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From one amino acid to another: tRNA-dependent amino acid biosynthesis

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ABSTRACT

Aminoacyl-tRNAs (aa-tRNAs) are the essential substrates for translation. Most aa-tRNAs are formed by direct aminoacylation of tRNA catalyzed by aminoacyl-tRNA synthetases. However, a smaller number of aa-tRNAs (Asn-tRNA, Gln-tRNA, Cys-tRNA and Sec-tRNA) are made by synthesizing the amino acid on the tRNA by first attaching a non-cognate amino acid to the tRNA, which is then converted to the cognate one catalyzed by tRNA-dependent modifying enzymes. Asn-tRNA or Gln-tRNA formation in most prokaryotes requires amidation of Asp-tRNA or Glu-tRNA by amidotransferases that couple an amidase or an asparaginase to liberate ammonia with a tRNA-dependent kinase. Both archaeal and eukaryotic Sec-tRNA biosynthesis and Cys-tRNA synthesis in methanogens require *O*-phosophoseryl-tRNA formation. For tRNA-dependent Cys biosynthesis, *O*-phosphoseryl-tRNA synthetase directly attaches the amino acid to the tRNA which is then converted to Cys by Sep-tRNA: Cys-tRNA synthase. In Sec-tRNA synthesis, *O*-phosphoseryl-tRNA kinase phosphorylates Ser-tRNA to form the intermediate which is then modified to Sec-tRNA by Sep-tRNA:Sec-tRNA synthase. Complex formation between enzymes in the same pathway may protect the fidelity of protein synthesis. How these tRNA-dependent amino acid biosynthetic routes are integrated into overall metabolism may explain why they are still retained in so many organisms.

INTRODUCTION

To maintain the fidelity of protein synthesis, pairing an amino acid with its cognate tRNA to form aminoacyl-tRNA (aa-tRNA) is essential. Usually, in cells, this is achieved by a group of enzymes known as aminoacyl-tRNA synthetases (aaRSs). Each aaRS specifically recognizes an amino acid and its corresponding set of tRNA isoacceptors in a cell. Accordingly, for each of the 20 canonical amino acids there is a matching aaRS found in nature. aa-tRNA formation catalyzed by the aaRSs occurs in a two-step process (1). First, the amino acid is activated in an ATP-dependent manner forming an enzyme-bound aminoacyl-adenylate. Second, the activated aa is transferred onto the 3′ terminal adenosine of the tRNA.

A full complement of all 20 aaRSs was thought to be essential for the survival of most species. Exceptions to the concept of 20 canonical amino acids/20 aaRSs in a species have been known about since 1968 (2). Only recently, functional genomic analyses confirmed by biochemical and genetic experiments have revealed only eukaryotes and a handful of bacteria have a full set of aaRSs (Table 1). The majority of bacterial and all known archaeal genomes do not encode a glutaminyl-tRNA synthetase (GlnRS) (3,4). Most prokaryotes do not have an asparaginyl-tRNA synthetase (AsnRS) (3–5) and a number of a methanogenic archaea lack a cysteinyl-tRNA synthetase (CysRS) (6–8). In addition, no aaRS exists to date for Sec, the 21st amino acid used in protein synthesis in a number of species across all three domains of life (9).

These organisms (Table 1) take advantage of indirect pathways to form the full complement of aa-tRNA species required for protein synthesis by synthesizing the amino

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Table 1. Prevalence of the indirect and direct pathways for the synthesis of the aa-tRNA species listed in all three domains of life

aa-tRNA	Prevalence of		Refs.
	Indirect pathway	Direct pathway	
Gln-tRNA^{Gln}	All known archaea, most bacteria, and chloroplasts	All known eukaryotes and a minority of bacteria	3, 4, 17, 18
Asn-tRNA^{Asn}	Most bacteria and archaea	All known eukaryotes and a number of bacteria and archaea	3–5, 38
Cys-tRNA^{Cys}	Methanogenic archaea (except *M. smithii* and *M. stadtmanae*) and *A. fulgidus*	All known eukaryotes and bacteria, and most archaea	6–8, 11, 77, 80–82
Sec-tRNA^{Sec}	All known Sec-decoding eukaryotes, archaea and bacteria	None known	107–110, 118

acids (Gln, Asn, Cys and/or Sec) when bound to their cognate tRNAs (2,10–16). This is accomplished by relaxed-specificity aaRSs that form misacylated aa-tRNA species; they will be subsequently converted to the correct aa-tRNA by remarkable RNA-dependent modifying enzymes. By synthesizing the amino acids on the tRNA, these organisms are able to directly link amino acid metabolism with protein synthesis. The purpose of this article is to summarize recent advances in our understanding of the key enzymes involved in these tRNA-dependent amino acid transformation pathways.

tRNA-dependent transamidation

In all known archaea (3), most bacteria (4) and chloroplasts (17,18) GlnRS is absent and Gln-tRNA^{Gln} is formed by synthesizing Gln on the tRNA (Table 1) (Figure 1A). First, these organisms attach Glu to tRNA^{Gln} to form the mischarged species, Glu-tRNA^{Gln}, by taking advantage of a non-discriminating glutamyl-tRNA synthetase (ND-GluRS) (19), an aaRS with relaxed tRNA specificity (20). The Glu moiety on the tRNA^{Gln} is then amidated by a Glu-tRNA^{Gln} amidotransferase (Glu-AdT) in the presence of ATP and an amide donor to form the properly aminoacylated species, Gln-tRNA^{Gln} (2). Similarly, in the many bacteria and archaea lacking an AsnRS (Table 1) (3–5), Asn-tRNA^{Asn} is synthesized by the concerted action of a non-discriminating aspartyl-tRNA synthetase (ND-AspRS) (20,21) and an aspartyl-tRNA^{Asn} amidotransferase (Asp-AdT) (Figure 1B) (10,22).

In the mitochondria of many eukaryotes the indirect pathway may also be used to form Gln-tRNA^{Gln}. Homologs of AdT subunits are encoded in the nuclear genomes of numerous eukaryotes including *Saccharomyces cerevisiae* (23) and *Homo sapiens* (24). In *S. cerevisiae*, the homologs (Pet112 and YMR293C) are essential for mitochondrial function (25,26) and Glu-tRNA^{Gln} is found in the organelle (27). However, *in vitro* the yeast mitochondrial GluRS was unable to form the mischarged tRNA species (28). The presence of a Glu-AdT activity in yeast mitochondria was noticed already 30 years ago (29), and recent work characterizes this activity for mammalian (T. Suzuki, unpublished data) and plant mitochondria (A.M. Duchêne and H. Becker, unpublished data). In addition, it was shown that both cytoplasmic GlnRS and tRNA^{Gln} are imported into the

