Extremophiles (2017) 21:73–83
DOI 10.1007/s00792-016-0884-0

SPECIAL FEATURE: ORIGINAL PAPER

11th International Congress on Extremophiles

Novel stand-alone RAM domain protein-mediated catalytic control of anthranilate phosphoribosyltransferase in tryptophan biosynthesis in *Thermus thermophilus*

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Received: 30 September 2016 / Accepted: 10 October 2016 / Published online: 19 October 2016

Abstract Regulation of amino acid metabolism (RAM) domains are widely distributed among prokaryotes. In most cases, a RAM domain fuses with a DNA-binding domain to act as a transcriptional regulator. The extremely thermophilic bacterium, *Thermus thermophilus*, only carries a single gene encoding a RAM domain-containing protein on its genome. This protein is a stand-alone RAM domain protein (SraA) lacking a DNA-binding domain. Therefore, we hypothesized that SraA, which senses amino acids through its RAM domain, may interact with other proteins to modify its functions. In the present study, we identified anthranilate phosphoribosyltransferase (AnPRT), the second enzyme in the tryptophan biosynthetic pathway, as a partner protein that interacted with SraA in *T. thermophilus*. In the presence of tryptophan, SraA was assembled to a decamer and exhibited the ability to form a stable hetero-complex with AnPRT. An enzyme assay revealed that AnPRT was only inhibited by tryptophan in the presence of SraA. This result suggests a novel feedback control mechanism for tryptophan biosynthesis through an inter-RAM domain interaction in bacteria.

Keywords RAM domain · Tryptophan biosynthesis · Anthranilate phosphoribosyltransferase · Feedback inhibition · *Thermus thermophilus*

Abbreviations

PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RAM domain	Regulation of amino acid metabolism domain
Lrp	Leucine-responsive regulatory protein
AS	Anthranilate synthase
AnPRT	Anthranilate phosphoribosyltransferase
PRPP	Phosphoribosylpyrophosphate

Introduction

Amino acid biosynthesis is regulated at two different levels: enzyme activity and the expression of genes responsible for biosynthesis. The former mechanism functions well for an immediate response to changes in environmental amino acid concentrations, while the latter has the ability to control biosynthesis before enzyme production. As protein machineries that regulate amino acid biosynthesis, two functional domains, the aspartate kinase–chorismate mutase–TyrA (ACT) domain and regulation of amino acid metabolism (RAM) domain, are well known (Chipman and Shaanan 2001; Ettema et al. 2002). The ACT domain is

Communicated by H. Atomi.

T. Kubota and H. Matsushita contributed equally to this work.

This article is part of a special feature based on the 11th International Congress on Extremophiles held in Kyoto, Japan, September 12–16, 2016.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-016-0884-0) contains supplementary material, which is available to authorized users.

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often found in amino acid biosynthetic enzymes that catalyze the first step of the biosynthetic pathway (Grant 2006). In most cases, two ACT domains, each consisting of a β-α-β-β-α-β fold, form an effector-binding unit and transmit the effector signal to the catalytic domain by binding its effector molecule. The RAM domain also adopts a similar β-α-β-β-α-β fold, but possesses a distinct binding site for effector molecules (Ettema et al. 2002). Most RAM domains are fused with helix-turn-helix (HTH)-type DNA-binding domains. Thus, ACT and RAM domains appear to be used in the regulation of enzyme activity and gene expression, respectively. A typical example of a RAM domain-containing protein family member is the transcriptional regulator, leucine-responsive regulatory protein (Lrp). Lrp-type proteins are distributed among various bacteria and archaea and sense various kinds of amino acids via RAM domains as environmental signals (Ernsting et al. 1992; Enoru-Eta et al. 2000; Brinkman et al. 2002; Knoten et al. 2011; Song et al. 2016). For example, Lrp regulates the expression of approximately 10% of all genes on the genome in *Escherichia coli*, which includes those for amino acid biosynthesis/degradation, the transport of amino acids, formation of pili, and other transcriptional regulators, in a positive or negative manner (Ernsting et al. 1992).

As another type of RAM domain-containing protein, stand-alone RAM domain proteins that do not possess any additional functional domains are also distributed in prokaryotes (Ettema et al. 2002). While the functions of most stand-alone RAM domain proteins are unknown, the DM1 protein from *Pyrococcus* sp. OT3 is the only example of a stand-alone RAM domain protein whose function has been elucidated (Okamura et al. 2007). DM1 interacts with FL11, which is a Lrp-type transcription regulator containing a RAM domain, to modify the function of FL11. FL11 alone senses lysine and regulates the transcription of approximately 200 transcriptional units by binding target promoters in response to lysine (Yokoyama et al. 2007). When lysine is limited but arginine is abundant in the environment, DM1 senses arginine, forms a hetero-octamer with FL11, and increases the DNA-binding affinity of FL11. The hetero-octamer formed in turn represses the transcription of *fl11* and regulates the expression of lysine biosynthetic genes and other targets involved in different metabolic pathways through DNA-binding ability depending on FL11 (Okamura et al. 2007). Thus, DM1 lacking a DNA-binding domain acts as an indirect transcriptional regulator that modifies the DNA-binding ability of FL11 through the formation of a hetero-complex, which enables FL11 to respond to non-innate environmental signals. As shown for DM1, stand-alone RAM domain proteins may function in a hetero-complex with other Lrp-like proteins containing a RAM domain. *Thermus thermophilus* HB8 is an extremely thermophilic bacterium that has been used as a biological model, for which structural genomics have been developed (Cava et al. 2009). This bacterium carries the gene, *TTHA0845* (*sraA*), which encodes a stand-alone RAM domain protein, but not any other genes coding for RAM domain-containing proteins on the genome. The structure of TTHA0845 has been determined through the structural genomics project; however, a functional role has not yet been elucidated for this protein (Nakano et al. 2006). This finding prompted us to clarify whether SraA functions as a sensor of amino acids in cells, and also if amino acid-bound SraA interacts with other proteins lacking a RAM domain to control their functions. In the present study, we analyzed the function of SraA encoded by *TTC0493*, the ortholog of *TTHA0845*, in *T. thermophilus* HB27 and demonstrated that SraA interacts directly with anthranilate phosphoribosyltransferase (AnPRT), which is the enzyme catalyzing the second step of tryptophan biosynthesis, in *T. thermophilus* and regulates the activity of AnPRT. This study provides a novel regulatory mechanism for tryptophan biosynthesis.

