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Unusual Activities of the Thioesterase Domain for the Biosynthesis of the Polycyclic Tetramate Macrolactam HSAF in *Lysobacter enzymogenes* C3

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Supporting Information

ABSTRACT: HSAF is an antifungal natural product with a new mode of action. A rare bacterial iterative PKS-NRPS assembles the HSAF skeleton. The biochemical characterization of the NRPS revealed that the thioesterase (TE) domain possesses the activities of both a protease and a peptide ligase. Active site mutagenesis, circular dichroism spectra, and homology modeling of the TE structure suggested that the TE may possess uncommon features that may lead to the unusual activities. The iterative PKS-NRPS is found in all polycyclic tetramate macrolactam gene clusters, and the unusual activities of the TE may be common to this type of hybrid PKS-NRPS.

HSAF (dihydromaltophilin) is an antifungal metabolite produced by the biological control agent *Lysobacter enzymogenes* C3.[1] Strain C3 has shown efficacy in control multiple fungal pathogens infecting wheat and barley.[2−4] HSAF exhibits strong activity against a wide range of fungi and exhibits a novel mode of action.[5−7] HSAF is a polycyclic tetramate macrolactam (PTM) (Figure 1), which is distinct

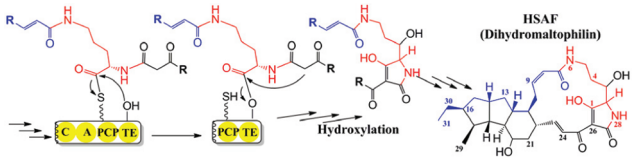

Figure 1. Biosynthetic mechanism for the tetramate macrolactam functionalities in HSAF.

from any existing fungicides.[8] One of the intriguing features of HSAF is that it has two amide bonds that are formed between two separate polyketide chains and the two amino groups of ornithine.[9] This is distinct from other tetramic acid-containing polyketides, such as equisetin,[10] fusarin C,[11] tenellin,[12] and cyclopiazonate.[13] Formation of the tetramate macrolactam leads to the release of the two polyketide chains bound to the hybrid polyketide synthase (PKS)-nonribosomal peptide synthetase (NRPS) that is responsible for the assembly of the HSAF skeleton.[9,14] This hybrid PKS-NRPS contains nine domains, including a C-terminal thioesterase (TE). The structural features of HSAF and our previous studies[8,9] suggest that the TE domain uses a carbon nucleophile (carbanion), instead of an oxygen or nitrogen nucleophile as seen in typical PKS-NRPS, to attack the carbonyl group of the acyl-*O*-synthase to release the acyl chain. The determination of the reactions catalyzed by the PKS-NRPS domains could provide insights into the mechanism of formation of unusual functionalities.

We previously purified the four-domain (C-A-PCP-TE, 149 kDa) NRPS that was heterologously expressed in *Escherichia coli*.[9] To directly show amide bond formation, we first performed the [^{14}C]ornithine labeling of C-A-PCP-TE following the established methods.[15,16] The protein was preincubated with Svp, a 4′-phosphopantetheinyl (PPT) transferase, to tether the PPT group to the PCP.[17] In the presence of ATP, ornithine was expected to be recognized by the A domain and loaded onto the holo-PCP to form [^{14}C]aminoacyl-*S*-PCP. During these assays, we found an unusual phenomenon. As part of the standard procedure, the protein samples, after the desired reactions, were boiled for 5−10 min before being loaded and analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and exposed to X-ray film. Surprisingly, we found that ∼50% of the 149 kDa band disappeared after the sample had been boiled for 5 min; concurrently, a band at ∼300 kDa appeared on the gel (Figure 2A). Other proteins, such as BSA (66.8 kDa) and lysozyme (14.3 kDa), under the same conditions remained unchanged. Interestingly, the 149 and ∼300 kDa NRPS bands completely disappeared when the boiling time was more than 15 min. A similar phenomenon was also observed when the NRPS was co-incubated with other proteins (shown as BSA in Figure 2A). The 66.8 kDa BSA band disappeared upon boiling, and new bands (putative oligomers) in the high-mass region appeared. One possible explanation of this phenomenon is that this NRPS possesses a peptide ligase-like activity as well as a protease-like activity at the elevated temperature.

