Structure of the *Pseudomonas aeruginosa* transamidosome reveals unique aspects of bacterial tRNA-dependent asparagine biosynthesis

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Many prokaryotes lack a tRNA synthetase to attach asparagine to its cognate tRNA<sub>Asn</sub>, and instead synthesize asparagine from tRNA<sup>Asn</sup>-bound aspartate. This conversion involves two enzymes: a nondiscriminating aspartyl-tRNA synthetase (ND-AspRS) that forms Asp-tRNA<sup>Asn</sup>, and a heterotrimERIC amidotransferase GatCAB that amidates Asp-tRNA<sup>Asn</sup> to form Asn-tRNA<sup>Asn</sup> for use in protein synthesis. ND-AspRS, GatCAB, and tRNA<sub>Asn</sub> may assemble in an ∼400-kDa complex, known as the Asn-transamidosome, which couples the two steps of asparagine biosynthesis in space and time to yield Asn-tRNA<sup>Asn</sup>. We report the 3.7-Å resolution crystal structure of the *Pseudomonas aeruginosa* Asn-transamidosome, which represents the most common machinery for asparagine biosynthesis in bacteria. We showed that, in contrast to a previously described archaeal-type transamidosome, a bacteria-specific GAD domain of ND-AspRS provokes a principally new architecture of the complex. Both tRNA<sub>Asn</sub>-molecules in the transamidosome simultaneously serve as substrates and scaffolds for the complex assembly. This architecture rationalizes an elevated dynamic and a greater turnover of ND-AspRS within bacterial-type transamidosomes, and possibly may explain a different evolutionary pathway of GatCAB in organisms with bacterial-type vs. archaeal-type Asn-transamidosomes. Importantly, because the two-step pathway for Asn-tRNA<sup>Asn</sup> formation evolutionarily preceded the direct attachment of Asn to tRNA<sup>Asn</sup>, our structure also may reflect the mechanism by which asparagine was initially added to the genetic code.

Accurate translation of the genetic code into a protein sequence relies on a covalent attachment of amino acids to cognate tRNAs that are later used in protein synthesis (1). This attachment is catalyzed by aminoacyl-tRNA synthetases (aaRSs), each specific to one amino acid and a set of tRNA isoacceptors (2). However, the majority of prokaryotes lack several tRNA synthetases, particularly asparaginyl-tRNA synthetase (AsnRS), which ligates asparagine to tRNA<sup>Asn</sup> (3, 4). In these organisms, asparagine is synthesized in a two-step, tRNA-dependent pathway (5). First, a nondiscriminating aspartyl-tRNA synthetase (ND-AspRS) attaches aspartate to tRNA<sup>Asn</sup> to form Asp-tRNA<sup>Asn</sup> (6, 7). Then the tRNA-bound aspartate is converted to asparagine by the amidotransferase (AdT) GatCAB to yield the final product, Asn-tRNA<sup>Asn</sup> (6, 8–13). Likewise, in prokaryotes lacking glutaminyl-tRNA synthetase (GlnRS), Gln-tRNA<sub>Gln</sub> is formed by the sequential actions of a nondiscriminating glutamyl-tRNA synthetase (ND-GluRS) (14) and an AdT (5). In bacteria, the role of AdT is played by GatCAB (15), whereas in archaea, it is played by GatDE (16, 17).

More than 25 years ago (18), it was proposed that ND-aaRSs and AdTs may form a complex—now called a transamidosome—to couple the two steps of Asn-tRNA<sup>Asn</sup> formation in space and time and allow efficient transfer of Asp-tRNA<sup>Asn</sup> from the aaRS to the AdT. The first characterized transamidosome was the Asn-transamidosome from *Thermus thermophilus* (19, 20). This complex was identified as a tRNA-dependent association of AspRS2 (TiAspRS2) and GatCAB in a 2:2:2 ratio. It was shown that transamidosome formation stabilizes interactions between subunits of GatCAB (21) and protects Asn-tRNA<sup>Asn</sup> from hydrolysis, with product release being rate-limiting (19). In the complex, the AspRS forms a dimer with only one catalytic site active at a time (21). It was suggested that the key advantages of asparagine formation by the transamidosome compared with separate enzymes are enhanced asparylation of tRNA<sup>Asn</sup> and better prevention of the misacylated Asp-tRNA<sup>Asn</sup> from use in translation, because this would compromise the fidelity of protein synthesis (19–21).

