BIOCHEMISTRY
including biophysical chemistry & molecular biology

Rapid Report

pubs.acs.org/biochemistry

Broad Substrate Specificity of the Loading Didomain of the Lipomycin Polyketide Synthase

Satoshi Yuzawa,[†] Clara H. Eng,[†,‡,∥] Leonard Katz,[†,∥] and Jay D. Keasling*[,†,‡,∥,§,⊥,@]

[†]QB3 Institute, University of California, Berkeley, California 94270, United States
[‡]Department of Chemical and Biomolecular Engineering, University of California, Berkeley, California 94270, United States
[§]Joint BioEnergy Institute, 5885 Hollis Street, Emeryville, California 94608, United States
[∥]Synthetic Biology Engineering Research Center, 5885 Hollis Street, Emeryville, California 94608, United States
[⊥]Physical Bioscience Division, Lawrence Berkeley National Laboratory, Berkeley, California 94270, United States
[@]Department of Bioengineering, University of California, Berkeley, California 94270, United States

Supporting Information

ABSTRACT: LipPks1, a polyketide synthase subunit of the lipomycin synthase, is believed to catalyze the polyketide chain initiation reaction using isobutyryl-CoA as a substrate, followed by an elongation reaction with methylmalonyl-CoA to start the biosynthesis of antibiotic α-lipomycin in *Streptomyces aureofaciens* Tü117. Recombinant LipPks1, containing the thioesterase domain from the 6-deoxyerythronolide B synthase, was produced in *Escherichia coli*, and its substrate specificity was investigated *in vitro*. Surprisingly, several different acyl-CoAs, including isobutyryl-CoA, were accepted as the starter substrates, while no product was observed with acetyl-CoA. These results demonstrate the broad substrate specificity of LipPks1 and may be applied to producing new antibiotics.

Multimodular polyketide synthases (PKSs) are among the largest (2−7 MDa) and most complex enzymes known. They conduct a programmed, stepwise catalysis that leads to the generation of poly-β-ketones with varying degrees of reductions at the β-carbonyl centers. Polyketides are widely used as antibiotics and other pharmaceutical agents.

Multimodular PKSs are composed of several large polypeptides. Each polypeptide contains one or more modules, each of which minimally consists of a ketosynthase (KS) domain, an acyltransferase (AT) domain, and an acyl carrier protein (ACP) domain that are together responsible for a single round of decarboxylative condensation (polyketide chain elongation reaction). The module may contain a loading AT (AT_L) and a loading ACP (ACP_L) to catalyze the polyketide chain initiation reaction. It may also contain a ketoreductase (KR) domain, a dehydratase (DH) domain, and an enoyl reductase (ER) domain to reduce the newly generated β-ketone on the growing polyketide chain tethered to the ACP domain. The extended polyketide chain is then translocated to the KS domain in the next module in the same polypeptide or to the KS in another protein for subsequent condensation. Alternatively, chain growth is terminated by a thioesterase (TE) domain. Understanding the mechanism for this orderly process represents a fundamental challenge in assembly line enzymology.[1]

The fidelity and efficiency of the entire process are dictated by two levels of molecular recognition: protein−protein recognition and substrate specificity. To address the issue of intrinsic substrate specificity, we studied LipPks1, a PKS subunit of the lipomycin synthase from *Streptomyces aureofaciens* Tü117, which is a PKS nonribosomal peptide synthase hybrid that catalyzes the biosynthesis of β-lipomycin, the aglycone of acyclic polyene antibiotic α-lipomycin (Figure 1A).[2] LipPks1 is composed of an AT_L domain, an ACP_L domain, a KS domain, an AT domain, a KR domain, and an ACP domain, a simple PKS that catalyzes polyketide chain initiation and elongation reactions. Bihlmaier, Bechthold, and co-workers have described a model in which LipPks1 initiates the synthesis using isobutyryl-CoA and elongates once with methylmalonyl-CoA.[2] The model was based on the structure of the corresponding segment of α-lipomycin and the amino acid sequence similarity between the AT_L domain of LipPks1 and the AT_L domain of the avermectin PKS, which is believed to initiate polyketide synthesis using 2-methylbutyryl-CoA or isobutyryl-CoA as the starter substrate.[3−5]

