A single amidotransferase forms asparaginyl-tRNA and glutaminyl-tRNA

in Chlamydia trachomatis

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Running title: tRNA-dependent amino acid transformations in Chlamydia

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Aminoacyl-tRNA is generally formed by aminoacyl-tRNA synthetases, a family of twenty enzymes essential for accurate protein synthesis. However, most bacteria generate one of the two amide aminoacyl-tRNAs, Asn-tRNA or Gln-tRNA, by transamidation of mischarged Asp-tRNA^{Asn} or Glu-tRNA^{Gln} catalyzed by a heterotrimeric amidotransferase (encoded by the \textit{gatA}, \textit{gatB} and \textit{gatC} genes). The \textit{Chlamydia trachomatis} genome sequence reveals genes for eighteen synthetases while those for asparaginyl-tRNA synthetase and glutaminyl-tRNA synthetase are absent. Yet the genome harbors three \textit{gat} genes in an operon-like arrangement (\textit{gatCAB}). We reasoned that \textit{Chlamydia} uses the \textit{gatCAB}-encoded amidotransferase to generate both Asn-tRNA and Gln-tRNA. \textit{C. trachomatis} aspartyl-tRNA synthetase and glutamyl-tRNA synthetase were shown to be non-discriminating synthetases that form the misacylated tRNA^{Asn} and tRNA^{Gln} species. A preparation of pure heterotrimeric recombinant \textit{C. trachomatis} amidotransferase converted Asp-tRNA^{Asn} and Glu-tRNA^{Gln} into Asn-tRNA and Gln-tRNA, respectively. The enzyme used glutamine, asparagine or ammonia as amide donors in the presence of either ATP or GTP. These results suggest that \textit{Chlamydia trachomatis} employs the dual-specificity \textit{gatCAB}-encoded amidotransferase and eighteen aminoacyl-tRNA synthetases to create the complete set of twenty aminoacyl-tRNAs.
The codons of messenger RNA are paired on the ribosome with aminoacyl-tRNAs (AA-tRNAs) during the process of protein biosynthesis. As there are twenty canonical amino acids in proteins, a corresponding set of twenty AA-tRNAs is required. Since many organisms contain twenty aminoacyl-tRNA synthetases (AARSs) each capable of acylating the cognate tRNA with the correct amino acid (1), it was believed that this is the only path to AA-tRNA formation as first proposed in the adaptor hypothesis (2). However, the idea that there are twenty AARSs in all organisms has been challenged for many years beginning with the discovery of an alternative pathway to generate Gln-tRNA (3). Initially this was thought to be an interesting abnormality. Recent biochemical and functional genomic studies have made it clear that the “20 AARS rule” is preserved only in the eukaryotic cytoplasm while most organisms have less than twenty synthetases (4). The absence of glutamyl-tRNA synthetase (GlnRS) is by far the most common exception to the “20 AARS rule.” Most bacteria and all archaea known to date lack this enzyme. In addition, asparaginyl-tRNA synthetase (AsnRS) is absent in many archaea and also in some bacteria. Organisms lacking either AsnRS or GlnRS use a tRNA-dependent amino acid transformation pathway to generate Asn-tRNA or Gln-tRNA (Fig. 1). This alternate pathway is based on two extraordinary enzyme activities. First, it requires the presence of two non-discriminating aminoacyl-tRNA synthetases, a glutamyl-tRNA synthetase (GluRS) and an aspartyl-tRNA synthetase (AspRS). Such a non-discriminating enzyme differs from the canonical synthetase by having relaxed tRNA specificity that enables it to acylate the cognate and a non-cognate tRNA with the cognate amino acid. For instance, *Bacillus subtilis* GluRS produces Glu-tRNA^Gln^ in addition to Glu-tRNA^Glu^ (5). Non-discriminating AspRS enzymes have been shown in a number of archaea and bacteria (e.g., 6-8). Second, a tRNA-dependent amidotransferase (AdT) must amidate the mischarged aspartate or glutamate to form the correctly acylated tRNAs (6,9-12).

