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Complete Reconstitution of a Highly-Reducing Iterative Polyketide Synthase

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Abstract

Highly-reducing iterative polyketide synthases are large multifunctional enzymes that make important metabolites in fungi, such as lovastatin, a cholesterol-lowering drug from *Aspergillus terreus*. We report efficient expression of LovB (the Lovastatin Nonaketide Synthase) from an engineered strain of *Saccharomyces cerevisiae*, and complete reconstitution of its catalytic function in the presence and absence of cofactors (NADPH, SAM) and its partner enzyme, the enoyl reductase LovC. The results demonstrate that LovB retains correct intermediates until completion of synthesis of dihydromonacolin L, but off-loads incorrectly processed compounds as pyrones or hydrolytic products. Experiments replacing LovC with analogous MlcG from compactin biosynthesis demonstrate a gate-keeping function for this partner enzyme. This study represents a key step in the understanding the functions and structures of this family of enzymes.

Nature uses an amazing array of enzymes to make natural products (1). Among these metabolites, polyketides represent a class of over 7000 known structures of which more than 20 are commercial drugs (2). Among the most interesting but least understood enzymes making these compounds are the highly-reducing iterative polyketide synthases (HR-IPKSs) found in filamentous fungi (3). In contrast to the well-studied bacterial type I PKSs that operate in an assembly-line fashion (4), HR-IPKSs are megasynthases that function iteratively by using a set of catalytic domains repeatedly in different combinations to produce structurally diverse fungal metabolites (5). One such metabolite is lovastatin, a cholesterol-lowering drug from *Aspergillus terreus* (6). This compound is a precursor to simvastatin (ZocorTM), a semi-synthetic drug that had annual sales of over \$4.3 billion prior to loss of patent protection in 2006 (7).

Biosynthesis of lovastatin proceeds via dihydromonacolin L (acid form **1**; lactone form **2**), a product made by the HR-IPKS, LovB (Lovastatin Nonaketide Synthase), with assistance of a separate enoyl reductase, LovC (8) (Fig. 1). LovB is a 335 kDa protein that contains single copies of ketosynthase (KS), malonyl-CoA:ACP acyltransferase (MAT), dehydratase (DH),

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Reconstitution of catalytic function provides insight into how multifunctional enzymes synthesize important natural products.

methyltransferase (MT), ketoreductase (KR), acyl-carrier protein (ACP) domains and a section that is homologous to the condensation (CON) domain found in Nonribosomal Peptide Synthetases (NRPSs) (9). It also contains a domain that resembles an enoyl reductase (ER) but lacks that activity. LovB must catalyze ~35 reactions and use different permutations of tailoring domains after each of the eight chain extension steps to yield the nonaketide, dihydromonacolin L **2**. This enzyme also catalyzes a biological Diels Alder reaction during the assembly process to form the decalin ring system (10). *In vitro* studies of LovB (11) have been hampered by inability to obtain sufficient amounts of the functional purified megasynthase from either *A. terreus* or heterologous *Aspergillus* hosts. As a result, the programming that governs metabolite assembly by LovB or other HR-IPKSs is not understood. Key aspects that remain to be elucidated include: 1) the catalytic and structural roles of each domain in the megasynthase; 2) substrate specificities of the catalytic domains and their tolerance to perturbation in megasynthase functions; and 3) factors governing the choice of different combinations of domains during each iteration of catalysis. To initiate such studies, we engineered an expression system in yeast to produce large amounts of LovB and examined the influence of cofactors and the enoyl reductase partner (e.g. LovC) on product formation.

