Bacterial Product Template Domain: An Evolutionary Intermediate between Dehydratase and Aldol Cyclase of Type I Polyketide Synthases

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Abstract

The product template (PT) domains act as an aldol cyclase to control the regiospecific aldol cyclization of the extremely reactive poly- β -ketone intermediate assembled by an iterative type I polyketide synthases (PKSs). Up to now, only the structure of fungal PksA PT that mediates the first-ring cyclization via C4-C9 aldol cyclization is available. We describe here the structural and computational characterization of a bacteria PT domain that controls C2-C7 cyclization in orsellinic acid (OSA) synthesis. Mutating the catalytic His949 of the PT abolishes production of OSA and results in a tetraacetic acid lactone (TTL) generated by spontaneous O-C cyclization of the acyl carrier protein (ACP)-bound tetraketide intermediate. Crystal structure of the bacterial PT domain closely resembles dehydrase (DH) domains of modular type I PKSs in the overall fold, dimerization interface and catalytic "His-Asp" dyad organization, but is significantly different from PTs of fungal iterative type I PKSs. QM/MM calculation reveals that the catalytic His949 abstracts a proton from C2 and transfers it to C7 carbonyl to mediate the cyclization reaction. According to the structural similarity to DHs and the functional similarity to fungal PTs, we propose that the bacterial PT represents an evolutionary intermediate between the two tailoring domains of type I PKSs.

Introduction

Polyketides are structurally diverse natural products, constituting one of the most important sources of bioderived pharmaceuticals, for example, antibiotics, antitumor agents, and immunosuppressants [1, 2]. They are biosynthesized from coenzyme A (CoA)-activated carboxylic acids by multifunctional megaenzymes or multisubunit enzyme complexes called polyketide synthases (PKSs) that resemble fatty acid synthases (FASs), using a β -ketoacyl synthase (KS), acyl transferase (AT) and acyl carrier protein (ACP). The resulting ACP-bound poly- β -keto thioester intermediates are ultimately converted to highly diversified compounds by optional tailoring domains. PKSs are grouped into different types according to their biosynthetic machinery architectures, including modular type I PKSs, iterative type I PKSs, iterative type II PKS, and the ACP-independent freestanding iterative type III PKSs. Modular type I PKSs are widely distributed in bacteria, comprising multiple sets of domains that are organized into modules. In contrast, the iterative type I PKSs are usually found in fungal species, repeatedly reusing one module that is highly programmed to generate polyketides with diverse structural features by using different domain combinations in each iteration.

Iterative type I PKSs are further classified as non-reducing (NR), partial-reducing (PR), and highly-reducing (HR) PKSs according to the function and phylogeny[3-5]. NR-PKSs synthesize aromatic polyketides that have important biological activities, exemplified by the environmental carcinogen aflatoxin B1. The poly- β -ketone backbone assembled by a NR-PKS is extremely reactive. Spontaneous cyclization must be suppressed to form the unique cyclization pattern observed in the final product. PksA involved in biosynthesis of aflatoxin B1 is a model system to investigate NR-PKSs. Functional dissection of PksA reveals a unique product template (PT) domain between AT and ACP domains, which functions as an aldol cyclase mediating the regioselective cyclization of a hexanoyl-primed octaketide intermediate via

C4-C9/C2-C11 aldol condensations to generate a key intermediate of aflatoxin biosynthesis, norsolorinic acid (Figure 1) [6, 7].

NR-PKSs are divided into three basal subclades (subclades I–III) according to a phylogenetic analysis of KS sequences [8]. PTs embedded in subclades I and II are classified into five groups based on a phylogenetic analysis of those associated with known aromatic polyketides [9]. The well-studied PksA PT and other NR-PKS PTs involved in C4-C9/C2-C11 cyclization belong to Group IV. PTs of group I perform the regioselective C2-C7 cyclization of a tetraketide primed by different starter units and are embedded in NR-PKSs that synthesize polyketides containing a single aromatic ring, exemplified by the orsellinic acid synthase (OSAS) from *Aspergillus nidulans* [10, 11]. PTs of group II catalyze the C2-C7 cyclization of pentaketide intermediates to form the first aromatic ring of tetrahydroxynaphthalene (THN) compounds, of which the second ring is formed by thioesterase-catalyzed Claisen cyclization [12]. PTs of group III and group V mediate cyclization of longer intermediates to form polyketides containing multiply fused-ring structures. Group III PTs have C2-C7 regioselectivity while group V PTs cyclize the nascent polyketide via C6-C11/C4-C13 regioselectivity [9]. NR-PKSs in subclade III contain an additional methyltransferase (CMeT) domain compared to those in subclade I and II and produce aromatics with C2-C7 cyclization regioselectivity. The PTs of subclade III NR-PKSs are classified into groups VI and VII [13]. Recently, phylogenetic analysis of all known fungal NR-PKSs reveals a new group VIII PT domains [14].