Bacterial AdTs are, in general, heterotrimeric enzymes (10,12,13). Their corresponding subunits are encoded by the *gatC, gatA*, and *gatB* genes, which are arranged in an operon-like
manner in the chromosomes of some bacteria. Biochemical characterization of these enzymes is still sketchy. However, the data suggest that GatA is the AdT’s catalytic subunit, that GatB is involved in recognition of the mischarged AA-tRNA, and that GatC is essential for proper expression and/or folding of a fully active GatA protein (10). All $gatCAB$-encoded bacterial AdTs studied to date are responsible only for \textit{in vivo} synthesis of one of the two possible amide AA-tRNAs (employing Asp-AdT or Glu-AdT activity) while the other amide AA-tRNA is formed by direct aminoacylation by AsnRS or GlnRS. Looking at the available genomic sequences, eight of thirteen archaea lack both AsnRS and GlnRS, but encode two different AdT enzymes for formation of Asn-tRNA and Gln-tRNA. The discovery of an archaeal heterodimeric GatDE amidotransferase specific for Gln-tRNA formation suggests that the two amide AA-tRNA species in archaea are very likely synthesized by two different AdTs (4). Bacterial genomes do not encode this latter enzyme. Nevertheless, complete genomes lacking identifiable GlnRS and AsnRS genes are known, such as \textit{Campylobacter jejuni}, all known \textit{Chlamydia} strains, \textit{Helicobacter pylori}, \textit{Mycobacterium tuberculosis}, and \textit{Rickettsia prowazekii} (14). It has been shown \textit{in vitro} that, when provided with suitable heterologous substrates, the \textit{B. subtilis}, \textit{Deinococcus radiodurans} and \textit{Thermus thermophilus} GatCAB amidotransferases can synthesize both Asn-tRNA and Gln-tRNA, even though they are only required to synthesize one of these AA-tRNAs \textit{in vivo} (12,13). Using a heterologous substrate, the \textit{Acidithiobacillus ferrooxidans} amidotransferase has been described as dual-specific. However, its genome sequence is not yet complete, thus this organism might still contain the canonical AsnRS or GlnRS activities although they were not seen in cell extracts (15). These data suggested that in organisms lacking both GlnRS and AsnRS the $gatCAB$-encoded amidotransferase is an Asp/Glu-AdT that might provide both Asn-tRNA and Gln-tRNA for protein synthesis. Therefore, we decided to test this idea using \textit{Chlamydia trachomatis} as a model. Here we report that pure \textit{Chlamydia trachomatis} amidotransferase can amidate \textit{Chlamydia} Asp-tRNA$^{\text{Asn}}$ and Glu-tRNA$^{\text{Gln}}$ generated by the homologous AspRS and GluRS enzymes. This suggests that this
single amidotransferase is responsible for both Asn-tRNA and Gln-tRNA formation in this human pathogenic parasite.

EXPERIMENTAL PROCEDURES

General—Chlamydia trachomatis genomic DNA was a gift of L. Olinger (16). Oligonucleotide synthesis and DNA sequencing were performed by the Keck Foundation Research Biotechnology Resource Laboratory at Yale University. The pBAD expression vector was from Invitrogen. The Ceramic Hydroxyapatite Type I (5 mL) column was from BioRad. HiTrap Heparin (5 mL) and HiTrap Q (5 mL) columns were from Amersham Pharmacia Biotech. Cellulose thin layer chromatography plates were from Macherey-Nagel. Epicurian Coli® BL21-CodonPlus™ Competent Cells were purchased from Stratagene. Preparations of Clostridium acetobutylicum AsnRS and AspRS2 and D. radiodurans AspRS2 were kindly given by Bokkee Min, Benfang Ruan and Joanne Pelaschier (Yale University), D. radiodurans GluRS and E. coli GlnRS were obtained from Dylan Chan, Hiroyuki Kobayashi and Debra Tumbula-Hansen (Yale University). Nucleoside triphosphates were of sequencing grade and HPLC analysis showed no cross-contamination.

Preparation of in vivo Overexpressed C. trachomatis tRNA species—The tRNA genes (tRNAAsp, tRNAAsn, tRNAGlu, tRNAGln) were constructed in the pKK223-3 vector (Pharmacia) by cassette cloning of oligonucleotides synthesized according to the tRNA sequence and their complement, and subsequent ligation into the HindIII-BamHI digested vector generating pKtRNAAsn and pKtRNAGln. Positive clones were sequenced and used for tRNA overexpression in E. coli.