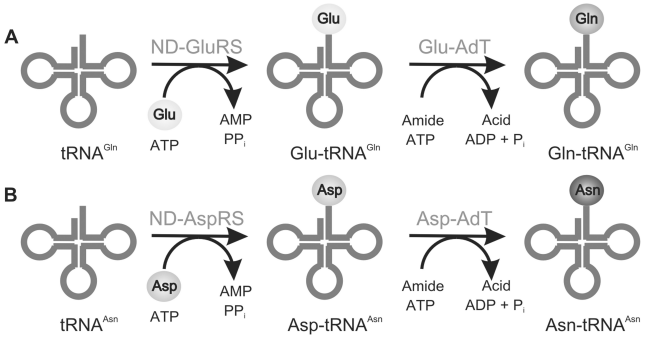

Figure 1. Indirect pathways for **(A)** Gln-tRNA^{Gln} and **(B)** Asn-tRNA^{Asn} formation. (A) First a ND-GluRS glutamylates tRNA^{Gln} to form Glu-tRNA^{Gln}. The mischarged species is then amidated by a Glu-AdT to form Gln-tRNA^{Gln}. (B) First a ND-AspRS aspartylates tRNA^{Asn} to form Asp-tRNA^{Asn}. The mischarged species is then amidated by an Asp-AdT for form Asn-tRNA^{Asn}.

S. cerevisiae mitochondrion (28) and that in *H. sapiens* tRNA^{Gln} is imported into the organelle (J. Alfonzo, unpublished data). The role (e.g. additional coding functionality) of such dual pathways of mitochondrial Gln-tRNA^{Gln} formation remains to be established.

Two different tRNA-dependent amidotransferases (AdT) exist, the heterotrimeric GatCAB (30) and the heterodimeric GatDE (3). The latter is an archaeal signature protein and serves as the Glu-AdT for Gln-tRNA^{Gln} synthesis in *Archaea* (3). GatCAB is found in both bacteria and archaea (3,30). In archaeal genomes, GatCAB is encoded only when an AsnRS is not (3,5). This and the fact that the *Methanothermobacter thermautotrophicus* GatCAB is unable to transamidate archaeal Glu-tRNA^{Gln} *in vitro* (31), strongly indicates that the role of the heterotrimeric AdT in *Archaea* is as an Asp-AdT.

All bacterial GatCAB enzymes studied to date are able to serve as both a Glu-AdT and an Asp-AdT *in vitro* (4,32–36). The exact activity/activities assumed by GatCAB in bacteria *in vivo* is determined by the presence and nature of the non-discriminating aaRS (ND-GluRS and/or ND-AspRS) in the organism. For example, bacteria like *Bacillus subtilis* (19) that have a ND-GluRS but lack a ND-AspRS use their GatCAB only as a Glu-AdT (30). In bacteria possessing a ND-AspRS but lacking a ND-GluRS (e.g. *Pseudomonas aeruginosa*, *Neisseria meningitidis*, *Thermus thermophilus* and

Deinococcus radiodurans) GatCAB serves only as an Asp-AdT (21,22,32,33,37–40). In bacteria carrying both non-discriminating aaRSs [e.g. *Chlamydia trachomatis* (34) and *Helicobacter pylori* (41–43)], GatCAB serves a dual function as both a Glu-AdT and an Asp-AdT (4,34,36).

tRNA substrate recognition by the AdTs

The AdTs must discriminate their mischarged tRNA substrates (Glu-tRNA^{Gln} and/or Asp-tRNA^{Asn}) from the cognate aa-tRNA species (Glu-tRNA^{Glu} and Asp-tRNA^{Asp}). Both AdTs achieve this without recognizing the anticodon of their tRNA substrates (Figure 2) (40,44–46). The elements that the bacterial GatCAB, GatDE and the archaeal GatCAB recognize in their aa-tRNA substrates are summarized here. The *Staphylococcus aureus* GatCAB positively recognizes the U1-A72 base pair in the acceptor stem of tRNA^{Gln}, while discriminating against tRNA^{Glu} based on the presence of a supernumerary base in the D-loop of tRNA^{Glu} (45). Biochemical studies with the *N. meningitidis* GatCAB revealed that the enzyme uses similar elements to distinguish Asp-tRNA^{Asn} from Asp-tRNA^{Asp} (40). These elements are conserved among bacteria using the indirect pathways for Gln-tRNA^{Gln} and/or Asn-tRNA^{Asn} formation suggesting a general substrate discrimination mechanism for all bacterial GatCAB enzymes (40,45).

The cocrystal structure of the *M. thermautotrophicus* GatDE with tRNA^{Gln} demonstrated that the enzyme makes contact with the acceptor stem, TΨC-stem/loop and D-loop of the tRNA (Figure 2) (44). The structural results coupled with biochemical analysis revealed GatDE recognizes the A1-U72 base pair found in the acceptor stem of archaeal Glu-tRNA^{Gln} and discriminates against Glu-tRNA^{Glu} based on extra bases in the D-loop of tRNA^{Glu} (44). In addition, the results indicated that the *M. thermautotrophicus* GatDE uses the first base-pair of the acceptor stem and positions 19 and 20 in the D-loop to distinguish Glu-tRNA^{Gln} from Asp-tRNA^{Asn} (44).

In contrast to GatDE and bacterial GatCAB, archaeal GatCAB does not recognize the first base pair of the acceptor stem of its tRNA substrate, Asp-tRNA^{Asn} (40,46). The *M. thermautotrophicus* GatCAB instead relies on antideterminants in tRNA^{Asp} (the D-loop and position 49) to distinguish Asp-tRNA^{Asn} from Asp-tRNA^{Asp} (46). In addition, archaeal GatCAB discriminates Asp-tRNA^{Asn} from archaeal Glu-tRNA^{Gln} and Asp-tRNA^{Asp} based on the length of the variable loops of the tRNA species (40,46). Both the *Methanosarcina barkeri* and *M. thermautotrophicus* GatCAB enzymes were able to transamidate aa-tRNA species with variable loops five nucleotides in length, as is found in archaeal tRNA^{Asn} isoacceptors, but not ones with variable loops four nucleotides in length, as is conserved in archaeal tRNA^{Gln} and tRNA^{Asp} species (31,40,46). The differences in tRNA recognition by GatDE and archaeal GatCAB may enable archaea lacking a GlnRS and an AsnRS to encode one AdT (GatDE) as a Glu-AdT and the other (GatCAB) as an Asp-AdT (31). Why archaea lacking both aaRSs encode two AdTs (3), while bacteria in a same situation encode only GatCAB for synthesis of both Gln-tRNA^{Gln} and Asn-tRNA^{Asn} (4,34,36) remains an open area of investigation.

