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CONCISE ARTICLE

Importance of the MbtH-like protein TioT for production and activation of the thiocoraline adenylation domain of TioK†‡

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The 3-hydroxyquinaldic acid (3HQA) chromophores of thiocoraline are essential for the biological DNA bisintercalating function of this antitumor agent. The 3HQA units are also proposed to play a critical role in the resistance mechanism of the thiocoraline-producing organism against this natural product. Because of their important functions, there is a great interest in understanding the 3HQA formation from L-Trp. The first proposed committed steps during 3HQA biosynthesis consist of conversion of L-Trp into L-Trp-AMP by the adenylation domain of TioK followed by installation of the activated amino acid onto the thiolation domain of this didomain enzyme. However, testing this series of events has been hindered by the inability to heterologously express soluble TioK. Here, we demonstrated that the MbtH-like protein TioT is required for production and activation of TioK. With soluble functional TioK in hand, we established the amino acid substrate profile and kinetically characterized this enzyme. By site-directed mutagenesis of TioT, we also investigated the significance of three Pro residues that are universally conserved in MbtH-like proteins.

Introduction

Many biologically active natural products of nonribosomal peptide (NRP) origin have been at the forefront of the drug discovery process. These secondary metabolites are biosynthesized on nonribosomal peptides synthetase (NRPS) assembly lines, which comprise multiple modules that each contain three core domains: a condensation (C), an adenylation (A), and a thiolation (T) domain. The A domains are responsible for dictating the identity of the amino acid or amino acid-like building blocks to be activated for incorporation into the growing peptides. Earlier work demonstrated that in addition to the NRPS machinery, *mbtH*-like genes were essential for the production of secondary metabolites such as coelichelin[1] and clorobiocin.[2] In 2010, two groundbreaking biochemical studies performed independently by the Thomas[3] and Walsh[4] groups confirmed that MbtH-like proteins are indeed integral components of NRPSs that play an important role in stimulating the adenylating activity of A domains. Last year, the role of MbtH-like proteins in the adenylation of L-Tyr during the early steps of the biosynthesis of the aminocoumarin antibiotics novobiocin, clorobiocin, and simocyclinone D8, as well as of the glycopeptide antibiotic vancomycin was demonstrated (Fig. 1A).[5] These previous studies also demonstrated that MbtH-like proteins could not only help the expression of one specific A domain, but also that of several A domains. The thiocoraline biosynthetic gene cluster also contains a putative MbtH-like protein, TioT, which could potentially play a role in activation of L-Trp by the adenylating portion of the A–T didomain TioK as the first step in the formation of 3-hydroxyquinaldic acid (3HQA) chromophores en route towards thiocoraline production (Fig. 1B).

Thiocoraline is a thiodepsipeptide antitumor agent that was originally isolated from two marine actinomycetes *Micromonospora* sp. ML1 and *Micromonospora* sp. ACM2-092,[6,7] and more recently discovered in a marine *Verrucosispora* sp. isolated from the sponge *Chondrilla caribensis f. caribensis*.[8] The architecture of this nonribosomal bisintercalator natural product consists of two identical peptidic macrocycles decorated with planar 3HQA intercalating units.[9] The 3HQA moieties have been proposed to play a critical role in binding of thiocoraline (i) to duplex DNA for biological activity, and (ii) to TioX as a sequestration mechanism for protection of the producing organisms against thiocoraline itself.[10] Because of the key functions of the 3HQA chromophores, we are interested in deciphering their biosynthetic logic of assembly. In the thiocoraline gene cluster, there are nine enzymes (TioK, T, I, Q, F, L or M, G,

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‡ Electronic Supplementary Information (ESI) available: Plot showing the conversion of apo- to [5-^{3}H]L-Trp-*S*-TioK as well as Michaelis–Menten plots for the kinetic parameters for the TioK-catalyzed adenylation of L-Trp and L-Phe when using TioK that is co-expressed and purified with TioT-wt and TioT mutants. See DOI: 10.1039/c2md20131c