A 30-mL culture of E. coli JM105 (Amersham), harboring one of the pKtRNA clones, in Luria-Bertani (LB) medium supplemented with 75 µg/mL ampicillin was incubated at 37°C overnight, and then used as inoculum for a 750 mL culture. Once A600 of 0.5 was reached, tRNA expression was induced by addition of 1 mM IPTG and the culture supplemented with 50 µg/mL ampicillin. After 8 hr incubation, cells were harvested by centrifugation at 4,000 × g for
10 min at 4°C and suspended in Buffer A [20 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 10 mM 2-mercaptoethanol] to a final volume of 12.5 mL. Total nucleic acids were recovered by extraction with 12.5 mL buffer (pH 4.6)-saturated phenol. After 20 min agitation at 23°C, and 10 min centrifugation at 4,000 × g, the aqueous phase was removed and saved. The phenol phase was re-extracted with 12.5 mL of buffer A. The pooled aqueous phases were extracted with an equal volume of phenol, and the aqueous layer was recovered. DNA was partially removed by precipitation with 20% (v/v) of 2-propanol. After centrifugation for 15 min at 4,500 × g, the supernatant was adjusted to 60% (v/v) of 2-propanol. The tRNA precipitate was harvested by centrifugation for 25 min at 4,500 × g. After one wash the pellet was suspended in 5 mL of 200 mM Tris-acetate, pH 8.5, and incubated at 37°C for 1 hr to deacylate the tRNA. The RNA was then chromatographed over DEAE-cellulose (0.01 mL DE52/A₂₆₀). The sample was loaded onto the column, washed with several volumes of 0.01 M sodium acetate, pH 5.2, 0.2 M NaCl, 0.01 M MgCl₂. The tRNA was eluted with 1 M NaCl and recovered by ethanol precipitation (10) and then resuspended in 1.7 mL water. The heterologous expression of the *Chlamydia* tRNA genes was very good. For instance, expression of the tRNA\(^{\text{Asn}}\) gene gave a ‘total *E. coli* tRNA’ sample that could be aspartylated to 145 pmoles/A₂₆₀ compared to 25 pmoles/A₂₆₀ of the *E. coli* tRNA before expression. Given that there are 15 pmoles/A₂₆₀ of *E. coli* tRNA\(^{\text{Asn}}\) in the normal tRNA preparation, the tRNA\(^{\text{Asn}}\) in the ‘total *E. coli* tRNA’ sample is comprised mainly of the *Chlamydia* species (approx. 90%). The four *Chlamydia* tRNA species relevant for this study (tRNA\(^{\text{Asp}}\), tRNA\(^{\text{Asn}}\), tRNA\(^{\text{Glu}}\), tRNA\(^{\text{Gln}}\)) were purified from the tRNA preparation after aminoacylation and biotinylation as previously described (17). Aminoacylation showed the individual tRNA preparations to be approximately 95% pure.

**Construction of C. trachomatis AspRS, GluRS and AdT Overexpression Clones**—DNA sequences encoding the AspRS, GluRS, and GatCAB amidotransferase as identified in the genome analysis (16) were used to design primers for PCR amplification of the open reading frames (starting with ATG). After cloning the DNA fragments into pBAD expression vectors
(with and without His$_6$ sequence) and confirmation of their sequences, the plasmids were transformed into the *E. coli* BL21CodonPlus$^\text{TM}$ strain.

**Overexpression and Purification of C. trachomatis Enzymes**—The *E. coli* strain harboring a plasmid containing the *gatCAB* operon (with a C-terminal thioredoxin fusion for enhanced expression) was grown in a 5 L culture. At a cell density of $A_{600} = 0.5$ expression of the minor tRNAs was induced with 1 mM IPTG while AdT expression was induced with 0.02% (v/v) L-arabinose. After growth for 12 hr the culture was centrifuged (5 min, $4,000 \times g$, 4°C) to harvest the cells (30 g), which were then suspended in 15 mL buffer (10 mM potassium phosphate, pH 6.8, 5 mM 2-mercaptoethanol, 0.1 mM Na-EDTA, 0.1 mM benzamidine, 10% (v/v) glycerol). Following cell disruption by sonication (15 × 20 s, 60 V) and removal of cell debris by low-speed centrifugation, an S-100 fraction was prepared (centrifugation at 100,000 × g for 1 hr). All subsequent steps were performed at 4°C; all buffers contained 5 mM 2-mercaptoethanol, 0.1 mM Na-EDTA, 0.1 mM benzamidine, and 10% (v/v) glycerol.

The S-100 extract (30 mL) was applied to a CHT Type I column (5 mL) equilibrated and washed with 10 mM potassium phosphate, pH 6.8. Proteins were eluted with a linear gradient (500 mL, 2.0 mL/min) of 10-50 mM potassium phosphate, pH 6.8. Active fractions (253 mL) were pooled, dialyzed against a solution of 10 mM NaCl, 1mM MgCl$_2$ and applied to a CHT Type I column (5 mL) equilibrated with the same buffer solution. Basic and neutral proteins were removed by extensive washing (150 mL, 2 mL/min) with 10 mM NaCl, 1mM MgCl$_2$. Additional proteins were eliminated by washing with a linear gradient (100 mL, 2 mL/min) of 0.001-1M MgCl$_2$ in 10 mM NaCl. The column was then equilibrated with 10 mM potassium phosphate, pH 6.8, and proteins were eluted with a linear gradient (200 mL, 2mL/min) from 10-250 mM potassium phosphate, pH 6.8. Active fractions (110 mL) were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, loaded onto a HiTrap Heparin (2 × 5 mL, in series) column equilibrated with the same buffer, and the protein eluted with an isocratic flow (50 mL, 2 mL/min) of 100 mM KCl. Active fractions (15 mL) were dialyzed against 50 mM Hepes-KOH,
pH 7.2, and applied to a HiTrap Q column (5mL). Proteins were eluted with a linear gradient (100 mL, 2 mL/min) of 10-500 mM NaCl. Pure AdT fractions (20 mL) were dialyzed against 50 mM Hepes-KOH, pH 7.2, containing 50% (v/v) glycerol, and stored at -20°C. The purity of the enzyme was determined by SDS-PAGE and native-PAGE.