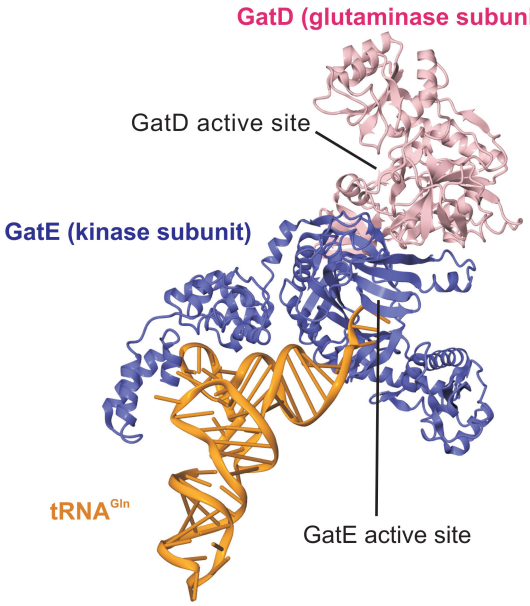

Figure 2. Crystal structure of the *M. thermautotrophicus* GatDE complexed with tRNA^{Gln}. The AdT forms an $\alpha_2\beta_2$ tetramer, with two GatE subunits binding a GatD homodimer. Each GatE subunit binds one tRNA^{Gln} molecule. For clarity only one monomer of GatD and GatE are shown. The glutaminase active site of the D-subunit and the kinase active site of the E-subunit are connected by a 40 Å long molecular tunnel (44). Adapted from Polycarpo,C. *et al.* (2007). In Cavicchioli,R. (ed.) *Archaea: Molecular and Cellular Biology*. ASM Press, Washington, DC USA with permission from ASM Press.

AdTs couple a tRNA-dependent kinase with a glutaminase

Regardless of the tRNA substrate recognized, both AdTs are thought to catalyze three distinct reactions to transamidate their mischarged substrates (Figure 3): (i) AdTs act as kinases to phosphorylate the Glu or Asp attached to the tRNA, forming an activated intermediate, γ-phosphoryl-Glu-tRNA^{Gln} (18,47–49) or β-phosphoryl-Asp-tRNA^{Asn}, respectively. The formation of the latter is still speculative as this point as biochemical evidence for existence of β-phosphoryl-Asp-tRNA^{Asn} has not been demonstrated as it has for γ-phosphoryl-Glu-tRNA^{Gln} (18,47–49) possibly due to the unstable nature of the putative β-phosphoryl-Asp-tRNA^{Asn} (49); (ii) AdTs are glutaminases, liberating ammonia from amide donors such as Gln or Asn; (iii) AdTs amidate the activated intermediate using the liberated ammonia to form the properly aminoacylated species, Gln-tRNA^{Gln} or Asn-tRNA^{Asn}. Thus, transamidation requires coupling of a tRNA-dependent kinase (GatB or GatE) with a glutaminase (GatA or GatD).

Recognition of the tRNA substrates of GatCAB and GatDE is the purview of the B and E-subunits of the respective AdTs (44,45,49). GatB and GatE are paralogs (24). The core of the two subunits belongs to an isolated protein family (3,44,45,50). Appended to the catalytic core of both subunits is a C-terminal extension that is

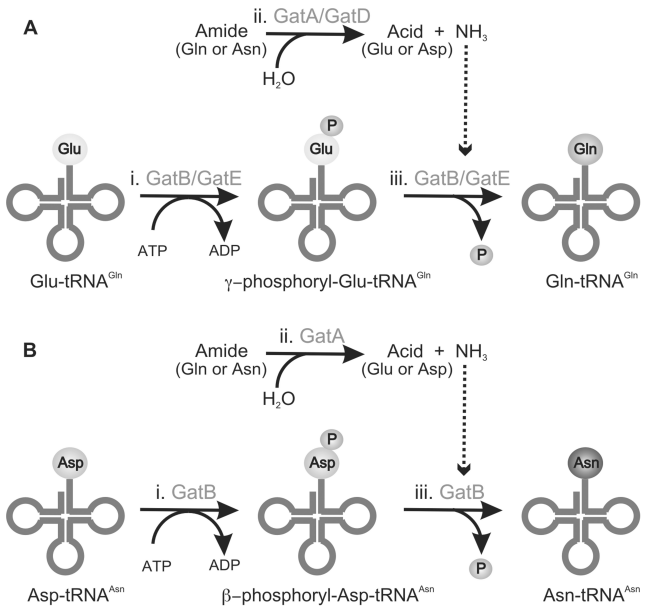

Figure 3. Both GatCAB (**A** and **B**) and GatDE (A) catalyze three distinct reactions in order to transamidate their mischarged tRNA species, (A) Glu-tRNA^{Gln} and/or (B) Asp-tRNA^{Asn}: (i) the kinase subunit of the respective AdT (GatB or GatE) phosphorylates the mischarged tRNA species to form an activated intermediate, (A) γ-phosphoryl-Glu-tRNA^{Gln} or **(B)** β-phosphoryl-Asp-tRNA^{Asn}; (ii) the glutaminase subunit (GatA or GatD) hydrolyzes an amide donor such as Gln or Asn to release ammonia. A molecular tunnel connects the glutaminase and kinase active sites of the respective AdTs, allowing ammonia liberated from the glutamianse subunit (GatA or GatD) to flow to the kinase subunit (GatB or GatE) (denoted by the dashed arrow); (iii) the liberated ammonia is then used by the kinase subunit (GatB or GatE) to amidate the activated intermediate to form the product aa-tRNA, (A) Gln-tRNA^{Gln} or (B) Asn-tRNA^{Asn}.

homologous to the YqeY family of enzymes (24,44,45,51). The YqeY C-terminal extension of the *D. radiodurans* GlnRS enables the enzyme to productively bind to its tRNA substrate (51). For the AdTs, the YqeY-like domain serves a similar function interacting with the D-loop of the tRNA (44,45).

The 3′ terminal CCA end of the tRNA binds in the core of the kinase subunit (GatB or GatE) (44) placing the aminoacyl-moiety attached to the tRNA in the proper position in the active site to be phosphorylated (44,45,49). In the same active site, GatB and GatE catalyze the subsequent amidation of the activated intermediate to form the cognate aa-tRNA (Gln-tRNA^{Gln} or Asn-tRNA^{Asn}) in their respective AdTs (Figure 3) (44,45). The phosphorylation step is thought to be Mg^{2+} dependent (44,45). Mutation of any of the three conserved residues that coordinate the Mg^{2+} in the catalytic pocket of the *M. thermautotrophicus* E-subunit (His15, Glu157 and Glu184) to Ala rendered the resulting GatDE mutant enzymes both as kinase and as transamidase inactive (44).

GatE pairs with GatD (3). The complex of the two is a $\alpha_2\beta_2$ tetramer with two GatE polypeptides binding to a GatD homodimer (44,50). The D-subunit shares homology with type I L-asparaginases (3,50) and serves as the glutaminase domain for the heterodimeric AdT, liberating ammonia from an amide donor (Figure 3) (49). The *M. thermautotrophicus* GatDE is able to use Gln nearly as well as Asn as the donor (31). Like other L-asparaginases (52–55), GatD catalyzes the hydrolysis of Gln or Asn by making use of Thr as the nucleophile coupled with a Thr-Lys-Asp triad in the active site (49,50).

