Terminal Alkene Formation by the Thioesterase of Curacin A Biosynthesis

**STRUCTURE OF A DECARBOXYLATING THIOESTERASE**

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Curacin A is a polyketide synthase (PKS)-non-ribosomal peptide synthetase-derived natural product with potent anticancer properties generated by the marine cyanobacterium *Lyngbya majuscula*. Type I modular PKS assembly lines typically employ a thioesterase (TE) domain to off-load carboxylic acid or macrolactone products from an adjacent acyl carrier protein (ACP) domain. In a striking departure from this scheme the curacin A PKS employs tandem sulfotransferase and TE domains to form a terminal alkene moiety. Sulfotransferase sulfonation of β-hydroxy-acyl-ACP is followed by TE hydrolysis, decarboxylation, and sulfate elimination. The atomic coordinates and structure factors (code 3QIT) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, for the curacin A (4) generates several unusual chemical groups in addition to the terminal alkene. In a notable exception to this off-loading paradigm, the curacin A final product contains a terminal alkenel moiety, Curacin A, from the marine cyanobacterium *Lyngbya majuscula*, is a mixed polyketide/non-ribosomal peptide with antimitic properties (3). The hybrid PKS/NRPS assembly line pathway for curacin A (4) generates several unusual chemical groups in addition to the terminal alkene, including a cyclopropyl ring, a thiazoline ring, and a cyclic peptide. We have investigated the biosynthetic steps leading to several of these segments (5–9). In this study we investigated the structural basis for the unique off-loading strategy leading to the terminal alkene in the curacin A molecule.

Natural products display a remarkable chemical diversity, providing advantages for the producing plants and microbes to survive and thrive in particular ecological niches. These secondary metabolites and their derivatives have important applications as pharmaceuticals (1), and some have the potential to be developed as biofuels (2). Gene clusters encoding assembly line biosynthetic pathways for polyketide and polypeptide natural products are ubiquitous in bacterial and fungal genomes. Polysynthase (PKS) and non-ribosomal peptide synthetase (NRPS) pathways have a common modular organization in which intermediates tethered to carrier domains by a thioester linkage pass sequentially through modules of the assembly line. The final step in the assembly line is typically a thioesterase-catalyzed off-loading from the final carrier domain to produce a carboxylic acid, macrolactone, or cyclic peptide.

In a notable exception to this off-loading paradigm, the curacin A final product contains a terminal alkene moiety. Curacin A, from the marine cyanobacterium *Lyngbya majuscula*, is a mixed polyketide/non-ribosomal peptide with antimitic properties (3). The hybrid PKS/NRPS assembly line pathway for curacin A (4) generates several unusual chemical groups in addition to the terminal alkene, including a cyclopropyl ring, a thiazoline ring, and a cyclic peptide. We have investigated the biosynthetic steps leading to several of these segments (5–9). Herein we investigate the structural basis for the unique off-loading strategy leading to the terminal alkene in the curacin A molecule.

The curacin PKS has an unusual terminal module, the CurM protein, with a C-terminal tridomain comprised of an acyl carrier protein (ACP), a sulfotransferase (ST), and a thioesterase (TE) (Fig. 1A). Annotations of both ST and TE were based on weak sequence similarity to characterized enzymes. The prediction of an ST within a PKS was unprecedented (4). STs are widely distributed and are known to have detoxification, hormone regulation, or signaling functions (10). They catalyze transfer of a sulfonate group from the donor PAPS to a hydroxyl or amine of an acceptor small molecule or protein. CurM TE,
Structure of CurM TE

Although identifiable as a thioesterase, does not resemble any of the previously established PKS or NRPS TE subfamilies (11).