AspRS and GluRS were partially purified. These enzymes were overexpressed in pBAD vector with or without an N-terminal His6-tag. The His-tagged enzymes were purified on a nickel-nitrilotriacetate resin (Qiagen), while native enzymes were chromatographed on Q-Sepharose (Pharmacia).

Formation of Aminoacyl-tRNA—For amidotransferase assays, a total of 5 nmoles of unfractionated tRNA from an *E. coli* stain which expresses the *C. trachomatis* tRNA\textsubscript{Gln} or tRNA\textsubscript{Glu} was charged with [\textsuperscript{14}C]glutamate (50 µM, 260 mCi/mmol) by 4 µg of partially purified native or His\textsubscript{6}-tagged GluRS. Charging curves were generated as described (10,12) to check the activity with purified *C. trachomatis* tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Gln} expressed in *E. coli*. GluRS from *D. radiodurans*, *B. subtilis* (10), and *E. coli* were also used. *C. trachomatis* tRNA\textsuperscript{Asn} or tRNA\textsuperscript{Asp} (4 nmoles of unfractionated tRNA from an *E. coli* strain expressing the tRNA, or the purified tRNA\textsuperscript{Asp} and tRNA\textsuperscript{Asn} species) was charged with [\textsuperscript{14}C]aspartate (50 µM, 213 mCi/mmol) by 4 µg of partially purified native or His\textsubscript{6}-tagged *C. trachomatis* AspRS. AspRS from *E. coli*, *D. radiodurans* (AspRS2), and *C. acetobutylicum* (AspRS1 and AspRS2) were also used. The charging reaction was performed at 37°C for 30 min in a 200 µl standard reaction mixture containing 50 mM Hepes-KOH, pH 7.2, 10 mM ATP, 25 mM KCl, 15 mM MgCl\textsubscript{2}, 5 mM DTT. For kinetic analyses, time points were taken in the initial velocity range in triplicate, testing seven different concentrations of tRNA. \( K_M \) for the two tRNA substrates was calculated by non-linear regression fitting of data to the Michaelis-Menten equation. The AA-tRNA to be used as substrate to examine the amidotransferase reaction was extracted with acid-buffered phenol, followed by a chloroform extraction and an ethanol precipitation. The [\textsuperscript{14}C]AA-tRNA was dried completely and stored at -80°C until needed.
Amidotransferase Activity Assay—The activity assay was adapted from previous work (10,18). Aminoacyl-tRNA was suspended in 20 µl (2 ×) amidation buffer (20 mM Hepes-KOH, pH 7.2, 10 mM KCl, 2 mM DTT). The AdT was characterized in the absence or presence of the following: 3 mM MgCl₂, 2 mM NTP, 2 mM amide group donor, 0.2 mM sulfhydryl reducing reagent. An equal volume of AdT sample (0.1 nmol) was added and the mixture was incubated at 37°C for 10–30 min. The reaction was stopped by the addition of 50 µl 0.6 M sodium acetate, pH 5.2, and followed by extraction with an equal volume of buffer (pH 5.2)-saturated phenol, followed by extraction with an equal volume of chloroform. The aqueous phase was removed and AA-tRNA was ethanol precipitated and pelleted by centrifugation (15,000 × g, 4°C, 30 min). The dried pellet was suspended in 50 µl 25 mM KOH and incubated at 65°C for 15 min to deacylate the AA-tRNA. The mixture was neutralized by addition of 1.3 µl 100 mM HCl, and then vacuum-dried. Samples were suspended in 6 µl of double distilled water, and a 1 µl aliquot was spotted on a cellulose TLC plate. After chromatography in ammonia: water: chloroform: methanol (2:1:6:6) the plate was dried at 65°C and then exposed to an activated phosphor-imaging plate for 8-12 hr. [14C]amino acids were detected by scanning the image plate using a Fuji or Storm 860 Bioimager and analyzed with FUJI IMAGE GAUGE V3.3 software or MOLECULAR DYNAMICS IMAGEQUANT V4.0. Localization of [14C]amino acids was confirmed by ninhydrin assay using 50 nmol of unlabeled standards.

