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博士学位論文

Structural insight into tRNA channeling on bacterial Asn-transamidosome

(真正細菌型 Asn-トランスアミドソーム
における tRNA チャネリング機構の構造基盤)

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1. Introduction

1.1 Aminoacyl-tRNA synthetase

Accurate synthesis of aminoacyl-tRNA is essential for translational fidelity. Seventeen of twenty canonical aminoacyl-tRNAs are synthesized directly by aminoacyl tRNA synthetases (aaRS), whereas glutamyl-, asparaginyl-, and cysteinyl-tRNA ($\text{Gln-tRNA}^{\text{Gln}}$, $\text{Asn-tRNA}^{\text{Asn}}$, and $\text{Cys-tRNA}^{\text{Cys}}$, respectively) are synthesized indirectly by two step process in some bacteria and archaea (Wilcox & Nirenberg, 1968, Curnow *et al.*, 1996, Sauerwald *et al.*, 2005). In the case of $\text{Asn-tRNA}^{\text{Asn}}$ formation, aspartic acid (Asp) is first mischarged on the tRNA^{Asn} by non-discriminate AspRS (ndAspRS), and then $\text{Asp-tRNA}^{\text{Asn}}$ is amidated to $\text{Asn-tRNA}^{\text{Asn}}$ by a heterotrimeric amidotransferase, GatCAB composed of GatA, GatB, and GatC subunit (Curnow *et al.*, 1996, 1997) (Fig 1-1). Similar to $\text{Asn-tRNA}^{\text{Asn}}$ synthesis, glutamic acid (Glu) is first mischarged on the tRNA^{Gln} by ndGluRS, and then $\text{Glu-tRNA}^{\text{Gln}}$ is amidated by GatCAB for $\text{Gln-tRNA}^{\text{Gln}}$ synthesis.

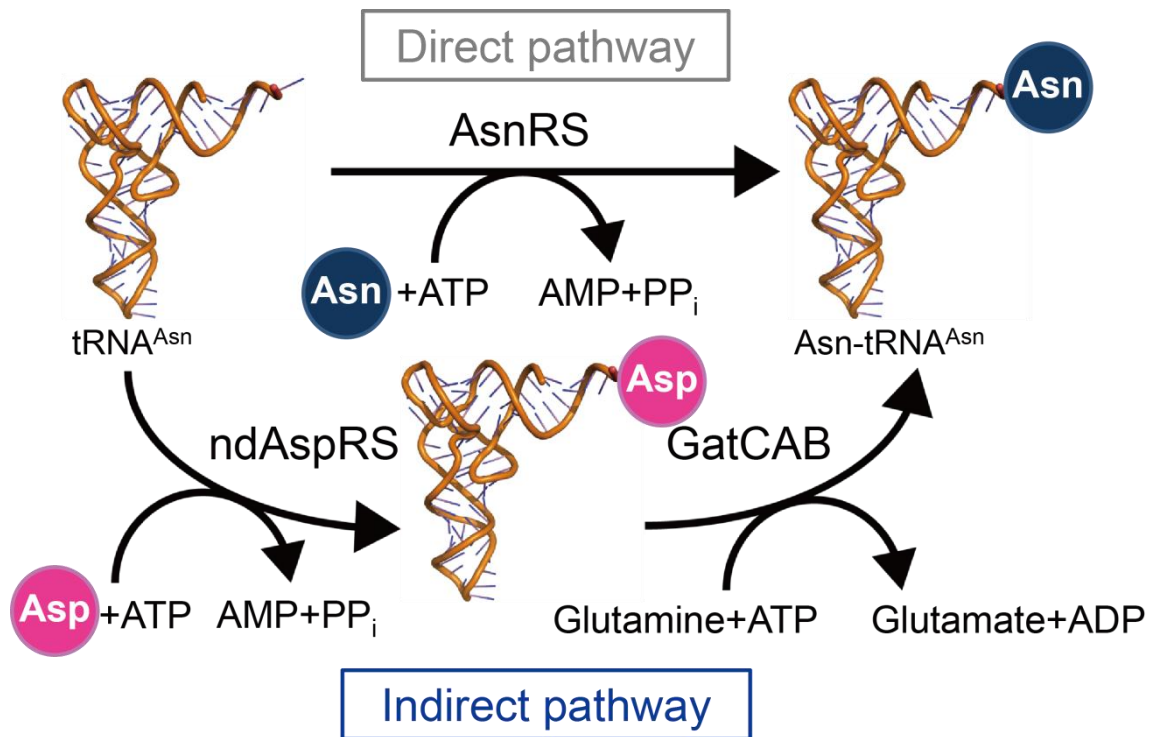


Figure. 1-1. Asn-tRNA^{Asn} synthetic pathway.

AsnRS directly synthesizes Asn-tRNA^{Asn} in direct pathway. On the other hand, initial aspartylation by ndAspRS and subsequent amidation by GatCAB synthesize Asn-tRNA^{Asn} in indirect pathway.

1.2 AspRS

In archaea and bacteria, two types of AspRS exist. One is a discriminate AspRS (dAspRS) and the other is non-discriminate AspRS (ndAspRS). The dAspRS aminoacylates only tRNA^{Asp} while the ndAspRS aminoacylates both tRNA^{Asp} and tRNA^{Asn} (Nameki *et al.*, 1992, Chuawong & Hendrickson, 2006, Bernard *et al.*, 2006, Becker & Kern, 1998) (Fig 1-2). The molecular mechanism explaining this different substrate specificity is still unclear. The species having AsnRS and lacking GatCAB gene is presumed to have dAspRS. On the other hand, the species lacking AsnRS and having GatCAB gene is presumed to have ndAspRS.

AspRS is basically composed of N-terminal anticodon binding domain and C-terminal catalytic domain (Ruff *et al.*, 1991). In addition, eukaryotic AspRS has extra sequence in upstream region of N-terminal anticodon binding domain which enhances the tRNA binding ability (Frugier *et al.*, 2000). On the other hand, bacterial AspRS has an additional ~16-kDa insertion domain named GAD domain within the catalytic domain (Delarue *et al.*, 1994) (Fig 1-3A, B). It is reported that GAD domain contacts with tRNA acceptor stem region via a water molecule but precise role of this domain is still unclear (Eiler *et al.*, 1999).

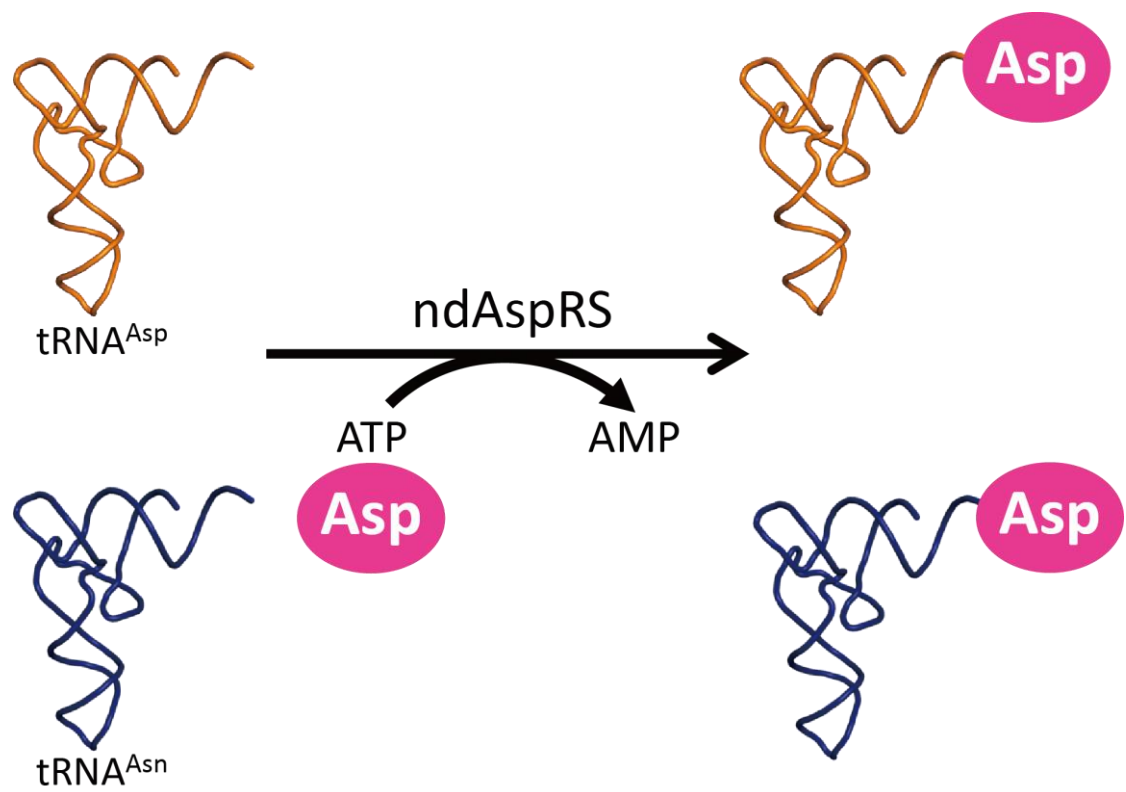


Fig. 1-2. Function of *ndAspRS*

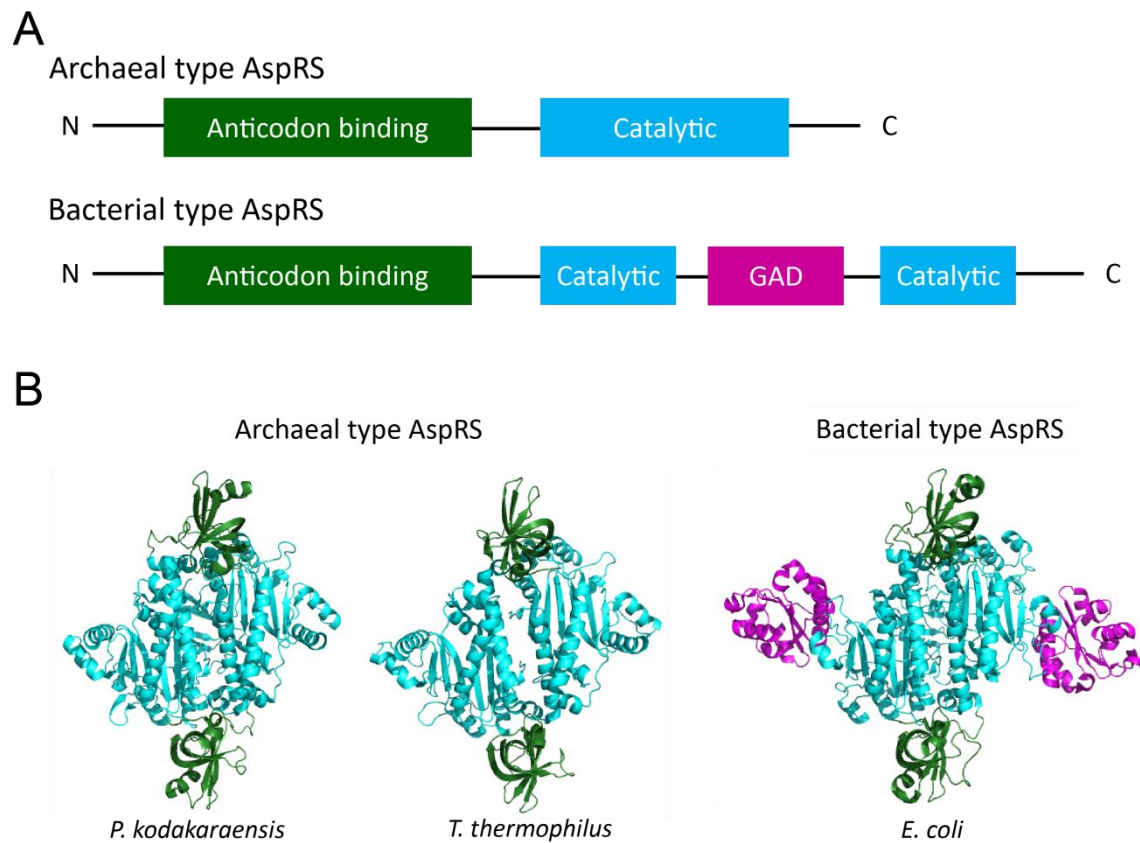


Figure. 1-3. (A) Domain structures of archaeal and bacterial type AspRS. (B) Crystal structures of archaeal and bacterial type ApRS. Each domain is colored as above domain structures.