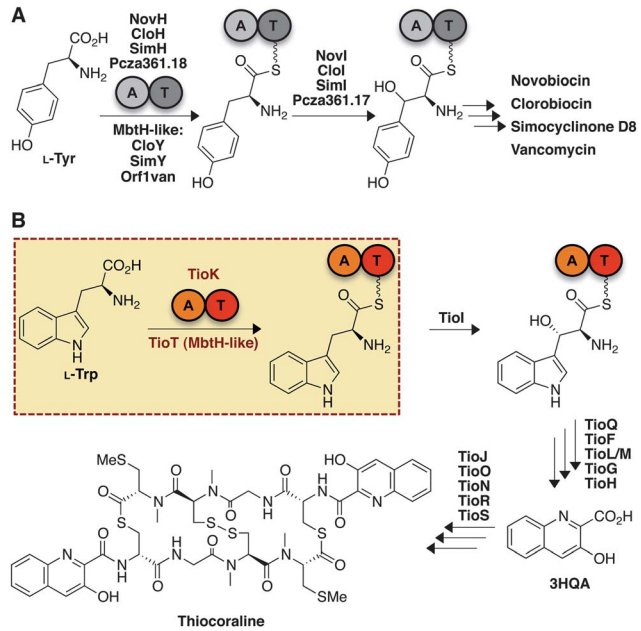

Fig. 1 Representation of the proposed role of (A) the A–T didomain/MbtH-like protein pairs involved in aminocoumarin (novobiocin, clorobiocin, and simocyclinone D8) and vancomycin antibiotic biosynthesis, and (B) the TioK (A–T didomain)/TioT (MbtH-like protein) pair of enzymes in the formation of 3HQA during thiocoraline biosynthesis.

and H) that are proposed to be involved in converting L-Trp into 3HQA (Fig. 1B). We previously demonstrated that TioF is a Trp 2,3-dioxygenase that displays the broad substrate promiscuity required for the conversion of β-hydroxy-L-Trp into β-hydroxy-*N*-formylkynurenine.[11] More recently, by phenotype of *tioQ* and *tioK* gene deletion as well as by biochemical characterization, we confirmed the requirement of the type II thioesterase TioQ and of the A–T didomain TioK in 3HQA formation.[12] However, during these studies we had access only to the T domain of TioK as the A–T didomain expressed in *E. coli* was either obtained in its insoluble form or not expressed at all.

Here, we report further efforts towards understanding the first steps during 3HQA biosynthesis. We show the importance of the MbtH-like protein TioT in the production of the full-length protein TioK in soluble form and in the activation of its A domain. We establish the substrate profile and determine the kinetic parameters of TioK. By site-directed mutagenesis of TioT, we also explore the importance of three universally conserved L-Pro residues in MbtH-like proteins.

Results and discussion

Heterologous co-expression and purification of the A–T didomain TioK with the MbtH-like protein TioT

To evaluate the proposed role of the discrete TioK A–T didomain in 3HQA formation, we set to heterologously overproduce and purify the 64 kDa enzyme from *E. coli* cells. Unfortunately, despite our efforts at varying tags (His, GST, MOCR), tag locations (N-terminal *versus* C-terminal), growth temperature and time, and IPTG-induction conditions, the full-length TioK alone was either not expressed in *E. coli* or expressed only in the insoluble form. Previous work on the vicibactin[13] and glidobactin[14] biosynthetic pathways demonstrated that it was optimal to co-express A domain-containing modules with MbtH homologues found in the corresponding NRPS gene clusters. Additional recent investigations of the founding member of this family of small (~8 kDa) proteins, the *Mycobacterium tuberculosis* MbtH protein, confirmed the ability of MbtH to enhance the solubility of A domains of a variety of modules of the mycobactin biosynthetic NRPS machinery.[15] Inspired by these studies, we successfully co-expressed in tandem the TioK A–T didomain and the MbtH-like protein TioT, and purified them in soluble form by Ni^{2+}-affinity chromatography (Fig. S1‡).

Activity and substrate specificity of TioK

TioK is predicted to behave as a free-standing A–T didomain with L-Trp specificity. The A domain of TioK is proposed to be responsible for the ATP-dependent activation of L-Trp into L-Trp-AMP prior to transfer of the activated amino acyl moiety onto the T domain of the enzyme (Fig. 1B). The A domains are known to generally display strong specificity towards a single amino acid and sometimes to accept, to a lesser extent, close analogues of their preferred substrate. In order to probe the activity of the A domain of TioK and to establish the substrate specificity profile of the enzyme, we performed an assay monitoring the amino acid-dependent exchange of radioactivity from [^{32}P]PP_i into ATP (Fig. 2). By doing so, we validated that L-Trp is the substrate of choice of TioK. Even though thiocoraline analogues containing L-Phe in place of L-Trp have never been isolated, we also identified L-Phe as a good *in vitro* substrate of TioK. All other amino acids tested were found to be poor substrates of the enzyme.

