Enzymatic Synthesis of 1,3,6,8-Tetrahydroxynaphthalene Solely from Malonyl Coenzyme A by a Fungal Iterative Type I Polyketide Synthase PKS1†

Isao Fujii,‡ Yuichiro Mori,‡ Akira Watanabe,‡ Yasuyuki Kubo,§ Gento Tsuji,§ and Yutaka Ebizuka*,‡

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, and Faculty of Agriculture, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan

Received March 21, 2000; Revised Manuscript Received May 16, 2000

ABSTRACT: The *Colletotrichum lagenarium PKS1* gene encoding iterative type I polyketide synthase of 1,3,6,8-tetrahydroxynaphthalene (T4HN) was overexpressed in *Aspergillus oryzae.* SDS−PAGE analysis of the cell-free extract prepared from the transformant showed an intense band of 230000 which corresponded to the molecular weight of the deduced PKS1 protein. By using this cell-free extract, in vitro synthesis of T4HN was successfully confirmed as the first example of the fungal multi-aromatic ring polyketide synthase activity ever detected. To identify the starter unit for T4HN synthesis, ^{14}C-labeled acetyl CoA and/or ^{14}C-labeled malonyl CoA were used as substrates for T4HN synthase reaction. Observed was the incorporation of ^{14}C label into T4HN solely from malonyl CoA even in the absence of acetyl CoA and not from acetyl CoA. This in vitro result unambiguously identified that malonyl CoA serves as the starter as well as extender units in the formation of T4HN by fungal polyketide synthase PKS1.

Polyketide metabolites are known as one of the most important groups of natural products with huge structural diversity. Many of them show significant biological activities such as antibiotic, immunosuppressant, antiparasitic, insecticidal, toxins and so forth (*1*, *2*). Polyketide carbon skeletons are assembled by so-called polyketide synthases (PKSs)[1] that utilize acyl CoAs, mostly acetyl CoA and malonyl CoA, as their building blocks. Reactions of PKSs proceed in a quite similar way to that of fatty acid synthase (FAS), but β-carbonyl groups generated through condensation of C_2 units remain, in most cases, unreduced during chain-elongation of PKS reactions (*3*, *4*).

Thus far, PKSs are classified into three types. Type I PKSs are large multifunctional enzymes with multidomains necessary for polyketide assembly such as β-ketoacyl synthase (KS), acyltransferase (AT), acyl carrier protein (ACP) and in some cases, β-ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and thioesterase (TE) (*5*, *6*). Type II PKSs, in contrast, are multienzyme complex system consisted of proteins with mono- or bifunctional domains (*4*). The third ones, sometimes designated as type III, are plant chalcone synthase (CHS) family (*7*, *8*).

Bacterial PKSs, especially those of actinomycetes, have been studied extensively and found that modular type I PKSs elaborate reduced complex type polyketides such as macrolides (*5*, *6*). For aromatic compounds, iterative type II systems are involved as exemplified in the biosynthesis of actinorhodin (*9*) and tetracenomycin (*10*).

Along with bacteria and plants, fungi produce a great number of compounds of polyketide origin (*11*, *12*). Studies on fungal PKS began in 1960s by Lynen and co-workers. Their works crystallized as a landmark paper on 6-methylsalicylic acid synthase (MSAS) from *Penicillium patulum* (*13*). Later, its gene cloning followed as the first fungal PKS gene ever cloned (*14*). Since then, several fungal PKS genes have been cloned. They are *atX* from *Aspergillus terreus* for MSAS (*15*), *wA* from *Aspergillus nidulans* for spore pigment biosynthesis (*16*), *PKS1* and *alb1* for melanin biosynthesis from *Colletotrichum lagenarium* (*17*) and *Aspergillus fumigatus* (*18*), respectively, PKS genes for sterigmatocystin/aflatoxin biosynthesis from *Aspergillus* species (*19*−*21*), *lovB* and *lovF* from *A. terreus* for lovastatin biosynthesis (*22*, *23*), *PKS1* for T-toxin biosynthesis from *Cochliobolus heterostrophus* (*24*), and *FUM5* for fumonisin biosynthesis from *Gibberella fujikuroi* (*25*). All fungal PKS genes so far known encode multidomain type I PKSs for both aromatic and nonaromatic reduced compounds (*26*, *27*). Their protein architectures are more closely related to mammalian FASs than fungal ones (*28*) and might be classified as an independent group of microbial PKSs as iterative type I PKSs since each domain is assumed to be involved iteratively in polyketide chain assembly.

Although the functions of fungal PKSs encoded by these genes have been assumed, direct PKS product compounds have been rarely identified. We have reported successful

† This research was supported in part by the "Research for the Future" Program (JSPS-RFTF 96I0032), The Japan Society for the Promotion of Science. A.W. and G.T. are recipients of JSPS young research fellowship.

* To whom correspondence should be addressed. Phone: +81-3-5841-4740. Fax: +81-3-5841-4744. E-mail: yebiz@mol.f.u-tokyo.ac.jp.

