Leishmania tarentolae contains distinct cytosolic and mitochondrial glutaminyl–tRNA synthetase activities

(Aminoacyl–tRNA synthetases/trypanosomatids/mitochondrial RNA import/evolution)

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ABSTRACT  The intracellular distribution of glutaminyl–tRNA synthetases and their role in mitochondrial tRNA import were evaluated in the ancient eukaryote Leishmania tarentolae. The following results were obtained: (i) Glutaminyl–tRNA synthetase was detected in leishmanial mitochondria. This was unexpected because it has been postulated that, in organelles, Gln–tRNA synthetase is not formed by direct acylation of tRNA but by enzymatic transamidation of misacylated Glu–tRNA. (ii) Whereas the cytosolic extract is able to charge cytosolic and mitochondrial tRNAs, the mitochondrial matrix extract does not aminoacylate the cytosol-specific tRNA. This indicates that mitochondrial and cytosolic glutaminyl–tRNA synthetases are distinct. (iii) Seven of the 11 nucleotides that differ between the cytosolic and the mitochondrial tRNA are sufficient to convert the cytosol-specific tRNA into an optimal substrate for the mitochondrial enzyme. These nucleotides are arranged in three groups consisting of the nucleotides flanking the anticodon stem, the 5′ nucleotide of the anticodon, and four nucleotides within the acceptor stem. And (iv), it was shown that the identity elements for recognition by the mitochondrial glutaminyl–tRNA synthetase do not overlap with a previously identified sequence segment required for mitochondrial import of the tRNA.

Generally, 20 functionally different aminoacyl–tRNA synthetases (aaRS), one for each amino acid, are required for protein synthesis. However, there are exceptions; in certain organisms some, tRNAs are formed by transformation of acylated tRNAs rather than by direct acylation. One example is the formation of Gln–tRNA because it can be achieved by different pathways. tRNA can either be charged directly with glutamine by glutaminyl–tRNA synthetase (GlnRS), or formation of Gln–tRNA is achieved by a two-step enzymatic reaction (1). In this case, the tRNA is first mischarged with glutamate by glutamyl–tRNA synthetase (GluRS), resulting in Glu–tRNA. The glutamate attached to the tRNA is then converted by a specific amidotransferase into a glutamine, yielding a correctly charged tRNA. Absence of GlnRS and usage of the transaminidation pathway to form Gln–tRNA has been demonstrated in a large number of organisms, including Archaea (2), Gram-positive bacteria (3, 4), and organelles of eukaryotes (5). In agreement with this, GlnRS was lacking in Rhizobium meliloti (6), a member of the α-subdivision of purple bacteria and, according to the endosymbiont theory, the ancestors of present day mitochondria.

L. tarentolae belongs to the earliest diverging cells in eukaryotic evolution that have mitochondria (7). This is illustrated by a number of unique features of their mitochondria, including a bipartite, topologically interlocked genome, guide RNA-mediated RNA editing, and the lack of mitochondrial tRNA genes (8, 9). Mitochondrial biogenesis in trypanosomatids therefore not only involves import of proteins, as in all other eukaryotes, but also import of the whole set of nuclearily encoded mitochondrial tRNAs (10–13). However, mitochondrial tRNA import is not restricted to protozoa but has also been shown in plants and yeast (14, 15). aaRS have been implicated in the import process. Whereas there is only indirect evidence for their involvement in plants (16), participation of a precursor of a mitochondrial aaRS in tRNA import has been shown directly in yeast (17). In L. tarentolae, in most cases, one gene codes for both mitochondrial and cytosolic tRNA isoforms, resulting in almost identical sets of tRNAs in the two compartments (10). This raises the questions whether, in L. tarentolae, the aaRS as well are identical in the cytosol and the mitochondrial fraction and whether these enzymes play a role in tRNA import.