To achieve the initial *in vitro* reconstitution of the activity of the T domain of TioK, we isolated the enzyme in its apo form. We initially confirmed the activity of TioK by monitoring the conversion of the enzyme from its apo to its [^{3}H]acetylated form by the trichloroacetic acid (TCA) precipitation assay. To confirm the loading of L-Trp onto TioK, we first completely converted the apo T domain of the enzyme into its holo form by using CoA and the phosphopantetheinyltransferase Sfp, and then monitored the attachment of [5-^{3}H]L-Trp onto the holo T domain of TioK by using the TCA assay after adenylation of the amino acid by the A domain (Fig. S2‡). No holo T domain TioK was present in the

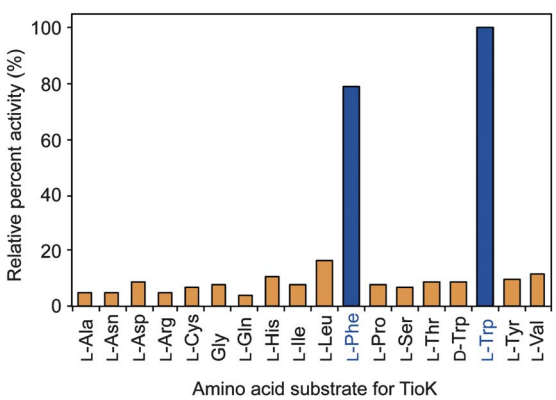

Fig. 2 The relative substrate specificity determined by ATP-[^{32}P]PP_i exchange assay catalysed by TioK co-expressed with TioT-wt. The data correspond to 2 h end-point assays.

apo enzyme purified as confirmed by the absence of loading of [5-^{3}H]L-Trp when no Sfp was added to the above reaction.

MbtH-like proteins

MbtH-like proteins are a family of small (~8 kDa) proteins that are present in a large number of gene clusters responsible for the production of NRPs.[16] The reported signature sequence for functional MbtH-like proteins is NXEXQXSXW**P**-X5-**P**XGW-X13-L-X7-WTDXR**P** (Fig. 3A, yellow circles).[16] It was originally hypothesized that the hydrophobic face of MbtH homologues containing strictly conserved Trp residues (underlined in the above signature sequence) could play an important role in moderating protein–protein interactions.[17] By generating single (W22A) and double (W22A/W32A) PacJ mutants (Fig. 3A, orange circles), Walsh and co-workers confirmed the importance of these Trp residues in the activity of the PacL partner protein.[4] In addition to these two Trp residues, three universally conserved Pro residues (in bold in the above signature sequence, Fig. 3A and B, highlighted in turquoise), which are often found to play a critical structural role, are present in the signature sequence of functional MbtH-like proteins. Based on homology modelling, in TioT these Pro residues are found at the end of a β-sheet (P27), in a loop (P33), and at the beginning of an α-helix (P61) (Fig. 3B). To probe the effect of these three Pro residues on the solubility and activity of the A domain of TioK, we constructed single TioT mutants P27A, P33A, and P61A, as well as a double mutant TioT-P27A/P33A (Fig. 3A, turquoise circles). We co-expressed and purified TioK with the TioT mutants exactly as we did with TioT-wt and in every case, obtained the TioK protein in the soluble active form in yields varying between 5 and 8 mg per liter of culture.

Kinetic characterization of TioK in the presence of wild-type TioT or its mutants

We next determined the kinetic parameters of TioK co-expressed with the TioT-wt and the TioT mutants for L-Trp and L-Phe substrates, by holding the concentration of the catalyst TioK constant at 2.5 μM and by varying the concentration of the amino acids. In all cases, for both substrates the data exhibited classical hyperbolic Michaelis–Menten saturation behaviour (Fig. S3‡ and Table 1). As expected, for TioK co-expressed and purified with TioT-wt the catalytic efficiency of adenylation of L-Trp (49 ± 8 mM^{-1} min^{-1}) was higher than that of L-Phe (4.4 ± 0.5 mM^{-1} min^{-1}). In general, we observed that mutation of TioT at positions P33 and P61 did not have any effect on the catalytic efficiency of adenylation of both amino acids. Interestingly, the catalytic efficiency of activation of L-Trp (14 ± 2 mM^{-1} min^{-1}) was reduced by ~3.5-fold when TioK was co-expressed with the TioT-P27A mutant. However, no difference in catalytic efficiency of activation of L-Phe (4.0 ± 1.1 mM^{-1} min^{-1}) was

