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# Structural elements of an NRPS cyclization domain and its intermodule docking domain

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**Epothilones are thiazole-containing natural products with anticancer activity that are biosynthesized by polyketide synthase (PKS)-nonribosomal peptide synthetase (NRPS) enzymes EpoA–F. A cyclization domain of EpoB (Cy) assembles the thiazole functionality from an acetyl group and L-cysteine via condensation, cyclization, and dehydration. The PKS carrier protein of EpoA contributes the acetyl moiety, guided by a docking domain, whereas an NRPS EpoB carrier protein contributes L-cysteine. To visualize the structure of a cyclization domain with an accompanying docking domain, we solved a 2.03-Å resolution structure of this bidomain EpoB unit, comprising residues M1–Q497 (62 kDa) of the 160-kDa EpoB protein. We find that the N-terminal docking domain is connected to the V-shaped Cy domain by a 20-residue linker but otherwise makes no contacts to Cy. Molecular dynamic simulations and additional crystal structures reveal a high degree of flexibility for this docking domain, emphasizing the modular nature of the components of PKS-NRPS hybrid systems. These structures further reveal two 20-Å-long channels that run from distant sites on the Cy domain to the active site at the core of the enzyme, allowing two carrier proteins to dock with Cy and deliver their substrates simultaneously. Through mutagenesis and activity assays, catalytic residues N335 and D449 have been identified. Surprisingly, these residues do not map to the location of the conserved HHxxxDG motif in the structurally homologous NRPS condensation (C) domain. Thus, although both C and Cy domains have the same basic fold, their active sites appear distinct.**

crystal structure | molecular dynamics | epothilone | natural product

**E**pothilones are hybrid polyketide/nonribosomal peptide natural products indicated for treatment of metastatic or locally advanced breast cancers that are taxane resistant (1, 2). They contain a large macrocycle with a thiazole-containing side chain, which is important for stabilizing microtubules and impairing cell division (Fig. 1A) (3, 4). Azole heterocycles, such as thiazoles, are commonly found in many natural products and in different oxidation states (i.e., azolines and azolidines). They are more resistant to hydrolysis than a peptide bond, and their incorporation can increase a compound's affinity for a target biomolecule (5). Despite their importance, there is still much to learn about the structure and mechanism of the enzymes involved in their biosynthesis (6, 7). Here we explore the structural basis of activity of the catalytic domain that generates the 2-methylthiazoline precursor of epothilone natural products.

The epothilone biosynthetic gene cluster from *Sorangium cellulosum* is encoded by *epoA–K* (Fig. 1A). EpoA, a polyketide synthase (PKS), and EpoB, a nonribosomal peptide synthetase (NRPS), are responsible for making the 2-methylthiazole functionality (8–11). 2-methylthiazole derives from the condensation of an acetyl group with L-cysteine (Fig. 1B). Before condensation, the acetyl moiety and L-cysteine are covalently attached to the carrier T domains of EpoA (acetyl-S-EpoA-T) and EpoB (cysteinyl-S-EpoB-T), respectively, via phosphopantetheine (Ppant) linkers (Fig. 1A and Fig. S1). Instead of

a traditional condensation or C domain, EpoB has a cyclization (Cy) domain that performs both the amide bond forming condensation reaction and the cyclization/dehydration to form the five-membered ring structure of 2-methylthiazoline (10). Two mechanisms have been proposed that differ in whether the amide bond forms first or second to the cyclization reaction (12, 13) (Fig. 1B). Subsequent oxidation by the flavin-dependent oxidase domain (EpoB-Ox) results in 2-methylthiazole (8, 10, 11). PKS-NRPS docking domains (14), referred to as EpoAdd and EpoBdd for the upstream donor and the downstream acceptor proteins, respectively, serve to localize the T domains to the appropriate intermodule junction, facilitating what has often been referred to as an assembly line biosynthetic process.

