The asparagine-transamidosome from *Helicobacter pylori*: a dual-kinetic mode in non-discriminating aspartyl-tRNA synthetase safeguards the genetic code

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ABSTRACT

*Helicobacter pylori* catalyzes Asn-tRNA<sub>Asn</sub> formation by use of the indirect pathway that involves charging of Asp onto tRNA<sub>Asn</sub> by a non-discriminating aspartyl-tRNA synthetase (ND-AspRS), followed by conversion of the mischarged Asp into Asn by the GatCAB amidotransferase. We show that the partners of asparaginylation assemble into a dynamic Asn-transamidosome, which uses a different strategy than the Gln-transamidosome to prevent the release of the mischarged aminoacyl-tRNA intermediate. The complex is described by gel-filtration, dynamic light scattering and kinetic measurements. Two strategies for asparaginylation are shown: (i) tRNA<sub>Asn</sub> binds GatCAB first, allowing aminoacylation and immediate transamidation once ND-AspRS joins the complex; (ii) tRNA<sub>Asn</sub> is bound by ND-AspRS which releases the Asp-tRNA<sub>Asn</sub> product much slower than the cognate Asp-tRNA<sub>Asp</sub>. This kinetic peculiarity allows GatCAB to bind and transamidate Asp-tRNA<sub>Asn</sub> before its release by the ND-AspRS. These results are discussed in the context of the interrelation between the Asn and Gln-transamidosomes which use the same GatCAB in *H. pylori*, and shed light on a kinetic mechanism that ensures faithful codon reassignment for Asn.

INTRODUCTION

Accurate expression of genetic information is essential to all living organisms. Integrity of the message encoded within a gene is safeguarded by mechanisms that cells have evolved at each step of gene expression. In protein synthesis, tRNA aminoacylation is a crucial step where each tRNA species is bound to its cognate amino acid in reactions catalyzed by the aminoacyl-tRNA synthetases (aaRSs) (1,2). The amino acids are first activated as aminoacyl-C<sub>24</sub>AMPs and then transferred onto their cognate tRNAs to synthesize the final aminoacyl-tRNA products. The latter are then carried to the ribosome by an elongation factor where they are used to decode the codons on the mRNA. Fidelity of translation depends on accurate tRNA aminoacylation, i.e. correct matching of an amino acid to its set of isoacceptor tRNAs. This coupling is determined by the existence of a particular aaRS for each amino acid/tRNA pair. The capacity to select homologous substrates is conferred by active site geometry and by correction processes that are used by aaRSs when the wrong aminoacyl-tRNAs are formed.
Moreover, aaRSs are split into two classes, according to sequence/structure (5) and kinetic (6–8) properties. Members of class I bind tRNA on the minor groove side and acylate the 2'-OH of the terminal adenosine whereas the members of class II approach tRNA from the major groove side and transfer the amino acid moiety on the 3'-OH of the terminal adenosine. Further, it seems that most class I aaRSs display a rate-limiting step for the release of aminoacyl-tRNA, whereas the transfer of the activated amino acid onto tRNA is rate-limiting for class II aaRSs (8).

Global genome analyses and biochemical data indicate that about half of prokaryotes are deprived of asparaginyl-tRNA synthetase (AsnRS), and that the vast majority lack a glutaminyl-tRNA synthetase (GlnRS). This pattern negates the old dogma that postulated the presence of a specific aaRS for each amino acid and tRNA pair (9,10). These organisms, including mitochondria (11), all synthesize amidated aminoacyl-tRNAs thanks to an enzymatic system that includes a non-discriminating (ND) aaRS, ND-aspartyl-tRNA synthetase (ND-AspRS) and/or ND-glutamyl-tRNA synthetase (ND-GluRS), providing a misacylated aminoacyl-tRNA species (Asn-tRNA\textsubscript{Asn} or Glu-tRNA\textsubscript{Gln}); these aminoacyl-tRNAs are then modified into Asn-tRNA\textsubscript{Asn} or Gln-tRNA\textsubscript{Gln} through a phosphorylation/transamidation mechanism catalyzed by a tRNA-dependent amidotransferase called GatCAB in bacteria (12–20). The synthesis of Asn-tRNA\textsubscript{Asn} and Gln-tRNA\textsubscript{Gln} is ensured by two separate bi-enzymatic systems, consisting in functionally related enzymes brought together by a scaffold/substrate tRNA. The resulting aaRS/tRNA/GatCAB complexes are named transamidosomes. These particles vary in cohesive strength, with the Asn-transamidosome of Thermus thermophilus (21) showing the highest stability while the Gln-transamidosomes of Thermotoga maritima and Helicobacter pylori are more dynamic (22–24). Within all of these complexes, the tRNA moiety can be used either as a scaffold for the nucleoprotein to form Asn or Gln or as a substrate for their synthesis. This makes the misacylation/transamidation system a matrix-assisted amino acid biosynthesis pathway of particular interest in H. pylori, a well-known and widespread human pathogen, which uses these indirect pathways to form Asn, Gln-tRNA\textsubscript{Gln} and Asn-tRNA\textsubscript{Asn} (24–28).