To address these questions, we focused on the tRNAs in L. tarentolae. Two highly homologous tRNAs have been characterized in L. tarentolae: the tRNA with the anticodon UUG, which, like most other leishmanial tRNAs is found in the cytosol as well as in mitochondria (18), and the tRNA with the anticodon CUG, which is the only known cytosol-specific tRNA in that organism (19). The intracellular distribution and the substrate specificities of GlnRSs were determined. Unexpectedly, GlnRS activity was found in mitochondrial matrix fractions of L. tarentolae, showing that GlnRS is not universally absent from mitochondria. In addition, it was shown that the mitochondrial GlnRS of L. tarentolae is distinct from its cytosolic counterpart. Furthermore, it could be demonstrated that the sequence elements within mitochondrial tRNAs that are identity determinants for the mitochondrial GlnRS are distinct from a previously identified import signal in the D loop (20).

MATERIALS AND METHODS

Cells. L. tarentolae (UC strain) was grown at 27°C in Difco brain heart infusion medium containing 10 µg/ml hemin to late log phase (0.5 × 10^6–1.5 × 10^8 cells/ml) and used immediately. Cells were washed once in cold 20 mM sodium-phosphate buffer (pH 7.9) containing 150 mM NaCl and 20 mM glucose.

Isolation of tRNA. Washed L. tarentolae (3–10^11 cells) were resuspended in 10 ml of 20 mM Tris-HCl/20 mM magnesium acetate, pH 7.4. Cells were extracted twice with an equal volume of phenol and equilibrated in 25 mM sodium acetate (pH 4.5) containing 50 mM NaCl. Sodium acetate (pH 5.2) was added to 0.3 M and total RNAs were precipitated by the addition of an equal volume of isopropanol. The RNA pellet

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Abbreviations: aaRS, aminoacyl–tRNA synthetase; GlnRS, glutaminyl–tRNA synthetase; GluRS, glutamyl–tRNA synthetase.

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was resuspended in 4 ml of 0.2 M Tris-acetate (pH 9.0) and deacylated for 30 min at 37°C. After addition of sodium acetate to 0.3 M, the RNAs were precipitated by the addition of 2 vol of ethanol. The resulting pellet was resuspended in 2 ml of 10 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane-HCl (pH 7.0), 1 mM MgCl₂, and 1 mM 1,4-dithiothreitol and treated with 10 units of RNasin (Promega) for 20 min at 37°C. Subsequently, the mixture was phenol-extracted and precipitated with ethanol as before. The pellet was dissolved in 3 ml of 1 M NaCl and centrifuged for 10 min at 16,000 × g to remove high molecular weight RNAs. The supernatant containing the tRNAs was ethanol precipitated, resuspended in 1 ml of H₂O, and loaded on a 0.6 ml DEAE-cellulose column equilibrated in buffer A (10 mM sodium acetate/0.2 M NaCl/10 mM MgCl₂, pH 5). The column was washed with 4 ml of buffer A and eluted by 2 ml of buffer A containing 0.7 M NaCl. The tRNAs (yield 2–3 mg) were precipitated, resuspended in H₂O, and frozen at −70°C until further use.