Considering the composition of the NRPS, we concluded that the TE domain is most likely responsible for this unusual activity. To test this idea, we expressed the TE domain in *E. coli* and purified the 28.3 kDa protein (Figure S1). When TE was boiled, the magnitude of the 28.3 kDa band was gradually reduced while the magnitude of a band at ∼56 kDa gradually increased upon SDS−PAGE (Figure 2B). In addition, the originally sharp 28.3 kDa TE band became a smear, implying a

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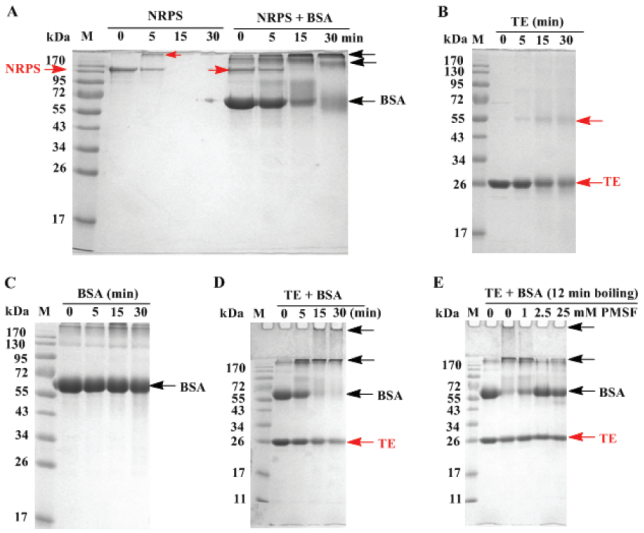

Figure 2. SDS–PAGE of the four-domain (C-A-PCP-TE) NRPS and the TE domain to show the peptide ligase-like activity: (A) NRPS alone or with BSA, (B) TE, (C) BSA, (D) TE incubated with BSA, and (E) TE incubated with BSA in the presence of serine protease inhibitor PMSF. The samples were loaded onto the gels without being boiled or were boiled at 100 °C for 5–30 min.

partial degradation and/or ligation may have taken place. When TE was co-incubated with BSA, the 66.3 kDa BSA band gradually disappeared while the bands in the high-mass region appeared again (Figure 2C,D). In the presence of BSA, the magnitude of the 28.3 kDa TE band was only slightly decreased. To test if the bands in the high-mass region were protein aggregates due to a heat denaturation, BSA alone was treated under the same conditions. However, no band corresponding to those putative oligomers was formed. Furthermore, when the serine protease inhibitor phenylmethanesulfonyl fluoride [PMSF (Figure 2E)] was co-incubated with TE and BSA under the same conditions, the 66.8 kDa BSA band reappeared on the gel. The reappearance of BSA was dependent on PMSF concentration. The effect was also observed in the presence of other inhibitors such as TPCK and TLCK (data not shown). In addition to BSA, other proteins (lysozyme and acyl carrier protein[18]) exhibited similar results when co-incubated with the TE. These results clearly showed that the observed phenomenon is due to a peptide ligase/protease-like activity of the TE domain, rather than a random aggregation of the proteins.

The BSA band shifted to a higher mass only when the temperature was >65 °C (Figure S2). Because the activity is temperature-dependent, we measured the TE's circular dichroism spectral changes at different temperatures (Figure S3). From 20 to 100 °C, the content of α-helices and unordered structures decreased while that of β-sheets and turns increased. Nevertheless, the TE appeared to retain part of its secondary structure even at 100 °C. Moreover, the secondary structure elements were partly restored when the temperature gradually shifted from 100 to 20 °C (Figure S3). In agreement with the observations, the TE maintained the ligase-like activity on SDS–PAGE when it was preheated at 100 °C for 5–15 min and then co-incubated with BSA (Figure S4). To exclude the possibility that the observed activity is due to a contaminated enzyme, we expressed surfactin TE and enterobactin TE in *E. coli*.[19,20] Both of the TE domains belong to non-PTM-type NRPS. When these TE domains were purified and tested under the same conditions that were used for HSAF TE, no activity was observed (Figure S5).

To further investigate this unusual phenomenon, we analyzed the TE using MS. While the control protein gave the expected mass, the purified TE did not provide the expected molecular mass of 28345 Da but rather produced a number of minor components over a broad region (26–36 kDa). TE belongs to the α/β-hydrolase superfamily that includes lipases and proteases.[14] A conserved catalytic triad, Ser-His-Asp, is present in these enzymes. We mutated the TE's active site Ser91 to alanine and expressed the mutant TE-S91A in *E. coli* (Figure S1). The purified TE-S91A produced by MS a 28329 Da molecular species, with a value identical to the calculated mass. Next, we searched for potential self-cleaved products resulting from the protease activity using LC–MS. Indeed, we detected peptide fragments in freshly prepared native TE samples at room temperature (Figure S6). The specificity of the cleavage site appears to be the C-terminus of polar amino acids, such as S, D, R, C, and T. Notably, these fragments were not observed in TE-S91A. Interestingly, TE-S91A still exhibited the same ligase-like activity as wild-type TE as shown by SDS–PAGE (Figure S7). To test the possibility that another Ser in this TE may compensate for the mutated Ser91, we generated a second mutant, TE-S119A. Ser119 was chosen because it is close to Ser91 in the TE homology model (see below). This mutant behaved in the same manner as native TE, with the exception that its activity was only slightly inhibited upon PMSF treatment (even up to 100 mM) (Figure S8). We then generated a doubly mutated TE, TE-S91A/S119A. Surprisingly, this double mutant behaved just like the wild type on SDS–PAGE (Figure S8). Finally, a double mutant, TE-R71S/S119A, with the active site Ser91 unchanged, also showed activity (Figure S8). It appears likely that another Ser or a water molecule could act as the nucleophile when the mutants exhibited the peptide ligase-like activity (Figure 3).