Although it was shown that mischarged Asp-tRNA\textsubscript{Asn} and Glu-tRNA\textsubscript{Gln} species are deprived of significant affinity for the elongation factor EF-Tu (29,30), several investigations have shown that they can lead in vivo to incorporation of Asp (31,32) or Glu (33–35) in place of Asn and Gln into proteins. Overexpression of either misacylating aaRS is toxic in Escherichia coli (31–35) while in yeast a disabled amidotransferase resulted in significant incorporation of Glu at Gln codons in mitochondrial proteins (36). Asn- and Gln-transamidosomes, which control the release of misacylated intermediates, may therefore be another layer of aminoacyl-tRNA quality control.

Here, we report how this quality control is achieved for Asn-tRNA\textsubscript{Asn} synthesis in H. pylori through the formation of an Asn-transamidosome. Moreover, we show that ND-AspRS exhibits surprisingly sensitive kinetic properties for Asp-tRNA\textsubscript{Asp} and Asp-tRNA\textsubscript{Asn} formation: fast release of Asp-tRNA\textsubscript{Asp} for translation and slow release of Asp-tRNA\textsubscript{Asn} to allow transamidosome formation by GatCAB binding. This safeguard mechanism resembles an editing process and may shed light on evolutionary processes that led to Asn infiltration into the present-day genetic code. Our results suggest that, in H. pylori, ND-AspRS and GatCAB co-evolved to generate an appropriate kinetic system able to reduce translational error level and to ensure a faithful codon reassignment for Asn.

**MATERIALS AND METHODS**

**Overproduction and purification of enzymes and tRNAs**

The *H. pylori* (Hp) ND-AspRS and GatCAB were overproduced from the cloned *aspS, gatC, gatA* and *gatB* genes as described (28,32). For protein purification, the cells from an overnight culture were harvested by centrifugation at 4000g, washed in cold Tris–HCl buffer 100 mM, pH 8.0, sedimented and suspended in the cell disruption buffer (1/3, w/v) formed by supplementing the above buffer with 5 mM 2-mercaptoethanol, 0.1 mM Na\textsubscript{2}EDTA, 1 mM benzamidine and 1 mM of protease inhibitors [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, AEBSF, Pefabloc]. The cell extract, obtained by sonication of the cell suspension and centrifugation at 105 000g, was dialyzed against potassium phosphate buffer 20 mM, pH 7.2, containing 0.5 mM Na\textsubscript{2}EDTA and 5 mM 2-mercaptoethanol, and adsorbed on a DEAE-cellulose column followed by elution with a linear gradient of potassium phosphate from 25 to 250 mM pH 7.2.

The AspRS-containing fractions, eluted between 130 and 150 mM salt, were then adsorbed on a Ni-NTA column in 50 mM Tris–HCl buffer pH 7.5 before elution of the proteins with a linear gradient from 25 to 450 mM of imidazole. Pure AspRS (10 mg at 95% purity) were obtained from 30 g cells. The GatCAB containing fractions eluted on DEAE-cellulose from 160 to 190 mM potassium phosphate were adsorbed on a Ni-NTA column in 50 mM Tris–HCl buffer pH 7.5. After washing with the buffer containing 25 mM imidazole, GatCAB was eluted with 450 mM imidazole. Pure GatCAB (50 mg at 95% purity) was obtained from 80 g of cells.

The reconstituted tRNA\textsubscript{Asn} gene from *T. thermophilus* was cloned in the pKK223 vector and purified as described (21). The *E. coli* (Ec) tRNA\textsubscript{Asp} gene was amplified from genomic DNA and cloned into the pUC18 vector for expression in the *E. coli* JM103 strain. Transfer RNA\textsubscript{Asp} (800 mg from 120 g of cells; accepting capacity 30.6 nmol mg\textsuperscript{-1}) was obtained by phenol extractions followed by chromatographies on DEAE-cellulose and nmmol mg Sepharose 4B as described (37). All tRNA\textsubscript{Asp} and tRNA\textsubscript{Asn} mutants used in this study were cloned into the pUC18 vector downstream a T7 promoter, transcribed and purified as described (38).
Gel-filtration

Experiments were conducted as described (21) using an ÄKTA Purifier and a 24-ml Superdex G200 column (GE Healthcare) equilibrated with 50 mM Na–HEPES buffer pH 7.2 containing 30 mM KCl, 6 mM MgCl2, 0.1 mM Na2EDTA and 5 mM 2-mercaptoethanol at 12°C. Samples of 0.2 ml containing the enzyme and tRNA partners at the indicated concentrations were diluted in the equilibration buffer and analyzed. \( K_D \) values [free enzyme] \( \times \) [free tRNA\(^{\text{Asn}}\)]/[enzyme/tRNA\(^{\text{Asn}}\) complex] were determined by evaluation of the areas of enzyme-bound tRNA and free tRNA peaks, the sum of which equals the total tRNA present in the sample. The contaminating non-specific tRNA was subtracted from free tRNA. The quantity of free enzyme was determined by subtracting the quantity of enzyme-bound tRNA from total amount of enzyme in the sample.