In the heterotrimeric AdT, the glutaminase subunit is GatA (4,45,56). The AdT subunit belongs to the amidase protein family (4,30,45,56) that uses a Ser-cisSer-Lys catalytic triad to hydrolyze amides (57). The cocrystal structure of *S. aureus* GatCAB with either Gln or Asn suggested Gln as a better substrate than Asn for GatA (45). Gln bound in the active site of the A-subunit forming a covalent bond with Ser178 (*S. aureus* GatA numbering), consistent with the predicted role of the residue as the nucleophile in the amidase catalytic triad (45). However, Asn bound into the same active site was unable to form the covalent intermediate, most likely due to the shorter length of the Asn side chain (45). Consistent with the structural results, the *H. pylori* GatCAB is 130-fold more efficient using Gln as the amide donor in transamidation than Asn, mostly due to a difference in k_{cat} (4). Other mesophilic bacterial GatCAB enzymes studied have been shown to also be more active preferring Gln to Asn (18,58). On the other hand, the *M. thermautotrophicus* GatCAB is about equally efficient using Asn or Gln as amide donors suggesting that the active site of the archaeal GatA may differ slightly from that of *S. aureus* GatA (31). It appears that GatA requires a small helper protein, GatC (approximately 100 amino acids long), to fold properly (30,45) and to form a complex with GatB (45).

Gated tunnel couples ammonia release to transamidation

A molecular tunnel (30 Å and 40 Å long in GatCAB and GatDE, respectively) connects the glutaminase and kinase active sites in both AdTs (Figure 2) (44,45). It is proposed that the ammonia liberated by the glutaminase subunit of the AdT travels down the tunnel to the kinase active site where it is used to amidate the activated intermediate (44,45,50). Given that both tunnels are lined with hydrophilic residues it is speculated that ammonium is transported through the tunnel via a series of protonations and deprotonations (44,45), possibly akin to potassium transport through the potassium channel, KcsA (59).

The tunnel may provide the mechanism by which the glutaminase activity of GatA can be coupled to overall transamidation (45). Biochemical evidence has shown that the glutaminase activity of GatCAB is enhanced in the presence of mischarged substrate; it is further stimulated by ATP (4,60). In the *S. aureus* GatCAB structures (all in the absence of tRNA substrate), the tunnel is gated closed (45). It is plausible that upon binding to Glu-tRNA^{Gln} or Asp-tRNA^{Asn} the tunnel opens, allowing ammonium to flow through it and in turn enhance the glutaminase activity of the A-subunit (45). Whether it is opening of the tunnel alone or if there are accompanying structural rearrangements awaits further investigations;

in particular, a cocrystal structure of GatCAB with either Asp-tRNA^{Asn} or Glu-tRNA^{Gln} along with ATP.

In the case of GatDE the coupling of the glutaminase activity of the enzyme with overall transamidation is tighter such that the AdT is glutaminase inactive in the absence of Glu-tRNA^{Gln} (49). In the crystal structure of the *Pyrococcus abyssi*, GatDE apo-enzyme not only is the molecular tunnel closed but the catalytically important Thr in the D-subunit is positioned 7 Å away from the subunit's active site (50). This Thr is located on a β-hairpin that molecular dynamic simulations predict to be flexible (50). It is hypothesized that Glu-tRNA^{Gln} binding to GatE induces conformational changes in the holoenzyme that open the molecular tunnel and also move the β-hairpin to correctly position the Thr into the GatD active site, thus enabling the subunit to catalyze the release of ammonia from an amide donor (50). Such a mechanism would prevent unproductive hydrolysis of Gln or Asn by GatDE in the absence of Glu-tRNA^{Gln} (49,50).

Substrate channeling between non-discriminating aaRS and AdT

Aminoacylation and transamidation may also be coupled. While a possible complex between ND-aaRS, AdT and tRNA was proposed almost two decades ago (17), it was only recently that evidence has emerged for such a complex that would enable substrate channeling (61) of a mischarged tRNA from the aaRS to the AdT (44,62,63). In the case of the *T. thermophilus* GatCAB and ND-AspRS, the complex between the AdT and ND-AspRS requires tRNA^{Asn} (63). *In vitro*, the complex between ND-AspRS, AdT and tRNA^{Asn} has been shown to enhance the aminoacylation activity of the aaRS (63). In addition, the complex appears to stabilize Asp-tRNA^{Asn} and in particular Asn-tRNA^{Asn} (62,63). The structural modeling of the complex suggests that the 3′ terminal CCA end of the tRNA flips from the aminoacylation site in the aaRS to the kinase center of the AdT after aminoacylation (63), analogous to the movements in the tRNA seen in certain aaRSs with editing domains (64,65). Thus, the Asp moiety of the mischarged tRNA formed by the aaRS can be rapidly amidated to Asn, *de facto* making this complex an AsnRS formed *in trans*. It is speculated that the complex between ND-AspRS, GatCAB and tRNA^{Asn} enables formation of Asn-tRNA^{Asn} without the risk of Asp-tRNA^{Asn} being used in protein synthesis, while also protecting the product aa-tRNA from deacylation until it can be bound by EF-Tu to be used in translation (62,63).

Structural modeling also predicts a complex between ND-GluRS, tRNA^{Gln} and either Glu-AdT (GatCAB or GatDE) may also be possible (44,63). Given that AspRS, a class II aaRS, binds to the major groove side of the acceptor stem of the tRNA and GluRS, a class I aaRS, binds to the minor groove side indicate the complexes formed by GatCAB with ND-AspRS and tRNA^{Asn} or ND-GluRS and tRNA^{Gln} will differ significantly from one another (63). In the case of GatDE, modeling suggests that the AdT would be able to form a complex with ND-GluRS but not ND-AspRS due to sterical clashes between the class II aaRS and an insertion domain found in GatE but not GatB (44). Whether such complexes exist between tRNA^{Gln}, ND-GluRS, and AdTs awaits further investigations.

Why have the indirect pathways for amide aa-tRNA formation been retained?

Both GlnRS and AsnRS were absent in the last universal communal ancestor (LUCA) and most likely Gln-tRNA^{Gln} and Asn-tRNA^{Asn} were formed via the indirect pathways (5,66–69). Why the indirect pathways for amide aa-tRNA formation have been retained in so many prokaryotes remains an open question. The unique archaeal tRNA^{Gln} favored by GatDE may be a barrier to acquistion of GlnRS in *Archaea* (3,24) as neither the *Escherichia coli* nor *S. cerevisiae* GlnRS can aminoacylate archaeal tRNA^{Gln} (3). Recognition of tRNA though does not seem to be as a significant barrier preventing more bacteria from acquiring GlnRS. For example, the *B. subtilis* tRNA^{Gln} can serve as a substrate for the *E. coli* GlnRS (19).