The engineered *Saccharomyces cerevisiae* strain BJ5464-NpgA, which contains a chromosomal copy of the *Aspergillus nidulans* phosphopantetheinyl (ppant) transferase gene *npgA* (12), was the expression host. A C-terminus hexahistidine tagged LovB was placed under the control of the *S. cerevisiae ADH2* promoter (13,14) on an episomal plasmid (YEplovB-6His). Significant amounts of the intact LovB could be purified from the soluble fraction to near homogeneity with a final yield of ~ 4.5 mg/L (Fig. S1). The identity of the recombinant LovB was verified using mass analysis of tryptic digest fragments. The ACP domain of LovB was determined to be nearly completely phosphopantetheinylated by using a ppant ejection assay with high resolution Q-TOF mass spectrometry (Fig. S2). To ascertain activity of the resulting LovB and to examine the necessity for cofactors, malonyl-CoA alone was first added to the purified enzyme in buffer. Whole cell feeding studies of doubly [¹³C, ²H]-labeled acetate to cultures of *A. terreus* showed that all three acetate hydrogens were incorporated into the acetate-derived starter units for both the nonaketide and diketide moieties in lovastatin (15). Interestingly, the purified LovB can use malonyl-CoA for both chain priming and chain elongation, loading malonate with decarboxylation to make the acetyl starter unit. While LovB is able to prime with, and elongate the chain by two further condensations with malonyl-CoA, in the absence of NADPH no ketoreduction occurs. The dominant product is lactone **3** (Fig. 2A, trace i), which forms by enolization and cyclization with off-loading of the unreduced triketide. Addition of NADPH to this system enables function of the KR domain. In this and subsequent experiments, the malonyl-CoA could be conveniently synthesized *in situ* by malonyl-CoA synthase (MatB) from *Rhizobium trifolii* using free malonate and coenzyme A (16). With KR enabled, LovB makes penta-, hexa- and heptaketide pyrones **4-6** as well as ketones **7** and **8** (Fig. 2A, trace ii). The structures were confirmed by chemical synthesis of authentic standards except for heptaketide **6**, which proved very unstable. However, the mass increase of 26 amu for **6** and its red shift in the UV spectrum when compared to **5** are consistent with its proposed heptaketide pyrone structure (Table S3). Compounds **7** and **8** result from thioester hydrolysis of penta- and hexaketides stalling on the ACP at the β -keto stage. The resulting β -keto acids spontaneously decarboxylate to afford **7** and **8**. Formation of **4-8** illustrates that derailment in the normal programmed steps, namely lack of methylation due to absence of *S*-adenosylmethionine (SAM), stalls chain elongation and promotes off-loading from the ACP. This occurs either by addition of two acetate units without reduction followed by pyrone formation (**4-6**, major route) or by addition of one acetate unit and hydrolysis/decarboxylation of the shunt intermediates (**7-8**, minor route) (Fig. 2B). In either case the shut-down of chain elongation and reductive processing is not absolute: LovB is able to create longer homologs (i.e. **5**, **6**, **8**) after failure of methylation at the tetraketide stage.

SAM was then added to the system to enable construction of the natural intermediate, methylated tetraketide **11**. However, as enoyl reduction cannot occur in the absence of LovC, methylated hexaand heptaketide pyrones **9** and **10** are the primary products (Fig. 2A, trace iii). This is consistent with previous results where **9** and **10** are generated when LovB is expressed in *A. nidulans* in the absence of LovC, or when LovC is inactivated in the producer *A. terreus* (8).

Methylated tetraketide **11** must undergo enoyl reduction enroute to **2**. To examine the influence of the enoyl reductase, active LovC was added to LovB, NADPH and malonyl-CoA in the absence of SAM. It might be expected that a des-methyl version of dihydromonacolin L (i.e. **12**) would be produced, but **5-8** are obtained instead. This suggests that the α -methyl substitution, while not required for the function of KR and DH, is a prerequisite for enoyl reduction of the tetraketide. One possibility is that LovC has stringent substrate specificity at the tetraketide stage for an α -methyl substituted chain. Alternatively, absence of SAM or of a methylated substrate chain could prevent LovB from interacting with LovC.

We then attempted to reconstitute the synthesis of dihydromonacolin L **1** *in vitro*. Equimolar amounts (25 μ M) of purified LovB and LovC were incubated at 25°C for 12 hours with all cofactors (NADPH, SAM) and malonyl-CoA. The resulting sample was extracted under acidic conditions to convert any **1** into **2**. However, LC-MS analysis revealed **2** was not present in the extract (Fig. 3A, trace ii). Interestingly, no truncated products are observed. This suggests that LovB may not be able to release **1** because it lacks a thioesterase (TE) domain to free the completed product. To examine if the polyketide remains covalently attached to the ACP domain of LovB, in an analogous experiment the mixture was treated with base to hydrolyze thioester bonds prior to acidic extraction. LC-MS analysis revealed the emergence of an m/z [M+H]⁺ 307 ion eluting at the exact retention time of the standard **2** (Fig. 3A, trace iii, Fig S11). As expected, repetition of the experiment with [2-¹³C]malonate gives a peak at the same retention time with m/z [M+H]⁺ 316 (Fig. 3A, trace iv, Table S3). Time course analysis reveals that ~30 ng of **2** can be recovered at the plateau level (Fig. S12), which corresponds to ~3% of the theoretical yield of the single turnover experiment and suggests that some of the LovB is inactive. The reconstituted system possesses the entire range of catalytic activities and does not generate truncated products.