Dynamic light scattering

Dynamic light scattering (DLS) experiments were conducted as described (21) at 20 ± 0.1°C in a Zetasizer NanoS instrument (Malvern, UK). The 20- to 40-μl samples contained 10 μM ND-AspRS (20 μM subunits) and/or 20 μM GatCAB with or without 20 μM tRNA\(^{\text{Asn}}\) in the gel-filtration buffer. When present, ATP, Asp and Gln were at a concentration of 2 mM. Hydrodynamic diameters were corrected for solvent refractive index \( (n = 1.3353) \) and absolute viscosity \( (\eta = 1.041 \text{ mPa.s}) \).

Aminoacylation of tRNA

The reaction mixture contained 100 mM Na–HEPES buffer pH 7.2, 30 mM KCl, 12 mM MgCl2, 2 mM ATP, 20–50 μM \([^{14}\text{C}] \text{L-Asp}\) (330 cpm.pmol\(^{-1}\), GE Healthcare), 0.1 mg.ml\(^{-1}\) bovine serum albumin, 10 μM \( T. \) thermophilus \( (Tt) \) tRNA\(^{\text{Asn}}\) or \( E. \) coli tRNA\(^{\text{Asp}}\), or 0.04–6 mM \( Tt \) tRNA\(^{\text{Asn}}\) for \( K_M \) measurements, and 0.01–0.5 μM \( H. \) pylori ND-AspRS. The reactions were conducted at 37°C unless otherwise indicated. The rate-limiting step was determined with 0.5, 1.0 and 1.5 μM \( H. \) pylori ND-AspRS and 10 μM tRNA\(^{\text{Asp}}\) or tRNA\(^{\text{Asp}}\) without or with GatCAB at the indicated concentrations in the presence of 15% glycerol at 4°C. For \( K_M \) measurements of Asp and ATP in the presence of tRNA\(^{\text{Asp}}\) or tRNA\(^{\text{Asn}}\), the small substrate concentrations varied from 10 to 300 μM and tRNA was present at a 10 μM concentration. Inhibition experiments with aspartol-adenylate (Asp-ol-AMP), kindly provided by Prof. R. Chènevert (Université Laval, Québec), were performed with concentrations ranging from 10 to 200 μM. The inhibition constants were determined as described (39). The \([^{14}\text{C}] \text{aminoacyl-tRNA}\) formed in 10 or 20 μl aliquots withdrawn at various time intervals was determined as described (1).

ATP-PPI exchange

The reaction mixture contained 100 mM Na–HEPES buffer pH 7.2, 10 mM MgCl2, 2 mM L-Asp, 2 mM ATP, 2 mM \([^{32}\text{P}] \text{PPI}\) (2 cpm.pmol\(^{-1}\), Perkin Elmer) and 0.5 μM ND-AspRS. The \([^{32}\text{P}] \text{ATP}\) formed at 37°C was determined in 40 μl aliquots at various time intervals as described (1).

Transamidation of Asp-tRNA\(^{\text{Asn}}\)

Reactions were conducted at 37°C for 10 min in a mixture containing 100 mM Na–HEPES buffer pH 7.2, 30 mM KCl, 12 mM MgCl2, 2 mM ATP, 10 μM \([^{14}\text{C}] \text{L-Asp}\) (330 cpm.pmol\(^{-1}\)), 1 mM L-Gln, 0.1 mg.ml\(^{-1}\) bovine serum albumin and 10 μl of the transamidosome fraction eluted from size-exclusion chromatography. Transamidation was measured as described (21,24).