It should be noted that glutamine is not only used in cells for translation. The synthesis of Gln from Glu by glutamine synthetase is the primary mechanism for ammonium assimilation in all free-living organisms and Gln serves as the primary amide donor for a variety of vital cellular biosynthetic pathways (70). In *Firmicutes* such as *B. subtilis*, glutamine is an important signaling molecule for the regulation of nitrogen metabolism through the transcriptional regulator TnrA (71) as Gln is an indicator of the nitrogen and Glu status of the cell (70,71). Therefore, retention of the indirect pathway for Gln-tRNA^{Gln} formation may constitute a regulatory link between central carbon metabolism (levels of α-ketoglutarate) and the level of nitrogen availability, both key parameters in regulation of protein synthesis. In addition, a number of bacteria that use GatCAB as a Glu-AdT have elevated cellular levels of Glu (72). It is currently unknown how well a GlnRS under such *in vivo* conditions could discriminate Gln from Glu but it is conceivable that the indirect pathway is retained in these organisms to maintain translational fidelity (30).

Metabolic reasons may also explain why so many prokaryotes have retained the indirect paths for Asn-tRNA^{Asn} formation. For example, *T. thermophilus* and *D. radiodurans* encode an AsnRS in their genomes (73,74), while also retaining a ND-AspRS and GatCAB (21,22,32,33,37,38). The indirect path is the sole route for Asn biosynthesis in these species as they lack both synthetases responsible for free Asn formation (AsnA and AsnB) (38) a situation found in numerous other bacteria (4,38). Interestingly, all known bacterial and archaeal genomes encoding AnsA, the ammonia-dependent asparagine synthetase, use AsnRS and not GatCAB to form Asn-tRNA^{Asn} (4,5) enabling these organisms to form Asn-tRNA^{Asn} in a glutamine-independent fashion. How amide aa-tRNA formation is tied into other cellular needs for the amide amino acids is still unclear (70), but such investigations will likely enable a greater understanding into why

the indirect pathways for Gln-tRNA^{Gln} and Asn-tRNA^{Asn} are retained in so many bacteria and archaea.

tRNA-dependent cysteine biosynthesis

In certain archaea, a canonical class I CysRS is either absent or dispensable (75,76). In addition, some of these archaea lack homologs of enzymes involved in cysteine biosynthesis in bacteria and eukaryotes. Biochemical investigation and genetic analysis revealed that a tRNA-dependent indirect pathway is responsible for providing Cys-tRNA^{Cys} for ribosomal translation as well as free cysteine biosynthesis in these organisms (Table 1) (11). This process couples protein synthesis with cysteine production and is mostly observed in a large subset of the *Euryarchaeota* (77).

The tRNA-dependent pathway consists of a two-step pretranslational amino acid transformation on tRNA^{Cys} (Figure 4). The tRNA^{Cys} is initially aminoacylated with *O*-phosphoserine (Sep) by *O*-phosphoseryl-tRNA synthetase (SepRS). The Sep moiety is subsequently transformed to tRNA-bound cysteine by Sep-tRNA:Cys-tRNA synthase (SepCysS) (11). Sulfide was shown to be a sufficient sulfur donor *in vitro*. However, the nature of the sulfur donor *in vivo* awaits further investigation. This finding confirms an earlier report that Sep is the source of the carbon framework for cysteine biosynthesis in *M. jannaschii* (78). Furthermore, an archaeal Sep biosynthetic pathway was characterized and proven to provide a sufficient amount of Sep for serine, cystathionine and tRNA-dependent cysteine formation (79).

Homologs of SepRS and SepCysS are identifiable in the genomes of the sulfate reducing archaeon *Archaeoglobus fulgidus* (80) and all known methanogenic archaea (77) except *Methanobrevibacter smithii* (81) and *Methanosphaera stadtmanae* (82). In the majority of these species, the indirect SepRS/SepCysS pathway coexists with the direct pathway catalyzed by CysRS (77). In many of these organisms, the enzymes required for tRNA-independent cysteine biosynthetic pathways are not encoded in their genomes (11,77). Consequently, the tRNA-dependent SepRS/SepCysS-mediated pathway provides the only means for the *de novo* production of cysteine in these species. This was demonstrated for *Methanococcus maripaludis* where the deletion of SepRS resulted in cysteine auxotrophy (11).

SepRS aminoacylates tRNA^{Cys} with Sep

SepRS belongs to the subclass IIc of aaRSs and is most closely related to the α-subunit of PheRS based on its amino acid sequence and structural organization (11,83). Phylogenetic analyses show that SepRS and α-PheRS share a common ancestor (77). Crystal structures of SepRS from *M. maripaludis* (84), *M. jannaschii*, and *A. fulgidus* (85) supported by biophysical analyses in solution demonstrate that SepRS is a homotetrameric enzyme. The quaternary structure of the core region of this α_4 assembly, consisting mostly of the four catalytic domains, resembles closely the core region of PheRS (84).

In spite of the structural similarity of their active sites, amino acid recognition differs significantly in SepRS relative to PheRS (85). Sep, unlike any of the 20 canonical amino acids, has a highly negatively charged side chain; therefore, it is not surprising that its phosphate moiety is extensively recognized by SepRS. Each of the three non-bridging oxygen atoms of the phosphate group forms two hydrogen bonds to residues in the amino acid binding pocket and mutation of the corresponding residues in *M. maripaludis* SepRS resulted in complete loss of enzymatic activity (84). Two non-bridging oxygen atoms in the phosphate group are hydrogen-bonding with the α-amino group of active site residues. The recognition of the side chain of a substrate amino acid by protein main chain groups is unique to SepRS. Furthermore, structural data suggests that the polar side chain of Sep is stabilized by dipole interactions with the positively charged N-terminal end of a central α-helix in the active site (85). This amino acid recognition mechanism including the organization of the substrate side-chain binding pocket is an exceptional characteristic of SepRS, and is not observed in any of the canonical aaRSs (85).

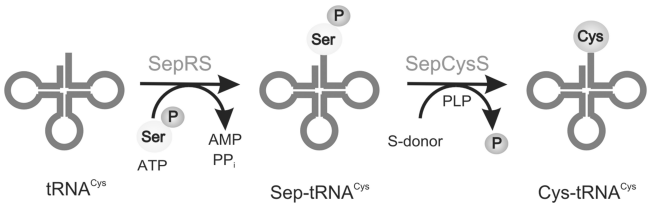

Figure 4. Indirect pathway for Cys-tRNA^{Cys} formation. First, SepRS aminoacylates tRNA^{Cys} with Sep to form Sep-tRNA^{Cys}. The Sep bound to the tRNA is then converted to Cys by SepCysS in the presence of a sulfur donor to form Cys-tRNA^{Cys}.