Using a simplified analog of the penultimate pathway intermediate, we recently demonstrated that off-loading and terminal alkene formation require ST-mediated sulfonation of the β-hydroxyl group from the PAPS cofactor (9) (Fig. 1A). This was the first observation of biological substrate activation by formation of a sulfate leaving group. CurM TE acts upon the β-sulfate intermediate to yield a decarboxylated product with a terminal double bond resulting from sulfate elimination (Fig. 1B). CurM TE catalyzes thioester hydrolysis 800-fold more slowly on the corresponding substrate bearing a terminal double bond resulting from sulfate elimination (Fig. 1B). CurM TE catalyzes thioester hydrolysis 800-fold more slowly on the corresponding substrate bearing a terminal double bond resulting from sulfate elimination (Fig. 1B). CurM TE catalyzes thioester hydrolysis 800-fold more slowly on the corresponding substrate bearing a terminal double bond resulting from sulfate elimination (Fig. 1B). CurM TE catalyzes thioester hydrolysis 800-fold more slowly on the corresponding substrate bearing a terminal double bond resulting from sulfate elimination (Fig. 1B).

EXPERIMENTAL PROCEDURES

Cloning and Site-directed Mutagenesis—A construct encoding the TE (CurM residues 1929–2211) was amplified from the cosmid pLM14 (9) and was inserted into pMoCR, a vector encoding the fusion protein His6-Mocr for enhanced solubility (21). Site-directed mutagenesis was performed using the QuikChange protocol (Stratagene). All constructs were verified by sequencing. The CurM ACP and ST expression plasmids were previously described (9).

Protein Expression and Purification—Escherichia coli strain BL21(DE3) was transformed with an expression plasmid, grown at 37 °C in 500 ml of TB with 4% glycerol to an A500 of 1.0, cooled to 18 °C, induced with isopropyl β-d-thiogalactopyranoside (final concentration, 0.2 mM), and grown for an additional 18 h. Selenomethionyl (SeMet) protein was produced in the same E. coli strain in SelenoMet medium (AthenaES) containing 100 μg/ml of seleno- DL-methionine.

All steps were performed at 4 °C. The cell pellet from 500 ml of cell culture was resuspended in 40 ml of Buffer A (20 mM Tris, pH 7.9, 500 mM NaCl, 20 mM imidazole, 10% glycerol), incubated 30 min with DNase (2 mg), lysozyme (5 mg), and MgCl2 (4 mM), lysed by sonication, and the soluble fraction loaded onto a 5-ml HisTrap Ni-NTA column (GE Healthcare). CurM TE was eluted with a linear gradient from 20 to 650 mM imidazole (Buffer B). The His6-Mocr fusion partner was removed by a 2-h incubation with 1 mM DTT and tobacco etch virus protease (1 mg of protease/50 mg of TE) at room temperature. After overnight dialysis at 4 °C in Buffer C (20 mM Tris, pH 7.9, 500 mM NaCl, 10% glycerol) with 1 mM DTT, the remaining His-tagged proteins were removed by nickel-affinity chromatography, followed by size exclusion chromatography with a HiLoad 16/60 Superdex 200 column (GE Healthcare) pre-equilibrated with...
Buffer C. CurM TE was concentrated to 5 mg/ml, flash frozen in liquid N\textsubscript{2}, and stored at \( -80^\circ \text{C} \). Of 14 TE variants purified as the wild type, only 6 yielded enough soluble protein for assay. SeMet TE was purified as the wild type with addition of 2 mM DTT to all buffers. Yields per 500 ml of culture were 5 mg of TE and 2 mg of SeMet TE.

Crystalization—Crystals grew at 4 °C within 24–48 h by vapor diffusion from a 1:1 mixture of protein stock (2 mg/ml of TE, 20 mM Tris, pH 7.9, 200 mM NaCl, 2.5% glycerol) and well solution (27–32% PEG3350, 100 mM Tris, pH 8.3–8.5). Microseeding was required for crystal growth of the SeMet protein in similar conditions with 1 mM DTT in the protein solution. Crystals were cryoprotected in a well solution with 15% glycerol, harvested in loops, and flash cooled in liquid N\textsubscript{2}.

