Crystal Structure of a Bacterial Type III Polyketide Synthase and Enzymatic Control of Reactive Polyketide Intermediates

Received for publication, June 11, 2004, and in revised form, July 12, 2004
Published, JBC Papers in Press, July 20, 2004, DOI 10.1074/jbc.M406567200

Michael B. Austin‡§, Miho Izumikawa¶, Marianne E. Bowman‡, Daniel W. Udvardy†, Jean-Luc Ferrer‡, Bradley S. Moore††‡‡§§, and Joseph P. Noel‡§ §§

From the ‡Structural Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037, the ¶Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92037-0634, the §Division of Medicinal Chemistry, University of Arizona, Tucson, Arizona 85721-0207, and the §§Institut de Biologie Structural Jean-Pierre Ebel/Laboratoire de Cristallographie et Cristallogenese des Proteines, 41 rue Jules Horowitz, 38027 Grenoble Cedex 1, France, and the ††Department of Chemistry, University of Arizona, Tucson, Arizona 85721

In bacteria, a structurally simple type III polyketide synthase (PKS) known as 1,3,6,8-tetrahydroxynaphthalene synthase (THNS) catalyzes the iterative condensation of five CoA-linked malonyl units to form a pentaketide intermediate. THNS subsequently catalyzes dual intramolecular Claisen and aldol condensations of this linear intermediate to produce the fused ring tetrahydroxynaphthalene (THN) skeleton. The type III PKS-catalyzed polyketide extension mechanism, utilizing a conserved Cys-His-Asn catalytic triad in an internal active site cavity, is fairly well understood. However, the mechanistic basis for the unusual production of THN and dual cyclization of its malonyl-primed pentaketide is obscure. Here we present the first bacterial type III PKS crystal structure, that of Streptomyces coelicolor THNS, and identify by mutagenesis, structural modeling, and chemical analysis the unexpected catalytic participation of an additional THNS-conserved cysteine residue in facilitating malonyl-primed polyketide extension beyond the triketide stage. The resulting new mechanistic model, involving the use of additional cysteines to alter and steer polyketide reactivity, may generally apply to other PKS reaction mechanisms, including those catalyzed by iterative type I and II PKS enzymes. Our crystal structure also reveals an unanticipated novel cavity extending into the “floor” of the traditional active site cavity, providing the first plausible structural and mechanistic explanation for yet another unusual THNS catalytic activity: its previously inexplicable extra polyketide extension step when primed with a long acyl starter. This tunnel allows for selective expansion of available active site cavity volume by sequestration of aliphatic starter-derived polyketide tails, and further suggests another distinct protection mechanism involving maintenance of a linear polyketide conformation.

1,3,6,8-Tetrahydroxynaphthalene (THN)1 is biosynthesized from polyketide intermediates in fungi and bacteria (Fig. 1A). In some fungi, THN is reduced to 1,8-dihydroxynaphthalene and undergoes polymerization to form UV-protective 1,8-dihydroxynaphthalene-melanin (1). Filamentous bacteria of the genus Streptomyces utilize THN not only for melanin production (2) but also incorporate the THN scaffold into pharmacologically active meroterpenoids such as neomarasmine (3).

Fungi and bacteria use dramatically different enzymatic systems to produce THN from five activated thioester-linked malonyl building blocks. In fungi, THN is synthesized by an iterative type I polyketide synthase (PKS), which functions as a large (−230-kDa) multidomain complex (4). Whereas type I PKSs and their fatty acid synthase ancestors have been intensely studied, their three-dimensional domain organization remains obscure due to their size and complexity. In stark contrast, bacteria use a structurally simple type III PKS architecturally organized as a homodimeric condensing enzyme (−40 kDa) to synthesize THN from five coenzyme A (CoA)-tethered malonyl units, without the use of additional enzymes or catalytic domains (5–8).

The type III PKS enzyme family (9–11), defined by homology to chalcone synthase (CHS) (12–15), is currently known to include at least 15 functionally divergent (11) β-ketosynthases (KSs) of plant (9–11) and bacterial origin (11, 16). As first revealed in the crystal structure of alfalfa CHS (17), each type III PKS monomer utilizes a Cys-His-Asn catalytic triad (Fig. 1B) within an internal active site cavity that is connected to the surrounding aqueous phase by a narrow CoA-binding tunnel. The catalytic triad and the buried active site cavity condense an acetyl unit (derived from the decarboxylation of a malonyl-CoA) to a preloaded starter molecule attached by a thioester bond to the catalytic cysteine (18). Subsequent structure-guided mutagenic and biochemical studies of CHS provided a

---

1 The abbreviations used are: THN, 1,3,6,8-tetrahydroxynaphthalene; PKS, polyketide synthase; THNS, THN synthase; CHS, chalcone synthase; STS, stilbene synthase; TAL, triacetic acid lactone (4-hydroxy-6-methyl-2H-pyran-2-one); 2-PS, 2-pyrone (TAL) synthase; PEG, polyethylene glycol; KS or KAS, β-ketoacyl synthase; MtFabH, an M. tuberculosis KAS III enzyme; MtPKS18, M. tuberculosis PKS18; HPLC, high performance liquid chromatography; MOPS, (3-N-morpholino)-2-hydroxypropanesulfonic acid.
clear picture of the conserved type III PKS polyketide extension mechanism (Fig. 1B) (19–21). Type III PKSs are both iterative and multifunctional, having evolved to catalyze an impressive repertoire of functionally divergent and mechanistically complex reactions. These remarkable enzymes typically perform three polyketide extensions of their preferred CoA-activated starter molecules (ranging in size from acetyl- to caffeoyl-CoA), prior to catalyzing six-membered ring formation via intramolecular Claisen-, aldol-, or lactone-forming cyclization reactions (11). Mutagenic analyses of plant PKSs based upon homology with CHS or upon the more recent crystal structures of daisy 2-pyrene synthase (2-PS) (22) and pine stilbene synthase (STS) (23), facilitated identification of much of the structural and mechanistic underpinnings for plant type III PKS substrate specificity and catalysis (11).

Despite these advances, the structural and mechanistic basis for the bacterial THNS reaction pathway remains frustratingly obscure. Whereas most type III PKSs cyclize linear tetraketide intermediates to form glucogulonic- or resorcinol-based products, THNS uniquely catalyzes two successive intramolecular carbon-carbon cyclization reactions of an all malonate-derived pentaketide chain (Fig. 1A). How THNS controls chain length and reactivity while avoiding the many possible derailment reactions available to its carboxylated polyketide intermediate is one of this enzyme’s most intriguing features. Furthermore, it is unclear which of several possible cyclization routes to THN are utilized by the enzyme (Fig. 1C), despite the perpetuation in the literature of a hypothetical STS-like initial C-2 → C-7 intramolecular aldol condensation (5). Our recent structural and mechanistic analysis of STS (23), considered in light of earlier biomimetic studies (24), instead implicates the C-1 thiolate carbanion (activated by the C-11 ester carbonyl and the C-10 methylene (activated by the C-11 carboxylate moiety) as the most likely electrophile and nucleophile, respectively, for intramolecular cyclization. Thus, an initial CHS-like C-6 → C-1 Claisen condensation could lead to a prearomatic chlorogluconol-like intermediate, or a C-10 → C-5 aldol condensation could lead to the corresponding resorcinol-like intermediate (Fig. 1C). It is also possible that the THNS active site may juxtapose these two reactive carbons, C-1 and C-10, to first facilitate an unprecedented C-10 → C-1 Claisen condensation. Although the resulting 10-membered polyketide ring seems energetically unfavorable relative to a more stable six-membered ring, in the context of the THNS active site, it could form and rapidly collapse via any one of five symmetric intramolecular aldol condensations to form the two stable fused six-carbon rings of the THN product (Fig. 1C). Significantly, no on-pathway monocyclic intermediates or their stable aromatic derivatives have ever been observed to result from the THNS-catalyzed reaction, suggesting that the unknown initial cyclization intermediate must indeed strongly facilitate enzymatic formation of the second ring.