‡ Graduate School of Pharmaceutical Sciences, The University of Tokyo.

§ Faculty of Agriculture, Kyoto Prefectural University.

[1] Abbreviations: ACP, acyl carrier protein; AT, acyltransferase; CHS, chalcone synthase; CoA, coenzyme A; DH, dehydratase; DHN, 1,8-dihydroxynaphthalene; DTT, dithiothreitol; ER, enoyl reductase; FAS, fatty acid synthase; HPLC, high performance liquid chromatography; KR, β-ketoreductase; KS, β-ketoacyl synthase; LC-APCIMS, liquid chromatography atmospheric pressure chemical ionization mass spectrometry; MSAS, 6-methylsalicylic acid synthase; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PKS, polyketide synthase; SDS, sodium dodecyl sulfate; TE, thioesterase; T4HN, 1,3,6,8-tetrahydroxynaphthalene.

10.1021/bi000644j CCC: $19.00
Published on Web 07/06/2000

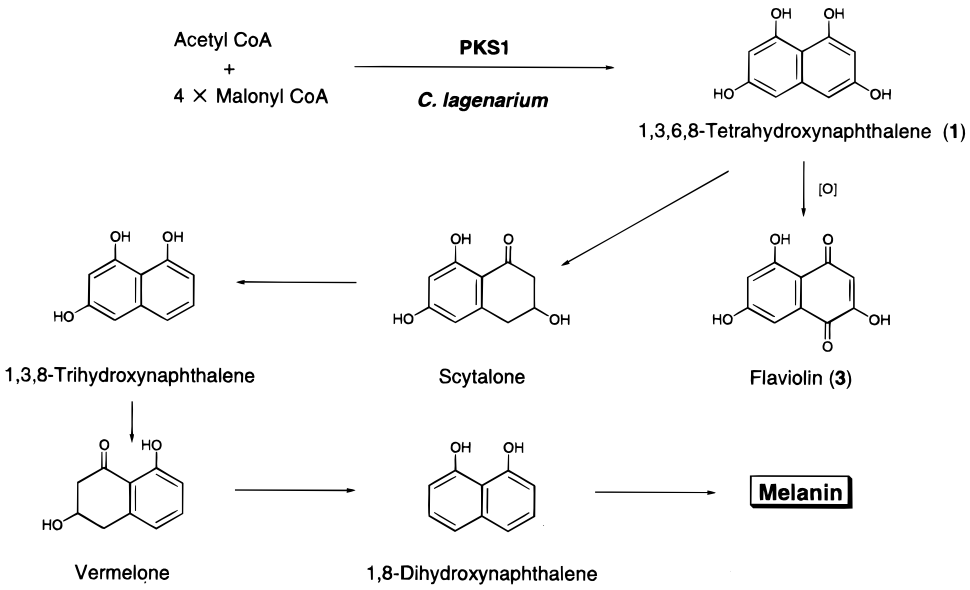

FIGURE 1: Biosynthesis of T4HN and DHN melanin.

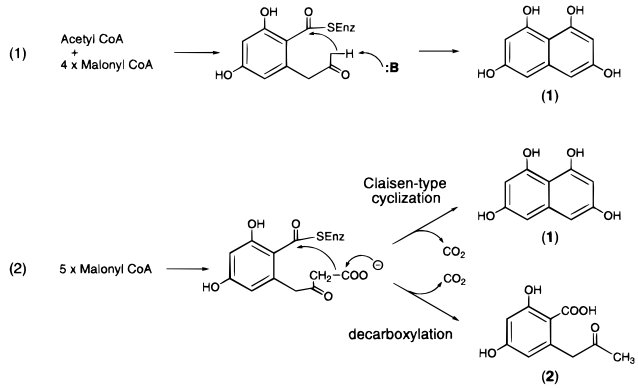

FIGURE 2: Proposed mechanisms for T4HN formation.

expression of *atX* (*15*), *wA* (*nwA*) (*29*, *30*), and *C. lagenarium PKS1* (*31*) using starch inducible α-amylase promoter in heterologous fungal hosts and identified their PKS products. Hutchinson and Vederas group also reported identification of products of Lov PKSs (*23*).

C. lagenarium is a phytopathogenic fungus, a causal agent of anthracnose of cucumber. 1,8-Dihydroxynaphthalene (DHN) melanin formation is one of the determinants for its infection (*32*). 1,3,6,8-Tetrahydroxynaphthalene (T4HN) (**1**) is an indispensable precursor to DHN melanin and is an unique symmetrical compound of polyketide origin with no apparent starter unit (*33*). (Figure 1) In our previous paper on the PKS1 expression experiment, we identified a pentaketide monocyclic carboxylic acid (**2**) as a possible byproduct of PKS1 PKS reaction in addition to the normal product T4HN (**1**) (*31*). Identification of this monocyclic compound (**2**) led us to propose the folding pattern of the pentaketide intermediate and two possible cyclization mechanisms for the second aromatic ring formation of T4HN (**1**) as shown in Figure 2. A key issue here is how the apparent terminal methyl group could be activated to facilitate Claisen-type cyclization. One possibility is a presence of a strong basic amino acid residue in the active site that can activate a terminal methyl group. The other one is that malonate serves as a starter unit instead of acetate that can generate carbanion by decarboxylation. To clarify the mechanism involved for T4HN formation, it was desirable to establish in vitro system synthesizing T4HN (**1**).