RESULTS

C. trachomatis Has Two Non-discriminating Aminoacyl-tRNA Synthetases—A prerequisite of the transamidation route of Asn-tRNA and Gln-tRNA formation is the presence of non-discriminating AspRS and GluRS enzymes able to synthesize the mischarged tRNA substrates for the AdT. Therefore, we cloned (based on the genome sequence), expressed and partially purified these enzymes as described in Experimental Procedures. Both chlamydial synthetases have comparable activity when expressed as native protein or with the His₆-tag (data not
Because of ease of purification, the His6-tagged proteins were used. The *C. trachomatis* genome also contains a single tRNA\textsuperscript{Asn} and a tRNA\textsuperscript{Gln} gene (19). These genes were cloned and overexpressed in *E. coli*. The charging efficiency of the chlamydial synthetases, using the *C. trachomatis* tRNA\textsuperscript{Asn} and tRNA\textsuperscript{Gln} preparations, were comparable to those obtained with the well-characterized non-discriminating *B. subtilis* GluRS (5) and *D. radiodurans* AspRS2 (8) enzymes. The purified *Chlamydia* tRNA species (tRNA\textsuperscript{Asp}, tRNA\textsuperscript{Asn}, tRNA\textsuperscript{Glu}, tRNA\textsuperscript{Gln}) could be charged well with *Chlamydia*, *E. coli*, *Deinococcus* and *Clostridium* synthetases (see Figs. 2A, 2B and 3A, 3B). In addition, formation of *Chlamydia* Asp-tRNA\textsuperscript{Asn} was efficient when *D. radiodurans* AspRS2, *C. acetobutylicum* AspRS2 or *Chlamydia* AspRS were used (Fig. 2C). Similarly, formation of Glu-tRNA\textsuperscript{Gln} was accomplished by *B. subtilis* and *Chlamydia* GluRS (Fig. 3C). As *Chlamydia* AspRS does not resemble the archaeal non-discriminating AspRS proteins (see below) we determined the $K_M$ for tRNA\textsuperscript{Asp} and tRNA\textsuperscript{Asn} (0.95 ± 0.4 µM and 2.76 ± 0.6 µM, respectively). These results show that the chlamydial GluRS and AspRS are non-discriminating AARSs and efficiently produce Glu-tRNA\textsuperscript{Gln} and Asp-tRNA\textsuperscript{Asn}.

*C. trachomatis* GatCAB Amidotransferase Has Both Glu-AdT and Asp-AdT Activities—Orthologs of the three AdT encoding genes (*gatA*, *gatB*, and *gatC*) were found in the *C. trachomatis* genome (16). Interestingly, these genes are adjacent and situated in an operon-like manner with the same arrangement as in *B. subtilis* (10). The whole operon was cloned into an *E. coli* expression vector as a thioredoxin fusion of the gatC subunit, overexpressed and the protein product was purified to homogeneity. The presence of heterologous ribosomal binding sites upstream of gatC, gatA and gatB genes does not seem to restrict the overexpression of GatCAB, suggesting that a chlamydial promoter region can be recognized by *E. coli*. Our purification procedure consisted of three chromatographic media (hydroxyapatite, Heparin sepharose, Q sepharose) and allowed purification of 8.4 mg GatCAB from 30 g of cells with a yield of approximately 10% (Table I). SDS-PAGE analysis of the purified enzyme corroborated the predicted molecular mass of the three ORFs: GatC 25.3 kDa (11.1 kDa GatC plus a 14.2 kDa...
thioredoxin fusion protein); GatA, 55.0 kDa; GatB 53.6 kDa (Fig. 4A). Native PAGE revealed only one band confirming an intact heterotrimeric enzyme (Fig. 4B). The intensity of the stain suggested an approximately equal ratio of the three subunits (Fig. 4A).

The purified recombinant AdT was assayed using *Chlamydia* Asp-tRNA\(^{\text{Asn}}\) and Glu-tRNA\(^{\text{Gln}}\) species (prepared with *Chlamydia* AspRS and GluRS) in the presence of ATP and the amide donor glutamine. Under our assay conditions about half of the two different substrates was converted into the desired products with equal efficiency (Fig. 5, lanes 2 and 4), while an *E. coli* S-100 extract was incapable of carrying out the conversion of the mischarged AA-tRNAs (Fig. 5, lanes 1 and 3). Additionally, removal (with enterokinase) of the thioredoxin part of the GatC fusion protein did not affect the Glu-AdT or Asp-AdT activities of the enzyme (data not shown). All these results demonstrated that *Chlamydia* possesses a dual-specificity Asp/Glu-AdT. Together with the fact that the organism also contains non-discriminating AspRS and GluRS enzymes, it is likely that the dual-specificity amidotransferase serves in Asn-tRNA and Gln-tRNA formation *in vivo*.

**Characterization of the C. trachomatis AdT** – With the availability of pure Asp/Glu-AdT we wanted to characterize the other substrates of the enzyme (Table II). Mg\(^{2+}\) is essential for the reaction. Amidotransferases use various amide donors (20), predominantly glutamine, asparagine, and ammonium chloride. As can be seen in Table II, they are all active in Asn-tRNA and Gln-tRNA formation with ammonium chloride being less effective. The usage is somewhat different from that of *B. subtilis* Glu-AdT (10); however, this will be clarified when both enzymes will be compared by detailed enzyme kinetics. On the other hand, the utilization of nucleoside triphosphates is significantly different by the two enzymes. While the *B. subtilis* Glu-AdT can only use ATP (10), the *C. trachomatis* Asp/Glu-AdT accepts GTP quite efficiently compared to ATP (Table II). In addition there appears to be an effect of the tRNA substrate on nucleoside triphosphate use: the *Chlamydia* enzyme’s Asp-AdT activity can also utilize CTP (Table II).
Many organisms contain tRNA-independent amidotransferases that use the amide nitrogen of glutamine or asparagine to form ammonia as the substrate for subsequent amination (20-23). Several of these enzymes contain a cysteine residue in their catalytic core. To probe the role of cysteine residues in the Chlamydia Asp/Glu-AdT, the enzyme was incubated with the sulfhydryl reagents N-ethylmaleimide (NEM), 5,5'-dithiobis(2-nitrobenzoic acid) (DTMB), and p-hydroxy-mecuribenzoate (PMB). As this treatment did not affect the enzyme activity (Table II), it appears that the solvent exposed cysteine residues are not required for enzymatic activity.

