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Structural basis of nonribosomal peptide macrocyclization in fungi

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Abstract

Nonribosomal peptide synthetases (NRPSs) in fungi biosynthesize important pharmaceutical compounds, including penicillin, cyclosporine and echinocandin. To understand the fungal strategy of forging the macrocyclic peptide linkage, we determined the crystal structures of the terminal condensation like (C_T) domain and the holo T-C_T complex of *Penicillium aethiopicum*

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Accession codes. The atomic coordinates and structural factors of C_T, C_T, C_TM, and the T-C_T complex have been deposited in the Protein Data Bank (www.rcsb.org) under accession codes 5DIJ, 5DLK, 5EGF, and 5EJD respectively.

Author contributions

J.Z., purified and crystallized protein samples and solved the X-ray structures; N.L. and R.A.C., measured the mutant activity assays in vivo and performed docking experiments; Z.G. and Z.L., prepared samples; W.Q., collected the X-ray diffraction data; J.Z., N.L., R.A.C., C.T., Y.T., and J.Z., designed the experimental approach, analyzed data and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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TqaA. The first structural depiction of the terminal module in fungal NRPS provides a molecular blueprint for generating novel macrocyclic peptide natural products.

Nonribosomal peptide synthetases (NRPSs) are multimodular enzymes that catalyze the biosynthesis of peptidyl natural products in a ribosomal-independent manner¹⁻⁷. An NRPS module minimally consists of a condensation (C) domain, an adenylation (A) domain, and a thiolation (T) domain that is posttranslationally modified with a phosphopantetheinyl (pPant) arm. Each C domain catalyzes the condensation reaction between a growing peptide that is tethered to the pPant of an upstream T domain and an aminoacyl thioester attached to the pPant of a downstream T domain.

Whereas bacterial NRPSs use the terminal thioesterase (TE) domains to perform cyclization through a nucleophilic serine, many fungal NRPSs use C-like domains (C_T) as the terminal domain to produce macrocyclic peptidyl products, including cyclosporine and echinocandin, two of the most clinically-relevant fungal NRP products⁸⁻¹¹. While the C_T domains share the conserved HXXXDXXS with canonical C domains in which the histidine serves as the catalytic base, phylogenetic analysis clearly places C_T domains in a separate clade, indicating functional divergence¹²⁻¹³. A further distinction between the bacterial TE-catalyzed and the fungal C_T-catalyzed reactions is that, while peptidyl-*S*-*N*-acetylcysteamine (SNAC) mimics can be accepted by the TE domain, C_T domains require specific protein-protein interactions with the upstream T domain for catalysis¹². A multitude of bacterial C and TE domain structures have been elucidated in recent years, but the absence of a C_T crystal structure has motivated us to explore the divergent bacterial and fungal NRPSs catalytic strategies used for the macrocyclization step^{3,14-16}.

The C_T domain from *Penicillium aethiopicum* TqaA was previously shown to catalyze the formation of a 10-membered macrocycle from the tripeptidyl thioester Ant (anthranilate)-D-Trp-L-Ala-*S*-T, followed by a spontaneous intramolecular annulation to yield the indole alkaloid fumiquinazoline F (FQF)^{12,13} (Fig. 1a, Supplementary Results, Supplementary Fig. 1a). To elucidate the molecular basis of the functional differences between canonical C and C_T domains, we obtained the crystal structure of the TqaA C_T domain. As observed in structures of bacterial C domains, each C_T chain is arranged in a pseudo-dimeric open sandwich (V shaped) structure with two chloramphenicol acyltransferase (CAT) like subdomains consisting of a total of 14 α -helices and 14 β -strands^{3,14-16} (**Fig. 1b**). A channel is formed at the interface of the two CAT subdomains and zigzags through the hinges of the V shape model from the acceptor side to the donor side, starting near the N-terminal helix α 2 and ending near the C-terminal β 13 and β 14. The three conserved residues in the active site motif HXXXDXXS are located in the channel and are in the β 7- α 7 loop (residues 3766-3773). Along this loop, the catalytic base H3766 lies at the bottom of the cavity, exposed to solvent with the imidazole side chain pointed toward the donor side (**Supplementary Fig. 2a,2b**).