In Vitro Transcription of tRNA. The tRNA<sub>Gln</sub> variants were produced by in vitro transcription using T7 polymerase (21). DNA-fragments containing the T7 promoter and the corresponding natural or synthetic tRNA<sub>Gln</sub> genes were produced by PCR using the following oligonucleotides: QM1, 5'-GAGTTTATGTTGATTGGTGTAC-3'; QM2, 5'-TTGGTTGTTGTATTCACTATG-3'; QC1, 5'-GGAGTTTATGTTGATTGGTGATG-3'; QC2, 5'-GGATTTATGTTGATTGGTGAG-3'; QCUUG, 5'-CTGCTTATAATACGACTCACTATAGCTCCTATAGTGTAGCTCCTATAGTGTAGCGG-3'; QZ1, 5'-TAATACGAGCTCTACTATAATGATGTGTTGATGGAGTCTCTGGACTCTTGAAT-3'; CQCUUC, 5'-TAATACGAGCTCTACTATAATGATGTGTTGATGGAGTCTCTGGACTCTTGAAT-3'; QC2, 5'-TAATACGAGCTCTACTATAATGATGTGTTGATGGAGTCTCTGGACTCTTGAAT-3'; ACST, 5'-TAATACGAGCTCTACTATAATGATGTGTTGATGGAGTCTCTGGACTCTTGAAT-3'. Sequences corresponding to the T7 promoter are underlined. As DNA templates to produce the variant tRNAs<sub>Gln</sub>, in addition to some of the PCR products listed below, the following PCR products were used: PCR-fragment GlmCyt was produced using the primers GNLT1 and GNLT2 and genomic leishmanial DNA as template; PCR-fragment GlmMit was produced using the primers LTQM1 and genomic leishmanial DNA as template. The fragment GlnMit was produced using the primers LTQM1 and genomic leishmanial DNA as template. The following PCR products were used: PCR-product of previous reaction); mitochondrial tRNA<sub>Gln</sub> containing the mitochondrial acceptor stem and anticodon, the nucleotide substitutions flanking the acceptor stem and the D loop substitution (ACST/ACST³', template GlmMit); cytosolic tRNA<sub>Gln</sub> containing all the previous changes except the D loop substitution (ACST³', template PCR product of previous reaction); mitochondrial tRNA<sub>Gln</sub> (QM1/QM2, template GlmMit); and tRNA<sub>Gln</sub>(D-Ile) (Q1/OC2, template GlmCyt).

Preparation of RNA-Free Cytosolic Fractions. Washed <i>L. tarentolae</i> cells were hypotonically lysed at 2.4 × 10⁶ cells/ml in 1 M Tris-HCl/1 mM EDTA, pH 8, by four passages through a 25-G syringe needle. A quarter volume of 5X acylation buffer (250 mM Tris-HCl, pH 7.5/125 mM KCl/40 mM MgCl₂/50 mM 2-mercaptoethanol), one-ninth volume of 100% glycerol was added, and the extract was centrifuged for 25 min at 150,000 × g at 4°C. NaCl was added to 150 mM, and the sample was applied on a DEAE cellulose column (13 mg protein/ml bed volume) equilibrated in the same buffer. The flow through fraction was concentrated using a Centricron-30 concentrator (Amicon) to 1 ml and loaded on a 10 ml Sephadex G-100 column (20 cm length) equilibrated in 1X acylation buffer containing 10% glycerol (22). Protein-containing fractions of the exclusion volume were pooled (yield 2.3 mg/10¹⁰ cells), frozen in liquid N₂, and stored at −70°C until further use in the charging assays.

Preparation of RNA-Free Mitochondrial Matrix Fractions. Mitoplasts of <i>L. tarentolae</i> were purified as described (23, 24). Isolated mitoplasts (40 mg protein) were resuspended in 20 ml Tris-HCl (pH 8), 250 mM sucrose, and 2 mM EDTA and frozen in liquid N₂. To prepare the mitochondrial matrix fraction, an equal volume of 16 mM MgCl₂ 100 mM Tris-HCl (pH 7.5), and 50 mM KCl containing 2 mM phenylmethylsulfonfylfluoride was added to thawed mitoplasts, and the suspension was sonicated on wet ice (10 bursts of 5 s using the microtip of a sonicator (MSE, Zivy, Switzerland), amplitude 3 at medium power setting). The extract was then subjected to another freeze thaw cycle, and the sonication step was repeated. After the addition of glycerol to 10% and 2-mercaptoethanol to 10 mM, the suspension was centrifuged at 180,000 × g at 4°C for 25 min. Approximately 35% of total proteins were recovered in the supernatant corresponding to the mitochondrial matrix fraction. The supernatant was adjusted to 150 mM NaCl and applied to a DEAE cellulose (12 mg matrix protein/0.5 ml bed volume) followed by Sephadex G-100 chromatography as described for the cytosol (see previous paragraph). Approximately 2.5 mg of mitochondrial matrix proteins was recovered in the final fraction.