Only the structure of PksA PT has been reported, displaying a distinct double hotdog (DHD) fold, a variant of that observed in the dehydrase (DH) domains of modular type I PKSs [7, 15]. PksA PT is dimeric in crystal structures, indicating the role of PT domains in dimerization of fungal NR-PKSs. A PT-specific sequence insertion is involved in dimerization of the two monomers and conserved in all known fungal NR-PKSs. Each monomer contains an internal pocket that extends 30 Å from the surface to the bottom for substrate binding and is visually divided into the phosphopantetheine (PPANT)-binding region, the cyclization chamber, and the hydrophobic hexyl-binding region. The PT active site is a His-Asp “catalytic dyad” located at the cyclization chamber. The His residue functions as a catalytic base, using its N δ as the basic nitrogen to deprotonate the substrate, while the Asp polarizes the His by hydrogen bond to N ϵ . A binary structure complexed with a bisisoxazole model of the experimentally inaccessible poly- β -ketone intermediate reveals additional protein-substrate interactions involved in orientation and stabilization during the multistep catalytic cycle. As the only PT domain that has been structurally characterized, PksAPT provides a vital model for understanding the high regioselective aldol cyclization of poly- β -ketone intermediates assembled by NR-PKSs.

Orsellinic acid (OSA) is synthesized in bacteria and, in most cases, functions as a building block that is further incorporated into biosynthesis of more complex natural products, for example, avilamycin produced by *Streptomyces viridochromogenes* [16] and calicheamicin produced by *Micromonospora echinospora* [17]. Cloning and analysis of biosynthetic gene clusters suggests that AviM and CalO5 are responsible for the assembly of OSA. Cultures of *Streptomyces lividans* TK24 and *Streptomyces coelicolor* CH999 containing AviM can produce OSA, confirming the function of AviM as OSAS [16]. Compared to fungal OSASs [11], AviM and CalO5 lack the starter-unit acyltransferase (SAT) domain

loading a primer for polyketide extension and the thioesterase (TE) domain releasing OSA from the PPANT group of the ACP domain (Figure 1A). Here, we confirm that AviM PT domain functions as an aldol cyclase to catalyze C2-C7 cyclization in OSA synthesis by comparing the products generated by AviM and its mutants. A tetraacetic acid lactone (TTL, 1) is generated by spontaneous O-C cyclization of the ACP-bound tetraketide backbone when the catalytic His949 of the PT domain is replaced by Ala or Phe. The structure of AviM PT, solved by selenomethionine incorporation and single-wavelength anomalous dispersion (SAD) phasing, closely resembles dehydratase (DHs) of modular type I PKSs in the overall fold, dimerization interface and catalytic “His-Asp” dyad organization. The catalytic His949 of AviM PT uses its N ϵ as the basic nitrogen to deprotonate the substrate, whereas the catalytic His1345 of PksA PT uses its N δ as the basic nitrogen [7, 15]. QM/MM calculation confirms that the catalytic His949 of AviM PT abstracts a proton from C2 and transfers it to C7 carbonyl in the aldol cyclization reaction. The structural similarity to DHs and the functional similarity to PTs suggest that the bacterial PT may represent an evolutionary intermediate between the two tailoring domains of type I PKSs.

Methods

General Methods and Materials

Strain cultivation and DNA manipulation followed standard methods. DNA sequencing and all primers were purchased from Tsingke Biotechnology Co., Ltd. (China). Primers used for PCR and site-directed mutagenesis are listed in Table S1. All Fast Digest enzymes were from Thermo Scientific. DNA polymerases were purchased from Invitrogen Trading Co., Ltd. (China). All the other chemicals and reagents were purchased from commercial suppliers and used without further purification. ClustalX was used in protein sequence alignments.

Cloning and Site-directed Mutagenesis

aviM was cloned from *S. viridochromogenes* Tü57 and digested with *Nde*I and *Hind*III restriction enzymes. The 3.9 kb DNA fragment was purified and ligated into pET28a to generate pET28-AviM. *aviM* PT was cloned and digested with *Nde*I and *Eco*RI restriction enzymes to obtain an 816 bp gene fragment, which was purified and ligated into pET28a to generate pET28-aviM-PT. The *sfp* gene was amplified from the genomic DNA of *Bacillus subtilis*. All constructs contain His₆ N-terminal tags for purification.