RESULTS

An Asn-transamidosome in \( H. \) pylori

Association of ND-AspRSs, tRNA\(^{\text{Asn}}\) and GatCAB from \( T. \) thermophilus leads to a stable ternary complex named Asn-transamidosome (21). Gel-filtration and DLS experiments conducted with the \( H. \) pylori partners show that the ND-AspRSs can bind both tRNA\(^{\text{Asp}}\) and tRNA\(^{\text{Asn}}\) but with different affinities \( (K_D \text{ values of 7.9 and 21.4 μM respectively, Figure 1A}) \) and that GatCAB can form a much more stable binary complex with tRNA\(^{\text{Asn}}\) than ND-AspRS \( (K_D \text{ value of 2.1 μM, Figure 1B}) \). These divergent \( K_D \) values differ from those obtained using the \( T. \) thermophilus partners, where tRNA\(^{\text{Asn}}\) bound to ND-AspRS with higher affinity than to GatCAB (21). The asymmetric \( K_D \) values determined with the \( H. \) pylori system were confirmed by DLS (Figure 2). Indeed, a higher-sized particle was clearly seen in the presence of ND-AspRS and tRNA\(^{\text{Asp}}\) (12.6 nm), compared to free ND-AspRS and tRNA \( (10.9 \text{ and 4.9 nm, respectively}) \) but not when ND-AspRS and tRNA\(^{\text{Asn}}\) were mixed (Figure 2, lane 1–4) (10.9 nm). Similarly, we detected an association between GatCAB and tRNA\(^{\text{Asn}}\) \( (10.9 \text{ nm}) \) compared to their isolated counterparts \( (9.4 \text{ and 4.9 nm, respectively}) \) (Figure 2, lanes 5 and 6). No association of the protein partners in the absence of tRNA was detected by either technique (data not shown). Finally, when the three partners were mixed, a new ribonucleoprotein (RNP) of significantly higher size \( (13.5 \text{ nm}) \) appeared (Figure 2, lane 7). Isolation of this complex by gel-filtration (Figure 1C) and analysis of its components by SDS-PAGE (Figure 1D) revealed the presence of ND-AspRS, the three GatCAB subunits and tRNA\(^{\text{Asn}}\). Functional analysis confirmed that it was fully able to synthetize Asn-tRNA\(^{\text{Asn}}\) in the presence of free Asp, ATP and Gln (Figure 1E). Because the association of the protein partners is tRNA\(^{\text{Asn}}\)-dependent, this complex constitutes a bona fide Asn-transamidosome according to previous arguments (21).

Dynamics of the \( H. \) pylori Asn-transamidosome

In \( T. \) thermophilus, the Asn-transamidosome is characterized by its remarkable stability in gel filtration (21,22), whereas for \( H. \) pylori, it is less stable (Figure 1C). Since this observation could reflect a difference in dynamic properties, we tested the stability of the \( Hp \) Asn-transamidosome by measuring its hydrodynamic properties.
diameter in the presence of small substrates, e.g. under catalytic conditions. The particle size decreased from 13.6 nm under non-catalytic conditions to 10.9 nm under aspartylation conditions (i.e. presence of Asp and ATP), and from 13.6 to 12.9 nm under transamidation conditions (i.e. presence of Asp, ATP and Gln). Furthermore, gel-filtration with a mix of GatCAB, ND-AspRS and preformed Asp-tRNAAsn revealed elution of the binary GatCAB/Asp-tRNAAsn complex and a separate ND-AspRS peak (data not shown). These results suggest that charging of tRNAAsn may trigger destabilization of the transamidosome (Figure 2, lanes 7–9), apparently through release of ND-AspRS. Hence the Hp Asn-transamidosome displays completely different dynamics than does its T. thermophilus counterpart (21).

**Helicobacter pylori** ND-AspRS, an enzyme with dual kinetic behavior

Table 1 summarizes the steady-state kinetic parameters of Hp ND-AspRS for aminoacylation of tRNAAsp and...
tRNA<sup>Asn</sup>. The enzyme aspartylates tRNA<sup>Asp</sup> 2.5-fold faster than tRNA<sup>Asn</sup> (0.33 and 0.14 s<sup>-1</sup>), as reported previously (32). However, pre-steady-state kinetics conducted under conditions that allowed an examination of the first catalytic cycles of the enzyme (4°C and in the presence of 10 or 15% of glycerol) revealed intriguing differences in aminoacylation of both tRNAs since in contrast to tRNA<sup>Asp</sup> the charging kinetics of tRNA<sup>Asn</sup> were biphasic. Aminoacylation of tRNA<sup>Asp</sup> remains linear after completion of the first catalytic cycle. Thus, this first cycle occurs with the same rate as subsequent ones (0.041 s<sup>-1</sup>) (Figure 3). Since ATP-Pi exchange rate is significantly faster than tRNA charging (15.9 and 0.33 s<sup>-1</sup> at 37°C), the steady-state rate of tRNA<sup>Asp</sup> aminoacylation is dictated by the rate of transfer of activated Asp onto tRNA<sup>Asp</sup>. Surprisingly, when tRNA<sup>Asn</sup> is used as a substrate, biphasic kinetics arise, which exhibit a burst of Asp-tRNA<sup>Asn</sup> formation (~0.04 s<sup>-1</sup>) followed by a significantly slower linear phase (0.0033 s<sup>-1</sup>) (Figure 3). Extrapolation of the linear phase at t<sub>0</sub> points to formation of one Asp-tRNA<sup>Asn</sup> per ND-AspRS active site during the fast phase (Figure 3) and suggests that the first tRNA<sup>Asn</sup> is aspartylated significantly faster than those following. Interestingly, the first tRNA<sup>Asn</sup> is aminoacylated with a rate equivalent to that observed for tRNA<sup>Asp</sup> (Figure 3). Burst and steady-state rate values are both dependent on enzyme concentration (Supplementary Figure S1A). In the slow phase, the steady-state rate of tRNA<sup>Asn</sup> charging increases with the pH but not significantly with the ionic strength (Supplementary Figure S1B). This kinetic behavior is consistent with the release of Asp-tRNA<sup>Asn</sup> being the rate-limiting step at the steady-state of the reaction (40). The slow dissociation of the Asp-tRNA<sup>Asn</sup> product agrees with the absence of detectable hydrolysis of its ester bond in the presence of ND-AspRS (28).