Data Collection and Structure Determination—Data were collected at GM/CA-CAT beamline 23ID-D at the Advanced Photon Source (APS) at the Argonne National Lab (Argonne, IL). Among 25 SeMet TE crystals, only one diffracted beyond 4 Å, but was visibly two crystals and had multiple lattices in the diffraction pattern. A region visually identified as a single crystal was probed in three 10-μm steps using a 20-μm mini-beam (22). The crystal was centered at the position with the strongest diffraction and the least interference from the second lattice, and Friedel data were collected in inverse-beam geometry (\( \phi = 0–90^\circ \) and 180–270° as wedges of 45° with 1° images) at the wavelength of peak absorption at the selenium edge. The diffraction images showed signs of decay at the end of the collection. The crystal was translated to expose a fresh region to the beam, and rotated 90° from the start of data collection. Each of two regions was probed with the 10-μm mini-beam in a 3 x 3 raster of 10-μm steps. Data were collected at the strongest diffracting position with a single lattice, again in inverse beam geometry (\( \phi = 90–150^\circ \) and 270–330° as 30° wedges with 0.5° images). The two partial datasets were integrated separately and scaled together using the HKL2000 suite (23). Using Phaser (24) in the PHENIX (25) software suite, selenium sites were found for all Met residues of the four polypeptides in the asymmetric unit. Nine Met side chains had two partially occupied selenium sites and another Met had three sites for 39 total selenium sites (average figure of merit = 0.401). After density modification and 4-fold noncrystallographic symmetry averaging in RESOLVE (26) (figure of merit = 0.81), an 86% complete initial model was built by AUTOBUILD (27) and completed manually in COOT (28). The construct encoding amino acids 1929–2211 of CurM (here denoted 1–283) yielded crystals after removal of the Mocr fusion partner. In the CurM TE crystal structure, solved by single anomalous diffraction phasing using selenomethionyl TE, 20 mM Tris, pH 7.9, at 30 °C. Complete loading was confirmed by reverse phase HPLC using a Jupiter C4 column (250 x 2.0 mm, 5 μm, 300 Å, Phenomenex) and a linear elution gradient from 30 to 90% CH\textsubscript{3}CN (0.1% CF\textsubscript{3}CO\textsubscript{2}H)/H\textsubscript{2}O (0.1% CF\textsubscript{3}CO\textsubscript{2}H) over 45 min. After exchange into Buffer C and concentration (Amicon Ultra 10-kDa concentrators Millipore), substrate-loaded ACP was flash frozen and stored at \( -80^\circ \text{C} \). To generate the sulfated substrate for the TE assay, 225 μM loaded ACP was incubated with 5 μM ST, 1.75 mM PAPS (Sigma), 100 mM Tris, pH 7.9, at room temperature for 10 min. Complete sulfonation was confirmed by HPLC. The TE reaction was initiated by addition of TE (4 μM). After 1 min the reaction was quenched with 10% formic acid. Conversion of loaded to holo-ACP was quantified by HPLC as described above. Assays with the non-sulfated substrate were performed by incubating 1 mM (3R)-hydroxy-5-methoxytetradecanoyl-CoA (9), 10 μM S. verticillus Svp (36), 10 mM MgCl\textsubscript{2}, 100 mM Tris, pH 7.9, at 80 °C. Of 14 TE variants purified as apo-ACP, 100 μM (3R)-hydroxy-5-methoxytetradecanoyl-CoA (9), 10 μM S. verticillus Svp (36), 10 mM MgCl\textsubscript{2}, 100 mM Tris, pH 7.9, at 30 °C. Complete loading was confirmed by reverse phase HPLC using a Jupiter C4 column (250 x 2.0 mm, 5 μm, 300 Å, Phenomenex) and a linear elution gradient from 30 to 90% CH\textsubscript{3}CN (0.1% CF\textsubscript{3}CO\textsubscript{2}H)/H\textsubscript{2}O (0.1% CF\textsubscript{3}CO\textsubscript{2}H) over 45 min. After exchange into Buffer C and concentration (Amicon Ultra 10-kDa concentrators Millipore), substrate-loaded ACP was flash frozen and stored at \( -80^\circ \text{C} \). To generate the sulfated substrate for the TE assay, 225 μM loaded ACP was incubated with 5 μM ST, 1.75 mM PAPS (Sigma), 100 mM Tris, pH 7.9, at room temperature for 10 min. Complete sulfonation was confirmed by HPLC. The TE reaction was initiated by addition of TE (4 μM). After 1 min the reaction was quenched with 10% formic acid. Conversion of loaded to holo-ACP was quanified by HPLC as described above. Assays with the non-sulfated substrate were performed by incubating 1 mM (3R)-hydroxy-5-methoxytetradecanoyl-CoA, 50 mM Tris, pH 7.9, with 40 μM TE for 16 h, including 6% glycerol and 300 mM NaCl for protein stability. The reaction was quenched with equal volume of 1 mM CH\textsubscript{3}CO\textsubscript{2}H and neutralized with 1 mM NaOH, and crotonyl-CoA was added as an internal standard. Hydrolysis was analyzed using a Luna C18 column (250 x 4.60 mm, 5 μm, 100 Å, Phenomenex) with a linear gradient from 10 to 90% CH\textsubscript{3}OH/H\textsubscript{2}O (10 mM CH\textsubscript{3}CO\textsubscript{2}NH\textsubscript{4}) over 20 min.