Site-directed mutations were generated by PCR using standard protocol according to the GeneTailor method (Invitrogen). Template plasmid was removed by *Dpn*I digestion at 37 °C for 1.5 hours. All constructed plasmids were confirmed by DNA sequencing.

Protein Expression and Purification

The *E. coli* BL21(DE3) cells transformed with corresponding plasmids were grown to an OD₆₀₀ of 0.4 in LB medium supplemented with 50 mg/mL kanamycin at 37 °C and induced with 0.3 mM IPTG for 12 h at

16 °C. The cells were harvested by centrifugation and resuspended in lysis buffer containing 500 mM NaCl, 50 mM Tris (pH 7.5). Following sonication, cell debris was removed by centrifugation at 15,000 g for 40 minutes. The supernatant was poured over a column of Nickel-NTA resin, which was then washed with 50 mL lysis buffer containing 30 mM imidazole and eluted with lysis buffer containing 300 mM imidazole. Proteins were further purified by using a Superdex 200/Superoose 6 gel filtration column (GE Healthcare Life Sciences) equilibrated with buffer containing 150 mM NaCl and 10 mM Tris (pH 7.5). All purification steps were carried out at 4 °C.

Heterologous Production of OSA

E. coli K207-3 was transformed with pET28a and its AviM derivatives. *E. coli* K207-3 containing pET-28a does not lead to any OSA production and served as a negative control. The corresponding overnight pre-culture was used for inoculation of 50 mL of LB medium. 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) was supplemented at OD₆₀₀ of 0.4. After 24h of incubation at 30°C and 220 rpm, the culture was acidified with concentrated HCl to pH 4 and extracted with ethyl acetate (v/v, 1:1). The organic layer was dried and dissolved in 0.5 mL of methanol. LC-MS was conducted with an Agilent 1290 Infinity Liquid chromatography and 6545 Quadrupole Time-of-Flight Mass Spectrometer by using negative electrospray ionization and a C18 reverse-phase column (TC-C18(2) 4.6 x 250 mm, 5 μ m, Agilent). The flow rate was 0.4 mL/min, and a binary solvent system gradient of 0.1% formic acid in water (A) and in acetonitrile (B) was used as follows: 0-10 min: 20-40% B, 10-20 min: 40% B, 20-30 min: 40-50% B, 30-32 min: 50-100% B, 32-35 min: 100% B, 35-36 min: 100-20% B, 36-45 min: 20% B. For MS/MS, HPLC system was coupled to a 6545XT AdvanceBio LC/Q-TOF mass spectrometer (Agilent Technologies) equipped with an ESI interface (Agilent Technologies) operating in negative ion mode using a capillary voltage of +4 kV. Other parameters were drying gas temperature, 325°C; drying gas flow, 13 L/min; and nebulizing gas pressure, 2 bar. Detection was carried out within a mass range of 50–1100 m/z. The MS/MS analyses were acquired by precursor ion scan: m/z 167.035, RT: 12.5 min, dwell time: 1 min. CE at 2 ev, 10 ev, 20 ev and 40 ev.

In vitro Enzymatic Assay of AviM

For the enzymatic assays of AviM, a typical 100 μ L reaction mixture contains 10 mM MgCl₂, 0.2 mM CoA, 50 μ M WT AviM or its mutant and 10 μ M sfp in 10 mM Tris, pH 7.4, 150 mM NaCl, and 10% glycerol. After incubation at 30°C for 1 h, the reaction mixture was supplemented with 1 μ L of acetyl-CoA (100 mM) and 2 μ L of malonyl-CoA (100 mM) and incubated at 25°C. The reaction was quenched with 5 μ L of 6 mM HCl and the solution was vortexed to precipitate the enzyme. Then the mixture was spun at 14,800 rpm for 10 min and the supernatant was subjected to LC-MS analysis. The method for LC-MS is as described as *in vivo* analysis.

TTL Synthesis

The synthesis of TTL has been described [18]. 2,2,6-Trimethyl-4H-1,3-dioxin-4-one **1** (3 mL, 22.5 mmol, 1.0 eq) and 1H-Benzotriazole **2** (1.0eq) in dry PhMe (80 mL) were heated for 11 h at 90 °C. Solvents were removed by rotary evaporator and a pale yellow solid **3** was generated. **1** (3.0 eq) was added dropwise to