**GatCAB modifies the kinetic properties for tRNA<sup>Asn</sup>**

Table 1 shows the effect of GatCAB on kinetic constants of Hp ND-AspRS for tRNA<sup>Asn</sup> and tRNA<sup>Asp</sup> aspartylation. The addition of GatCAB increases the steady-state rate of Asp-tRNA<sup>Asn</sup> formation according to a hyperbolic curve (Figure 4A), but has no effect on the steady-state rate of tRNA<sup>Asp</sup> charging (Figure 4A, inset and Table 1), indicating that this phenomenon is specific to tRNA<sup>Asn</sup>. When saturating concentrations of GatCAB are reached, the rate of tRNA<sup>Asn</sup> aspartylation increases 1.8-fold (0.14–0.25 s<sup>-1</sup>) and nearly fits that of tRNA<sup>Asp</sup> (0.33 s<sup>-1</sup>, Table 1), while the K<sub>M</sub> value of ND-AspRS for tRNA<sup>Asn</sup> increases 3.8-fold, leading to an increase in the overall charging efficiency (k<sub>cat</sub>/K<sub>M</sub>) of 1.8-fold (Table 1). Thus, in the presence of GatCAB, aminoacylation of tRNA<sup>Asn</sup> occurs with a similar efficiency as that of tRNA<sup>Asp</sup> (k<sub>cat</sub>/K<sub>M</sub>, respectively of 0.27 and 0.30 μM<sup>-1</sup>s<sup>-1</sup>). Pre-steady-state kinetics reveal that this GatCAB-mediated effect originates in an increase in the rate of aminoacylation during the slow phase (0.0033–0.012 s<sup>-1</sup>, Figure 5A and B). The rate of the fast phase does not seem to be affected (Figure 5B). Considering that tRNA<sup>Asn</sup> binds GatCAB and ND-AspRS with K<sub>D</sub> values of 2.1 and 21.4 μM, respectively (Figure 1), and that aspartylation of tRNA<sup>Asn</sup> is more efficient when it is ‘labeled’ with or ‘presented’ by GatCAB (Table 1), the GatCAB/tRNA<sup>Asn</sup> complex may constitute a better substrate for misacylation by the ND-AspRS. We named this complex the tRNA<sup>Asn</sup>-presentation complex (tRNPC). Since this complex is non-productive in absence of ND-AspRS and can also be a substrate, tRNPC can be considered as a bona fide transfer ribonucleoprotein (tRNP).

Interestingly, the tRNPC has opposing effects on the discriminating AspRS of E. coli (D-AspRS) (Figure 4B) in vitro. Ec D-AspRS is able to aspartylate tRNA<sup>Asn</sup>, in addition to its cognate tRNA<sup>Asp</sup>, albeit much less efficiently (14). As shown for other mischarging kinetics of non-cognate tRNAs, the transfer step is rate-limiting (41). GatCAB decreases the rate of aminoacylation of tRNA<sup>Asn</sup> by the D-AspRS (Figure 5B). This result can be

**Table 1. Kinetic constants for aspartylation of tRNA<sup>Asp</sup> and tRNA<sup>Asn</sup> and a tRNA variant with H. pylori ND-AspRS in the absence and presence of GatCAB**

