adding, as a PKS, malonate units without any reductive steps to generate a hypothetical enzyme-bound intermediate **1** [13]. Alternatively, the hexanoyl starter unit could be synthesized by a specialized FAS that releases, for example, hexanoylCoA or hexanoylACP. Such an enzyme could also channel its product to a distinct PKS with which it was complexed to lead on to the synthesis of intermediate **1** [21]. Although proposed intermediates such as **1** have never been detected, the central dogma of polyketide biosynthesis holds that highly reactive species such as these are generated before intramolecular aldol condensation, dehydration, aromatization and expulsion of the enzyme active-site thiol, which would result in the production of the anthrone **2** in a powerfully exothermic process. We can only speculate on the folding and exact sequence of reactions of the polyketide intermediates; for example, the reactions may occur in a stepwise manner to form first the A-ring, then the B-ring and, finally, the C-ring as each of the last two reactions with malonate take place.

Consistent with the second reaction mechanism involving two distinct enzymatic activities, genetic evidence has recently implicated two specialized FASs (FAS-1 and FAS-2) as being jointly responsible for the formation of the C_6 precursor, which would then serve as the substrate for a separately transcribed PKS. Insertional inactivation of the putative PKS gene (*pks*-A) gave a mutant unable to synthesize aflatoxin or any detectable pathway intermediate [26,27]. Sequence alignments indicate that this PKS contains four recognizable domains: an acylcarrier protein, a β-ketoacyl synthase, an acyltransferase and a thioesterase. Reduction/dehydration domains characteristic of FAS proteins are notably absent. Insertional inactivations of *fas-1* gave the mutants Dis-1 and Dis-2, which are unable to incorporate acetate into the aflatoxin pathway [28]. Sequence analysis shows that FAS-1 and FAS-2 have a high degree of identity to the yeast FAS β and α subunits, respectively [29], and together include all

Figure 2



An overview of aflatoxin biosynthesis. It is proposed that FAS enzymes synthesize the hexanoyl starter unit (in blue) to which a PKS homologates malonyl units (in red) to initiate anthrone formation and aflatoxin biosynthesis.

the catalytic domains required for fatty acid synthesis. Here, we provide biochemical evidence to support the roles of the PKS and the FASs in aflatoxin biosynthesis, which have been deduced based on these genetic studies. Experiments with the disrupted strains PKS-A, Dis-1 and Dis-2, and the deletion mutant Dis-3, demonstrate that addition of the NAC thioester of hexanoate can substitute for the synthetic functions of FAS-1 and FAS-2, but at significantly reduced efficiency. This observation and the results of three control experiments suggest the need for physical association of the PKS and at least one of the FAS proteins to initiate norsolorinic acid synthesis. Such a proposed PKS–FAS complex has not been demonstrated previously for multidomain type I PKS and FAS enzymes, although for the bacterial type II particulate FAS and PKS enzymes it is possible that FAS catalytic elements may be shared in both fatty acid and polyketide synthesis [30,31].

Results and discussion

The experimental approach used here was based on a series of earlier incorporation experiments. Suspended cells of a mutant strain of *Aspergillus parasiticus* (ATCC 24551) blocked in the ability to produce aflatoxin B_1 (compound **5**) accumulated the intermediate averufin (compound **4**), which contains the C_6 side chain present in norsolorinic acid (compound **3**). Under a defined set of conditions [1-^{13}C]hexanoic acid was observed to give an intact incorporation of 3–4 % at C-1′ in **4**, over a background of ~0.5 % at alternating sites derived from the C-1 of acetate [21–23]. The latter pattern of incorporation was expected to occur because of β-oxidation of [1-^{13}C]hexanoic acid to [1-^{13}C]acetate and its incorporation throughout the molecule [21–23]. Under identical conditions the NAC thioester of hexanoate gave an unprecedented 22 % specific incorporation, again superimposed on an acetate-derived background of ~0.5 % per site. In contrast, [1-^{13}C]butyrate gave only randomization of label whether administered as the free acid or NAC thioester [21].

