The Non-Heme Monooxygenase ThoJ Catalyzes Thioholgamide β -Hydroxylation

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Supporting Information Placeholder

ABSTRACT: Thioviridamide-like compounds, including thioholgamides, are ribosomally synthesized and post-translationally modified peptide natural products with potent anti-cancer cell activity and an unprecedented structure. Very little is known about their biosynthesis and we were intrigued by the β -hydroxy-N1, N3-dimethylhistidinium moiety found in these compounds. Here we report the construction of a heterologous host capable of producing thioholgamide with a 15-fold increased yield compared to the wild-type strain. A knock-out of *thoJ*, encoding a predicted non-heme monooxygenase shows that ThoJ is essential for Thioholgamide β -hydroxylation. The crystal structure of ThoJ exhibits a typical mono-/dioxygenase fold with conserved key active-site residues. Yet ThoJ possesses a very large substrate binding pocket that appears suitable to receive a cyclic thioholgamide intermediate for hydroxylation. The improved production of the heterologous host will enable the dissection of the individual biosynthetic steps involved in biosynthesis of this exciting RiPP family.

INTRODUCTION

Ribosomally-synthesized and post-translationally modified peptides (RiPPs) have emerged as a structurally and functionally diverse group of natural products.¹ RiPPs share a common biosynthetic strategy that is initiated by the ribosomal synthesis of a bipartite precursor peptide. It generally contains a C-terminal core region, which receives posttranslational modification(s) (PTMs) by one or more tailoring enzyme(s), as well as an N-terminal leader peptide, which directs some of these modifications. The leader is usually removed during the maturation process to deliver the final natural product.¹⁻³ Thioviridamide-like compounds represent a subgroup of RiPPs and were first isolated from *Streptomyces olivoviridis*.⁴ They display potent cytotoxic activity against a range of human cancer cells lines.⁵⁻¹⁰ Due to their promising bioactivity and unprecedented core structure, thioviridamide-like compounds are a focus of biotechnological and pharmaceutical research. Genome mining efforts have resulted in the identification of a number of thioviridamide-like compounds, including thioholgamides.^{6, 8-11}

Thioholgamides were isolated from the culture supernatant of *Streptomyces malaysiense* MUSC 136 57 and their biosynthetic gene cluster (Figure 1a) is similar to that of thioviridamides (Supplementary Figure 1).⁶ Thioholgamides contain extensive PTMs including four thioamides, a β -hydroxy-N1, N3-dimethylhistidinium (hdmHis), and a C-terminal *S*-(2-aminovinyl)-cysteine (AviCys) motif that forms part of the macrocycle (Figure 1b). Despite the identification and confirmation of numerous thioviridamide-like biosynthetic gene clusters (BGCs) by heterologous expression, little is known about the individual steps involved in biosynthesis.

Recently, the first biochemical and structural characterization of an enzyme from the thioviridamide biosynthetic pathway was reported: TvaF was shown to be responsible for the decarboxylation of the terminal cysteine en route to AviCys formation (Figure 1b).^{12, 13} We were intrigued by the novelty of the hdmHis moiety and set out to investigate its biosynthesis. Based on sequence homology, the two proteins involved in its biosynthesis were predicted to be

ThoG (putative methyltransferase) and ThoJ (putative monooxygenase) (Figure 1c).

Figure 1. a) Organization of the thioholgamide biosynthetic gene cluster in *Streptomyces malaysiense* MUSC 136 57. Intergenic regions not drawn to scale. **b)** The thioholgamide precursor peptide undergoes several PTMs, the order of which is still unknown. Flavin-dependent decarboxylase from the thioviridamide pathway has been shown to perform the oxidative decarboxylation of the C-terminal cysteine *in vitro* in the presence of flavin mononucleotide (FMN). The PTMs present in thioholgamides (A and B) are highlighted. **c)** HdmHis formation in thioviridamide-like compounds is thought to involve di-methylation and hydroxylation.