Since Chlamydia Asp/Glu-AdT evolved to recognize two misacylated tRNAs (tRNA<sup>Asn</sup> and tRNA<sup>Gln</sup>) it was important to check if the enzyme is able to specifically recognize only the non-cognate amide AA-tRNA. To test this, the correctly charged Asp-tRNA<sup>Asp</sup> and Glu-tRNA<sup>Glu</sup> were used in the amidation reaction and found to be unsuitable substrates (Table II). Apparent initial velocity kinetic parameters of amidation by the chlamydial enzyme were determined under conditions of great substrate excess relative to the enzyme. The initial velocity of Gln-tRNA formation was 6.1 pmol/min and Asn-tRNA formation was 3.5 pmol/min. It appears that the rates for conversion of Glu to Gln are about twice as fast as the rate of Asp to Asn conversion. This is consistent with the total activity profile presented in Table I. This difference may reflect the relative importance of Gln-tRNA formation to Asn-tRNA formation in the cell (see below).

**DISCUSSION**

This is the first detailed investigation of AA-tRNA synthesis in a genome that lacks two canonical AARSs. To make up for the lack of AsnRS and GlnRS, the non-discriminating AspRS and GluRS enzymes and the heterotrimeric Asp/Glu-AdT constitute the transamidation pathway for the synthesis of Asn-tRNA and Gln-tRNA in Chlamydia. The same complement of genes is also found in the genome sequence of Helicobacter pylori (24) where knockout experiments established the essentiality of the amidotransferase<sup>2</sup>. Thus, there is a group of
organisms where transamidation provides the essential synthetic route to both amide aminoacyl-tRNAs. While Asn-tRNA and Gln-tRNA are a prerequisite for protein synthesis, under certain metabolic situations *Chlamydia* may also require them for asparagine or glutamine synthesis; the organism appears to lack the genes encoding both asparagine synthetase and glutamine synthetase (*asnA/asnB* and *glnA*, respectively). A similar route to asparagine formation has been suggested for *Deinococcus* and *Thermus* (9,12).

Biochemical and genomic analyses have shown a great diversity of AspRS enzymes in the living world. Sequence-based alignments reveal three types according to their taxonomic origin (25). Bacterial-like AspRS proteins are characterized by the presence of a C-terminal extension and a 100 amino acid insertion domain located between the conserved class II motifs 2 and 3. These features are missing in the archaeal and eukaryal enzymes. A number of bacteria (e.g., *Deinococcus* and *Thermus* AspRS2) (7,8,12) contain, in addition to their bacterial AspRS, a copy of an archaeal-like AspRS, able to form Asp-tRNA^Asn^ (i.e., non-discriminating) and being significantly smaller than the bacterial-type enzyme. To date the latter enzymes are believed to be solely discriminating. However, as show above, the *C. trachomatis* AspRS is a non-discriminating enzyme of the bacterial genre.

Little is known about roles of the three subunits of the GatCAB Asp/Glu-AdT. The GatA polypeptide contains a well-known amidase signature sequence [GGSSGGSAAVSAR-FCPIALGSDTGGSRQPA, (positions 150-183)] (26); thus, this is likely to be the catalytic subunit with glutaminase and amidotransferase activity (10,27). The binding of tRNA is thought to be a property of the GatB protein, which is a member of an isolated protein family with no known function. GatC is the most divergent subunit for which no function can be suggested by homology searches. It was proposed that GatC is required for proper expression or folding of the GatA subunit (10), but appears dispensable for active Asp-AdT purified from *Thermus thermophilus* (9). Genetic analysis and biochemical study of partial reactions with the isolated subunits is needed to clarify this.
What happens with the misacylated AA-tRNA? Incorrectly charged tRNA in free form is probably detrimental to the cell as it will cause errors in protein synthesis (28). It was shown that elongation factor-Tu (EF-Tu) from *Thermus* or from spinach chloroplasts has only weak affinity for Asp-tRNA$^{Asn}$ or Glu-tRNA$^{Gln}$ (9,29) and that this ‘rejection’ of misacylated tRNA guarantees the maintenance of translational fidelity. *Chlamydia* provides an additional challenge to EF-Tu, which has to discriminate against two different tRNAs. While chlamydial EF-Tu may be capable of doing this, there could also be another mechanism that takes the misacylated tRNA out of circulation. Should the non-discriminating synthetases form a complex with the Asp/Glu-AdT, then the misacylated tRNA formed by the synthetase could be ‘handed off’ to the amidotransferase, thus eliminating free diffusion of this AA-tRNA. Such a ‘channeling mechanism’ may involve the GatC subunit for which there is yet no known role (30).