Unlike PheRS, where the catalytically active domain and the tRNA anticodon recognition site are located on different subunits, each SepRS monomer harbors both sites and tRNA recognition and aminoacylation occur in *cis*. However, the crystal structure of the *A. fulgidus* SepRS:tRNA^{Cys} complex indicates that two tRNA^{Cys} molecules are bound per SepRS α_4 tetramer (85). The stoichiometry of tRNA and SepRS in solution is unclear. The binding of tRNA does not induce significant conformational changes in SepRS based on the crystal structure and four tRNAs per α_4 complex can also be accommodated *in silico* (85).

A comparative biochemical study revealed that *M. maripaludis* SepRS and CysRS both recognize the same set of major identity elements in tRNA^{Cys}: the discriminator base U73 and the anticodon (G34, C35 and A36) (86). In addition, nucleotides G15 and A47 serve as minor identity elements for both enzymes, whereas G37, A59 and the base pairs G1:C72 and G10:C25 are minor identity elements for SepRS but do not affect aminoacylation by CysRS. While SepRS approaches the tRNA from the major groove, CysRS approaches it from the minor groove side, and it has been speculated that a ternary complex of SepRS:tRNA^{Cys}:CysRS could be formed (85). Class II aaRSs generally aminoacylate the 3′-terminal adenosine of their cognate tRNA at the 3′-OH group, with PheRS being a notable exception (87).

tRNA-dependent transformation of Sep to Cys

The conversion of Sep-tRNA^{Cys} to Cys-tRNA^{Cys} by SepCysS is pyridoxal phosphate (PLP)-dependent (11). In the resting state, the PLP cofactor was shown to covalently attach to the conserved lysine residue in the active site of SepCysS through a Schiff-base linkage (88). The side chain of a conserved asparagine rather than an aspartate residue observed in most other PLP enzymes, hydrogen-bonds with the nitrogen atom in the ring structure of PLP (88). The PLP cofactor is expected to be intimately involved in the catalysis of the β-replacement reaction by stabilizing the negative charge developed in the transition state as described in the basic mechanism for almost all PLP-dependent proteins (89).

The crystal structure (2.4 Å resolution) of *A. fulgidus* SepCysS shows that the enzyme forms a homodimer (88). Conserved residues from both subunits form the catalytic center located deep within the large, basic cleft near the dimer interface. Structurally, SepCysS belongs to the fold type I family (90). Each monomer contains two domains and the N-terminal large domain has a characteristic seven-stranded β-sheet for this family of protein. A model of the complex between SepCysS with its substrate Sep-tRNA^{Cys}, suggested that three conserved amino acid residues (Arg79, His103 and Tyr104) interact with the phosphate moiety of the Sep in the substrate and one of the three highly conserved cysteine residues in *A. fulgidus* SepCysS may be essential in the catalysis by carrying persulfide sulfur for cysteine formation (Figure 5) (88). The detailed mechanism for SepCysS catalyzing tRNA-dependent conversion of Sep to Cys awaits further biochemical investigation.

In *M. maripaludis*, two pathways are present for Cys-tRNA^{Cys} formation: the indirect SepRS/SepCysS pathway mentioned above and the direct charging of tRNA^{Cys} by CysRS. The indirect pathway is also the sole route for free cysteine formation. Interestingly, when the organism is grown in the presence of exogenous cysteine, SepRS can be deleted (11) while SepCysS is still indispensable (T. Major, M. Hohn, D. Su, W.B. Whitman, unpublished data) suggesting SepCysS may possess an alternative essential function in *M. maripaludis*.

Cysteine biosynthesis in archaea and the evolutionary view of the indirect pathway

To date, four different routes are shown to be present for cysteine biosynthesis in archaea: the eukaryotic pathway with cystathionine as the precursor (91), the bacterial pathway with *O*-acetylserine as the precursor (92–94), the modified bacterial pathway with *O*-phosphoserine as the precursor (95,96) and the tRNA-dependent pathway (11). Free cysteine has been shown to be the sulfur source for multiple biosynthetic processes, including Fe-S cluster formation, tRNA modification and cofactor biosynthesis in bacteria (97). Methanogens encode an unusually high number of Fe-S cluster proteins (98). Organisms that rely on the tRNA-dependent pathway for cysteine biosynthesis are thought to regulate the amount of free cysteine versus tRNA-bound cysteine by controlling the deacylation of Cys-tRNA^{Cys}, in order to maintain the balance between protein synthesis and other sulfur-related biosynthetic processes. Alternatively, considering that the natural habitat of methanogens is rich in reduced sulfur compounds, these organisms may have evolved to use inorganic sulfur directly for other sulfur-related biosynthetic processes.

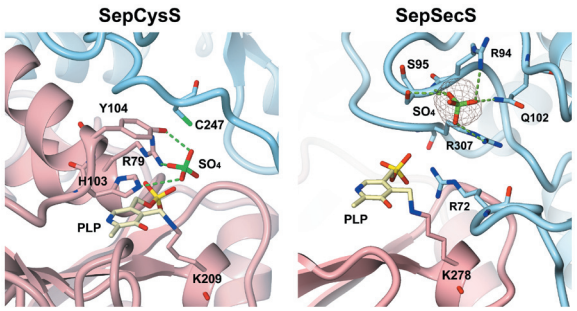

Figure 5. The crystal structures of the active sites of *A. fulgidus* SepCysS and *M. maripaludis* SepSecS. In both, the different monomers of the respective enzyme are colored pink and blue. PLP and residues in the catalytic centers are shown as ball-and-stick models (adapted from 136).

The discovery of the SepRS/SepCysS pathway raised questions as to whether this mechanism is a recent evolutionary invention or if it is more ancient and possibly predates direct tRNA charging by CysRS. Phylogenetic analyses, using structure-based amino acid alignments (77,83) demonstrate that the indirect aminoacylation pathway for tRNA^{Cys} and the direct pathway mediated by CysRS were already present at the time of LUCA. Although both pathways developed contemporarily, CysRS was initially only vertically inherited in the bacterial lineage, whereas SepRS was vertically inherited in *Archaea*. Later in evolution, CysRS was horizontally transferred to *Archaea* and replaced the indirect mechanism in some archaeal lineages, whereas in the methanogenic archaea the indirect pathway either was retained or coexisted with CysRS (77). The question why the SepRS/SepCysS pathway has been preserved in some archaea is still unclear. It has been speculated that some yet unknown link between indirect cysteine formation, sulfur metabolism and methanogenesis might exist (77). Further experimental evidence in this direction is required.

tRNA-dependent selenocysteine biosynthesis

Selenium, an essential dietary trace element, has a beneficial effect on the functions of four major organ systems in the human body as well as is involved in cancer prevention in a dose-dependent manner (99,100). Sec, the major biological form of selenium, is cotranslationally incorporated into proteins as the 21st amino acid in a number of organisms from all three domains of life (9,101). Under physiological conditions (pH 7), Sec is more stable in its ionized form due to the lower redox potential of selenium and thus the lower pKa of the selenol group compared to the thiol group in Cys (5.2 versus 8.5) (102). The extra electrons in the side chain of selenocysteine make it an extraordinary nucleophile in the