Materials and methods

Strains, media, and chemicals

Escherichia coli DH5α (Sambrook et al. 1989) was used for DNA manipulation, and *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA) was used as the host to express the *sraA* and *trpD* (encoding AnPRT) genes. 2 × YT medium (Sambrook et al. 1989) was generally used for the cultivation of *E. coli* cells, whereas TM medium (Koyama et al. 1986) and MM medium (Tanaka et al. 1981) were used for the cultivation of *T. thermophilus* HB27 and its recombinant derivatives. All chemicals were purchased from Sigma-Aldrich (St. Louis, USA), Wako Pure Chemical (Osaka, Japan), or Kanto Chemicals (Tokyo, Japan). Enzymes for DNA manipulation were purchased from Takara Bio (Kyoto, Japan) and TOYOBO (Osaka, Japan).

Preparation of recombinant strains of *T. thermophilus* HB27

We constructed several plasmids for the transformation of *T. thermophilus* HB27; pNstrepSraA for the production of SraA with a Strep tag at the N-terminus, and pNHisAS for the production of component I of anthranilate synthase (AS) with a $(His)_{12}$ tag at the N-terminus. These plasmids were designed as illustrated in Supplementary Fig. S1 and the oligonucleotide primers used for these constructions are listed in Supplementary Table. S1. pNstrepSraA was constructed as follows. First PCR was performed with the primers SraA-up-fw and SraA-up-rv to amplify the upstream region of the *sraA* gene of 700 bp. Second

PCR was performed with the primers Ntaghyg10-fw and Ntaghyg10-rv to amplify the *hyg10* gene encoding thermostable hygromycin B phosphotransferase (Nakamura et al. 2005). Third PCR was performed with the primers NtagslpA-fw and NtagslpA-rv to amplify the *slpA* promoter of *T. thermophilus* (Fujita et al. 2013). Fourth PCR was performed with the primers NstrepSraA-fw and NstrepSraA-rv to amplify coding and downstream regions of 700 bp of the *sraA* gene. The four amplified fragments were digested with *Xba*I/*Spe*I, *Spe*I/*Pst*I, *Pst*I/*Hin*dIII, and *Hin*dIII/*Kpn*I, respectively, and cloned separately into pBlueScript II KS(+) for sequence verification. The four fragments with the correct sequences were digested with appropriate enzymes and ligated together with pBlueScript II KS(+) digested with *Xba*I/*Kpn*I to yield the plasmid, pNstrepSraA. The resulting plasmid was used for the transformation of *T. thermophilus* (Koyama et al. 1986) to generate the *T. thermophilus* strain, Tt27NStrepSraA. Colonies that were resistant to 160 μg/mL hygromycin on TM medium were picked up, and the knockout was confirmed by colony PCR using NstrepSraAcheck-fw and NstrepSraAcheck-rv as well as successive DNA sequencing of the PCR fragment. The other plasmids were constructed in the same manner as pNstrepSraA using the primers listed in Supplementary Fig. S1, and were used for the integration of the modified genes into the *T. thermophilus* HB27 genome. The *T. thermophilus* strain was named Tt27NHisAS.

Pull-down assay using NstrepSraA as a bait protein

Tt27NstrepSraA cells were grown in 800 mL of TM medium supplemented with 40 μg/mL hygromycin at 70 °C for 12–14 h. Approximately 10 g of cells were washed and suspended in 16 mL of buffer A (20 mM Tris–HCl, pH 8.0, 150 mM NaCl). Cells were then disrupted by sonication. The supernatant prepared by centrifugation at 40,000×*g* at 4 °C for 15 min was loaded onto a column with Strep-Tactin MacroPrep® Resin (Novagen, Darmstadt, Germany), equilibrated with buffer A. After being washed with the same buffer, the adsorbed proteins were eluted with buffer A supplemented with 2.5 mM desthiobiotin. The eluates were concentrated to approximately 200 μl with a VIVASPIN 20 (MWCO3000) concentrator (Sartorius Japan, Tokyo, Japan) and subjected to SDS-PAGE. Gel pieces containing a protein spot that was co-purified with SraA were washed twice with distilled water followed by 50% acetonitrile, incubated with 50 mM dithiothreitol in 100 mM NH_4HCO_3 at 56 °C for 30 min, and then incubated with 100 mM iodoacetamide in 100 mM NH_4HCO_3 at room temperature for 30 min in the dark. Proteins were digested with mass spectrometry-grade trypsin (Promega KK, Tokyo, Japan) at 37 °C overnight. The resulting peptides were analyzed by a nano HPLC–MS/MS analysis using an Advance nanoLC (Michrom Bioresources, Auburn, CA) system connected to an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific KK, Tokyo, Japan). Peptides dissolved in solvent A (0.1% formic acid) were separated with a 25-min gradient from 5 to 55% solvent B (acetonitrile containing 0.1% formic acid) at a flow rate of 500 nL/min. Full-scan MS spectra were obtained using the LTQ ion trap mass spectrometer.

Peak lists were searched against the NCBI *T. thermophilus* HB27 database (2013_07, 5773 protein sequences) (Henne et al. 2004) using a MASCOT server version 2.4.1 (Matrix Science KK, Tokyo, Japan). The following search parameters in MASCOT were used: peptide mass tolerance, 2 Da; fragment mass tolerance, 1 Da; trypsin cleavage with a maximum of one missed cleavage; fixed modifications, *S*-carbamidomethylation (Cys); variable modification, oxidation (Met); charge state, +1, or +2 or +3; ion score cut-off, 30; and false discovery rate cut-off, <5%. Only peptides with a MASCOT rank of 1 were accepted.