Importantly, TiAspRS2 was acquired through horizontal gene transfer from archaea (10), and lacks the GAD domain typical of bacterial AspRSs (22). The TiAsn-transamidosome crystal structure suggests that a complex between bacterial ND-AspRS and GatCAB should be less stable and more structurally different than the *T. thermophilus* complex, owing to the presence of the GAD domain in bacterial ND-AspRS (21, 23). Consistent with this notion, the stable association of the *Helicobacter pylori* ND-AspRS (HpND-AspRS) with GatCAB requires the presence of an auxiliary factor, Hp0100 (23, 24). In the complex, the activity of the HpND-AspRS is unchanged, although the activity of GatCAB increases (23); however, Hp0100 is phylogenetically limited to e-proteobacteria (23). Therefore, most bacteria have

transamidosome | asparagine biosynthesis | aspartyl-tRNA synthetase | GatCAB

The present structure reveals the architecture of the *Pseudomonas aeruginosa* bacterial-type asparagine-transamidosome, the most common macromolecular assembly required for asparaginyl-tRNA<sup>Asn</sup> formation in bacteria. We show that the presence of an additional GAD domain in the aspartyl-tRNA synthetase, common in most bacteria but missing in the archaeal-type *Thermus thermophilus* transamidosome, results in a complex with a distinct architecture and stoichiometry. Furthermore, our kinetic studies reveal that bacterial transamidosomes have distinct kinetic properties compared with the archaeal complex, with rapid release of the Asp-tRNA<sup>Asn</sup> product, leading to improved turnover by the bacterial-type aspartyl-tRNA synthetase in the complex. Overall, our study provides a structural basis for understanding tRNA-dependent asparagine biosynthesis found in the in majority of bacterial species.

Significance

The present structure reveals the architecture of the *Pseudomonas aeruginosa* bacterial-type asparagine-transamidosome, the most common macromolecular assembly required for asparaginyl-tRNA<sup>Asn</sup> formation in bacteria. We show that the presence of an additional GAD domain in the aspartyl-tRNA synthetase, common in most bacteria but missing in the archaeal-type *Thermus thermophilus* transamidosome, results in a complex with a distinct architecture and stoichiometry. Furthermore, our kinetic studies reveal that bacterial transamidosomes have distinct kinetic properties compared with the archaeal complex, with rapid release of the Asp-tRNA<sup>Asn</sup> product, leading to improved turnover by the bacterial-type aspartyl-tRNA synthetase in the complex. Overall, our study provides a structural basis for understanding tRNA-dependent asparagine biosynthesis found in the in majority of bacterial species.
a structurally and, possibly, functionally distinct class of transamidosomes than those described by the *T. thermophilus* and *H. pylori* complexes.

In the bacterium *Pseudomonas aeruginosa*, Asn-tRNA\textsubscript{Asn} formation is catalyzed by GatCAB and bacterial ND-AspRS, and thus represents the most common type of bacterial Asn-transamidosome (25). Here we report the crystal structure of the *P. aeruginosa* Asn-transamidosome (PaAsn-transamidosome), which represents the transamination state of the Asn-tRNA\textsubscript{Asn} formation. The structure suggests that the additional GAD domain within the ND-AspRS changes the overall architecture of the complex relative to the previously described TiAsn-transamidosome. Consistent with the structure, our in vitro measurements show that PaAsn-transamidosome has unique kinetic properties and functions primarily to enhance tRNA\textsubscript{Asn} turnover and facilitate Asp-tRNA\textsubscript{Asn} handoff from AspRS to GatCAB.