The failure of LovB to release **1** is surprising because the heterologous host *Aspergillus nidulans* expressing LovB and LovC produces substantial quantities of dihydromonacolin L (8). Cleavage of the LovB ACP-thioester of **1** may be achieved by TEs involved in fatty acid biosynthesis or unrelated PKS pathways. To test this, an excised fungal TE domain from the *Gibberella zeae* PKS13 (17), which has broad specificity, was added to the *in vitro* reaction. Interestingly, this leads to release of **1** and enzyme turnover, which can be detected by production of lactone **2** after mild acid extraction (no base added) (Fig. 3A, trace v). Time-course analysis (Fig. S12) demonstrates that PKS13 TE can support generation of **1** linearly for ~12 hours to give a >10-fold increase in product. Turnover is dependent on the catalytic triad of PKS13 TE, as mutation of its active site histidine 2009 to alanine completely abolishes the release of **1** (Fig. S12). Other fungal TE domains, such as the *Gibberella fujikuroi* PKS4 TE domain (18), also allow liberation of **1**, albeit at 50% the rate of PKS13 TE. In contrast, TE domains from bacterial type I PKSs, such as that from the erythromycin PKS (19), do not show any detectable release of **1** (Fig. S12). These experiments suggest that cleavage of **1** from LovB in *Aspergillus* can proceed via thioesterases not present in the gene cluster for lovastatin biosynthesis.

We next examined whether the CON domain in LovB is required for synthesis of **1**. This portion of LovB could be an evolutionary relic derived from a fungal PKS-NRPS hybrid in which an entire NRPS module (consisting of CON, Adenylation and Peptidyl Carrier Protein domains)

is present (20,21). Using *S. cerevisiae* BJ5464-NpgA, a soluble truncated variant LovB-ΔC was expressed that terminates at the end of the ACP boundary (Ser2542) and lacks the CON domain. A combination of purified LovB-ΔC (in place of full length LovB) and LovC, PKS13 TE and the other required cofactors does not produce detectable amounts of **2** (Fig. S14). A single turnover experiment (using base hydrolysis workup) with LovB-ΔC, LovC and required cofactors but no PKS13 TE did not give **2** (Fig. S14). Removal of the CON domain does not affect the functions of the minimal PKS and the tailoring domains. The synthesis of **3**, **4-8**, or **9-10** proceeds as efficiently as with full length LovB using LovBΔC alone, LovB-ΔC with NADPH, or LovB-ΔC with NADPH and SAM, respectively. The standalone CON protein (22) was then added in equimolar amount to the LovB-ΔC. Remarkably, the CON domain can interact with LovB-ΔC *in trans* and afford **2** in both the single turnover (base hydrolysis workup) and the PKS13 TE-mediated release assays (Fig. S14). The yields of **2** in both assays are lower than with the full length LovB, which may be due to the less efficient *in trans* protein-protein interactions. These *in vitro* experiments indicate a critical but still enigmatic role for this domain in controlling correct biosynthesis of **1**.

To examine whether replacement of the *in trans* interaction of LovB with LovC is possible, we cloned MlcG, an analogous, dissociated ER from the compactin biosynthetic gene cluster in *Penicillium citrinum* (Fig. S3) (23). Compactin is a 6-desmethyl analog of lovastatin, and **12** is a proposed intermediate in its biosynthesis. Hence, the compactin nonaketide synthase MlcA and its ER partner MlcG are programmed to function normally in the absence of methylation at the tetraketide stage, in contrast to the LovB/LovC complex. Interestingly, substitution of LovC with MlcG (72% identity, 83% similarity to LovC) in the absence of SAM affords desmethyl-dihydromonacolin L **12** *in vitro* instead of pyrones **5-8** (Figure 3B, trace iv). Chemical synthesis of optically pure **12** as a standard confirmed its identity. The yield and the turnover rate (with PKS13 TE) for **12** are similar to those for **2**, indicating that skipping the methylation step has little effect on later stages of LovB synthesis. Addition of SAM to LovB and MlcG affords **2** in similar yield as the native enzyme pairing of LovB and LovC (Figure 3B, trace i and ii), thereby demonstrating that MlcG is tolerant of both methylated and unmethylated tetraketide substrates (Figure 3C). Thus LovC is highly specific towards a *methylated* tetraketide, which is surprising considering it must be capable of reducing α-unmethylated pentaketide and heptaketide intermediates during the biosynthesis of **1**. It is clear from the ability of MlcG and LovB to produce **12** in the absence of SAM, or **2** when this cofactor is added, that the gate-keeping mechanism for the normal synthesis in *A. terreus* resides with the enoyl reductase LovC.