RESULTS AND DISCUSSION

To determine the function of ThoJ we planned gene inactivation experiments. Due to the low production yield of thioholgamide in the native producer strain we decided to heterologously express the thioholgamide BGC in a suitable host strain. For this, the cluster was cloned from the genome of *S. malaysiense* MUSC 136 57 by transformation associated recombination (TAR) in *Saccharomyces cerevisiae* BY4742. The vector pTho with the intact thioholgamide cluster was then heterologously expressed in the *S. lividans* delYA8 host strain.¹⁴ The successful expression of the cluster in *S. lividans*:pTho led to the production of fully modified thioholgamide A (Figure 2 and Supplementary Figure 2) with a molecular ion $[M]^+$ of m/z 1305.49524, consistent with the molecular formula $C_{56}H_{85}N_{14}O_{10}S_6^+$ (calculated exact mass: 1305.48974; error 4.2 ppm) and the same retention time as thioholgamide A isolated from the native producer. Furthermore, the production level for thioholgamide A was improved 15-fold, from 2 mg L⁻¹ in the native producer to 30 mg L⁻¹ (Supplementary Figure 3). To explore the enzymatic function of ThoJ, an in-frame deletion of the *thoJ* monooxygenase gene was performed on the pTho vector. The

resulting vector pTho-delThoJ was introduced into the *S. lividans* host strain. The new strain *S. lividans*:pTho-delThoJ produced a thioholgamide derivative (**2**; Supplementary Figures 2 and S4) with a molecular ion $[M]^+$ of m/z 1289.49729 Da, indicating the loss of oxygen from **1** (molecular formula: $C_{56}H_{85}N_{14}O_9S_6^+$, exact mass: 1289.49482; error 2.0 ppm). Liquid chromatography tandem mass spectrometry (LC-MS²) places the loss of oxygen in the macrocyclic ring, which implicates loss of the hydroxyl at hdmHis (Supplementary Figure 4). These results indicate that the putative monooxygenase ThoJ is responsible for the β -hydroxylation of the His-derived moiety in thioholgamide A biosynthesis.

Figure 2. LC-ESI-MS analysis of **1** and **2** purified from the extracts of the *S. lividans* host expressing pTho (top) and delThoJ (bottom). Total ion chromatograms as well as the mass spectrum (small window) are shown. MS² characterization can be found in Supplementary Figures S2 and S4.

To confirm the function of ThoJ *in vitro*, we planned to incubate either the core peptide (ThoA^{core}) or the precursor peptide ThoA with ThoJ. The start codon of ThoA is not defined yet and a closer look at the sequence allowed us to identify multiple putative in-frame start codons upstream of the core peptide. To avoid missing a crucial recognition element for ThoJ we recombinantly expressed and purified the longest conserved precursor peptide sequence (ThoA, Supplementary Figure 6). Incubation of neither substrate with ThoJ in the presence of iron (II), α -ketoglutarate (α -KG) and ascorbic acid led to observable hydroxylation (Supplementary Figures 5 and 6). In the absence of an *in vitro* route to thioholgamides many pathway intermediates that could be ThoJ substrates are inaccessible. However, the purified product of our heterologous host featuring the *thoJ* deletion, **2**, was available in sufficient quantities. Upon overnight incubation of **2** with ThoJ we again failed to see any hydroxylated species (Supplementary Figure 7). These data suggest that histidine/N1, N3-dimethylhistidinium hydroxylation is likely neither the first nor the last PTM installed during thioholgamide biosynthesis.