Preparation of recombinant SraA and AnPRT from *E. coli* cells

The *sraA* and *AnPRT* genes were amplified by PCR using *T. thermophilus* genomic DNA as the template and oligonucleotide primers as listed in Supplementary Table S2. The amplified DNA fragment was digested with appropriate restriction enzymes and ligated into pBlueScriptII KS(+) (Novagen), which was prepared by digestion with the same enzymes. After DNA sequence verification, these fragments were digested by appropriate restriction enzymes and ligated with pET-26b(+) (Novagen). The resulting plasmids, pET-NstrepSraA, pET-NstrepSraA/AnPRTChis, and pET-AnPRTChis, were used for the transformation of *E. coli* BL21-CodonPlus (DE3)-RIL cells.

SraA was purified as follows. The *E. coli* transformant harboring pET-NstrepSraA was inoculated into 2 × YT media supplemented with kanamycin at 50 and 30 μg/mL chloramphenicol, and pre-cultured at 37 °C for 10 h. Sixteen milliliters of the culture broth was transferred into 1.6 L of the same fresh media and cultured at 37 °C for an additional 3 h. Isopropyl β-D-thiogalactopyranoside (IPTG) was supplemented at 0.1 mM and cultivation was continued at 25 °C for 12–14 h. Cells were harvested by centrifugation at 5000×*g* for 10 min and washed with the buffer A. Cells were resuspended in the same buffer and lysed by sonication. The supernatant was prepared by centrifugation at 40,000×*g* for 15 min and then heated at 70 °C for 30 min. After the removal of denatured proteins by centrifugation at 40,000×*g* for 15 min, the supernatant was applied to the Strep-Tactin column pre-equilibrated with buffer A. After being washed with the same buffer, the adsorbed SraA protein was eluted with buffer A supplemented with 2.5 mM

desthiobiotin. Every fraction was applied to SDS-PAGE to check the purity of the SraA protein, and the SraA fraction with a purity of more than 95% was pooled. In the preparation of SraA used for the AnPRT enzyme assay, gel filtration chromatography with HiLoad 26/60 Superdex 200 pg (GE Healthcare Japan, Tokyo, Japan), using buffer A as the eluent, was additionally performed. The eluted fractions were concentrated with VIVASPIN 20 (MWCO3000) (Sartorius). Protein concentrations were measured using a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA).

AnPRT with the $(His)_6$ tag at the C-terminus was purified as follows. The *E. coli* cell lysate prepared in the same manner was heated at 70 °C for 30 min, followed by the removal of denatured proteins by centrifugation at 40,000×*g* for 15 min. The supernatant was applied to the Ni^{2+}-affinity column pre-equilibrated with buffer A supplemented with 20 mM imidazole. After being washed with the same buffer, AnPRT was eluted with buffer A supplemented with 500 mM imidazole. Purity was checked by SDS-PAGE. Gel filtration chromatography for the preparation of AnPRT and AS for the enzyme assay was performed in the same manner as described above. AnPRT was concentrated with a VIVASPIN (MWCO10000) concentrator (Sartorius) prior to the enzyme activity assay.

Preparation of his-tagged AS from *T. thermophilus*

Tt27NHisAS cells were grown in 1.6 L of TM medium supplemented with 40 μg/mL hygromycin at 70 °C for 12–14 h. Approximately 10 g of cells were washed, suspended in 32 mL of buffer A, and disrupted by sonication. The supernatant of the cell lysate prepared by centrifugation at 40,000×*g* for 15 min was applied to the Ni^{2+}-affinity column pre-equilibrated with buffer A supplemented with 20 mM imidazole. After washing with the same buffer, AS with the $(His)_6$-tag at the N-terminus of component I was eluted with buffer A supplemented with 500 mM imidazole. Purity was checked by SDS-PAGE. Prior to the enzyme activity assay, protein was concentrated with a VIVASPIN (MWCO10000) concentrator (Sartorius).

Analysis of the subunit assembly of SraA and interaction between SraA and AnPRT

To analyze the subunit assembly of SraA and interaction between SraA and AnPRT, purified SraA, AnPRT, and the SraA/AnPRT hetero-complex were subjected to gel filtration with HiLoad 26/60 Superdex 200 pg (GE Healthcare Japan) equilibrated with buffer A in the presence or absence of 1 mM tryptophan or isoleucine. Flow rates were set at 2.5 mL/min. Protein assembly and protein–protein interactions were analyzed by molecular weight calibration using molecular weight markers (Gel filtration Calibration Kits HMW and LMW, GE Healthcare Japan) and SDS-PAGE of the eluates.

Enzyme assay

AnPRT catalyzes the condensation of anthranilate and phosphoribosylpyrophosphate (PRPP) to generate phosphoribosylanthranilate and pyrophosphate. AnPRT activity was assayed by measuring released pyrophosphate using a Pyrophosphate reagent (Sigma-Aldrich Japan, Tokyo, Japan). After the reaction mixture (792 μL 50 mM HEPES–NaOH, pH 8.0) containing 1 mM $MgCl_2$, various concentrations of tryptophan (0, 1, 10, 100, or 1000 μM), and 266 μL of the pyrophosphate reagent was pre-incubated at 30 °C for 5 min, the reaction was started by adding 8 μL of solution containing AnPRT that gave a final concentration of 10 μg/mL and SraA that gave a final concentration of 0, 30, 60, 90, or 120 μg/mL. Decreases in the absorbance at 340 nm were monitored with a UV–VIS spectrophotometer UV-2600 (Shimadzu, Kyoto, Japan). One unit of enzyme activity was defined as the amount of the enzyme that released 1 μmol of the product per 1 min.