Although homology-based analysis of bacterial THNSs predicts a number of unusual active site residues (11, 25), including four or five exposed cysteine side chain thiol groups that might contribute to these enzymes’ unusual physiological reaction, sequence divergence between bacterial CHS-like enzymes and the structurally characterized plant PKSs increases the likelihood of significant model bias and error in homology models derived from distantly related plant PKS structures. In order to illuminate the mechanistic features responsible for these enzymes’ catalytic pathway, we undertook a structural and biochemical analysis of THNS from the model actinomycete Streptomyces coelicolor A3 (2, 26). Toward this end, we present here the 2.2-Å crystal structure of THNS from S. coelicolor A3 (2), accompanied by additional mutagenic and biochemical investigations into the complicated THNS reaction mechanism.

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Mutagenesis**—The construction and characterization of the S. coelicolor THNS construct in the pHiHS vector was previously reported (8). Mutations were introduced using mutagenic oligonucleotides (Table I) and the QuikChange (Stratagene) system. The entire coding sequence of the mutant enzyme was amplified by nucleotide sequencing. Wild type and mutant proteins were expressed and purified as previously described (8). For crystallization, THNS was overexpressed in *Escherichia coli* BL21(DE3) cells containing the pLYS plasmid (Invitrogen), purified, buffer-exchanged, and concentrated as described for CHS (19), resulting in final enzyme concentrations of 5–50 mg/ml in 12 mM HEPES (pH 7.5), 25 mM NaCl, and 5 mM dithiothreitol. All crystals were stored at −30 °C.

**Crystallization and Data Collection**—THNS was crystallized by vapor diffusion in hanging drops consisting of a 1:1 mixture of purified protein solution and crystallization buffer. The crystallization buffer contained 14% (w/v) PEG 8000, 200 mM MgCl₂, 100 mM Na⁺-MOPS buffer (pH 7.0), 5 mM dithiothreitol, and 3% (w/v) sucrose. A 30-μl crystal seed prior to freezing employed a cryogenic solution differing from the crystallization buffer due to an increased PEG 8000 concentration (16% (w/v)) and the inclusion of 0.1 mM K₂PtCl₄ prior to cryogenic freezing as before. Several unsuccessful heavy atom soaks were screened for anomalous diffraction at the European Synchrotron Radiation Facility, whereas the 2.2-Å native and derivative data were collected at the Stanford Synchrotron Radiation Laboratory. Images were indexed and integrated with DENZO (27), reflections were merged with SCALEPACK (27), and data reduction was completed with CCP4 programs (28). THNS crystallizes in the P2₁(1) space group, with unit cell dimensions of a = 76.68 Å, b = 69.88 Å, c = 81.14 Å, α = γ = 90°, and β = 95.42°.

**Structure Determination and Refinement**—The S. coelicolor THNS structure was solved from the 2.9-A platinum derivative data set. Experimental multiple wavelength anomalous dispersion phases were obtained using SOLVE (29), based upon two K₂PtCl₄ sites. The experimental electron density maps were improved by bulk solvent density modification and automated building with RESOLVE (29). Inspection of electron density maps and subsequent model building were performed in O (30). The asymmetric unit contains one physiological THNS dimer. Two copies of a monomeric THNS homology model, generated by MOD-ELLER (31) from an alignment with the alfalfa CHS2 crystal structure (17), were manually inserted into the density-modified electron density maps and rebuilt in O. Following the success of a single round of refinement in CNS (32) of this rebuilt protein model against the same 2.9-Å platinum derivative data set, subsequent iterative rounds of refinement in CNS utilized the higher quality 2.2-Å native data set. Coordinate and structure factor statistics for this model are described in Table S1. Several alternative models were obtained from the HIP-U (Hetero-compound Information Centre-Uppsala) site on the World Wide Web (x-ray.bmc.uu.se/hicup/). PROCHECK (in CCP4) analysis of the final refined crystal structure (see Table II) revealed 86.6% of residue conformations to be in the most favored region of the Ramachandran plot, with 11.2% in additional allowed, 2.2% in generously allowed, and no residues in disallowed regions. THNS residues in generously allowed conformations are located in poorly ordered surface loops. Coordinates and structure factors for THNS have been deposited in the Protein Data Bank (1U0M).

Structures were overlaid for comparison using MOLMOL (33). Structural illustrations were prepared with MOLSCRIPT (34) and rendered with POV-Ray (persistance of vision ray tracer; available on the World Wide Web at www.povray.org).

**Enzyme Activities**—THNS activity was monitored by reversed-phase HPLC (8) and TLC. Reversed-phase TLC analysis of flavilin/TAL product ratios were obtained from standardization 50-μl room temperature reactions containing 100 μM Tris-HCl buffer (pH 7.5), 10 μM THNS, and 20 μM [2-¹³C]malonyl-CoA (PerkinElmer Life Sciences). Reactions were quenched with 5 μl of concentrated HCl and extracted twice with 50 μl of ethyl acetate. The extracts were dried, dissolved in 50 μl of H₂O, applied to TLC plates, developed in MeOH/H₂O/ACOH (60:40:1, v/v/v), and visualized with radiography film. Apparent kinetic constants kₚ and Kₗₚ for THN production (Table III) were determined from Eadie-Hofstee plots, using an average of three or more independent assays (8).

**Flavilin Labeling and ¹³C NMR Analysis**—E. coli BL21(DE3)pLysS cells (Invitrogen) harboring the pHiHS-THNS construct were grown at 37 °C in 1.2 liters of Terrific broth containing 50 μg/ml kanamycin and
37 μg/ml chloramphenicol until A_600 reached 0.7. Following induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside and the addition of 120 mg of [1,2-13C]AcONa (Cambridge Isotope Laboratories, Inc.), cultures were shaken at 28 °C overnight, with additional 120-mg aliquots of [1,2-13C]AcONa added 3 and 6 h after induction for a total of 360 mg of labeled acetate. Cells were removed by centrifugation. The media supernatant was acidified with concentrated HCl and extracted with ethyl acetate. The crude organic extract was concentrated and sepa-
nately, a 2.9-Å data set of a K2PtCl4 soak of our native crystals did not result in stable and active protein preparations. Fortunately, a 2.5 mg of enriched flaviolin in Me2SO-THNS crystal structure by molecular replacement using a E. coli enomethionine into theoroacetic acid in water to methanol over 60 min; flow rate reversed-phase column with a linear solvent gradient of 0.15% triflu-INADEQUATE NMR on a Bruker 600-MHz spectrometer. was further purified by preparative HPLC (YMC ODS-AQ 10
rated by oxalic acid-treated silica flash chromatography employing a

| Mutant    | Mutagenic oligonucleotide sequence |
|----------|-----------------------------------|
| C106S    | 5′-G ATC ATC TAC GTG GCC TGC TCC |
| C168S    | 5′-G ATC ATC TAC GTG GCC TGC TCC |
| C171S    | 5′-G ATC ATC TAC GTG GCC TGC TCC |
| C184S    | 5′-G ATC ATC TAC GTG GCC TGC TCC |

