J|A|C|S COMMUNICATIONS

Published on Web 02/26/2003

Epothilone C Macrolactonization and Hydrolysis Are Catalyzed by the Isolated Thioesterase Domain of Epothilone Polyketide Synthase

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The epothilone family of compounds is a group of polyketide natural products produced by the myxobacterium *Sorangium cellulosum*.[1] These small molecules are highly potent stabilizers of microtubules. Prolonged exposure to epothilones results in mitotic arrest followed by apoptotic cell death in a wide range of cell lines, making them particularly interesting as anticancer agents.[2]

Epothilone C is produced by the combined action of one nonribosomal peptide synthetase (NRPS) and nine polyketide synthase (PKS) modules in a multienzyme system.[3] These proteins catalyze the formation of the epothilone carbon−carbon backbone in a linear fashion starting with the heteroaromatic side chain.[4] The final step in the biosynthesis is the thioesterase (TE)-catalyzed cyclorelease of epothilone from the EpoF protein (Figure 1). This occurs first by transacylation of the linear epothilone-acyl carrier protein (ACP) thioester to the active site serine residue of the TE domain. Intramolecular nucleophilic attack of the C-15 hydroxyl group effects macrocyclization and release of the product from the enzyme.

Here we demonstrate that the excised epothilone TE domain can catalyze the efficient cyclization of the *N*-acetylcysteamine thioester of seco-epothilone C. Given the plethora of such macrocycle forming TE domains in nature, and the ease of producing these TEs via recombinant means, our finding provides a foundation for using these enzymes as environmentally friendly catalysts in macrolide synthesis.

The thioesterase-catalyzed macrocyclization/enzyme release strategy is used in many PKS[5] and NRPS systems.[6] While NRPS TE domains have recently been shown to catalyze the in vitro macrolactamization of linear peptides,[7] it has remained unclear whether excised PKS TE domains exhibit the corresponding macrolactonization activity.[8]

PKS TE domains, when fused to upstream PKS modules out of their normal context, have been shown to catalyze macrolactonization.[9] When separated from the upstream modules, PKS TE domains have been observed only to catalyze hydrolysis of thioester substrates. To date, though, only non-native substrates have been used.[8,10] Two hypotheses could account for the lack of macrocyclization activity. Either the TE domain does catalyze macrocyclization, but the substrates used in the assay cannot be processed by the enzyme, or the TE domain cannot perform macrocyclization unless attached to upstream PKS modules.

We were able to address these hypotheses using the epothilone biosynthesis system because of the ease of preparation of the substrates and enzyme (TE domain). The linear acyl chain substrate can be easily generated from the parent compound epothilone C.

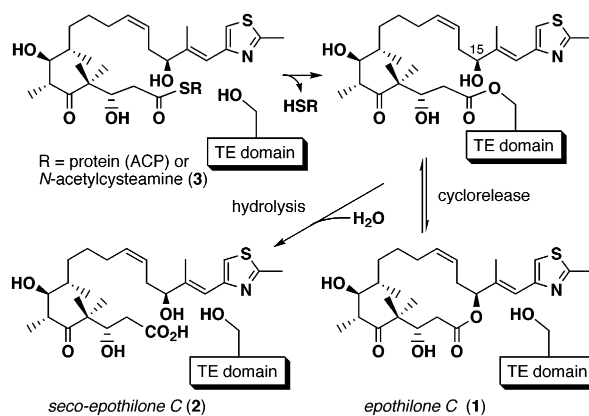

Figure 1. The thioesterase domain from the epothilone biosynthesis pathway catalyzes macrocyclization of the linear polyketide chain, releasing epothilone C from the enzyme.

The macrolactone can be opened by treatment under aqueous basic conditions in excellent yield (LiOH, MeOH, H_2O; 93% yield). The free acid **2** can then be activated by converting it into the *N*-acetylcysteamine thioester **3** (DCC, HSNAC; 65% yield) to mimic the *S*-ACP substrate context.

The epothilone TE domain of the EpoF subunit[3] was cloned into an *E. coli* expression vector under the T7 promoter with an *N*-terminal His-tag. Overexpression and purification via affinity chromatography gave epothilone TE domain at >90% purity as measured by SDS-PAGE analysis with a yield of approximately 20 mg/L of culture.

Treatment of thioester **3** with the purified TE domain generated a mixture of the cyclization product, epothilone C (**1**), and the hydrolysis product, seco-epothilone C (**2**). The formation of epothilone C was confirmed by ^{1}H NMR. The two products were separated by analytical reverse phase HPLC and quantified by their absorbance at 254 nm (Figure 2).

Hydrolysis rates increased with pH, while cyclization rates showed a maximum at neutral pH. Thus, the partitioning of the reaction pathway for cyclization over hydrolysis increased as pH decreased. Steady-state kinetic analysis of the epothilone TE domain was conducted at pH 6.5, where partitioning between cyclization and hydrolysis was maximized to a ratio of 5:1 while still maintaining fast cyclization rates (see Supporting Information). At pH 8, for example, the initial macrocyclization rate doubled, but the partition ratio fell to 3.5:1, reflecting increased flux to hydrolysis.