AS activity was measured using fluorometric assay with the fluorescence of anthranilate according to a previously described method (Grove and Levy 1975). Fluorescence at 395 nm by excitation at 310 nm was detected with the fluorescence spectrometer SpectraMax i3 (Molecular Devices Japan, Tokyo, Japan). After the reaction buffer (594 μL 50 mM Tris–HCl, pH 7.8) containing 2 mM $MgCl_2$, 100 μM chorismate, and 20 mM glutamate was pre-warmed at 45 °C for 5 min, the reaction was initiated by adding 6 μL of solution that gave a final concentration of 10 μg/mL AS and various concentrations of tryptophan giving a final concentration of 0, 1, 2, 5, 10, or 50 μM to the mixture, and fluorescence at 395 nm was monitored for 1 min. One unit of enzyme activity was defined as the amount of the enzyme that released 1 μmol of the product per 1 min.

Results

Screening of partner proteins of SraA

The RAM domain has often been found in a protein composed of multiple functional domains. Lrp is a typical example that contains DNA-binding and RAM domains at the N- and C-termini, respectively. SraA is unique in that it possesses a RAM domain, but does not have any additional domains. Since the RAM domain of SraA was expected to have the ability to bind the effector molecule, SraA, which bound the effector, may have the ability to interact with a

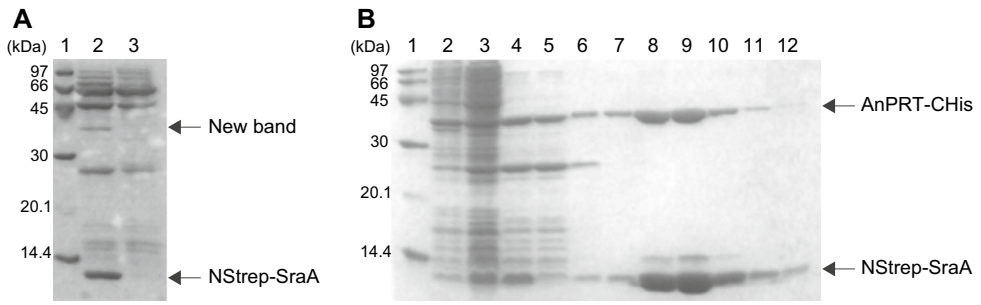

Fig. 1 Interaction of SraA with AnPRT. **a** Pull-down assay using a Strep-Tactin column with Strep-tagged SraA as the bait in *T. thermophilus*. *Lane 1* Molecular size markers; *lane 2* Tt27NstrepSraA strain producing SraA with the N-terminal Strep tag; *lane 3* wild-type *T. thermophilus* HB27 (negative control). **b** Co-purification from *E. coli* cells of His-tagged AnPRT with Strep-tagged SraA by a Strep-Tactin column. *Lane 1* Molecular size markers; *lane 2* precipitant of the sonicate of *E. coli* cells producing His-tagged AnPRT and Strep-tagged SraA; *lane 3* supernatant of the sonicate of *E. coli* cells producing His-tagged AnPRT and Strep-tagged SraA; *lane 4* heated fraction of the supernatant; *lane 5* passing fraction of *lane 4* through a Strep-Tactin column; *lane 6* washing fraction. *Lanes 7–12* fractions eluted with desthiobiotin from the column. *Lane 7* elute 1; *lane 8* elute 2; *lane 9* elute 3; *lane 10* elute 4; *lane 11* elute 5; *lane 12* elute 6

partner protein to modify its function. Based on this scenario, we performed a pull-down assay to screen for interacting partner proteins using SraA with a Strep tag at the N-terminus as the bait. When Strep-tagged SraA was produced in Tt27NstrepSraA and the cell lysate was applied to a Strep-Tactin column, a unique protein showing a molecular weight of 36 k on SDS-PAGE was co-purified with SraA (Fig. 1a). Using a successive MALDI-TOF–MS analysis, the protein co-purified with SraA was identified as TTC1491. TTC1491 is annotated as anthranilate phosphoribosyltransferase (AnPRT), which is an enzyme that catalyzes the second step of tryptophan biosynthesis. To confirm the interaction between SraA and AnPRT, SraA with the Strep tag at the N-terminus was co-produced with AnPRT with the $(His)_6$ tag at the C-terminus in *E. coli* and purified with a Strep-Tactin column. As expected, the eluate from the column contained SraA and also AnPRT, demonstrating that these proteins interact with each other (Fig. 1b).

Effects of tryptophan on the assembly of SraA and its interaction with AnPRT

In Lrp family transcriptional regulators, binding of an amino acid to a RAM domain induces a change in the oligomeric state of Lrp, which, in turn, changes the affinity of Lrp to the target DNA (Brinkman et al. 2003). Since AnPRT is involved in tryptophan biosynthesis, we hypothesized that tryptophan induces alterations in the oligomeric state of SraA and changes the affinity of SraA to AnPRT. To examine the oligomeric state of SraA, we performed a gel filtration chromatography analysis of SraA in the presence or absence of tryptophan (Fig. 2a). In the absence of tryptophan, SraA was eluted at two different elution volumes, corresponding to molecular weights of approximately 30 and 130 k, respectively. SraA with the N-terminal Strep tag is composed of 105 amino acid residues with a molecular weight of 11.5 k. Since TTHA0845, possessing 99% of amino acid sequence identity to SraA from *T. thermophilus* HB27, was previously reported to adopt a decameric ring structure composed of five sets of the SraA dimer (Nakano et al. 2006), these two peaks were inferred as a dimer and decamer of SraA, respectively. In the presence of tryptophan, it was eluted as a single peak of 130 k, corresponding to a decamer. This result suggests that tryptophan binding stabilized SraA in the decameric form. We then analyzed the oligomeric state of AnPRT and found that it was eluted as a single peak (70 k), irrespective of tryptophan, indicating that the addition of tryptophan did not change the oligomeric state of AnPRT (Fig. 2b). We also analyzed the fraction containing the co-purified proteins, SraA and AnPRT. In the absence of tryptophan, proteins were eluted at three different elution volumes corresponding to 30, 70, and 130 k (Fig. 2c). In SDS-PAGE of each elution, the 30-, 70-, and 130-k fractions were suggested to be an SraA dimer, an AnPRT dimer, and an SraA decamer, respectively (Fig. 2d). In the presence of tryptophan, the two protein peaks corresponding to 30 and 70 k disappeared in the elution profile, and a new peak of 200 k emerged with the 130-k peak remaining. SDS-PAGE showed that the 200-k fraction contained both SraA and AnPRT (Fig. 2e). These results suggest that an SraA decamer of 130 k interacted with an AnPRT dimer of 70 k to yield the 200-k hetero-complex. When similar experiments were performed with isoleucine as an additive instead of tryptophan, the decamer formation of SraA was induced, even by isoleucine; however, this SraA decamer did not form a hetero-complex with the AnPRT dimer (Supplementary Fig. S2). This result indicates that although the decamer formation of SraA was induced by isoleucine, the SraA decamer binding isoleucine did not take the proper conformation to interact with AnPRT.