RESULTS

Overall Structure—The curacin TE is the C-terminal domain of the 2211-residue CurM polypeptide. Because low sequence identity to other TEs prevented accurate definition of the domain N terminus, expression plasmids were constructed with three different start sites corresponding to amino acids 1917, 1929, and 1934. For all three domain variants, an N-terminal fusion of the protein Mocr (21) was necessary to obtain sufficient soluble protein for purification and crystallization. The construct encoding amino acids 1929–2211 of CurM (here denoted 1–283) yielded crystals after removal of the Mocr fusion partner. In the CurM TE crystal structure, solved by single anomalous diffraction phasing using selenomethionyl CurM TE (Table 1), the polypeptide chain was ordered to the ends of the construct. CurM TE possesses the α/β-hydrolase fold, as expected, with residues 1–132 and 215–283 comprising the structurally conserved core domain (Fig. 2, A and B). Residues of the catalytic triad (Ser\textsuperscript{106}, Glu\textsuperscript{124}, and His\textsuperscript{266}) are located at the top of the core domain as in other TEs, but with a Glu in place of the more
common Asp (Fig. 2B). The catalytic triad faces into a cleft between the core and lid. The lid subdomain (residues 133–214) is ~20 residues longer than the analogous region in other TEs, and includes three helices (αL1, αL2, and αL3) and a small β-hairpin (βL1 and βL2) (Fig. 2, A and B). The first lid helix (αL1) is designated the “protruding helix” because it has few contacts with the lid and none with the core of the polypeptide.

The orientation of the lid with respect to the core is identical in the four independent copies of the CurM TE polypeptide in the asymmetric unit of crystals (root mean square deviation = 0.29 Å for 216 Ca atoms, supplemental Fig. S1A), demonstrating that the active site cleft is identically open in all four polypeptides. The position of the lid is maintained by complementar-y surface contacts of lid helix αL3 (residues 176–186) with several loops in the core. Most of the contacts are hydrophobic. Specificity for the fixed lid-core orientation is provided by an “arginine anchor” in which the side chain of Arg185 in lid helix αL3 extends into the core domain where it forms a full set of five hydrogen bonds with core residues, including a buried salt bridge Asp57 and hydrogen bonds with Gln35 and Glu3 (supplemental Fig. S2). Additionally, the Gln35 side chain amide is hydrogen bonded with the backbone carbonyl of Leu182 in lid helix αL3. In contrast to the remarkable lid-core surface complementarity on one side of the active site cleft, the linker peptides (residues 129–136 and 205–215) on the opposite side of the cleft are dynamic with some residues disordered in some subunits (supplemental Fig. S1A).