1.3 GatCAB

GatCAB is composed of GatA, GatB, and GatC. GatA has glutaminase activity and supplies NH_3 by hydrolyzing Gln with GatB to amidate the Asp. GatB has amidase activity and tRNA binding activity. GatC is a molecular belt to stabilize overall structure of GatCAB complex. Glutaminase and amidase sites are connected by a hydrophilic ammonia channel 30 Å in length (Nakamura *et al.*, 2006) (Fig 1-4). The tRNA recognition is performed by GatB which is composed of three domains named Cradle, Helical and Tail domain (Nakamura *et al.*, 2010) . U1-A72 base pair is recognized by 3_{10} turn in Cradle domain and shoulder region of tRNA is recognized by Helical and Tail domain. In bacteria, synthesis of both Asn-tRNA^{Asn} and Gln-tRNA^{Gln} is carried out by GatCAB. On the other hand, GatCAB undertakes the Asn-tRNA^{Asn} synthesis only and GatDE which is a homologue of the GatAB does Gln-tRNA^{Gln} synthesis in archaea (Tumbula *et al.*, 2000, Sheppard *et al.*, 2008).

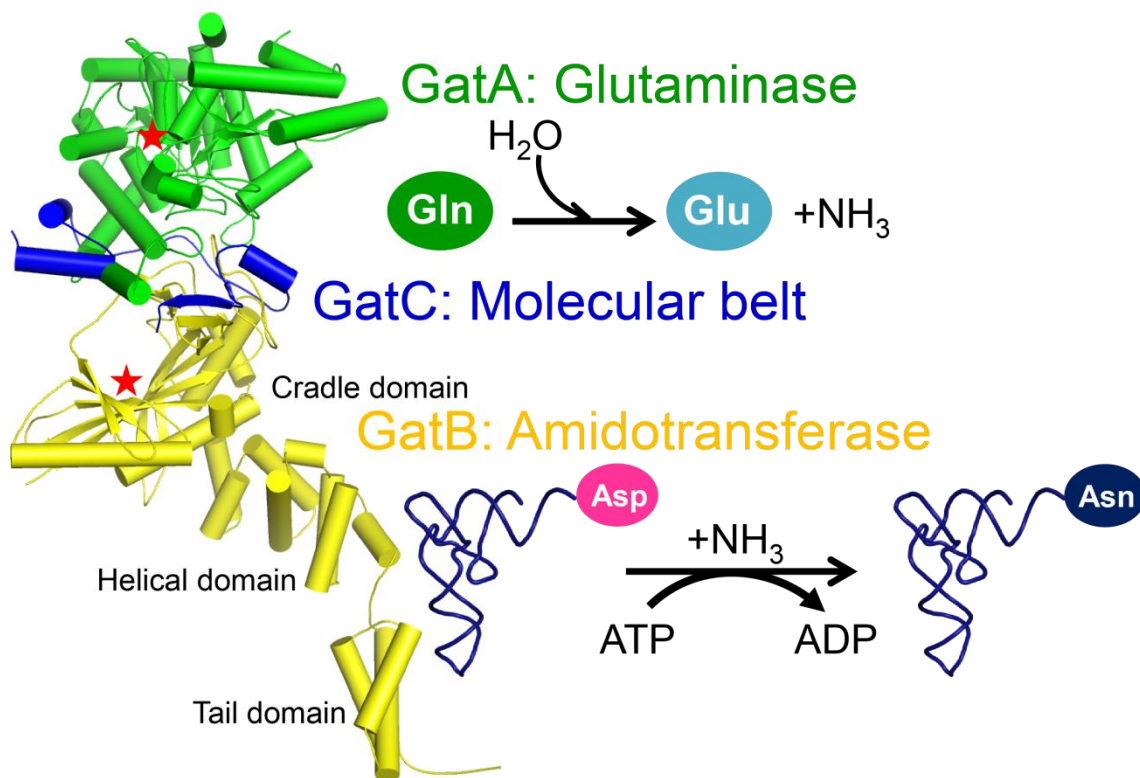


Fig. 1-4. Overall structure and function of GatCAB.

GatA, GatC and GatB are colored green, blue and yellow. The active sites of GatA and GatB are represented as red stars.

1.4 Transamidosome

During the two step process, the mistranslation occurs in ribosome and aberrant protein is synthesized if the mischarged Asp-tRNA^{Asn} is released to the cytoplasm. In fact, it is reported that the expression of ndAspRS in *E. coli* which does not have GatCAB makes its growth impossible (Chuawong & Hendrickson, 2006). Therefore, it is suggested that there is some mechanism to prevent the release of Asp-tRNA^{Asn} to cytoplasm. In accordance with this idea, it is reported that GatCAB, AspRS, and tRNA^{Asn} assemble into a ribonucleoprotein particle designated as the transamidosome and two step reaction progresses in this complex (Bailly *et al.*, 2007). A similar ribonucleoprotein particle is also formed for Gln-tRNA^{Gln} synthesis, in which GatCAB, GluRS, and tRNA^{Gln} participate (Ito & Yokoyama, 2010). Crystal structures of Asn-transamidosome from *Thermus thermophilus* (*Tt*Asn-transamidosome) (Blaise *et al.*, 2010) (Fig 1-4) and Gln-transamidosome from *Thermotoga maritime* (*Tm*Gln-transamidosome) (Ito & Yokoyama, 2010) were reported, in which two GatCAB, two archaeal-type AspRS dimers, and four tRNA^{Asn} assembled in the Asn-transamidosome though two GatCAB, one archaeal-type AspRS dimers, and two tRNA^{Asn} assembled in solution, whereas one GatCAB, one GluRS, and one tRNA^{Gln} are present in the Gln-transamidosome. It is reported that the property of bacterial Asn-transamidosome from *H. pylori* was less stable and its structure would be distinct from *Tt*Asn-transamidosome due to the presence of the GAD domain (Fischer *et al.*, 2012).

Furthermore, novel protein HP0100 takes part in the *H. pylori* Asn-transamidosome and increases the efficiency of Asp-tRNA^{Asn} transamidation (Silva *et al.*, 2013). However, Hp0100 is phylogenically limited to ϵ -proteobacteria. Therefore, the structure of Asn-transamidosome in most bacteria must differ from not only *T. thermophilus* but also the *H. pylori*.

1.5 Purpose

Bacterial AspRSs commonly possess an additional ~16-kDa insertion domain named the GAD domain, within the catalytic domain, compared with archaeal or eukaryotic counterparts (Wolf *et al.*, 1999). Superposition of bacterial AspRS on archaeal Asn-transamidosome causes steric hindrance between the GAD domain and GatCAB, suggesting that the presence of the GAD domain may cause marked structural changes. Furthermore, during the two-step process, how the aminoacylated CCA terminus of tRNA was channeled from ndAspRS to GatCAB active sites is still unclear. To obtain the insight into the molecular mechanism of the two step process and the precise role of the GAD domain, we tried the structure analysis of the γ -proteobacteria *Pseudomonas aeruginosa* Asn-transamidosome.

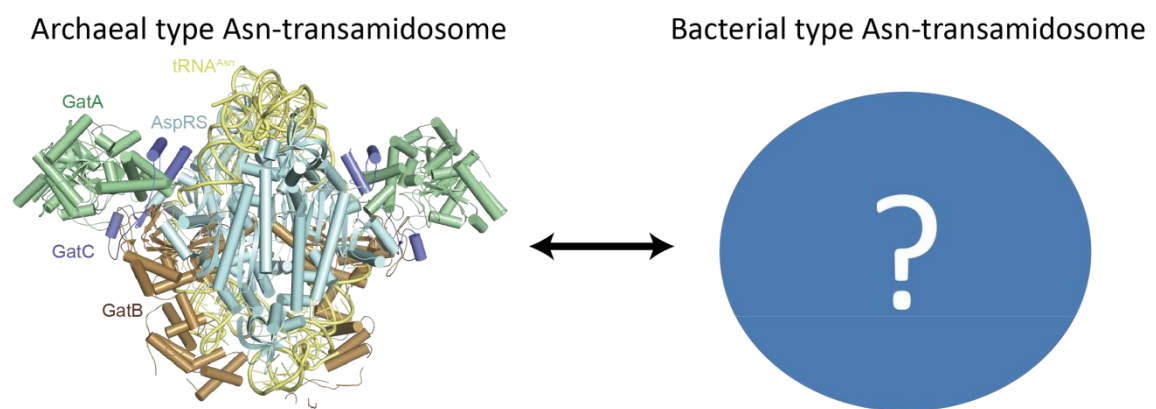


Figure. 1-5. Crystal structure of archaeal type Asn-transamidosome from *T. thermophilus*.

2. Materials and methods

2.1 Expression and purification of proteins

The genes of the GatCAB operon and ndAspRS from *P. aeruginosa* PAO1 (*Pa*) were amplified by PCR from genomic DNA. The DNA fragments encoding *Pa*GatCAB and *Pa* ndAspRS were then severally cloned between *Nde*I and *Xho*I restriction sites in modified pET28b vector with an N-terminal His-tag.

The plasmid carrying *Pa*GatCAB was transformed into *Escherichia coli* strain B834 (DE3)-pRARE2 by electroporation, and 3-L cultures were grown in LB media containing 25 µg/mL kanamycin and 34 µg/mL chloramphenicol at 310 K until OD₆₀₀ = 0.6. The cultures were then induced by addition of isopropyl-β-thiogalactose (IPTG) to a final concentration of 10 µM, and shifted to 298 K for approximately 20 h before harvesting. The cells were harvested by centrifugation at 4500 × *g* for 25 min at 277 K and resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1 mM β-mercaptoethanol (BME), 0.5 mg/mL lysozyme, 0.1 mg/mL DNase). After sonication and centrifugation at 40000 × *g* for 30 min, the His₆-tagged protein was purified by immobilized metal-ion affinity chromatography using a HisTrap HP column (GE Healthcare). The sample was washed with 15 mM imidazole and eluted with 20 – 250 mM imidazole gradient in buffer B (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1 mM BME). Then, the collected fractions were dialyzed

against buffer C (25 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 10% glycerol, 1 mM BME) and purified on a HiTrap Q HP column (GE Healthcare) by elution with 0 – 800 mM NaCl gradient in buffer C. The collected fractions were diluted twice with buffer D (25 mM HEPES-NaOH, pH 7.5, 5 mM MgCl₂, 5% glycerol, 2 M (NH₄)₂SO₄, 1 mM BME) and further purified on a HiTrap Phenyl column (GE Healthcare) by elution with a gradient of 1 – 0 M (NH₄)₂SO₄. Finally, the protein was loaded onto HiLoad 26/60 Superdex 200pg (GE Healthcare) equilibrated with buffer E (25 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, and 1 mM DTT). The protein was concentrated to 27 mg/mL using an Amicon Ultra 30000 MWCO (Millipore), flash-frozen, and stored at 193 K.

As *Pa* ndAspRS is toxic for the *E. coli* expression system by causing the depletion of free tRNA^{Asn} by mischarging with Asp (Bernard *et al.*, 2006), we used the co-expression method for preparing *Pa* ndAspRS. A plasmid encoding *Pa* ndAspRS was co-transformed into *E. coli* strain B834 (DE3)-pRARE2 by electroporation with a plasmid encoding GatCAB from *Staphylococcus aureus* (*Sa*) in modified pCDFDuet-1 vector without any tag. Culture was performed according to a method similar to that described for *Pa*GatCAB, but LB medium containing 25 µg/mL kanamycin, 34 µg/mL chloramphenicol, and 50 µg/mL streptomycin was used. After sonication and centrifugation at 40000 × g for 30 min, the His₆-tagged protein was purified by immobilized metal-ion affinity chromatography on a HisTrap HP column (GE

Healthcare). The sample was washed with 20 mM imidazole and eluted with a gradient of 20 – 250 mM imidazole in buffer B. The collected fractions were then diluted to 75 mM NaCl with buffer C and purified on a HiTrap Heparin HP column (GE Healthcare) by elution with a gradient of 75 – 800 mM NaCl in buffer C. Finally, the protein was loaded onto HiLoad 16/60 Superdex 200pg (GE Healthcare) equilibrated with buffer E. The protein was concentrated to 16 mg/mL using an Amicon Ultra 30000 MWCO (Millipore), flash-frozen, and stored at 193 K.

2.2 Preparation of tRNA^{Asn}

PatRNA^{Asn} was transcribed *in vitro* as described previously (Nakamura *et al.*, 2006). The oligo DNAs used to construct *P. aeruginosa* tRNA^{Asn} were as follows: 5'-primer: TAATACGACTCACTATATCCGCGATAGCTCAGTC (forward); middle template: CACTATATCCGCGATAGCTCAGTCGGTAGAGCAAATGACTGTTAATCATTGGGTCCCT GGTTCGAGTCC (forward); 3'-primer: TGmGCTCCGCGACCTGGACTCGAACCAGGGAC (reverse); in which the T7 promoter sequence is underlined. Gm represents 2'-O-methyl modification, used for manipulating the uniform 3'-end of transcribed products (Sherlin *et al.*, 2001). Transcribed tRNAs were purified by denaturing urea-polyacrylamide gel electrophoresis and extracted with an Elutrap Electroelution system (Whatman). Pooled tRNAs were precipitated with ethanol and resuspended in buffer F (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 20 mM MgCl₂). tRNAs were heated at 353 K for 5 min, then slowly cooled at room temperature over 5 min and purified on a Resource Q column (GE Healthcare) by elution with a gradient of 300 – 700 mM NaCl in buffer G (20 mM Tris-HCl, pH 7.6, 8 mM MgCl₂). Pooled fractions were precipitated with ethanol and stored at –30°C.