Yeast mitochondria originating from the wild-type strain D273-10B were purified using Nycodenz gradients (25). Mitochondrial RNAs were isolated as described (26). From 20 mg of mitochondrial RNA, 75 µg of RNA was obtained. RNA-free mitochondrial matrix fraction was prepared as described for <i>L. tarentolae</i>. Fifty milligrams of purified mitochondria yielded 4 mg of RNA-free matrix fraction.

Charging Assays. Aminocloning reactions for <i>L. tarentolae</i> were performed in 100 µl of 1x acylation buffer supplemented with 4 mM ATP containing 20 µg of total leishmanial tRNA or 1.8 µg of the corresponding in vitro transcription tRNA, 60 µg of crude cytosolic or mitochondrial aaRS fraction and 19 µM of 14C-glutamine (258 mCi/mmol) or 14C-glutamate (265 mCi/mmol (Dupont) respectively. For the competition assays an additional 1.8 or 9 µg of the competitor tRNA [cytosolic tRNA<sub>Gln</sub> or tRNA<sub>Gln</sub>(D-Ile)] were added to a standard charging reaction. Charging assays for yeast were done under identical conditions with the exception that 50 µg of total mitochondrial RNA and 100 µg of crude mitochondrial aaRS-containing matrix fraction were used. All samples were incubated for 15 min at 37°C and spotted onto dried Whatman filter paper (2.5 × 2.5 cm) which had been pretreated with 5% TCA containing either 0.1 mM glutamine or glutamate. The filters were washed three times (15 min each) on ice in 4 ml of 0.1 M HCl. After a final wash in cold ethanol (96%) the filters were dried and processed for scintillation counting.

Thin Layer Chromatography. After incubation at 37°C, a standard charging reaction containing total tRNAs, leishmanial mitochondrial matrix extract, and 14C-glutamate was phenol-extracted, ethanol-precipitated, and washed with 75% ethanol. The resulting pellet was decayed in 50 µl of 10 M KOH for 10 min at 65°C. Subsequently, the pH was adjusted (pH 6–7) by the addition of 0.1 M HCl and dried under vacuum. Finally, the pellet was resuspended in 3 µl of water.
and spotted onto a cellulose thin layer chromatography plate. The plate was developed for ~3 h in a mixture of isopropanol/formic acid/water (77:18.2:4.8) and exposed on x-ray film.

**Miscellaneous.** Antiserum directed against yeast Hsp60 was a generous gift of G. Schatz, Biozentrum, Basel. Polyclonal antiserum specific for leishmanial pyruvate kinase was kindly provided by P. Michels, International Institute of Cellular and Molecular Pathology, Brussels. Both sera were used at a dilution of 1/500. The signals were visualized using peroxidase-conjugated second antibody and the enhanced chemiluminescence detection kit (Amersham). Protein concentrations were determined using the BCA assay (Pierce).

**RESULTS**

**Mitochondrial GlnRS in L. tarentolae.** In vitro charging assays using labeled glutamine or glutamate were performed to measure GlnRS and GluRS activities in the mitochondria and the cytosol of *L. tarentolae*. Because of the nuclear origin of mitochondrial tRNAs in *L. tarentolae*, their cytosolic and mitochondrial sets of tRNAs overlap to a great extent (10). Therefore, tRNAs isolated from total cellular extract were used as substrates for both cytosolic and mitochondrial aaRS.

Similar specific activities of GlnRS and GluRS were detected in the mitochondria and the cytosol of *L. tarentolae* (11 nucleotide positions and in their intracellular localization). tRNAsGln have been characterized in lower than in cells. Measured in the mitochondrial matrix of trypanosomes (data not shown), the specific activities were significantly lower than in *Leishmania*. These results demonstrate the existence of GlnRS in the mitochondria of trypanosomatids, indicating that this enzyme is not universally absent in mitochondria.