There is much that is unknown about Cy domains; the molecular basis for the differentiation of C and Cy domain activity is not established, and the key catalytic residues have not been identified. Keating et al. predicted that the C and Cy domains would adopt similar structures (15), and early sequence alignments identified a DxxxxDxxS Cy domain sequence that replaces the C domain HHxxxDG catalytic motif (Fig. S2). Although conservation of the

## Significance

Here we investigate the structural basis for cyclization activity in hybrid polyketide synthase-nonribosomal peptide synthetases. This first structure of a cyclization (Cy) domain reveals an unexpected location for the enzyme active site, providing a fresh perspective on past mutational studies. Our structures also depict two 20-Å-long channels that create routes for the two tethered substrates to simultaneously reach the buried active site, affording substrate condensation and cyclization. Along with the Cy domain, these structures contain a covalently attached docking domain, providing insight into how protein modules work together to achieve uni-directionality in the biosynthesis of natural products.

Author contributions: D.P.D., Y.K., A.K.C., K.T., and C.L.D. designed research; D.P.D., Y.K., A.K.C., and K.T. performed research; W.L.K. and C.T.W. contributed new reagents/analytical tools; D.P.D., Y.K., A.K.C., K.T., and C.L.D. analyzed data; and D.P.D. and C.L.D. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, [www.pdb.org](http://www.pdb.org) (PDB ID codes 5T81 and 5T72).

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docking domain structure with this fold, TubCdd from the *Angiococcus disciformitus* tubulysin system (14). This docking domain functions at an NRPS-NRPS junction and shares with EpoBdd 33% sequence identity and an RMSD of 3.6 Å for 52 C $\alpha$  atoms (Fig. S3) (14). The  $\beta$ -hairpin and the third  $\alpha$ -helix ( $\alpha 3'$ ) of the  $\alpha\beta\alpha$  fold show the largest deviation between these two docking domain structures (Fig. S3). In EpoBdd, the  $\beta$ -hairpin is tipped inward slightly toward  $\alpha 2'$ , whereas  $\alpha 3'$  is swung slightly away from the structural core (Fig. S3D). This difference in the  $\beta$ -hairpin is interesting given that this region is predicted to be a binding site of the cognate recognition sequence (14, 25). However, it is not currently clear if these variations are mechanistically significant or an artifact of a difference in oligomeric state. In solution and without its cognate protein, TubCdd is a dimer, whereas EpoBdd is monomeric in our structure. Notably, the  $\beta$ -hairpin and the third  $\alpha$ -helix ( $\alpha 3'$ ) are both involved in the TubCdd dimer interface. Unfortunately, no structure of TubCdd with a partner protein is available that could clarify the relevance of the dimeric unit and provide additional information about protein interaction specificity.

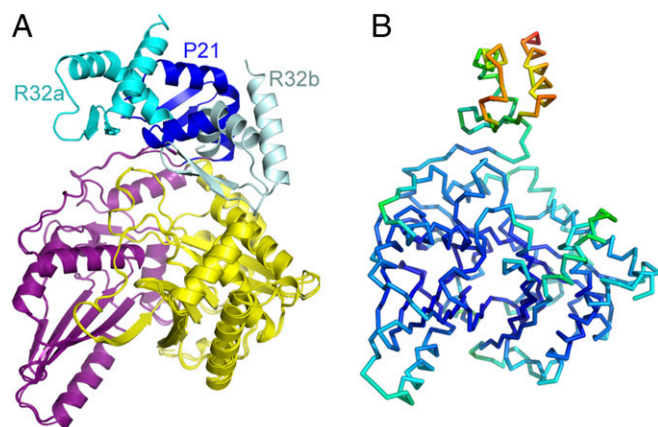
**Structures and MD Simulations Reveal Conformational Flexibility of Docking Domain.** Three different conformations of the docking domain are observed in our structures, consistent with the presence of a flexible rather than rigid linker between the docking and Cy domains, and the existence of very little buried surface at the domain–domain interface in any of the structures (Fig. 3A and Fig. S4). The R32 crystal form reveals two conformations of the docking domain, with each molecule in the asymmetric unit adopting a different conformation. A third orientation is visible in the P<sub>21</sub>-EpoBcy structure (Fig. 3A). An alignment of these three docking domain structures with each other shows strong agreement (RMSD of less than 0.7 for all C $\alpha$  atoms), indicating that this domain moves as a rigid body (Fig. S3C). To further explore the flexibility of the docking domain, a 20-ns molecular dynamics (MD) simulation of the fully hydrated protein was run. Little movement of the Cy domain was observed in comparison with the docking domain, which sampled a larger range of positions especially during the first 5 ns of the simulation (Fig. 3B and Movie S1). None of these conformations were observed to make substantial interactions with

the Cy domain; thus, the connection between domains appears largely dependent on the covalent linker.