Results

Overall Structure of the Bacterial Asn-Transamidosome. The structure of the PaAsn-transamidosome determined at 3.7-Å resolution (Table S1) reveals the architecture of a 413-kDa symmetric complex comprising ND-AspRS, GatCAB, and tRNA\textsubscript{Asn} in a 2:2:2 stoichiometry. Each GatCAB is bound to a different tRNA\textsubscript{Asn} and ND-AspRS, and all are related by a noncrystallographic twofold axis at the interface of the ND-AspRS monomers (Fig. 1L). The complex is stably formed without the auxiliary factor Hp0100 (Fig. S1), although an association of ND-AspRS with GatCAB is tRNA\textsubscript{Asn}-dependent, as described previously (19, 24). Consistent with the crystal structure, an ∼400-kDa complex was detected in solution by gel filtration (Fig. S2).

The structure reveals the interface between ND-AspRS and GatCAB. The α3 helix and β-hairpin connecting β-strands 13 and 14 in GatB contacts the C-terminal loop of ND-AspRS with an average interaction surface of ∼553 Å\textsuperscript{2} (Fig. 1B, Fig. S3A, and Table S2). In addition, Arg583 and Arg585 in ND-AspRS are in a salt bridge distance from Glu236, Glu239, and Asp240 in GatB and seem to further stabilize the complex. Notably, these residues are largely conserved in organisms that use GatCAB (Fig. S4A) with a ND-AspRS (Fig. S4B), suggesting a common mode of GatB–ND-AspRS interaction across bacterial species.

The two tRNA\textsubscript{Asn} molecules are bound to the complex in an identical manner (Fig. 1C). The ND-AspRS anticodon-binding domain (ABD) recognizes the anticodon of the tRNA, whereas the 3′ CCA terminus of tRNA\textsubscript{Asn} is accommodated by the active site of GatCAB with the tRNA\textsubscript{Asn} U1-A72 base pair near the 3π turn in the GatB cradle domain. Consistent with previous studies, recognition of the U1-A72 base pair by the 3π turn enables the bacterial AdT to distinguish its tRNA substrates (tRNA\textsubscript{Asn} and tRNA\textsubscript{Ade}) from tRNA\textsubscript{Asp} and tRNA\textsubscript{Glu} (21, 26–28). As seen in the *Staphylococcus aureus* GatCAB structure (27), the tail domain of GatB is positioned to size the D-loop of the tRNA, another major tRNA recognition element of bacterial GatCAB (26, 28). Accordingly, the structure likely represents the transamination state of the bacterial Asn-transamidosome.

Unique Structural Features of the Bacterial Asn-Transamidosome. The key difference between bacterial and archaeal-type transamidosomes is the presence of an additional GAD domain in the ND-AspRS proteins. The structural superposition of the PaAsn-transamidosome with the TiAsn-transamidosome (21) reveals how the GAD domain alters the overall architecture of the bacterial complex.

The previously described TiAsn-transamidosome comprises two dimers of the archaeal type TiAspRS2, two GatCABs, and four tRNA\textsubscript{Asn} molecules, although one TiAspRS2 dimer seems to dissociate from the complex in solution, according to small angle X-ray scattering analysis (21). The structure likely represents a transitional step in Asn-tRNA\textsubscript{Asn} formation. It was initially proposed that tRNA-dependent Asn biosynthesis in *T. thermophilus* requires binding of two tRNA molecules to the TiAspRS2 dimer (21). Two GatCAB molecules are then recruited and induce conformational changes in the TiAspRS2 dimer in such a way that makes only one TiAspRS2 monomer active at a time. In this complex, one tRNA molecule (cattRNA\textsubscript{Asn}) is bound to the active TiAspRS2 monomer to be aspartylated (Fig. 24), whereas the other tRNA molecule (cattRNA\textsubscript{Asn}) binds to the inactive monomer and plays the role of a scaffold that stabilizes the complex. In agreement with the structure, TiAspRS2 activity in the TiAsn-transamidosome has biphasic kinetics, with only one-half of the catalytic sites active at a time (19, 21).