LiN(SiMe₃)₂ (3.1eq) in THF (750 ml) at -78 °C and stirred for 1.5 h. Then, **3** was added and the mixture was warmed up to room temperature and stirred overnight. The reaction mixture was quenched with 1M aqueous HCl to pH 4. The mixture was extracted with EtOAc (3 x 250 ml), followed by washing with brine and drying with MgSO₄. The solvent was removed by rotary evaporator and the residual was dissolved in CH₂Cl₂ (250 ml) and washed with the Sigma-Aldrich 456101 buffer solution (pH 9.0, 5 x 50 ml). The organic layer was dried with MgSO₄ and the solvents were removed by rotary evaporator. The crude product was purified by silica gel column chromatography with hexanes/EtOAc (8:1 to 2:1) to give diketone-1,3-dioxinone **4** as a clear oil. **4** (100 mg, 0.44 mmol) in PhMe (30 ml) was heated for 8 h at 110 °C. The solvents were removed by rotary evaporator and the products were separated by silica gel column chromatography (hexanes:EtOAc=2:1) to provide **TTL** as a yellowish oil. **TTL**: ¹H NMR (400 MHz, DMSO-d₆): δ (ppm) 2.17 (s, 3H), 3.75 (s, 2H), 5.27 (d, *J* = 4 Hz, 1H), 6.05(d, *J* = 4 Hz, 1H); ¹³C NMR (100 MHz, DMSO-d₆): δ (ppm) 30.23, 47.73, 89.38, 103.08, 160.35, 164.20, 170.66, 203.31.

Phylogenetic Analysis

A dataset of 36 proteins was selected, including 27 PTs (2 from bacteria (AviM PT, CalO5 PT), 25 from fungi) with a supplement of a Branching domain from *rhizoxin* PKS as the outgroup[19]. The sequences were aligned with MAFFT-linsi (v7.480)[20] and trimmed with trimAL (v1.2)[21]. The final alignments have 279 trimmed columns. A phylogenetic tree was constructed by IQ-TREE (v1.6.12)[22] with “LG+G4” model and 1000 ultrafast bootstraps. The phylogenetic tree is edited by iTol.

Crystallization and Structure Determination

Crystallization of the Avim PT was performed using 1 µl of protein solution (8 mg/mL in 150 mM sodium chloride, 10 mM Tris-HCl pH 7.5) mixed with 1 µl of reservoir solution by sitting drop vapor diffusion at 20 °C. The best crystals were obtained in a solution containing 0.1 M Tris-HCl (pH 8.5), 1 M lithium chloride, 28% PEG 4000 (w/v) within 5 days. Selenomethionyl crystals were obtained in a similar condition. The selenomethionyl and native data were collected at wavelengths of 0.97853 Å at 100 K. All the datasets were collected at the beamline BL-18U1, Shanghai Synchrotron Radiation Facility (SSRF), China, and processed using HKL2000. Crystals belonged to space group P2₁ with similar unit-cell dimensions for the native and selenomethionyl crystals. Experimental phases were determined using AutoSol in Phenix. The model was iteratively built in Coot[23] and refined in Refmac[24].

Computational Details

The tetraketide-PPANT substrate was docked into the active site of AviM PT by AutoDock software[25]. The parameters for tetraketide-PPANT were generated at the HF/6-31G(d) level with the Gaussian 16 program[26]. The restrained electrostatic potential (RESP)[27] charge fitting procedure was employed on the tetraketide-PPANT and the missing parameters were generated by the Antechamber package. The complex was solvated in a cubic box of TIP3P water molecules with 10 Å thickness and Na⁺ counterions were added to achieve charge neutralization. MD simulations of the complex were run with the AMBER

software package (version 18)[28] . The enzymes and substrate were described under ff12SB and GAFF force fields from the AMBER18 software package. The Particle Mesh Ewald (PME) [29] method was conducted to account for long-range electrostatic interactions. The SHAKE algorithm was employed to fix angles and bonds involving hydrogen atoms[30]. Prior to production MD simulations, the solvated system was treated with minimization and progressive heating, with the system temperature increasing from 0 to 298 K over 50 ps. After 50 ps equilibration, the complex conducted six-times 50 ns production simulations with a different random number without any restraints under NPT conditions. Then, the conformation that favored the reaction taking place was selected as the initial structure for the QM/MM calculations. The QM calculations were performed with the M06-2x/6-31G(d) method and the MM region was under the AMBER Parm99 force field, utilizing a two-layered ONIOM method in the Gaussian16 program. We selected the side chain of the H949, D1104 as well as a portion of the backbone of A959 and V1123. We also selected the entire tetraketide, the sulphur atom and the two adjacent CH₂ groups of the substrate as QM region. Additionally, the H₂O that was positioned between the substrate C7 carbonyl and the D1104 side chain was retained at the QM layer. The rest of the system was treated as the MM region.

