A Double-Hotdog with a New Trick: Structure and Mechanism of the trans-Acyltransferase Polyketide Synthase Enoyl-isomerase

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Supporting Information

ABSTRACT: Many polyketide natural products exhibit invaluable medicinal properties, yet much remains to be understood regarding the machinery responsible for their biosynthesis. The recently discovered trans-acetyltransferase polyketide synthases employ processing enzymes that catalyze modifications unique from those of the classical cis-acetyltransferase polyketide synthases. The enoyl-isomerase domains of these megasynthases shift double bonds and are well-represented by an enzyme that helps forge the triene system within the antibiotic produced by the prototypical bacillaene synthase. This first crystal structure of an enoyl-isomerase, at 1.73 Å resolution, not only revealed relationships between this class of enzymes and dehydratases but also guided an investigation into the mechanism of double bond migration. The catalytic histidine, positioned differently from that of dehydratases, was demonstrated to independently shuttle a proton between the γ- and α-positions of the intermediate. This unprecedented mechanism highlights the catalytic diversity of divergent enzymes within trans-acetyltransferase polyketide synthases.

Polyketides are a structurally diverse class of natural products manufactured by a broad spectrum of bacteria, fungi, and plants. The clinical importance of such polyketides as the antibiotic erythromycin and the cholesterol-lowering agent lovastatin has led to the structural, functional, and mechanistic investigation of the enzymatic machinery responsible for their synthesis. Type I polyketide synthases (PKSs) employ biosynthetic logic similar to the metazoan fatty acid synthase, which iteratively condenses and reduces acetate units into fatty acyl chains. In contrast to fatty acid synthesis, PKSs operate in an assembly line fashion, where modules (sets of domains responsible for a single round of condensation and processing) are structured in a linear arrangement, each obtaining a ketide extender unit selected by an acyltransferase domain (AT), condensing that extender unit to a growing polyketide chain with the ketosynthase domain (KS) and processing the resulting β-ketoacyl chain depending on which processing domains are associated with the module. These domains include a ketoreductase (KR) that catalyzes stereoselective reduction of the β-carbonyl, a dehydratase (DH) that yields an α,β double bond through the elimination of water, and an enoylreductase (ER) that reduces the double bond to yield a completely saturated acyl intermediate.

Type I PKSs have been subdivided into two classes depending on whether ATs are encoded within modules (cis-AT PKSs) or discretely encoded as separate polypeptides that dock to the synthase (trans-AT PKSs). While each of the domains from cis-AT PKSs have been structurally characterized, only recently has structural information become available for domains found within trans-AT PKS assembly lines. The earliest discovered and archetypal trans-AT PKS is encoded within the Bacillus subtilis and Bacillus amyloliquefaciens genomes (PksX and Bae, respectively) and is responsible for the biosynthesis of the polyketide bacillaene, a polyene diamide that inhibits prokaryotic protein synthesis. This PKS is illustrative of the unusual combinatorial logic in trans-AT PKS systems, which are commonly fused with nonribosomal peptide synthetases (NRPS) and employ processing domains such as methyltransferases, enoyl-CoA hydratases, pyran peptide synthetases (NRPS) and employ processing domains such as methyltransferases, enoyl-CoA hydratases, pyran synthases, and β-branching domains (B), rarely or not observed in cis-AT PKSs. The evolutionary acquisition of these additional processing domains has permitted trans-AT PKSs to explore a vast realm of possible molecular configurations, yielding natural products with functionalities not accessible to PKSs restricted to the traditional processing reactions employed in fatty acid biosynthesis.

One of the additional processing domains available to trans-AT PKSs is the enoyl-isomerase domain (EI), which has been previously annotated as an inactive DH or DH* due to sequence alignments that predicted a DH double-hotdog fold. This domain is located in what has been termed “shift-modules” that yield intermediates with β,γ double bonds, in contrast to the classic α,β-unsaturated products of DH catalysis (Figure 1). Olefins within polyketides impact the sterically available conformations of the final product more than any other functional group, and therefore several studies have recently been conducted to determine if this domain is...
responsible for the isomerization of double bonds to positions “out-of-register” with classical PKS biosynthetic logic. Some DH domains contain intrinsic isomerase activity, which has been shown for DHs from the bacillaene PKS (Figure 1B).\textsuperscript{10} Both dehydration and $\alpha\beta \rightarrow \beta\gamma$ double bond isomerization for C5–C6 and C7–C8 in bacillaene were demonstrated to occur within the first two DHs of PksN, without the participation of a dedicated EI domain. Whether the isomerization of the $\beta\gamma$ double bond at C3–C4 of bacillaene was also catalyzed by the inherent isomerase activity of the third DH remained undetermined, and sequence alignments revealed that the following module could potentially be a shift-module.

