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RESEARCH ARTICLE

Cross-module Enoylreduction in the Azalomycin F Polyketide Synthase

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Abstract: The colinearity of canonical modular polyketide synthases, which creates a direct link between multienzyme structure and the chemical structure of the biosynthetic end-product, has become a cornerstone of knowledge-based genome mining. Here we report genetic and enzymatic evidence for the remarkable role of an enoylreductase in the polyketide synthase for azalomycin F biosynthesis. This internal enoylreductase domain, previously identified as acting only in the second of two chain extension cycles on an initial iterative module, is shown here also to catalyse enoylreduction *in trans* within the next module. The mechanism for this rare deviation from colinearity appears to involve direct cross-modular interaction of the reductase with the longer acyl chain, rather than backtransfer of the substrate into the iterative module, suggesting an additional and surprising plasticity in natural PKS assembly-line catalysis.

Introduction

Modular polyketide synthase (PKS) multienzymes are versatile biosynthetic machines catalyzing the formation of a very large number of antibiotics and related bioactive compounds via an assembly line mechanism.[1] The assembly-line paradigm, first established for the erythromycin-producing PKS,[2,3] established that each step is catalysed by a distinct enzyme domain and that these domains are arrayed in the multienzyme essentially in the order they are used. This colinearity has been found to hold for a large number of bacterial modular PKS. In each round of chain elongation, an extender unit is selected by a specific acyltransferase (AT) domain, transferred from acyl-CoA onto the acyl carrier protein (ACP) domain, and then condensed with the growing polyketide chain by the β-ketoacyl synthase (KS) domain. The elongated intermediate remains tethered to ACP and is successively and selectively processed by the (optionally present) ketoreductase (KR) domain, dehydratase (DH) domain and enoylreductase (ER) domain to adjust appropriately the extent of reduction of the β-keto group, and subsequently transferred to the downstream module for the next round of chain elongation. Finally, the full-length intermediate is released by a C-terminal thioesterase (TE) domain, usually by hydrolysis or macrocyclisation.[1]

A second important group of modular PKSs has evolved that also adopts an assembly-line mechanism, but here the extension modules lack an integral AT domain, and all of the extension modules are serviced by a free-standing AT enzyme acting *in trans*.[4,5] A free-standing enzyme may even act selectively at different stages of chain growth, as in the action of the ER LovC during lovastatin biosynthesis on a fungal iterative unimodular PKS.[6] The *trans*-AT PKSs of bacteria also tend to show other obvious deviations from colinearity, making the deciphering of their assembly lines far from easy.

Strikingly, certain canonical (or *cis*-AT) PKSs also exhibit rare deviations from colinearity. For example, an extension module may 'stutter', that is, catalyse more than one successive identical chain elongation.[7-12] In other cases, a module is 'skipped' to provide a shortened polyketide.[9c,13,14] In certain PKS systems, the aberrant behaviour concerns a single domain. For example, the function of a single "missing" DH domain may actually be accomplished by a DH domain residing in a later-acting module.[15-17] There are also cases of a PKS AT domain working iteratively on other modules.[9,18]

In previous work, we identified an unusual toggling ER domain in the first iterative extension module during PKS-catalysed biosynthesis of the 36-membered macrocyclic azalomycin F (AZL) (Figure 1A), in which the ER is inactive in the first but active in the second chain-elongation, respectively.[19] This stuttering module is referred to here as module 1/2. We wished to examine a further non-colinearity in the operation of the AZL PKS: during the third elongation, catalysed by extension module 3, the polyketide chain should be fully reduced based on the known AZL structure. However, module 3 contains no ER domain (Figure 1A). This function is unlikely to be supplied by an external ER enzyme since the AZL biosynthetic gene cluster alone produces AZL when