Results

AviM PT Catalyzes C2-C7 Cyclization of OSA

AviM is previously shown to synthesize OSA in *S. lividans* TK24 and *S. coelicolor* CH999 [16]. Here we used *E. coli* K207-3 [31], an *E. coli* B-derived strain developed for the heterologous expression of polyketide biosynthetic genes, to investigate the function of AviM *in vivo*. The DNA fragments encoding AviM was amplified by PCR and ligated into pET28a. The resulting pET28a-*aviM* was transformed into *E. coli* K207-3 to give the recombinant strain for OSA production, while the *E. coli* K207-3 carrying the vector pET28a was used as the control strain. LC-MS analysis revealed that cultures of *E. coli* K207-3 containing pET28a-*aviM* were able to produce OSA (Figure 2).

A conserved H⁹⁴⁹XXXGXXXXP⁹⁵⁸ motif was found in the N-terminus of AviM PT domain by sequence alignment of AviM with various fungal NR-PKSs (Figure S1). The His949 residue, corresponding the catalytic His1345 of PksA PT, was replaced by site-specific mutagenesis with Ala or Phe. LC-MS analysis showed that the mutations of His949Ala and His949Phe completely abolished OSA production of AviM in *E. coli* K207-3. However, the extracted ion chromatography (EIC) revealed a new product that had the same molecular weight but different retention time relative to OSA. MetFrag was used to identify the new product with a relative mass deviation of 5 ppm and an absolute mass deviation of 0.002 Da as thresholds [32]. Tetraacetic acid lactone (TTL, **1**) was retrieved from PubChem as a potential candidate (Figure S2). Subsequently, the new product was identified as TTL by comparing with the standard prepared by organic synthesis (Figure 2A). Obviously, the functions of the KS, AT, and ACP domains are not affected by mutations in the PT domain. The new TTL product is generated by spontaneous O-C cyclization of the ACP-bound tetraketide backbone in the absence of PT domain (Figure 1B). In contrast, a C-C bond is formed between C2 and C7 by aldol cyclization in the presence of PT domain, followed by

aromatization, to yield final OSA product. Analysis of the LC-MS data of the wild type AviM also revealed the presence of **TTL**, whereas the production of TTL was significantly increased in H949A and H949F mutants (Figure 2A). Interestingly, a triacetic acid lactone (TAL, **2**) released after two condensation steps was also observed in the cultures of the wild-type AviM and mutating the catalytic H949 also increased the production of the TAL (Figure S3)

In vitro functional assays of AviM were carried out to exclude the possible effects of other enzymes encoded in *E. coli* K207-3 genome. The recombinant AviM was expressed in *E. coli* BAP1 containing a *sfp* gene from *Bacillus subtilis* [33]. The purified AviM was further incubated with coenzyme A (CoA) and *sfp* enzyme to ensure the phosphopantetheinylation of all proteins. Trace amount of OSA was indeed produced when AviM was incubated with acetyl-CoA and malonyl-CoA *in vitro* (Figure 2B). These *in vitro* and *in vivo* results confirm that AviM alone can mediate the assembly and release of OSA. Replacing the putative catalytic His949 by the Ala residue destroyed the aldol cyclase activity of AviM PT domain. TTL instead of OSA was produced by the AviM His949Ala mutant. In short, these *in vivo* and *in vitro* assays establish that the PT domain of AviM functions as an aldol cyclase to control the C2-C7 cyclization in OSA biosynthesis. Consequently, we proposed a OSA biosynthesis pathway presented in (Figure 1B). The poly- β -keto chain elongation first starts with an acetyl-CoA building block. After two iterative rounds of decarboxylative Claisen condensations using malonyl building blocks, some intermediates are released by spontaneous O-C cyclization to yield a shunt product TAL. The remaining intermediates are condensed with the third malonyl-CoA molecule to form the ACP-bound tetraketide intermediate which were cyclized between C2 and C7 by the PT domain and released as OSA.

Overall Structure of AviM PT Resembles DHs instead of PksA PT

PT domains catalyze the first-ring cyclization via three common patterns (C2-C7, C4-C9, and C6-C11). Up to now, only PksA PT (group IV) that cyclize the first ring via C4-C9 regiospecificity has been structurally characterized, hindering attempts to rationally control the cyclization patterns of poly- β -keto backbones. Our *in vivo* and *in vitro* functional assays revealed that AviM PT controls the C2-C7 cyclization in OSA biosynthesis. To understand the molecular basis of the aldol cyclization step, we solved the crystal structure of the AviM PT domain.