We show that, unlike the TiAsn-transamidosome, the PaAsn-transamidosome comprises only one ND-AspRS dimer bound to two tRNA\textsubscript{Asn} molecules and two GatCAB molecules (Fig. L4). Both tRNA molecules adopt a uniform cat-tRNA\textsubscript{Asn} conformation, suggesting that both ND-AspRS monomers can be active at the same time. The differences between the two Asn-transamidosomes likely are related to the bacteria-specific GAD insertion domain. Superposition of the ND-AspRS in the PaAsn-transamidosome and TiAsn-transamidosome reveals a steric clash between the GAD domain of the PaND-AspRS and the TiGatB (Fig. 2B). In the PaAsn-transamidosome, the PaGatCAB bends away from the rest of the complex to accommodate the PaND-AspRS GAD domain. This particular orientation of GatCAB in the PaAsn-transamidosome enables both tRNA\textsubscript{Asn} molecules bound to the ND-AspRS dimer to adopt cat-tRNA\textsubscript{Asn} conformations.

![Fig. 1](image-url)
The lack of a GAD insertion in the \( \text{TrAspRS} \) allows the \( \text{TgCatCAB} \) to bend toward the catalytic core of \( \text{TrAspRS} \), facilitating the binding of one tRNA\(^{\text{Asn}}\) in the \( \text{scafRNA}^{\text{Asn}} \) and tRNA\(^{\text{Asn}}\) from hydrolysis. The lack of \( \text{scafRNA}^{\text{Asn}} \) from stabilizing the complex suggests that the \( \text{Pa}^{\text{Asn-transamidosome}} \) can readily release tRNA\(^{\text{Asn}}\) after product formation. Our measurements show that, consistent with that hypothesis and unlike the \( \text{TrAsn-transamidosome} \), the \( \text{P. aeruginosa} \) complex does not protect Asp-tRNA\(^{\text{Asn}}\) from hydrolysis (246 min vs. 246 min, respectively). However, \( \text{Pa}^{\text{aspartyl-tRNA-synthetase}} \) does protect Asp-tRNA\(^{\text{Asn}}\) from deacylation (1/2 of 246 min vs. 39 min with no enzyme present and 41 min with ND-AspRS present), but offers minimal protection of Asn-tRNA\(^{\text{Asn}}\) (1/2 of 29 min vs. 22 min without enzyme), consistent with \( \text{CatB}^{\text{aspartyl-tRNA-synthetase}} \) binding Asp-tRNA\(^{\text{Asn}}\) to generate and release Asn-tRNA\(^{\text{Asn}}\) for protein synthesis.

In agreement with the results of the protection assay, we did not detect burst phase kinetics with the \( \text{Pa}^{\text{aspartyl-tRNA-synthetase}} \) and tRNA\(^{\text{Asn}}\) that had been observed with the \( \text{TrAsn-transamidosome} \) (19, 21). The association of \( \text{CatB}^{\text{aspartyl-tRNA-synthetase}} \) with ND-AspRS did increase ND-AspRS turnover by 3.2-fold; however, the presence of CatB also increased the \( K_{\text{m}} \) of ND-AspRS for tRNA\(^{\text{Asn}}\) by a similar factor, leading to no difference in ND-AspRS catalytic efficiency (Table 1). Taken together, the kinetic data show that the \( \text{Pa}^{\text{aspartyl-tRNA-synthetase}} \) can readily release tRNA\(^{\text{Asn}}\) after its formation. This biochemical property may be a reflection of the GAD insertion rather than the lack of \( \text{scafRNA}^{\text{Asn}} \) to stabilize the complex.

We attempted to determine whether the bacterial \( \text{Asn-transamidosome} \) can behave like the archaeal \( \text{Asn-transamidosome} \) when the GAD insertion domain is deleted. Unfortunately, none of the \( \text{Pa}^{\text{ND-AspRS}} \) deletion mutant constructs (including a replacement of the GAD domain with the loop found in \( \text{TrAspRS} \)) produced sufficient protein amounts for analysis.

**Distinct Biochemical Properties of the Bacterial Asn-Transamidosome.** To test whether a distinct architecture of the complex results in distinct kinetic properties, we measured the \( \text{Pa}^{\text{Asn-transamidosome}} \) protects Asp-tRNA\(^{\text{Asn}}\) from hydrolysis by a similar factor. The concentration of tRNA\(^{\text{Asn}}\) varied between 0.1 and 10.1 \( \mu \text{M} \). The conditions of tRNA\(^{\text{Asn}}\) varied from 0.1 and 10.1 \( \mu \text{M} \).