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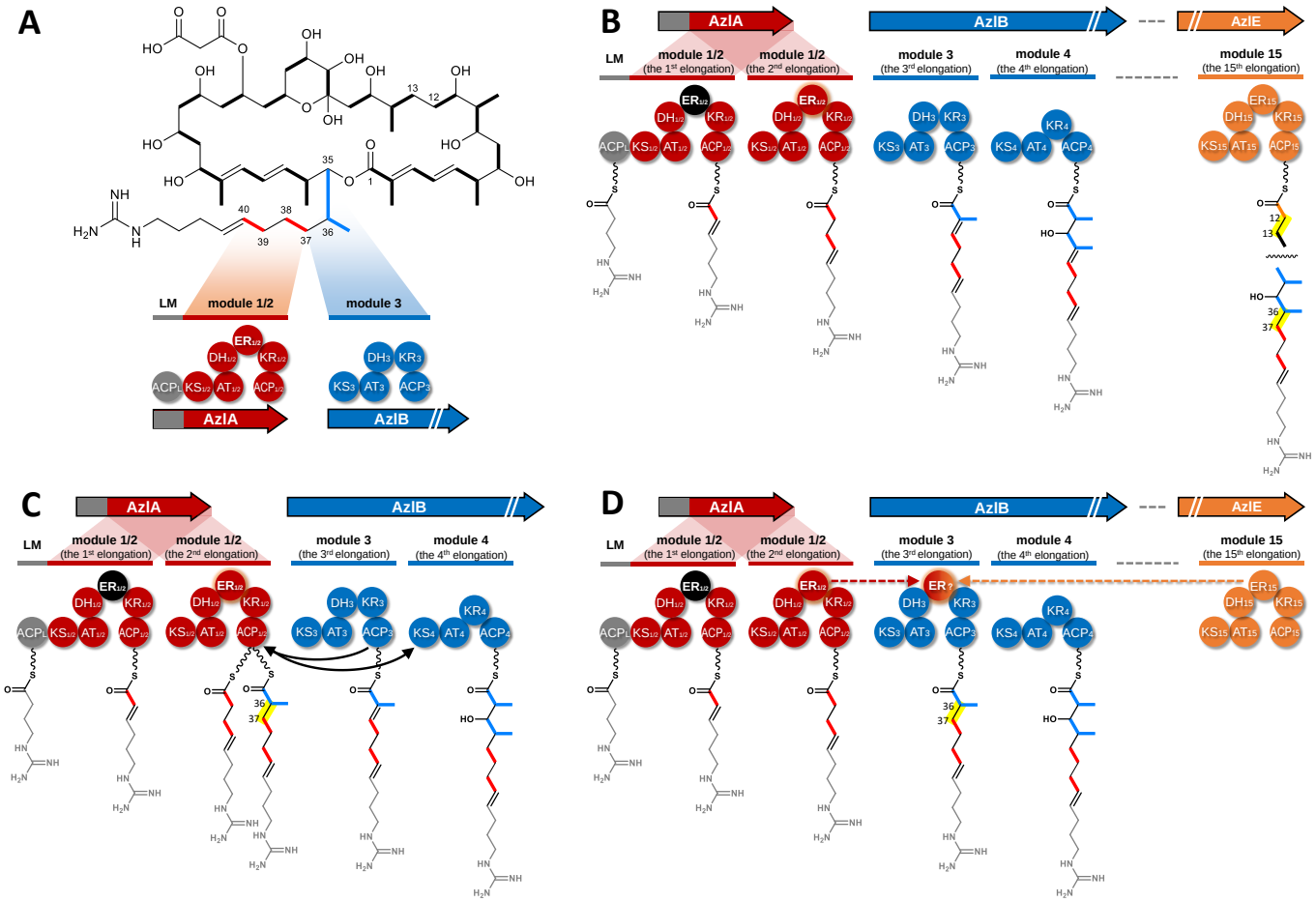

Figure 1. Alternative models for non-colinear enoylreduction in the AZL PKS. A) The non-colinearity between the AZL F3a structure and domain organization in the initial extension modules of the AZL PKS. B) In this model, the intermediate α,β-unsaturated tetraketide product of module 3 is further elongated until it reaches ACP_{15} where ER_{15} catalyses enoylreduction of both C12-C13 and C36-C37. C) In this model, the unreduced intermediate of module 3 is transferred back to $ACP_{1/2}$ for reduction by $ER_{1/2}$ then transferred forward (without condensation) onto KS_4 for further elongation. D) $ER_{1/2}$ and/or ER_{15} catalyses enoylreduction *in trans* of the α,β-unsaturated intermediate of module 3 when it is tethered to ACP_3 during the third round of elongation.