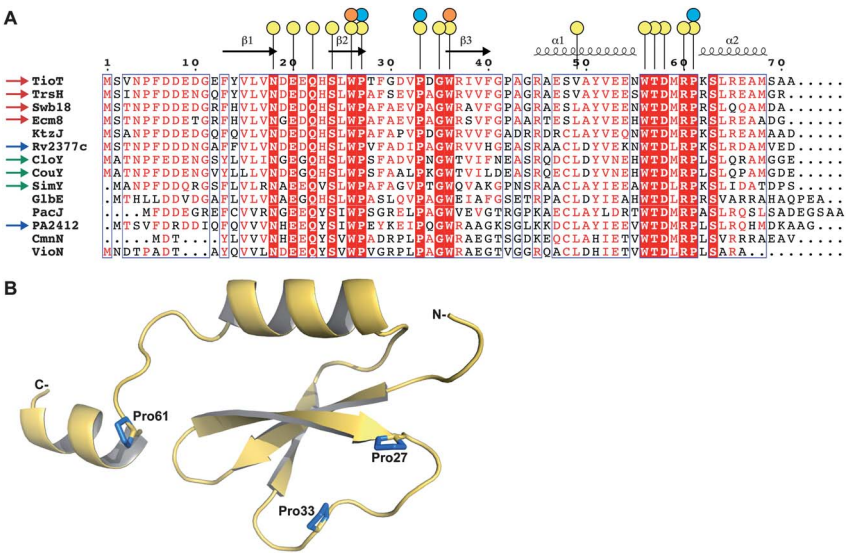

Fig. 3 (A) Multiple sequence alignment of some MbtH-like proteins for which biological, biochemical, or structural studies have been performed. Residues in solid red boxes are fully conserved whereas the residues in the red font are highly conserved. The yellow circles indicate the signature sequence previously reported for predicted functional MbtH-like proteins.[16] The turquoise circles indicate the residues mutated in this study. The orange circles indicate Trp residues proposed to moderate protein–protein interactions that were mutated in another study.[4] The red arrows indicate direct homolgues of TioT predicted to play a role in L-Trp adenylation prior to β-hydroxylation. The green arrows indicate homologues of TioT showed to play a role in L-Tyr adenylation prior to hydroxylation during aminocoumarin biosyntheses. The blue arrows indicate MbtH-like proteins for which the structures have been determined. The proteins are from the following bacterial species and have the following accession numbers: TioT (CAJ34376; *Micromonospora* sp. ML1), TrsH (BAH04160; *Streptomyces triostinicus*), Swb18 (BAI63290; *Streptomyces* sp. SNA15896), Ecm8 (BAE98157; *Streptomyces lasaliensis*), KtzJ (ABV56590; *Kutzneria* sp. 744), Rv2377c (NP_216893; *Mycobacterium tuberculosis* H37Rv),[18] CloY (AAN65223; *Streptomyces roseochromogenes* subsp. *oscitans*),[2,5] CouY (AAG29779; *Streptomyces rishiriensis*), SimY (AAG34186; *Streptomyces antibioticus*),[5] GlbE (CAL80823; [*Polyangium*] *brachysporum*),[14] PacJ (ADN26246; *Streptomyces coeruleorubidus*),[4] PA2412 (NP_251102; *Pseudomonas aeruginosa* PA01),[17] CmnN (ABR67757; *Saccharothrix mutabilis* subsp. *capreolus*),[3] and VioN (AAP92504; *Streptomyces vinaceus*).[3] (B) The conserved Pro residues (as turquoise sticks) mutated in this study in a TioT homology model (see panel (A) for proposed β-sheets and α-helices boundaries). The model was built by homology modelling based on the structure of the *P. aeruginosa* PA2412 (PDB:2PST) in SwissModel.