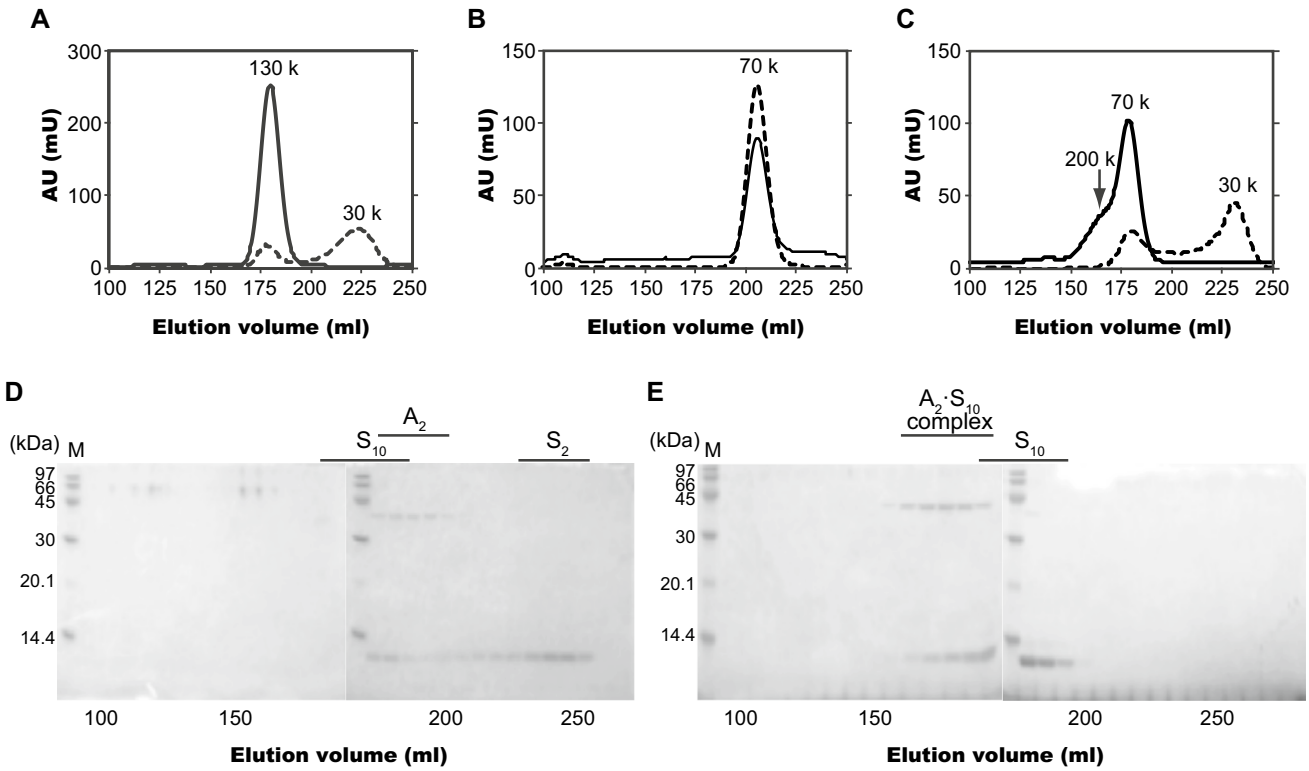

Fig. 2 Analysis of oligomeric states using gel filtration chromatography. **a** Gel filtration chromatogram of SraA in the presence (*solid line*) and absence (*dotted line*) of 1 mM tryptophan. **b** Gel filtration chromatogram of AnPRT in the presence (*solid line*) and absence (*dotted line*) of 1 mM tryptophan. **c** Gel filtration chromatogram of SraA and AnPRT co-purified from *E. coli* cells in the presence (*solid line*) and absence (*dotted line*) of 1 mM tryptophan. The shoulder peak corresponding to the hetero-oligomer composed of SraA and AnPRT is indicated with an *arrow*. **d** SDS-PAGE of the chromatography fractions containing SraA and AnPRT co-purified from *E. coli* cells in the absence of tryptophan. Putative oligomeric states are shown as follows: S_2 SraA dimer, A_2 AnPRT dimer, S_{10} SraA decamer. **e** SDS-PAGE of the chromatography fractions containing SraA and AnPRT co-purified from *E. coli* cells in the presence of 1 mM tryptophan. Putative oligomeric states are shown as follows: A_2S_{10} *complex* AnPRT dimer·SraA decamer complex, S_{10} SraA decamer

Regulation of AnPRT by SraA and tryptophan

Based on the results described above, we hypothesized that SraA regulates the activity of AnPRT in the presence of tryptophan. We subsequently examined the effects of SraA and tryptophan on the AnPRT activity (Fig. 3). AnPRT activity was measured under the conditions containing various concentrations of SraA in the presence or absence of 1 mM tryptophan (Fig. 3a). AnPRT activity was not affected by the sole addition of SraA or tryptophan; however, it was inhibited by increasing amounts of SraA in the presence of tryptophan. When SraA was added at 120 μg/mL, which was an approximately 37-fold higher concentration than that of AnPRT used in the assay in terms of the monomer–monomer molar ratio, AnPRT activity was inhibited by up to 50% of the basal activity. We then analyzed the effective concentrations of tryptophan needed to inhibit AnPRT. When activity was measured under conditions containing various concentrations of tryptophan in the presence of 90 μg/mL SraA (approximately 28-fold higher than that of AnPRT), it decreased to approximately 50% in the presence of tryptophan at concentrations greater than 0.1 mM (Fig. 3b). We also measured AnPRT activity in the presence of other amino acids. As expected, amino acids, except for tryptophan, did not affect the AnPRT activity, suggesting that tryptophan acts as a specific inhibitor of AnPRT by binding to SraA (Fig. 3c).

