Mitochondrial tRNA Import in *Toxoplasma gondii* 

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Apicomplexan parasites have the smallest known mitochondrial genome. It consists of a repeated element of ~6–7 kb in length and encodes three mitochondrial proteins, a number of tRNA fragments, but no tRNAs. It has therefore been postulated that in apicomplexans all tRNAs required for mitochondrial translation are imported from the cytosol. To provide direct evidence for this process we have established a cell fractionation procedure allowing the isolation of defined organellar RNA fractions from the apicomplexan *Toxoplasma gondii*. Analysis of *T. gondii* total and organellar RNA by Northern hybridization showed that except for the cytosol-specific initiator tRNA Met all nucleus-encoded tRNAs tested were present in the cytosol and in the mitochondrion but not in the plastid. Thus, these results provide the first experimental evidence for mitochondrial tRNA import in apicomplexans. The only other taxon that imports the whole set of mitochondrial tRNAs are the trypanosomatids. Interestingly, the initiator tRNA Met is the only cytosol-specific tRNA in trypanosomatids, indicating that the import specificity is identical in both groups. In agreement with this, the *T. gondii* initiator tRNA Met remained in the cytosol when expressed in *Trypanosoma brucei*. However, in contrast to trypanosomatids, no thio-modifications were detected in the tRNA Met of *T. gondii* indicating that, unlike what is suggested in *Leishmania*, they are not involved in regulating import.

*Toxoplasma gondii* is an obligate intracellular parasite of humans and animals. Whereas in immunocompetent individuals a *T. gondii* infection is generally asymptomatic, immunosuppressed patients are at risk of severe disease (1). *T. gondii* and *Plasmodium falciparum*, the causative agent of human malaria, belong to the apicomplexans and are evolutionarily closely related. Thus, in addition to being an important pathogen of its own, *T. gondii* serves as a model system for *Plasmodium* to study some experimentally less accessible aspects of apicomplexan biology (2). Interestingly, apicomplexans contain a non-photosynthetic plastid whose genome shares many features with chloroplast DNA (3). As expected for eukaryotes, the mitochondrial genome with the most limited coding capacity is surprising, because their biology is not only expected to be of medical relevance but, due to some unique features, is also of great interest for basic science. It has been proposed that the contribution of mitochondrial ATP synthesis to the total cellular pool is minimal (4). However, the presence of a mitochondrial membrane potential has been demonstrated in both intracellular *T. gondii* tachyzoites (5) and intraerythrocytic *Plasmodium yoelii* (4). Furthermore, it has been suggested that the anti-apicomplexan drug atovaquone acts by interfering with the electron transport chain of the parasites through inhibition of cytochrome b in the bc1 complex (4). This is supported by the detection of mutated cytochrome b genes in the mitochondrial DNA of atovaquone-resistant *T. gondii* cell lines (6). Thus, the mitochondrial electron transport must be essential for some apicomplexan life cycle stages that are found in the vertebrate host.

The most striking difference between mitochondrial of the apicomplexan and that of another of the species is its DNA. It consists of a tandemly repeated element of ~6–7 kb and encodes subunits I and III of cytochrome c oxidase, cytochrome b, and a number of short fragments representing the small and large subunit rRNAs (7–9). This makes it the shortest mitochondrial genome with the most limited coding capacity known. Most interestingly, the apicomplexan mitochondrial genome does not encode any tRNAs. The lack of apparently essential mitochondrial tRNA genes has been reported in other organisms, such as plants, some fungi, and other protozoa. It is known that, in these organisms, the lacking mitochondrial tRNA genes are compensated for by import of the corresponding cytosolic tRNAs (10). The number of tRNAs that are imported depends on the species. *Saccharomyces cerevisiae* mitochondria import a single tRNA only. Plants import a significant fraction of their mitochondrial tRNAs but still have mitochondrial-encoded ones. The most extreme situation is found in trypanosomatids (such as *Trypanosoma brucei* and *Leishmania*) (11, 12), a group of parasitic protozoa that evolutionarily are not related with the apicomplexans but whose mitochondrial genomes, like those of apicomplexans, completely lack mitochondrial tRNA genes. Whereas nothing is known about mitochondrial tRNA import in apicomplexans, the process has been studied in quite some detail in trypanosomatids. It was shown that an imported nucleus-encoded mitochondrial tRNA always represents only a small fraction (typically 1–7%) of a normal cytosolic tRNA (13). Although all mitochondrial tRNAs derive from the cytosol, a single cytosol-specific tRNA has been identified in *T. brucei*. This tRNA corresponds to the eukaryotic initiator tRNA Met (tRNA Met-i), and its cytosol-specific localization--

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* The abbreviations used are: tRNA Met-i, initiator tRNA Met; tRNA Met-e, elongator tRNA Met; LSU rRNA, large subunit ribosomal RNA; MTF, tRNA Met-formyl transferase.