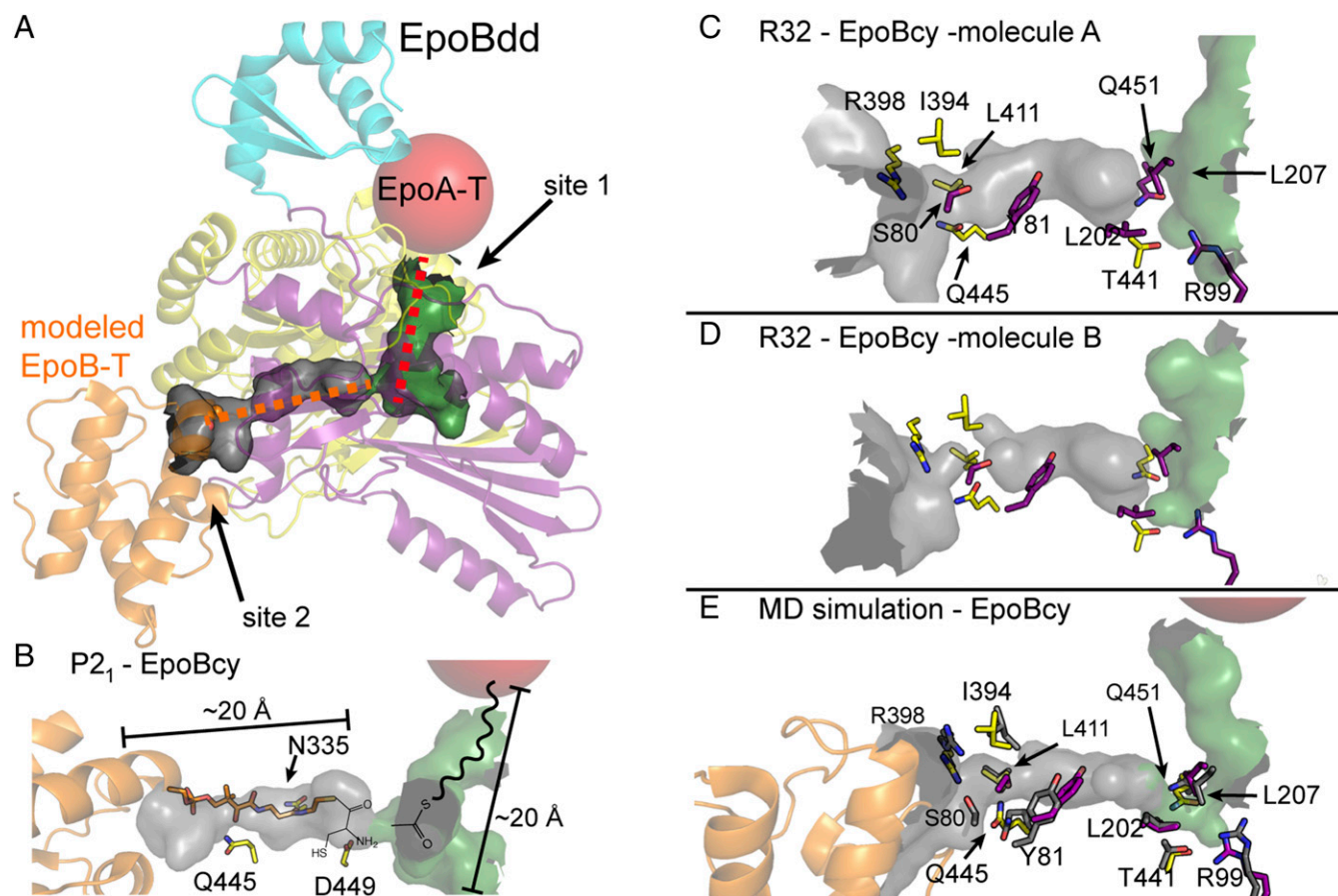
**Structures Suggest T-Domain Binding Sites.** The EpoB Cy domain has the challenging task of interacting with carrier T domains from two separate proteins, each of which supply a different component to make 2-methylthiazoline (Fig. 1). Because EpoBdd facilitates interactions with the PKS T-domain of EpoA, the location of the docking domain in our structures identifies the approximate binding site for EpoA-T (referred to as site 1 in Fig. 4A). Of the three observed conformations of EpoBdd (Fig. 3A), R32b is the closest to site 1, whereas the P<sub>21</sub> domain position is  $\sim 10$  Å farther removed and R32a is yet another  $\sim 10$  Å farther. Superimposition of EpoBcy with the recently reported structure of a holoenzyme T–E bidomain from gramicidin synthetase (23) provides further support for this upstream T domain binding site (Fig. S5A). The T domain in the gramicidine synthetase is adjacent to the R32b-position of EpoBdd when the EpoB Cy domain and gramicidine synthase E domains are superimposed.