To identify structural elements that may lead to the unique macrocyclization activities of the C_T domain, we compared it to three canonical C domains^{3,14,15}. Although C_T shares low protein sequence homology (less than 20% identity) with VibH (PDB ID: 1L5A), TycC-C6 (PDB ID: 2JGP), and SrfA-C (PDB ID: 2VSQ), the overall fold of C_T is very similar to

these canonical bacterial C domains. (**Supplementary Fig. 3a**). The most dramatic difference between the C_T and C domains is the replacement of the N-terminal loop in C domains by a unique α 1 helix in the C_T domain (**Supplementary Fig. 3b**). Secondary structure based comparison of the C_T domains and C domains also indicated an insertion of helix α 1 in the N-terminal loop region of the C_T domain (**Supplementary Fig. 3c**). Helix α 1 pushes the first β sheet (consisting of the β 1, β 3, and β 4 strands) and helix α 2 toward the solvent channel. Helix α 2 (9 residues) is significantly shorter than the corresponding helices in the previously determined C domains, in which the helix is 13 to 14 residues long. This truncation allows for tighter packing of helix α 2 against the CAT-like subdomains. The helix truncation is also observed in the structure of tyrocidine epimerase (PDB ID: 2XHG), where the 8 residue helix α 1 completely blocks the acceptor side of the epimerase domain¹⁷. The β 12- β 13 loop (residues 4014-4019) forms substantial contact with helix α 2; in particular, the salt bridge between E4019 of the β 12- β 13 loop and R3652 of helix α 2 forms a lid on the acceptor side of the C_T domain (**Supplementary Fig. 4**). This is in stark contrast to the known structures of canonical C domains, in which the corresponding loop does not interact with the acceptor side helix α 1, enabling the acceptor T domain to contact the helix α 2 during the peptide elongation step. These conformational and topological differences result in the compaction of the two subdomains of C_T near the acceptor side and the blocking of the solvent channel. This structural feature is therefore consistent with the catalytic role of C_T, for which no downstream acceptor substrate is required for cyclization of the donor substrate (**Supplementary Fig. 5**).

To probe the molecular basis of the specific recognition between the C_T and T domains, we purified the T-C_T fusion protein in the holo form in which pPant arm is present (holo T-C_T), and determined its crystal structure at 2.49 Å resolution using an MR method (**Fig. 2a**). The structure of the C_T domain in holo T-C_T is nearly identical to that of the standalone C_T, with an RMSD of less than 0.4 Å over 430 C α atoms, suggesting that the binding of the T domain does not cause any obvious conformational changes to C_T under crystallographic conditions. To further investigate the interaction between the C_T and T domains, the holo T-C_T complex was aligned with the C-PCP di-domains of holo AB3403 (PDB ID: 4ZXH) from the human pathogen *Acinetobacter baumannii*. Whereas the pPant-attached PCP domain in holo AB3403 interacts with the upstream C domain at its acceptor side, helix α 2 of the C_T domain clearly blocks entrance of the pPant arm into the active pocket in the C_T structure¹⁸ (**Supplementary Fig. 6**).

The pPant arm is attached to the conserved S3560 of the T domain and is inserted to the C_T domain through the donor side cavity formed by loop 2 (residue 3862-3869), loop 4 (residues 3991-4003), and the N-terminus of α 7 (**Supplementary Fig. 6,7a, 7b**). The entrance of this solvent channel has a highly cationic environment that is conducive for the binding of the pPant phosphate group (**Supplementary Fig. 7c**). The H3978T mutation, located here, caused a significant decrease in FQF production (**Supplementary Table 1 and Supplementary Fig. 7d, 8**). In addition, the pantothenic amide portion of the pPant molecule, forms an extensive hydrogen bonding network with T3870, N3890, N3899 and Y3945 of the C_T domain, giving credence to the preference of the C_T domain for the T-domain bound peptidyl substrate over the SNAC-tethered substrate. The backbone amide of

G3771 and the ϵ^2 -N atom of the catalytic residue H3766 are proposed to polarize the thioester oxygen of the peptidyl intermediate, and are 3.1 Å and 4 Å away from the sulfur atom of the pPant thiol, respectively (**Fig. 2b**). This is in agreement with the previous findings showing that a H3766A mutation resulted in failure to produce any FQF product in *S. cerevisiae* or *in vitro* using the purified enzyme¹². The F3649A mutation also failed in to produce FQF (**Supplementary Table 1**), indicating a key role of F3649 in FQF catalysis through stabilization of the cationic H3766 via π - π interactions (**Fig. 2b**). Besides H3766, F3649, and W3769, residues positioned around the cavity between the pPant arm and the donor side are mostly aromatic in character; these include Y3658, F3918, F4014, W4021, and Y4023. This hydrophobic pocket can thus accommodate the aromatic substrate for macrocyclization (**Supplementary Fig. 9a**).