The intercellular physiology of *Chlamydia* may be tied to the multifaceted activities of an AdT. After inoculation of host tissue with the *C. trachomatis* elementary body (EB), the environment changes to one that is depicted as hostile for invading parasites. Bacterial infection begins a cascade of events in the body leading to inflammation and immune response coordinated by lymphocytes, macrophages and neutrophils. The role of glutamine utilization by these immune cells has recently been described and reviewed (31). The intercellular milieu surrounding the EB is depleted in glutamine and has normal levels of glutamate, which does not affect the internalization of the EB into the host cell since this is dependent upon intrinsic proteins on the outer membrane of the EB and not *de novo* protein synthesis (32,33). However, once inside the host cell, it is necessary for the parasite to express certain early gene products to intersect an exocytic pathway avoiding lysosomal degradation (33) and transform from the non-metabolic EB form to the metabolically active reticulate body. It is plausible that this glutamine deficient parasitophorous vacuole would necessitate the *Chlamydia* Asp/Glu-AdT to generate correctly charged tRNA and also supply the cell with glutamine and/or asparagine using
ammonium chloride as a amide donor since the parasite is dependent on the host for amino acids (34) and nucleotides (35).

Detailed biochemical studies of this amidotransferase will further our understanding of protein synthesis in this human pathogen. Because of its unique dual tRNA specificity, this enzyme may have potential as a species-specific therapeutic drug target.

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FOOTNOTES

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1 The abbreviations used are: AdT, amidotransferase; AA, amino acid; AA-tRNA, aminoacyl-tRNA; AARS, aminoacyl-tRNA synthetase; AsnRS, asparaginyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase; CHT, ceramic hydroxyapatite; GlnRS, glutaminyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; EB, elementary body; RB, reticulate body; tRNA, transfer RNA; NEM, N-ethylmaleimide; DTNB, 5’5’-dithiobis-(2-nitrobenzoic acid); PMB, p-hydroxymercuribenzoate.

2 A. Buhmann, D. Tumbula-Hansen, D. Söll, and K. Melchers, unpublished observation.
FIGURE LEGENDS

FIG 1. tRNA-dependent transamidation pathway of Asn-tRNA and Gln-tRNA formation. Non-discriminating AspRS or GluRS form Asp-tRNA\textsubscript{Asn} and Glu-tRNA\textsubscript{Gln}. The mischarged aminoacyl-tRNAs are then transamidated to the correctly charged tRNAs by an amidotransferase (AdT) in the presence of ATP and an amide donor.

FIG 2. Aminoacylation of \textit{C. trachomatis} tRNA\textsuperscript{Asp} and tRNA\textsuperscript{Asn}. The tRNAs, enzymes (amino acids) used are: (A) tRNA\textsuperscript{Asn} with \textit{C. acetobutylicum} AsnRS (Asn) (\(\textbullet\) \(\rightarrow\)) and \textit{C. trachomatis} AspRS (Asp) (\(\rightarrow\)). (B) tRNA\textsuperscript{Asp} with \textit{C. trachomatis} AspRS (Asp) (\(\rightarrow\)) and \textit{E. coli} AspRS (Asp) (\(\rightarrow\)). The activity of the \textit{C. trachomatis} AspRS in this experiment was lower than what we normally observed. (C) tRNA\textsuperscript{Asn} with \textit{C. trachomatis} AspRS (Asp) (\(\rightarrow\)), \textit{D. radiodurans} AspRS2 (Asp) (\(\rightarrow\cdot\rightarrow\)), and \textit{C. acetobutylicum} AspRS2 (Asp) (\(\rightarrow\cdot\rightarrow\)). Background is reaction without tRNA (\(\rightarrow\cdot\rightarrow\)).

FIG 3. Aminoacylation of \textit{C. trachomatis} tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Gln}. The tRNAs, enzymes (amino acids) used are: (A) tRNA\textsuperscript{Gln} with \textit{E. coli} GlnRS (Gln) (\(\rightarrow\)) and \textit{C. trachomatis} GluRS (Glu) (\(\rightarrow\)). (B) tRNA\textsuperscript{Glu} with \textit{D. radiodurans} GluRS (Glu) (\(\rightarrow\cdot\rightarrow\)) and \textit{C. trachomatis} GluRS (Glu) (\(\rightarrow\cdot\rightarrow\)). (C) tRNA\textsuperscript{Gln} with \textit{B. subtilis} GluRS (Glu) (\(\rightarrow\)) and \textit{C. trachomatis} GluRS (Glu) (\(\rightarrow\)). Background is reaction without tRNA (\(\rightarrow\cdot\rightarrow\)).