The *A. parasiticus* mutants Dis-1, Dis-2 and Dis-3 were generated by mutagenesis of a norsolorinic acid accumulating mutant and were incapable of producing norsolorinic acid or any other later intermediate in aflatoxin biosynthesis [28]. As Dis-1 and Dis-2 were generated by insertional mutagenesis at *fas-1*, the gene encoding one of the FAS enzymes necessary for aflatoxin biosyntheis, the PKS should be unaffected; it was thus expected that in these mutants hexanoic acid, and particularly hexanoyl-NAC, could substitute for the defective FAS apparatus and restore norsolorinic acid production. To determine the extent of conversion of the putative C_6 starter units, the substrates were directly supplied to liquid cultures of the respective mutants. Dis-1, Dis-2 and Dis-3 were initially fermented on Adye and Mateles medium [25] for 48 h. After 48 h of growth, the mycelia were vacuum filtered and transferred (10 g 125 ml^{-1}) into replacement medium [25], at which time the precursors were also added. The cell suspensions were allowed to grow an additional 48 h, after which the products of fermentation were isolated by extraction, analyzed by thin-layer chromatography (TLC) and quantified by high pressure liquid chromatography (HPLC) analysis.

The method used in these experiments was comparable to that used for earlier whole-cell incorporations with the averufin-accumulating mutant [21], and we were therefore

surprised to find that hexanoic acid failed to support norsolorinic acid production in any of the three FAS-1 mutants (Table 1). In contrast, hexanoylNAC, the more effective C_6 precursor in the previous experiments, afforded a 0.4 % and 2 % conversion to norsolorinic acid in Dis-1 and Dis-2, respectively, and a trace of aflatoxin B_1 synthesis in both. Unlike these insertionally inactivated mutants, Dis-3 arises from an apparent double crossover event, and contains a large deletion within *fas-1* that extends beyond *pks*-A (see below). It failed to support detectable synthesis of any anthraquinone. As a control, averufin (compound 4), an intermediate occuring later in the pathway after the requirement for any of the modified gene products, was efficiently converted to aflatoxin by all three of these mutants (Table 1). In sum, these results show that, although the transformation of C_6 units was unexpectedly poor in the two insertionally inactivated mutants, the later enzymes of the biosynthetic pathway were fully functional.

β-oxidation is expected to cause competing degradation of the administered C_6 units. To control for this effect, incubations with suspended Dis-2 cells were repeated in the presence of two degradation inhibitors, 4-pentynoic acid (compound 6) and 3-(tetradecylthio)propionic acid (compound 7) [32] (Fig. 3). These inhibitors had no effect on the transformation of hexanoic acid and only compound 7 gave minor improvement in the conversion of hexanoylNAC to norsolorinic acid (compound 4) (Table 2). The low efficiency of hexanoylNAC conversion to norsolorinic acid in these strains is thus unlikely to be caused by enhanced β oxidation.

The parent strain of Dis-1, Dis-2 and Dis-3 is a UV-generated mutant of wild-type *A. parasiticus* SU-1 selected

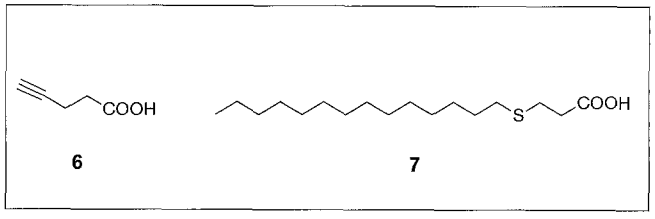
Table 1

Use of hexanoic acid/NAC by FAS mutant strains.