**Structural Basis of the CCA Terminus Translocation During the Transamidation Cycle.** The \( \text{Pa}^{\text{Asn-transamidosome}} \) structure likely represents the transamidation state of the complex; thus, we determined the co-crystal structure of \( \text{Pa}^{\text{ND-AspRS}} \) with tRNA\(^{\text{Asn}}\) at 3.3 Å resolution to provide insight into the transamidation state of \( \text{Pa}^{\text{ND-AspRS}} \) with tRNA\(^{\text{Asn}}\) (Fig. 3A and B and Table S1). Overall, the \( \text{Pa}^{\text{ND-AspRS}}:\text{tRNA}^{\text{Asn}} \) binary complex is similar to the \( \text{Escherichia coli} \) D-AspRS (EcD-AspRS) enzyme bound to tRNA\(^{\text{Asp}}\) (PDB ID code 1COA) (29) and reveals subtle differences in the tRNA structures that may explain tRNA specificity of the enzyme (SI Text and Figs. S5–S7).

The \( \text{Pa}^{\text{Asn-transamidosome}} \) molecules bound to the \( \text{Pa}^{\text{aspartyl-tRNA-synthetase}} \) are positioned at the \( \text{CatCAB}^{\text{transamidase}} \) active site. However, for tRNA-dependent Asn biosynthesis, the tRNA first must be aspartylated by ND-AspRS. Thus, the \( \text{Pa}^{\text{Asn-transamidosome}} \) and tRNA must bind to the ND-AspRS active site and then flip ~40 Å up into the \( \text{CatCAB}^{\text{transamidase}} \) catalytic site after Asp-tRNA\(^{\text{Asn}}\) formation. To clarify how the ND-AspRS accommodates the flipping of the CCA tRNA\(^{\text{Asn}}\) terminus, we superposed the \( \text{Pa}^{\text{ND-AspRS}} \) bound to tRNA\(^{\text{Asn}}\) onto that in the \( \text{Pa}^{\text{aspartyl-tRNA-synthetase}} \) (Fig. 4A). This superposition revealed that when the tRNA acceptor end flips from the active site of ND-AspRS to CatB, the catalytic domain shifts away from the tRNA. The shift is possible because of a hinge between the AB and catalytic domain of ND-AspRS. In addition, the GAD insertion and helix bundle appends to the catalytic domain shift to open up the PaND-AspRS catalytic site (Fig. 4B). Presumably, this movement facilitates the flipping of the tRNA acceptor stem from ND-AspRS to CatB.

These structural rearrangements in the ND-AspRS are accompanied by an ~14–18 Å shift of the tRNA\(^{\text{Asn}}\) elbow and an ~40–45 Å shift of the tRNA\(^{\text{Asn}}\).

**Table 1. Kinetic data for Asp-tRNA\(^{\text{Asn}}\) formation by \( \text{P. aeruginosa} \) ND-AspRS**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( k_{\text{cat}} ), ( \mu \text{M} ), mean ± SD</th>
<th>( k_{\text{cat}}/K_{\text{m}} ), ( s^{-1} \mu \text{M} ), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AspRS</td>
<td>0.61 ± 0.07</td>
<td>0.111 ± 0.006</td>
</tr>
<tr>
<td>AspRS + CatCAB*</td>
<td>2.0 ± 0.2</td>
<td>0.36 ± 0.01</td>
</tr>
</tbody>
</table>

Measurements are from three separate experiments. Reactions with \( \text{P. aeruginosa} \) ND-AspRS (5 nM) were carried out at 37 °C in the presence of excess ATP (4 mM), Asp (3.3 mM), and Gln (2 mM), as described in Materials and Methods. The concentration of tRNA\(^{\text{Asn}}\) varied between 0.1 and 10.1 \( \mu \text{M} \).