Diffraction-quality crystals were obtained by sitting-drop method using polyethylene glycol 4000 as a precipitant. The protein crystallized in space group $P2_1$ with two independent monomers per asymmetric unit. The structure was solved to 2.0-Å resolution through selenomethionine incorporation and SAD phasing (Table S2). The histidine-tag and the first ten residues are invisible in both monomers. Residues 946-950 and 1055-1069 are invisible in monomer B but visible in monomer A. The two monomers in the asymmetric unit have essentially identical folds with a rmsd of 0.4 Å for Ca positions (Figure S4). An automatic search of AviM PT for structurally homologous proteins using the Dali server revealed low sequence identity (less than 20%) to all previously reported structures. Interestingly, AviM PT shows more structural similarity to dehydratase (DH) domains than to PksA PT. Each monomer comprises a DHD fold of DH domains (Figure 3), characterized by a continuous antiparallel β -sheet curving around two α -helices

(α HD1 from the N-terminal and α HD2 from the C-terminal hotdog folds). The C-terminal hotdog (residues 1056-1174) possesses an additional helix (α C) compared to the N-terminal hotdog (residues 915-1032). These two hotdogs are connected by a 23-residue-long flexible linker containing two short helices (α L1 and α L2). AviM PT most closely resembles the DH domain from CurF module of curacin PKS (Protein Data Bank (PDB) codes 3KG6, 2.4 Å Ca rmsd; 19% sequence identity) [34]. The most notable difference is in the C-terminal hotdog of CurF DH, which contains an additional 3_{10} helix (Figure S4). The PksA PT is ~70-residue larger than the AviM PT. They are superimposed with a rmsd of 3.2 Å for 147 (of 260 possible) Ca positions. Compared to AviM PT, four obvious differences are observed in PksA PT (Figure 3B): 1) the N-terminal hotdog and the C-terminal hotdog are connected by a bent long helix; 2) the α HD1 from the N-terminal hotdog is two turns longer; 3) the β N1 and β N2 strands of the N-terminal hotdog are much longer; and 4) the ten C-terminal residues of the PksA PT form a helix that cap the substrate binding tunnel.

The two monomers in the asymmetric unit dimerize through N-terminal β strands and bury a surface area of 665 Å² (calculated by PDBe PISA). The relatively large dimer interface and the observed migration behavior as a dimer on the size exclusion column suggest that the PT domain is involved in oligomerization of AviM (Figure S5). The β -hairpin turns between β N1 and β N2, and between β N5 and β N6 contribute significantly to the dimer interface (Figure 3A). Deleting the PT domain of AviM (residues 915-1174) abolished the production of both OSA and TTL (Figure 2A), suggesting that the deletion of PT probably caused an architectural change rendering the megaenzyme inactive. The angle between the two monomers is ~180° in CurF DH and this extended conformation has been observed in all previously reported structures of PKS DH domains. However, the two monomers of AviM PT are obviously in a different relative orientation, forming an angle of ~150° (Figure S4C and D). The observed conformation of the excised PT domain may reflect the conformation in the complete AviM megacomplex. Interestingly, the dimer interface of AviM PT is completely different from that observed in PksA PT, of which the two monomers dimerize through the C-terminal hotdog, involving the α C helix, the β strand following the bent long helix linker, and the linker connecting α HD2 and β 10 (Figure 3A).

AviM PT Possesses a Substrate Tunnel Distinct from DHs and PksA PT

The “catalytic dyad” of AviM PT is in the similar orientation as observed in DH domains. The His949 is from the “cap loop” of the N-terminal hotdog and the Asp1104 from the helix α HD2 of the C-terminal hotdog. The N δ of His949 is involved in a hydrogen bond with the backbone carbonyl oxygen of Ile956, indicating that the catalytic His949 uses its N ϵ as the basic nitrogen to deprotonate the substrate (Figure 4). The Asp1104 is 5 Å from the His949 and the Asp1104Ala mutant shows decreased production of OSA (Figure 2A), indicating that the Asp1104 may be involved in catalysis indirectly. In contrast, the catalytic Asp1543 directly polarizes the catalytic His1345 by a hydrogen bond to N ϵ in PksA PT and mutating Asp1543 results in no detectable activity (Figure 4x) [7, 15]. The active site is positioned at the middle of a 25 Å substrate binding tunnel that is located on top of two DHD helices and closed off by the cap loop (Figure 4B). The substrate binding tunnel starts at the junction of the N- and C-terminal hotdog and extends into the C-terminal hotdog fold. The entrance is bounded by the β N3b, the β C3, and the C-

terminal end following the β C6, while the bottom is bounded by the C-terminal of the α C and the N-terminal of the β C1. The volume of the AviM PT substrate binding tunnel ($761 \text{ \AA}^3$) is as same as that of CurF DH ($762 \text{ \AA}^3$), but is significantly smaller than that of PksA PT ($1196 \text{ \AA}^3$), which contains an additional hydrophobic binding region lying at the bottom of the tunnel to accommodate the hexyl group derived from the hexanoyl-CoA starter unit. Compared to the tunnel of PksA PT that is closed off at the bottom by the β strands of the C-terminal hotdog (Figure 4C), the tunnels of AviM PT and CurF DH are open at the bottoms (Figure 4B and 4D). Interestingly, the bottom of the tunnel is differently positioned in AviM PT and CurF DH due to the alternative orientation of the α C helix. The bottom is positioned on top of the middle of the α HD2 in AviM PT. However, in CurF DH, the bottom is positioned at different side of α C helix and located on top of the C-terminal end of the α HD2. As a result, the tunnel of AviM PT is straight while the tunnel of CurF DH is bent almost 90° at the catalytic His1720.