The EI domain of the rhizoxin PKS (at the N-terminus of RhiE) has been shown to be responsible for $\alpha\beta \rightarrow \beta\gamma$ double bond isomerization, and a strain with an EI knockout prevented polyketide transfer downstream of the disruption.\textsuperscript{9} Whether this is due to KS selectivity for the properly isomerized product or interference with docking between RhiD and RhiE is unclear. A mechanism was proposed for EI-catalyzed isomerization that involved a histidine conserved between DH and EI domains, as well as an unknown residue that could act as a general base. More recently, the EI domain from the corallopyronin A synthase was isolated and studied.\textsuperscript{11} In vitro reconstitution of isomerase activity coupled with site-directed mutagenesis revealed that the proposed active site histidine is indeed required for catalysis; however, the unknown residue that acts in cooperation with this histidine could not be determined through mutagenic knockouts of proposed active site residues.

Here we report the 1.73 Å resolution crystal structure and reconstituted in vitro activity of the EI domain from the 14th module of the bacillaene PksX synthase (PksEI14). The structure reveals how a key active site substitution of a proline residue conserved among PKS DH domains permits the catalytic histidine to act as both a general base and general acid in the isomerization of a double bond. Experiments conducted in deuterated solvent reveal that the mechanism proceeds through the relocation of a substrate $\gamma$-proton to the $\alpha$-position without solvent interference, similar to the mechanism of double bond isomerization catalyzed by ketosteroid isomerase.\textsuperscript{12} The structure also shows that the EI domains may not contribute significantly to the dimerization of trans-AT PKS polypeptides, in contrast to what has been observed for cis-AT PKSs.\textsuperscript{13--15} The structural and biochemical evidence presented here reveals an unprecedented mechanism for double bond isomerization and suggests that PksEI14 is responsible for the

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**Figure 1.** Polyketides with shifted double bonds. (A) Several polyketides with isomerized double bonds are highlighted with blue circles. The C3-C4 double bond of bacillaene, isomerized by PksEI14, is annotated. (B) The last two PKS subunits responsible for the final catalytic steps of bacillaene biosynthesis are PksN and PksR. The proposed route involves the installation of $\beta\gamma$ double bonds by the DH domains of modules 11 and 12 (PksDH11 and PksDH12), while the EI domain of module 14 is a dedicated isomerase for the $\alpha\beta$ double bond installed by the DH from module 13 (PksDH13). The KS domain of the terminal module (PksKS15) is not condensationally competent and likely acts as a gatekeeper to ensure that the isomerization has taken place before the final product is released from the synthase. The small red circles appended to the KS domains indicate the presence of a flanking subdomain.
final step in the formation of the C3–C8 triene system characteristic of bacillaene.

■ RESULTS AND DISCUSSION

The boundaries chosen for PksEI14 were based on the boundaries of domains upstream and downstream within PksR (PksKS14 and PksACP14, respectively). Crystals provided data enabling a model of PksEI14 to be refined to a resolution of 1.73 Å, with phasing obtained by single-wavelength anomalous dispersion from crystals soaked in methyl mercury acetate (Table 1). The structure revealed a double-hotdog fold with an overall architecture very similar to that of cis-AT PKS DH domains (Cα rmsd: 2.4 Å over 250 Cα when aligned with theDH from the fourth module of the erythromycin synthase, EryDH4; PDB code 3EL6) (Figure 2A).13 In contrast to all known cis-AT DH structures, PksEI14 crystallized as a monomer, without the ∼20-residue N-terminal region observed in those DHs that mediates their dimeric interfaces.