Strain	Substrate[a]	Norsolorinic acid (% converted)[b]	Aflatoxin B_1 (% converted)
Dis-1	Control (no substrate)	–	–
Dis-1	Hexanoic acid	–	–
Dis-1	HexanoylNAC	0.4 %	0.3 %
Dis-1	Averufin	–	33 %
Dis-2	Control (no substrate)	–	–
Dis-2	Hexanoic acid	–	–
Dis-2	HexanoylNAC	2 %	0.4%
Dis-2	Averufin	–	45 %
Dis-3	Control (no substrate)	–	–
Dis-3	Hexanoic acid	–	–
Dis-3	HexanoylNAC	–	–
Dis-3	Averufin	–	59 %

[a]Hexanoic acid/NAC (15 mg) or averufin (10 mg) was dissolved in 1.5 ml of dimethylformamide (DMF).
[b]Dashes indicate <0.05 % conversion for norsolorinic acid and <0.01 % conversion for aflatoxin B_1.

Figure 3



Inhibitors of β-oxidation degradation of fatty acids.

for its ability to accumulate norsolorinic acid (NOR-1, ATCC 24690) [33]. To ensure that the low conversions of hexanoylNAC by Dis-1 and Dis-2 were not the result of an inherent limitation of NOR-1, hexanoic acid and its NAC thioester were administered to this strain. To distinguish between the endogenous production of norsolorinic acid and that derived from the externally supplied C_6 units, the C_6 units were administered in ^{13}C form to suspended NOR-1 cells, exactly as had been done for Dis-1 and Dis-2. The total amount of norsolorinic acid produced in each experiment was accurately assessed by quantitative HPLC analysis, and the amount resulting from incorporation of the C_6 precursor was judged by the proportion of the product bearing ^{13}C label. Addition of [1-^{13}C]hexanoic acid gave a specific incorporation of 2 %; when the corresponding NAC thioester was added, 16 % incorporation was observed. The percent conversions into norsolorinic acid, calculated as the ratio of the [^{13}C]norsolorinic acid produced to the labeled C_6 unit administered, were 3 % and 39 % for hexanoic acid and its corresponding NAC thioester, respectively. As these data were obtained by the same method as that used in Tables 1 and 2, they can be directly compared. The conversion is considerably more efficient in the parent NOR-1 strain, in complete accord with earlier experiments [21].

These incorporations and calculated conversions are entirely consistent with the earlier reported experiments with the averufin-accumulating mutant [21], and indicate no impairment of either the FASs or the PKS in NOR-1. The poor conversions of hexanoylNAC into norsolorinic acid in Dis-1 and Dis-2 must have some other explanation. The results with β-oxidation inhibitors exclude competing degradation. Mapping experiments show that the promoter region between the divergently transcribed *fas-1* and *fas-2* genes is unaffected by the insertional inactivation event, and Southern blots demonstrate unequivocally that only *fas-1* is disrupted, so it is unlikely that lowered expression of FAS2 protein could explain the low efficiencies of hexanoylNAC transformation. Having discounted all of these possible explanations, we suggest that hexanoylNAC cannot effectively 'load' onto the PKS by itself, as would be anticipated based on extensive experimentation with NAC thioesters in other polyketide

Table 2

Effect of β-oxidation inhibitors on FAS mutant strain Dis-2.

Substrate[a]	β-oxidation inhibitor[b]	Norsolorinic acid (% converted)[c]	Aflatoxin B_1 (% converted)[c]
Control (no substrate)	None	–	–
HexanoylNAC	None	5 %	0.3 %
HexanoylNAC	4-Pentynoic acid	5 %	0.2 %
HexanoylNAC	3-(Tetradecylthio)-propanoic acid	8 %	0.3 %

[a]15 mg of hexanoic acid/NAC was dissolved in 1.5 ml of DMF.
[b]50 μmol of 4-pentynoic acid or 3-(tetradecylthio)propanoic acid was solubilized in DMF with the appropriate substrate.
[c]Dashes indicate <0.05 % conversion for norsolorinic acid and <0.01 % conversion for aflatoxin B_1.

biosynthetic systems. Physical association of FAS-1, PKS and perhaps also FAS-2 is proposed to be necessary for efficient synthesis of norsorlorinic acid (compound 3) (Fig 4). Disruption of *fas-1* gives rise to the synthesis of a protein defective in its ability to correctly assemble and contribute to the efficient transfer of the hexanoyl unit to the PKS to begin aflatoxin biosynthesis. Of possible importance in this connection, it is recalled that, while other highly similar fungal PKSs have been observed to have two ACP domains, *pks-A* contains only one [27].