2.3 Electrophoretic mobility shift assay (EMSA)

EMSA was performed in 6 μ L reaction mixture containing 20mM HEPES-NaOH, pH7.5, 5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 20 or 40 μ M enzyme and 20 μ M in vitro transcribed *P. aeruginosa* tRNA^{Asn}. Reaction mixtures were incubated at 298 K for 30 min, and were loaded on a 5% non-deneaturing polyacrylamide gel composed of 43 mM imidazole, 35 mM HEPES, 10 mM magnesium acetate, 1 mM DTT, and run at 277 K for 3 hour at a constant 60 V. The gel was stained with ethidium bromide.

2.4 Gel filtration assay

Samples for Gel filtration assay were prepared in 150ul reaction mixture containing 20mM Hepes-KOH pH7.2, 5mM MgCl₂, 5% glycerol, 1 mM DTT, 2mM ATP, 50uM ndAspRS monomer, 50uM GatCAB, 50uM tRNA^{Asn}. Reaction mixtures were incubated at 298 K for 30 min, and were loaded to a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with buffer H (20mM Hepes-KOH pH7.2, 5mM MgCl₂, 5% glycerol, 1 mM DTT).

2.5 Crystallization and data collection

Asn-transamidosome was prepared by mixing ndAspRS dimer, GatCAB, and tRNA^{Asn} each at a final concentration of 4.8 μ M in buffer I (20 mM HEPES-NaOH, pH7.5, 10 mM MgCl₂, 5% glycerol). For crystallization, 500 μ L of Asn-transamidosome solution was concentrated to 120 μ L using an Amicon Ultra-0.5 Centrifugal Filter Device (Millipore).

Crystallization was carried out by the sitting-drop vapor-diffusion method in 96-well plates at 293 K. Initial screening for the crystallization conditions of Asn-transamidosome was performed with crystal screen kits of JCSG+ Suite and PACT Suite (Qiagen) by mixing 1 μ L of Asn-transamidosome solution with an equivalent volume of reservoir solution. Crystals of Asn-transamidosome were obtained with reservoir solution containing 100 mM MES-NaOH (pH 6.0), 200 mM lithium sulfate, and 25% (w/v) PEG3350 (Fig 2-1B). Although we prepared the sample as Asn-transamidosome, crystals of ndAspRS-tRNA^{Asn} binary complex were obtained with reservoir solution containing 1.4 M Tri-sodium citrate pH 6.7 (Fig 2-1A).

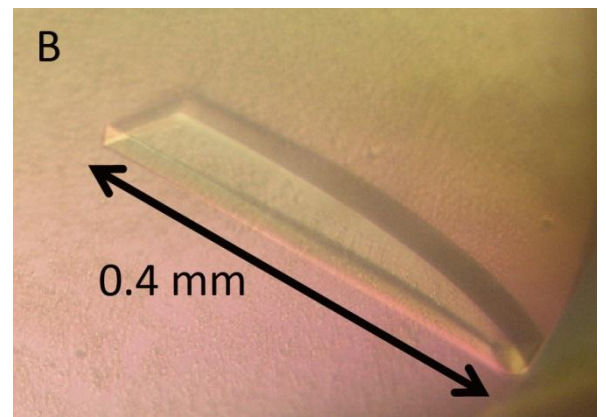
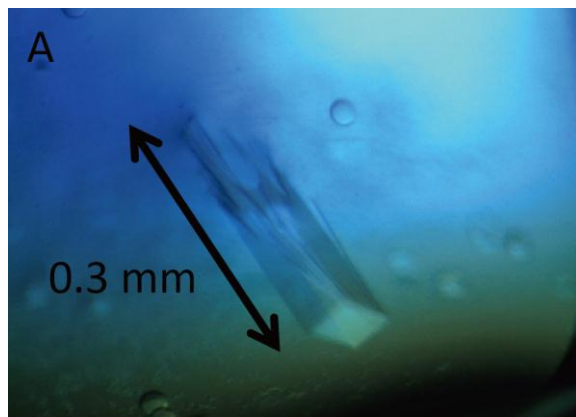


Fig. 2-1. (A) Crystal of ndAspRs-tRNA^{Asn} binary complex.

(B) Crystal of Asn-transamidosome.

2.6 Data collection, structure determination and refinement

For the X-ray diffraction experiment, the crystals of ndAspRS-tRNA^{Asn} binary complex were soaked with cryoprotectant consisting of reservoir solution containing 8% glycerol before flash cooling under cryostream. Diffraction data were collected on beamline BL44XU of SPring-8 (Harima, Japan) (Fig 2-2A). The data were indexed, integrated, scaled, and merged using *XDS* (Kabsch, 2010). The statistics of data collection are summarized in Table 1. The crystal belonged to the *P*4₁22 space group with unit cell parameters *a*=*b*=157.9, *c*=146.3. The phases for ndAspRS-tRNA^{Asn} binary complex were determined by the molecular replacement method using *Phaser* (McCoy *et al.*, 2007). The coordinates of dAspRS-tRNA^{Asp} complex from *E. coli* (PDB entry 1c0a; Eiler *et al.*, 1999) were used as a search model. The single solution of ndAspRS-tRNA^{Asn} binary complex was successfully obtained with final TFZ score of 30. Initial model was refined manually with *coot* (Emsley & Cowtan, 2004). Structure refinement was performed using *phenix.refine* (Afonine *et al.*, 2012) and *autoBUSTER* (Blanc *et al.*, 2004) with secondary structure restraints. After several times of refinement, *R*_{work} and *R*_{free} factors converged to 19.56% and 23.13%, respectively.

The crystals of Asn-transamidosome were soaked with cryoprotectant consisting of reservoir solution containing 15% glycerol before flash cooling under cryostream. Diffraction data were collected on beamline BL41XU of SPring-8 (Harima, Japan) (Fig 2-2B). The data were indexed,

integrated, scaled, and merged using *XDS* (Kabsch, 2010). The statistics of data collection are summarized in Table 1. The crystals belonged to the $P2_1$ space group with unit cell parameters $a=93.1$, $b=185.7$, $c=290.4$, $\beta=93$. The phases for Asn-transamidosome complex were determined by the molecular replacement method using *Phaser* (McCoy *et al.*, 2007). The coordinates of GatCAB from *Staphylococcus aureus* (PDB entry 2g5h; Nakamura *et al.*, 2006), AspRS dimer from *E. coli* (PDB entry 1eqr; Rees *et al.*, 2000), and tRNA^{Asp} in dAspRS-tRNA^{Asp} complex from *E. coli* (PDB entry 1c0a; Eiler *et al.*, 1999) were used as search models, respectively. The four solutions of GatCAB and two solutions of ndAspRS dimer were successfully obtained with final TFZ score of 28.9, although no solution was obtained for tRNA molecules. The models of GatCAB and ndAspRS were subjected to rigid-body refinement using *phenix.refine* (Afonine *et al.*, 2012) and followed by several cycles of jelly-body refinement with local NCS restraints by *REFMAC5* (Murshudov *et al.*, 2011). The resultant $2F_o-F_c$ and F_o-F_c electron density maps clearly showed four tRNA molecules and models of tRNA (PDB entry 1c0a) were placed at each site. Structure refinement was performed using *phenix.refine* and autoBUSTER (Blanc *et al.*, 2004) with noncrystallographic symmetry restraints and secondary structure restraints. The resultant model was refined manually with *coot* (Emsley & Cowtan, 2004). After several times of refinement, R_{work} and R_{free} factors converged to 29.21% and 32.95%, respectively.

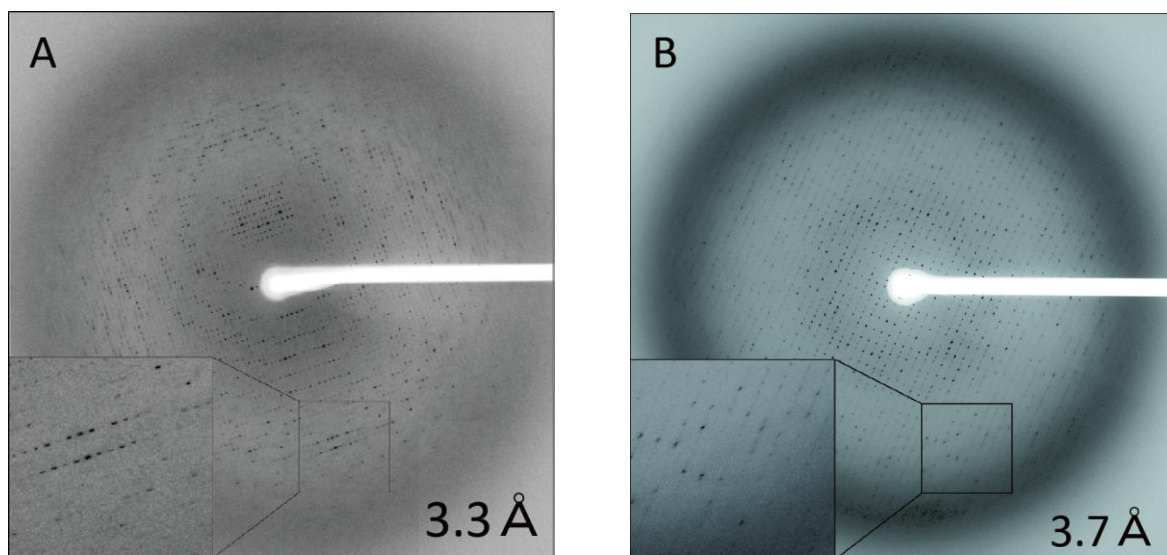


Fig. 2-2. (A) Diffraction image of ndAspRS-tRNA^{Asn} binary complex crystal.

(B) Diffraction image of Asn-transamidosome crystal.

Table 1

Statistics of data collection

Values in parentheses are for the highest resolution shell.

Dataset	ndAspRS-tRNA ^{Asn} binary complex	Asn-transamidosome
Beamline	BL44XU, SPring-8	BL41XU, SPring-8
Detector	MX225HE	MX225HE
Crystal-to-detector distance (mm)	450	320
Wavelength (Å)	0.9000	1.0000
No. of images	600	180 [†]
Oscillation range (°)	0 – 180	0 – 180
Resolution range (Å)	47.26 – 3.29	48.12 – 3.70 (3.93 – 3.70)
No. of total reflections	415324 (64947)	251360 (33868)
No. of unique reflections	28534 (4481)	96512 (14673)
Space group	<i>P</i> 4 ₁ 22	<i>P</i> 2 ₁
Unit cell parameters (Å, °)	<i>a</i> = <i>b</i> = 157.91, <i>c</i> = 146.26	<i>a</i> = 93.1, <i>b</i> = 185.7, <i>c</i> = 290.4, <i>β</i> = 93.0
Completeness (%)	99.8 (99.5)	92.1 (87.5)
$R_{\text{meas}}^{\ddagger}$ (%)	16.8 (184.2)	18.4 (57.2)
$\langle I/\sigma(I) \rangle$	14.53 (1.92)	6.36 (2.15)
Mosaicity (°)	0.191	0.509
Wilson B-factor (Å ²)	100.16	55.78
Refinement		
$R_{\text{work}} / R_{\text{free}}$	19.56 / 23.13	29.21 / 32.95

[†]55 out of 180 frames were omitted in data processing.

$R_{\text{meas}}^{\ddagger} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ and $N(hkl)$ are the mean intensity of a set of equivalent reflections and the multiplicity, respectively.