Novel Dimer Interface—CurM TE is dimeric in solution (supplemental Fig. S3) and also in the crystal structure (Fig. 2C). The mode of dimer formation is radically different from other PKS off-loading TEs even though the core structures and catalytic triads are similar (Fig. 3A). The primary dimer contact is between the protruding helix (αL1) in the lid of one subunit and three helices (α2, α3, and α4) in the core of the partner subunit (Fig. 2C), with additional core-to-core contacts of the β4-α2 loops of the two subunits. The subunit interface is predominantly hydrophobic and large (buried surface area of 1220 Å² per monomer). The N termini are distant from the dimer interface (Fig. 2C), allowing for fusion to the monomeric ST domain as well as dimerization of the TE domain within the dimeric CurM module. This is consistent with the observation that the natural CurM ST-TE di-domain is dimeric in solution (data not shown).

The crystal asymmetric unit contains two dimers that differ by a slight flexure (3°) at the dimer interface, accounting for the poorer overall fit of dimers (root mean square deviation = 1.05 Å for 486 Ca atoms) compared with monomers. The protruding lid helix, αL1, moves with the partner subunit as the subunits flex (supplemental Fig. S1B). Thus, the lid-to-core dimer contact helps fix the lid in an open orientation (Fig. 3B). This is surprising and unique compared with off-loading TEs of other PKSs in which lid-to-lid dimer contacts fix the lid in a closed orientation. In these other TEs, the lid-to-lid dimer contact stabilizes an open-ended tunnel with the active site at its center (Fig. 3C). In contrast, the dimer-enforced open lid of CurM TE results in a highly exposed, open cleft active site (Fig. 3B). Thus, the dimer interface creates a very different active site environment in CurM TE and the other off-loading PKS TEs.

CurM TE Is Not Alone—We identified five open reading frames in the sequence data base with substantial identity (33 to 51%) to the CurM ACP-ST-TE tridomain. The sequences are uncharacterized protein products from genomes of cyanobacteria (Synechococcus and Cyanothoece) and proteobacteria (Pseudomonas and Haliangium). Based on the conservation of all three domains, including, for example, the TE catalytic triad, we assume that the other gene products also catalyze sequential sulfonation, hydrolysis, decarboxylation, and sulfate-elimination reactions, and that conserved residues within these sequences may illuminate areas of the structure that are important for function. Conserved residues in CurM TE and the five other putative TE sequences (Fig. 4) were mapped onto the CurM TE structure, revealing a dense area of conservation in the active site cleft (Fig. 5A). Sequence conservation indicates that CurM TE and the other gene products have the same lid structure and orientation and the same dimer interface. The essential features of the lid-to-core arginine anchor (Arg185, Asp57, and Gln35) are conserved. The protruding helix (αL1) has a conserved hydrophobic character, as do the surfaces of the core that it contacts in the dimer. The lid-to-core TE dimer interface, the open active site cleft, and the surface of the cleft are likely conserved among these TEs.

Active Site—The open cleft active site and intact catalytic triad (Fig. 3) appear poised to hydrolyze a broad array of thioester substrates. To understand how CurM TE selects a sulfated ester substrate, we modeled the acyl enzyme intermediate of the hydrolytic reaction (Scheme 1) using our knowledge of catalytic triad catalysis and geometry and the structure of affinity-labeled Pik TE (12) (Fig. 5B). Constraints on the model included acylation of the Ser190 nucleophile, carbonyl-oxygen binding in the oxanion hole (NHs of Ile32 and Met103), the (R)-β-sulfate isomer, contact of sulfate with the enzyme, and placement of