Cell-free enzymological studies on microbial PKSs have advanced since recombinant systems have become available as first exemplified by Shen and Hutchinson (*34*). Bacterial type I PKS for 6-deoxyerythronolide B (*35*, *36*) and type II PKSs for tetracenomycin (*37*) and actinorhodin (*38*) have been purified and extensively studied. However, no successful preparation of active cell-free synthesis systems of iterative type I PKSs from fungi have been reported except MSAS and orsellinic acid synthase. Both are PKSs for single aromatic ring compounds (*39*−*41*).

We report here the preparation of active cell-free system of T4HN synthesis from the *Aspergillus oryzae* transformant overexpressing *C. lagenarium* PKS1 and identification of T4HN starter unit by in vitro analysis.

MATERIALS AND METHODS

Materials. [2-^{14}C]Malonyl CoA (2.1 GBq/mmol) and [1-^{14}C]acetyl CoA (2.2 GBq/mmol) were obtained from NEN Life Science Products, Inc. Acetyl CoA and malonyl CoA were from Sigma. All other chemicals used were of the highest purity commercially available grade.

Strain and Culture Condition. The *A. oryzae* transformant with the PKS1 expression plasmid pTAPSG was from previous work (*31*). The transformant was precultured in Czapek-Dox medium containing glucose as carbon source and then transferred into induction medium (Czapek-Dox medium containing starch). After 3 days induction culture at 30 °C on a rotary shaker at 200 rpm, mycelia were harvested on a Buchner funnel, washed with distilled water, and then used for cell-free extraction or stored at −80 °C after liquid nitrogen flush freezing.

Cell-Free Extract Preparation. Mycelia, freshly harvested or stored deep frozen, were flash frozen in liquid nitrogen and pulverized in a mortar with a pestle. The mycelial powder was then suspended in 50 mM potassium phosphate buffer, pH 7.5, containing 30% glycerol, 2 mM β-mercaptoethanol, 1 mM EDTA, and 0.1 mM benzamidine. The mixture was occasionally stirred on ice for 20 min and

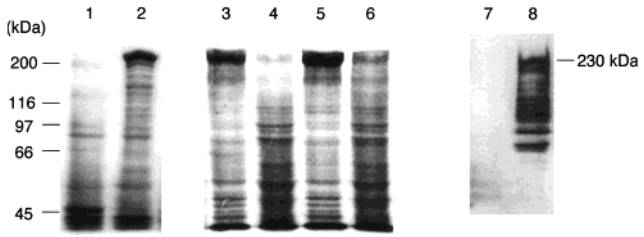

FIGURE 3: SDS−PAGE and Western blot analysis of the cell-free extract of *A. oryzae* transformant. (A) SDS−PAGE: Lane 1, crude extract of *A. oryzae* M-2-3; lane 2, crude extract of *A. oryzae*/pTAPSG transformant; lane 3, 50000*g* ppt fraction; lane 4, 50000*g* sup fraction; lane 5, 210000*g* ppt fraction; lane 6, 210000*g* sup fraction. (B) Western blot: lane 7, crude extract of *A. oryzae* M-2-3; lane 8, crude extract of *A. oryzae*/pTAPSG transformant.

subsequently centrifuged at 10000*g* for 20 min. The supernatant was then filtered through four layered gauze to remove residual cell debris. Thus prepared crude cell-free extract was then subjected to ultracentrifugation at 50000*g* for 1 h. The supernatant was further fractionated by ultracentrifugation at 210000*g* for 2 h.

HPLC and LC-APCIMS. Reverse phase HPLC condition used for enzymatic product analysis was as follows: ODS-80Ts column (4.6 × 150 mm; Tosoh), maintained at 40 °C; mobile phase, linear gradient from 5% CH_3CN in H_2O to 40% CH_3CN in H_2O (each contained 2% acetic acid) over 30 min with detection at 254 nm; flow rate, 0.8 mL/min. LC-APCIMS was measured on LCQ (Thermo Quest) under the same condition as for the HPLC analysis.

Preparation of anti-PKS1 Antibody. Polyclonal antiserum against PKS1 protein was obtained by immunizing rabbits with SDS-gel purified PKS1 protein expressed in *Escherichia coli* using pET expression system (Novagen). IgG fraction was obtained by passage through a Protein A affinity column.