heterologously expressed.[11] There are only two ER domains in the AZL PKS, residing in module 1/2 and module 15, respectively. We therefore considered alternative models for enoylreduction in module 3 involving these enzymes. In one mechanism, similar to the dehydration in module 4 of the iso-migrastatin PKS by the DH domain of module 10,[16] the intermediate product of module 3 is elongated until it reaches module 15, where ER_{15} catalyses two successive enoylreductions to give the expected product (Figure 1B). Alternatively, the α,β-unsaturated intermediate product of elongation on module 3 is transferred back to the ACP of module 1/2 for enoylreduction, catalysed by $ER_{1/2}$ and is then transferred forward without condensation (i.e. module 3 is skipped) onto KS_4 of module 4 for subsequent elongation (Figure 1C). A further possibility is that either $ER_{1/2}$ or ER_{15} catalyses the enoylreduction required in module 3, acting directly *in trans* independent of the assembly-line (Figure 1D).

Results and Discussion

To discriminate between the alternative models (Figure 1) for enoylreduction of the tetraketide product of AZL module 3, the NADPH binding site was mutated from GGVG to AAVA in either the $ER_{1/2}$ or the ER_{15} domain of the AZL gene cluster in *Streptomyces* sp. 211726, (Figures S1-S2). Analysis of the fermentation products of these mutants (referred to here as $\Delta ER_{1/2}$ and ΔER_{15}, respectively) by liquid chromatography coupled with electrospray ionization high-resolution mass spectrometry (LC-ESI-HRMS) showed that inactivation of either $ER_{1/2}$ or ER_{15} completely abolished the production of AZL. For ΔER_{15} this led to the appearance of a new peak whose molecular weight was 2 Da less than that of AZL F3a from the wild-type strain (Figure 2). In contrast, for the $\Delta ER_{1/2}$ mutant the new peak had a molecular weight 4 Da less than that of AZL F3a (Figure 2). Large-scale fermentation was conducted to obtain enough of these F3a-related compounds for NMR analysis. The ^{13}C NMR chemical shift changes of C12 (δ_C from 33.7 to 133.2) and C13 (δ_C from 30.7 to 134.6) revealed the presence of a double bond between C12 and C13 in the structure of **1** from ΔER_{15} compared with F3a of wild-type (Figures S3-S9 and Table S1). This result revealed that inactivation of ER_{15} only affected the enoylreduction on the intermediate of module 15, which unequivocally ruled out the possibility that ER_{15} is involved in the enoylreduction on module 3 (Figure 1B). In contrast, the structure of **2** purified from

10.1002/anie.202011357

$\Delta ER_{1/2}$ contained two extra double bonds compared to wild-type, in conjugation with the C40-C41 double bond, evidenced by the changes in ^{13}C NMR chemical shift of C36 (δ_C from 35.1 to 135.1), C37 (δ_C from 34.6 to 130.2), C38 (δ_C from 28.0 to 127.7), and C39 (δ_C from 33.7 to 135.1); and the corresponding changes in ^{13}C NMR chemical shift of the adjacent carbon atoms, C35 (δ_C from 80.7 to 84.3), C40 (δ_C from 130.2 to 133.1), and C41 (δ_C from 132.6 to 134.7) (Figures S3, S10-S15 and Table S2). As well as being fully consistent with the previously-reported role of the toggling $ER_{1/2}$ domain in the iterative module 1/2,[19] these results provide direct genetic evidence that $ER_{1/2}$ is the necessary and sufficient catalyst for double bond reduction at C36-C37.

