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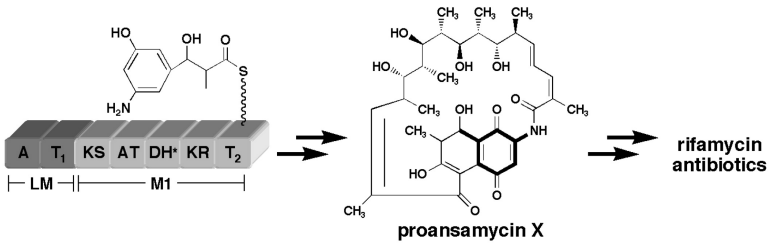

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A Switch for the Transfer of Substrate between Nonribosomal Peptide and Polyketide Modules of the Rifamycin Synthetase Assembly Line

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Rifamycin (Rif) synthetase is a nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) comprising an NRPS-like loading module and 10 PKS elongation modules.[1] Each module is a functional unit that incorporates one aryl or acyl monomer into the elongating product, and the 11 modules are distributed among five polypeptides. The linear undecamer encoded by the synthetase is released and cyclized to yield the proansamycin X precursor of the rifamycin class of antitubercular agents. These antibiotics inhibit transcription by binding to the β subunit of bacterial RNA polymerase.[1,2]

A distinguishing feature of modular NRPS, PKS, and mixed NRPS−PKS protein assemblies is the series of covalent intermediates on their reaction pathways.[3] Intermediates remain linked to these biosynthetic proteins as thioesters via the phosphopantetheine (Ppant) arm of thiolation domains or the active-site cysteine of ketosynthase domains. For example, the initial steps in the pathway of Rif synthetase are shown in Figure 1. The loading module (LM) of Rif synthetase activates 3-amino-5-hydroxybenzoate (AHB) as AHB-AMP and links it to the Ppant arm of its thiolation domain (T_1). AHB is then transferred across the NRPS−PKS interface to the active-site cysteine of the ketosynthase domain (KS) of PKS module 1 (M1), and this second intermediate reacts with a methylmalonyl moiety to form an aryl ketide covalently bound to the M1 thiolation domain (T_2). Downstream PKS modules further extend the polyketide backbone of the molecule, always maintaining the elongating chain in a thioester linkage. This assembly-line strategy presents a challenge for probing the molecular mechanisms of individual reactions, as intermediate products remain attached to proteins.

In this study we have probed the two earliest intermediates on the Rif synthetase pathway using a protein construct that encompasses its loading and initial elongation modules (LM−M1). This work demonstrates methods to assess the processing of assembly-line intermediates and provides insight into the timing and kinetics of early enzymatic steps in the biosynthesis of rifamycin antibiotics.

Previous work has shown that benzoate (B) and a variety of substituted benzoates are accepted as slow alternatives to the biological substrate AHB and processed with C−C bond formation into the corresponding aryl ketides by LM−M1;[4] consequently, reactions of B were investigated for convenience. To isolate covalent intermediates (E−B), varying amounts of radiolabeled B and saturating Mg·ATP were added to wild-type LM−M1 in the absence of the M1 substrate methylmalonyl-CoA (mmCoA). At various times the protein in the reactions was precipitated with TCA, and the radiolabeled E−B that had formed was quantitated by scintillation counting. A representative reaction is shown in Figure 2A (open circles), where the amount of E−B formed relative to the total amount of LM−M1 present is plotted as a function of time. The stoichiometry of E−B relative to total E is less than one (0.6), suggesting that this fraction of the enzyme molecules is covalently modified with only one B molecule.