SDS−PAGE and Western Blot Analysis. SDS−PAGE was performed essentially as described by Laemmli (*42*). Gels of 5% acrylamide and 0.5% bisacrylamide were used and electrophoresed in a modified Laemmli buffer system (0.025 M Tris base, 0.192 M glycine, 0.05% SDS). Gels were stained with GelCode Blue stain reagent (Pierce). After separation on SDS−PAGE, proteins were blotted onto nitrocellulose membrane (Schleicher and Schuell) by Bio-Rad electroblotting apparatus according to the manufacturer's instructions. The filter was detected by enzyme-linked immunodetection with anti-PKS1 antibody and anti-rabbit IgG-peroxidase conjugate using Konica immunostain horseradish peroxidase kit (Konica, Japan).

RESULTS

Fractionation and SDS−PAGE Analysis of the Cell-Free Extract. SDS−PAGE analysis of the crude cell-free extract prepared from the starch induced *A. oryzae* transformant mycelia indicated overexpression of 230000 band which well corresponded to the deduced molecular weight of PKS1 protein. This band was immunoreactive to antiserum raised against PKS1 protein expressed in *E. coli* although a ladder of smaller bands, possible degradation products of PKS1 protein, could be observed in Western analysis (Figure 3). By fractionation with ultracentrifugation, this large protein sedimented even at 50000*g* for 1 h and was mainly recovered in the precipitates by ultracentrifugation at 210000*g* for 2 h, providing a highly concentrated PKS1 fraction. Although

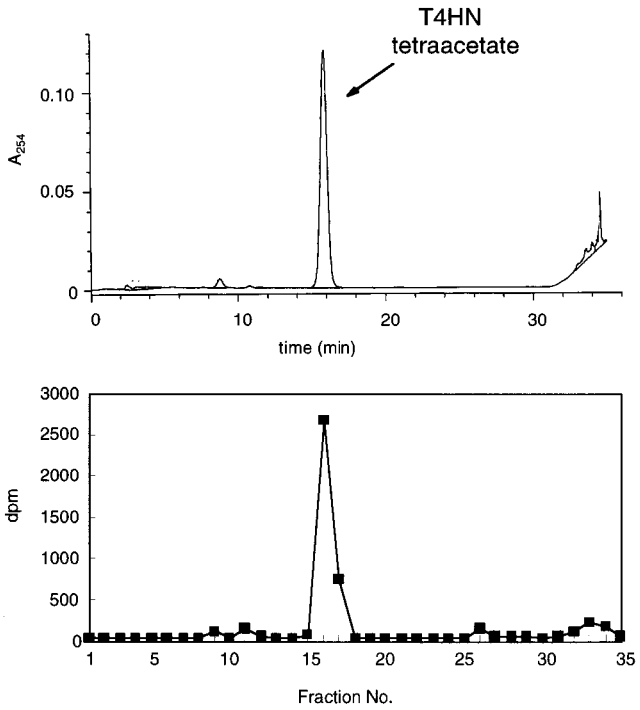

FIGURE 4: HPLC analysis of acetylated PKS1 cell-free product with UV absorbance and ^{14}C radioactivity.

native molecular weight of PKS1 has not been estimated yet, precipitation by ultracentrifugation might be ascribed to nonspecific aggregation while extraction procedures.

In Vitro Synthesis of T4HN. Enzymatic synthesis of T4HN (**1**) was first established by the following experiment. Reaction mixture containing 50 μL of 50 mM potassium phosphate buffer, pH 7.5, 25 μL of 100 μM acetyl CoA, 25 μL of 180 μM [2-^{14}C]malonyl CoA (5.55×10^5 dpm), and 150 μL of enzyme solution (210000*g* ppt fraction) was incubated at 30 °C for 30 min. After acidification with addition of 50 μL of 6 M HCl, the reaction mixture was extracted with ethyl acetate and then acetylated with acetic anhydride and pyridine. The acetylated product was analyzed by silica gel TLC. Radioautogram indicated the formation of T4HN tetraacetate (**4**), which was further confirmed by HPLC analysis (Figure 4). None of T4HN tetraacetate (**4**) was detected in the control reaction with the cell-free extract prepared from nontransformant.

Direct Detection of T4HN and Flaviolin. Direct detection of T4HN (**1**) enzymatically formed by PKS1 PKS was attempted since high enzyme activity was expected in the 210000*g* ppt fraction. The PKS1 PKS assay mixture (250 μL) contained 100 μM acetyl CoA, 150 μM [2-^{14}C]malonyl CoA (5.55×10^5 dpm), 2 mM DTT, and 20 μL of ultracentrifugation fraction in 0.1 M potassium phosphate buffer, pH 7.5. Reaction was initiated by adding malonyl CoA, incubated at 25 °C for 60 min, and terminated by adding 100 μL of 1 M HCl. Products were extracted with 400 μL of ethyl acetate, dried up by flushing of nitrogen gas, and dissolved in 100 μL of acetonitrile for HPLC analysis. Standard T4HN (**1**), carboxylic acid (**2**), and flaviolin (**3**) were eluted with retention time at 15, 16, and 21 min, respectively. Radioactivity was monitored by liquid scintillation counting of fractions collected every 1 min. As shown in Figure 5 A, two radioactive peaks were observed and their retention times corresponded to those of T4HN (**1**)

