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Role of a Conserved Arginine Residue in Linkers between the Ketosynthase and Acyltransferase Domains of Multimodular Polyketide Synthases

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Supporting Information

ABSTRACT: The role of interdomain linkers in modular polyketide synthases is poorly understood. Analysis of the 6-deoxyerythronolide B synthase (DEBS) has yielded a model in which chain elongation is governed by interactions between the acyl carrier protein domain and the ketosynthase domain plus an adjacent linker. Alanine scanning mutagenesis of the conserved residues of this linker in DEBS module 3 led to the identification of the R513A mutant with a markedly reduced rate of chain elongation. Limited proteolysis supported a structural role for this Arg. Our findings highlight the importance of domain−linker interactions in assembly line polyketide biosynthesis.

Multimodular polyketide synthases (PKSs) are modular enzymatic assembly line proteins that catalyze the biosynthesis of numerous polyketide antibiotics.[1] Each catalytic module minimally consists of a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain. The KS domain receives the growing polyketide chain from the ACP domain of the previous module, while the AT domain transfers an α-carboxyacyl extender unit onto the phosphopantetheine arm of the ACP domain. The KS then catalyzes decarboxylative condensation between the growing chain and the extender unit, leading to the formation of a β-ketoacyl thioester−ACP intermediate (chain elongation reaction). Following varying degrees of β-carbon modification by auxiliary enzymes in the module, this intermediate is eventually translocated to the KS domain of the next module. Alternatively, chain growth is terminated by a thioesterase (TE) domain. Understanding the mechanism for this orderly progress of the growing polyketide chain represents a fundamental challenge in assembly line enzymology.

The 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea* is perhaps the most well-studied multimodular PKS.[2,3] Its ACP domains show marked specificity toward their cognate KS partners during chain elongation and intermodular chain translocation.[4,5] We have recently demonstrated that the specificity of these two catalytic reactions is controlled by distinct protein−protein interfaces.[6−8] Whereas the ACP recognition elements for both reactions have been mapped in considerable detail, less is known about their precise sites of interaction with the KS-AT partner protein. For example, it was proposed that, during chain elongation, the ACP domain interacts with the KS domain as well as the linker connecting the KS and AT domains (KS-AT linker).[6] We have subjected our model to further interrogation, using DEBS module 3 harboring an appended TE domain (M3+TE) as a test case (Figure 1). The advantages of DEBS module 3 are twofold. First, it lacks any apparent active auxiliary enzyme and is therefore a bona fide "minimal" PKS module. Second, the majority of this 367 kDa homodimer has been structurally characterized.[9]

The KS-AT linkers in individual modules of DEBS are approximately 100 residues long and consist of a three-stranded β-sheet packed against two α-helices on one side, representing a unique fold in the Protein Data Bank (PDB).[10] Guided by the assumption that mechanistically important residues in these linkers were also evolutionarily conserved, we compared the sequences of the KS-AT linkers of DEBS with those of other multimodular PKSs (Figure 1 of the Supporting Information). On the basis of the resulting alignment, we selected the most conserved Pro-473, Val-475, Val-476, Ser-477, Arg-479, Leu-484, Gln-487, Ile-491, Leu-509, Arg-513, His-516, His-518, Arg-519, Leu-534, and Ile-537 (numbering based on PDB entry 2QO3) and constructed the corresponding alanine mutants in M3+TE. All of the mutants were expressed as soluble proteins and purified at yields of 15−20 mg/L. The rate of triketide lactone formation by each mutant protein was measured at 23 and 30 °C (Table 1). Most mutants showed initial rates comparable to that of the wild-type enzyme (0.1 min^{-1}). The R513A mutant, however, showed a 7-fold decrease in activity (0.014 min^{-1}) at 23 °C. Its rate was even more severely attenuated (>20-fold relative to that of the wild-type enzyme) at 30 °C. To rule out gross protein structural changes, we determined the rates of both KS- and AT-catalyzed self-acylation in the R513A mutant. Both rates were comparable to the corresponding reaction rates of the wild-type enzyme. Moreover, both acylation reactions (0.5 and 1 min^{-1}, respectively) were substantially faster than the overall M3+TE turnover rate (Figure 2 of the Supporting Information). These results support a model in which chain elongation is the rate-

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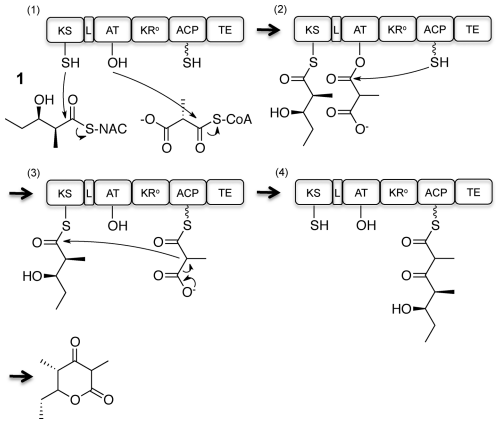