3. Results

3.1 The confirmation of the formation of *Pa*Asn-transamidosome

In order to confirm whether *Pa*Asn-transamidosome is formed by mixing components of AspRS, GatCAB and tRNA^{Asn}, gel mobility shift and gel filtration assay were performed. The results of gel shift assay showed that the additional band was appeared when the concentration of each component was higher than 20 μ M, indicating the formation of *Pa*Asn-transamidosome (Fig 3-1). In the gel filtration assay, a new peak which can be considered as *Pa*Asn-transamidosome was appeared when the concentration of each component was higher than 50 μ M. The sample for crystallization of *Pa*Asn-transamidosome was prepared based on these complex formation conditions.

tRNA ^{Asn} (μM)	20	20	20	20	20
GatCAB (μM)		40		20	20
AspRS (μM)			40	20	40

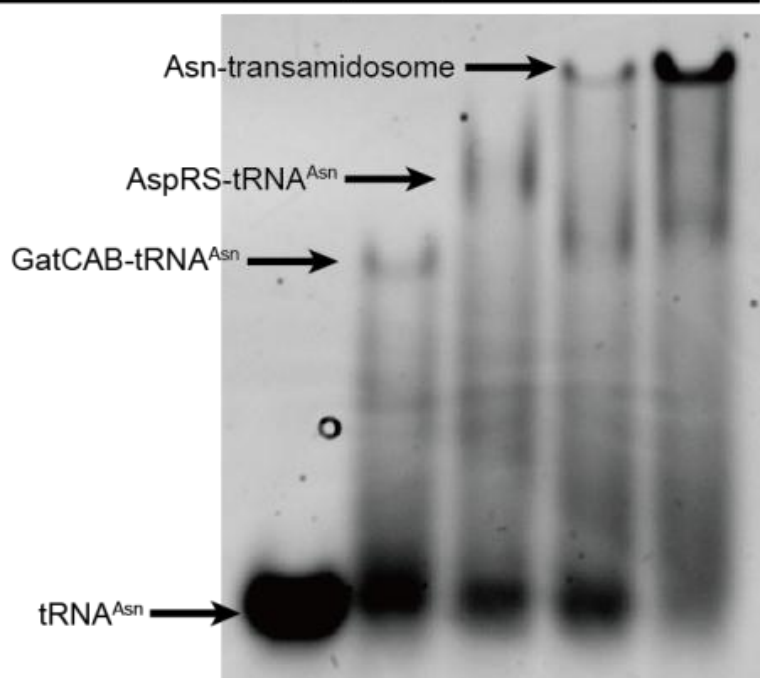


Fig. 3-1. Gel mobility shift assay.

The final concentrations of samples are indicated at the top.

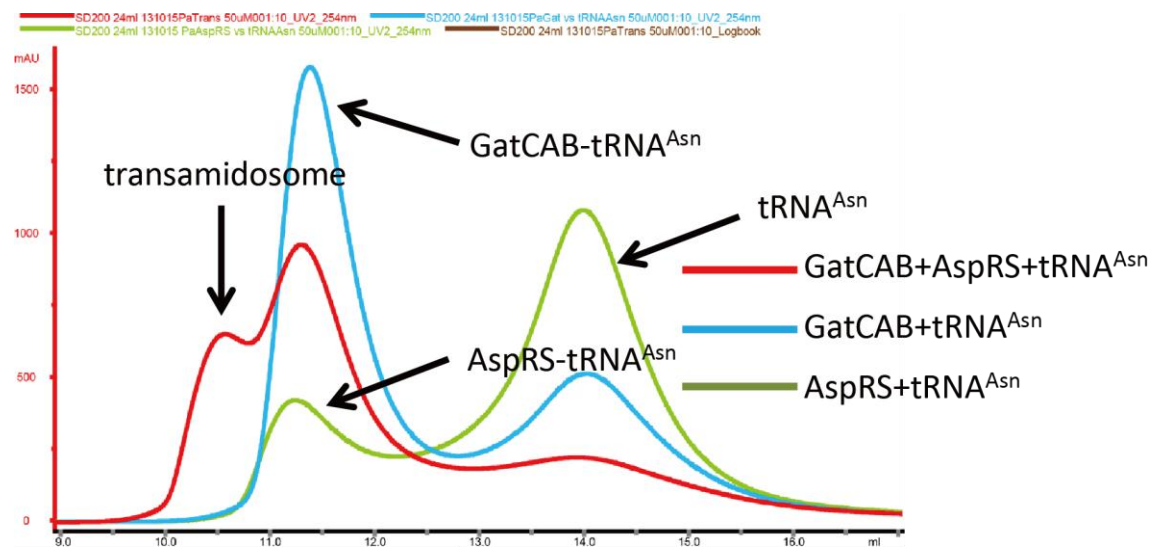


Fig. 3-2. Gel filtration assay to confirm the formation of Asn-transamidosome.

Green line: mixture of AspRS and tRNA^{Asn}

Blue line: mixture of GatCAB and tRNA^{Asn}

Red line: mixture of AspRS, GatCAB and tRNA^{Asn}

3.2 Overall structure of ndAspRS-tRNA^{Asn} binary complex from *P. aeruginosa*

Obtained crystals of ndAspRS-tRNA^{Asn} complex were exposed to X-ray using beam-line BL44XU on SPring-8, and 3.3 Å resolution data set was collected (Table 1). The structure of ndAspRS-tRNA^{Asn} complex was determined by molecular replacement method using dAspRS-tRNA^{Asp} complex structure from *E. coli* (PDB ID: 1C0A) as a search model. Interestingly, only one ndAspRS molecule and one tRNA^{Asn} exists in an asymmetric unit (Fig 3-3B). The ndAspRS dimer which is the biological unit of AspRS was formed by crystallographic 2-fold axis (Fig 3-3A). Similar to the bacterial dAspRS structure, the structure of ndAspRS consists of N-terminal anticodon binding domain, C-terminal catalytic domain, GAD domain which is appended to catalytic domain, and hinge region connecting anticodon binding and catalytic domain. The acceptor stem and CCA terminus of tRNA^{Asn} was recognized by catalytic domain, while anticodon of tRNA^{Asn} was recognized by anticodon binding domain. Moreover, there is a blob of Fo-Fc electron density map in active site of ndAspRS (Fig 3-4). Structural comparison with ndAspRS-Asp complex (PDBID: 4O2D) shows that the position of this blob corresponds to the Asp molecule, indicating that an Asp molecule was bound to ndAspRS-tRNA^{Asn}. Because, the Asp was not added to the crystallization sample, this Asp was probably captured from disrupted *E. coli* cell extract.

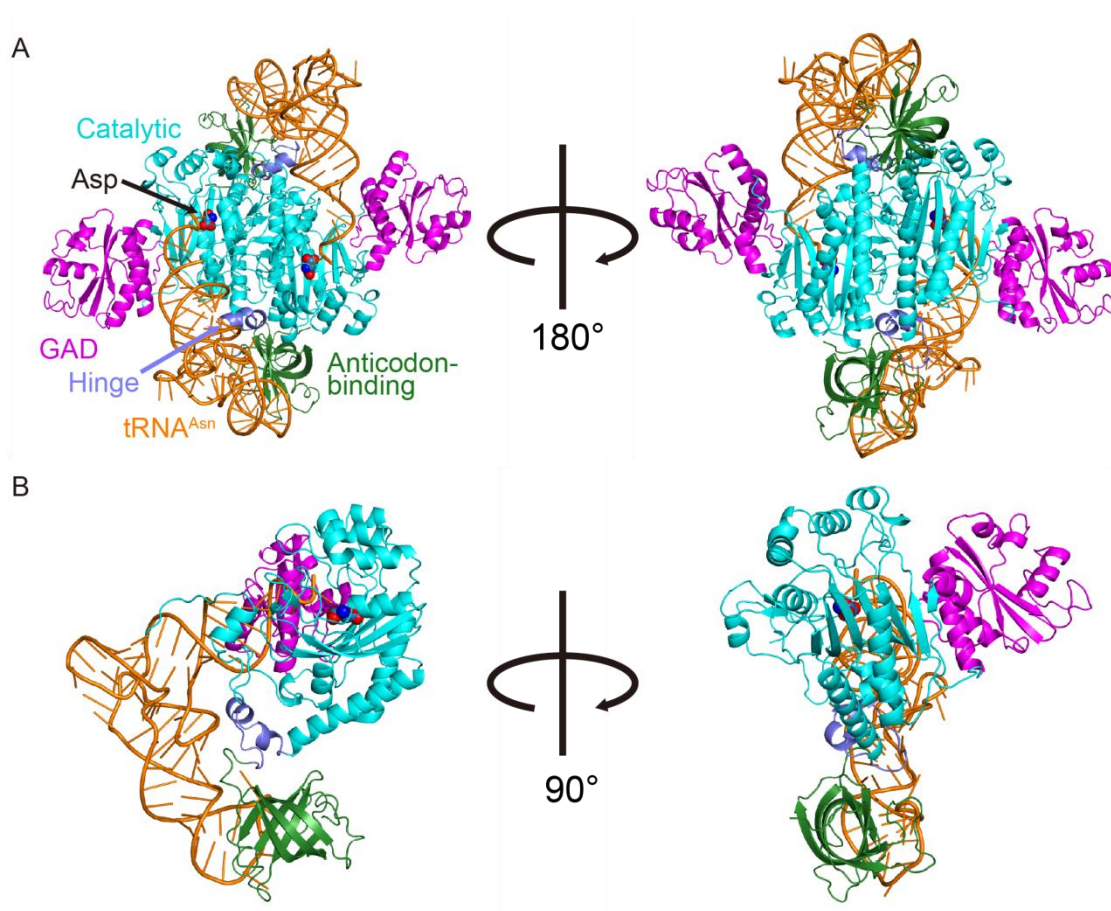


Fig. 3-3. Overall structure of ndAspRS-tRNA^{Asn} binary complex from *P. aeruginosa*

(A) Dimeric structure of ndAspRS-tRNA^{Asn} binary complex. Each monomer is related by crystallographic 2-fold axis. (B) Monomeric structure of ndAspRS-tRNA^{Asn} binary complex in an asymmetric unit. The anticodon binding domain, catalytic domain, insertion GAD domain, and hinge region of ndAspRS are colored green, cyan, magenta, and blue. tRNA^{Asn} is colored orange.

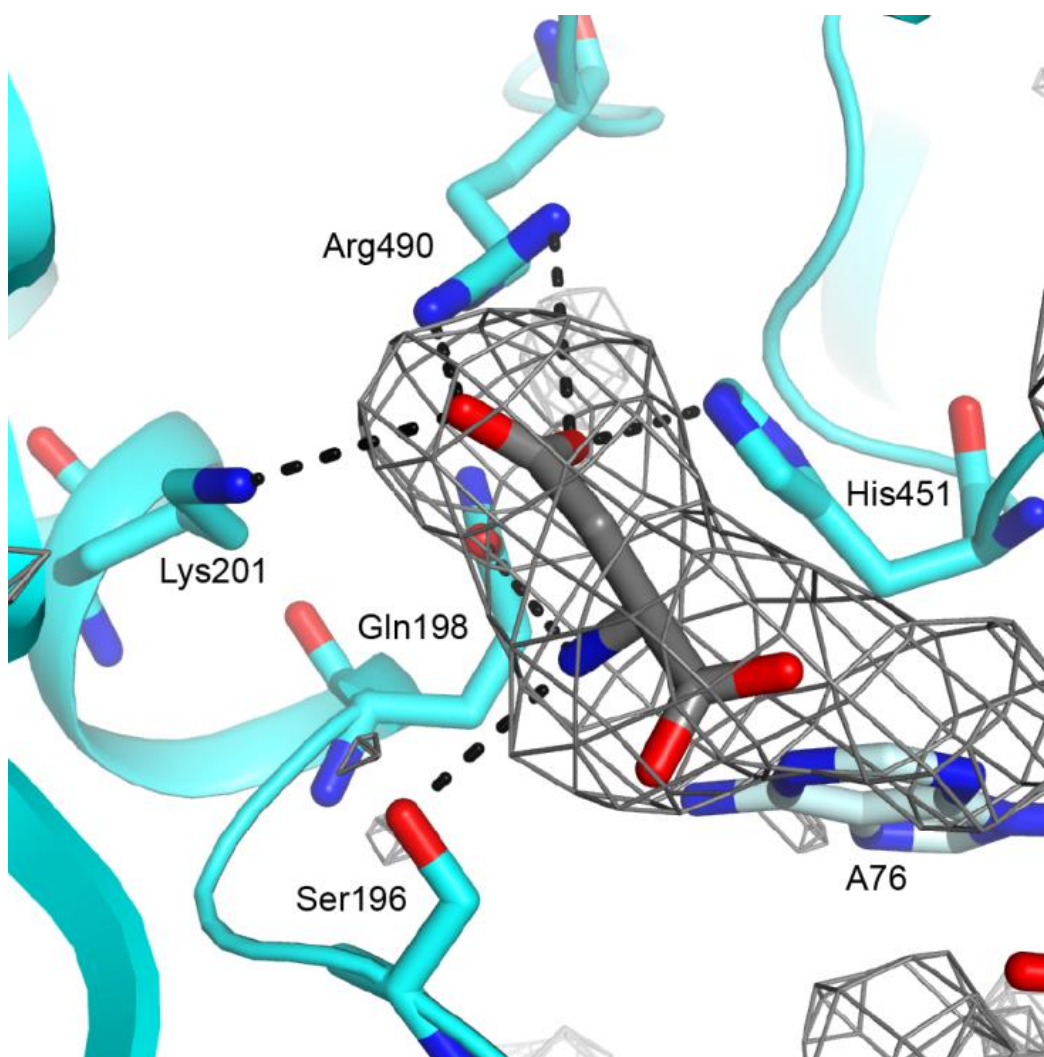


Fig. 3-4. Aspartate recognition by ndAspRS from *P. aeruginosa*.