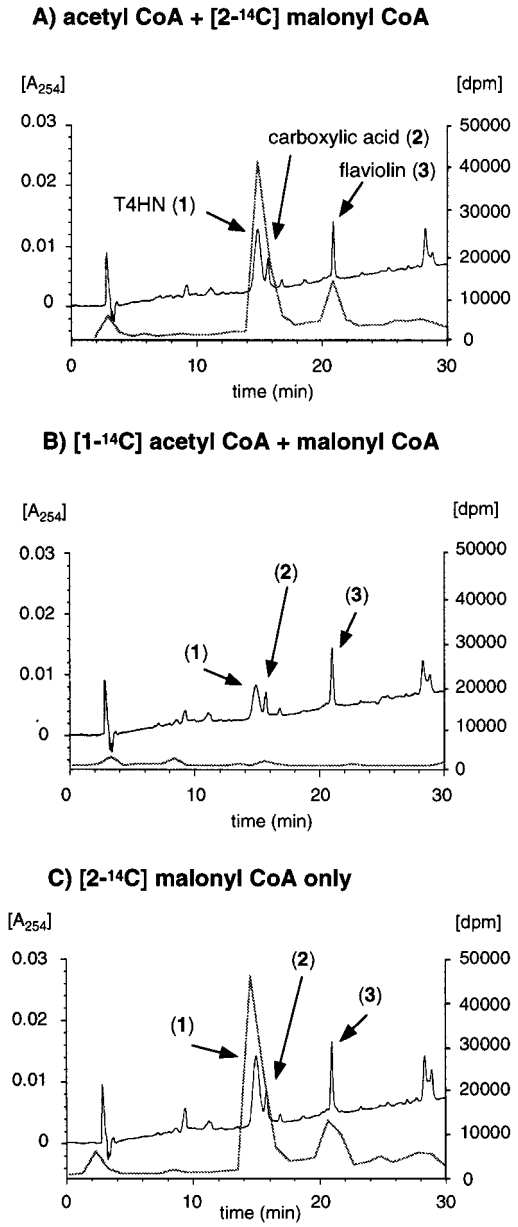

FIGURE 5: HPLC analysis of enzymatically formed PKS1 products with UV absorbance and ^{14}C radioactivity. Substrate(s) used in each reaction: (A) acetyl CoA and [2-^{14}C]malonyl CoA, (B) [1-^{14}C]-acetyl CoA and malonyl CoA, (C) [2-^{14}C]malonyl CoA.

and flaviolin (**3**), the latter being an autoxidation product of T4HN (**1**). Furthermore, these products could be detected even with UV monitoring at 254 nm alone. Rigorous identification of products was obtained by LC-APCIMS. This strong T4HN synthase activity provided us a desirable in vitro system to identify the starter unit of T4HN (**1**) formation.

Determination of T4HN Synthase Starter Unit. As proposed in our previous paper (*31*), malonyl CoA instead of acetyl CoA might be a starter unit in PKS1 T4HN synthase reaction. Determination of the starter unit of this reaction was presumed to be difficult since acetyl CoA could arise from the decarboxylation of malonyl CoA to serve as the starter unit even though malonyl CoA alone is used as the substrate.

The strong activity of PKS1 enzyme preparation (210000*g* ppt fraction) enabled us to monitor the in vitro formation of PKS1 products by UV absorption at 254 nm and the incorporation of ^{14}C label from [1-^{14}C]acetyl CoA or [2-^{14}C]-malonyl CoA on HPLC simultaneously (Figure 5). As summarized in Table 1, amounts of PKS1 products [T4HN (**1**), carboxylic acid (**2**), and flaviolin (**3**)] formed in vitro from acetyl CoA plus malonyl CoA (reaction A and B) or malonyl CoA alone (reaction C) were found to be in the similar range as estimated by peak area at UV 254 nm. Radioactivity incorporation from [2-^{14}C]malonyl CoA was comparable in reaction A and C irrespective of the presence of cold acetyl CoA. On the other hand, negligible level of ^{14}C incorporation from [1-^{14}C]acetyl CoA was detected in reaction B with [1-^{14}C]acetyl CoA and malonyl CoA. Relative ^{14}C specific incorporation from [1-^{14}C]acetyl CoA (**b**/**a** of reaction B in Table 1) was found to be less than 1% of those from [2-^{14}C]malonyl CoA (**b**/**a** of reaction A in Table 1). Theoretical ^{14}C specific incorporation from [1-^{14}C]-acetyl CoA should be 25% of those from [2-^{14}C]malonyl CoA if acetyl CoA serves as a starter unit of PKS1 pentaketide synthase reaction.

From these results, we could conclude that the fungal type I PKS, T4HN synthase (PKS1), utilizes not acetyl CoA but malonyl CoA as the starter unit.