The sensitivity of the HPLC assay is sufficient that ~0.05 % conversion to norsolorinic acid could be detected. Although we failed to observe hexanoic acid use in Dis-1 and Dis-2 under any conditions, it remains possible that it occured at efficiencies that were too low to be observed. Alternatively, the reaction may have been complicated by hindered association of the activated C_6 units (presumably as hexanoylCoA) with the putative PKS–FAS complex. That hexanoic acid is incorporated into norsolorinic acid (compound 3) in NOR-1 with about the same efficiency as its NAC thioester is in Dis-1 and Dis-2 suggests that incorporation does occur in Dis-1 and Dis-2, but at undectable levels. As would be expected, controls using PKS-A, the comparably disrupted PKS mutant [34], failed to convert either hexanoic acid or hexanoylNAC to an anthraquinone.

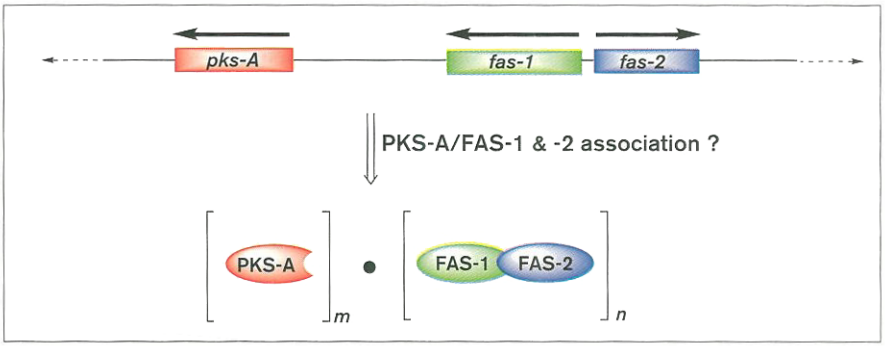
Significance

Type I polyketide synthases are high molecular weight proteins containing multiple domains, each capable of catalyzing one of a sequence of individual reactions in the biosynthesis of a given natural product. In contrast, type II polyketide synthases, typically found in bacteria, form multienzyme complexes in which each component enzyme catalyzes a reaction that is used reiteratively. The biosynthesis of the potent environmental carcinogen aflatoxin B_1 shows an interesting combination of these characteristics. It requires a simple type I PKS that is apparently capable of only non-reductively homologating a C_6 primer by reaction with seven malonates to give the anthrone 2. This C_6 primer is synthesized by a pair of independently transcribed type I FASs. Two mutants, Dis-1 and Dis-2, generated by insertional inactivation of one of the FAS genes, *fas-1*, are incapable of producing norsolorinic acid (compound 3) or aflatoxin (compound 5). However, in the presence of hexanoylNAC, a viable surrogate of hexanoylCoA — the presumed product of the specialized FASs — production of compounds 3 and 5 was restored, but at a much reduced efficiency compared to that seen in the parent strain NOR-1.

On the basis of control experiments, it is proposed that FAS-1 (and possibly also FAS-2) is required to mediate transfer of the synthetic C_6 primer to the PKS (perhaps by direct transfer without the need for conversion to the CoA ester) to initiate aflatoxin biosynthesis. Although the nature of the proposed channeling is not clear, the reason for the poor efficiencies of anthraquinone synthesis in Dis-1 and Dis-2 mutants may be explained by the production of a modified FAS-1. This would fail to complex with PKS or form suboptimal complexes with the PKS, perhaps by faulty geometry, reducing the