$F_o - F_c$ map contoured at 3.0σ is colored gray. Hydrogen bonds are represented as dash lines.

ndAspRS-tRNA^{Asn} binary complex is colored cyan and aspartate molecule is colored gray.

3.3 Recognition of tRNA^{Asn} by ndAspRS

ndAspRS can recognize both tRNA^{Asp} and tRNA^{Asn} while dAspRS can only tRNA^{Asp}. The apparent difference between tRNA^{Asp} and tRNA^{Asn} is sequence of anticodon. That is 34GUC36 for tRNA^{Asp} while 34GUU36 for tRNA^{Asn}.

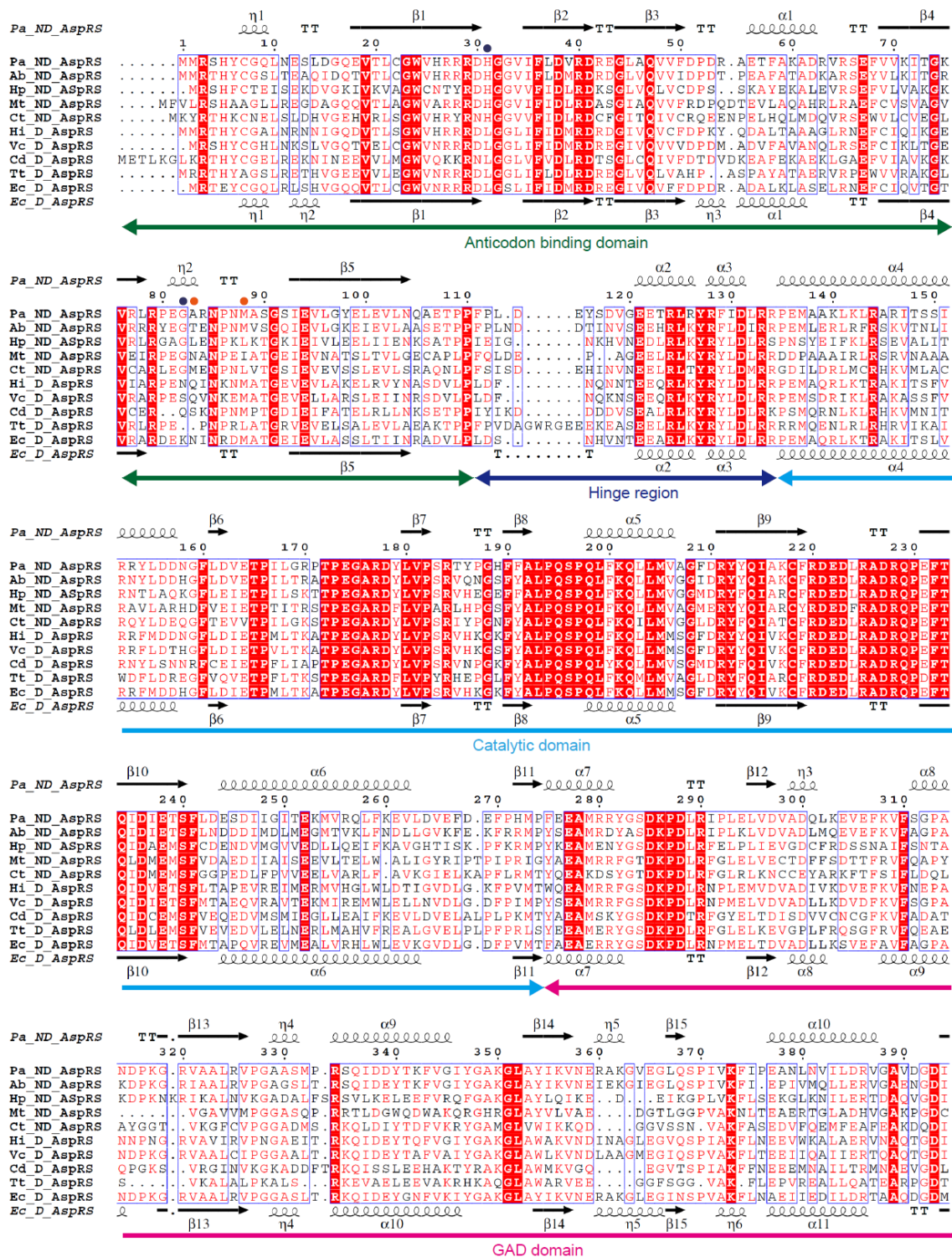
The structure of *Pa* ndAspRS-tRNA^{Asn} binary complex showed that N1 and N2 of G34 formed hydrogen bonds with side chain of Glu94, and O6 formed hydrogen bonds with side chain of Arg77. Furthermore, guanine base is involved in stacking interaction with Phe36. O2 and N3 of U35 formed hydrogen bonds with side chains of Arg29 and Gln47, and O4 formed hydrogen bonds with side chains of Gln47 and Arg79. O2 of U36 formed hydrogen bonds with side chains of Arg79 and Asn85, and N3 formed hydrogen bond with main chain carbonyl group of Ala83. Taken together, the recognition of G34 and U35 by *Pa* ndAspRS is almost the same as that of *Ec* dAspRS and the both C36 and U36 bases are recognized by the same residues between d and ndAspRS (Eiler *et al.*, 1999) (Fig.3-6A, B). This anticodon recognition manner suggests another identity elements are needed for differentiating tRNA^{Asp} and tRNA^{Asn}.

Several residues in the anticodon binding domain giving ndAspRS the tRNA^{Asn} recognition property have been reported (Bernard *et al.*, 2006, Chuawong & Hendrickson, 2006) (Fig 3-5). Among these residues, most conserved residue is a H31 and its counterpart in dAspRS is a leucine. The H31L mutant kept the comparable activity to wild type for *Ec* tRNA^{Asp}, whereas

reduced the activity by 0.28-fold for *Ec* tRNA^{Asn}. The imidazole ring of His31 stacked with the adenine base of A38 in the crystal structure of *Pa* ndAspRS-tRNA^{Asn} while the side chain of Leu30 contacts C38 in that of *Ec* dAspRS-tRNA^{Asp} (Fig.3-6C, D). Furthermore, NE2 of His31 hydrogen bonded with O4' of U35 and this interaction seems to increase the affinity for tRNA^{Asn}. Superposition of *Ec* dAspRS-tRNA^{Asp} on *Pa* ndAspRS-tRNA^{Asn} structure according to three β -strand region in anticodon binding domain of AspRS (residues 18-50 in *Pa* ndAspRS) suggested that H31 in *Pa* ndAspRS can also stack with C38 of tRNA^{Asp} by rotating its imidazole ring. On the other hand, leucine residue in *Ec* dAspRS forms hydrophobic interaction with C38 of tRNA^{Asp} but this interaction may be weakened and insufficient for recognizing A38 of tRNA^{Asn} because of being distant. To elucidate the details of the recognition mechanism, the structure of ndAspRS complexed with tRNA^{Asp} will be indispensable.

The A76 conformation of tRNA^{Asn} in *Pa* ndAspRS-tRNA^{Asn} binary structure is different from that of tRNA^{Asp} in *Ec* dAspRS-tRNA^{Asp} and inactive *Ec* dAspRS-yeast tRNA^{Asp} heterologous complex structure (Moulinier *et al.*, 2001). In the *Ec* dAspRS-tRNA^{Asp} structure, A76 sits between flipping loop (residues 170-176 in *Pa* ndAspRS) and aspartyl-adenylate and its 3'-OH group locates near the α -carbonyl carbon of aspartyl-adenylate for the nucleophilic attack. N6 of adenine base is recognized by Ser193 in LXQ(S/A)PQXXKQ motif and N7 is recognized

by Thr169 and Pro170 in the flipping loop, which forms open conformation (Fig 3-7B). The flipping loop is considered as the door which only opens when the right tRNA is bound to the synthetase. In *Ec* dAspRS-yeast tRNA^{Asp} heterologous structure, the terminal adenosine is stabilized by Met447 and Glu482 and points outward from the active site by the rotation of the backbone dihedral angle. In this structure, aspartic moiety of aspartyl-adenylate interacts with Glu171 in flipping loop, which forms closed conformation (Fig 3-7C). In *Pa* ndAspRS-tRNA^{Asn} structure, 2'-OH group of A76 interacts with Ala448 and His451 in histidine loop (residues 438-451 in *Pa* ndAspRS) and adenine base pointed outward from the active site. However, the conformation of the flipping loop was similar to the open conformation without any interactions with adenine base of A76 and aspartate (Fig 3-7A). Subsequent ATP binding and formation of aspartyl-adenylate may induce the shift of A76 to the active site.



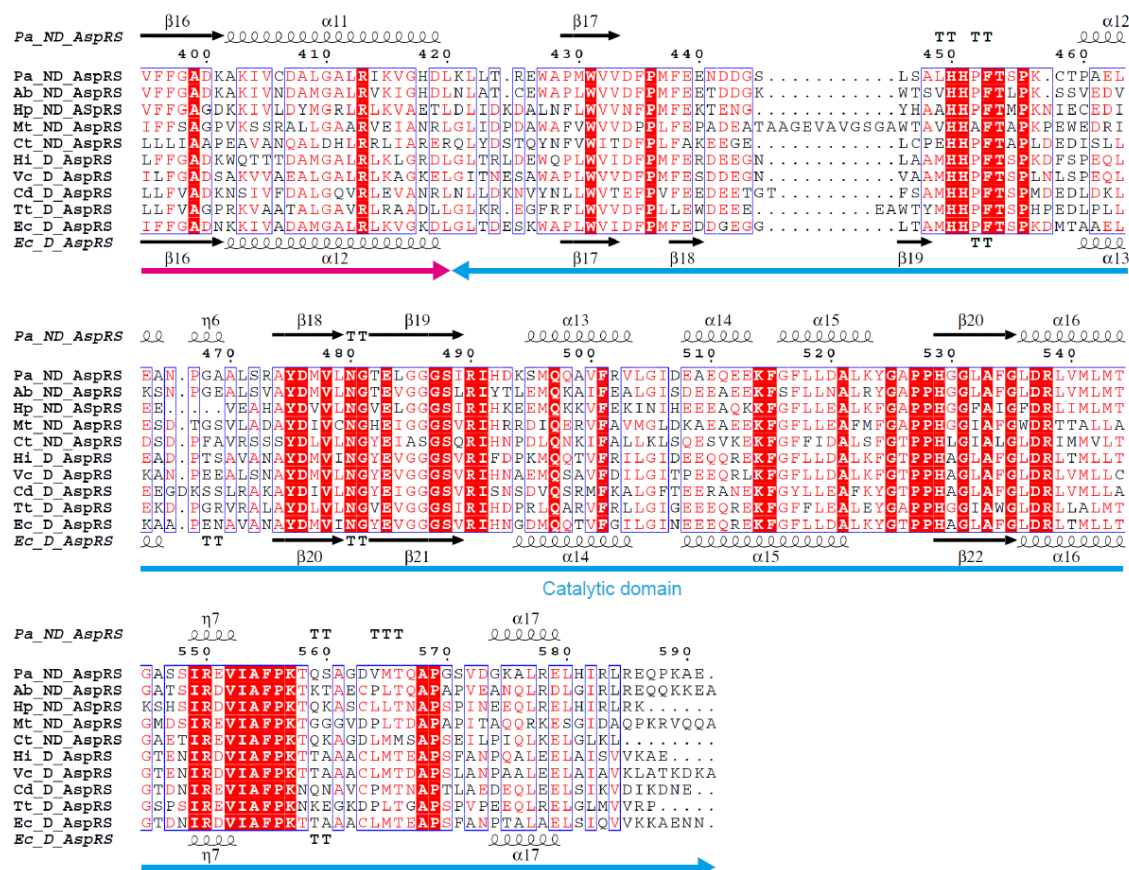


Fig. 3-5. Multiple alignments of bacterial AspRS

The blue rounds mark the residues of ndAspRS from *P. aeruginosa* used for mutant analysis.

The red rounds mark the residues of ndAspRS from *H. pylori* used for mutant analysis.