To test the model biochemically, we constructed an expression vector that encodes N-terminal hexahistidine tagged LipPks1 with the TE domain from the 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea* (LipPks1+TE) to release intermediates tethered to the ACP domain (Figure 1B). We also constructed expression vectors encoding the protein that lacks the proline and arginine-rich N-terminal linker [(−NL)LipPks1+TE] and the TE-truncated version, which is essentially LipPks1. We used *Escherichia coli* K207-3 as a host for expression of the different PKS gene sets. *E. coli* K207-3 is an engineered strain whose genome encodes *sfp* from *Bacillus subtilis* that encodes the substrate promiscuous surfactin phosphopantetheinyl transferase that converts the expressed PKS apoproteins to their corresponding holo forms.[6]

Protein production was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. LipPks1+TE was almost exclusively produced in the soluble fraction (Figure 1A of the Supporting Information). (−NL)LipPks1+TE was also pro-

Received: April 26, 2013
Revised: May 19, 2013
Published: May 21, 2013

dx.doi.org/10.1021/bi400520t | *Biochemistry* 2013, 52, 3791−3793

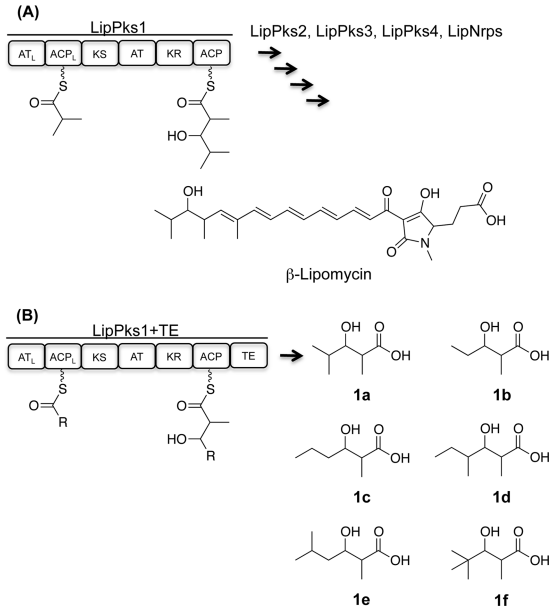

Figure 1. (A) Polyketide chain initiation and elongation reactions catalyzed by LipPks1. LipPks1 consists of an AT_L domain, an ACP_L domain, a KS domain, an AT domain, a KR domain, and an ACP domain. The phosphopantetheine prosthetic arm is shown as a wavy line. The extended polyketide chain tethered to the ACP domain is translocated to the downstream LipPks2 to complete the *β*-lipomycin biosynthesis. (B) Broad substrate specificity of LipPks1+TE. Enzymatic production of **1a**–**f** was confirmed by liquid chromatography–mass spectrometry analysis.

duced in the soluble fraction, although a significant amount was insoluble. In addition, the lysate displayed a large amount of soluble protein running in the gel between 160 and 260 kDa, suggesting that the proline and arginine-rich linker is contributing to stable protein folding, at least in *E. coli*. Surprisingly, a relatively low level of production was observed for LipPks1. This protein corresponds to a natural LipPks1 but lacks the C-terminal linker that is proposed to bind the N-terminal linker of the LipPks2 subunit. This result may suggest that the linker itself stabilizes the LipPks1 structure. Structural studies of DEBS, which is the most extensively studied multimodular PKS, demonstrated that each module forms a homodimer and that homodimerization is driven by the KS domains and the TE domain.[7−9] One possible explanation of the soluble and relatively stable production of LipPks1+TE is that adding a TE domain stabilized the homodimeric structure of LipPks1. LipPks1+TE was purified by Ni affinity chromatography followed by anion exchange chromatography to yield 2 mg of protein from 1 L of *E. coli* culture (Figure 1C of the Supporting Information).