Inhibition of AS by tryptophan

Tryptophan biosynthesis is generally regulated at the first step of biosynthesis catalyzed by AS via feedback inhibition by tryptophan (Belser et al. 1971; Bae and Crawford 1990; Tutino et al. 1997); however, AS from *T. thermophilus* has not yet been characterized so far. To evaluate the significance of the regulation of AnPRT by SraA and tryptophan in cells, we analyzed the effects of tryptophan on the activity of AS from *T. thermophilus*. We found that AS was inhibited to 32% by 1 μM tryptophan and 2% by 10 μM tryptophan (Fig. 3d). This result indicates that the

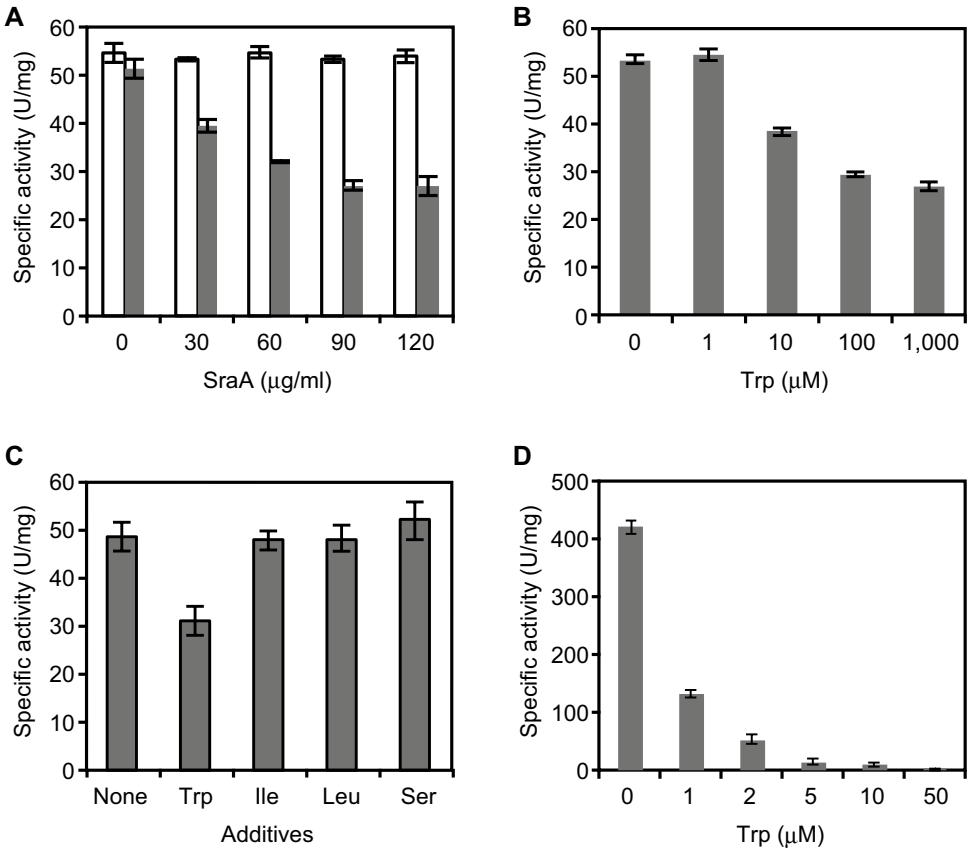

Fig. 3 Inhibition of AnPRT and AS by tryptophan and/or SraA. **a** Effects of various concentrations of SraA on the specific activity of AnPRT in the presence (*gray bar*) and absence (*white bar*) of 1 mM tryptophan. **b** Effects of various concentrations of tryptophan on the specific activity of AnPRT in the presence of 90 μg/mL SraA. **c** Effects of various amino acids on AnPRT activity in the presence of 60 μg/mL SraA. **d** Effects of various concentrations of tryptophan on the specific activity of AS

AS reaction is the most critical step in the regulation of tryptophan biosynthesis in *T. thermophilus*.

Discussion

RAM domains are often fused to the helix-turn-helix (HTH)-type DNA-binding domain, and function as effector-binding domains of Lrp-type transcriptional regulators in prokaryotes (Brinkman et al. 2003; Peeters and Charlier 2010). There is another type of RAM domain-containing protein called a stand-alone RAM domain protein, a single-domain protein that is not fused to any other functional domains. This type of RAM domain protein is known to function by interaction with other Lrp-type transcriptional regulators (Okamura et al. 2007). In contrast, SraA from *T. thermophilus* interacts with AnPRT, which does not possess a RAM domain. Thus, this study provides the first instance for a RAM domain-containing protein interacting with proteins lacking a RAM domain.