A structural alignment of PksEI14 with EryDH4 reveals that the α-carbon of the catalytic histidine (His18 of PksEI14) conserved in both PKS DH and EI domains superposes with the α-carbon of the catalytic histidine of EryDH4; however, the imidazole rings are offset by ∼1.7 Å (as measured between Nε atoms) (Figure 2B). In PKS DHs, the catalytic histidine lies in a highly conserved HX8P motif, in which its imidazole stacks against the proline ring.13 This motif is HX8V in PksEI14, with the Cγ methyl group of the valine sterically repositioning the imidazole ring. In other PKS EI domains, leucine is observed to substitute for the proline (Supplementary Figure S1). The previously suggested mechanism of α,β → β,γ double bond isomerization by the EI domain involves an unknown residue that acts as a general base and abstracts a proton from the γ-carbon, similar to the isomerization mechanism for FabA.9,11,16 However, the structure reveals an active site pocket dominated by hydrophobic residues that cannot participate in acid−base catalysis. The only ionizable residue within the PksEI14 active site in position to play this role is Tyr185, although it is not conserved among available EI sequences. To determine whether Tyr185 is involved in catalyzing double bond isomerization, an assay was developed in which in vitro activity from the isolated PksEI14 domain was reconstituted. The previously suggested mechanism of α,β → β,γ double bond isomerization by the EI domain involves an unknown residue that acts as a general base and abstracts a proton from the γ-carbon, similar to the isomerization mechanism for FabA.9,11,16 However, the structure reveals an active site pocket dominated by hydrophobic residues that cannot participate in acid−base catalysis. The only ionizable residue within the PksEI14 active site in position to play this role is Tyr185, although it is not conserved among available EI sequences. To determine whether Tyr185 is involved in catalyzing double bond isomerization, an assay was developed in which in vitro activity from the isolated PksEI14 domain was reconstituted. As obtaining an analogue of the natural polyketide substrate to observe the reaction in the forward direction was challenging natural polyketide substrate (a long, highly functionalized

Table 1. Crystallographic Data and Refinement Statistics**

|                                | native                  | CH3-Hg derivative |
|--------------------------------|-------------------------|-------------------|
| **Data Collection**            |                         |                   |
| wavelength (Å)                 | 1.0332                  | 0.9763            |
| space group                    | P212121                 | P212121           |
| cell dimensions, a, b, c (Å)   | 48.8, 68.3, 77.8        | 48.7, 68.2, 78.1  |
| resolution (Å)                 | 35.37–1.73              | 51.3–1.88         |
| Rmerge                         | 0.113 (0.702)           | 0.066 (0.520)     |
| I/σ(I)                         | 48.3 (2.5)              | 26.1 (5.1)        |
| no. of reflections             | 25889 (1429)            | 21801 (3088)      |
| completeness (%)               | 97.6 (74.4)             | 99.5 (98.9)       |
| redundancy                     | 3.5 (2.4)               | 14 (13.7)         |
| Wilson B value (Å²)            | 41.3                    | 24.7              |
| no. of heavy atom sites        |                         |                   |
| figure of merit                |                         |                   |
| resolution (Å)                 | 35.37–1.73              |                   |
| no. of reflections             | 25889 (1429)            |                   |
| Rwork/Rfree                    | 0.214/0.239             |                   |
| no. of atoms                   |                         |                   |
| protein                        | 1932                    |                   |
| water                          | 131                     |                   |
| αv B factors (Å²)              |                         |                   |
| protein                        | 41.1                    |                   |
| water                          | 50.2                    |                   |
| RMS deviations                 |                         |                   |
| bond lengths (Å)               | 0.010                   |                   |
| bond angles (deg)              | 1.424                   |                   |
| Ramachandran statistics (%)   |                         |                   |
| preferred regions              | 98.72                   |                   |
| allowed regions                | 1.28                    |                   |
| outliers                       | 0.0                     |                   |

**Values in parentheses refer to the highest resolution shell (1.76–1.73 Å).
polyene diamide), an analogue of the reaction product, (E)-hex-3-eny1-S-pantetheine, was prepared instead. PksEI14-mediated isomerization yielding (E)-hex-2-eny1-S-pantetheine would thus resemble the anticipated reverse direction for double bond migration, namely, $\beta\gamma \rightarrow \alpha\beta$ double bond isomerization. The reverse of the biological reaction is anticipated to be thermodynamically more favorable for this analogue since the double bond of the product is conjugated with the thioester carbonyl. Incubation of (E)-hex-3-enyl-S-pantetheine with PksEI14 followed by reversed-phase HPLC analysis revealed substrate conversion to a distinct species with properties consistent with a synthetic standard of (E)-hex-2-enyl-S-pantetheine, the $\beta\gamma \rightarrow \alpha\beta$ isomerized product. An identical reaction replacing wild-type PksEI14 with the Tyr185Phe mutant is shown in red, exhibiting increased isomerase activity over the same time course. See also Supplementary Figure S2.