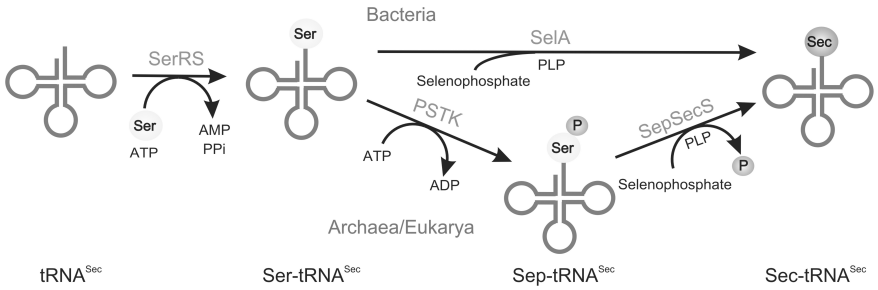

Figure 6. Indirect pathways for Sec-tRNA^{Sec} formation. In all known Sec-decoding organisms, first SerRS aminoacylates tRNA^{Sec} with Ser to form Ser-tRNA^{Sec}. In Sec-decoding bacteria, the Ser bound to the tRNA is directly converted to Sec in the presence of selenophosphate by SelA to form Sec-tRNA^{Sec}. In Sec-decoding eukaryotes and *Archaea*, the Ser-moiety on tRNA^{Sec} is first phosphorylated by PSTK to form Sep-tRNA^{Sec}. The Sep bound to the tRNA is then converted to Sec in the presence of selenophosphate by SepSecS to form Sec-tRNA^{Sec}.

catalytic center of selenoproteins for oxidation–reduction reactions.

The ribosomal selenoprotein biosynthesis uses selenocysteinyl-tRNA^{Sec} (Sec-tRNA^{Sec}) as a substrate. To date, a selenocysteinyl-tRNA^{Sec} synthetase (SecRS), an enzyme that could directly aminoacylate tRNA^{Sec} with Sec, has not been identified in any organism (Table 1). Free selenocysteine can be activated and ligated to tRNA^{Cys} by CysRS from plants (103) and bacteria (104). To prevent mis-incorporation of Sec in Cys codons and the consequent malfunction of proteins (105), the free Sec concentration most likely has to be well controlled and kept at a low level. In addition, a Sec to Cys mutation in a selenoprotein can result in a drastic reduction of enzyme activity (106). Due to the lack of mechanisms differentiating Cys and Sec, the invention of SecRS might be undesirable. Therefore, a pathway to synthesize Sec on tRNA using selenophosphate as the selenium source rather than directly charging tRNA with Sec (Figure 6) may help to maintain the accuracy of Sec and Cys decoding (107).

In all Sec-decoding organisms (108–110), the first step in the Sec biosynthetic pathway is the serylation of tRNA^{Sec} with serine by seryl-tRNA synthetase (111–113). In bacteria, Sec biosynthesis and its incorporation into proteins have been extensively worked out in the nineties (9). Selenocysteine synthase (SelA) converts Ser-tRNA^{Sec} to Sec-tRNA^{Sec}, which is then incorporated into proteins in the presence of a RNA element (facilitating UGA recognition) and Sec-specific elongation factor SelB (9). In *Archaea* and eukaryotes, Ser-tRNA^{Sec} is not directly converted to the final product Sec-tRNA^{Sec} (Figure 4). Instead, it is first converted to Sep-tRNA^{Sec} by *O*-phosphoseryl-tRNA kinase (PSTK) (114), and then the resulting tRNA-bound Sep is transformed to Sec by Sep-tRNA:Sec-tRNA synthase (SepSecS) in the presence of selenophosphate (15,16).

Both SelA and SepSecS are PLP-dependent proteins. They use the same selenium donor, selenophosphate, to produce Sec *in vitro* and *in vivo* (15,16,115–117). The bacterial SelA also converts Sep-tRNA^{Sec} to selenocysteine *in vitro* (16), which may not be biologically relevant as PSTK, the enzyme producing Sep-tRNA^{Sec}, is only found in *Archaea* and eukaryotes (15). In contrast, SepSecS only recognizes Sep-tRNA^{Sec} but not Ser-tRNA^{Sec} as a substrate. SepSecS genes are always copresent with PSTK genes in the complete genomes of all known Sec decoding archaea and eukaryotes (118). Sep-tRNA^{Sec} has a more stable carboxyl ester bond between the amino acid and the tRNA than Ser-tRNA^{Sec} (114). Additionally, the phosphate group in the side chain of Sep is expected to be a better leaving group compared to the hydroxyl group in the serine side chain. Therefore, the increased stability of the substrate and the decrease of activation energy make Sep-tRNA^{Sec} a better precursor for Sec formation.

PSTK phosphorylates serine in a tRNA^{Sec}-dependent manner

The enzymatic phosphorylation of Ser-tRNA^{Sec} was first observed in rat and rooster liver and lactating bovine mammary gland in the 1970s (119,120). A partially purified enzyme from bovine liver was characterized and shown to have high affinity for tRNA^{Sec} (121). It was not until recently that the protein responsible for this activity was identified in mouse as PSTK (114). Later, the archaeal homolog of mouse PSTK was also shown to have the same activity *in vitro* (122). PSTK catalyzes the transfer of the γ-phosphate of ATP to the serine moiety in the presence of magnesium ions (114,118). The N-terminal kinase domain of PSTK consists of a phosphate-binding loop (P-loop), a Walker B and RxxxR motif which are generally conserved in the P-loop kinase superfamily (118,123). Like T4 polynucleotide kinase (124), PSTK has relaxed NTP specificity with a preference for ATP *in vitro* (118).

Comprehensive biochemical studies of wild type and mutant *M. jannaschii* PSTK also uncovered several unique aspects of this protein (118). Most notably, PSTK binds to unacylated tRNA^{Sec} and its substrate Ser-tRNA^{Sec} with similar affinities ($K_d \cong 40$ nM) (118). As the *in vivo* concentration of tRNA^{Sec} is much lower than that of other tRNA species (e.g. it is <10% of tRNA^{Ser}) (125,126), it is not surprising that PSTK has about 20-fold higher affinity for its substrate than aaRSs. The equally high affinity for unacylated tRNA^{Sec} suggests a scavenger role of PSTK for tRNA^{Sec}; this may assist other enzymatic reactions in the pathway such as the misacylation by SerRS and the final conversion step by SepSecS (118). PSTK may

also prevent mis-incorporation by sequestering the mis-acylated $tRNA^{Sec}$ intermediates and channeling them to SepSecS for the cognate Sec-$tRNA^{Sec}$ production (118). Moreover, the tRNA-dependent activation of ATPase activity in PSTK (118) is another example of substrate-assisted catalysis in this kinase superfamily (123).