**TABLE II**

Crystallographic data and refinement statistics for the S. coelicolor THNS structure

|                     | Native       | Platinum derivative |
|---------------------|--------------|---------------------|
| Wavelength (Å)      | 0.773        | 1.0718              |
| Resolution (Å)      | 2.2          | 2.95                |
| Space group         | P2(1)        | P2(1)               |
| Unit cell dimensions (Å) | a = 76.7   | a = 76.5            |
|                     | b = 69.7     | b = 69.9            |
|                     | c = 81.1     | c = 81.2            |
| Unit cell dimensions (degrees) | β = 95.4 | β = 95.3 |
| Total reflections   | 139,869      | 102,360             |
| Unique reflections  | 37,666       | 32,802              |
| Completeness (%)    | 89.8 (20.6)  | 87.8 (52.0)         |
| Rmerge (%)          | 25.5 (4.9)   | 15.0 (2.3)          |
| Average B-factor, protein (Å²) | 54.0 | 10.4 (50.7) |
| Average B-factor, solvent (Å²) | 67.0 | 10.6 (49.1) |

|                     | λ1           | λ2           | λ3           |
|---------------------|--------------|--------------|--------------|
| Refinement statistics | Rcryst/Rfree (%) | 25.2 / 29.3  |              |
| Protein atoms       | 5308         |              |              |
| Ligand atoms        | 50           |              |              |
| Water molecules     | 148          |              |              |
| Root mean square deviation bond lengths (Å) | 0.008 | 1.4 |
| Root mean square deviation bond angles (degrees) | 1.4 |
| Average B-factor, protein (Å²) | 54.0 |              |
| Average B-factor, solvent (Å²) | 67.0 |              |

|                     | Native       | Platinum derivative |
|---------------------|--------------|---------------------|
| Wavelength (Å)      | 0.773        | 1.0718              |
| Resolution (Å)      | 2.2          | 2.95                |
| Space group         | P2(1)        | P2(1)               |
| Unit cell dimensions (Å) | a = 76.7   | a = 76.5            |
|                     | b = 69.7     | b = 69.9            |
|                     | c = 81.1     | c = 81.2            |
| Unit cell dimensions (degrees) | β = 95.4 | β = 95.3 |
| Total reflections   | 139,869      | 102,360             |
| Unique reflections  | 37,666       | 32,802              |
| Completeness (%)    | 89.8 (20.6)  | 87.8 (52.0)         |
| Rmerge (%)          | 25.5 (4.9)   | 15.0 (2.3)          |
| Average B-factor, protein (Å²) | 54.0 | 10.4 (50.7) |
| Average B-factor, solvent (Å²) | 67.0 | 10.6 (49.1) |

|                     | λ1           | λ2           | λ3           |
|---------------------|--------------|--------------|--------------|
| Refinement statistics | Rcryst/Rfree (%) | 25.2 / 29.3  |              |
| Protein atoms       | 5308         |              |              |
| Ligand atoms        | 50           |              |              |
| Water molecules     | 148          |              |              |
| Root mean square deviation bond lengths (Å) | 0.008 | 1.4 |
| Root mean square deviation bond angles (degrees) | 1.4 |
| Average B-factor, protein (Å²) | 54.0 |              |
| Average B-factor, solvent (Å²) | 67.0 |              |

* The number in parenthesis is for the highest resolution shell.

**TABLE III**

Steady state kinetic constants for THN production for wild type THNS and the Cys to Ser mutants

|                     | Wild type    | C106S        | C168S        | C171S        | C184S        |
|---------------------|--------------|--------------|--------------|--------------|--------------|
| kcat × 10³ (min⁻¹)  | 27.4 ± 2.4   | NA⁶          | ND⁶          | 27.9 ± 1.3   | 47.5 ± 5.4   |
| Kₘ (μM)             | 2.3 ± 0.6    | NA           | ND           | 8.5 ± 1.0    | 1.4 ± 0.5    |
| kcat/Kₘ (s⁻¹ M⁻¹)   | 1.970        | NA           | ND           | 549          | 5,530        |

|                     | λ1           | λ2           | λ3           |
|---------------------|--------------|--------------|--------------|
| Refinement statistics | Rcryst/Rfree (%) | 25.2 / 29.3  |              |
| Protein atoms       | 5308         |              |              |
| Ligand atoms        | 50           |              |              |
| Water molecules     | 148          |              |              |
| Root mean square deviation bond lengths (Å) | 0.008 | 1.4 |
| Root mean square deviation bond angles (degrees) | 1.4 |
| Average B-factor, protein (Å²) | 54.0 |              |
| Average B-factor, solvent (Å²) | 67.0 |              |

* NA, no activity.

**RESULTS**

THNS Crystal Structure—We were unable to solve the THNS crystal structure by molecular replacement using a 2.2-Å native data set and homology models based on plant type III PKS structures. Moreover, the in vivo incorporation of sel-nemothionine into the E. coli-expressed S. coelicolor THNS did not result in stable and active protein preparations. Fortunately, a 2.9-Å data set of a K2PtCl4 soak of our native crystals produced an anomalous signal suitable for phasing the structure using multiple anomalous diffraction with SOLVE (29). Two platinum sites producing strong peaks on anomalous difference Patterson maps permitted phasing as well as phase extension and phase improvement by density modification using RESOLVE (29). The quality of the phase information deter-riorated significantly beyond 3.9 Å, but the initial figure of merit-weighted 2Fo – Fs electron density map was interpretable. Whereas RESOLVE automatic model building using this low resolution phasing information produced only a few erroneous polyalanine chains, their symmetrical distribution in the asymmetric unit facilitated identification of electron density corresponding to the THNS conserved type III PKS aβaβa-fold core domain and expected physiological homodimeric interface (11). A CHS-derived THNS homology model was then globally positioned in this experimental electron density map, and each residue with interpretable electron density was individually repositioned using O. Following a single round of successful CNS refinement of our manually placed and partially rebuilt model against the 2.9-Å derivative data, the resulting 2Fo – Fs and Fo – Fs electron density maps indicated a number of regions needing to be rebuilt. In order to move rapidly and achieve a higher quality final model, subsequent rounds of refinement utilized our original native data set extending to 2.2-Å resolution. Portions of the model with no apparent electron density (discussed below) were initially deleted, but most
were eventually replaced, since later electron density maps greatly improved following iterative rounds of building and refinement using the 2.2-Å native data set.

Although previous plant PKS-based homology modeling failed to predict the many subtle backbone differences observed throughout this first bacterial type III PKS crystal structure reported here, there are no drastic rearrangements of the conserved αβαβα-fold or dimer interface in THNS. Our crystal structure shows that homology-based assignments of the THNS catalytic triad and other active site residues are essentially accurate, as previously supported by in vitro assays of *Streptomyces griseus* THNS point mutants (7).