*CatCAB (2.0 \( \mu \text{M} \)) was added to the reaction mixture.*
movement of the tRNAAsn 3′ CCA end during the transition from the aminoacylation to transamidase state (Fig. 4C). Superposition of the T-arms of the tRNA in the two states revealed rearrangements within the tRNAAsn D-loop (Fig. 4D and Fig. S3 B and C). The shift results in C17 being flipped out of the D-loop and U20 pointing down toward the anticodon instead of up toward the TP-loop. Accordingly, G18 is shifted so it no longer hydrogen-bonds with U55. Similar structural changes are seen in the GatDE-tRNAGln structure (17).

**Bacterial Asn-Transamidosome Structure Reveals the GatCAB Transamidation State.** With the tRNAAsn 3′ end bound in the GatCAB active site, the PaAsn-transamidosome likely represents the transamidation state of the complex. Apart from association with ND-AspRS, bacterial GatCAB can form a ternary complex with ND-GluRS and tRNAGln, known as a Gln-transamidase with ND-AspRS, bacterial GatCAB can form a ternary complex. Our work supports the transamidation state of the complex. Apart from association with ND-AspRS, bacterial GatCAB can form a ternary complex with ND-GluRS and tRNAGln, known as a Gln-transamidase with ND-AspRS, bacterial GatCAB can form a ternary complex. Similar to the case of ND-AspRS:tRNAAsn binary complex, the Gln-transamidase is better described by a ternary complex. The catalytic domain (cyan), GAD domain (magenta), and hinge region (blue) of the ND-AspRS are shown with tRNAAsn (orange). ND-AspRS-bound Asp is shown as a sphere model. (b) Dimeric structure of the PaND-AspRS:tRNAAsn binary complex. Each monomer is related by a crystallographic twofold axis.

**Fig. 3.** (A) Monomeric structure of the P. aeruginosa ND-AspRS:tRNAAsn (PaND-AspRS:tRNAAsn) binary complex in an asymmetric unit. The ABD (green), catalytic domain (cyan), GAD domain (magenta), and hinge region (blue) of the ND-AspRS are shown with tRNAAsn (orange). ND-AspRS-bound Asp is shown as a sphere model. (b) Dimeric structure of the PaND-AspRS:tRNAAsn binary complex. Each monomer is related by a crystallographic twofold axis.

**Discussion**

**Complexes of Translation Components for Improved Protein Synthesis.** Multi-aaRS complexes, or complexes of aaRSs with other translation machinery components, are known in all domains of life (32). Such higher-order structures have been shown to contribute to translational fidelity and to increased catalytic efficiency of aaRS reactions (33–35). The allure of substrate channeling (36), preventing the participation of misacylated aminoacyl-tRNA in protein synthesis, led to the suggestion of a multienzyme complex for tRNA-dependent Gln-tRNA synthesis (18). This has been borne out by detailed analyses of Gln-tRNA and Asn-tRNA formation by transamidosomes in bacteria and archaea (this work and refs. 19 and 30), although exceptions might exist (37). The transamidosome architecture nicely explains substrate channeling and efficient aa-tRNA formation by the transamidation route. Another multimeric complex (consisting of SepRS, SepCysS,
the enzyme PaAspRS binds tRNAAsn with its anticodon positioned in the acylation state (Fig. 6), as observed in the PaAspRS complexed with tRNAAsn. The GatB tail domain moves into position to bind the tRNAAsn acceptor stem and adopt the transamidation state seen in the PaAspRS-transamidosome crystal structure (Fig. 6C). The 310 turn in the GatB cradle domain selectively binds the tRNAAsn U1-A72 base pair; this provides a second check to ensure that Asp-tRNAAsn is amidated and not Asp-tRNAAsp. Now the tRNAAsn-bound Asp is positioned in the GatCAB transamidase active site for amidation. Following Asn-tRNAAsn production, the complex dissociates, releasing Asn-tRNAAsn, consistent with the complex’s inability to protect Asn-tRNAAsn from hydrolysis. Given that the association of the PaGatCAB and PaND-AspRS is tRNA-dependent, GatCAB is also likely released from ND-AspRS when Asn-tRNAAsn dissociates.