DISCUSSION

To the best of our knowledge, this is the first report on the successful detection of cell-free activity of multi-aromatic ring PKS from fungi. The *C. lagenarium* PKS1, T4HN synthase, was overexpressed in a heterologous fungus *A. oryzae*, and its cell-free activity was confirmed. Although fungal PKS study has a long history, MSAS and orsellinic acid synthase, both for single aromatic ring polyketides, have been the only two fungal PKSs whose activities have been detected in vitro (*39*−*41*). MSAS was purified and its gene was cloned by Schweizer's group only a decade ago (*14*). Since then, several fungal PKS genes have been cloned for both aromatic and reduced polyketides. However, no other cell-free activity of PKS has been reported (*26*, *27*).

Acetate or its equivalent acetyl CoA has been believed to be a C_2 starter unit of polyketide biosynthesis. However, bacterial PKSs for actinorhodin (*38*) and tetracenomycin (*37*) from streptomycetes have been shown to utilize malonyl CoA as a starter of polyketide synthesis. Starter unit for T4HN (**1**) has been discussed for a long time since it is a symmetric compound with no apparent starter. Identification of monocyclic carboxylic acid (**2**) as a byproduct of PKS1 reaction in vivo (*31*) suggested that accidental decarboxylation of malonyl starter or misloading of acetate as a starter forced to form the compound (**2**). Thus, the malonyl starter mechanism was favored. As malonyl CoA can be converted to acetyl CoA in vitro by enzymatic or nonenzymatic decarboxylation and contamination of each other compound is inevitable, great caution should be paid to identify the starter unit by in vitro PKS reaction. In T4HN synthesis by the *C. lagenarium* PKS1, its starter unit was unambiguously identified to be malonyl CoA by virtue of high enzyme activity of the cell-free preparation. As shown in Figure 2, the mechanism of decarboxylative Claisen-type cyclization well explains the naphthalene ring formation. In vitro formation of monocyclic carboxylic acid (**2**) as a minor product (HPLC retention time at 16 min) was also observed

Table 1: In Vitro Incorporation of ^{14}C Label from Acetyl CoA or Malonyl CoA into PKS1 Products[a]

	T4HN (**1**) + carboxylic acid (**2**)			flaviolin (**3**)		
substrate	(**a**) rel peak area	(**b**) ^{14}C incorp	**b**/**a**	(**a**) rel peak area	(**b**) ^{14}C incorp	**b**/**a**
(A) acetyl CoA + [2-^{14}C]malonyl CoA	443	63 257	143 (100)	112	14 436	129 (100)
(B) [1-^{14}C]acetyl CoA + malonyl CoA	235	259	1.1 (0.7)	119	52	0.4 (0.3)
(C) [2-^{14}C]malonyl CoA only	487	69 790	143 (100)	141	12749	90 (70)

[a] (**a**) Relative peak area of each compound (**1**, **2**, **3**) were monitored on HPLC at UV 254 nm. (**b**) ^{14}C incorporation (dpm) were deteremined by liquid scintilation counting of HPLC fractions collected every 1 min.

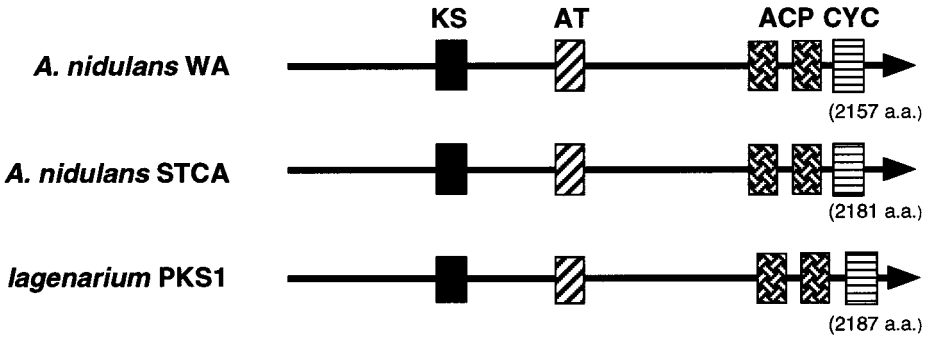

FIGURE 6: Architecture of fungal type I polyketide synthases of Claisen-type cyclization. KS, β-ketoacyl synthase; AT, acyltransferase; ACP, acyl carrier protein; CYC, putative Claisen-type cyclase.

with scarce incorporation of label from [1-^{14}C]acetyl CoA. This result indicates that starter of byproduct (**2**) is also malonate and its decarboxylation might occur while chain elongation to first ring closure on the PKS1 enzyme that leads to formation of (**2**). However, it should be noted that these data could not discriminate whether decarboxylative carbanion generation and the second ring formation of naphthalene occur in a concerted manner or by distinct stepwise way.