Interestingly, THNSs feature unusual (~25-residue) extensions of their C termini, not found in plant or most other bacterial CHS-like sequences examined to date. These additional residues may facilitate in vivo protein-protein interactions with upstream and downstream enzymes, including the P450 or ORF3 proteins encoded next to the THNS gene in *S. coelicolor* and *S. griseus*. The elimination of this C-terminal extension was previously shown to have no detrimental effect upon the in vitro activity of *S. coelicolor* THNS (8). Although we crystallized the full-length protein, this C-terminal extension is completely disordered in the electron density maps calculated from the most recent THNS model obtained after multiple rounds of rebuilding and refinement. Moreover, several adjacent solvent-exposed short loops on the αβαβα core domain are also somewhat disordered, with interpretable electron density for many of these residues emerging only in the final stages of refinement. Whereas the conformational homogeneity of these exposed loops is no doubt locally perturbed by the adjacent disordered C termini of the THNS physiological dimer, the THNS unit cell also lacks stabilizing crystal lattice contacts in this “upper” half of the physiological dimer. Our refined THNS crystallographic B (temperature) factors indicate that this segregation of lattice contacts allows subtle global rigid body pivoting movements that uniformly increase conformational heterogeneity as a function of the distance from stabilizing crystal contacts (Fig. 2A).

In contrast to these particular surface-exposed regions adjacent to the disordered C termini, the electron density maps near the active site cavity were unambiguous and provided no hint of conformational disorder. Interestingly, the electron density associated with the interior of THNS clearly indicated the unexpected presence of two different small molecules bound near the traditional “floor” of the type III PKS active site cavity (if the Cys-His-Asn catalytic triad is considered to project from the active site “ceiling” formed by the upper αβαβα-fold core domain; see Fig. 2A). Subsequent refinement confirmed these serendipitous small molecules to be a polyethylene glycol (PEG) heptamer, present in the crystallization solution, and a glycerol molecule, subsequently introduced to the PEG-bound THNS crystal during a final 30-s cryoprotection treatment immediately prior to freezing in liquid nitrogen. Although neither of these bound molecules are a physiological substrate, intermediate, or product of the THNS reaction, the bound location of PEG is surprising and relevant to the functional interpretation of our static structure.

One end of this PEG heptamer protrudes into the THNS active site cavity, but most of the molecule is sequestered in a newly discovered and buried tunnel that extends into the traditionally solid “floor” of the type III PKS active site cavity (Figs. 2, A and B, and 3, A and B). Not predicted by homology, this novel cavity is formed in THNS by the subtle tertiary repositioning (relative to plant type III PKS structures; see Fig. 2B) of structural elements that come together “under” the active site cavity, including the outward rigid body displacement of three conserved helices (Fig. 2, A and B). One of these helices forms the lower threshold of the type III PKS-conserved CoA-binding tunnel’s intersection with the active site cavity (Fig. 2B), whereas the other two helices form an adjacent and structurally conserved helix-loop-helix motif, whose solvent-exposed outer surface contains the phosphate-binding basic residues that mediate CoA complex formation in all CHS-like enzymes examined to date (11). Significantly, this latter helix-loop-helix motif also participates in THNS crystal lattice contacts, which probably select for and stabilize the observed PEG-binding conformation of THNS. Whereas this PEG-bound tunnel conformation is probably not a static apo (non-ligand-bound) conformation in solution, it supports earlier predictions that this area of type III PKSs might be subject to dynamic motion in solution (11). This novel tunnel also suggests the first mechanistic hypothesis for the cryptic in vitro activity of THNS when primed with long acyl starter molecules (35).

Remarkably, the *S. griseus* THNS (RpaA) catalyzes an additional (fifth) polyketide extension step in vitro when primed with octanoyl-CoA, thus generating a hexaketide from this larger, nonphysiological starter molecule (35). This unprecedented result contradicts existing (and well supported) models of both physiological and “unnatural” type III PKS catalysis, which correlate polyketide size limits to active site cavity volume (i.e., the promiscuous incorporation of a significantly larger starter within a given active site cavity should result in fewer polyketide extension steps). Nonetheless, we were able to duplicate this previous *S. griseus* THNS result using our *S. coelicolor* enzyme (data not shown). In CHS-like enzymes, linear reaction intermediates are covalently attached to either the catalytic cysteine or bound CoA through thioester linkages at the C-1 carboxyl carbon. The distance between Cys138 and the novel PEG-binding tunnel precludes all but the longest (i.e., pentaketide) of the malonyl-primed linear physiological reaction intermediates from accessing the THNS PEG-binding tunnel. However, the PEG-like aliphatic tail of polyketide intermediates generated from an octanoyl-CoA starter is easily long enough to access, bind to, and stabilize the open conformation of this tunnel should it become even transitorily available due to dynamic fluctuations during priming and/or polyketide chain extension (Fig. 3A). Notably, the depth of the observed tunnel is such that it could progressively envelop the aliphatic tail of a growing C-1-tethered polyketide intermediate, thus effectively (and selectively) increasing the active site volume for the octanoyl-primed reaction. Such progressive binding would maintain a relatively taut linear polyketide intermediate conformation inconsistent with the juxtaposition of atoms necessary for early termination of polyketide extension by the typical intramolecular Claisen or lactone cyclization routes. Efforts to probe the mechanistic importance of this novel THNS cavity are underway, but initial indications suggest, as expected due to its distance from the nucleophilic Cys138, that the PEG tunnel is not important for the physiological production of THN (data not shown).

We also considered whether the observed interaction of typically buried polar residues (Asp176 and Asn185) with the bound glycerol ligand might be mechanistically informative. Although again slightly too distant from Cys138 to interact with physiological C-1-tethered linear intermediates, we reasoned that an initial CHS-like thioester-cleaving intramolecular C-6 → C-1 Claisen condensation could liberate a phloroglucinol cyclization intermediate whose secondary cyclization to form THN might be catalyzed within this polar glycerol-binding lower pocket. However, subsequent point mutations of the residues binding glycerol (Asp176 and Asn185) did not derail THN production (data not shown). We thus concluded that neither PEG nor
glycerol binding were relevant to the malonyl-primed physiological THNS reaction and turned our attention to the traditional type III PKS active site cavity for insights into the structural and chemical control of polyketide length and reactivity by THNS. As predicted by homology modeling, the THNS active site cavity contains a number of unusual amino acid substitutions relative to plant type III PKSs. Notably, these residues are conserved in all of the functionally confirmed bacterial THNS sequences discovered to date (5–8). Funa and co-workers recently identified the importance of Tyr224 for THNS starter specificity (7). Although mutation to Phe or Trp was tolerated by THNS, nonconservative mutations at this position abrogated the enzymatic loading of the physiological malonyl starter. These results are not surprising, since the substitution of bulky side chains in place of the spatially equivalent Gly256 residue of alfalfa CHS was shown to affect both starter specificity and the number of catalyzed polyketide chain extension steps (22, 36). As first observed in the crystal structure of 2-PS (22), which loads an acetyl moiety as a starter, variation of