The crystal structure of holo T-C_T showed that the T domain approaches the C_T domain by interacting with loop 2 (residues 3862-3869) and loop 3 (residues 3902-3907) (**Fig. 2c and Supplementary Fig. 9b**). F3554 and F3555 of the T domain have hydrophobic interactions with G3868 and I3869 from the C_T domain. Moreover, the hydroxyl group of T3870 in the C_T domain form hydrogen bonds with the phosphate oxygen in pPant, further anchors the T domain in position (**Supplementary Fig. 9b**). Mutation of T3870 nearly completely abolished FQF production (**Supplementary Table 1**). In addition, D3906 from loop 3 of the C_T domain and R3571 from the T domain forms a salt bridge (2.8 Å) and mediates anchoring the pPant-bearing helix at the donor side of the C_T, which explains the steep attenuation of FQF production in the R3571A mutant of the T domain (**Fig. 2c and Supplementary Table 1**). Complete removal of loop 3 from TqaA, while not affecting the solubility of the megasynthetase, led to loss of FQF production (**Supplementary Table 1**).

With the holo structure and well-defined positioning of the pPant arm in hand, we next investigated the potential configuration of the cyclic product in the active site using molecular docking simulations. Simulated docking results of FQF with the C_T domain suggest both that ϵ -NH₂ of K4010 forms hydrogen bonds with the Trp-carbonyl oxygen of FQF and that H3656 potentially forms T-stacking interactions with FQF (**Supplementary Fig. 10a**). Mutation studies show that these two residues have different degrees of importance in the production of FQF (**Supplementary Table 1**), with the H3656A mutation proving to be relatively much more deleterious. In the docking model of the holo T-C_T complex with FQF, the product FQF is located in the active pocket near the acceptor side of C_T and is exposed to the solvent through an exit channel, suggesting that product release occurs here (**Supplementary Fig. 10b**).

The C_T structure manifested several distinct features that enable it to effectively catalyze the cyclization of FQF. First, the C_T active site is lined with aromatic residues that can potentially stabilize FQF, as suggested by both simulated docking results and attenuation of FQF synthesis in the H3656A mutant. Second, α 2 helix forms extensive contacts with the β 12- β 13 loop, thus excluding access of water or other competing nucleophiles from entering the active site. Third, replacement of the N-terminal loop region in the C domain with the corresponding helix α 1 in the C_T domain results in the inward shift of helix α 2 toward the acceptor site, thereby preventing the T domain attached substrate loading onto the acceptor site of C_T domain for a new round of peptidyl chain elongation. Fourth, the flexible loop 4

(residues 3991–4003) acts as a lid above the active pocket. The truncation mutation of this loop results in partial loss of the FQF yield, suggesting that this loop functions as a tether for controlling the process of substrate entrance (**Supplementary Table 1**). Our structural insights explain the fungal strategy to peptide macrocyclization, and provide a molecule blueprint for engineering novel macrocyclic natural products of various sizes and structures.

ONLINE METHODS

Cloning and Mutagenesis for crystallization

The gene encoding the C_T domain (residues 3591–4074) of *P. aethiopicum* TqaA was amplified from the biosynthetic gene cluster and cloned into the expression vector pET37b (Novagen, USA)¹². The gene encoding the upstream T domain (residues 3523–3590) and the downstream C_T domain (residues 3591–4074) was cloned into pET28a (Novagen, USA) for expressing the T-C_T fusion protein. The double mutant K3622A/E3623A (C_TM) was designed by the Surface Entropy Reduction prediction (SERp) server¹⁹. Mutations were generated by PCR using the QuickChange method (Stratagene, USA) and verified by DNA sequencing. All primers used in this work were listed in **Supplementary Table 2**.