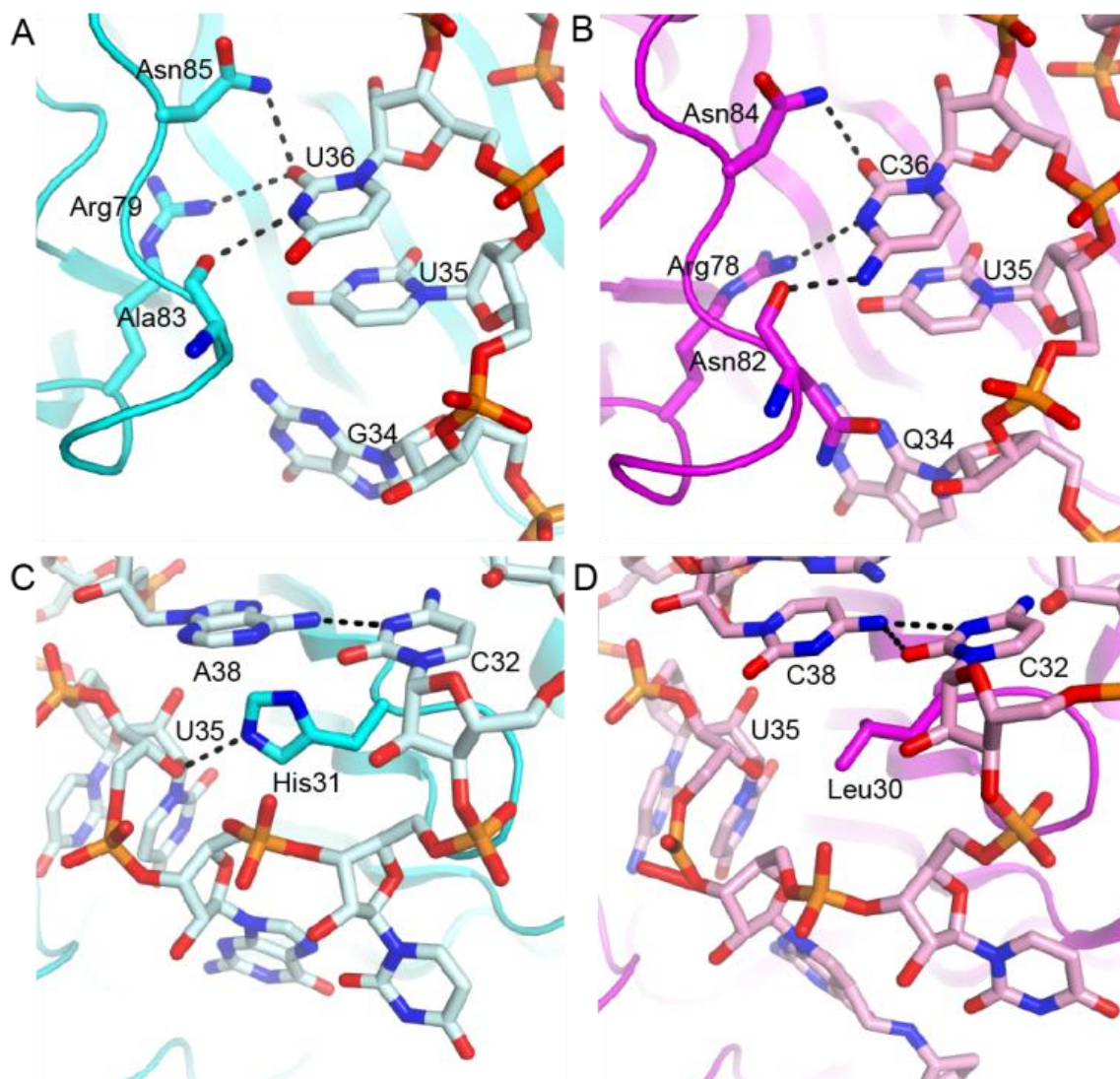


Fig. 3-6. The anticodon loop recognition by anticodon binding domain of nd and dAspRSs.

The residues recognizing respective base are shown as stick and hydrogen bonds are shown as dashed line. (A) The U36 recognition by *Pa* ndAspRS. (B) The C36 recognition by *Ec* dAspRS. (C) The U35 and A38 recognition by *Pa* ndAspRS. (D) The C38 recognition by *Ec* dAspRS.

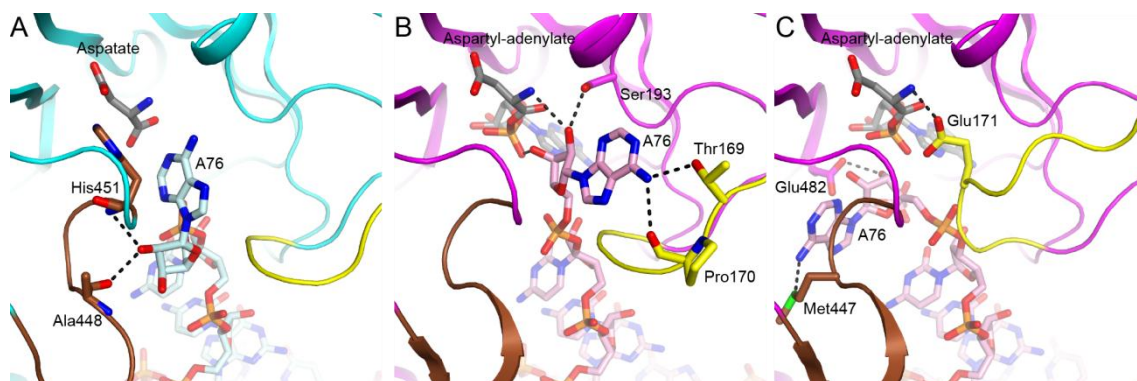


Fig. 3-7. The conformation of A76 and flipping loop in (A) the *Pa* ndAspRS-tRNA^{Asn} complex, (B) the *Ec* dAspRS-tRNA^{Asp} complex, (C) the *Ec* dAspRS-yeast tRNA^{Asp} heterologous complex. The flipping and the histidine loops are colored yellow and brown, respectively. The aspartate and aspartyl-adenylate are colored gray. The hydrogen bonds are shown as dashed line.

3.4 Overall structure of *PaAsn*-transamidosome

The crystal of *PaAsn*-transamidosome was exposed to X-ray using beam-line BL41XU on SPring-8, and 3.7 Å resolution data set was collected (Table 1). The structure of *PaAsn*-transamidosome was determined by molecular replacement method. There are two *PaAsn*-transamidosomes in an asymmetric unit. Each *PaAsn*-transamidosome is consisted of one AspRS dimer, two GatCAB complexes and two tRNA^{Asn} molecules (Fig 3-8A). One ndAspRS protomer, one GatCAB complex, and one tRNA^{Asn} molecule formed one catalytic unit (Fig 3-8 B). Two catalytic units in one Asn-transamidosome are related by non-crystallographic 2-fold axis which existed at the interface of two ndAspRS protomers.

The direct interactions between ndAspRS and GatCAB were limited in narrow area like *TtAsn*-transamidosome and *TmGln*-transamidosome (Fig 3-9A). Basic residues like R583 and R585 in the C-terminal loop of AspRS (residue 580-591) formed ionic and hydrogen bond interactions with acidic residues like E236, E239 and D240 in α6 helix and β-hairpin composed of β13 and 14 strands (residue 220-262). These residues are conserved among the organisms in which Asn-transamidosome is formed (Fig 3-9B, C) and stabilize the formation of the Asn-transamidosome.

The structure of *PaAsn*-transamidosome showed that tRNA^{Asn} did not interact with ndAspRS except an anticodon which was bound to anticodon binding domain of ndAspRS. Such binding

manner is similar to that of *Tt* dAspRS-*Ec* tRNA^{Asp} complex, but different from *Tt*Asn-transamidosome. In addition to the anticodon, several nucleotides in D-stem are interacted with hinge region of ndAspRS in *Tt*Asn-transamidosome.

Similar to other bacterial GatCAB, the structure of GatCAB in *Pa*Asn-transamidosome is consisted of three subunits: glutaminase GatA, amidotransferase GatB, and GatC which functions as a molecular belt. The GatB is composed of three domains: Cradle, Helical and Tail domain. A loop between $\beta 8$ and $\beta 9$ of Cradle domain, and a linker loop connected Helical and Tail domain are not able to build because poor electron density. Moreover, because poor electron density, the side chains of Tail domain also could not be built.

The tRNA^{Asn} binding manner of GatB in *Pa*Asn-transamidosome is similar to that of *Tt* Asn-transamidosome. GatB recognized T-loop and D-loop by helical and tail domain and CCA terminus and U1-A72 base pair by cradle domain. The CCA terminus which accepts amino acid was not positioned in aminoacylation reaction site of ndAspRS, but in amidation reaction site of GatB Cradle domain (Fig 3-8B). Such structure indicates that the obtained crystal structure reflects amidation reaction state during Asn-tRNA^{Asn} synthesis process in indirect path way.

3.5 Structural comparison with *Tt*Asn-transamidosome

*Tt*Asn-transamidosome consists of two AspRS dimers, two GatCAB complexes and four tRNA^{Asn} molecules in the crystal structure, while SAXS analysis shows that it consists of one AspRS dimer, two GatCAB complexes and two tRNA^{Asn} in solution (Blaise *et al.*, 2010) (Fig 3-8C). Crystal structure and SAXS analysis shows that the biological unit of *Tt*Asn-transamidosome can be divided into catalytic and scaffold unit. Each unit contains one ndAspRS monomer, one GatCAB complex, and one tRNA^{Asn}. Interestingly, the tRNA^{Asn} binding manner is different between catalytic and scaffold unit. While anticodon of both tRNA^{Asn} interacted with the anticodon binding domain of ndAspRS in the same way, acceptor stem of two tRNA^{Asn} were recognized by different way. In the catalytic unit, the acceptor stem of tRNA^{Asn} is buried in GatB active site. On the other hand, the acceptor stem is trapped in GatA and GatB interface.

Superposition of ndAspRS in *Pa*Asn-transamidosome on ndAspRS in *Tt*Asn-transamidosome showed that GAD domain of ndAspRS in *Pa*Asn-transamidosome clashes with GatCAB of scaffold unit in *Tt*Asn-transamidosome (Fig 3-10), which explains that scaffold unit does not exist in *Pa*Asp-transamidosome.

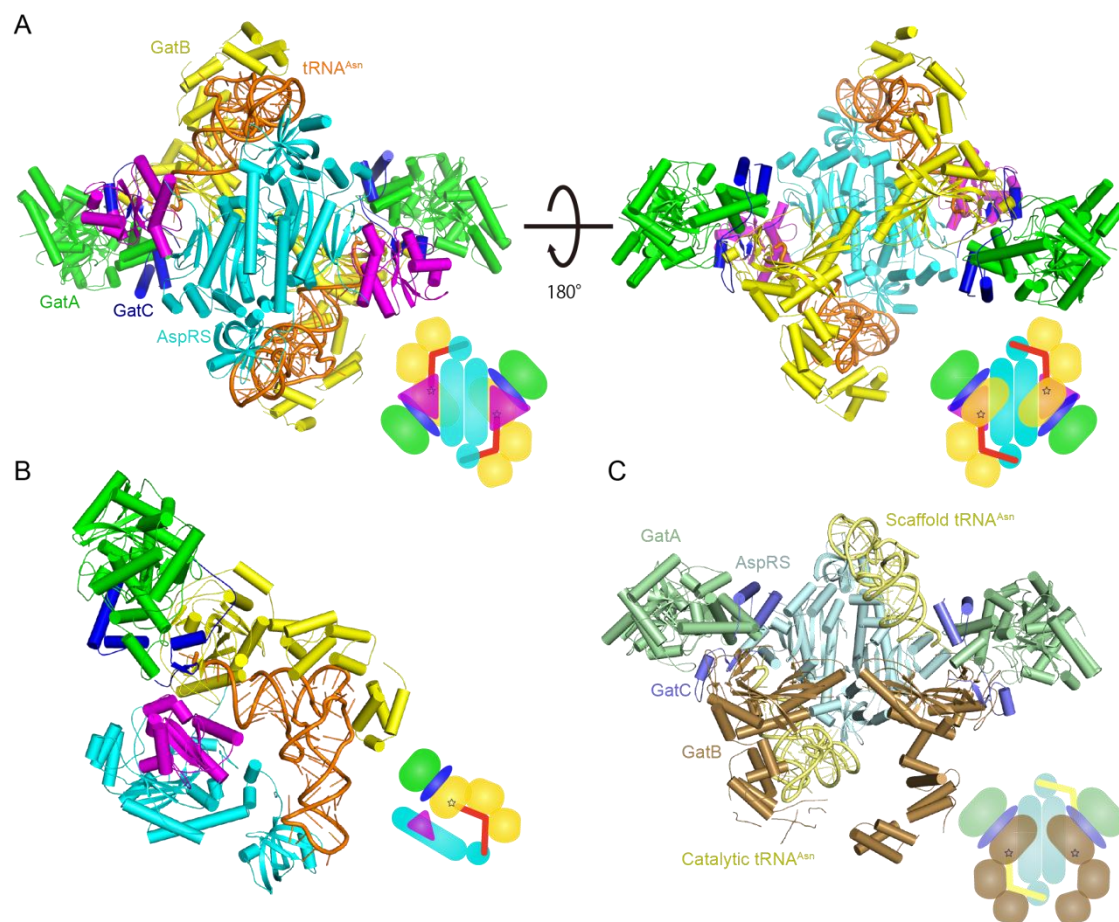


Fig. 3-8. (A) Overall structure of the bacterial Asn-transamidosome. (B) One catalytic unit of *Pa*Asn-transamidosome. The tRNA^{Asn} was recognized by both ndAspRS and GatCAB. (C) The solution structure of archaeal Asn-transamidosome, which is consisted of the catalytic and scaffold unit.