FIG. 2. Overall structure of THNS and comparison with evolutionarily related enzymes. A, view of the THNS physiological homodimer and serendipitously bound small molecules. Oxygen atoms of these CPK-depicted ligands are red, whereas carbon atoms of PEG and glycerol are gold and green, respectively. Blue portions of each monomer correspond to three conserved α-helices whose subtle rearrangement in THNS relative to previously described plant type III PKSs contributes to PEG tunnel formation. Inset, B-factor plot (blue to red from low to high B-factors) of the refined THNS structure, reflecting the lack of crystal contacts near the disordered 25-residue C termini of each monomer. B, comparison of the three-dimensional architectures of a plant type III PKS (alfalfa CHS; green) and the bacterial type III PKS (S. coelicolor THNS; rose) determined here. Type III PKS-conserved access of CoA-tethered substrates to the active site cavity cysteine is demonstrated by depiction of CHS-bound CoA (ball-and-stick). C, similar comparison of the three-dimensional architectures and unusual active site cavity extensions of the bacterial type III PKS determined here (THNS; rose) and a Mycobacterium enzyme MtFabH (blue), a divergent member of the distantly related KAS III enzyme family involved in fatty acid biosynthesis in bacteria and plants. Water molecules demonstrating the location of the unique MtFabH acyl-binding cavity extension are red, whereas the PEG molecule occupying the similar but distinct tunnel of THNS is gold. CoA is modeled (based on its CHS-bound position shown in B). The position of the nucleophilic cysteine from each enzyme’s dyad-related monomer illustrates slight changes in dimerization. Important structural differences (see “Results”) are also labeled. The figure was prepared with MOLSCRIPT (34) and rendered with POV-Ray (persistence of vision ray-tracer; available on the World Wide Web at www.povray.org).
steric bulk at this particularly well placed active site position is quite effective for horizontal modulation of the type III PKS active site cavity volume. These prior studies also surprisingly revealed that a too spacious active site cavity can actually decrease the ability of a type III PKS to utilize smaller starter molecules, possibly due to excessive conformational freedom of the loaded starter (11). Our current structure confirms that Tyr224 indeed contributes to a steric constriction along a horizontal direction of the THNS active site cavity relative to CHS (17) and STS (23), no doubt limiting the conformational freedom of a Cys138-loaded malonyl starter.

Horizontal constriction by Tyr224 is the THNS active site’s only similarity to 2-PS, which features two additional bulky substitutions relative to alfalfa CHS (T197L and S338I). This complementary vertical constriction of the 2-PS active site cavity results in this enzyme’s catalysis of only two polyketide extensions of its small acetyl starter (22). In contrast, THNS features less bulky residues relative to CHS at these (T197C and S338A) and other (T132C and T194C) sterically important active site positions. Thus, the THNS active site cavity combines a 2-PS-like horizontal constriction with slight expansions in volume toward the back and bottom of the cavity (with this latter vertical expansion possibly accentuated by the dynamic flexibility suggested by the appearance of a PEG binding tunnel). This architectural strategy seems ideal for maintaining the ability to utilize a small starter molecule (via horizontal constriction) while providing adequate volume for the additional polyketide chain extension steps and dual cyclization reactions necessary for THN production (via vertical expansion). Interestingly, another CHS-like enzyme that extends polyketides past the typical tetra ketide length was reported during the preparation of this manuscript. This rhubarb aloe sone synthase catalyzes six polyketide extensions of an acetyl-CoA starter, apparently terminated by a CHS-like C-6 → C-1 Claisen cyclization to produce a phloroglucinol ring (37). The reported aloe sone synthase sequence appears to mirror the architectural strategy evident in the THNS active site, by combining a horizontally restricting (2PS-like) G256L substitution with a downward expanding T197A substitution, relative to alfalfa CHS.

As chemical reasoning predicted (see Introduction), the THNS active site does not possess the STS-like “aldol switch” thioesterase-like hydrogen bond network we recently linked to the intramolecular C-2 → C-7 aldol condensation cyclization specificity of STS (23), reinforcing our conclusion that the physiological THNS polyketide is likely to be offloaded via an intramolecular Claisen condensation severing the C-1 thioester linkage. However, other than suggesting a vertical orientation of the THNS fused ring cyclization product, as opposed to the horizontal orientation of CHS and STS product complexes, the observed THNS active site topology does not seem to preclude any of the plausible cyclization pathways leading to THN (depicted in Fig. 1C); nor does this newly described topology indicate how THNS prevents the multitude of derailment cyclization reactions available to its reactive pentaketide intermediate. With retention of the starter malonyl’s carboxylate, even the first six-carbon polyketide intermediate product should be susceptible to lactonization. Prior to the structural elucidation of THNS, these factors led us to expect that the THNS active site cavity would probably exert dramatic steric restrictions on the polyketide intermediates’ conformational freedom. However, when we began to model cysteine-linked linear polyketide intermediates in the THNS active site cavity, we were struck by the low degree of apparent steric control of polyketide conformation imposed by the enzyme. In fact, we were able to manually model a surprising range of polyketide conformations, including those leading to typical lactone derailment products. Unfortunately, whereas automated docking programs are often useful to address substrate, intermediate, and product binding, our experience with this and previous PKS structures indicates that these programs are unable to accurately predict productively folded polyketide conformations in type III PKS active sites. Although we pursued co-crystallization and crystal soaking with nearly a dozen linear,
monocyclic, and fused ring polyketide and cyclization interme-
diate analogs, none of these compounds succeeded in binding to
the active site and displacing PEG and glycerol, even in cases
where co-crystallization altered the dimensions of the crystal-
lographic unit cell (data not shown).

$^{13}$C NMR Analysis of the THN Cyclization Pattern—We con-
ducted labeling experiments to elucidate the folded confor-
manon of linear polyketide intermediates leading to the THN
skeloton, hoping to further illuminate the bacterial THNS cy-
cyclization pattern (see Fig. 1 C). The expected effects of the
symmetric THN product’s spontaneous oxidation to flavilin upon label
distribution are depicted, as is our result: the observation of U-THN
derived flavilin.

Previously, the mutation of each additional S. griseus THNS
active site cysteine to alanine was shown to abolish THN pro-
duction (7). The S. coelicolor THNS also contains a fifth active
site cysteine, not conserved in other THNSs, at position 184
(CHS Gly211). We carried out more conservative serine muta-
tions of all four additional S. coelicolor THNS active site cy-
siteines in order to probe their specific contribution to THN
production. Notably, whereas the $pK_a$ value of serine’s alcoholic
proton ($\sim 14$) (38) is similar to that of threonyne, the cysteine
and serine side chains are otherwise nearly identical in polar-
ity, steric bulk, and hydrogen-bonding ability.