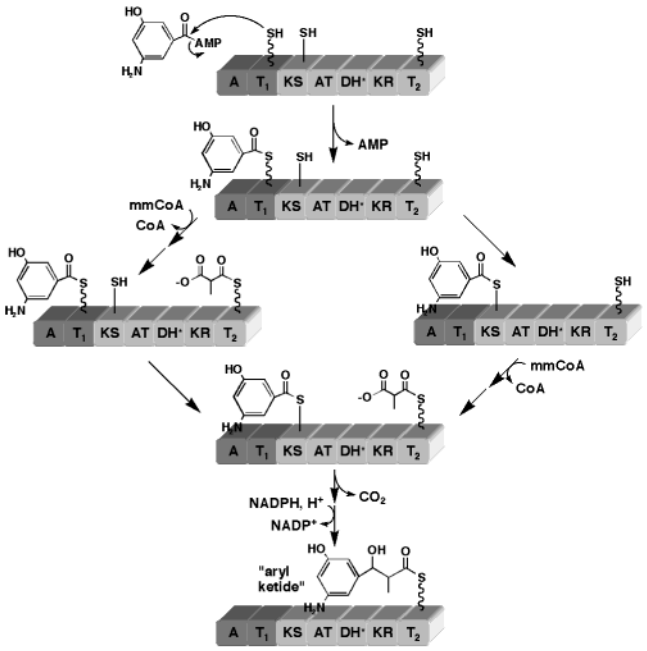

Figure 1. Initial steps in the reaction pathway of Rif synthetase. Selected reactions and intermediates are shown and described in the text, and the active sites denote adenylation (A), thiolation (T), ketosynthase (KS), acyltransferase (AT), β-ketoreductase (KR), or dehydratase (DH) domains. The two domains of the NRPS-like LM are shaded more darkly than the five domains of PKS M1, and enzymatic thiol nucleophiles are depicted schematically. The two models represented by the right and left limbs of the pathway are described in the text. The starred DH domain is inactive due to active site amino acid deletions.[1]

The simplest model for the covalent association of one B molecule with one LM−M1 molecule is that the enzyme can be stably loaded with B via the Ppant arm of T_1 but not via the active-site cysteine of KS. To test this idea the loading experiment described above was repeated using a site-directed mutant in which the cysteine nucleophile of KS had been replaced by alanine, preventing this mutant from forming an E−B intermediate at KS. The B loading reactions of wild-type and KS mutant enzymes are indistinguishable over the entire range of B concentrations investigated, giving identical loading stoichiometries (Figure 2A and data not shown) and kinetic parameters (Figure 2B), within error. This strongly suggests that both sets of loading experiments monitor formation of an E−B intermediate at T_1 only. This conclusion is strengthened by the observation that the same results are obtained

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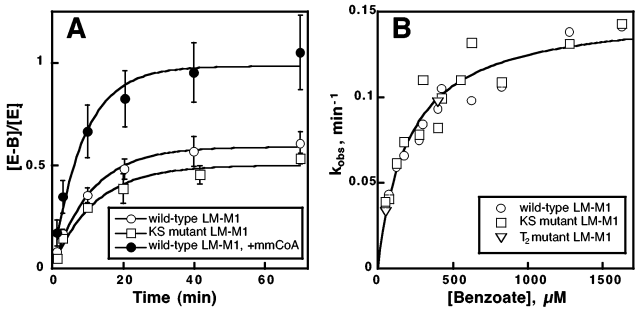

Figure 2. Covalent modification of LM−M1 by benzoate (B). **(**A**)** Time course of E−B formation in representative reactions that contained 400 μM B, 5 mM tris(2-carboxyethyl)phosphine hydrochloride, 5 mM ATP, 5 mM NADPH, 15 mM $MgCl_2$, 100 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 7.4), and 5 μM wild-type or KS mutant LM−M1 in the absence or presence of 1 mM methylmalonyl-CoA (mmCoA). Error bars represent one standard deviation from the mean of at least three data points. Lines are best exponential fits to each data set and give k_{obs} of 0.09 min^{-1} and an endpoint stoichiometry of 0.6 for the wild-type reaction, k_{obs} of 0.08 min^{-1} and an endpoint stoichiometry of 0.5 for the KS mutant reaction, and k_{obs} of 0.12 min^{-1} and an endpoint stoichiometry of 1.0 for the wild-type reaction containing mmCoA. **(**B**)** Dependence of E−B formation on the concentration of B for wild-type, KS mutant, and T_2 mutant LM−M1. The line is the best fit of the combined data to a simple saturation model and gives k_{cat} of 0.15 min^{-1} and K_M of 200 μM. The same kinetic parameters are obtained when the individual data sets for wild-type and KS mutant LM−M1 are fit separately.

for a T_2 mutant of LM−M1 (Figure 2B and Supporting Information), which renders unlikely the possibility that T_2 is modified by B.

Covalent loading of LM−M1 with B at only T_1 has implications for the timing of synthetase reactions. For product to be formed from this assembly line, B must be transferred from the Ppant thiol of T_1 to the cysteine thiol of KS. There are two models for the timing of this transthiolation event. In the first model, B is stalled at T_1 until mmCoA, the downstream substrate of M1, is available for reaction (Figure 1, left). In the second model, transfer of B occurs irrespective of the covalent occupancy of the downstream module (Figure 1, right). The B loading experiments described above are consistent with the former model, and thus B loading was investigated in the presence of mmCoA to determine if the E−B intermediate at KS could then be detected. Addition of mmCoA to reactions containing wild-type LM−M1 results in increased formation of radiolabeled intermediate (Figure 2A, solid circles), whereas its addition has no effect on reactions containing the KS mutant (see Supporting Information). Alkaline hydrolysis followed by TLC analysis revealed only a small amount of the new intermediate, ~20%, to be the phenyl ketide condensation product linked to T_2 under these conditions. To account for the remainder of the increased B incorporation by wild-type LM−M1, we propose that B is transferred from T_1 to KS in the presence of mmCoA, whereupon a second molecule of B is activated and loaded onto T_1 to form the doubly benzoylated intermediate. Furthermore, addition of mmCoA to reactions containing a T_2 mutant did not have a significant effect on the amount of E−B formed (see Supporting Information). Thus, the intermodular transfer of B from the T_1 domain of LM to the KS domain of M1 appears to require covalent loading of the methylmalonyl moiety of mmCoA onto the T_2 domain of M1, not simply initial binding and loading of mmCoA onto the AT domain.