Isobutyryl-CoA, methylmalonyl-CoA, and NADPH were incubated with or without LipPks1+TE, and the production of 3-hydroxy-2,4-dimethylpentanoate (**1a**) was monitored by liquid chromatography and mass spectroscopy (LC–MS). The MS measurements were taken in the selected ion monitoring mode (*m*/*z* 145). As shown in Figure 2B, a strong signal was observed in the presence of LipPks1+TE at 9.0 min. To confirm the production, we chemically synthesized **1a**. The ^{1}H NMR data indicated that the compound was a mixture of diastereomers (26:1 syn:anti).[10] We analyzed the authentic standard by LC–MS and observed two peaks at 8.2 and 9.1 min (Figure 2C). The product generated by LipPks1+TE

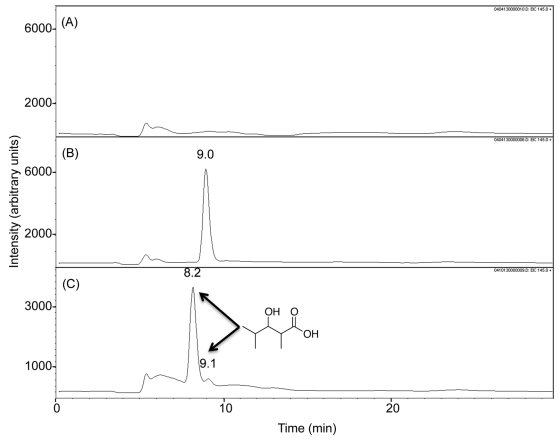

Figure 2. LC–MS analysis of the *in vitro* biosynthesis of **1a**. Isobutyryl-CoA (320 *μ*M) and methylmalonyl-CoA (200 *μ*M) were incubated for 17 h at 23 °C with 500 *μ*M NADPH in the absence (A) or presence (B) of LipPks1+TE (0.5 *μ*M). The resulting reaction mixtures were analyzed by LC–MS in the selected ion monitoring mode (*m*/*z* 145). (C) LC–MS analysis of the chemically synthesized authentic standard (16 *μ*M). The two peaks indicated by arrows are diastereomers of **1a**. Retention times are shown atop the peaks.

appears to have the same stereochemistry as the second peak, which is (2*S*,3*S*)-**1a** and/or (2*R*,3*R*)-**1a**. The amino acid sequence of the KR domain suggests the 2*S*,3*S* product (Figure 2 of the Supporting Information). We also monitored the *β*-keto product of **1a** by LC–MS (*m*/*z* 143). No product was observed in the presence of NADPH (data not shown). The steady-state kinetic parameters for the reaction were determined: k_{cat} = 0.053 min^{-1}; K_M(isobutyryl-CoA) = 2.9 *μ*M; and K_M(methylmalonyl-CoA) = 1.3 *μ*M (Table 1).

Table 1. Steady-State Kinetic Parameters for the Formation of 1a–f by LipPks1+TE

substrate	product	k_{cat} (min^{-1})	K_M (*μ*M)	k_{cat}/K_M (M^{-1} s^{-1})
isobutyryl-CoA	**1a**	0.053	2.9	304.1
propionyl-CoA	**1b**	0.056	13.4	70.3
n-butyryl-CoA	**1c**	0.036	26.4	22.7
2-methylbutyryl-CoA	**1d**	0.126	8.8	237.0
isovaleryl-CoA	**1e**	0.290	128.1	38.0
pivaloyl-CoA	**1f**	0.002	8.8	4.1