Product specificities of these four isosteric Cys $\rightarrow$ Ser mu-
tations are shown in Fig. 5 A. Unlike mutations of the nucleo-
philic Cys$^{138}$, none of these mutations abolish malonyl loading
or polyketide chain extension. Furthermore, serine point mu-
tants of Cys$^{168}$, Cys$^{171}$, or Cys$^{184}$ (CHS positions 194, 197, and
211) are still able to produce THN (Fig. 5 A and Table III), albeit
with slightly increased partitioning of the reaction to form the
derailed triketide lactone (relative to wild type THNS). These
results indicate that the interactions of these three
THNS active site cysteine side chains with covalently bound
polyketide intermediates are similar to the roles played by the
plant PKS threonines (i.e. steric, polar, and/or hydrogen bond-
ing). Conversely, the isosteric mutation of Cys$^{106}$ (CHS position
32) to serine abolishes THN production, with complete parti-
tioning of the overall reaction to derailment of the triketide
intermediate to form triacetic acid lactone (TAL) (Fig. 5 A).
This latter C106S mutant assay result implicates the importance of
these thiol moiety’s $pK_a$ value in wild type THNS, indicating that
transfer or loss of the more labile thiol proton of Cys$^{106}$ is
crucial for steering the THNS reaction pathway toward the pro-
duction of THN. Moreover, since the C106S mutant pro-
duces only a triketide derailment product, this residue’s chemi-
cal role must occur prior to either THN cyclization event,
neither of which can take place before the tetraketide or pent-
taketide stage of polyketide chain extension. These results
imply that partitioning between two competing mechanistic
pathways determines the ultimate fate of the physiological
malonyl-primed triketide intermediate of THNS (Fig. 5). One of
these competing branch point triketide reactions facilitates the
dual polylactide chain extension reactions and dual
cyclization reactions necessary to biosynthesize THN, whereas
the competing branch of this mechanism leads to triketide
lactone derailment in the form of TAL production.

Partial derailment to TAL in wild type THNS (and mutants
conserving cysteine at position 106) indicates that the presence of
the Cys$^{106}$ thiol does not guarantee additional polylactide
chain extensions of the reactive triketide intermediate to form
THN. The complete partitioning of the C106S mutant’s reaction
toward TAL derailment could in theory be achieved in this
mutant THNS either by promotion of the derailment reaction
or inhibition of the THN mechanistic pathway. Notably, as in

![Fig. 4. In vivo THN labeling experiment using heterologously
expressed S. coelicolor THNS to determine the S- or U-shaped
THN cyclization pattern (see Fig. 1 C). The expected effects of the
symmetric THN product’s spontaneous oxidation to flavilin upon label
distribution are depicted, as is our result: the observation of U-THN
derived flavilin.](http://www.jbc.org/Downloaded from)
other iterative type III PKS enzymes, the third THNS polyketide extension step is almost certainly catalyzed in a conventional manner by the conserved (and spatially separate) Cys-His-Asn catalytic triad. Indeed, both wild type and C106S THNS enzymes are capable of extension to the tetraketide stage when primed with an acyl starter (data not shown), further implying that the increased intrinsic reactivity of the malonyl-primed triketide is probably the driving force behind TAL derailment of the wild type THNS reaction pathway. THN or TAL product-based kinetic constants, dependent upon the rate-limiting steps of each product’s branched multistep pathway, are unlikely to illuminate the product-determining enzymatic partitioning ratios we focus upon here. This issue is further clouded by uncertainty as to the timing of the THN biosynthetic pathway’s decarboxylation of the starter-derived β-keto acid or subsequent enolate tautomerization of the resulting triketide carbanion (Fig. 5B). Although the presumed mechanism of TAL formation employs decarboxylation at (or prior to) the triketide stage (Fig. 5C), it is unclear whether the TAL pathway branches from THN biosynthesis as the result of triketide decarboxylation (Fig. 5D) or alternatively by failing to quench the resulting lactonization-prone enolate tautomer (Fig. 5E).

The theoretical pKₐ of a cysteine residue’s thiol is ~8.7 (38), implying protonation at physiological pH. However, proximity to reactive residues and other local environmental effects can significantly lower this pKₐ, as previously demonstrated for the CHS catalytic triad Cys¹⁶⁴ (pKₐ = 5.5) (20). Interestingly, the implicated THNS position 106 corresponds to CHS Thr¹³², which is conserved in STS enzymes but repositioned (via protein backbone conformational changes) to form a hydrogen-bonding network that shuttles electrons from the adjacent Glu¹⁹² (universally conserved in all type III PKS enzymes) to activate a hydrolytic water poised next to the catalytic cysteine (23). This emergent thioesterase-like hydrogen bonding network promotes the C-2 → C-7 aldol-based cyclization which distinguishes the STS reaction from that of CHS. The CHS/STS “aldol switch” likewise acts by modulating the partitioning of a common intermediate, in this case a tetraketide, between two competing mechanistic paths that are in fact each accessible to both CHS and STS wild type enzymes. However, the THNS crystal structure shows the immediate environment and back-
bone conformation of Cys\textsuperscript{106} to be distinctly CHS-like, with no STS-like interactions of the Cys\textsuperscript{106} thiol with the adjacent glutamate, nor any other immediate environmental modification of this cysteine’s theoretical pK\textsubscript{a} value. This conclusion is qualitatively supported by iodoacetamide labeling experiments of the various THNS cysteine mutants, which indicate the catalytic triad cysteine at position 138 to be the only strong nucophile in the THNS active site at neutral pH (data not shown). The abundance of these thiols in the cysteine-rich THNS active site cavity may facilitate some indirect modification of the pK\textsubscript{a} of Cys\textsuperscript{106}, as might the proximity of two nearby potential helix dipoles (11).

The THNS Cys\textsuperscript{138} and Cys\textsuperscript{106} thiol groups are too far apart (\textsim 6.4 Å) to allow direct interaction with a Cys\textsuperscript{138}-loaded starter malonyl group. However, modeling of physiological intermediates indicates that Cys\textsuperscript{106} can easily access the C-5 position of the reaction’s malonyl starter, seemingly contradicting models that correlate polyketide chain length to type III PKS active site volume (11, 17, 22, 36). The THNS PEG-binding tunnel demonstrates how the enzyme could selectively increase (in a starter substrate-specific manner) the active site volume available to the resulting polyketides. The biological incorporation of THN into prenylated naphthoquinones (3, 5) makes it tempting to speculate that this tunnel could, by binding isoprenoid moieties of prenylated THN derivatives, facilitate some in vivo regulatory inhibition of THNS.

Although clearly reinforced by crystal lattice contacts, the PEG-binding conformation supports previous hypotheses that this region of CHS-like enzymes might undergo some degree of dynamic motion in solution (11). It seems possible that other type III PKS enzymes could transiently form a similar tunnel. Notably, previous CHS-like enzyme crystal structures (17, 22, 23) reveal smaller pockets of buried water in this “subfloor” region, and this area is completely disordered in one crystal form of this family’s noniterative KAS III evolutionary ancestor (41). Whereas typical KAS III enzymes catalyze one malonyl-ACP-mediated extension of a preloaded acetyl starter using the same catalytic triad conserved in CHS-like enzymes, one divergent Mycobacterium tuberculosis KAS III (MtFabH) displays specificity for a long aliphatic acyl starter (42). This noniterative ketosynthase binds its acyl starter in a unique pocket at the dimer interface (42) that is distinct from the THNS PEG-binding tunnel we observe here (Fig. 2C). This MtFabH binding pocket is created by the appearance and dimerization, in an otherwise typical KAS III fold, of a unique helical motif resulting from an insertion into a KAS III \beta-finger loop already significantly longer than the equivalent \beta-finger in type III PKS enzymes. Long acyl starters gain access to this tunnel from the active site cavity through steric reduction of the only residue lining each traditional active site cavity that is contributed by the dyad-related monomer (equivalent to THNS Met\textsuperscript{111} and CHS Met\textsuperscript{137}). Recently, one of three M. tuberculosis CHS-like enzymes (MtPKS18) was found to catalyze the extension of long acyl starters \textit{in vitro} (43). These authors predicted the existence of a MtFabH-like acyl-binding tunnel in MtPKS18, based upon that enzyme’s MtFabH-like steric reduction of CHS position 137. However, the primary sequence of MtPKS18 clearly clusters with other type III PKSs, with MtPKS18 showing more sequence identity with conserved plant PKSs than do the remaining two M. tuberculosis type III PKS sequences. More specifically, no significant sequence deviation from other CHS-like enzymes is apparent in the relevant MtFabH-modified \beta-finger region. Furthermore, even if a MtFabH-like insertion were to occur in a corresponding type III PKS \beta-finger loop, the dimerization of such a hypothetical extension would be prevented by the presence of a type III PKS-conserved long \alpha-helix that is much shorter in KAS III enzymes (Fig. 2, B and C). Therefore, MtPKS18 (or any other hypothetical aliphatic acyl-binding type III PKS enzyme) seems much more likely to access a THNS-like tunnel than an MtFabH-like one.