Insight into the binding location for the NRPS EpoB-T domain comes from two structures of NRPS modules: the terminal surfactin A module (18) and the terminal holo-AB3403 module (19), in which the internal T domain of each module is positioned to interact with its C domain. Structural superimposition (Fig. S5C and D) identifies the EpoB-T binding site as site 2 in Fig. 4A, a position that is at the interface of the N- and C-terminal halves of the Cy domain and is proximal to  $\alpha 1$  and  $\alpha 10$  (Fig. S2). Putative upstream and downstream T domain binding sites 1 and 2 (Fig. 4A) are nonoverlapping, consistent with the proposal that both T domains bind EpoBcy at the same time (15). Intriguingly, the P<sub>21</sub>-EpoBcy structure shows an extended L-shaped channel that connects putative T domain binding site 1 to the active site and the active site to putative T domain binding site 2 (Fig. 4A). The distance between each putative T domain binding site and the active site is  $\sim 20$  Å, the length of an extended Ppant arm. This physical relationship suggests an acetyl moiety tethered via a Ppant arm from the EpoA-T domain would sit juxtaposed to a L-cysteinyll moiety tethered via a Ppant arm from the EpoB T domain (Fig. 4B). Interestingly, a superimposition of EpoBcy with the terminal holo-AB3403 module (19) positions the Ppant arm bound in the holo-AB3403 structure into the EpoBcy site 2 channel (gray in Fig. 4B), demonstrating conservation of the channel position at least between these two structures. In contrast to this conservation, superimposition of EpoBcy with gramicidine synthase E domain does not result in the placement of the Ppant arm bound to the E domain into the EpoBcy site 1 channel (green in Fig. 4B and Fig. S5B). Inspection of the structural superimposition (Fig. S5B) shows that four  $\beta$ -strands in the E domain structure are shifted with respect to the equivalent strands in EpoBcy creating an alternative channel that is positioned next to, and not overlapping with, the channel in EpoBcy. The latter provides an example of how small structural differences may influence substrate positioning.

The three EpoBcy structures show channels with different degrees of openness due to variations in surface loop positions and in side chain positions (Fig. 4B–D and Fig. S6). To investigate the conformational dynamics of residues near the active site that may allow for channel widening and clamping, we carried out MD simulations on the R32 structure that had the contracted cavity (shown in Fig. 4D). As mentioned above, in a 20-ns MD simulation, the Cy domain does not move substantially; however, certain protein residues located on both sides of the active site appear to undergo small movements of their backbone and slightly larger movements of side chains, with the result being that the cavity opens to resemble the contiguous channel observed in the P<sub>21</sub> structure (Fig. 4E). These data suggest that small movements of residues can alternately contract and widen channels between the two T domain binding sites and the active site without the need for large movements of the protein backbone.