FIG 4. Purification of \textit{C. trachomatis} Asp/Glu-AdT. (A) Denaturing PAGE in a 4-20% gradient gel containing 1% SDS. (B) Native PAGE in a 10% gel. Lanes: 1, S-100 extract from overexpressing \textit{E. coli} strain; 2, pooled fractions from first CHT chromatography; 3, pooled fractions from second CHT chromatography; 4, pooled fractions from HiTrap Heparin chromatography; 5, final fraction of pure AdT after HiTrap Q chromatography.
Fig 5. **Glu-AdT and Asp-AdT activities of the *C. trachomatis* GatCAB enzyme.** Phosphorimages of thin-layer chromatographic separation of [¹⁴C]-labeled glutamine, glutamate, asparagine and aspartate. For details see Experimental Procedures. tRNA^{Gln} in lanes 1 and 2, tRNA^{Asn} in lanes 3 and 4. Lanes 1 and 3: No AdT, but *E. coli* S-100. Lanes 2 and 4: *Chlamydia* Asp/Glu-AdT.
### TABLE I

*Purification of C. trachomatis Asp/Glu-amidotransferase*

| Purification Step | Total Protein (mg) | Total activity* (units × 10²) | Specific activity (units/mg) | Yield (%) | Relative Purification (fold) |
|-------------------|--------------------|--------------------------------|-----------------------------|-----------|-----------------------------|
|                   |                    | AspAdT | GluAdT | AspAdT | GluAdT | AspAdT | GluAdT | AspAdT | GluAdT |
| S-100§             | 2500               | 27     | 58     | 1.1    | 2.3    | 100    | 100    | 1      | 1      |
| Hydroxyapatite-1   | 1100               | 20     | 43     | 1.7    | 3.8    | 74     | 74     | 1.5    | 1.7    |
| Hydroxyapatite-2   | 380                | 9.1    | 17     | 2.4    | 4.5    | 34     | 29     | 2.2    | 2.0    |
| HiTrap Heparin     | 30                 | 2.4    | 3.5    | 8.1    | 12     | 9      | 6      | 7.4    | 5.2    |
| HiTrap Q           | 8.4                | 2.9    | 4.0    | 35     | 48     | 11     | 7      | 32     | 21     |

*One unit is defined as 1 pmol asparagine or glutamine produced per minute at 37°C using the assay conditions in Experimental Procedures.

§ Due to contamination of background amino acids and nucleic acids in the whole cell extract, we estimated the total activity and assumed a 1-fold purification.
### TABLE II

**Requirements of the amidation reaction**

| Enzyme/Substrate Combination* | [\(^{14}\)C]Gln recovered (pmol) | Enzyme/Substrate Combination* | [\(^{14}\)C]Asn recovered (pmol) |
|-------------------------------|---------------------------------|-------------------------------|---------------------------------|
| Glu-tRNA\(^{Glu}\) + Gln + ATP + Mg\(^{2+}\) | 8.1 | Asp-tRNA\(^{Asn}\) + Gln + ATP + Mg\(^{2+}\) | 6.4 |
| + Asn | 8.4 | + Asn | 7.0 |
| + NH\(_4\)Cl | 2.5 | + NH\(_4\)Cl | 6.3 |
| + NH\(_4\)Cl & - Mg\(^{2+}\) | 0.01 | + NH\(_4\)Cl & - Mg\(^{2+}\) | 0.01 |
| - ATP | 0.01 | - ATP | 0.01 |
| - ATP + CTP | 0.2 | - ATP + CTP | 3.3 |
| - ATP + GTP | 5.7 | - ATP + GTP | 5.8 |
| - ATP + UTP | 0.4 | - ATP + UTP | 1.4 |
| + NEM | 8.7 | + NEM | 6.9 |
| + DTNB | 9.2 | + DTNB | 7.0 |
| + PMB | 9.0 | + PMB | 6.8 |
| - Glu-tRNA\(^{Glu}\) + Glu-tRNA\(^{Glu}\) | 0.01 | - Asp-tRNA\(^{Asn}\) + Asp-tRNA\(^{Asp}\) | 0.01 |

* All reactions include AdT and were performed as described in “Experimental Procedures”.
Figure 1.
Figure 2.

A

B

C

[Graphs showing the relationship between time (min) and the concentration of various types of tRNA.

AA-tRNA<sub>Asn</sub> (pmol)

Asp-tRNA<sub>Asp</sub> (pmol) vs. Time (min) for different conditions.]
Figure 3.
Figure 4.
Figure 5.
A single amidotransferase forms asparaginyl-tRNA and glutaminyl-tRNA in
Chlamydia trachomatis
Gregory Raczniak, Hubert D. Becker and Dieter Söll

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