In the Introduction, we pointed out the numerous plausible intramolecular polyketide cyclization routes leading to THN (Fig. 1C). Our crystal structure supports the proposal that an initial STS-like C-2 \rightarrow C-7 aldol condensation (23) is unlikely in the bacterial THNS pathway, although the resulting resorcinolic acid was previously (but not conclusively) identified as a fungal THNS intermediate cyclization product (44). Our \textit{S. coelicolor} THNS crystal structure, supported by previous mutations in \textit{S. griseus} THNS (7), suggests that a “horizontal restriction/vertical expansion” trend in active site topology is responsible for maximizing polyketide extension steps while preserving type III PKS specificity for small starter molecules. This model is distinct from an older one arising from previous studies of 2-PS, where multidirectional restriction (22) or horizontal restriction alone (36) decreased starter size as well as
the number of polyketide extension reactions. This new adden-
dum to the older “steric modulation” model (11) is independ-
ently supported by a similar trend in the recently reported
sequence of a rhubarb aloesone synthase, which synthesizes a
heptaketide using an acetyl starter (37).

Unfortunately, our crystal structure did not illuminate the
specific bacterial THN cyclization pathway. The failure of nu-
merous potential intermediate and product analogues to dis-
place PEG and glycerol from the THNS active site indicates
that further crystallographic analysis of the THNS reaction
will require a different crystal form. However, our NMR anal-
ysis of 13C-enriched flaviolin conclusively demonstrated that
this heterologically expressed S. coelicolor type III PKS medi-
ates a U-shaped (rather than S-shaped) THN cyclization pat-
ttern (Fig. 4). This eliminates two of the theoretical routes to
rates a U-shaped (rather than S-shaped) THN cyclization pat-
ttern. The same labeling pattern was pre-
tern (Fig. 4). This eliminates two of the theoretical routes to
ates a U-shaped (rather than S-shaped) THN cyclization pat-
ttern. The same labeling pattern was pre-
tern (Fig. 4). This eliminates two of the theoretical routes to
ates a U-shaped (rather than S-shaped) THN cyclization pat-
ttern. The same labeling pattern was pre-
tern (Fig. 4). This eliminates two of the theoretical routes to
ates a U-shaped (rather than S-shaped) THN cyclization pat-
ttern. The same labeling pattern was pre-

The isosteric Cys → Ser mutations of the THNS cysteine-rich
active site indeed implicate either the Cys106 thiol proton or its
conjugate base as crucial for THN formation (Fig. 5A). These
results further suggest that reaction partitioning between the
competing physiological THN and derailed TAL pathways
occurs at a single critical branch point acting at the triketide
stage of polyketide chain extension. Recently, another type III
PKS example of enzymatic partitioning between competing
reactions at a single mechanistic branch point was discovered
to be similarly responsible for modulating the cyclization spe-
cificities of STS and CHS enzymes (23). In this previous case,
overall (product-based) kinetic comparisons of these multistep
biosynthetic reactions failed to illuminate the critical (but ap-
parently not rate-limiting) mechanistic differences between
them. This is not surprising, since the subtle inhibition or
promotion of one of two competing reaction rates can easily
modulate the ratio (partitioning) between competing multistep
pathways without affecting either pathway’s overall kcat, un-
less the affected branch point reaction also happens to be that
pathway’s rate-limiting step. With our present data, it is im-
possible to ascertain whether the specific mechanistic role of
Cys106 in facilitating THNS triketide chain extension to the
tetraketide stage occurs via promotion of this extension reac-
tion or instead via inhibition of the competing derailed reac-
tion. Since modulating either reaction’s rate constants di-
rectly impacts the partitioning ratio, while also altering
substrate availability for the competing reaction, this question
might be argued to be something of a semantic issue.

It is not known exactly when in the THN biosynthetic path-
way’s decarboxylation of the starter-derived β-keto acid or sub-
sequent enolate tautomerization of the resulting triketide car-
banion occurs (Fig. 5B). Although the presumed mechanism of
TAL formation employs decarboxylation at (or prior to) the
triketide stage (Fig. 5C), it is unclear whether the TAL path-
way branches from THN biosynthesis as the result of triketide
decarboxylation (Fig. 5D) or alternatively by failing to quench
the resulting lactonization-prone enolate tautomer (Fig. 5E).
Due to these complications, we were forced to consider several
mechanistic roles for Cys106 that seemed capable of modulating
the balance of competing THN/TAL pathway branch point
reactions.

We also considered mechanisms utilizing either the proton-
ated thiol or charged thiolate form of Cys106, since its pKa
value has not yet been experimentally determined and may
approach physiological pH due to influences in the THNS ac-
tive site cavity, such as the abundance of other cysteines,
proximity to two helical dipoles, or even the positioning of
nearby polyketide intermediates. We arrived at three compet-
ing and chemically plausible mechanistic hypotheses for the
role of Cys106 involving triketide intermediates in the THNS
reaction mechanism that are consistent with the current bio-
chemical and structural data (Fig. 6).

We initially considered a fourth possible mechanistic sce-
nario, involving Cys106 protonation of the diketide or triketide
(starter malonyl-derived) terminal carboxylate moiety. Such a
protonation event, should it occur, would indeed inhibit decar-
boxylation of the triketide intermediate (necessary for TAL for-
tmation) and thus has the potential to alter reaction parti-
tioning at the THN/TAL triketide branch point. However, be-
cause protonated carboxylates are significantly more acidic
(pKa 4–5) (46) than the typical ~8.7 pKa of a cysteine thiol, this
hypothetical proton transfer seems much less probable than
the following three alternative proposals.

Whereas TAL formation clearly requires decarboxylation at
or prior to the triketide stage, it is unclear if premature decar-
boxylation is in fact the watershed branch point reaction doom-
ing THN pathway intermediates to TAL derailment. It is pos-
ible that “early” decarboxylation sometimes (or always) accompa-
nyes THN biosynthesis but that protonation of the decarboxylated triketide’s enolate anion (the tautomeric nu-
cleophile leading to TAL lactone formation; see Fig. 5B) by the
Cys106 thiol quenches this reactive, triketide enolate anion,
thus allowing polyketide chain extension to continue past the
triketide stage (Figs. 5E and 6A).