Protein Expression and Purification

Recombinant wild-type and mutant proteins were overexpressed at 16°C in *E. coli* BL21(DE3) strain with induction by 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Proteins were purified by one Ni-NTA column and one size exclusion column (Superdex 75 16/600, GE Health Care). Selenomethionine-substituted (SeMet) C_TM was expressed in *E. coli* B834 (DE3) plysS (Novagen, USA) strain at 16°C in modified M9 medium and purified similarly to the wild-type protein²⁰. The T-C_T complex protein in apo form (named T-C_T) was expressed at 16°C with induction of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) by transforming the pET28a derivative plasmid in *E. coli* BL21 (DE3). The T-C_T complex protein in holo form (named holo T-C_T) was expressed similarly but by co-transformation with pSU20-Sfp, an expression plasmid which harbors the gene encoding Sfp, the phosphopantetheinyl transferase from *Bacillus subtilis*²¹. Mass spectrum analysis showed that holo T-C_T was successfully produced by covalently attaching 4'-phosphopantetheinyl (pPant) onto T-C_T (**Supplementary Fig. 11**).

Crystallization and data collection

Diffraction-quality crystals were obtained by either using the double mutant K3622A/E3623A (C_TM) or co-crystallizing the wild-type protein with the substrate analog (*S*)-2-((*R*)-2-(2-aminobenzamido)-3-(1H-indol-3-yl) propanamido) propanoic acid (FS) (**Supplementary Fig. 1b**). Crystals of C_TM and selenomethionine (SeMet) labeled C_TM were grown at 4°C by using the sitting-drop vapor diffusion method and soaked in reservoir solution containing 10% ethylene glycol before flash frozen in liquid nitrogen. In the presence of tetraethyleneglycol monoethyl ether (C8E4), the SeMet C_TM crystals belonged to space group *C*2. Three sets of single wavelength anomalous diffraction data of the SeMet C_TM crystals were collected at BL17U1 beamline in Shanghai Synchrotron Radiation Facility (SSRF) at the wavelength of 0.9791 Å, 0.9790 Å and 0.9792 Å respectively. One data set for C_TM crystal diffracted to 2.29 Å was collected at BL5A beamline in Photon

Factory and processed with the HKL2000 package²². Crystals of C_T were grown at 4°C and soaked in reservoir solution containing 10% glycol and then flash frozen in liquid nitrogen. One data set for C_T crystal diffracted to 2.70 Å was collected at BL17U1 beamline in SSRF and processed with the HKL2000 package²². Truncation of residues 3992-4001 of C_T resulted in C_T crystal with diffraction resolution to 1.80 Å. Micro-crystals of holo T-C_T were grown at 4°C by using the sitting-drop vapor diffusion method. Crystals qualified for X-ray diffraction after micro-seeding and were soaked in reservoir solution containing 10% glycol, then flash frozen in liquid nitrogen. One data set for holo T-C_T crystal diffracted to 2.49 Å was collected at BL19U1 beamline in National Protein Science Facility Shanghai and processed with the HKL3000 package²².

Structural Determination and Refinement

Three XDS_ASCII.HKL files obtained by XDS were scaled into an XDS_ASCII file, which was further converted into an MTZ format file by XDSCONV²³. The locations of 24 selenium sites were identified in one asymmetric unit by Hybrid Substructure Search (HySS) in the PHENIX package and used for model building through Autobuild²⁴. The structure of C_TM was determined by molecular replacement using the program PHASER and the atomic coordinates of one chain with the highest completion after Autobuild as the search model²⁵. The detergent molecule C8E4 mediated the protein-protein interaction and separated the two SeMet C_TM dimers that were present in one asymmetric unit. Structures of C_T, C_T and holo T-C_T were determined by molecular replacement using PHASER and the atomic coordinates of C_TM as the search model. Although the addition of the substrate analog FS facilitated the growth of C_T crystals, affording a space group of *I*422, the electron density of FS was not observed. The model of T domain was built manually in COOT after MR according to the electron density map²⁶. Iterative cycles of model building and refinement were carried out using COOT, Refmac and PHENIX. A random selection of 5% reflections was set aside for cross-validation^{27,28}. PROCHECK and MolProbity were used to access the overall quality of the structural models^{29,30}. All statistics for data collection and structural refinement were listed in **Supplementary Table 3,4**. Structure figures were made using PyMol 1.3 (Schrödinger, LLC) and Discovery Studio Visualizer 4.0 (Accelrys Software, Inc).