Table 1 Kinetic parameters for the A domain of TioK in the presence of TioT-wt or TioT-mutants with L-Trp and L-Phe

Substrate	Protein pair	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
L-Trp	TioK/TioT-wt	0.095 ± 0.016	4.66 ± 0.13	49 ± 8
	TioK/TioT-P27A	0.150 ± 0.018	2.13 ± 0.05	14 ± 2
	TioK/TioT-P33A	0.050 ± 0.012	2.37 ± 0.09	48 ± 12
	TioK/TioT-P27A/P33A	0.090 ± 0.026	3.86 ± 0.18	43 ± 13
	TioK/TioT-P61A	0.067 ± 0.009	4.41 ± 0.09	66 ± 9
L-Phe	TioK/TioT-wt	0.883 ± 0.087	3.90 ± 0.11	4.4 ± 0.5
	TioK/TioT-P27A	0.394 ± 0.113	1.58 ± 0.11	4.0 ± 1.1
	TioK/TioT-P33A	0.568 ± 0.050	2.99 ± 0.07	5.3 ± 0.5
	TioK/TioT-P27A/P33A	0.663 ± 0.054	2.53 ± 0.06	3.8 ± 0.3
	TioK/TioT-P61A	0.694 ± 0.071	2.27 ± 0.07	3.3 ± 0.4

observed for TioK grown with this mutant. As we did not observe any significant effect on TioK production or activity by mutating the three conserved Pro residues of TioT, we concluded that the exact identity of these residues is not essential for TioT structure or TioT–TioK interactions.

Conclusions

In summary, we have presented evidence consistent with the importance of MbtH homologues in expression of soluble A–T didomain enzymes during NRP biosynthesis. We confirmed that TioT is required to express soluble and active TioK, consistent with the proposed role of TioK in for the first committed steps during 3HQA biosynthesis. Interestingly, the three strictly conserved Pro residues in the signature sequence of functional MbtH-like proteins do not appear to be absolutely required for the structure or interaction of these proteins with their A–T didomain partners. Nevertheless, a role of these residues in the context of a larger NRPS assembly line cannot be ruled out. In conjunction with our previous reports on TioF and TioQ, this study provides essential tools for the completion of the *in vitro* reconstitution of 3HQA biosynthesis.

Materials and methods

Bacterial strains, plasmids, and materials

Chemically competent *E. coli* TOP10 cells were purchased from Invitrogen (Carlsbad, CA). The BL21(DE3)*ybdZ*:*aac(3)IV* bacterial strain used for over-co-expression of TioT and TioK was generously provided by Prof. Michael G. Thomas (University of Wisconsin-Madison, WI).[3] Restriction endonucleases, T4 DNA ligase, and Phusion DNA polymerase were purchased from New England BioLabs (NEB; Ipswich, MA). DNA primers for PCR were bought from Integrated DNA Technologies (IDT; Coralville, IA). DNA sequencing was performed at the University of Michigan DNA Sequencing Core. All chemicals and buffer components were purchased from Sigma-Aldrich (Milwaukee, WI) and used without any further purification. [^{32}P] PP_i as well as [^{3}H]acetyl-CoA and [5-^{3}H]L-Trp were purchased from Perkin Elmer (Waltham, MA) and American Radiolabled Chemicals (St. Louis, MO), respectively.

Preparation of TioK/TioT-wt and TioK/TioT mutants over-co-expression constructs

The primers used for the amplification of the *tioK*, *tioT*, and *tioT* mutant genes are listed in Table 2. PCRs were carried out using Phusion DNA polymerase as described by NEB. Using *Micromonospora* sp. ML1 genomic DNA,[11] wild-type (wt) TioK and TioT were constructed in the pET28a and pACYCDuet-1 vectors (Novagen), respectively. TioT mutants were constructed using the splicing by overlap extension (SOE) method.[19] In the first round of PCR, the sequence downstream and upstream of the mutation(s) were separately amplified using the *Micromonospora* sp. ML1 genomic DNA as a template in conjunction with the 5′ primer for the *mutant* with the 3′ primer for *tioT-wt* and the 5′ primer for

Table 2 Primers used for the PCR amplification of the *tioK* and *tioT* genes[a,b] for various constructs used in this study[c]