### Table 1

| Crystallographic summary | CurM TE (SeMet) | CurM TE (native) |
|--------------------------|----------------|------------------|
| **Diffraction data**     |                |                  |
| Space group              | P2₁            | P2₁              |
| X-ray source             | APS 23-ID-D    | APS 23-ID-D      |
| a, b, c (Å)              | 74.1, 86.9, 87.1 | 74.5, 86.9, 87.6 |
| α, β, γ (%)              | 90, 90, 90     | 90, 90, 90       |
| Wavelength (Å)           | 0.97948        | 1.0322           |
| d<sub>i</sub> (Å)        | 2.14 (2.22-2.14) | 1.68 (1.74-1.68) |
| Average I/osl            | 16.3 (5.5)     | 18.1 (2.1)       |
| R<sub>free</sub>         | 0.113 (0.362)  | 0.059 (0.384)    |
| Completeness             | 99.5 (99.9)    | 90.2 (49.8)      |
| Average redundancy       | 6.4 (6.3)      | 3.4 (2.0)        |
| Unique reflections       | 60,147         | 114,584          |

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*a* Outermost shell in parentheses.

*b* Including anomalous differences.

*c* The R<sub>free</sub> data set included a random 5% of reflections.
the long acyl chain away from the catalytic His\textsuperscript{266}. Given these constraints, the acyl-enzyme was easily modeled with favorable bond rotations and without change to the protein structure. The acyl chain lies in a narrow cleft between the lid and core domains, and the (R)-\(\beta\)-sulfate occupies a niche adjacent to Arg\textsuperscript{205} (Fig. 5A). The model is consistent with the strong stereoselectivity of CurM TE for the (R)-\(\beta\)-sulfate over the corresponding (S) isomer (9), as modeling of the (S)-\(\beta\)-sulfate form of the substrate resulted in sulfate contacts with the hydrophobic surface of the protein. The model also suggests that the hydrogen-bonded side chains of Arg\textsuperscript{205} and Asn\textsuperscript{211} may recognize the \(\beta\)-sulfate moiety. To explore these issues further, the functions of the presumed sulfate-recognition residues and others in the active site cleft were probed by site-directed mutagenesis. Three substitutions were made at Arg\textsuperscript{205} (Gln, Glu, and Ala), as well as Ala substitutions at Asn\textsuperscript{267}, which is adjacent to the catalytic His, and at Asn\textsuperscript{211}, which is hydrogen bonded with Arg\textsuperscript{205} (Fig. 5B).

The TE wild type and variants were tested using a one-pot, multistep assay (9) in which recombinant CurM ACP was loaded with a synthetic substrate mimic (Fig. 1B) and reacted sequentially with recombinant CurM ST and CurM TE. ACP substrates and ACP hydrolysis products were analyzed by HPLC (supplemental Fig. S3). Activities of the CurM TE variants were normalized to the activity of the wild type (Table 2). The three substitutions at Arg\textsuperscript{205} (R205Q, R205E, and R205A) resulted in significantly reduced activity, comparable with a negative-control substitution in the catalytic triad (H266R). Substitutions at two Asn residues (N211A and N267A) that are conserved in the ACP-ST-TE group of
sequences but not in the wider TE family resulted in only 2–3-fold reduced catalytic activity. To test the role of Arg205 in sulfate recognition, the Arg205 variants were assayed with a non-sulfated substrate. Due to the ~800-fold lower activity of wild type TE on non-sulfated substrates than on sulfated substrates (9), these assays were performed with a CoA-linked β-OH substrate available at a higher concentration than the ACP-linked substrate. In striking contrast to their lack of activity with sulfated substrates, the Arg205 variants had activity comparable with the wild type and non-sulfated substrate (supplemental Table S1). This result supports a vital role for Arg205 in sulfate recognition.

**DISCUSSION**

* A New Thioesterase Subfamily—The CurM TE structure defines a new thioesterase subfamily with the remarkable catalytic activity of hydrolysis, decarboxylation, and sulfate elimination to form alkene products from β-sulfated acyl-ACP substrates. The structural features that distinguish CurM TE from other thioesterase subfamilies are conserved in sequences of five other putative ACP-ST-TE tridomains, indicating that all of them catalyze terminal alkene formation. The CurM TE lid subdomain, which sits above the active site, is substantially longer than the analogous region in other TE subfamilies. The lid creates an open active site cleft, and is fixed in an open orientation by key interactions unique to this TE subfamily (Figs. 2C and 3B). One side of the lid is secured by the surface complementarity of lid helix αL3 with the core domain and by an arginine anchor in which Arg185 in the lid extends deep into the core domain to form a salt bridge with Asp97. The other side of the lid is secured by dimer contacts of lid helix αL1 with the core domain of the partner subunit.