To analyze the substrate specificity of the AT domain, we tested malonyl-CoA as a potential extender substrate. Malonyl-CoA was not accepted (data not shown), indicating the AT domain is specific for methylmalonyl-CoA. We also monitored production of 3-hydroxy-2-methylpentanoate (**1b**) by LC–MS (*m*/*z* 131) because DEBS1 was previously shown to start the catalytic cycle using methylmalonyl-CoA as a sole substrate where the methylmalonyl moiety on the ACP is decarboxylated by the KS domain, followed by transfer of the resulting propionyl group back to the KS domain where it could act as a primer for condensation with a new methylmalonyl-ACP exender (Figure 3 of the Supporting Information).[11−13] Production of **1b** was not observed (data not shown), indicating that either the backtransfer reaction is slower than the rate-limiting step for **1a** production or the KS does not accept a propionyl group. However, as descried below, the KS

dx.doi.org/10.1021/bi400520t | *Biochemistry* 2013, 52, 3791−3793

domain can accept the propionyl group as a starter, which refutes the latter hypothesis.

The amino acid sequence of the AT_L domain of LipPks1 is 50% identical to that of the AT_L domain of the avermectin PKS, which is known to accept a variety of starter substrates.[2] Previous studies showed that an engineered *Streptomyces avermitilis* produced more than 40 avermectin analogues when the culture was fed different carboxylic acids.[14,15] To analyze the substrate tolerance of the AT_L domain of LipPks1, we incubated the enzyme with acetyl-CoA, propionyl-CoA, *n*-butyryl-CoA, 2-methylbutyryl-CoA, isovaleryl-CoA, or pivaloyl-CoA at the saturation concentration of methylmalonyl-CoA (200 μM) with NADPH. No product was observed when acetyl-CoA was used as a starter substrate (data not shown). The other acyl-CoAs, however, gave the corresponding products, which are 3-hydroxy-2-methylpentanoate (**1b**), 3-hydroxy-2-methylhexanoate (**1c**), 3-hydroxy-2,4-dimethylhexanoate (**1d**), 3-hydroxy-2,5-dimethylhexanoate (**1e**), and 3-hydroxy-2,4,4-trimethylpentanoate (**1f**), which were confirmed by LC−MS analysis (m/z 131 for **1b**, m/z 145 for **1c**, and m/z 159 for **1d**−**f**) with chemically synthesized authentic standards (Figures 4−8 of the Supporting Information). The steady-state kinetic parameters for the reactions are listed in Table 1. The k_{cat}/K_M values for isobutyryl-CoA and 2-methylbutyryl-CoA were comparable, which correlates well with avermectin biosynthesis in *S. avermitilis* in which isobutyryl- and 2-methylbutyryl-started products were isolated.[3,5] Interestingly, the α-lipomycin analogue that would use 2-methylbutyryl-CoA as a starter was not observed in *S. aureofaciens* Tü117.[2,16] One possibility is that the intracellular concentration of 2-methylbutyryl-CoA is far below the K_M for LipPks1. The k_{cat} values for all substrates investigated, except pivaloyl-CoA, were similar; the k_{cat} value for pivaloyl-CoA was substantially lower than the others. The basis for the relatively low k_{cat} with pivaloyl-CoA is not understood but may be related to the different rates of nucleophilic attack of the carbanion on the KS-linked substrates in the chain elongation reactions. These data suggest that production of α-lipomycin is tightly controlled by regulation of intracellular acyl-CoA concentrations.

The substrate specificity of the loading didomains of multimodular PKSs is usually assumed from the structure of the corresponding segment of polyketide products. However, this methodology depends on the products isolated under particular culture conditions, which might not reflect the actual substrate preference. The intrinsic substrate specificity can be determined by only *in vitro* kinetic analysis.[17] Our kinetic studies revealed broad substrate specificity of the loading didomain of LipPks1, highlighting the importance of using biochemistry to determine substrate flexibility.

In summary, analyzing substrate specificity is crucial to our understanding of polyketide biosynthesis by multimodular PKSs and to the design of hybrid PKSs. In this work, we focus on the LipPks1 subunit of the lipomycin synthase and provide the first insights into substrate specificity. We have uncovered broad substrate specificity of the loading didomain, which was not anticipated from the study of the PKS in its native host and led us to speculate that novel lipomycin analogues can be produced by increasing intracellular acyl-CoA concentrations.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: keasling@berkeley.edu.