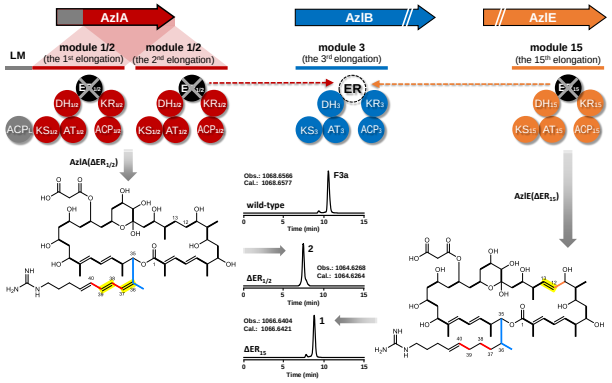

Figure 2. LC-ESI-HRMS analysis of $\Delta ER_{1/2}$ and ΔER_{15} mutants. Inactivated domains are labelled in black with a cross, and the corresponding changes in the main products are highlighted in yellow. The structures of azalomycin F3a and its derivatives from $\Delta ER_{1/2}$ and ΔER_{15} were confirmed by LC-ESI-HRMS and NMR (Figures S4-S15 and Tables S1-S2).

To examine the role of $ER_{1/2}$ *in vitro*, the PKS multienzyme AzlA, comprising the loading module (LM) and module 1/2, and module 3 of multienzyme AzlB with an intact *N*-terminus were each expressed as recombinant proteins in *Escherichia coli* BAP1 (Figure S16).[20] In our previous work, module 1/2 chain elongation was reconstituted *in vitro*,[19] by using Azl4 (ligase) and Azl5 (acyltransferase) to activate and deliver the starter unit to AzlA. Here, we synthesised the product of module 1/2 as the *N*-acetylcysteamine thioester (**3**) (Scheme S1and Figures S17-S22) and used it as a triketide substrate mimic for module 3 (Figure 3A). An assay containing **3** and AZL PKS module 3 was performed in the presence of methylmalonyl-CoA and NADPH and the thioester-bound product was subsequently released by alkaline hydrolysis (Figure 3B).[19,21] LC-ESI-HRMS and MS/MS fragmentation[22] analysis revealed a peak corresponding to the tetraketide 10-guanidino-2-methyl-2,6-dien-decanoic acid (**4**), which represents one round of chain elongation without enoylreduction (Figure 3B and Figures. S23A-S23B). When the same reaction was carried out in the presence of AzlA, a major compound (10-guanidino-2-methyl-6-en-decanoic acid, **5**) was detected whose mass corresponds to one round of chain elongation with ensuing enoylreduction (Figure 3C and Figures S23C-S23D). Even though AzlA comprises seven domains ($ACP_{Loading}$-$KS_{1/2}$-$AT_{1/2}$-$DH_{1/2}$-$ER_{1/2}$-$KR_{1/2}$-$ACP_{1/2}$), the obvious catalyst for the enoylreduction is the ER domain. To confirm its role in this conversion, we inactivated the $ER_{1/2}$ of AzlA by mutating the NADPH binding site sequence GGVG to AAVA (Figure S1). When the assay containing **3** and module 3 was