There were eight chains in the asymmetric unit of T-C_T. Although NCS restraints was not applied for structure refinement, eight chains were quite similar to each other, with the root-mean-square deviation (RMSD) less than 0.25 Å. The structures of C_TM, C_T and C_T can be superimposed within a root-mean-square deviation (RMSD) of 0.49 Å, verifying that there was little conformational change in C_T resulting from surface entropy mutations or truncations of residues 3992-4001. We therefore used C_TM to represent C_T for structural analysis. In the C_T structure, the densities of the loop 3833-3853 (loop 1) between α9 and β8, and loop 3991-4003 (loop 4) between β11 and β12 at the apex of the V shape are missing. The electron density of loop 3992-4001 was improved when the substrate analog FS was used for soaking the C_T crystal, suggesting that this region might participate in substrate binding. The densities of loop 1 and loop4 were also missing from T-C_T structures.

Plasmid Construction and Protein Purification for Mutant Activity

TqaA mutants were generated using various cloning methods. The YEpLac195-TqaA was digested with *Age*I and *Swa*I to remove the sequence encoding the C_T domain. PCR amplification was used to introduce each point mutation or loop deletion for each mutant to generate two PCR products with overlapping regions with the YEpLac195-TqaA vector and the each other. The two PCR fragments and digested backbone of YEpLac195-TqaA were transformed using the EasyComp Transformation Kit (Invitrogen) into *S. cerevisiae* strain BJ5464-NpgA to allow assembly of the mutant gene in vivo. Plasmids were then extracted and checked through PCR and sequencing to confirm the correct mutation was generated. *S. cerevisiae* cultures with mutant plasmids were used to check for protein expression of the TqaA mutants.

Mutant Activity Assays in Vivo

Verified assembled plasmids were then transformed back into *S. cerevisiae* and grown for 2 days in YPD media. Seed cultures were grown to the same cell density before inoculation (checked by OD₆₀₀ values). Metabolites were extracted with equal volume ethyl acetate after 2 days of growth then dried and dissolved with methanol for LC-MS injection. LC-MS samples were run on a Shimadzu 2020 EVLC-MS using both positive and negative electrospray ionization and a C-18 reverse phase column (Phenomenex Luna, 5 μ m, 2.0 $\times$ 100 mm) and a linear gradient of 5-95% acetonitrile-H₂O in 15 min and 95% acetonitrile for 5 min at a flow rate of 0.3 mL/min. The production of FQF was checked by measuring the A₂₆₄ and comparing to purified FQF standards. Aliquots of the culture were also taken to lyse the yeast cells through mechanical disruption using 0.5 mm silica beads. The soluble fractions of the lysate were run on SDS-PAGE gels to check for relative protein production levels (Supplementary Fig. 8).

Computational Modeling

Simulated docking of the cyclization product fumiquinazoline F (FQF) to TqaA C_T domain was performed using Autodock Vina³¹. The FQF ligand, with the C α -C β bond and C β -C γ bond of the tryptophan moiety allowed to rotate, was docked to the TqaA C_T structure in a grid box with a size of 20 Å on each dimension centered on the catalytic histidine. Nine different possible binding modes were obtained from the docking simulation with a maximum energy difference of 0.3 kcal/mol and maximum upper bound RMSD difference of 7.24 Å. The binding modes that have the anthranilic acid oriented towards the interior of the cavity were chosen for further analysis to determine residues that may play a role in binding and catalysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure. 1. The overall structure of the C_T domain and comparison to canonical C domains
(a) Schematic depiction of fumiquinazoline (FQF) biosynthesis catalyzed by the T₃-C_T complex. The T-C_T di-domains located in the third NRPS module are responsible for the termination of the biosynthesis step and the cyclization of FQF respectively. **(b)** The overall structure of C_T is shown as a cartoon, and the secondary structures are numbered sequentially. The α helices are colored in red; β sheets are cyan; loops are green. Missing loops are shown as black dashed lines. The α helix 2 of C_T and the conserved histidine are colored in magenta.

Figure. 2. The crystal structure of the T-C_T complex in the holo form

The pPant molecule and the conserved histidine are drawn as magenta sticks. **(a)** The crystal structure of the T-C_T complex shows the pPant-binding environment in the activity site. The T domain is colored in yellow and the C_T domain is colored in orange. The loop 2 (loop 3862-3869) and loop 3 (loop 3902-3907) of the C_T domain involved in interacting with T domain are highlighted as red circles. **(b)** The interaction between pPant and residues in the active pocket. The e²-N atom of the catalytic residue H3766 is 4 Å away from the sulfur atom of the pPant thiol. **(c)** R3571 from the T domain and D3906 from loop 3 of the C_T domain form a salt bridge.