Structural aspects of SraA for sensing tryptophan as a signal

The crystal structures of selenomethionine- or isoleucine-bound DM1 have already been determined (Okamura et al. 2007). In each structure, the α-amino group of the bound amino acid is recognized by Asp39 and the α-carboxyl group is recognized by Thr69 and Thr71 of DM1. When the amino acid sequence of SraA was compared with those of DM1 and the other RAM domain-containing proteins, these residues were conserved in many RAM domain-containing proteins including SraA (Fig. 4), suggesting that SraA shares the mechanism to recognize the α-amino and α-carboxyl groups with these RAM domain-containing proteins. On the other hand, the residues surrounding the side chains of these amino acids bound to DM1 are not conserved. For example, Glu15, Tyr35, and Arg61 of DM1 are replaced with Val15, Thr35, and Leu61, respectively, in SraA. These replacements appear to enlarge the size of the amino acid-binding pocket of SraA, which allows SraA to recognize the larger amino acid, tryptophan. Among Lrp homologs, those that have the ability to recognize tryptophan or related metabolites have been identified. An Lrp homologue from *Mycobacterium tuberculosis* recognizes tryptophan as one of the alternative ligand molecules (Song et al. 2016). Also, KynR from *Pseudomonas aeruginosa* senses kynurenine by its RAM domain, which is one of the metabolites of tryptophan, to induce quorum-sensing control (Knoten et al. 2011). However, the residues responsible for the recognition of the side chain and even of the main

Fig. 4 Amino acid sequence alignment of SraA and related RAM domain proteins. Amino acid residues responsible for the recognition of the α-amino and α-carboxyl groups of bound amino acids in the structure of DM1 are indicated by *black circles*. Amino acid residues around the side chain moiety of bound amino acids in the structure of DM1 are indicated by *black squares*. *TTC0493* SraA from *T. thermophilus* HB27, *TTHA0845* SraA from *T. thermophilus* HB8, *EcLrp* Lrp from *E. coli*, *MtLrp* Lrp from *Mycobacterium tuberculosis*, *PaKynR* KynR from *Pseudomonas aeruginosa*, *SYN_02590* fusion protein of the RAM domain and AnPRT domain from *Syntrophus aciditrophicus*, *SAV_6058* Stand-alone RAM domain protein from *Streptomyces avermitilis*, *R. equi* Stand-alone RAM domain protein from *Rhodococcus equi*, *K. setae* Stand-alone RAM domain protein from *Kitasatospora setae*, *POT3DM1* DM1 from *Pyrococcus* sp. OT3, *POT3FL11* FL11 from *Pyrococcus* sp. OT3

chain of the ligand amino acids are not conserved in these RAM domain-containing proteins, suggesting that there is an alternative mode to bind tryptophan or its metabolite in RAM domain-containing proteins.

Distribution of the SraA-dependent regulation of AnPRT in other organisms

We searched the homologous proteins of SraA using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/) and found that *Syntrophus aciditrophicus*, an anaerobic bacterium, possessed the RAM domain-containing protein, SYN_02590, which is annotated as an AnPRT. This is a fused protein of a RAM domain at the N-terminus and AnPRT domain at the C-terminus (Fig. 5a). This finding suggests the presence of another example showing RAM domain-dependent AnPRT regulation in nature. Moreover, the *SYN_02590* gene is located adjacent to *SYN_02591* (*trpC* homolog) and SYN_02592 (*trpF* homolog), possibly forming an operon in this bacterium (Fig. 5a). This gene organization suggests that SYN_02590 is also involved in tryptophan biosynthesis in *S. aciditrophicus*. However, the functions of these tryptophan biosynthetic gene homologs remain unclear, because *S. aciditrophicus* possesses the complete tryptophan biosynthetic gene cluster (SYN_01941—SYN_01947) on a different genome locus adjacent to SNY_01924—SYN10940, which may be

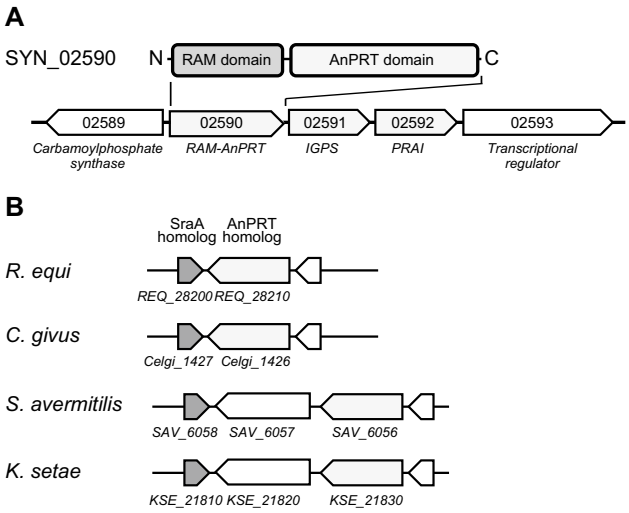

Fig. 5 Possible functional relationship of AnPRT with SraA. **a** Schematic drawing of the SYN_02590 structure and flanking region of the *SYN_02590* gene encoding AnPRT fused with the RAM domain of *S. aciditrophicus*. **b** Close gene location of *AnPRT* homologs and *sraA* homologs in some actinobacteria

responsible for the shikimate pathway. We also found that several *Streptomyces* species carry the *sraA* homolog at the flanking region of the *AnPRT* gene on the genome (Fig. 5b). These *Streptomyces* strains except for *Kitasatospora setae*, only possess a single gene encoding AnPRT, suggesting that

these gene products function as AnPRTs and are presumably regulated through stand-alone RAM domains. Thus, the biological roles of the SraA-like proteins remain unknown; however, this gene arrangement suggests a functional relationship between the SraA and AnPRT homologs.

Biological significance of the regulation of AnPRT by SraA

Tryptophan biosynthesis is known to be regulated at two levels: enzyme inhibition and the repression of gene expression. Enzyme-level regulation has been reported in AS from *E. coli* and *Salmonella typhimurium*, which catalyzes the first reaction in tryptophan biosynthesis (Henderson et al. 1970; Jackson and Yanofsky 1974). Mutational and crystallographic analyses of AS from *S. typhimurium* identified Leu38, Glu39, Ser40, Lys50, Pro291, Tyr292, Met293, Gly454, Tyr455, Asp463, and Cys465 of the component I (the translational product of the *trpE* gene) as residues that bind tryptophan for inhibition (Caligiuri and Bauerle 1991; Morollo and Eck 2001). Most of these residues are conserved among AS from various organisms including AS from *T. thermophilus*. This finding suggests that AS from *T. thermophilus* shares a mechanism for feedback inhibition with the counterparts from other organisms. AS from *T. thermophilus* was inhibited to 1% by 10 μM tryptophan, which was more effective than the inhibition of AnPRT (50% by 100 μM tryptophan) mediated by SraA.