<table>
<thead>
<tr>
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<th>−GatCAB</th>
<th>Burst</th>
<th>+GatCAB</th>
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<tr>
<td>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>K&lt;sub&gt;M&lt;/sub&gt; (μM)</td>
<td>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>K&lt;sub&gt;M&lt;/sub&gt; (μM)</td>
</tr>
<tr>
<td>tRNA&lt;sup&gt;Asp&lt;/sup&gt;</td>
<td>0.33 ± 0.01 n.d.</td>
<td>–</td>
<td>0.31</td>
</tr>
<tr>
<td>tRNA&lt;sup&gt;Asn&lt;/sup&gt;</td>
<td>0.14 ± 0.02 0.08 ± 0.01</td>
<td>–</td>
<td>0.25 ± 0.03 0.3 ± 0.03</td>
</tr>
<tr>
<td>yeast tRNA&lt;sup&gt;Asp&lt;/sup&gt;</td>
<td>0.29 ± 0.03 n.d.</td>
<td>–</td>
<td>n.d.</td>
</tr>
<tr>
<td>tRNA&lt;sup&gt;Asn&lt;/sup&gt;</td>
<td>0.26 ± 0.02 n.d.</td>
<td>+</td>
<td>0.28 ± 0.02 n.d.</td>
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interpreted through the strong binding of tRNA<sub>Asn</sub> on GatCAB and the low affinity of the D-AspRS for tRNA<sub>Asn</sub>. In contrast to the <i>H. pylori</i> system, GatCAB decreases the rate of Asp-tRNA<sub>Asn</sub> formation by Ec D-AspRS presumably through sequestration of tRNA<sub>Asn</sub> limiting the free tRNA<sub>Asn</sub> available for aminoacylation. Thus, the enhancing effect of GatCAB is restricted to tRNA<sub>Asn</sub> aspartylation by ND-AspRS.

In vivo experiments confirmed that expression of <i>Hp</i> ND-AspRS in the transformed <i>E. coli</i> ER Asn-auxotrophic strain was toxic presumably because of the accumulation of Asp-tRNA<sub>Asn</sub> (Supplementary Figure S2), as previously shown (32). An antibiogram-like test with an IPTG-soaked paper disk clearly triggers a growth defect phenotype compared to controls (Supplementary Figure S2A–C). Co-expression of <i>Hp</i> GatCAB reduces this toxic effect, since the enzyme is able to convert Asp-tRNA<sub>Asn</sub> into Asn-tRNA<sub>Asn</sub> allowing growth of the recombined strain (Supplementary Figure S2D), although with lesser efficiency.

**GatCAB helps to release Asp-tRNA<sub>Asn</sub> from the ND-AspRS active site**

The acceleration of the steady-state rate of tRNA<sub>Asn</sub> aspartylation by GatCAB (Figure 5) suggests that GatCAB controls the release of Asp-tRNA<sub>Asn</sub> from the ND-AspRS active site. To test this hypothesis, we used a strategy to nullify this effect without altering GatCAB or ND-AspRS. In other words, we asked whether this effect was solely due to the ability of GatCAB to bind Asp-tRNA<sub>Asn</sub>. To this end, we investigated aspartylation of tRNA<sub>Asn</sub> variant mutated at positions known to determine recognition by GatCAB (38). The U<sub>1</sub>–A<sub>72</sub> base-pair constitutes the major transamidation determinant, but recognition of tRNA<sub>Asn</sub> is further favored by the absence of the U<sub>20A</sub> position in the D-loop (38).

Importantly, these positions are not critical for ND-AspRS recognition (38,42). Thus, we constructed a G<sub>1</sub>–C<sub>72</sub>:U<sub>20A</sub>-containing tRNA<sub>Asn</sub> variant and verified that it is a suitable substrate for ND-AspRS but is not recognized by GatCAB (Figure 6 and 38). The aminoacylation kinetics of this tRNA<sub>Asn</sub> variant remained biphasic and saturating levels of GatCAB had no effect on the steady-state rate of aminoacylation (Figure 6A and B). Thus, removing the ability of GatCAB to bind this mutant Asp-tRNA<sub>Asn</sub> eliminated the impact of GatCAB on ND-AspRS kinetics. These results indicate that GatCAB enhances ND-AspRS kinetics by recognizing the wild-type aspartylated extremity of tRNA<sub>Asn</sub> according to the transamidation determinants, consequently freeing the ND-AspRS active site. As a
Consequence, the overall turnover of ND-AspRS increases in the presence of GatCAB.