Since the structure does not reveal any other active site residues that could catalyze acid—base chemistry, the general base in the biological reaction could simply be a solvent-supplied water molecule, similar to the isomerization mechanism proposed for the crotonase domain of multifunctional enzyme, type-1. The reverse in vitro reaction described herein would require water to play the role of a general acid in donating a proton to the $\gamma$-carbon. The PksEI14-catalyzed reaction described above was conducted in D2O (with the more catalytically proficient Tyr185Phe mutant) to observe whether a solvent-supplied deuterium would label the $\gamma$-carbon in the product, (E)-4-deutero-hex-3-enyl-S-pantetheine. The reaction afforded sufficient (~3 mg) isomerized product for analysis by $^1$H NMR, revealing a characteristic $\beta$-carbon proton splitting pattern that corresponded to a completely protonated methylene $\gamma$-carbon (Figure 4). As the $\alpha\beta$-unsaturated reaction product was not purified from the $\beta\gamma$-unsaturated substrate before $^1$H NMR analysis, (E)-hex-3-enyl-S-pantetheine was still clearly visible in the spectrum (1:0.52 molar ratio by proton signal integration). Greater than 95% of the $\alpha$-protons of (E)-hex-3-enyl-S-pantetheine had exchanged for deuterons during the course of the reaction, confirming that the degree of contamination by residual solvent protons was insignificant (Supplementary Figure S3C). The rate of solvent-catalyzed $\alpha$-proton exchange is on approximately the same time scale as the concurrent background hydrolysis of the labile thioester in both substrate and product, complicating the analysis of reactions incubated longer than 24 h (Supplementary Figure S3D).

To determine if the remarkable decrease (>10-fold) in catalytic rate of the reaction in D2O was the result of an
Figure 4. PksEI14-catalyzed isomerization in D₂O. To determine whether PksEI14 shuffles a proton within the substrate or employs water in the catalytic mechanism, (E)-hex-2-enyl-S-pantetheine (1) was incubated with PksEI14 in D₂O, and the reaction was analyzed by NMR. The negative control (top spectrum), in which PksEI14 was not added, shows only uncatalyzed α-hydrogen exchange. When PksEI14 is supplied (middle spectrum), isomerization to (E)-hex-2-enyl-S-pantetheine (2) is observed. If the mechanism of isomerization involved a solvent- or enzyme-donated proton, the γ-position would be monodeuterated, and different splitting patterns would be observed. The bottom spectrum is of synthetically prepared (E)-hex-2-enyl-S-pantetheine (2). Each of the four possible olefinic proton signals are aligned with the corresponding region of the spectrum and denoted with red circles. Pantetheine moieties are represented by “Pant”. See also Supplementary Figure S3.

abnormally large kinetic isotope effect, (E)-2,2-dideutero-hex-3-enyl-S-pantetheine was incubated with PksEI14 in H₂O. Although isomerization to (E)-hex-2-enyl-S-pantetheine was observed, it could not be excluded that the product was generated primarily from a substrate species that had first undergone background exchange of α-deuterons for solvent protons, compromising any conclusions drawn from analysis of the product. The reduced catalytic rate observed for the reaction conducted in D₂O may have resulted from an alteration of the PksEI14 active site geometry caused by deuteration, as observed for haloalkane dehalogenase.¹⁹

To our knowledge, the only polyketide from cis-AT PKSs that contains a βγ double bond is ansamitocin (Figure 1A). Intriguingly, the ansamitocin PKS does not contain an EI domain, yet studies have shown that the formation of the βγ double bond occurs while the polyketide is tethered to the synthase, and the positioning of this double bond is critical for downstream transfer of the growing intermediate.²⁰ A sequence alignment of DH domains reveals that the highly conserved catalytic aspartic acid in the responsible DH (AsmDH3) is replaced by a glutamic acid, which may help enable an unusual βε-dehydration of the substrate. Although the migration of double bonds by polyunsaturated fatty acid synthases has also been proposed to be catalyzed by dedicated shift modules, no cis-AT PKS pathway has been discovered that employs a dedicated EI domain to catalyze αβ → βγ double bond isomerization.²¹