We wanted to better understand ThoJ substrate specificity and thus crystallized the protein as described in the materials and methods section. The ThoJ apo structure was determined to 2.09 Å resolution (Figure 3a and Supplementary Figure S8) and the data collection and refinement statistics can be found in Table S1. The overall structure of ThoJ is representative of other Fe²⁺ / α -KG-dependent mono- and dioxygenases (α -KGDs) and consists of a double-stranded β -helix core, which is surrounded and stabilized by a number of α -helices (Figure 3a and Supplementary Figure S9).¹⁵ This jelly roll fold is usually formed by four-stranded anti-parallel β -sheets forming a distorted barrel-like structure open at one end. In ThoJ only seven out of the eight β -strands of the jelly roll are ob-

served (Supplementary Figure S8). Instead of an N-terminal loop, ThoJ has an extended α -helix (Supplementary Figures 8 and 9). Additional secondary structure elements at the N or C-terminus of the jelly roll fold are not uncommon in α -KGDs and have been reported to play a role in substrate binding.¹⁵ Fortuitously, the tartrate ions present in the ThoJ crystallization condition shed light on the residues important for co-factor binding because it is structurally similar to succinate - the product of the α -KGD monooxygenase reaction. The carboxylic ends of tartrate form hydrogen bond interactions with His108, Trp127, Ser200 and a salt-bridge with Arg209 (Figures 3c, 3d and Supplementary Figure 10B). While the canonical α -KGD metal-binding motif (HxD/E...H) is present in ThoJ (His108, Asp110 and His198), attempts to obtain a ThoJ-Fe²⁺ crystal structure were unsuccessful. When using a selection of different divalent metal ions, we obtained a complex structure with Ni²⁺ by soaking the apo crystals with excess NiCl₂.¹⁶⁻¹⁸ Ni²⁺ forms a distorted octahedral coordination geometry in which it is coordinated by the metal-binding motif, water and by tartrate as a bi-dentate ligand through carboxyl groups (Figures 3b and 3d).

A DALI¹⁹ search against the Protein Data Bank²⁰ to identify structural homologs of ThoJ showed that the enzyme shares structural similarities with a number of α -KGDs, including the recently characterized leucine hydroxylase, GriE (PDB ID: 5NCH; Supplementary Figure 10A, C α RMSD of 1.47 Å).²¹ Superposition of the ThoJ and GriE structures in the presence and absence of cofactors and substrate reveal key structural differences, which may be important for catalysis and substrate recognition. While ThoJ residues 157-165 were disordered, the corresponding region is well defined in the GriE/cofactor/substrate or product complexes (Supplementary Figure 9). This region is involved in GriE-substrate binding, and has also been implicated in closing the active site to prevent the release of harmful reactive oxygen species generated and/or shield the active site from solvent during hydroxylation.²¹⁻²³ Therefore, it is likely that substrate binding will stabilize this loop in ThoJ to perform a similar function.

To analyze the relationship of ThoJ to related α -KGDs, we generated a sequence similarity network (SSN) for its closest 1000 homologs (Supplementary Figure 11A).²⁴ Apart from ThoJ none of the proteins in the network have been functionally characterized. The node containing ThoJ contains its homologs from other thioviridamide-like BGCs except the monooxygenase from the thioviridamide BGC, TvaJ (Supplementary Figure 11A). It is a somewhat surprising feature of the family of thioviridamide-like BGCs that the overall sequence conservation at the amino acid level is quite low (Only 35% between ThoJ and TvaJ with 91% coverage). Members of the ThoJ node were individually analyzed and only the sequences which were found to be a part of thioviridamide-like BGCs were selected. These sequences were then mapped onto the structure of ThoJ with Consurf.²⁵⁻²⁸ This analysis revealed the residues in the ThoJ binding site to be highly conserved (Supplementary Figure 11B), compared to the rest of the protein. In contrast, the closest structural homologues identified by a DALI¹⁹ search show very low sequence conservation (29% or less) (Supplementary Figure 11C). This is not necessarily surprising because the structural homologs identified through DALI have much smaller substrates and correspondingly have smaller substrate binding pockets (Figures 3d and Supplementary Figure 12). Based on these

data it is likely that ThoJ has evolved to accept a cyclic thioholgamide intermediate as a substrate for hydroxylation.