Origin of the rate-limiting step

What are the structural elements that trigger different aminoacylation mechanisms with respect to tRNAAsp and tRNAAsn? The major differences between bacterial tRNAAsp and tRNAAsn lie within the acceptor arm (displaying a G1–C72 or a U1–A72 base pair, respectively) and the anticodon loop (positions C36 and U36 and C38 and A38 in tRNAAsp and tRNAAsn, respectively). Yeast cytoplasmic tRNAAsp provides a natural variant that displays a GUC ‘Asp’ anticodon and a bacterial ‘tRNAAsn-like’ acceptor-end that almost avoids aminoacylation by EcD-AspRS (43). Similar to bacterial tRNAAsp, aminoacylation of yeast tRNAAsp is monophasic (data not shown). Thus, the elements leading to the biphasic kinetics for aminoacylation of tRNAAsn by the HpND-AspRS are absent in yeast tRNAAsp. Further, substitution of C36 by U36 in bacterial tRNAAsp leading to a GUU ‘Asn’ anticodon did not trigger a biphasic kinetic response (data not shown). Hence, the divergent kinetic responses of HpND-AspRS for tRNAAsp versus tRNAAsn aminoacylation are probably triggered by different overall conformations of the aminoacyl-tRNA end-products, rather than by distinct affinities for the tRNA substrates. This hypothesis is in line with the fact that the two kcat’s of aspartylation (Table 1) show opposing trends with respect to the affinities of the enzyme for the two tRNAs (Figure 1). Moreover, the steady-state rate of tRNAAsn aspartylation is more affected by increase of the pH than by that of the ionic strength (Supplementary Figure S1), suggesting that the aminoacylated tRNAAsn extremity, and mostly the Asp moiety, plays a prevalent role in the slow dissociation of Asp-tRNAAsn, in accordance with other studies on the rate-limiting step of tRNA aminoacylation (40) and the inhibition properties of Asp-ol-AMP. Interestingly, this non-hydrolyzable Asp-AMP mimic, competitive with respect to Asp and ATP, shows different inhibition patterns for aminoacylation of tRNAAsp and tRNAAsn (39 and Supplementary Table S1). Indeed K_i values of 8 and 32 μM were determined in presence of tRNAAsp and tRNAAsn, respectively (Supplementary Table S1). This 4-fold increase in the K_i value when Asp-tRNAAsn is formed suggests a strong binding of the tRNAAsp-bound Asp moiety within ND-AspRS active site, compared to Asp-tRNAAsp. This is further confirmed with differential inhibition patterns using Asp-ol-AMP (Supplementary Table S1) in presence of tRNAAsp or tRNAAsn, the latter tRNA protecting the active site from inhibition more efficiently as a consequence of its retention. This is in agreement with a previous work on Pseudomonas aeruginosa ND-AspRS (39) showing that the limiting step for Asp-tRNAAsn synthesis could be general for ND-AspRS of bacterial type.

To obtain further insights into the origin of the distinct dissociation rates of the two aminoacyl-tRNAs from ND-AspRS, we performed Arrhenius activation energy (E_A) measurements (Supplementary Figure S3). The E_A values are the same for aminoacylation of tRNAAsp (68.6 ± 0.6 kJmol⁻¹K⁻¹) and tRNAAsn (66.9 ± 1.9 kJmol⁻¹K⁻¹). Thus, both E_A are determined by the same step, presumably the transfer step, since the release of aminoacyl-tRNA does not contribute significantly to E_A. From these similar E_A values, one can suggest that the steps preceding the release of Asp-tRNA are equivalent and that the differences occur after the transfer step. It is therefore reasonable to assume that the formation of Asp-tRNAAsp, or Asp-tRNAAsn trigger different structural arrangements of the active site. This conformational difference may explain the difference in Asp-tRNA release and in inhibition patterns by Asp-ol AMP.

DISCUSSION

The Asn-transamidosome in H. pylori

Previous work has suggested that an Asn-transamidosome should exist in H. pylori (28). Indeed, this organism does...
ND-AspRS displays a dual mode of release for its two Asp-tRNA products

ND-AspRS recognizes and aspartylates both tRNA^{Asp} and tRNA^{Asn} equivalently, which makes it a ND enzyme for substrate recognition and aminoacylation. Nevertheless, kinetic studies clearly show a difference in product release. Asp-tRNA^{Asn} is retained upon aminoacylation, while Asp-tRNA^{Amp} is not (Figure 3). This difference seems to be triggered by the overall conformation of tRNA rather than by specific identity elements. Our results suggest that the shapes of tRNA^{Amp} and tRNA^{Asn} could induce different active site configurations that lead to these divergent modes of release. According to the predominant effect of pH on the steady-state rate (slower phase) (Supplementary Figure S1), the CCA-Asp moiety may play an important role in Asp-tRNA^{Asn} retention on ND-AspRS. This is further confirmed with differential inhibition patterns using Asp-ol-AMP (Supplementary Table S1) in presence of tRNA^{Amp} or tRNA^{Asn}, the latter tRNA protecting the active site from inhibition more efficiently as a consequence of its retention. This is in agreement with a previous work on P. aeruginosa ND-AspRS (39) showing that the limiting step for Asp-tRNA^{Asn} synthesis could be general for ND-AspRS of bacterial type.

This scheme strongly shares analogy with editing mechanisms, where a tRNA gets misacylated with a wrong or near-cognate amino acid and retained on the enzyme to be edited. However, here, a cognate amino acid is loaded onto a ‘non-cognate’ tRNA^{Asn} and the Asp-tRNA^{Asn} product gets retained, perhaps to statistically favor further processing of the misacylated intermediate by GatCAB prior to its release (Scheme 1). We named this a ‘rescue mechanism’; it may be essential under conditions where GatCAB is not found in sufficient amounts within the cell or when it is occupied in the glutaminyl pathway that also exists in H. pylori.