Funding

This work was funded by the Advanced Research Projects Agency-Energy (ARPA-E), U.S. Department of Energy, via Grant DE-AR0000091, by the National Science Foundation, via Grant EEC-0540879 to the Synthetic Biology Research Center, and by the Joint BioEnergy Institute (JBEI), which is funded by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, under Contract DE-AC02-05CH11231.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Edward Baidoo and Sean Poust for assistance with LC−MS analysis and Tristan de Rond and Woncheol Kim for assistance with chemical synthesis.

■ REFERENCES

(1) Khosla, C., Tang, Y., Chen, A. Y., Schnarr, N. A., and Cane, D. E. (2007) *Annu. Rev. Biochem. 76*, 195−221.

(2) Bihlmaier, C., Welle, E., Hofmann, C., Welzel, K., Vente, A., Breitling, E., Muller, M., Glaser, S., and Bechthold, A. (2006) *Antimicrob. Agents Chemother. 50*, 2113−2121.

(3) Schulman, M. D., Valentino, D., and Hensens, O. (1986) *J. Antibiot. 39*, 541−549.

(4) Chen, T. S., Arison, B. H., Gullo, V. P., and Inamine, E. S. (1989) *J. Ind. Microbiol. 4*, 231−238.

(5) Cane, D. E., Liang, T.-C., Kaplan, L. K., Nallin, M. K., Schulman, M. D., Hensens, O. D., Douglas, A. W., and Albers-Schonberg, G. (1983) *J. Am. Chem. Soc. 105*, 4110−4112.

(6) Murli, S., Kennedy, J., Dayem, L. C., Carney, J. R., and Kealey, J. T. (2003) *J. Ind. Microbiol. Biotechnol. 30*, 500−509.

(7) Staunton, J., Caffrey, P., Aparicio, J. F., Roberts, G. A., Bethell, S. S., and Leadlay, P. F. (1996) *Nat. Struct. Biol. 3*, 188−192.

(8) Tsai, S. C., Miercke, L. J., Krucinski, J., Gokhale, R., Chen, J. C., Foster, P. G., Cane, D. E., Khosla, C., and Stroud, R. M. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 14808−14813.

(9) Tang, Y., Kim, C. Y., Mathews, I. I., Cane, D. E., and Khosla, C. (2006) *Proc. Natl. Acad. Sci. U.S.A. 103*, 11124−11129.

(10) Van Draanen, N. A., Arseniyadis, S., Crimmins, M. T., and Heathcock, C. H. (1991) *J. Org. Chem. 56*, 2499−2506.

(11) Pieper, R., Ebert-Khosla, S., Cane, D., and Khosla, C. (1996) *Biochemistry 35*, 2054−2060.

(12) Jacobsen, J. R., Cane, D. E., and Khosla, C. (1998) *Biochemistry 37*, 4928−4934.

(13) Weissman, K. J., Bycroft, M., Staunton, J., and Leadlay, P. F. (1998) *Biochemistry 37*, 11012−11017.

(14) Hafner, E. W., Holley, B. W., Holdom, K. S., Lee, S. E., Wax, R. G., Beck, D., McArthur, H. A., and Wernau, W. C. (1991) *J. Antibiot. 44*, 349−356.

(15) Dutton, C. J., Gibson, S. P., Goudie, A. C., Holdom, K. S., Pacey, M. S., Ruddock, J. C., Bu'Lock, J. D., and Richards, M. K. (1991) *J. Antibiot. 44*, 357−365.

(16) Kunze, B., Schabach, K., Zeeck, A., and Zahner, H. (1972) *Arch. Mikrobiol. 86*, 147−174.

(17) Liou, G. F., Lau, J., Cane, D. E., and Khosla, C. (2003) *Biochemistry 42*, 200−207.