Figure 4

Model for the organization of FAS-1, FAS-2 and PKS-A proteins and the genes that encode them. Divergent promoters drive the transcription of the *fas-1* and *fas-2* genes, whereas the *pks-A* gene is located further away. It is postulated that the proteins that are produced associate to initiate aflatoxin biosynthesis.



effectiveness of primer binding and transfer. Yeast FAS is known to form an $\alpha_6\beta_6$ structure. Neither the quaternary state of the related *Aspergillus* FASs nor the relative stoichiometry of the postulated PKS/FAS-1/FAS-2 complex involved in aflatoxin biosynthesis are known. By contrast the modular type I PKSs found often in *Streptomyces* more closely resemble animal FASs and, like these multienzymes, appear to function as homodimers [35].

Materials and methods

Hexanoic acid and *N*-acetylcysteamine were purchased from Aldrich (Milwaukee, WI). Labeled [1-^{13}C]hexanoic acid was obtained from Isotec (99 % ^{13}C; Miamisburg, OH). The β-oxidation inhibitor 4-pentynoic acid was purchased from Aldrich and 3-(tetradecylthio)propanoic acid was a generous gift of Professor J.C. Vederas (University of Alberta). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian Unity^{plus} 400 spectrometer. Chemical shifts for ^{1}H- and ^{13}C-NMR spectra are reported in ppm referenced to d_1-chloroform (7.26 ppm) and d_6-dimethylsulfoxide (39.7 ppm), respectively.

Synthesis of substrates

The synthesis of hexanoylNAC was carried out as described elsewhere [21]; reported melting point (mp) 44–46 °C [21], found mp 43–45 °C.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.0–5.9 (m, 1H, NH), 3.43 (d x t, J = 6.3, 5.9 Hz, 2H, $-CH_2NH-$), 3.02 (t, J = 6.6 Hz, 2H, $-CH_2S-$), 2.57 (t, J = 7.5 Hz, 2H, $-CH_2CO-$), 1.97 (s, 3H, CH_3CO-), 1.71–1.63 (t, J = 7.4 Hz, 2H, $-CH_2CO-$), 1.33–1.28 (m, 6H, $-CH_2CH_2CH_2-$), 0.90 (J = 6.3 Hz, 3H, $-CH_3$).

Organisms

The *Aspergillus parasiticus* norsolorinic acid accumulating mutant (NOR-1, ATCC 24690) was purchased from the American Type Culture Collection (ATCC; Rockville, MD). The *A. parasiticus* disruption mutant strains PKS-A, Dis-1 and Dis-2 and the deletion mutant Dis-3 were a gift of Professor J.E. Linz and co-workers [28].

Media

The basal medium [25] used to initially culture the *A. parasiticus* strains contained per liter: sucrose, 50 g; potassium phosphate monobasic, 10 g; ammonium sulfate, 3 g; magnesium sulfate (anhydrous), 1 g; and trace metals [25], 2 ml. The replacement medium of Adye and Mateles was modified to contain per liter: glucose, 1.62 g; potassium phosphate monobasic, 5 g; potassium chloride, 0.5 g, magnesium sulfate (anhydrous), 0.25 g; and trace metals, 2 ml. All media were autoclaved (140 °C, 20 psi) for 20 min and cooled to room temperature prior to use.

Culture techniques

The fungal strains were grown on potato agar plates for 3–7 d at 28–30 °C. The potato agar plates contained: yeast extract, 2.5 g; Bacto-agar, 2.5 g; potato dextrose agar, 19 g (Difco; Detroit, MI); and distilled water, 500 ml (20 plates). The spores were suspended in 9 ml of a solution of Tween 80 (0.05 % v/v), which was subsequently diluted by the addition of 9 ml of NaCl solution (0.85 % w/v). A fraction of the cultured spore suspension (2 ml) was then transferred to 1 l of Adye and Mateles medium in 4-l Erlenmeyer flasks. The flasks were incubated at 28–30 °C in the dark at 175 rpm as described below.