The inhibition of AnPRT has been reported for enzymes from *E. coli*, *S. typhimurium*, and *Corynebacterium glutamicum* (formally called *Brevibacterium flavum*) (Henderson et al. 1970; Jackson and Yanofsky 1974; Sugimoto and Shiio 1983; O'Gara and Dunican 1995). The first two enzymes are fused with AS and their AnPRT activities are inhibited through the binding of tryptophan to the AS domain (Caligiuri and Bauerle 1991). AnPRT from *S. typhimurium* was shown to be partially inhibited by tryptophan, with a maximum of approximately 70% at a tryptophan concentration of 5 μM and higher (Henderson et al. 1970). In contrast, *C. glutamicum* AnPRT, which is not fused with AS, was inhibited by 50% following the addition of 150 μM tryptophan (Sugimoto and Shiio 1983). The degree of inhibition by 100–150 μM tryptophan was similar to the case of AnPRT from *T. thermophilus*; however, the *C. glutamicum* enzyme was almost completely inhibited by an increase in the tryptophan concentration, whereas *T. thermophilus* AnPRT was not inhibited further even by increase in the concentration of tryptophan.

Since the effective concentration of tryptophan in the SraA-mediated inhibition of AnPRT was higher than that of AS and the maximal inhibition of AnPRT was approximately 50%, it appears unlikely that the inhibition of AnPRT functions under normal conditions. However, the concentration of tryptophan may be elevated when the chorismate concentration is increased because the inhibition of AS by tryptophan was found to be competitive with chorismate in *S. typhimurium* (Henderson et al. 1970). In *C. glutamicum*, tryptophan biosynthesis has been suggested to be controlled by three different levels: the feedback inhibition of AS (Matsui et al. 1987), the *E. coli*-type attenuation of tryptophan biosynthetic gene expression (Heery and Dunican 1993), and the feedback inhibition of AnPRT (Sugimoto and Shiio 1983). It is important to note that the mutated *trpD* gene from tryptophan-overproducing *C. glutamicum* was isolated on the basis of its ability to confer resistance to 5-methyltryptophan on the wild-type strain (O'Gara and Dunican 1995). The AnPRT of the wild-type *C. glutamicum* strain was sensitive to inhibition by

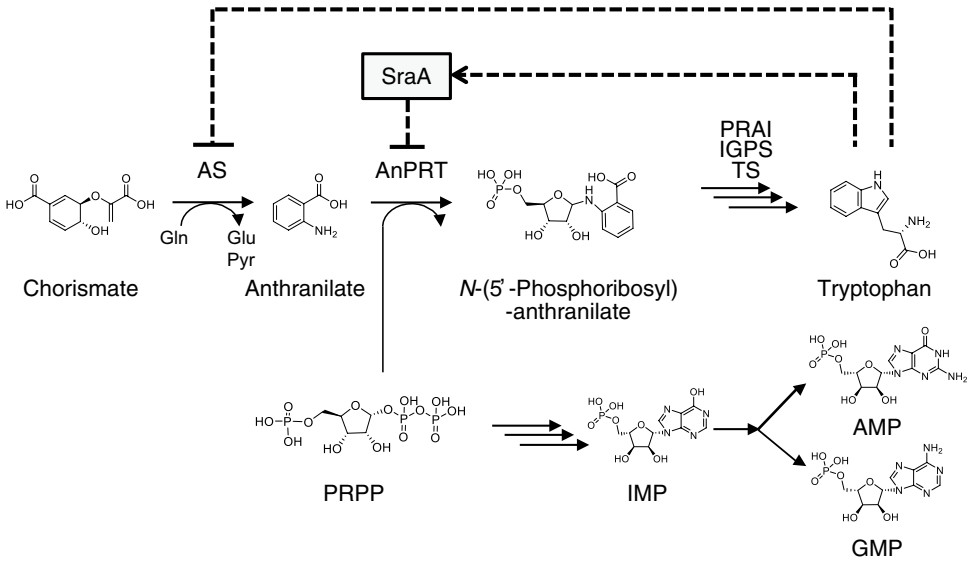

Fig. 6 Biosynthetic pathway of tryptophan and its regulation in *T. thermophilus* HB27. AS, PRAI, IGPS, and TS stand for anthranilate synthase, phosphoribosyl anthranilate isomerase, indole-3-glycerol phosphate synthase, and tryptophan synthase, respectively

tryptophan, while that of the strain carrying the *trpD* mutation was 3.6-fold more active than its wild-type counterpart in the presence of tryptophan. These findings suggest that the feedback inhibition of AnPRT is effective for controlling tryptophan biosynthesis in *C. glutamicum*.

AnPRT catalyzes the transfer of the phosphoribosyl moiety from PRPP to anthranilate. PRPP also acts as one of the key intermediates in nucleotide biosynthesis (Hove-Jensen 1989). Therefore, we assume that the inhibition of AnPRT by tryptophan through SraA contributes to some extent to the avoidance of the unfavorable consumption of PRPP under tryptophan-rich conditions (Fig. 6).

Conclusion

In the present study, we showed a novel regulatory mechanism for AnPRT mediated by the stand-alone RAM domain protein, SraA, in tryptophan biosynthesis in *T. thermophilus*. We have not yet elucidated the molecular mechanism underlying the binding of SraA to tryptophan or how tryptophan-bound SraA regulates AnPRT. We expect that further studies including crystallographic analyses of the AnPRT–SraA complex will clarify the molecular mechanisms responsible for the regulation of tryptophan biosynthesis mediated by SraA.

Acknowledgements This work was supported in part by JSPS KAKENHI Grant Nos. 24580137 (T.T.) and 15K07382 (T.T.). We thank Kaori Ohtsuki, Masaya Usui, and Aya Abe at the RRC center of RIKEN-BSI for their technical assistance with the MS analysis.

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