Taking into account the hypothesis forged from a kinetic study (8), the rate-limiting step of ND-AspRS, like all class II aaRSs, is expected to be the transfer of activated Asp onto tRNA. In contrast, in class I aaRSs, the rate-limiting step is typically product release. Clearly, tRNA^{Amp} ND-AspRS shifts to a class I-like kinetic behavior with tRNA^{Amp} but retains its class II character with tRNA^{Asn}. Previously, it has been demonstrated that the class II phosphoseryl-tRNA synthetase (SepRS) used in the Cys-tRNA^{Ays} biosynthesis indirect pathway in Methanococcales janaschii, also has a rate-limiting step for the release of the aminoacyl-tRNA (46). The authors hypothesized that this characteristic could have evolved from modifications of the motifs implicated in aminoacylation, accelerating the transfer step over release, leading to a new rate-limiting step compared to other class II enzymes that is more similar to class I. ND-AspRS is a new example of an aaRS that can shift from a class II to a class I-like kinetic behavior, according to its tRNA substrate. This phenomenon fully correlates with the fates of Asp-tRNA products, since Asp-tRNA^{Asn} should be retained on ND-AspRS until GatCAB transforms it into Asn-tRNA^{Asn}, while Asp-tRNA^{Amp} should be released in line with its ‘cognate’ status. Together, these results allow us to make an analogy between the misacylation/transamidation pathway and editing strategies encountered with several classical aaRSs.
Scheme 1. Formation of Asn-tRNA^Asn in H. pylori. (A) The major pathway used in H. pylori (the main ‘Asn-transamidosome pathway’) starts with (1) the formation of the tRNPC, linking GatCAB and tRNA^Asn, onto which (2) ND-AspRS can then dock to form the Asn-transamidosome. In this ribonucleoprotein, tRNA^Asn gets aspartylated more efficiently (3), and the aspartylated CCA-end of tRNA^Asn shifts from the ND-AspRS active site to the GatCAB phosphorylation/amidation active site. Asn-tRNA^Asn biosynthesis proceeds thanks to amide donor hydrolysis (here free Gln) to provide the ammonia moiety necessary for transamidation (4) (28). When Asn-tRNA^Asn is formed, it is released together with ND-AspRS, and used in protein synthesis (5). (B) When GatCAB is not in sufficient amounts to produce enough tRNPC, free tRNA^Asn binds to ND-AspRS (6) and gets aminoacylated less efficiently (7). The dissociation of Asp-tRNA^Asn is rate-limiting, but if Asp-tRNA^Asn is released (8), GatCAB can still recognize it (9) and transamidate it (10) (28) into Asn-tRNA^Asn, which can be released to fuel translation (11). (C) Since the release of Asp-tRNA^Asn from ND-AspRS is rate-limiting, Asp-tRNA^Asn remains within the ND-AspRS active site for longer times, enabling free GatCAB to bind to the ND-AspRS/Asp-tRNA^Asn complex (12) to form an Asn-transamidosome, in which Asn-tRNA^Asn can be synthesized through transamidation (4) rejoining the main Asn-transamidosome cycle. This latter mechanism can be qualified as a ‘rescue mechanism’. The table summarizes the different pathway characteristics. The aspartylation and transamidation steps are highlighted to stress the fact that the Asn-transamidosome pathway couples both reactions, while they are sequential when GatCAB is absent or when the tRNPC is not involved.
GatCAB is a node for transamidation

*Helicobacter pylori* belongs to a subclass of organisms that lack both GlnRS and AsnRS, in which GatCAB plays a dual role in glutaminyltransferase and asparaginyltransferase. In contrast, archaea possess two tRNA-dependent amidotransferases each specific for one pathway (57). In *H. pylori*, GatCAB is able to bind very efficiently to uncharged tRNA^{Asn} (this study) but also to tRNA^{Gln} with equivalent affinities (2.1 and 2.0 μM, respectively) (Supplementary Scheme S1). This capacity provides two types of tRNA-presentation complexes: tRNAPC and the tRNA^{Gln}-presentation complex (tRQPC), which may represent a way to target GatCAB toward ND-AspRS and asparaginyltransferase on one hand and GluRS2 (the enzyme specific for tRNA^{Gln} misacylation in *Hp*) (25,34) and glutaminyltransferase on the other hand (Supplementary Scheme S1). This need to share GatCAB between two distinct aaRSs could explain the reason why the Asn- and Gln-transamidosomes are both more dynamic in *H. pylori* than in *T. thermophilus* (22) or *T. maritima* (23). Hence, transamidosomes may be adapted to the metabolic context and physiology of the organism from which they originate.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Table 1, Supplementary Scheme 1 and Supplementary Figures 1–3.

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