QM/MM Calculation of C2-C7 Cyclization Catalyzed by AviM PT

The proposed mechanism for AviM PT-catalyzed cyclization involves formation of an enolate intermediate by deprotonation of C2 and subsequent aldol addition to the C7 carbonyl (Scheme 1). Electrostatic potential surface indicates the substrate tunnel is negatively charged (Figure S6). The polar microenvironment favors the C7 carbonyl in its electrophilic keto tautomeric form. We used QM/MM calculation to understand the molecular basis of the C2-C7 cyclization. The structure of PksA PT complexed with the bisisoxazole guided the placement of the tetraketide-PPANT substrate in the active site of AviM PT [15]. The resulting complex was subjected to MD simulations. The “active” conformer was selected according to two distances ($d(\text{Ne-H}\alpha)$ and $d(\text{C2-C7})$) and optimized at the ONIOM(M06-2X/6-31G(d): Amber) level. In the resulting reactant, the C1 and C3 carbonyl of the substrate form hydrogen bonds with the backbone NH of A959 and V1123 respectively. In addition, a water molecule is positioned between the substrate C7 carbonyl and the D1104 side chain (Figure 5). In the first stage, the C2 is deprotonated by the Ne of the catalytic H949. The first transition state (TS1) was observed when the H was located at $1.53 \text{ \AA}$ from the C2 and at $1.23 \text{ \AA}$ from the Ne (Figure 5B). The C1-C2 and C2-C3 bonds decrease from $1.51 \text{ \AA}$ in reactant to $1.40 \text{ \AA}$ in the first intermediate (IM1), while the C=O bonds of C1 and C3 increase from $1.21 \text{ \AA}$ in reactant to $1.23 \text{ \AA}$, suggesting the formation of an enolate structure. Consequently, the negative charge is distributed between the C2 and two adjacent carbonyls. Next, the nucleophilic C2 launches an attack on the C7. Simultaneously, the C7 carbonyl deprotonates the H949 Ne to form a hydroxy. During the process, the H of the H949 Ne gradually turns to the C7 carbonyl accompanying the decrease of the distance between C2 and C7. In TS2, the $d(\text{C2-C7})$ and $d(\text{H-O7})$ distances are $2.56 \text{ \AA}$ and $1.15 \text{ \AA}$ respectively, whereas they are further decreased to $1.56 \text{ \AA}$ and $0.99 \text{ \AA}$ in IM2. The deprotonation of C2 conquers a 14.0 kcal/mol energy barrier and the subsequent ring cyclization overcomes a 14.1 kcal/mol barrier (Figure 5C and Figure S9). The two steps are feasible with low reaction barriers. Besides, the deprotonation and aldol addition are exergonic with 7.5 kcal/mol . Following aromatization could occur with a loss of hydroxide by an E1cb stepwise mechanism as proposed for PksA PT [7].

Discussion

NR-PKSs utilize PT domains to mediate the regioselective cyclization of highly reactive poly- β -ketone intermediates. According to the phylogenetic analysis, fungal PTs are divided into 8 groups. OSAS PTs belong to the group I and perform the regioselective C2-C7 cyclization. Compare to their fungal counterparts, bacterial OSASs lack the SAT and TE domains. Our *in vitro* assays indicate that the AviM alone can synthesize OSA from acetyl-CoA and malonyl-CoA, but is inefficient (Figure 2B). It's possible that other enzymes help the initiation and/or release steps *in vivo*. AviM PT has low sequence similarity to fungal PTs. We confirmed the aldol cyclase of AviM PT by mutating its catalytic His949. The TTL derived from spontaneous O-C cyclization of the ACP-bound tetraketide was produced both *in vivo* and *in vitro*.