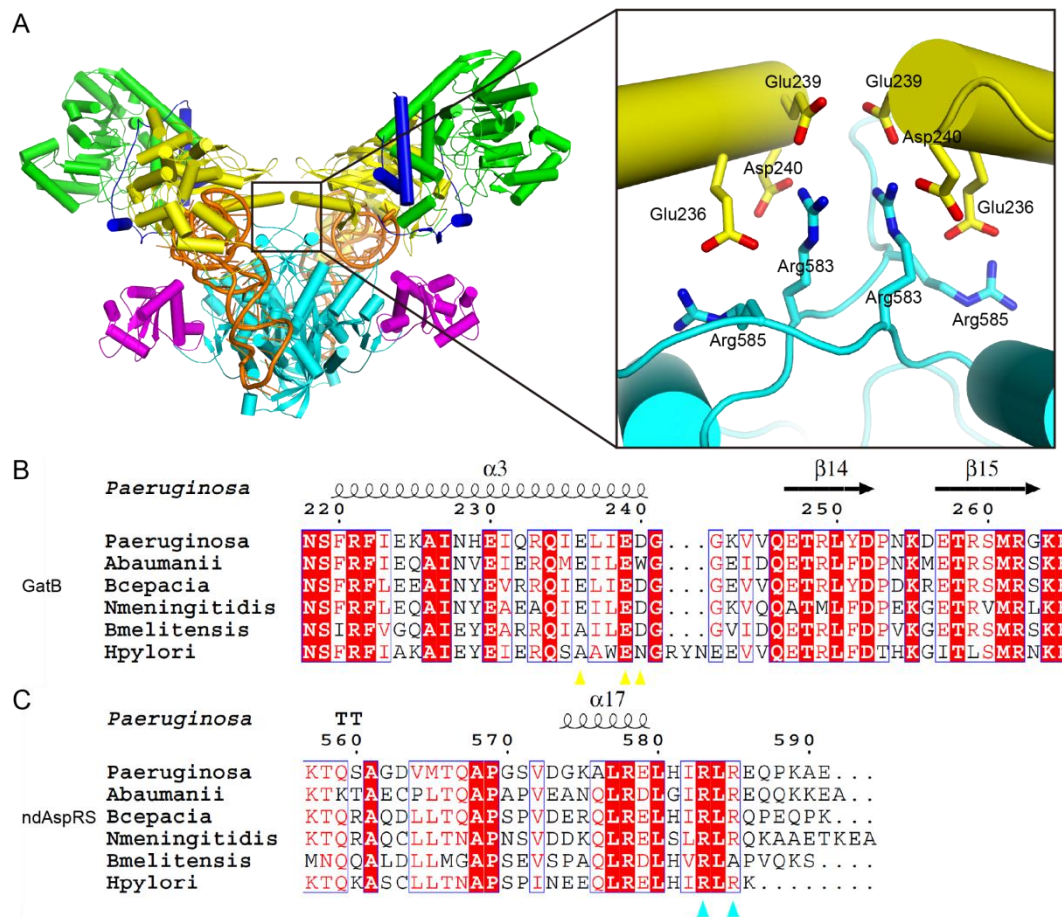


Fig. 3-9. The residues involved in the interaction between GatB and ndAspRS. (A) Close-up view of the GatB and ndAspRS interface region. (B) Multiple sequence alignment of the cradle domain of GatB. (C) Multiple sequence alignment of the C-terminal region of ndAspRS. The number at top is corresponding to *P. aeruginosa* GatB and ndAspRS sequence, respectively. The residues represented in (A) are marked with yellow and cyan triangles, respectively.

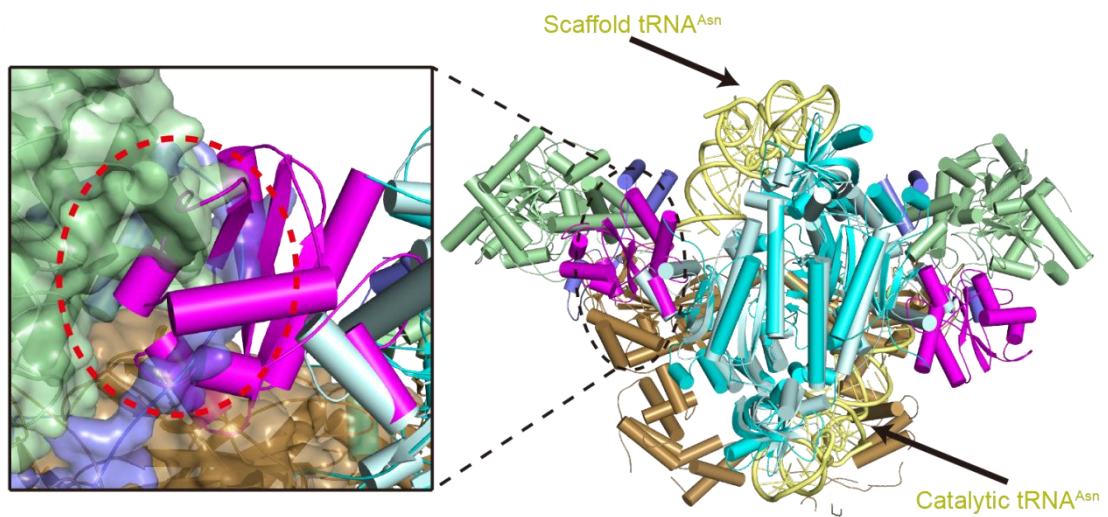


Fig. 3-10. The superposition of ndAspRS in bacterial Asn-transamidosome on ndAspRS in archaeal one. The GAD domain of bacterial ndAspRS causes steric hindrance with GatCAB in scaffold unit of archaeal Asn-transamidosome.

3.6 The conformation changes of ndAspRS and the function of GAD domain

The CCA end of tRNA^{Asn} molecule in *Pa*Asn-transamidosome is positioned in the GatB active site. For tRNA-dependent Asn biosynthesis, the tRNA however first has to be aspartylated by ndAspRS. Thus the CCA end must bind in the ndAspRS active site and then flip into the GatB active site after Asp-tRNA^{Asn} formation. In order to understand how aminoacylated CCA terminus is channeled to active site of GatB, structural comparison of *Pa* ndAspRS-tRNA^{Asn} binary complex (aminoacylation state) and ndAspRS-tRNA^{Asn} in *Pa*Asn-transamidosome (amidation state) was carried out.

Two ndAspRS-tRNA^{Asn} complexes were superposed by using anticodon binding domain. The superposition revealed that when the tRNA acceptor end shifts from active site of ndAspRS to that of GatB, catalytic domain of ndAspRS shifted away from the anticodon binding domain (Fig 3-11A). In addition, the superposition of ndAspRS catalytic core which is composed of 7 β -strands showed that the GAD domain and helix bundle shifts to open up the ndAspRS catalytic site (Fig 3-11B). This movement presumably facilitates the channeling of the tRNA acceptor stem from ndAspRS to GatB.

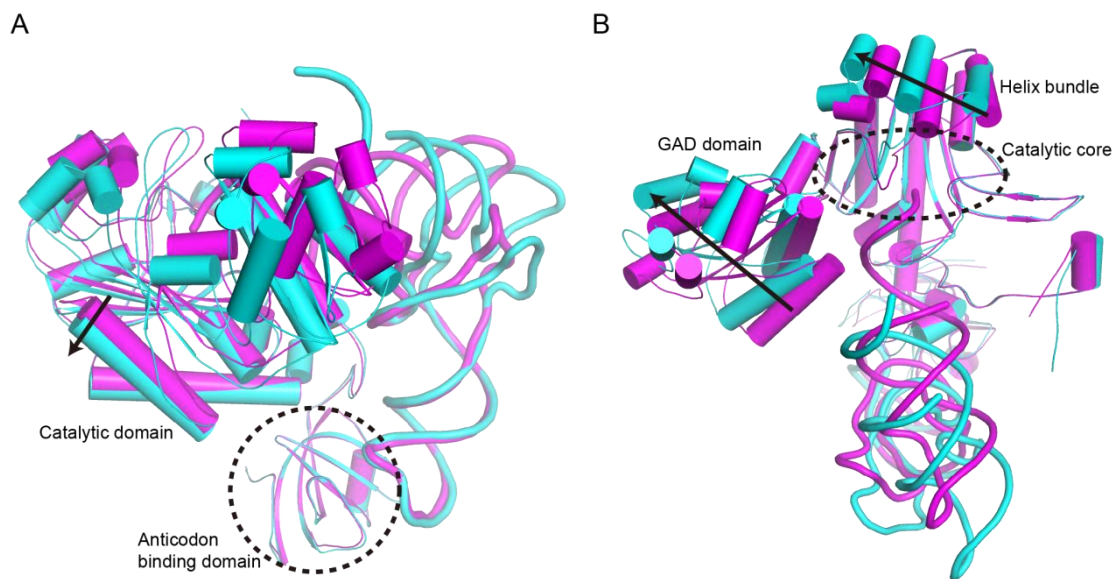


Fig. 3-11. Structure comparison of ndAspRS-tRNA^{Asn} in binary complex and that in Asn-transamidosome (A) Superposition of two ndAspRS-tRNA^{Asn} structures with respect to the anticodon binding domain. (B) Superposition of ndAspRS-tRNA^{Asn} structures with respect to the catalytic core. The anticodon binding domain and catalytic core are indicated by dotted circle. The structural rearrangements are indicated by black arrow.

3.7 The conformational changes related to the function of GatCAB

In order to reveal the possible conformational change of GatB during tRNA channeling, the structural comparisons of GatB in *Pa*Asn-transamidosome with that in *Tt*Asn-transamidosome (PDBID: 3KFU), *Tm*Gln-transamidosome (PDBID: 3AL0) and tRNA free *S. aureus* GatCAB (PDB ID: 3IP4) by using the helical domain were performed.

As description above, GatB can be divided into three domain; Cradle, Helical, and Tail domain and the result of structural comparison showed that GatB has two flexible hinges in each domain boundary (Fig 3-12). One exists between the Cradle and Helical domain, and the other exists between the Helical and Tail domain. The former one seems to be used for the conformational change of GatB needed to the transition from aminoacylation to amidation state of transamidosome. The latter one is used for the recognition of D- loop by the Tail domain.