Gene	5′ Primer[d]	3′ Primer
tioK-wt	ATGGAGCATATGCCCACCGTTGCGGAAT TGTTTGAGTCCG	TCCTCGCTCGAGTCAGGGGCGCCC GTTGGGGATGGATG
tioT-wt	TACGGAGAATTCATGAGCGTCAACCCG	AGCCCAAAGCTTTCATGCTGCCGACATC
tioT-P27A	GCACTCGCTGTGGgcgACCTTCGGTGACG	CGTCACCGAAGGTcgcCCACAGCGAGTGC
tioT-P33A	CTTCGGTGACGTGgcgGACGGCTGGCG	CGCCAGCCGTCcgcCACGTCACCGAAG
tioT-P27A/P33A	CTGTGGgcgACCTTCGGTGACGTGgcgGACGGC	GCCGTCcgcCACGTCACCGAAGGTcgcCCACAG
tioT-P61A	GACATGCGGgcgAAGAGCCTGCGTGAG	CTCACGCAGGCTCTTcgcCCGCATGTC

[a] The introduced restriction sites are underlined for each primer. The underlined portion of the 5′ primers for TioK-wt and TioT-wt introduced *Nde*I and *Eco*RI restriction sites, respectively. The underlined portion of the 3′ primers for TioK-wt and TioT-wt introduced *Xho*I and *Hind*III restriction sites, respectively. [b] The mutations are in lower-case for each primer. [c] The TioK and all TioT constructs encode $NHis_6$-tagged proteins. [d] All primers are listed in the 5′ to 3′ direction.

tioT-wt with the 3′ primer for the *mutant*, respectively (Table 2). The resulting amplified PCR fragments were gel-purified and subjected to a second round of PCR using the forward and reverse primers for *tioT-wt* (Table 2). The newly amplified fragment for *tioK-wt* gene was then digested with *Nde*I and *Xho*I, whereas the *tioT-wt*, *tioT-P27A*, *tioT-P33A*, *tioT-P27A/P33A*, and *tioT-P61A* genes were digested with *Eco*RI and *Hind*III. The *tioK-wt* gene was subcloned into the linearized pET28a vector *via* the corresponding *Nde*I/*Xho*I. The *tioT-wt* and *tioT-mutants* genes were subcloned into the linearized pACYCDuet-1 vector *via* the corresponding *Eco*RI/*Hind*III restriction sites, to give TioT-wt as well as the single and double TioT mutants P27A, P33A, P27A/P33A, and P61A. All cloning experiments were performed in *E. coli* TOP10 cells. All expression clones (pTioK-pET28a, pTioT-wt-pACYCDuet-1, pTioT-P27A-pACYCDuet-1, pTioT-P33A-pACYCDuet-1, pTioT-P27A/P33A-pACYCDuet-1, and pTioT-P61A-pACYCDuet-1) were characterized by DNA sequencing (University of Michigan DNA Sequencing Core) (Accession numbers CAJ34367 (TioK) and CAJ34376 (TioT)).

Co-overproduction and purification of TioK and TioT (wt or mutants)

The purified plasmid pTioK-pET28a was co-transformed with either pTioT-wt-pACYCDuet-1, pTioT-P27A-pACYCDuet-1, pTioT-P33A-pACYCDuet-1, pTioT-P27A/P33A-pACYCDuet-1, or pTioT-P61A-pACYCDuet-1 into chemically competent *E. coli* BL21(DE3)*ybdZ*::*aac(3)IV* cells for protein co-overexpression and purification. All proteins were grown in LB medium (3 × 1 L) supplemented with kanamycin (50 μg mL^{-1}) and chloramphenicol (25 μg mL^{-1}). An overnight culture (4 mL) prepared from fresh transformants was used to inoculate each L of LB broth. The cells were grown (28 °C, 200 rpm) to an attenuance at 600 nm of 0.5, at which point they were induced with IPTG (0.1 mM final concentration). After an additional 16–18 h of growth (20 °C, 200 rpm) the cells were harvested by centrifugation (6000 rpm, 4 °C, 5 min, in a Beckman Coulter Avanti JE centrifuge with F10 rotor) and resuspended in buffer A (25 mM Tris pH 8.0, 400 mM NaCl, 10% glycerol). The resuspended cells were lysed by sonication (for 5 min in 10 s pulses alternating with 20 s pauses) and the cell debris was removed by centrifugation (16 000 rpm, 4 °C, 45 min, in a Beckman Coulter Avanti JE centrifuge with JA-17 rotor). Imidazole (2 mM final concentration) was added to the supernatant before incubation (4 °C, 2 h with gentle rocking) with Ni^{2+}-NTA agarose resin (3 mL; Qiagen). The resin was then loaded onto a column and washed with buffer A containing increasing concentrations of imidazole (2 mM (10 mL), 5 mM (10 mL), 20, 40, and 60 mM (5 mL each), as well as 200 and 500 mM (2 × 5 mL each)). As determined by SDS-PAGE, fractions containing the pure proteins of interest were combined and dialyzed for 14–18 h at 4 °C against 6 L of buffer B (40 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol). All proteins were concentrated, flash-frozen in liquid nitrogen, and stored at −80 °C. Protein concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific). Protein yields (in mg L^{-1} of culture) for TioK when co-overexpressed with TioT-wt and TioT mutants were 5.4 (TioT-wt), 6.6 (TioT-P27A), 8.1 (TioT-P33A), 7.4 (TioT-P27A/P33A), and 7.3 (TioT-P61A). A picture of the SDS-PAGE gel showing the co-overexpression of TioK and TioT-wt is presented in Fig. S1.‡