**Cytosolic and Mitochondrial GlnRS Are Distinct.** Two tRNAsGln have been characterized in *L. tarentolae*. They differ in 11 nucleotide positions and in their intracellular localization (Fig. 2A). The tRNA\(^{\text{Gln}}\)(CUG), unlike most other leishmanial

**Fig. 1.** Mitochondrial GlnRS in *L. tarentolae*. (A) Leishmanial total tRNAs were charged with \(^{14}\text{C}-\text{glutamine (Gln)}\) or \(^{14}\text{C}-\text{glutamate (Glu)}\) using 60 \(\mu\text{g}\) each of RNA-depleted cytosolic (Cyt) or mitochondrial matrix (Mit) fractions of *L. tarentolae*. Bars indicate mean values (±SD) of independent aminoacylation reactions (\(n=4–8\)) using at least two independently prepared cytosolic or mitochondrial extracts. (B) Yeast mitochondrial tRNA was charged with \(^{14}\text{C}-\text{glutamin}e \text{(Gln)}\) or \(^{14}\text{C}-\text{glutam}ate \text{(Glu)}\) using 100 \(\mu\text{g}\) of RNA-depleted yeast mitochondrial matrix extract. The reactions were incubated in the presence (hatched columns) or as a control in the absence of the corresponding tRNAs (empty columns). The y axis indicates the specific activities corresponding to picomolars of aminoacylated tRNA formed per \(\mu\text{g}\) of proteins. (C) Equal amounts (50 \(\mu\text{g}\)) of RNA-depleted cytosolic (Cyt) and mitochondrial matrix (Mit) fraction used for the charging experiments were analyzed by immunoblotting using antisera specific for mitochondrial heat shock protein 60 (HSP60) or the cytosolic protein pyruvate kinase (PYK). (D) A charging reaction containing total tRNAs, \(^{14}\text{C}-\text{glutamin}e\) and mitochondrial matrix extract was deacylated, and the released amino acid (Mit) was analyzed by thin layer chromatography. \(^{14}\text{C}-\text{glutamin}e\) (Gln) and \(^{14}\text{C}-\text{glutam}ate\) (Glu) served as markers.
tRNAs, is found exclusively in the cytosol (19). The tRNA Gln (UUG), however, has a dual location and is found in both the cytosol and the mitochondria (18). We will refer in this study to the tRNAGln(CUG) as the cytosolic and the tRNA Gln-(UUG) as the imported mitochondrial tRNA Gln, even though the latter also is found in the cytosol.

In vitro charging assays were performed using in vitro-transcribed cytosolic or mitochondrial tRNAs Gln as substrates and cytosolic or mitochondrial extract as source of enzyme. Cytosolic extract aminoacylated both substrates whereas the mitochondrial extract was only able to charge the mitochondrial but not the cytosolic tRNAGln (Fig. 2B). These results prove that the mitochondrial GlnRS activity cannot be caused by cytosolic contamination of the mitochondrial fraction, and, most importantly, they show that cytosolic and mitochondrial GlnRS activities are distinct.

Identity Elements Recognized by Mitochondrial GlnRS. Cytosolic and mitochondrial tRNA Gln differ by 11 nucleotides that are distributed in six groups all over the molecule (Fig. 2A). To determine which groups of nucleotides, or which combination of groups, are critical for recognition by the mitochondrial GlnRS, the cytosolic tRNAGln(CUG) sequences were successively replaced by the corresponding mitochondrial tRNAGln sequences (Fig. 3). The resulting hybrid molecules corresponded to the cytosolic tRNAGln containing (i) the mitochondrial acceptor stem, (ii) the mitochondrial anticodon, (iii) the mitochondrial acceptor stem and the anticodon, (iv) the mitochondrial acceptor stem, the anticodon, and the 5' nucleotide of the anticodon stem, and (v) all of the previous changes and the D loop substitution. In vitro transcripts of all of these tRNA Gln variants were tested for charging by the mitochondrial extract. Three elements required for recognition of the mitochondrial GlnRS were identified: (1) the nucleotides flanking the anticodon stem that contribute approximately half and (2) the 5' nucleotide of the anticodon and (3) the acceptor stem each contributing approximately one-fourth to the conversion of the cytosolic tRNAGln into a substrate for the mitochondrial GlnRS. If all three elements are present, the same level of activity was reached as with the natural substrate, the mitochondrial tRNAGln. The nucleotide substitutions in the D loop or the T loop therefore are not identity elements.