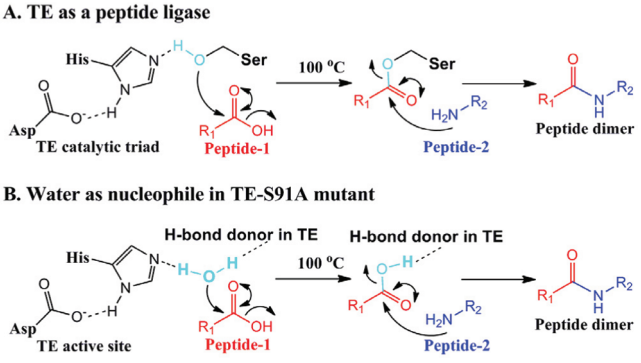

Figure 3. Proposed mechanism for the peptide ligase-like activity observed in HSAF TE and mutant TE-S91A.

The structure of several PKS-NRPS TE domains has been determined.[21–25] Our efforts to obtain an HSAF TE crystal structure have so far been unsuccessful. However, homology modeling of known structures suggested that HSAF TE has a typical α/β-hydrolase fold common to this family of enzymes. Two NRPS TEs (fengycin[21] and surfactin TE[22]) and two PKS TEs (DEBS[23] and picromycin TE[24]) were chosen for this study because their sequences are most similar to that of HSAF TE. The predicted secondary structure of HSAF TE showed the typical α/β-hydrolase fold, with a central six-stranded β-sheet surrounded by six helices (Figure S9). The predicted three-

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dimensional structure of HSAF TE superimposed well with the known TE structures, with a Z score of 16.3−30.4 and a root-mean-square deviation of 1.4−3.3 Å (Figure S10). The catalytic triad (Ser91-Asp118-His218) of HSAF TE was well positioned in the substrate pocket and nearly superimposable with the triad of fengycin TE and surfactin TE, except that Asp118 appeared to deviate from the known structures (Figure S10). Further studies are needed to determine whether this deviation or any other structural feature of HSAF TE contributes to the observed unusual activities.

To the best of our knowledge, this is the first example in which a TE exhibits both a protease-like activity and a peptide ligase-like activity. Recently, a TE1−TE2 tandem in the NRPS for lysobactin biosynthesis, which is also from a species of *Lysobacter*, was found to have a protease-like activity.[26] The biochemical data presented here provide a foundation for further investigations that aim to uncover the molecular basis for these unusual activities. HSAF belongs to a group of emerging polycyclic tetramate macrolactams (PTM), including frontalamides,[27] alteramide A,[28] cylindramide,[29] discodermide,[30] ikarugamycin,[31] aburatubolactam A,[32] and geodin A.[33] This group of metabolites has unique structural features and diverse biological activities. The biochemical and molecular mechanisms for their biosynthesis remain largely unclear. Clardy et al. recently showed that PTMs from phylogenetically diverse bacteria have common biosynthetic origins.[27] Within the numerous uncharacterized PTM gene clusters, a TE is always present at the C-terminus of a hybrid PKS-NRPS. The unusual activities found in HSAF TE could be a common feature of the PTM-type TE. Finally, the amide-bond formation/cleavage activity of this TE implies that this terminal domain could catalyze the formation of one of the two amides in PTM, in addition to the final product release.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary experimental methods, SDS−PAGE, MS data, CD spectra, sequence alignment, and homology modeling (Figures S1−S10). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written by L.L. and L.D. All authors have given approval to the final version of the manuscript.