**Fig. 3.** The PKS-NRPS docking domain is flexibly tethered to EpoBcy. (A) Structural alignment of the three monomeric forms of EpoBcy observed from the P<sub>21</sub> and R32 crystal structures. The Cy domain is colored as in Fig. 2, and the three observed orientations of the docking domain are colored cyan, pale cyan, and blue. (B) A 20-ns MD simulation of EpoBcy with increased protein movement indicated by the color of the protein trace, from blue (least motion) to red (greatest motion). The EpoBcy  $\alpha$  trace is displayed in ribbons. Also see Movie S1.



**Fig. 4.** Proposed binding sites for substrate T domains. (A) P<sub>21</sub>-EpoBcy (colored as in Fig. 2) is displayed in ribbons with the putative active site channels displayed in green and gray (calculated by HOLLOW) (32). The binding of EpoA-T at site 1 is predicted by proximity to the docking domain and is shown as a red sphere; EpoB-T (orange) is modeled at site 2 from the structural alignment of EpoBcy with AB3403 (19) that is shown in Fig. S5. Cavities are the approximate length of the Ppant prosthetic group (dashed orange and red lines). (B) Close-up view of active site channels from P<sub>21</sub>-EpoBcy structure. Locations of the EpoA-T and EpoB-T domains represented as in A. A Ppant arm is modeled in the site 2 channel (gray) based on the superimposition of EpoBcy with holo-AB3403, and a squiggly line represents the Ppant in the site 2 channel (green). The location of the catalytically important residues D449, N335, and Q445 are indicated. (C) Channel calculation for R32-EpoBcy molecule A shows less connectivity between the site 1 channel (green) and the site 2 channel (gray) than in the most open channel, which is shown in B. Residues shown as sticks have different positions in the open channel; carbons are colored purple if from the N-terminal domain and yellow if from the C-terminal domain. (D) Channel calculation for R32-EpoBcy molecule B shows the most restricted internal cavities. (E) The 20-ns MD simulation of most closed EpoBcy R32 structure (molecule B, shown in D), leads to an opening of the channel such that this channel now resembles the channel of the P<sub>21</sub> structure in B (residues from MD simulations are colored gray).

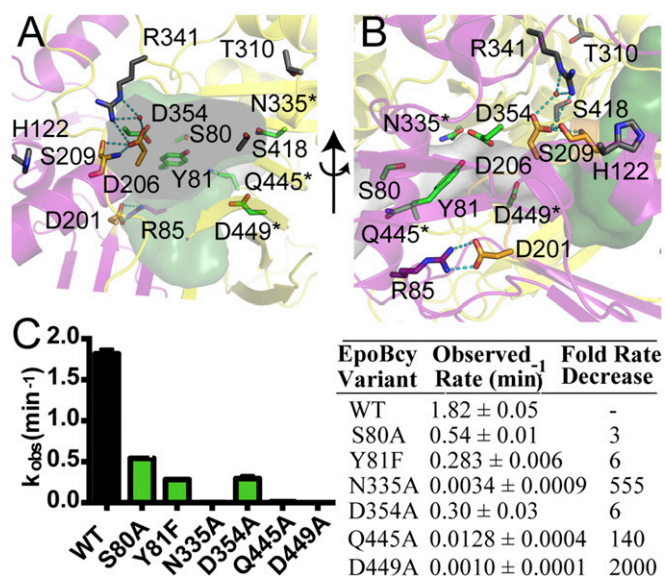
#### Structure and Mutagenesis Reveal Unexpected Location for Cy Active Site.