Import Signal and Identity Elements for Mitochondrial GlnRS Are Distinct. Mitochondrial tRNAGln must not only contain recognition elements for the mitochondrial GlnRS but also elements that specify its import into mitochondria. Recently Lima et al. (20) showed that, in L. tarentolae, the cytosolic tRNAGln is converted into an imported tRNA if its D stem loop is replaced with that of tRNA Ile, which is predominantly found in mitochondria. The five point mutations necessary to switch the tRNAGln D stem loop to the tRNA Ile D stem loop are indicated in Fig. 4A; the resulting molecule was called tRNAGln(D-Ile). From these results, it was concluded that at least one import signal must lie within the D loop region.

![Fig. 2. Cytosolic and mitochondrial GlnRS are distinct. (A) Inferred secondary structures of cytosol-specific (Cyt) and mitochondrial (Cyt/Mit) tRNAs Gln of L. tarentolae. Nucleotides in the mitochondrial tRNA Gln that are different from the cytosolic ones are boxed. (B) Cytosolic-specific tRNA Gln(CUG) and mitochondrial tRNA Gln(UUG) of L. tarentolae were charged with 14C-glutamine using 60 μg each of RNA-depleted cytosolic (Cyt) or mitochondrial matrix (Mit) fractions. As a control, the reactions were performed without adding the tRNAs (no tRNA). Bars indicate mean values (±SD) of independent aminoacylation reactions (n = 4–8) using at least two independently prepared cytosolic or mitochondrial extracts.](#)

![Fig. 3. Identity elements recognized by mitochondrial GlnRS.](#)
subjected to charging assays. The tRNA_{Gln}^{D-Ile} behaved identically to the cytosolic tRNA_{Gln}, in that both could be charged by the cytosolic but not by the mitochondrial GlnRS (Fig. 4B). Cytosolic tRNA_{Gln} and tRNA_{Gln}^{D-Ile}, however, differ in their intracellular localization; only tRNA_{Gln}^{D-Ile} is imported into mitochondria, indicating that charging by mitochondrial GlnRS is not required for import of tRNA_{Gln}^{D-Ile}. Nevertheless, the fact that charging and mitochondrial import tRNA_{Gln}^{D-Ile} are not coupled does not preclude the involvement of mitochondrial GlnRS in import because the imported tRNA_{Gln}^{D-Ile} may still interact with the tRNA charging network within the organelle. It is therefore concluded that no significant binding of tRNA_{Gln} to the domain of mitochondrial GlnRS, which is responsible for charging, is required for import. Mitochondrial GlnRS might, however, still be involved in import. In this case, one must postulate that it contains two distinct nonoverlapping binding sites for imported tRNA_{Gln}^{D-Ile}, one for charging and one for import.

**DISCUSSION**

There is overwhelming evidence that mitochondria arose from an endosymbiotic capture of a member of the α-subdivision of purple bacteria. It is generally assumed that the endosymbiotic event happened just once in eukaryotic evolution and that therefore all mitochondria are evolutionarily related (28). Recently, it was shown that *R. meliloti* (6), a member of the α-subdivision of purple bacteria, unlike other Gram-negative bacteria, uses the transamidation pathway for Gln-tRNA_{Gln} formation (6). In line with this, it was demonstrated also that mitochondria use the transamidation pathway like their presumed ancestors and therefore are devoid of mitochondrial GlnRS (5). The transamidation pathway is therefore considered to be a more ancestral pathway to form Gln-tRNA_{Gln} than direct acylation by GlnRS.

Using *in vitro* charging assays with radioactive glutamine and RNA-free cytosolic and mitochondrial extracts, we show here that GlnRS is not universally absent from mitochondria but is found in organelles of *L. tarentolae* and *T. brucei*. Evidence is presented that shows that, in *L. tarentolae*, mitochondrial and cytosolic GlnRS are distinct because they exhibit a different substrate specificity. One interpretation of these results is that the cytosolic GlnRS is able to charge both cytosolic and mitochondrial tRNA_{Gln}^{D-Ile}. The mitochondrial GlnRS, which has a more limited substrate specificity, would in this scenario be localized in mitochondria only. However, it cannot be excluded that cytosolic and mitochondrial GlnRS have nonoverlapping substrate specificities. In this case, one needs to postulate a dual location of the mitochondrial enzyme, in both cytosol and mitochondria.