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■ REFERENCES

(1) Giesler, L. J., and Yuen, G. Y. (1998) *Crop Prot. 17*, 509−513.
(2) Zhang, Z., and Yuen, G. Y. (1999) *Phytopathology 89*, 817−822.
(3) Yuen, G. Y., Steadman, J. R., Lindgren, D. T., Schaff, D., and Jochum, C. C. (2001) *Crop Prot. 20*, 395−402.
(4) Yuen, G. Y., Jochum, C. C., Osborne, L. E., and Jin, Y. (2003) *Phytopathology 93*, S93.
(5) Li, S., Du, L., Yuen, G., and Harris, S. D. (2006) *Mol. Biol. Cell 17*, 1218−1227.
(6) Li, S., Jochum, C. C., Yu, F., Zaleta-Rivera, K., Du, L., Harris, S. D., and Yuen, G. Y. (2008) *Phytopathology 98*, 695−701.
(7) Li, S., Calvo, A. M., Yuen, G. Y., Du, L., and Harris, S. D. (2009) *J. Eukaryotic Microbiol. 56*, 182−187.
(8) Yu, F., Zaleta-Rivera, K., Zhu, X., Huffman, J., Millet, J. C., Harris, S. D., Yuen, G., Li, X. C., and Du, L. (2007) *Antimicrob. Agents Chemother. 51*, 64−72.
(9) Lou, L., Qian, G., Xie, Y., Hang, J., Chen, H., Zaleta-Rivera, K., Li, Y., Shen, Y., Dussault, P. H., Liu, F., and Du, L. (2011) *J. Am. Chem. Soc. 133*, 643−645.
(10) Sims, J. W., Fillmore, J. P., Warner, D. D., and Schmidt, E. W. (2005) *Chem. Commun.,* 186−188.
(11) Song, Z., Cox, R. J., Lazarus, C. M., and Simpson, T. T. (2004) *ChemBioChem 5*, 1196−1203.
(12) Halo, L. M., Heneghan, M. N., Yakasai, A. A., Song, Z., Williams, K., Bailey, A. M., Cox, R. J., Lazarus, C. M., and Simpson, T. J. (2008) *J. Am. Chem. Soc. 130*, 17988−17996.
(13) Liu, X., and Walsh, C. T. (2009) *Biochemistry 48*, 8746−8757.
(14) Du, L., and Lou, L. (2010) *Nat. Prod. Rep. 27*, 255−278.
(15) Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C., and Walsh, C. T. (1996) *Chem. Biol. 3*, 923−936.
(16) Du, L., Chen, M., Zhang, Y., and Shen, B. (2003) *Biochemistry 42*, 9731−9740.
(17) Sanchez, C., Du, L., Edwards, D. J., Toney, M. D., and Shen, B. (2001) *Chem. Biol. 8*, 725−738.
(18) Gerber, R., Lou, L., and Du, L. (2009) *J. Am. Chem. Soc. 131*, 3148−3149.
(19) Tseng, C. C., Bruner, S. D., Kohli, R. M., Marahiel, M. A., Walsh, C. T., and Sieber, S. A. (2002) *Biochemistry 41*, 13350−13359.
(20) Frueh, D. P., Arthanari, H., Koglin, A., Vosburg, D. A., Bennett, A. E., Walsh, C. T., and Wagner, G. (2008) *Nature 454*, 903−906.
(21) Samel, S. A., Wagner, B., Marahiel, M. A., and Essen, L. O. (2006) *J. Mol. Biol. 359*, 876−889.
(22) Bruner, S. D., Weber, T., Kohli, R. M., Schwarzer, D., Marahiel, M. A., Walsh, C. T., and Stubbs, M. T. (2002) *Structure 10*, 301−310.
(23) 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.
(24) Tsai, S. C., Lu, H., Cane, D. E., Khosla, C., and Stroud, R. M. (2002) *Biochemistry 41*, 12598−12606.
(25) Akey, D. L., Kittendorf, J. D., Giraldes, J. W., Fecik, R. A., Sherman, D. H., and Smith, J. L. (2006) *Nat. Chem. Biol. 2*, 537−542.
(26) Hou, J., Robbel, L., and Marahiel, M. A. (2011) *Chem. Biol. 18*, 655−664.
(27) Blodgett, J. A., Oh, D. C., Cao, S., Currie, C. R., Kolter, R., and Clardy, J. (2010) *Proc. Natl. Acad. Sci. U.S.A. 107*, 11692−11697.
(28) Shigemori, H. B., Bae, M.-A., Yazawa, K., Sasaki, T., and Kobayashi, J. (1992) *J. Org. Chem. 57*, 4317−4320.
(29) Hart, A. C., and Phillips, A. J. (2006) *J. Am. Chem. Soc. 128*, 1094−1095.
(30) Gunasekera, S. P., Gunasekera, M., and McCarthy, P. (1991) *J. Org. Chem. 56*, 4830−4833.
(31) Paquette, L. A., Macdonald, D., Anderson, L. G., and Wright, J. (1989) *J. Am. Chem. Soc. 111*, 8037−8039.
(32) Bae, M. A., Yamada, K., Ijuin, Y., Tsuji, T., Yazawea, K., Tomono, Y., and Uemura, D. (1996) *Heterocycl. Commun. 2*, 315−318.
(33) Capon, R. J., Skene, C., Lacey, E., Gill, J. H., Wadsworth, D., and Friedel, T. (1999) *J. Nat. Prod. 62*, 1256−1259.