As suggested by the results presented here, the forward biological reaction (αβ → βγ double bond isomerization) would be thermodynamically unfavorable if the substrate did not harbor a moiety at the δ-position available for conjugation with a βγ double bond (e.g., double bond, amide, carbonyl). For PksEI14, this functional group is supplied by the δε double bond installed by PksDH12. This δε double bond is in conjugation with the double bond installed by PksDH11, which is in conjugation with the amide formed by the preceding NRPS module. While the amide likely sets a thermodynamically favorable foundation for the three subsequent double bond shifts, sequence alignments do not provide a clear explanation for how PksDH11 and PksDH12 are able to catalyze both dehydration and isomerization without a dedicated EI domain. These DHs could mediate isomerization after normal αβ-dehydration, similar to the mechanism proposed for FabA.¹⁶ Also possible is that the DHs of these modules directly dehydrate the βγ-positions through the elimination of a γ-proton and β-hydroxyl; this type of dehydration may be facilitated by the increased acidity of the γ-proton adjacent to the amide or double bond at the δε-position. In addition to the bacillaene EI domain, the EIs of both the rhizoxin and corallopyronin PKSs isomerize double bonds into conjugation with preexisting functional groups at the δε-position (a double bond and amide, respectively). Whether PksDH13 is capable of catalyzing isomerization is unknown; perhaps PksEI14 is more compatible with the substrate (e.g., due to its geometry or presence of an α-substituent).

Our results suggest that the mechanism for double bond isomerization by the EI domain proceeds via the shuttling of a substrate proton by a single active site histidine, similar to the mechanism of triose phosphate isomerase.²² An alignment of the six crystal structures of cis-AT PKS DHs solved to date reveal that the imidazole groups of the catalytic histidines superpose nearly perfectly,¹⁵ yet the imidazole group of PksEI14 is relatively shifted ~1.7 Å (as measured between Nε atoms) (Figure 2B). Since the EI domain possesses the same double-hotdog fold as the DH domain, the ACP-bound substrate may be presented to the active site residues of these two domains in a similar manner. The shifting of the catalytic histidine from its position in DH domains is...
approximately the distance of a carbon–carbon bond and could position the imidazole group between the sites of proton abstraction and donation. The shutting of a substrate proton catalyzed by a single residue to isomerize a double bond has been proposed for several other isomerases, including ketosteroid isomerase, human mitochondrial enoyl-CoA isomerase, cyclohexenylcarbonyl-CoA isomerase, and Δ^3,Δ^5,Δ^14-dienoyl-CoA isomerase.13–23

The possible intermediate of the mechanism proposed here involves either the transient protonation of the thioester carbonyl to an enol or the formation of an enolate that is stabilized by an oxyanion hole. To better understand how the single active site histidine catalyzes isomerization unaided and the possible intermediate of the mechanism proposed here involves either the transient protonation of the thioester carbonyl to an enol or the formation of an enolate that is stabilized by an oxyanion hole. To better understand how the single active site histidine catalyzes isomerization unaided and what the intermediate may be, substrate and product analogues were modeled into the active site of PksEI14. The location of the substrate and product were subjected to several restraints. First, a conserved positively charged residue and phenylalanine have been suggested to form the ACP-docking site for the substrate and product were subjected to several restraints. First, a conserved positively charged residue and phenylalanine have been suggested to form the ACP-docking site.
independently shuttling a substrate proton to catalyze $\alpha,\beta \rightarrow \beta,\gamma$ double bond isomerization.

## METHODS

**Cloning, Expression, and Purification.** The gene corresponding to PksEI14 was amplified from *B. subtilis* sp. 168, cloned into the LIC expression vector pGA2Zb, and expressed heterologously in *Escherichia coli* BL21(DE3).30 Hexahistidine-tagged PksEI14 was purified from cell lysate using immobilized metal affinity and size-exclusion chromatography. The mutation of the active site tyrosine to phenylalanine (Tyr185Phe) was accomplished using standard gene mutation techniques, and the resulting protein was purified in a similar manner to the wild-type.

**Crystallization and Structure Determination.** Crystals of PksEI14 grew over 1–4 days by sitting drop vapor diffusion at 22°C in a condition composed of ammonium sulfate and Tris-HCl. The structure was initially solved by single-wavelength anomalous dispersion using crystals soaked in a solution containing methyl mercuric acetate. The experimental phases provided an initial map, which was used to construct a model that was further refined with a 1.73 Å resolution native data set. The coordinates for PksEI14 have been deposited in the Protein Data Bank, accession code 4U3V.

## ASSOCIATED CONTENT

### Supporting Information
Complete details on protein purification, crystallization, syntheses of thioester substrates and activity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions
D.G. solved the native and derived crystal structures, synthesized thioester substrates, and conducted *in vitro* assays. P.S. cloned the PksEI14 gene, purified the protein, and optimized crystals. D.G. and A.T.K. designed the experiments and wrote the manuscript.

### Notes
The authors declare no competing financial interest.

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