The N- and C-terminal halves of the EpoB Cy domain form a stable interface, which in turn forms the putative substrate-binding channels that were described above. In addition to several hydrophobic residues that may mediate favorable interactions with the hydrophobic Ppant arm and acetyl moiety of substrate, the structure reveals a set of previously uncharacterized polar residues that may be involved in catalysis, including S80, Y81, D354, Q445, and D449 (Fig. 5 A and B and Fig. S7). We mutated these residues and also N335, which was studied previously in the homologous BacA system (Table S3) (13). These six residues were mutated individually and assayed for activity using LC/MS-MS detection, monitoring formation of 2-methylthiazole-4-carboxylic acid (2MTCA) (Fig. S1). Three active site variants have severely compromised rates of product formation: D449A, N335A, and Q445A have 2,000-, 555-, and 140-fold decreased activities compared with WT EpoBcy (Fig. 5C). The S80A, Y81F, and D354A variants of EpoBcy display only moderate effects with three-, six-, and sixfold decreased activities, respectively. Putting these results in context with the structure has allowed us to localize the active site to the C-terminal half of the Cy structure, where the channel is lined with residues N335, Q445, and D449 (Fig. 5 A and B).

Surprisingly, these active site residues occupy a site that is distal to the previously identified DxxxDxxS motif (15, 16), the latter of which is on the N-terminal half of Cy (Fig. 5 A and B). The DxxxDxxS motif aligns well with the catalytic HHxxxDG motif of NRPS C domains as predicted (13, 16, 17) (Fig. 2B), but instead of playing a catalytic role, our structures suggest that the DxxxDxxS motif (D201-LINVDLG-S209 in EpoBcy) may be important for maintaining the integrity of the substrate channels. Importantly, neither Asp is free to interact with substrate. Rather, D201 is involved in a salt bridge with R85, which provides structure to one side of the channel, and D206 forms a salt bridge with R341 and a hydrogen bond to S209, supporting another side of the channel (Fig. 5 A and B).

#### Discussion

Hybrid PKS-NRPSs are remarkable macromolecular assembly lines with carrier proteins delivering substrates from one enzyme module to the next and docking domains providing the intermodular communication that allows for the proper directionality (14, 25, 26). Our structures provide a visualization of the interactions between an N-terminal docking domain and a downstream enzyme within a NRPS module, and we find a bead-on-a-string type arrangement.



**Fig. 5.** Identification of EpoBcy active site residues. (A) Polar residues that were mutated in this work are shown in sticks and colored with green carbons, with asterisks denoting those mutations that most dramatically affected enzyme activity. The previously identified D201xxxD206xxS209 sequence is colored with orange carbons, and previously mutated residues have gray carbons (see Table S3 for summary of mutational studies). R85 is colored with purple carbons and has not been mutated. Channels colored as in Fig. 4A and ribbons colored as in Fig. 2A. Stereoview is shown in Fig. S7. (B) Same as A, but rotated  $\sim 90^\circ$  clockwise about the vertical axis to match orientation shown in Fig. 4A. (C) Calculated rates of 2-methylthiazole-4-carboxylic acid formation by LC/MS-MS for EpoBcy variants in this work. Data are the average  $\pm$  SD for three replicates.

Covalent attachment by a 20-residue linker is all that is involved; EpoBdd makes no other contacts to the Cy domain. Thus, any docking domain could be substituted for EpoBdd with no reengineering of the Cy domain protein surface required. This result expands on the previous finding that switching EpoA modules can result in transfer of the different substrate unit to EpoB (27). Although EpoBdd is highly flexible, allowing it to search for its partner proteins, its attachment point to Cy appears key to its function. When EpoBdd localizes the EpoA-T to Cy through interaction with EpoAdd, the T domain will end up positioned near to one of two channel openings on Cy (site 1), allowing a substrate linked by a Ppant arm to reach down into the core of the Cy domain.

A second channel from a second T domain binding site (site 2) has been identified that is at a right angle from the site 1 channel. The existence of two channels allows for simultaneous binding of the two substrate-loaded T domains. By physically isolating the binding sites of the upstream and downstream carrier proteins, NRPS systems have developed a directionality that is important for defining the generation of a specific natural product. The length of each channel ( $\sim 20$  Å) matches the length of an extended Ppant arm ( $\sim 20$  Å), allowing us to predict that the acetyl moiety from EpoA-T and L-cysteinyll moiety from EpoB-T will end up juxtaposed in the active site and also proximal to catalytic residues D449 and N335 (Fig. 4B and S7C). Notably, the EpoA channel appears a bit longer than necessary for an acetyl moiety to be accommodated (Fig. 4A and B), perhaps explaining the observation that larger substrates (propionyl-, isobutryl-, and benzoyl-EpoA-T) can be turned over by Cy, albeit more slowly (10).