A second partitioning scenario involves the Cys106-mediated
catalysis of intermediate polyketide cis-enoilization (Fig. 6B).
Although polyketides are biosynthetically formed (Fig. 1B) and
usually depicted as β-ketones, polyketide ketones readily un-
dergo acid- or base-catalyzed tautomerization to form anionic
and protonated enols (47). Whereas a comparison of the fundamen-
tal bond energies of keto and enol tautomers reveals the keto form to be slightly more thermodynamically stable, keto/enol equilibria
are influenced by a number of factors, including chain conjuga-
tion, pH, and polarity of solvent (47). The enol form of β-ketones
in aqueous solution is unusually favorable due to a combination
of enol π-bond conjugation and internal hydrogen bonding with
the adjacent β-keto moiety (47). Notably, it is not known what
effect the steric confines of the active site cavity has on tautomer-
ization rates and keto/enol equilibria of the various short lived
PKS linear polyketide intermediates.

When we positioned a modeled C-1-tethered carboxyl-bear-
ing triketide’s C-5 ketone near the Cys106 thiol moiety of
THNS, we noticed that the location of Cys106 at the back edge
of the active site cavity, in combination with the earlier noted
steric constrictions imposed by the bulky Tyr224 side chain of
THNS, predisposes the seven-carbon triketide to adopt a nearly
syn-periplanar conformation around the C-5 position. Further-
more, this cis-like conformation also positions both the C-5
carbonyl oxygen and adjacent C-6 methylene protons outward
and near the Cys106 thiol group. Hypothetical hydrogen bonds
between the Cys106 thiol and both the triketide’s C-5 carbonyl
oxygen and its adjacent C-6 methylene proton would facilitate
a proton shuttle as depicted in this second mechanistic pro-
posal for Cys106 (Fig. 6B). This ideally positioned thiol moiety
can now donate its proton to the C-5 oxygen while picking up the acidic C-6 methylene proton in a catalytic cycle that regenerates the thiol catalyst while accomplishing both acid/base catalytic events necessary for enolization. Since the polyketide conformation is already close to cis periplanarity, no significant barrier remains to the completion of enolization via C-5-C-6 π-bond formation. Moreover, the pKₐ of a polyketide methylene is ~9 (47), nearly identical to that of the nearby thiol, thereby supporting this hypothetical proton shuttle model. In addition to the reduction in rotational freedom associated with orbital rehybridization of the C-6 methylene position from sp² to sp³, this hypothetical C-5-C-6 cis-π-bond formation would additionally alter the bond angles involving the C-6 position from 109 to 120°. Although these subtle geometric effects may significantly impact conformational equilibria within the steric confines of the THNS active site, C-5-C-6 enolization more importantly directly impacts the reactivity of the C-5 keto group and C-6 methylene moiety positions, both of which probably play roles in TAL-forming derailment of the physiological malonyl-primed THNS reaction (Fig. 5B).

Our third mechanistic proposal for the THN/TAL partitioning role of Cys¹⁰⁶ involves the formation of a hemithioketal “protecting group” at the C-5 ketone position of the C-1-tethered triketide carboxylate intermediate (Fig. 6C). In this scenario, nucleophilic addition of the thiolate version of Cys¹⁰⁶ to the C-5 carbonyl carbon provides a stabilizing second cysteine tether to the growing intermediate, thus steering the reactive nascent chain toward an eventual aldol condensation reaction of the linear pentaketide. The C-5 hemithioketal group additionally serves to stabilize the adjacent β-keto acid and prevent spontaneous decarboxylation. Whereas we presume that Cys¹⁰⁶ is most likely protonated at physiological pH, hemithioketal formation also requires the carbonyl oxygen to pick up a proton. It is conceivable that nucleophilic attack on C-5 by Cys¹⁰⁶ could be accompanied by transfer of the thiol proton (perhaps via a water molecule) to this C-5 carbonyl oxygen, lowering a potential energetic barrier to this proposed reaction. Our modeling in the THNS active site predicts a malonate-like intramolecular interaction between the triketide’s C-7 terminal carboxylate and the resulting hemithioketal C-5 alcohol group (Fig. 6C) that is likely to stabilize both moieties during the remaining polyketide extension steps necessary for THN production.

Immobilizing the reactive starter-derived end of the triketide to Cys¹⁰⁶ at the back of the active site cavity would presumably facilitate the subsequent binding of malonyl-CoA, probably increasing the efficiency and therefore the rate of subsequent polyketide extension steps, thus allowing tetraketide formation to out-compete TAL derailment of the lactonization-prone triketide intermediate. Surprisingly, our modeling also indicates that the second of the two remaining THN pathway extensions of this hemithioketal-tethered triketide (to produce a C-1 thioester- and C-9 hemithioketal-tethered pentaketide) generates enough “slack” in the polyketide chain to allow a CHS-like C-6 → C-1 intramolecular Claisen condensation that would terminate chain extension by cleaving the C-1 thioester bond to Cys¹⁰⁶. The increased conformational freedom of the resulting phloroglucinol-like cyclization intermediate would then allow formation of the (U-shaped) THN skeleton via one of two available secondary aldol condensations (Fig. 1C). The resulting local disruption of the terminal carboxylate’s stabilization of this hypothetical hemithioketal, in combination with facile aromatization of the THN fused ring skeleton, would probably reverse the hemithioketal attachment to liberate the THN product.

Our recent structural and mechanistic elucidation of the STS “aldol switch” mechanism (23) suggests that most type III PKS enzymes, which typically catalyze the formation of single phloroglucinol- or resorcinol-like rings (11), probably modulate cyclization specificity by taking advantage of the different intrinsic reactivity of their tetraketide intermediates’ thiester-linked (C-6 → C-1 Claisen-promoting) or free carboxylate (C-2 → C-7 aldol-promoting) C-1 carbons (23). In contrast to these typical type III reactions, THNS produces a longer and more reactive polyketide intermediate, from which it produces the bicyclic THN skeleton with fidelity. Our current structural elucidation of THNS reveals a novel cavity extension that provides an explanation for the peculiar extra in vitro polyketide extension of a large aliphatic starter in THNS (35), whereas our mutagenic analysis of THNS implicates an additional active site cysteine residue at position 106 that is vital for THN biosynthesis. Aside from THNS, only Pseudomonas PhD en-
zymes conserve this additional cysteine residue (11), perhaps suggesting a similar mechanism in these related bacterial type III PKSs. Our subsequent modeling of the THNS reaction prompted three novel and plausible proposals (Fig. 6) for the mechanistic role of Cys106 in partitioning between the competing THN/TAL pathways. These mechanistic proposals might have implications for the biosynthesis of polyaromatic natural products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs. Generally, the quenching of reactive enolate anions, selective catalysis of enolate products by type I and II iterative PKSs.
Crystal Structure of a Bacterial Type III Polyketide Synthase and Enzymatic Control of Reactive Polyketide Intermediates
Michael B. Austin, Miho Izumikawa, Marianne E. Bowman, Daniel W. Udwary, Jean-Luc Ferrer, Bradley S. Moore and Joseph P. Noel

J. Biol. Chem. 2004, 279:45162-45174.
doi: 10.1074/jbc.M406567200 originally published online July 20, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406567200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 7 of which can be accessed free at http://www.jbc.org/content/279/43/45162.full.html#ref-list-1