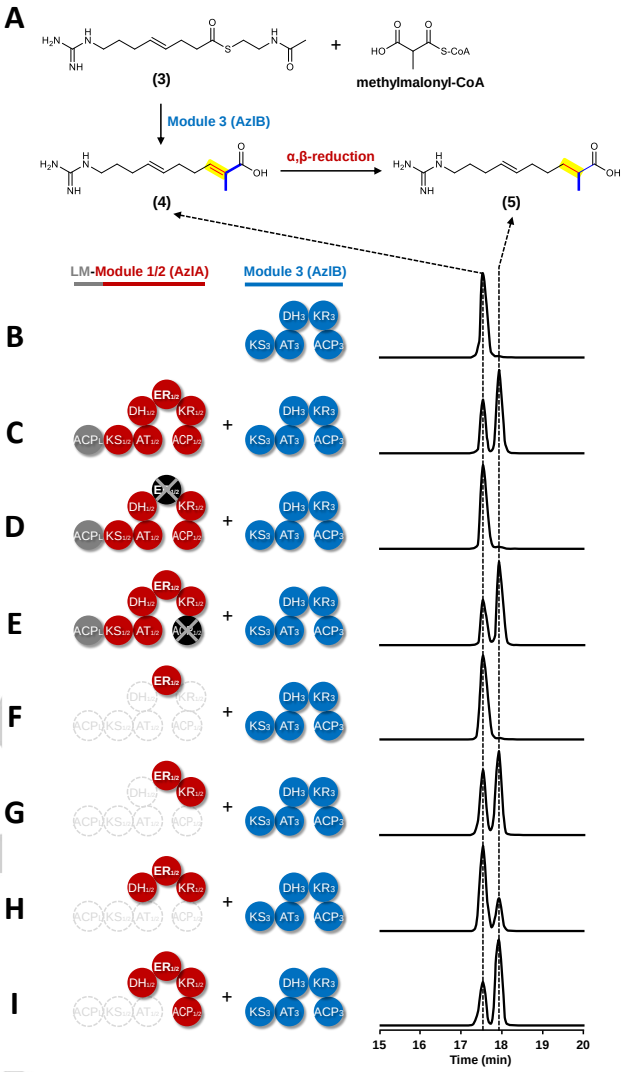

Figure 3. *In vitro* reconstitution of cross-module enoylreduction. A) The synthetic *N*-acetylcysteamine thioester **3** serves as a substrate mimic for module 3 and is condensed with elongation unit methylmalonyl-CoA. B) the tetraketide extension product was then released from module 3 by KOH hydrolysis and monitored by LC-ESI-HRMS using *m/z* 240.1706 for **4** and *m/z* 242.1863 for **5**. The same reaction was carried out in the presence of C) wild-type AzlA, D) AzlA($\Delta ER_{1/2}$) and E) AzlA($\Delta ACP_{1/2}$); and also with AzlA domain or domain sets F) $ER_{1/2}$, G) $ER_{1/2}$-$KR_{1/2}$, H) $DH_{1/2}$-$ER_{1/2}$-$KR_{1/2}$ and I) $DH_{1/2}$-$ER_{1/2}$-$KR_{1/2}$-$ACP_{1/2}$. As a negative control, methylmalonyl-CoA was omitted from the reaction mixture. Inactivated domains are shown in black with a cross.

performed in the presence of methylmalonyl-CoA, NADPH and the mutated AzlA, the reaction product was unsaturated tetraketide, with less than 1% α,β-saturated product detected (Figure 3D). Even this trace amount of reduced product was eliminated when a different AzlA mutant was used whose NADPH binding site was mutated by changing the sequence GGVG to SPVG (Figure S24).[23] These *in vitro* results further support the conclusion that $ER_{1/2}$ within AzlA is responsible for enoylreduction of the tetraketide intermediate in AZL biosynthesis.

These *in-vitro* results do not allow a distinction between a mechanism (Figure 1C) in which the α,β-unsaturated intermediate of module 3 is transferred back to the ACP of module 1/2 for

enoylreduction catalysed by $ER_{1/2}$, and one (Figure 1D) in which the ER acts *in trans* upon the unsaturated tetraketide while it remains tethered to the ACP of module 3. We therefore inactivated $ACP_{1/2}$ in the AzlA protein, by using site-directed mutagenesis to replace the active site serine residue by alanine (Figure S25).[24] AzlA($\Delta ACP_{1/2}$) was purified from *E. coli* BAP1 and supplied to the module 3 chain elongation reaction (Figure S16). The result (Figure 3E) showed that the elongated product of module 3 is reduced to the same extent as in the unmutated control (Figure 3C), indicating that the intermediate does not require back-transfer to $ACP_{1/2}$ and likely remains tethered to ACP_3 for enoylreduction. Taking the above genetic and enzymatic results together, $ER_{1/2}$ is recruited *in trans* by module 3 to catalyse α,β-double bond reduction of the intermediate tethered onto ACP_3. The factors that promote this - to our knowledge - unprecedented cross-modular catalysis remain to be determined, but may include a gatekeeping function of the KS domain of the downstream module 4, which may be highly selective for recruitment of the reduced tetraketide over the α,β-unsaturated tetraketide; and the presence of $ER_{1/2}$ on a separate protein may allow more flexibility than if modules 1-3 were housed in the same multienzyme.