Substrate specificity and determination of kinetic parameters for the A domain of TioK by ATP-[^{32}P]PP_i exchange assays

To determine the substrate specificity of the A domain of TioK, ATP-[^{32}P]PP_i exchange assays were performed at rt in reactions (100 μL) containing Tris–HCl (75 mM, pH 7.5 adjusted at rt), $MgCl_2$ (10 mM), TCEP (5 mM, pH 7.0 adjusted at rt), ATP (5 mM), an amino acid substrate (5 mM), and $Na_4P_2O_7$ (1 mM, spiked with 400 000 cpm of [^{32}P]PP_i per reaction). The reactions were initiated by addition of TioK (2.5 μM) (co-expressed and co-purified with TioT-wt) and incubated for 2 h prior to quenching with charcoal suspensions (500 μL) [1.6% (w/v) activated charcoal, 4.5% (w/v) $Na_4P_2O_7$, and 3.5% (v/v) perchloric acid in H_2O]. The charcoal was pelleted by centrifugation (13 000 rpm, rt, 7 min), washed twice with a wash solution (500 μL) [4.5% (w/v) $Na_4P_2O_7$, and 3.5% (v/v) perchloric acid in H_2O], resuspended in H_2O (500 μL), and counted by liquid scintillation counting. For the determination of the kinetic parameters (K_m and k_{cat}) for L-Trp and L-Phe, reactions were performed at rt with varying concentrations of the amino acid (0.05, 0.1, 0.25, 0.5, 1, 1.75, 2.5, 5, and 10 mM). The reactions were started by addition of TioK/TioT (2.5 μM) (either TioT-wt or a TioT mutant) and were stopped after 15 min. The experiments were carried out in duplicate for each substrate concentration. Michaelis–Menten plots for the kinetic parameters reported in Table 1 are presented in Fig. S3.‡

Characterization of T domain of TioK by trichloroacetic acid (TCA) precipitation assays

Incorporation of [5-^{3}H]L-Trp into holo-TioK was monitored over time by TCA precipitation assays. Conversion of the TioK from its apo to holo form was first achieved in a reaction mixture (150 μL) containing Tris–HCl (75 mM, pH 7.5 adjusted at rt), $MgCl_2$ (10 mM), TCEP (1 mM, pH 7.0 adjusted at rt), TioK (15 μM), CoA (100 μM), and Sfp (1 μM). Prior reactions performed with [^{3}H]acetyl-CoA had confirmed the complete loading of the phosphopantetheinyl arm onto to TioK (the T domain of the A–T didomain) in less than 20 min under similar reaction conditions. After 2 h of incubation at rt, L-Trp (2 mM, spiked with 350 000 cpm of [5-^{3}H]L-Trp per 25 μL reaction), and ATP (20 mM) were added. Samples (25 μL) were removed at 0, 2, 5, 10, and 15 min and added to 10% TCA (100 μL). The protein was pelleted by centrifugation (13 000 rpm, rt, 7 min), washed with 10% TCA (100 μL), and resuspended in 88% formic acid (100 μL). The radiolabeled product was counted by liquid scintillation counting (Fig. S2‡).

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