Claisen-type cyclization was assumed to be involved in other fungal PKS reactions, for example, naphthopyrone and norsolorinic acid anthrone synthesis by PKSs encoded by *A. nidulans wA* and *pksST* (*pksL*) of *Aspergillus* species. Interestingly, these Claisen-type PKSs including PKS1 have quite similar protein architectures as shown in Figure 6 (*27*). In Claisen-type cyclization, involvement of C-terminus region so far recognized as thioesterase (TE) domain was proposed in our previous publication on *wA* expression experiment (*30*). Compared with MSAS and reduced complex-type fungal PKSs which lack Claisen-type cyclization step, relatively long N-terminal sequences upstream of KS domains were also conserved in these type of fungal PKSs, suggesting possible importance of N-termini in these PKS reactions. Despite similarity in protein architecture, these PKSs show individual specificities such as choice of starter units (acetyl or malonyl for WA, malonyl for PKS1, and hexanoyl for STCA) and number of condensation cycles. To clarify the mechanism for these specificities, further enzymological studies including their three-dimensional structures are necessary. Detection of T4HN synthase cell-free activity reported here is an essential first step toward this purpose.

Recently, *rppA* was identified to code for a T4HN synthase (*43*). The gene was cloned from the gram-positive bacteria *Streptomyces griseus* and RppA protein shows 30% identity to plant CHSs, thus belonging to CHS-like Type III PKSs. Although most of PKSs of this type utilize CoA esters from the phenylpropanoid pathway as starter, malonyl CoA serves as both starter and extenders in RppA reaction. It is interesting to note that lower eukaryotic PKS1 and bacterial RppA catalyze the formation of the same compound T4HN (**1**), but mechanistically in quite different manners. The former catalyzes the elongation of polyketomethylene chain on the SH residue of phosphopantetheine group attached to the internal serine residue of acyl carrier domain. On the hand, the latter apparently does not use acyl carrier protein and carries out chain elongation probably as a CoA ester.

Fungal DHN melanin has critical role in pathogenicity to not only plants (*44*, *45*) but also mammals (*18*, *46*−*48)*. And T4HN formation is the indispensable first step of its biosynthesis. The establishment of cell-free system of fungal T4HN synthase in this study opens a frontier for detailed mechanistic studies on this important enzyme, such as choice of starter, control of condensation cycles, and controls of aldol and Claisen cyclization to form T4HN (**1**).

ACKNOWLEDGMENT

The authors are grateful to Prof. Katsuya Gomi (Graduate School of Agriculture, Tohoku University, Sendai, Japan) for his kind supply of the plasmid and fungal strain, and to Prof. C. Richard Hutchinson (School of Pharmacy, University of Wisconsin-Madison) for his critical reading and comments on the manuscript.

REFERENCES

1. O'Hagen, D. (1991) *The polyketide metabolites*, Ellis Horwood, New York.
2. Monaghan, R. L., and Tkacz, J. S. (1990) *Annu. Rev. Microbiol. 44*, 271−301.
3. Hopwood, D. A. (1997) *Chem. Rev. 97*, 2465−2497.
4. Hutchinson, C. R., and Fujii, I. (1995) *Annu. Rev. Microbiol. 49*, 201−238.
5. Khosla, C. (1997) *Chem. Rev. 97*, 2577−2590.
6. Staunton, J., and Wilkinson, B. (1997) *Chem. Rev. 97*, 2611−2629.
7. Schröder, J. (1997) *Trends Plant Sci. 2*, 373−378.
8. Schröder, J. (1999) in *Comprehensive Natural Products Chemistry* (Sankawa, U., Ed.) vol. 1, pp 749−771, Elsevier, Oxford.