In addition to providing information about the timing of intermodular transfer of B between LM and M1, these results also report on the kinetics of transfer. Although the endpoints for B incorporation differ as described above, the observed rates of B incorporation by the wild-type enzyme in the presence and absence of mmCoA are very similar, and the reactions follow reasonable first-order kinetics (Figure 2A). This suggests that the mmCoA-induced intermodular transfer of B from T_1 to KS is fast relative to initial activation and covalent loading of B, because biphasic kinetics reflecting build up of the E−B intermediate at T_1 followed by a slower accumulation of the E−B intermediate at KS and the doubly benzoylated intermediate would otherwise be expected for mmCoA-containing reactions.

In conclusion, occupancy of the T_2 domain of LM−M1 by a methylmalonyl moiety appears to trigger intermodular transfer of B from the T_1 domain to the KS domain, and this transthiolation event is fast relative to the initial loading of B onto the T_1 domain. The animal fatty acid synthase (FAS), closely related to PKSs, provides another example of an occupancy switch; decarboxylation of the malonyl moiety linked to the T domain of FAS, which generates a carbanion nucleophile for condensation with the cosubstrate acyl chain linked to the KS domain, is coupled to occupancy of the KS domain.[5] The Rif synthetase and FAS occupancy switches may exist to prevent formation of labile or highly reactive intermediates until cosubstrates are available for reaction. Although it remains to be seen whether these occupancy switches are a general feature of intact assembly lines, the putative switch for intermodular acyl transfer has been bypassed in several engineered PKS systems. In these cases, inactivation or absence of the upstream module facilitates the specific acylation of a KS domain by exogenous mimics of the natural acyl intermediate, even in the absence of a cosubstrate.[6]

Further analysis of intermediates on the reaction pathways of Rif synthetase and other assembly-line proteins promises to aid in the elucidation of their mechanisms and timing. It is expected that this information will guide the manipulation of these systems to efficiently generate novel products.

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Supporting Information Available: Procedures and data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) August, P. R.; Tang, L.; Yoon, Y. J.; Ning, S.; Müller, R.; Yu, T.-W.; Taylor, M.; Hoffmann, D.; Kim, C.-G.; Zhang, X.; Hutchinson, C. R.; Floss, H. G. *Chem. Biol.* **1998**, *5*, 69−79. (b) Tang, L.; Yoon, Y. J.; Choi, C.-Y.; Hutchinson, C. R. *Gene* **1998**, *216*, 255−265. (c) Schupp, T.; Toupet, C.; Engel, N.; Goff, S. *FEMS Microbiol. Lett.* **1998**, *159*, 201−207. (d) Floss, H. G.; Yu, T.-W. *Curr. Opin. Chem. Biol.* **1999**, *3*, 592−597. (e) Admiraal, S. J.; Walsh, C. T.; Khosla, C. *Biochemistry* **2001**, *40*, 6116−6123.

(2) Campbell, E. A.; Korzheva, N.; Mustaev, A.; Murakami, K.; Nair, S.; Goldfarb, A.; Darst, S. A. *Cell* **2001**, *104*, 901−912.

(3) (a) Cane, D. E.; Walsh, C. T.; Khosla, C. *Science* **1998**, *282*, 63−68. (b) Keating, T. A.; Walsh, C. T. *Curr. Opin. Chem. Biol.* **1999**, *3*, 598−606. (c) Du, L.; Sanchez, C.; Shen, B. *Metab. Eng.* **2001**, *3*, 78−95.

(4) Admiraal, S. J.; Khosla, C.; Walsh, C. T. *Biochemistry* **2002**, *41*, 5313−5324.

(5) (a) Witkowski, A.; Joshi, A. K.; Lindqvist, Y.; Smith, S. *Biochemistry* **1999**, *38*, 11643−11650. (b) Witkowski, A.; Joshi, A. K.; Smith, S. *Biochemistry* **2002**, *41*, 10877−10887.

(6) Tsukamoto, N.; Chuck, J.-A.; Luo, G.; Kao, C. M.; Khosla, C.; Cane, D. E. *Biochemistry* **1996**, *35*, 15244−15248.

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