Figure 1. Chain elongation cycle catalyzed by M3+TE. The module consists of a KS, a KS-AT linker (L), an AT, an inactive KR (KR°), and an ACP domain. In the absence of an upstream module, the KS can be primed by a synthetic mimic of an ACP-bound diketide, **1**, while the AT is acylated with a methylmalonyl extender unit (1), which is then transferred to the ACP domain (2). Decarboxylative condensation occurs (3), and then the extended polyketide chain is cyclized by the TE domain to generate a triketide lactone (4). The phosphopantetheine prosthetic arm is shown as a wavy line.

Table 1. Relative Rates of Triketide Lactone Formation of M3+TE Mutants[a]

protein	relative to M3+TE at 23 °C	relative to M3+TE at 30 °C
M3+TE	1.00	1.00[b]
P473A	0.95	0.93
V475A	0.96	0.88
V476A	1.03	0.94
S477A	1.06	0.85
R479A	0.83	0.68
L484A	1.16	1.14
Q487A	1.33	1.16
I491A	1.03	0.68
L509A	0.93	0.67
R513A	0.15	<0.05
H516A	0.87	0.80
H518A	0.90	0.98
R519A	0.82	1.01
L534A	0.75	0.82
I537A	0.79	1.02

[a]For each mutant, the initial rates were measured and compared with that of the wild-type enzyme. [b]Wild-type M3+TE was 3-fold less active at 30 °C than at 23 °C.

limiting step in the net turnover rate of M3+TE and interactions between the ACP and the KS-AT linker play a critical role in this rate-limiting step.

Inverse temperature dependence is a widely recognized property of systems in which hydrophobic interactions are dominant and is thought to be associated with a diminished amount of structured water around a nonpolar surface with an increasing temperature, along with a vibrational contribution that increases in magnitude with an increasing temperature.[11] Formation of triketide lactone by wild-type M3+TE has a turnover number of 0.1 min^{-1} at 23 °C versus a rate of 0.033 min^{-1} at 30 °C (Table 1 and Figure 3 of the Supporting Information). This observation suggests that hydrophobic interactions between the KS-AT didomain and the ACP may dominate during chain elongation.

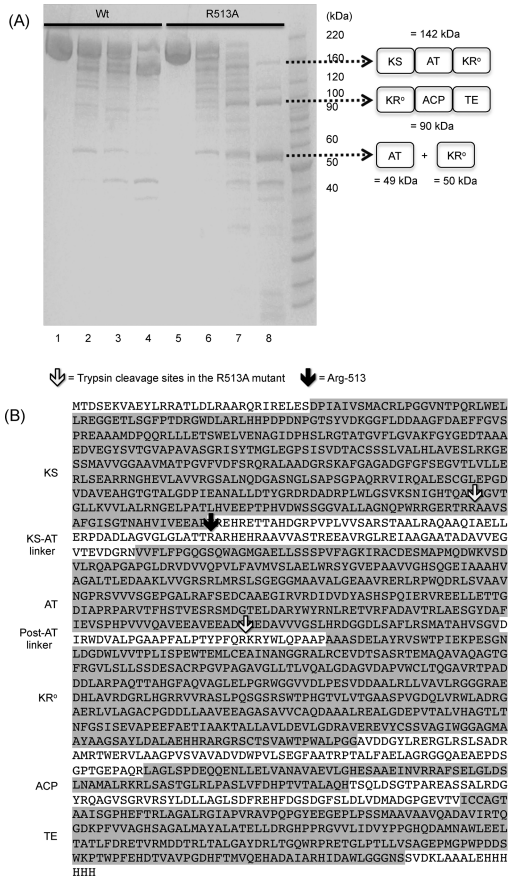

Figure 2. M3+TE and the R513A mutant were partially digested with 1 μg/mL trypsin at 30 °C. (A) Time course of limited trypsin digestion was monitored by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. Lanes 1−4 contained M3+TE (no digestion and digestion for 4, 16, and 64 min, respectively), and lanes 5−8 contained the R513A mutant (no digestion and digestion for 4, 16, and 64 min, respectively). The domain composition of the proteolytic fragments was inferred from the observed molecular mass and N-terminal sequencing. (B) Sequence of M3+TE annotated with domain and linker boundaries. Proteolyzed fragments between 50 and 60 kDa and between 90 and 100 kDa in the R513A mutant, which are indicated by black dashed arrows in panel A, were extracted, and their N-termini were identified by Edman degradation.