Our kinetic analysis clearly reflects enzyme catalysis of the macrocyclization and hydrolysis reactions from **3** as substrate. The k_{cat}/K_M value for macrocyclization ($\mathbf{3} \rightarrow \mathbf{1}$) is 0.41 ± 0.03 min^{-1} mM^{-1}. The limited solubility of thioester **3** (1 mM) in aqueous buffer precluded independent measurement of k_{cat} and K_M, but

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10.1021/ja0298646 CCC: $25.00 © 2003 American Chemical Society

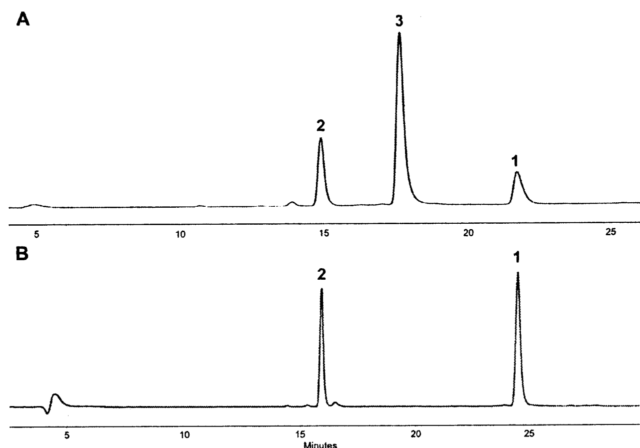

Figure 2. (A) An analytical HPLC trace showing the epothilone TE-catalyzed formation of epothilone C (**1**) and seco-epothilone C (**2**) from thioester **3**. Reaction conditions: 1.0 mM **3**, 3.3 μM TE, 50 mM sodium phosphate buffer, pH 6.5, 10% (v/v) DMSO, 3 h, 30 °C (Econosphere C_{18}, 5 μm, 4.6 × 250 mm). (B) An analytical HPLC trace showing the epothilone TE-catalyzed hydrolysis of epothilone C (**1**). Reaction conditions: 0.25 mM **1**, 3.3 μM TE, 50 mM sodium phosphate buffer, pH 6.5, 10% (v/v) DMSO, 1.0 h, 30 °C (Vydac C_{18}, 5 μm, 4.6 × 250 mm).

clearly indicated that the K_M is $\geq$1.0 mM. The concurrent hydrolysis reaction (**3** → **2**) was significantly slower, occurring with a turnover of $k_{cat} = 0.087 \pm 0.005$ min^{-1} and a $K_M = 291 \pm 53$ μM ($k_{cat}/K_M = 0.30 \pm 0.07$ min^{-1} mM^{-1}). Notably, the hydrolysis and macrocyclization reactions gave comparable k_{cat}/K_M values, supporting the proposed mechanism where an enzyme-acyl intermediate is formed in the first irreversible step in the enzyme pathway.

In contrast, the nonenzymatic hydrolysis reaction of **3** to **2** occurred with a background rate of 6×10^{-6} min^{-1}.[11] Thus, the enzyme accelerates the reaction 10 000-fold. In evaluating the relative acceleration of the TE for macrocyclization, no conversion of **3** to **1** was detected in HPLC assays in the absence of enzyme. We estimated that background cyclization, based on the detection limit of the HPLC assay, occurred at a rate less than 1×10^{-8} min^{-1}. The TE domain thus enhances the macrocyclization rate more than 7 orders of magnitude. This rate enhancement is comparable to those seen for NRPS TE-catalyzed macrocyclization reactions.[7a]

Additional assays showed that the TE domain could also catalyze the hydrolysis of macrolactone **1** to give seco-acid **2**, with saturation kinetics, at a $k_{cat} = 0.67 \pm 0.01$ min^{-1} and a $K_M = 117 \pm 5$ μM ($k_{cat}/K_M = 5.7 \pm 0.3$ min^{-1} mM^{-1}). Thus, the front (**3** → **2**) and back (**1** → **2**) reactions proceed with different catalytic efficiency, suggesting that the rate-limiting step varies between the two reactions. Epothilone D (C12-methyl) and epothilones A and B (C12−C13 epoxy)[1] were also substrates for macrolactone ring opening at comparable catalytic efficiency. Incubation of the TE domain with epothilone D and *N*-acetylcysteamine yielded enzyme-catalyzed formation of both seco-epothilone D and the *N*-acetylcysteamine thioester of epothilone D.

This work shows that an isolated PKS TE domain can catalyze macrocyclization, analogous to previous results with NRPS TE domains.[7] It should now be possible to probe the substrate tolerance of PKS TE domains, using synthetic and semisynthetic substrates. These studies, in combination with structural insights gained from other PKS TE domains,[12] will identify features in the substrate that are critical for the TE domain macrolactonization activity. Understanding the substrate tolerance of PKS TE domains will aid in rationally engineering new polyketide biosynthesis pathways.

Acknowledgment. Funding for this work was provided by the NIH GM20011 (C.T.W.) and CA 66736 (C.K.). C.N.B. is supported by a National Research Service Award (F32 CA091636) from the National Cancer Institute. T.L.S. is supported by Postdoctoral Fellowship Grant no. PF-02-023-01-CDD from the American Cancer Society. We acknowledge Kosan Biosciences, Inc. for providing the cosmid containing the epothilone TE domain and samples of epothilones.

Supporting Information Available: Experimental procedures, kinetic data, and characterization of compounds **1**, **2**, and **3** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0298646