GlnRS is not found in the α-subdivision of purple bacteria, the presumed ancestors of present day mitochondria (6). Could this mean that trypanosomatid mitochondria are derived from a different endosymbiont than other mitochondria? Indeed, mitochondria of trypanosomatids are unique in other respects as well. Their genome consists of a complex intercalated network of two genetic elements, the maxi- and the minicircles (29). Nowhere else in nature has a comparable structure been encoded by a single genetic element.
found. In addition, many mitochondrial genes in trypanosomatids represent cryptogenes, whose transcripts have to be extensively edited to become functional mRNAs (9). RNA editing was shown to be mediated by short RNAs, called guide RNAs (30), which have not been found in mitochondria of any other organism. These features would argue for a polyphyletic origin of mitochondria. However, a monophyletic origin of mitochondria is favored by the fact that the mitochondrial genes in trypanosomes code for the same set of proteins as most other mitochondria. In this case, mitochondrial GlnRS would have been acquired later in evolution after the endosymbiotic event. This could have been achieved by gene duplication of the cytosolic GlnRS and establishing of a mitochondrial targeting sequence. At present, it is not possible to decide whether mitochondrial GlnRS in trypanosomatids has been acquired early or late in evolution. Cloning and sequencing of mitochondrial GlnRS might help to answer this question.

It is interesting to note that mitochondrial GlnRS may also exist in other protozoa. Already more than 20 years ago, GlnRS activity was measured in Tetrahymena mitochondria (27). This work was done before the discovery of transamidation as the primary pathway to form Gln-tRNA\(^{\text{Gln}}\) in mitochondria, however, and therefore needs to be confirmed. The reason why trypanosomatid and maybe other protozoal mitochondria contain GlnRS and do not use the transamidation pathway is unknown at present. Of interest, both trypanosomatids and Tetrahymena (31, 32) import all or most of their mitochondrial tRNAs, including the tRNAs\(^{\text{Gln}}\), from the cytosol. However, we were able to show that, in L. tarentolae, there is no direct link between charging by mitochondrial GlnRS and import of tRNA\(^{\text{Gln}}\). This conclusion is based on the identification of the sequence elements necessary for charging by mitochondrial GlnRS, which can be separated from a previously identified import signal located in the D loop of the tRNA (20). Consequently, a mutated tRNA\(^{\text{Gln}}\) that cannot be charged by mitochondrial GlnRS can still be imported into mitochondria, provided that it contains an import signal in the D loop. This is in contrast with the plant system, in which a single point mutation within the acceptor stem concomitantly inactivated both charging by the cognate aaRS and mitochondrial import (16). In yeast, the situation is different. Mitochondrial import of the single imported tRNA\(^{\text{Gly}}\) is mediated by the mitochondrial precursor of lysyl–tRNA synthetase. Surprisingly, this enzyme is only able to bind, but not to aminoacylate, the imported tRNA\(^{\text{Gly}}\) (17). The same could potentially be true in L. tarentolae. However, neither an excess of cytosolic tRNA\(^{\text{Gln}}\) nor an excess of import competent tRNA\(^{\text{Gln}}\)(D-Hle) was able to inhibit charging of mitochondrial tRNA\(^{\text{Gln}}\) by mitochondrial extracts, indicating that the binding sites for charging and the putative binding sites for import do not overlap. In addition, it has been shown in T. brucei that intron-containing tRNA\(^{\text{Tyr}}\) that could not be charged could still be imported into mitochondria in vivo (33). These results, together with the observation that in vitro import of tRNA into mitochondria of Leishmania donovani does not require cytosolic factors (34, 35), make the involvement of aaRS in mitochondrial tRNA import in trypanosomatids unlikely.

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