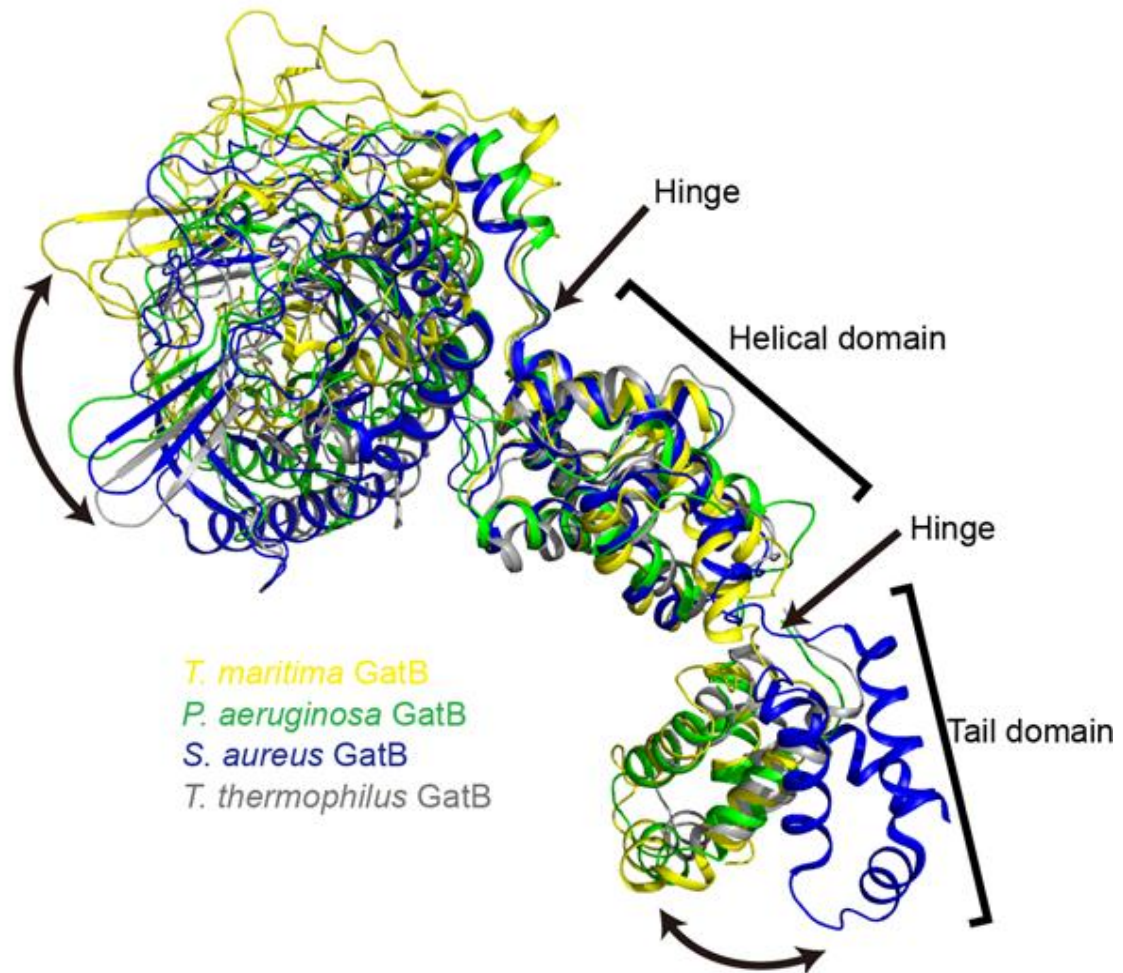


Fig. 3-12. Structure comparison of four GatB structures. (A) Superposition with respect to Helical domain show two hinges at the boundaries of each domain.

3.8 Structure comparison of tRNA^{Asp} and tRNA^{Asn}

To compare the structures of tRNA^{Asp} in dAspRS-tRNA^{Asp} and tRNA^{Asn} in ndAspRS-tRNA^{Asn}, these tRNAs were superposed by using the D- and T-arm region (Fig 3-13). As a result, it is shown that the structure around the C17, U20 and U47 are different from each other. The entire nucleotide of C17 in tRNA^{Asp} is directed outward from the D-loop while the same nucleotide in tRNA^{Asn} is directed inward. The entire nucleotide of U47 in tRNA^{Asp} is directed toward the inside of the tRNA core region and interacted with the adenine ring and phosphate of A21 while that in tRNA^{Asn} is directed outward and interacted with the phosphate of C50. Furthermore, there is U20A between U20 and A21 in tRNA^{Asp} which is absent in tRNA^{Asn}. This conserved nucleotide protrudes from D-loop and functions as the anti-determinant preventing the recognition by GatCAB (Bailly *et al.*, 2006).

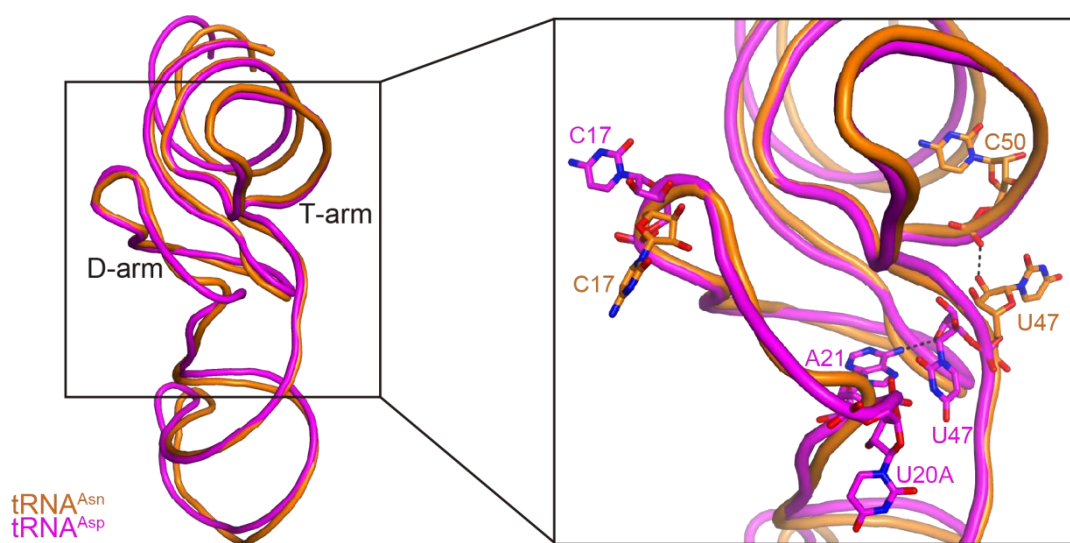


Fig. 3-13. Superposition of the tRNA^{Asp} in dAspRS-tRNA^{Asp} (magenta) and tRNA^{Asn} in ndAspRS-tRNA^{Asn} (orange) complex with respect to D- and T-arm region, where the structural differences of the nucleotides are indicated as stick models. The black-dashed lines indicate hydrogen bonds.

3.9 Conformational change of tRNA^{Asn} between ndAspRS-tRNA^{Asn} binary complex (aminoacylation state) and Asn-transamidosome (amidation state)

Next, we investigated the effect of the GatCAB binding to ndAspRS-tRNA^{Asn} binary complex and subsequent transition of transamidosome from aminoacylation to amidation state on tRNA^{Asn}. We superposed the tRNA^{Asn} in binary complex and that in transamidosome by using the anticodon arm region as the anticodon recognition manner is the same in both structure. The result showed the dramatic change in tRNA core and acceptor arm (Fig 3-13A). Furthermore, the superposition of the T-loop revealed the structural change in the D-loop, especially in C17 and U20. The entire nucleotide of C17 is directed outward from the D-loop in transamidosome (Fig 3-13B). As a result, the structure of the surrounding nucleotides, U16 and G18, are also different from the binary complex. Especially, the hydrogen bonds between G18 and U55 in binary complex are disrupted in transamidosome. As the structure of tRNA core is almost unchanged between ndGluRS-tRNA^{Gln} binary complex and aminoacylation state Gln-transamidosome, the transition from aminoacylation to amidation state may cause the drastic structural change of tRNA core region.

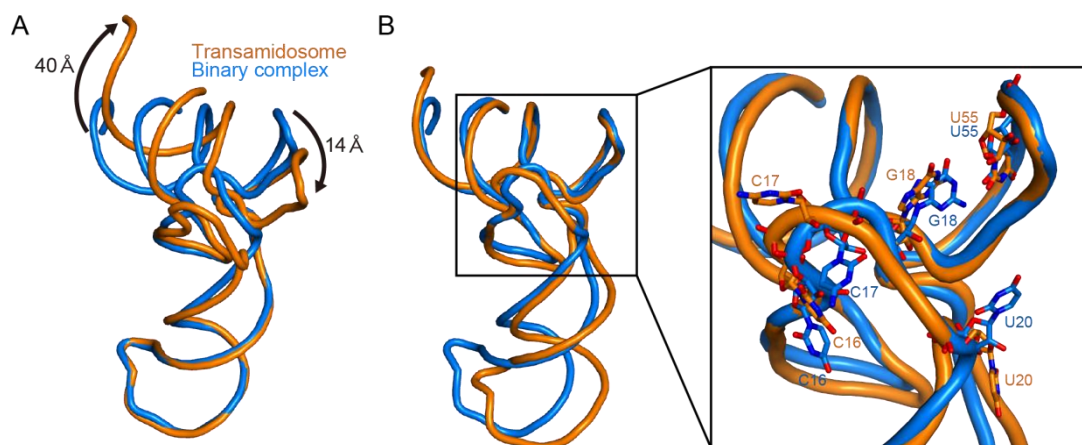


Fig. 3-13. Structural comparison of tRNA^{Asn} in ndAspRS-tRNA^{Asn} binary complex and that in transamidosome. (A) Superposition of the tRNA^{Asn} in ndAspRS-tRNA^{Asn} complex (blue) and that in transamidosome (orange) with respect to anticodon arm region. The structural differences in acceptor arm and tRNA core region are depicted by black arrow. (B) Superposition of above tRNAs according to the T-arm region. The structural differences of nucleotides in D-arm are indicated as stick models.

4. Discussion

In this study, we determined the crystal structure of ndAspRS-tRNA^{Asn} binary complex and Asn-transamidosome from *Pseudomonas aeruginosa* at 3.3 Å and 3.7 Å resolution, respectively. The crystal structure showed that tRNA binding mode were different from those of *T. thermophilus*. Based on the structure comparisons, we constructed the model of the aminoacylation state transamidosome to propose the two step reaction mechanism. The procedure for model construction is as follows. First, GatCAB-tRNA^{Asn} complex in *Pa*Asn-transamidosome was superposed on *Pa* ndAspRS-tRNA^{Asn} binary complex by using D- and T-arm of tRNA^{Asn} and then, tRNA^{Asn} in GatCAB-tRNA^{Asn} complex was deleted. Next, *Tm*GatCAB was superposed on *Pa*GatCAB by using Helical and Tail domain of GatB. Finally, GatC, GatA and Cradle domain of GatB in *Pa*GatCAB was shifted such that they overlap that in *Tm*GatCAB and then, *Tm*GatCAB was deleted. The resulted model represents an aminoacylation state transamidosome in which the CCA terminus is positioned at the active site of ndAspRS. From the above results, we propose following two step reaction mechanism. First, ndAspRS, GatCAB and tRNA^{Asn} form the transamidosome in aminoacylation state. After the aminoacylation of tRNA^{Asn}, GAD domain and catalytic domain rearrange to make the catalytic site more open like *Tt* dAspRS-*Ec* tRNA^{Asp} complex (Briand *et al.*, 2000), which enable the CCA terminus to move flexibly still retaining the anticodon recognition. At the

same time, the changes in relative positioning between anticodon binding domain and catalytic domain assure the space needed to recognize CCA terminal by GatB. After that, GatB recognizes the CCA terminal without any steric clash using a flexible hinge and synthesizes the Asn-tRNA^{Asn} by amidotransferase activity (Fig 4-1).

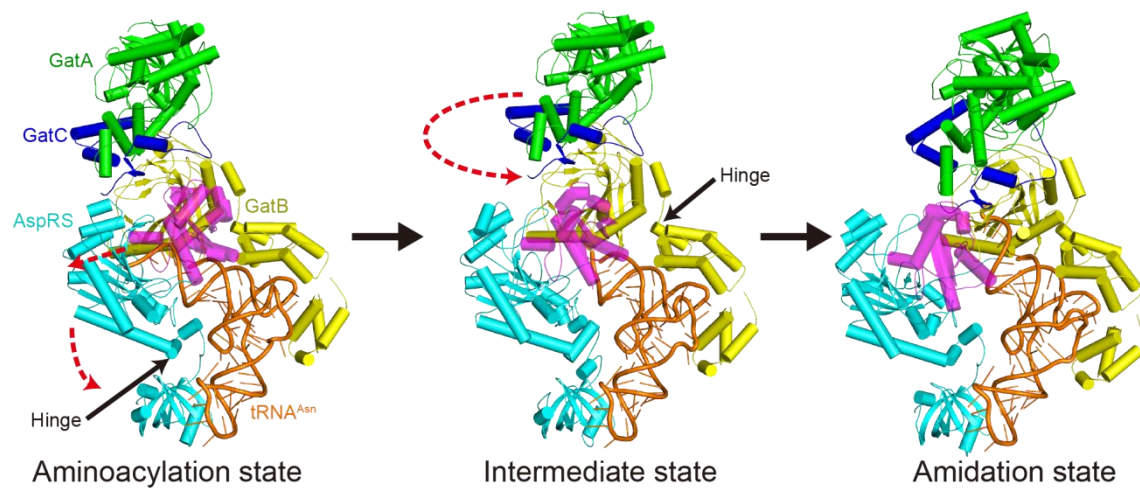


Fig. 4-1. tRNA channeling model for Asn-tRNA^{Asn} synthesis by transamidosome.

GAD domain of ndAspRS is colored magenta. After formation of transamidosome and aminoacylation of tRNA^{Asn} by ndAspRS, ndAspRS bends backward using the hinge and GAD domain becomes open. This conformational change of ndAspRS makes CCA terminus flexible and enable the GatCAB to recognize CCA terminal using the hinge of GatB without any steric hindrance.

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