To investigate the effect of the R513A mutation on intermodular chain translocation, we employed a back-transfer assay in which the KS domain was first completely acylated with ^{14}C-labeled **1** and then the rate of back-transfer of the acyl chain to a stand-alone ACP2 protein was monitored.[12] In contrast to the rate of intramodular chain elongation, the R513A mutant displayed an intermodular back-transfer rate comparable to that of wild-type M3+TE (Figure 4 of the Supporting Information). None of the other mutants were perturbed in their ability to catalyze this reaction (Figure 4 of the Supporting Information). The marked difference between the effects of the R513A mutant on chain elongation and chain translocation supports a model in which two ACP domains engage the same KS differently in the course of the two reactions.[6,8]

To further probe the role of the R513A mutation, wild-type and mutant M3+TE were subjected to limited proteolysis under identical conditions, and the reaction products were analyzed by sodium dodecyl sulfate−polyacrylamide gel

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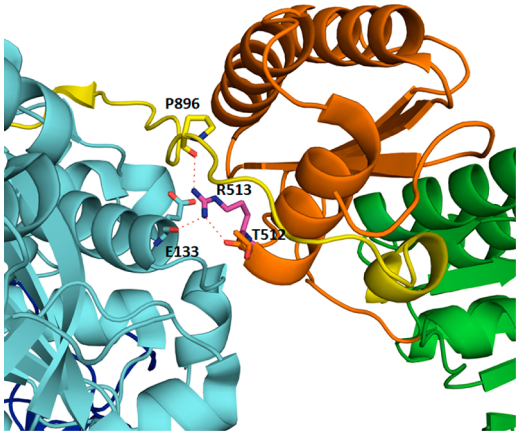

Figure 3. Arg-513 residue of DEBS module 3 (numbering based on PDB entry 2QO3). Arg-513 is located at the boundary between the KS domain (light blue) and the KS-AT linker (orange). Its side chain forms hydrogen bonds with the backbone amide groups of Glu-133 in the KS domain, Thr-512 in the KS-AT linker, and Pro-896 in the post-AT linker (yellow). The AT domain is colored green.

electrophoresis. The major product of prolonged proteolysis of the wild-type protein under the given conditions was the 142 kDa KS-AT-KR fragment (Figure 2A). In contrast, the corresponding fragment of the R513A mutant was susceptible to further proteolysis, suggesting that the mutant had a more flexible structure than the wild-type protein. N-Terminal sequencing of the two most abundant 50−100 kDa proteolytic fragments revealed the presence of two new proteolytically susceptible sites in the R513A mutant that were absent in the wild-type protein;[13] these sites were mapped near the C-terminal end of the KS domain and in the post-AT linker (see Figure 2B for proteolytic sites and definition of domain boundaries).

Analysis of the KS-AT structure suggests a possible explanation for these unexpected findings from limited proteolysis. As shown in Figure 3, the side chain of Arg-513 in the KS-AT linker of DEBS module 3 forms hydrogen bonds with the backbone amide groups of Glu-133 in the KS domain, Thr-512 in the KS-AT linker, and Pro-896 located in the post-AT linker.[9] Thus, the reduced turnover rate of the R513A mutant is not due to a direct role of the Arg-513 in the interaction with the ACP domain but instead is most likely due to the important role of this residue in stabilizing the demonstrated compact quaternary structure of the KS-AT didomain, which in turn shapes the ACP docking site during chain elongation. Pro-896 in particular is located immediately upstream of the YPFQRKRYW sequence (residues 898−906 in the post-AT linker), which is highly conserved in multimodular PKSs.[14,15] This sequence interacts with the side chains of Glu-126, Phe-106, Phe-107, and Trp-129 in the KS domain.[9] Interestingly, the YPFQRKRYW site underwent internal proteolysis in the R513A mutant, whereas trypsin cleaved the wild-type enzyme only after the sequence.[13] This suggests that R513 stabilizes the compact structure of this sequence. We also note that the post-AT linker has been shown to be required for triketide lactone synthesis by a combination of a stand-alone KS3 protein and a stand-alone AT3 protein.[16] Together, these findings lend further support to our model for ACP/KS-AT interaction during chain elongation.

In summary, the structural basis for protein−protein interactions is crucial to our understanding of the assembly line mechanism of multimodular PKSs and to the design of hybrid assembly lines. In this study, we have focused on the role of the relatively unconserved KS-AT linker. Our findings provide the first insights into the structural and mechanistic roles of this linker in polyketide chain elongation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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

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Notes

The authors declare no competing financial interest.

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