The characteristics of the CurM TE lid provide a striking contrast to other PKS and NRPS TE subfamilies. Dimeric PKS off-loading TEs have a closed lid, formed by a lid-to-lid dimer contact, which creates an open-ended tunnel with the active site at its center (Fig. 3C). Monomeric editing TE II enzymes and NRPS off-loading TEs have a flexible lid that may control access of the phosphopantetheine arm to the active site by its ability to open and close. The region of substrate entrance in these other TEs (the active site tunnel in other dimeric TEs and the flexible part of the movable lid of monomeric TEs) is analogous to the region in CurM TE secured by the arginine anchor. Hence, the usual entrance route for acyl-ACP substrates is blocked in CurM TE by the anchoring of lid helix αL3 to the core domain. Instead, we presume that the substrate enters the CurM TE active site via the conserved cleft that extends away from the catalytic triad toward the top of the lid (Fig. 5A). In contrast to the firm anchoring on the entrance side of the CurM TE active site cleft, the opposite side of the cleft has flexible lid-to-core linkers and less conservation.

Substrate Specificity—Despite the departure from precedent in the lid architecture and substrate entrance, the catalytic triad of CurM TE is fully formed and appears to be poised for hydrolysis (Fig. 3A). The question remains why CurM TE does not hydrolyze any thioester substrate given the positions of the Ser100 nucleophile, His266 base, and Ile32/Met101 oxyanion hole. An answer is found in the wide open architecture of the active site, which provides few obvious substrate recognition elements. CurM TE does not need exquisite recognition of a substrate that is present at a highly effective concentration via tethering to CurM ACP, which itself is fused to the TE. However, CurM TE must select the (R)-β-sulfated substrate and not other acylated forms of CurM ACP (malonyl, β-keto, and (R)-β-hydroxy), which are also present at a highly effective concentration before they are transformed by the other catalytic domains of CurM (ketosynthase, ketoreductase, and sulfotransferase, respectively). Our model of the TE acyl-enzyme intermediate
suggested that Arg^{205} recognizes the (R)-β-sulfate moiety (Fig. 5), and this was consistent with the site-directed mutagenesis data (Table 2, supplemental Table S1). The Arg^{205} variants lacked activity with a sulfated substrate but had activity comparable with the wild type in the much slower reaction with a non-sulfated substrate. Thus Arg^{205} may be the only positive substrate-recognition element, the active site may have little or no affinity for the rest of the acyl chain, and non-sulfated substrates may be only rarely positioned properly for catalysis. In this manner, the \((\text{R})/H\text{9252}\)-sulfate acts as a “handle” for Arg^{205} to assist in effective thioester binding to the catalytic machinery in the large open active site cleft, whereas non-sulfated substrates have no such guidance. Intriguingly, Arg^{205} is among the flexible residues in the linker connecting the CurM TE core and lid, and thus may provide an early interaction with incoming sulfated substrates. The flexibility of this region is consistent with the lack of conservation of Arg^{205} in the homolog from \(Haliangium ochraceum\), in which nearby arginine side chains may serve the same function.