General procedure for the administration of hexanoic acid/NAC starter-units to the A. parasiticus *strains*

The *A. parasiticus* mutant strains were initially cultured in 4-l flasks each containing 1 l of Adye and Mateles medium as described above. After 48 h of growth the mycelia were vacuum filtered and rinsed thoroughly with distilled water. The cells (10 g) were then transferred into 500-ml Erlenmeyer flasks each containing 125 ml of modified replacement medium. Typically substrates, hexanoic acid/NAC thioester (15 mg) or averufin (10 mg) together with 50 μmol of the desired β oxidation inhibitor, when appropriate, were dissolved in 1.5 ml of dimethylformamide and added to the mycelial suspensions, which were grown for an additional 48 h. The cells were harvested by vacuum filtration, flash frozen in liquid nitrogen and the metabolites extracted by steeping the cells in acetone for 1 h at 50 °C. The acetone extract was concentrated under vacuum and lyophilized. The biosynthesized products obtained from a single 125-ml culture were typically solubilized in 5 ml of methanol. Metabolite production was measured qualitatively by TLC (under long-wave UV). Norsolorinic acid (R_f = 0.93) and versicolorin A (R_f = 0.30) were detected using a ternary mixture of 8:1.8:0.2 of hexane:ethyl acetate:acetic acid while O-methylsterigmatocystin (R_f = 0.68) and aflatoxin B_1 (R_f = 0.56) were separated with a 6:3:1 mixture of chloroform:ethyl acetate:formic acid.

Analytical methods

Southern and northern blots were performed using standard procedures [36]. Quantitative determination of metabolite production was achieved by HPLC analysis. A reverse-phase Phenomenex C-18 column (250 x 4.60 mm; Torrance, CA) was used in conjunction with a Varian 5020 liquid chromatograph equipped with a 50-μl injection loop and an Applied Biosystems diode array UV detector (Model 1000S; Ramsey, NJ). Norsolorinic acid (t_r = 14.6 min) production was measured using an isocratic gradient (20 % of 0.1 % trifluoroacetic acid in water:acetonitrile) while amounts of versicolorin A (t_r = 41.3 min), O-methylsterigmatocystin (t_r = 29.6 min) and aflatoxin B_1 (t_r = 16.36 min) were determined with a gradient (70 % to 40 % in 55 min, 0.1 % trifluoroacetic acid in water:acetonitrile). Typically, metabolites generated from a 125-ml culture were solubilized in 5 ml of methanol, filtered (Alltech #2167 nylon 0.45 μm; Deerfield, IL) and 50 μl of this solution was analyzed by HPLC. Integrated peak areas were averaged based upon three injections and these data are entered in Tables 1 and 2.

Incorporation of [1-^{13}C]-labeled substrates into norsolorinic acid

The [1-^{13}C]-labeled fatty acid/NAC substrates were supplied to the *A. parasiticus* norsolorinic acid accumulating mutant strain NOR-1, the metabolites extracted following the procedure described above, and the total concentration estimated by UV spectroscopy. Following lyophilization, norsolorinic acid was most easily purified by direct recrystallization from acetone. In each case, approximately 4 mg of purified material was obtained from four 125-ml cultures.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.12 (s, 1H, H-4), 7.05 (d, J = 2.4 Hz, 1H, H-5), 6.53 (d, J = 2.4 Hz, 1H, H-7), 2.73 (t, J = 7.2 Hz, 2H, H-12), 1.59 (m, 2H, H-13), 1.25 (m, 4H, H-14/H-15), 0.81 (t, J = 6.8 Hz, 3H, H-16).

^{13}C NMR (100 MHz, d_6 dimethylsulfoxide) δ: 202.7 (C-1'), 188.3, 181.0, 165.1, 164.1, 162.2, 160.8, 134.8, 134.6, 121.4, 119.2 (?), 108.8, 108.4, 108.1, 43.6, 30.7, 22.6, 21.9, 13.9, C-2 not detected.

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