Evolution of Asn-Transamidosomes. In archaea, GatCAB is used only for Asn-tRNAAsn formation as GatDE forms Gln-tRNAGln (16, 17). Both AdTs likely were present in early archaea (40). The specificity of archaeal GatCAB for Asn-tRNAAsn synthesis might have enabled archaeal AspRS to coevolve with the AdT to form a thermostable complex in which GatCAB is not released following Asn-tRNAAsn synthesis. This stability likely was facilitated by the archaeal AspRS not retaining the GAD insertion domain found in bacterial-type AspRS. The thermostability of the archaeal complex may explain why Thermus thermophilus acquired an archaeal-type AspRS for tRNA-dependent Asn biosynthesis instead of using the bacterial-type AspRS that it also encodes in its genome (19, 21, 22, 41).

Early bacteria likely emerged from the last universal common ancestral state (LUCAS) with just one AdT—GatCAB—for both Gln-tRNAGln and Asn-tRNAAsn formation (40). After Asn-tRNAAsn formation in early bacteria, the release of GatCAB from bacterial ND-AspRS might be have been beneficial, because the free GatCAB could easily be repurposed for Gln-tRNAGln formation with GluRS. In that context, acquisition of the GAD insertion domain by bacterial AspRS might have facilitated GatCAB release from the Asn-transamidosome to better enable early bacteria to use GatCAB for both Gln-tRNAGln and Asn-tRNAAsn formation. Consistent with this idea, the GAD insertion was likely acquired early on in bacterial AspRS evolution and coevolved for an extended time with the rest of the enzyme (SI Text and Fig. S8).

In H. pylori, Hp0100 stabilizes GatCAB association with bacterial ND-AspRS even in the absence of tRNAA Asn (23). H. pylori also encodes two GluRS enzymes, with the second (GluRS2) complexing with GatCAB for Gln-tRNAGln formation (31, 42, 43). Hp0100 and GluRS2 may be adaptations for only Asn-tRNAAsn synthesis (25), retention of the GAD domain found in bacterial-type AspRS, and Fig. S8). In P. aeruginosa, which acquired GlnRS and uses GatCAB for only Asn-tRNAAsn synthesis (25), the GAD

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insertion in AspRS may be a remnant of the coevolution of the protein domains in early bacteria. The insertion domain may still be selected for to allow proper folding of PaND-AspRS and to enable simultaneous activity of both monomers of ND-AspRS in the complex, thereby improving ND-AspRS turnover of tRNA<sub>Asn</sub>. This coevolution may be preventing the generation of stable GAD domain deletion mutant PaND-AspRS enzymes.

Because the PaAsn-transamidosome allows Asp-tRNA<sub>Asn</sub> to be directly handed off from ND-AspRS to GatCAB, the misacylated tRNA<sub>Asn</sub> is protected from decay and use in protein synthesis, where it may compromise the fidelity of translation. Accordingly, under certain conditions, the complex of ND-AspRS with GatCAB may be less prone than AsnRS to mischarged tRNA<sub>Asn</sub> formation. In addition, the Asn-transamidosome allows <i>P. aeruginosa</i> to directly and efficiently couple the biosynthesis of Asn with its use in translation. As such, a similar complex between ND-AspRS and ancestral GatCAB pre-LUCAS might have enabled the addition of Asn to the genetic code (44).

**Materials and Methods**

Preparation of the PaAsn-transamidosome and PaND-AspRS:tRNA<sub>Asn</sub> and the details of crystallization and structure determination are summarized in SI Methods and Materials. Structures of the PaAsn-transamidosome and PaND-AspRS:tRNA<sub>Asn</sub> were solved by molecular replacement methods. Atomic coordinates and structure factors have been deposited in the Protein Data Bank under ID codes 4WJ3 for PaAsn-transamidosome and 4WJ4 for PaND-AspRS:tRNA<sub>Asn</sub>. Detailed descriptions of the gel-shift assay, gel filtration analysis, kinetic analysis, protection assay, and phylogenetic analysis using established methods are provided in SI Materials and Methods.

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