In our structures and MD simulations, we observe snapshots of more open and more closed states of the channel leading to the active site, and we expect that the binding of EpoA-T and EpoB-T domains at their respective binding sites will shift the equilibrium toward the more open state and that T domain departure will shift

toward the closed state, thus restricting active site access in the absence of substrates. Although conformational changes of protein backbone have been observed for C domain proteins that could contribute to channel opening and closing (20), here we see little to no movement of the backbone atoms of the three structures that display various degrees of channel openness. Also, MD simulations show that side chain motion is sufficient to open and close the internal protein cavities. Thus, for this Cy domain, there appears to be no need to invoke domain hinge motions in catalysis.

These studies have also allowed us to investigate how a Cy domain compares to a C domain. We find that the EpoB Cy domain adopts a similar protein fold as the NRPS C domain, validating previous predictions (15), but we also find that the catalytically important residues for Cy do not map to the location of the highly conserved sequence motifs (HHxxxDG for C domains and DxxxxDxxS for Cy domains). Importantly, residues of the DxxxxDxxS sequence motif are not free to interact with substrate, instead forming salt bridges and hydrogen bonding networks that stabilize the elaborate channels that run through the core of the protein fold. Although substrate binding might cause residues of the DxxxxDxxS motif to break their interactions and be available for catalysis, we instead propose that D449 is key to catalysis, potentially serving as a catalytic base. This proposal is consistent with the 2,000-fold effect on 2MTCA production when D449 is mutated to alanine.

Inspection of the proposed mechanisms in Fig. 1B shows a number of base-catalyzed steps that might be involved in this three step reaction: deprotonation of the amino group of cysteine for the peptide bond formation step with the acetyl moiety; deprotonation of the cysteine side chain for the cyclization reaction; and deprotonation of the ring NH for the dehydration reaction. It is possible that one residue may catalyze all three deprotonations, because the protonation state of the base could be reset after each step. Notably, the number of deprotonations equals the number of protonations, with the Ppant sulfur accepting one proton and the acetyl moiety oxygen accepting two protons as it is first reduced to a hydroxy group and then to water (Fig. 1B). Thus, from the perspective of stoichiometry, D449 could assist in all three reactions, but a structure with substrate bound would help to evaluate the geometric prospects of a single residue catalyzing all three different steps.

We also confirmed that N335 is catalytically important, having a 555-fold effect on 2MTCA production. Given that the corresponding asparagine residue in a chimeric engineered BacA Cy generated only an uncyclized dipeptide product when this asparagine was mutated to alanine (13), we expect that N335 is also involved in cyclization. Because N335 does not have a titratable side chain, we do not believe that it is a catalytic base. Instead, it may serve to position the substrates appropriately for cyclization or stabilize intermediates through hydrogen bonding. In short, the structures of EpoBcy explain much of the previous biochemical work and also reveal D449 as a key residue, although there is still much to be learned about this fascinating cyclization chemistry.

Since the discovery of NRPS natural products, researchers have been interested in manipulating these modular assembly lines for the bioproduction of novel chemical compounds (6, 26, 28, 29). For this to be achievable and efficient, we must increase our understanding of how these systems function at the molecular level (28). This work presents important structural information regarding two NRPS domains: the Cy domain that produces five-membered heterocycles for assembly into larger NRPS products and the docking domain that provides specificity between two different PKS-NRPS proteins to interact *in trans*.

## Methods

The EpoBcy protein construct was expressed and purified as previously described (17), with minor modifications detailed in SI Methods. EpoBcy site-specific mutagenesis was performed using standard protocols, and activity assays (17) were adapted for product detection by LC/MS-MS (SI Methods). Purified WT EpoBcy was crystallized using the vapor diffusion method, and X-ray diffraction

experiments, model building, and refinement are detailed in *SI Methods*. A 20-ns MD simulation was performed in GROMACS (30), and parameters are described in *SI Methods*.

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