Figure 3. Comparison of ThoJ and GriE crystal structures. **a)** ThoJ crystal structure is shown in cartoon representation. **b)** The electron density map (Fo – Fc contoured to 3 σ with phases calculated from a model that was refined without tartrate) for tartrate is shown as grey isomesh. Only residues that form hydrogen bonds and the salt bridge with tartrate are shown as sticks. **c)** Residues important for cofactor binding in ThoJ and GriE. Only residues that are conserved in both protein structures are depicted as sticks (Colour scheme: ThoJ/tartrate green, GriE/succinate cyan, and GriE/ α -KG magenta). The residues are labelled; GriE residue numbers are mentioned in parentheses. **d)** Active site metal coordination of ThoJ (left) and GriE complex (right; PDB ID: 5NCH). Water molecules and metal ions are shown as spheres (Color scheme: water red, Ni²⁺ magenta and Mn²⁺ yellow).

CONCLUSION AND PERSPECTIVES

We have demonstrated *in vivo* that the hydroxylation of hdmHis in the thioholgamide pathway is performed by ThoJ. Many α -KGDs are known to undergo self-inactivation, e.g. via auto-oxidation, in the absence of their primary substrate – however, self-inactivation is partially reversible in most cases.²⁹⁻³³ Keeping this in mind we cannot rule out the lack of observed *in vitro* activity of ThoJ in our hands due to this phenomenon. While we do not know the native substrate of ThoJ, we may speculate that dioxygen could bind to the Fe²⁺ bound at the active site and perform a nucleophilic attack on the carbonyl group of the co-factor, α -KG, resulting in the formation of a Fe(IV)-peroxy-hemiketal intermediate. Elimination of CO₂ from α -KG would result in the formation of an oxo-ferryl intermediate that could abstract a proton from the C β methylene group of either His or N1, N3-dimethylhistidinium to yield a radical and a Fe(III)-hydroxo species. The hydroxyl group could then react with the radical to result in hydroxylation and thus product formation. Determining whether the positive charge of the N1, N3-dimethylhistidinium could stabilize the radical intermediate will require further study. The crystal structure of ThoJ confirms that it belongs to the family of α -KGD enzymes known to carry out hydroxylations of varied substrates. β -hydroxylation is rare and to the best of our knowledge has only been reported in two other RiPP classes: thiopeptides and lantipeptides.³⁴⁻⁴¹ In most cases it is performed by a cytochrome P450 tailoring enzyme encoded in the BCG, with the exception of the two closely related lantipeptides duramycin and cinnamycin, which harbor an α -KGD tailoring enzyme (DurX and CinX: 89% identity with 97% coverage).^{35, 36, 42}

The β -hydroxylation of Asp15 in cinnamycin is performed by CinX (CinX and ThoJ are not homologous: 44% sequence identity with 7% sequence coverage).³⁵ This enzyme hydroxylates both the linear and cyclized precursor peptide in a leader-independent fashion.³⁵ In the absence of a CinX structure it is difficult to rationalize the differences in substrate preference between CinX and ThoJ. *In vivo* derivatization of prethioviridamide and neothioviridamide (structurally identical to thioholgamide) hints that ThoJ and its homologs may be specific for His, but that the residues surrounding this residues can be varied (Supplementary Figure 13).⁴³ The large putative substrate binding pocket of ThoJ appears suitable to accept His-AviCys-containing cyclic peptides as the potential substrate of ThoJ. Further studies are currently underway to elucidate the exact point at which ThoJ acts in thioholgamide biosynthesis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures and additional figures and tables are provided as a PDF file.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

No competing financial interests have been declared.

The structures of apo-ThoJ and Ni²⁺-ThoJ have been deposited in the protein databank (PDB IDs 6zyl and 6zyk, respectively).

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