The overall structure and the active site of AviM PT resemble DHs of modular type I PKSs instead of the PksA PT. Compared to AviM PT, PksA PT uses different structural elements to form the dimer interface and has an additional helix to cap the substrate binding tunnel. The His-Asp "catalytic dyad" is also oriented differently. In PksA PT, the side chain of the catalytic Asp1543 makes a hydrogen bond with the N ϵ of the catalytic His1345 [7]. Therefore, the polarized His1345 use its N δ as the basic nitrogen to deprotonate the substrate. In contrast, the catalytic His949 of AviM PT is polarized by a neighboring backbone carbonyl oxygen and use its N ϵ as the basic nitrogen, as observed in DHs of modular type I PKSs. The corresponding Asp1104 is ~ 5 Å from the His949 and the Asp1104Ala mutant still retains more 10% catalytic activity (Figure 2A). QM/MM calculations demonstrate that the His949 initiates the reactions by abstracting a proton from C2 and transfers it to C7 carbonyl during formation of the C2-C7 bond (Figure 5). We built a model of CalO5 PT, another bacterial OSAS PT, by using AlphaFold [35]. The structure is similar to AviM PT both in overall structure and in organization of His-Asp "catalytic dyad" (Figure S7). Models of four fungal OSAS PTs were also built and showed overall similarity to PksA PT. Iterative type I PKSs are usually found in fungi, whereas Wang *et al*/recently discovers numerous iterative type I PKSs in *Streptomyces* and proposes the horizontal-gene-transfer hypothesis on the origin of fungal iterative type I PKSs [36]. The observation, that AviM PT structurally resembles DHs of modular type I PKSs but catalytically acts as an aldol cyclase, suggests that bacterial PTs may be evolutionary intermediates between two tailoring domains of type I PKSs. The phylogenetic tree constructed by PTs and DHs shows that all the sequences are consistent with host species. Bacterial PTs nest within the clade containing DHs. These two groups of enzymes make up a large clade that is sister to fungal PTs. Bacterial PTs are phylogenetically distant from the fungal PTs but close to DHs, consistent with the functional and structural analysis (Figure S8).

In summary, AviM PT acts as an aldol cyclase, mediating C2-C7 cyclization in OSA biosynthesis. Its inactivation results in generation of TTL derived from spontaneous O-C cyclization of the ACP-bound tetraketide. Structural comparison reveals that the dimer interface, the overall architecture, and the "catalytic dyad" of AviM PT are similar to DHs of modular type I PKSs. These results suggest that bacterial PTs may represent an evolutionary intermediate between DHs and PTs of type I PKSs.

Declarations

Data Availability

Atomic coordinates of two structures have been deposited in PDB with accession codes 7VWK.

Supplementary Data

Supplementary Data are available Online.

Acknowledgements

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Figures

Figure 1

Biosynthesis reaction of OSA and Aflatoxin. (A) The domain compositions of bacterial and fungal OSASs. Bacterial OSASs lack the starter-unit acyltransferase (SAT) and the thioesterase (TE) domains. (B) Biosynthesis of OSA in bacteria. Triacetic acid lactone (TAL) released after two condensation steps and tetraacetic acid lactone (TTL) are generated in the absence of PT due to the spontaneous O–C

cyclization. AviM PT catalyzes the C2-C7 cyclization. (C) Biosynthesis reaction of Aflatoxin. PksA PT catalyzes the C4-C9 and C2-C11 cyclization.

Figure 2

In vivo and in vitro assays of AviM-catalyzed OSA biosynthesis. (A) *E. coli* K207-3 expressing AviM synthesizes OSA. Mutating the catalytic H949 abolishes OSA production, but results in TTL from spontaneous O-C cyclization of the ACP-bound tetraketide backbone. (B) OSA is produced when AviM is incubated with acetyl-CoA and malonyl-CoA in vitro, while TTL is produced by AviM mutant. The traces show the extracted ion counts (m/z: 167.0350) monitored in the negative mode.

Figure 3

Overall structure of of AviM PT. and PksA PT. (A) The dimerization interface of AviM PT is different from that of PksA PT. (B) Differences between AviM PT monomer and PksA PT monomer. (C) The topology structure of AviM PT. The monomer has a N-terminal hotdog, a C-terminal hotdog and a linker containing two short α helices.

Figure 4

The active site of AviM PT. (A) The N δ of the catalytic His949 forms a hydrogen bond with the backbone carbonyl of Ile956 in AviM PT. (B) The N ϵ of the catalytic H1345 forms a hydrogen bond with the side chain of the catalytic Asp1543 in PksA PT. (C) The substrate tunnel of AviM PT is different from that of PksA PT and CurF DH.

Figure 5

QM/MM calculation of AviM PT complexed with the substrate. (A) Optimized structure of reactant. (B) Structures of TS1, IM, TS2 and IM2 obtained with. (C) The energy profiles for the deprotonation of C2 and the ring formation at the M06-2x/6-31G(d): AMBER level. The distances are given in Å.

Supplementary Files

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