9. Richardson, M., and Khosla, C. (1999) in *Comprehensive Natural Products Chemistry* (Sankawa, U., Ed.) vol. 1, pp 474−494, Elsevier, Oxford.
10. Hutchinson, C. R. (1997) *Chem. Rev. 97*, 2525−2535.
11. Turner, W. B. (1971) *Fungal Metabolites*, Academic Press, London.
12. Turner, W. B., and Aldridge, D. C. (1983) *Fungal Metabolites II*, Academic Press, London.
13. Dimroth, P., Walter, H., and Lynen, F. (1971) *Eur. J. Biochem. 13*, 98−110.
14. Beck, J., Ripka, S., Signer, A., Schiltz, E., and Schweizer, E. (1990) *Eur. J. Biochem. 192*, 487−498.
15. Fujii, I., Ono, Y., Tada, H., Gomi, K., Ebizuka, Y., and Sankawa, U. (1996) *Mol. Gen. Genet. 253*, 1−10.
16. Mayorga, M. E., and Timberlake, W. E. (1992) *Mol. Gen. Genet. 235*, 205−212.
17. Takano, Y., Kubo, Y., Shimizu, K., Mise, K., Okuno, T., and Furusawa, I. (1995) *Mol. Gen. Genet. 249*, 162−167.
18. Tsai, H.-F., Chang, Y. C., Washburn, R. G., Wheeler, M. H., and Kwon-Chung, K. J. (1998) *J. Bacteriol. 180*, 3031−3038.
19. Yu, J.-h., and Leonard, T. J. (1995) *J. Bacteriol. 177*, 4792−4800.
20. Feng, G. H., and Leonard, T. J. (1995) *J. Bacteriol. 177*, 6246−6254.
21. Chang, P.-K., Cary, J. W., Yu, J., Bhatnagar, D., and Cleveland, T. E. (1995) *Mol. Gen. Genet. 248*, 270−277.
22. Hendrickson, L., Davis, C. R., Roache, C., Nguyen, D. K., Aldrich, T., McAda, P. C., and Reeves, C. D. (1999) *Chem. Biol. 6*, 429−439.
23. Kennedy, J., Auclair, K., Kendrew, S. G., Park, C., Vederas, J. C., and Hutchinson, C. R. (1999) *Science 284*, 1368−1372.
24. Yang, G., Rose, M. S., Turgen, B. G., and Yoder, O. C. (1996) *Plant Cell 8*, 2139−2150.
25. Proctor, R. H., Desjardins, A. E., Plattner, R. D., and Hohn, T. M. (1999) *Fungal Genet. Biol. 27*, 100−112.
26. Fujii, I., Watanabe, A., Mori, Y., and Ebizuka, Y. (1998) *Actinomycetology 12*, 1−14.
27. Fujii, I. (1999) in *Comprehensive Natural Products Chemistry* (Sankawa, U., Ed.) vol. 1, pp 409−441, Elsevier, Oxford.
28. Smith, S. (1994) *FASEB J. 8*, 1248.
29. Watanabe, A., Ono, Y., Fujii, I., Sankawa, U., Mayorga, M. E., Timberlake, W. E., and Ebizuka, Y. (1998) *Tetrahedron Lett. 39*, 7733−7736.
30. Watanabe, A., Fujii, I., Sankawa, U., Mayorga, M. E., Timberlake, W. E., and Ebizuka, Y. (1999) *Tetrahedron Lett. 40*, 91−94.
31. Fujii, I., Mori, Y., Watanabe, A., Kubo, Y., Tsuji, G., and Ebizuka, Y. (1999) *Biosci. Biotechnol. Biochem. 63*, 1445−1452.
32. Yamaguchi, I., and Kubo, Y. (1992) in *Target sites of fungicidal action* (Koller, W., Ed.) pp 101−118, CRC Press, London.
33. Sankawa, U., Shimada, H., Sato, T., Kinoshita, K., and Yamasaki, K. (1981) *Chem. Pharm. Bull. 29*, 3536−3542.
34. Shen, B. H., and Hutchinson, C. R. (1993) *Science 262*, 1535−1540.
35. Pieper, R., Gokhale, R. S., Luo, G., Cane, D. E., and Khosla, C. (1997) *Biochemistry 36*, 1846−1851.
36. Gokhale, R. S., Lau, J., Cane, D. E., and Khosla, C. (1998) *Biochemistry 37*, 2524−2528.
37. Bao, W., Wendt-Pienkowski, E., and Hutchinson, C. R. (1998) *Biochemistry 37*, 8132−8138.
38. Carreras, C. W., and Khosla, C. (1998) *Biochemistry 37*, 2084−2088.
39. Shoolingin-Jordan, P. M., and Campuzano, I. D. G. (1999) in *Comprehensive Natural Products Chemistry* (Sankawa, U., Ed.) vol. 1, pp 345−365, Elsevier, Oxford.
40. Gaucher, G. M., and Shepherd, M. G. (1968) *Biochem. Biophys. Res. Commun. 32*, 664−671.
41. Woo, E.-R., Fujii, I., Ebizuka, Y., Sankawa, U., Kawaguchi, A., Beale, J. M., Shibuya, M., Mocek, U., and Floss, H. G. (1989) *J. Am. Chem. Soc. 111*, 5498−5500.
42. Laemmli, U. K. (1970) *Nature 227*, 680−685.
43. Funa, N., Ohnishi, Y., Fujii, I., Shibuya, M., Ebizuka, Y., and Horinouchi, S. (1999) *Nature 400*, 897−899.
44. Woloshuk, C. P., Sisler, H. D., Tokousbalides, M. C., and Dutky, S. R. (1980) *Pestic Biochem. Physiol. 14*, 256−264.
45. Kubo, Y., Suzuki, K., Furusawa, I., and Yamamoto, M. (1985) *Pestic Biochem. Physiol. 23*, 47−55.
46. Kwon-Chung, K. J., Polacheck, I., and Popkin, T. J. (1982) *J. Bacteriol. 150*, 1414−1421.
47. Kwon-Chung, K. J., and Rhodes, J. C. (1986) *Infect. Immun. 51*, 218−223.
48. Wang, Y., Aisen, P., and Casadevall, A. (1995) *Infect. Immun. 63*, 3131−3136.

BI000644J