In conclusion, we demonstrated characterization of a purified HR-IPKS system by reconstituting *in vitro* the entire range of activities of LovB and its partner LovC. Together, these two enzymes catalyze the synthesis of **1** with excellent control of processivity, stereochemistry and regioselectivity. Addition of heterologous TE domains facilitates release of the final product. This approach opens the door to structural analysis of the proteins with partly assembled intermediates and provides a basis for understanding the programming rules of HR-IPKSs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Proposed mechanism of dihydromonacolin L **1** synthesis by LovB and the accessory ER LovC. LovB (335 kDa) consists of eight discrete domains and operates iteratively to condense nine malonyl-CoA equivalents to yield the nonaketide product **1**. Loading of the megasynthase by malonyl-CoA is presumably followed by decarboxylation to yield the acetyl starter unit (not shown). Each round of Claisen-condensation is catalyzed by the KS domain, while the growing polyketide is tethered to the phosphopantetheinyl (shown in squiggle line) arm of the ACP. After each condensation, the polyketide is subjected to a different combination of tailoring, which can include α -methylation by the MT domain, β -ketoreduction by the KR domain, β -dehydration by the DH domain, and α - β -enoylreduction by the dissociated LovC. The different tailoring permutations after each round of chain extension yield a triene-containing hexaketide thioester that can undergo a stereospecific Diels-Alder cyclization to yield the decalin portion of **1**. After formation of the nonaketide, the chain is released to yield the ring open form **1**. The acid form of **1** can undergo lactonization to yield **2**.

Fig. 2. Polyketides synthesized by LovB under different assay conditions in the absence of LovC. **(A)** Trace i: reaction with LovB afforded the triketide lactone **3** which confirms the function of the minimal PKS domains of LovB; Trace ii: reaction with LovB and NADPH (2 mM) afforded a number of highly conjugated compounds **4-8**; and Trace iii: reaction with LovB, NADPH (2 mM) and SAM (2 mM) afford the methylated, conjugated pyrones **9** and **10**. All reactions used MatB to regenerate malonylCoA, and were extracted with ethyl acetate (EA)/acetic acid (AcOH) (99/1); **(B)** The fate of the tetraketide intermediate in the absence of SAM and LovC. The correct tailoring yields the key intermediate **11**. In the absence of α -methylation, pyrones **4-6** can form readily through cyclization and release while the ketones **7-8** can form via hydrolysis and decarboxylation.

Fig. 3.

Synthesis of **2** and **12** by LovB and dissociated ER in vitro. **(A)** LC-MS analysis of the synthesis **2** using purified LovB and LovC. Typical reaction contains 25 μM of LovB and LovC, 2 mM NADPH, 2 mM SAM, and the MatB malonyl-CoA regeneration components (See SI for details). The traces shown are the selected ion monitoring of desired ions in the positive ionization mode. Trace i: standard of **2** purified from *A. nidulans* (8). Trace ii: products recovered from the reaction following extraction with EA/AcOH (99/1). Trace iii: the reaction mixture was first treated with 1 M KOH at 65°C for 10 minutes to hydrolyze all attached polyketides, followed by acidification with 1 N HCl to pH 2.0, followed by extraction. Emergence of **2** indicates the products are not released by LovB under reaction conditions; Trace iv: products recovered from base-treated sample that used $2\text{-}^{13}\text{C}$ -malonate as the precursor. The +9 mass shift confirms the nonaketide backbone of **2** synthesized by LovB; Trace v: release of **2** from LovB by heterologous fungal *G. zeae* PKS13 TE (25 μM). No base hydrolysis is required to observed product release; **(B)** Synthesis of **12** in the absence of SAM requires a different ER (MlcG) from the compactin biosynthetic pathway. All reactions contain LovB, PKS13 TE, ER, and 2 mM NADPH, and were extracted with EA/TFA (99/1). Products were detected using selected positive ion monitoring of 307 for **2**, 293 for **12**, and 302 for **12** synthesized from $2\text{-}^{13}\text{C}$ -malonate. Traces i and ii show that both LovC and MlcG can work with LovB to produce **2** in the presence of 2 mM SAM; Trace iii: in the absence of SAM, LovC cannot reduce the tetraketide intermediate and shunt products **5-8** were synthesized (not shown). Trace iv: in the absence of SAM, MlcG can perform the enoylreduction and lead to the synthesis of **12**; Trace v: when $2\text{-}^{13}\text{C}$ -malonate was used, the expected +9 mass increase was observed in **12** using LovB and MlcG. **(C)** The difference in substrate specificity between LovC and MlcG towards methylated and unmethylated tetraketide intermediate.