We were interested to determine whether the action of $ER_{1/2}$ on the neighbouring module would be seen with the isolated $ER_{1/2}$ domain; or whether other domains might be involved. Based on the known boundaries of PKS ER domains,[25,26] $ER_{1/2}$ was expressed as a recombinant protein, purified (Figure S16) and used in the module 3 chain-elongation assay. No reduced product was detected (Figure 3F). This result might reflect either incorrect folding of the recombinant protein, or a need for the presence of additional domains to provide protein:protein interactions essential for the intermodular activity. As confirmed by the crystal structure of the KR-ER didomain from the second module of the spinosyn type I PKS, the ER domain is sandwiched between a structural and a catalytic subdomain of the KR domain.[27] If the same intimate contacts are maintained in the AZL $ER_{1/2}$-$KR_{1/2}$ didomain, it may promote proper folding of the $ER_{1/2}$, allowing the didomain to be active when the $ER_{1/2}$ domain alone is not. The effects of inclusion of other flanking domains are less predictable. When recombinant subsets of module 1/2 containing additional domains ($ER_{1/2}$-$KR_{1/2}$, $DH_{1/2}$-$ER_{1/2}$-$KR_{1/2}$ and $DH_{1/2}$-$ER_{1/2}$-$KR_{1/2}$-$ACP_{1/2}$) were assayed (Figure S16), the LC-ESI-HRMS results showed that they all supported inter-modular enoylreduction (Figures 3G-3I). Notably, the presence of the $KR_{1/2}$ domain seems necessary for the in-trans $ER_{1/2}$ activity on the substrate analogue tethered to module 3. But the inactivation of $KR_{1/2}$ domain has no detectable effect on $ER_{1/2}$ domain catalysing the cross-module enoylreduction, which suggest that $KR_{1/2}$ domain is important in preserving contacts with $ER_{1/2}$ and stabilising the $ER_{1/2}$ in its native conformation (Figure S26). Also, the $ER_{1/2}$ can interact with its substrate tethered to module 3 even in the absence of specific ACP-KS contacts (Figure 3H); although the extent of reduction is greater when the $ACP_{1/2}$ domain is present (Figure 3I). The phylogenetic analysis and the 3D modelling revealed no obvious feature in which $ER_{1/2}$ differs structurally from typical PKS ER domains (Figures S27-S29). Further structural and functional experimentation will be required to identify the exact protein:protein interactions that contribute to this enhancement.

Conclusion

In summary, this study revealed an unprecedented modular PKS enoylreductase domain that is used in not only “switchable” but also cross-module enoylreduction during polyketide chain elongation, and provided evidence in favour of a direct intermodular interaction with the substrate anchored in the downstream module, instead of a "stuttering" mechanism in which the acyl chain is backtransferred for reduction. Our findings provide the starting point for detailed structural and mechanistic investigation of the factors favouring this mechanism, and add to our growing appreciation of the plasticity of assembly-line polyketide biosynthesis, and of the insights examples of non-colinearity may provide into plausible pathways by which these systems have evolved.[27]

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Keywords: natural products • biosynthesis • polyketide synthases • enoylreductase domain • iterative domain

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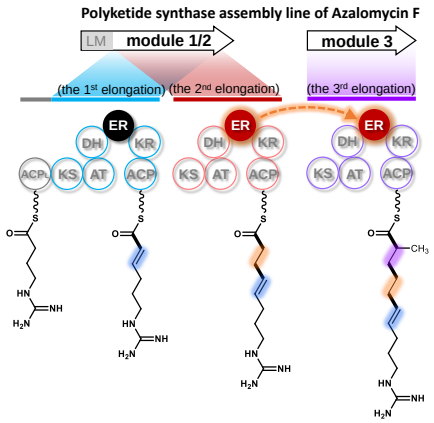

Borrowing: A switchable enoylreductase (ER) domain borrowed by neighbouring module in macrolide antibiotic Azlomycin F biosynthesis shows modular polyketide synthase (PKS)'s versatility that has never been known. This internal ER domain not only serves as an unusual switch in an iterative module for enoylreduction but also function surprisingly in a cross-module mode during polyketide chain elongation. This discovery further expands our understanding of the wonderful nature of PKS.