The recognition of Sep-$tRNA^{Sec}$ is different in *Archaea* and eukaryotes

The $tRNA^{Sec}$ has distinct structural features relative to other tRNA molecules: an elongated acceptor arm (acceptor stem + T-stem) and D-stem (127,128). Bacterial $tRNA^{Sec}$ has 8 bp and 5 bp in the acceptor stem and the T-stem, respectively, while archaeal and eukaryotic $tRNA^{Sec}$ has a 9 bp and 4 bp arrangement (127,129–131). For the serylation reaction, $tRNA^{Sec}$ shares the same major identity element as $tRNA^{Ser}$, namely the long variable arm and the discriminatory base for SerRS recognition (132). A different set of identity elements in $tRNA^{Sec}$ for the phosphorylation reaction catalyzed by PSTK is observed in archaea relative to eukaryotes. In eukaryotes, the major identity element for PSTK recognition is the length and the conformation of the D-stem (133), which is rather a minor identity element in archaea. Instead, the second and the third base pair (G2-C71 and C3-G70) in the acceptor stem of $tRNA^{Sec}$ serve as the major identity elements for archaeal PSTK phosphorylation (134). Interestingly, PSTK phylogeny shows a deep divide between the archaeal and the eukaryotic type enzyme (118). These findings may suggest the co-evolution of the kinase and its substrate $tRNA^{Sec}$ (134).

Despite the differences in the selenocysteine incorporation machinery between the bacterial system and the archaeal and eukaryotic systems, SepSecS and PSTK recognize *E. coli* $tRNA^{Sec}$ *in vivo* (15). Similarly, previous reports also showed that human $tRNA^{Sec}$ complements a $tRNA^{Sec}$ gene deletion in *E. coli* and that the eukaryotic $tRNA^{Sec}$ can be serylated and converted to Sec-$tRNA^{Sec}$ by *E. coli* enzymes *in vitro* (135). These findings suggest that $tRNA^{Sec}$ is functionally conserved between the two different selenocysteine incorporation systems.

SepSecS catalyzes the tRNA-dependent transformation of Sep to Sec

The archaeal and eukaryotic pathway for selenocysteine formation is reminiscent of the indirect pathway for cysteine biosynthesis in archaea, most notably in the final conversion step where Sep-tRNA is the immediate precursor for cysteine or selenocysteine production by SepCysS or SepSecS in the presence of a sulfur or selenium donor (Figures 4 and 6). SepSecS can also use thiophosphate as a substrate and produce Cys-$tRNA^{Sec}$ *in vitro* (136). The inability of differentiating selenophosphate *versus* thiophosphate by SepSecS indicates that the phosphate group in the selenium donor is the major recognition moiety by the enzyme.

Analogous to SepCysS, SepSecS catalyzes the β-replacement of phosphoserine to form selenocysteine in the presence of selenophosphate in a tRNA-dependent manner. Recently, the crystal structure of SepSecS from *M. maripaludis* (136) and mouse (137) were solved at high resolution. Both structures revealed a homotetrameric state $(\alpha_2)_2$ of SepSecS with two active sites per dimer (136,137). Each active site located at the dimer interface consists of catalytically crucial residues from both subunits (Figure 5). The tetramerization is mediated by the N-terminal extension of each SepSecS monomer. The elongated shape resulting from tetramerization together with the large patch of positive electronic potential on the surface of SepSecS are proposed to form an effective $tRNA^{Sec}$ binding surface (137). It appears that the disruption of the oligomeric state by deleting the N-terminal region abolishes the catalytic function of SepSecS (136). Similar to SepCysS (88), a conserved lysine and asparagine are involved in PLP binding by SepSecS (136). Conserved arginines, glutamine and serine are proposed to be essential for binding to the phosphate moiety of the substrates (Sep-$tRNA^{Sec}$ and/or selenophosphate) (Figure 5) (136). Mutations of these residues significantly reduce the catalytic activity of SepSecS *in vivo* and *in vitro* (136). An interesting filtering mechanism was proposed for excluding free amino acid including free Sep as a substrate, where the side chain of a conserved glutamate repels the carboxyl group of the free amino acid (137). Distinct from SepCysS, a perselenide intermediate is unlikely to be present in the reaction catalyzed by archaeal SepSecS due to the absence of a conserved cysteine residue near the active site. A structural phylogeny shows SepSecS is not closely related to other PLP-dependent proteins with solved structures in the fold type I family, while SepCysS belongs to the cysteine desulfurase group (136).

Cysteine and selenocysteine biosynthetic pathways are both ancient

Phylogenetic analysis demonstrated that bacterial, archaeal and eukaryotic selenocysteine incorporation machineries already existed at the time of LUCA (15) and so did the indirect pathway for cysteine formation (77). SepSecS and PSTK are strictly archaeal and eukaryotic enzymes and distinct from the bacterial SelA. The PSTK/SepSecS pathway for synthesizing selenocysteinyl-tRNA is reminiscent of the strictly archaeal tRNA-dependent pathway for cysteine formation in various aspects mentioned earlier. As both SepCysS and SepSecS use tRNA-bound phosphoserine as a substrate, it will be interesting to investigate if the functions of these two enzymes are interchangeable under certain conditions. Considering that most selenoproteins have Cys-containing homologs and a similar genetic code for Sec and Cys (UGA versus UGC/UGU), a dynamic evolutionary relationship may exist between selenocysteine and cysteine (138).

OUTLOOK

As discussed throughout this article, synthesis of an amino acid on its cognate tRNA requires the formation of a mischarged tRNA substrate. What prevents these

misacylated tRNA species from being used in protein synthesis? While elongation factors (EF-Tu and SelB) have been shown *in vitro* to have higher affinity for cognate aa-tRNA pairs over certain mischarged tRNA species (22,139–143), it still remains unclear the level of discrimination by the elongation factors *in vivo* (63,144). Substrate channeling of the misacylated tRNA from an aaRS to a tRNA-dependent modifying enzyme would provide an additional mechanism to maintain the fidelity of protein synthesis despite formation of the mischarged tRNA species (17,44,62,63,88). Intriguingly, structural modeling does suggest complexes between ND-GluRS, Glu-AdTs and $tRNA^{Gln}$, and SepRS, SepCysS and $tRNA^{Cys}$ are possible (44,63,88). Whether these enzymes do form complexes similar to that found for ND-AspRS, GatCAB and $tRNA^{Asn}$ awaits further investigations, as do their cellular implications.

It is tempting to speculate that such ancient complexes allowed Gln, Asn, Cys and Sec to be added to the genetic code (44,145), but that should be tempered by the fact that the indirect pathway for Cys-$tRNA^{Cys}$ formation may not predate the direct one (77). It may well be that use of tRNA-dependent amino acid biosynthetic routes arose and have been retained to maintain the fidelity of protein synthesis in environments in which an aaRS alone could not reliably discriminate between two similar amino acids (146), e.g. CysRS with Cys and Sec (103,104). Delving into such speculation will require a better understanding as to how these indirect pathways for aa-tRNA formation are integrated into overall metabolism and regulated under different cellular conditions.

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