**Catalytic Mechanism**—The CurM TE structure reveals an intact hydrolytic active site into which an acyl-enzyme intermediate was modeled easily without the need to move interacting groups (Fig. 5). This strongly suggests that acyl enzyme formation precedes other catalytic events. Therefore, we propose that hydrolysis of the acyl-enzyme precedes decarboxylation and sulfate group elimination, based on our...
detection of both the terminal alkene product and the substrate analog bearing intact β-sulfate and carboxylic acid groups (i.e. the initial TE serine ester hydrolysis product) (9). The CurM TE assures decarboxylation and sulfate group elimination in vivo, as the corresponding carboxylated and sulfated curacin A intermediate was not detected in cultures of L. majuscula (Scheme 1) (37). Through interactions with the oxyanion hole and Arg205, the carboxylic acid hydrolysis product remains bound to CurM TE long enough for the enzyme to promote loss of CO₂ and SO₄²⁻ in one concerted step. In the reaction scheme, CurM TE assists decarboxylation and sulfate elimination in at least three ways. First, the enzyme binds the carboxylic acid hydrolysis product in a conformation that prevents resonance of COOH with the C₁–C₂ bond to be broken upon decarboxylation. Arg 205, through its interaction with the sulfate at C₃, may assist in achieving the optimal conformation. Second, the catalytic His²⁶⁶ likely promotes deprotonation of the carboxylic acid hydrolysis product to facilitate loss of CO₂. Stabilization of the additional negative charge on the departing SO₄²⁻ offered

FIGURE 5. CurM TE active site. A, surface of the active site cleft. In the molecular surface view, the active site (magenta catalytic triad) is in a cleft in which the presumed phosphopantetheine entrance is lined with conserved residues (cyan), with other parts of the protein surface in green and the modeled acyl-enzyme intermediate in gray sticks. B, modeled acyl-enzyme intermediate (gray C). In this stereo view, the intermediate is surrounded by conserved amino acid residues (cyan C) and the catalytic triad (Ser¹⁰⁰, Glu¹²⁴, and His²⁶⁶) (magenta C). The carbonyl oxygen is bound in the oxyanion hole (hydrogen bonds to the NHs of the Ile⁵² and Met¹⁰¹).

SCHEME 1. Predicted reaction sequence for CurM TE hydrolysis, sulfate group elimination, and decarboxylation. Arg²⁰⁵ recognizes the (R)-β-sulfate, facilitating hydrolysis by the catalytic triad. The catalytic His²⁶⁶ is positioned to assist in decarboxylation followed by sulfate elimination.
by Arg
205
may further reduce energy barriers for initiating this remarkable decarboxylative elimination process.

**Potential for Hydrocarbon Production**—CurM TE is the first member of a branch of the TE family optimized to work in concert with a sulfotransferase to eliminate a carboxyl functional group and create a terminal double bond. The ST-TE system is the first example of a biological sulfonation employed for chemical activation (9). The ACP-ST-TE system exists as an uncharacterized open reading frame in E. coli, adapted for only those acyl-ACP substrates bearing a sulfate ester and optimized to work in concert with a fused sulfotransferase to generate a highly favorable leaving group. The CurM TE crystal structure reveals a fully developed hydrolytic active site, an arginine residue for (R)-β-sulfate recognition, and an active site cleft that may accommodate a wide variety of acyl substrates.

### Table 2

**Activity of CurM TE variants**

| Variant | Activity* (%) |
|---------|---------------|
| WT      | 100           |
| R205Q   | 1.8 ± 0.3     |
| R205E   | 2 ± 2         |
| R205A   | 6 ± 3         |
| N267A   | 34 ± 3        |
| N211A   | 55 ± 2        |
| H266R   | 1.4 ± 0.2     |

*Raw HPLC chromatogram peak area for the holo-ACP product with background subtracted and normalized with an internal standard to correct for differences in sample injection. The area for each mutant was normalized to the wild type within each replicate. Mean ± S.D. from duplicate experiments are shown.

### Acknowledgments

We thank Drs. Amol Kulkarni and Sarang Kulkarni for the synthesis of substrate analogs. Beamline 23ID-D is supported by the National Institutes of Health, National Institute of General Medical Sciences Grant Y1-GM-1104 and National Cancer Institute Grant Y1-CO-1020 through the GM/CA Collaborative Access Team at the APS, which is supported by the United States Department of Energy.

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