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tion is mediated by two nucleotide pairs in the T-stem of the tRNA (14). Furthermore, results obtained in Leishmania suggest that cytosol-specific 2-thiouridines, which are found in the anticond wobble position of tRNA\textsubscript{Met-i}(UUC) and tRNA\textsubscript{Met-o}(UUG), may regulate the extent of import because the two tRNAs carrying these modifications were prevented from being imported in vitro (15).

The aim of the present work was to directly demonstrate tRNA import in the apicomplexan T. gondii and to determine its specificity. Furthermore, we wanted to compare the features of mitochondrial tRNA import in T. gondii to the one of trypanosomatids.

**EXPERIMENTAL PROCEDURES**

Cells—Tachyzoites of T. gondii (RH strain) were maintained in Vero cell monolayers at 37 °C and 5% CO\textsubscript{2} in Roswell Park Memorial Institute medium containing 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2–10% immunoglobulin-free horse serum. Tachyzoites were harvested from their feeder cell cultures as previously described (16). The preparation containing tachyzoites and host cell debris was washed twice in cold phosphate-buffered saline and passed through a pre-equilibrated Sephadex G-25M column. The eluted purified parasites were centrifuged (3000 x g for 5 min at 4 °C), and the resulting pellets were used either directly, to isolate total RNA and for in vitro aminocaylations, or they were subjected to subcellular fractionation.

Procylic T. brucei cells, stock 427, were grown at 27 °C in SDM-79 medium supplemented with 5% fetal bovine serum. Cells were harvested at late log phase, corresponding to 2.5 x 10\textsuperscript{7} to 3.5 x 10\textsuperscript{7} cells/ml, and washed once in cold 20 mM sodium phosphate buffer (pH 7.9) containing 150 mM NaCl and 20 mM glucose. The resulting cellular pellets were used to isolate total RNA or to prepare mitochondria.

**Subcellular Fractionation of T. gondii**—Washed T. gondii cells (2 x 10\textsuperscript{6} cells) were subjected to ~0.5 mg of protein were resuspended in 5 ml of SoTE (0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA). Five percent of the sample (25 μl) was removed to isolate the total RNA. After the addition of 0.475 ml of SoTE containing either 0.1% (0.05% final) or 0.2% (0.1% final) of digitonin, the samples were mixed by pipetting and incubated on ice for 5 min. The centrifugation step was performed (8000 x g for 5 min at 4 °C), and the supernatants were discarded. The resulting pellets were resuspended in 500 μl of SoTE containing 1 μg of RNAase A and incubated on ice for 15 min to digest the contaminating cytosolic RNAs. The organelles fractions used to isolate RNA were recovered in the pellets after a final centrifugation step.

**Subcellular Fractionation of T. brucei**—Digitonin extraction (final concentration, 0.035%) was essentially performed as described previously (14). The obtained pellet was essentially free of cytosolic RNA and protein. Because T. brucei does not have a plastid, corresponded to a crude mitochondrial fraction.

**RNA Isolation and Northern Analysis**—RNA from total cells or digitonin-treated fractions of T. gondii or T. brucei was purified by using the acidic guaniinium isothiocyanate method as previously described (17). The samples containing the isolated RNAs were separated on 8% or 15% polyacrylamide gels containing 25 μM (N-acryloylamine)phenylmercuric chloride (18).

**Hybridization Probes**—Oligonucleotides recognizing the cytosolic 5.8 S rRNA (GAGCCAGACATCATATTG) (19), the plastid tRNA\textsubscript{Met}(AACC/TCTCCA/CCTGGA/CTGCT) (20), and the mitochondrial large subunit (LSU) rRNA fragment (GACAAGGATTTCCCTACCTT) of T. gondii (21) were designed to identify mitochondrial-encoded cytochrome b mRNA. The probe to detect mitochondrial-encoded cytochrome b mRNA (6) was prepared by PCR amplification using CGAGCGAGCTGGCAGATGTAC and CAGATACGTGAGAACCTCC as primers.

Oligonucleotides hybridizing to nucleus-encoded T. gondii tRNAs were designed using sequence information from the T. gondii databases (preliminary genomic and/or cDNA sequence data were accessed via ToxoDB.org and www.tigr.org/tdb/tg/tgdb). tRNA genes were identified as BLAST using tRNA genes from other organisms as templates. The following oligonucleotides were used: tRNA\textsuperscript{Met}(TGAG/GCACATGGGATTCG), tRNA\textsuperscript{Met}(TTGAGC/GCACATGGGATTCG), tRNA\textsuperscript{Met}(CAAGCGGCGACGAGCAGCAG), tRNA\textsuperscript{Met}(TGAGCGCGACGCGACGCGACG), tRNA\textsuperscript{Met}(CTGGCAAGGAGGTACGTCG), and tRNA\textsuperscript{Met}(CTGGCAAGGAGGTACGTCG).

In Vivo Aminocaylation Assay—Washed T. gondii (5 x 10\textsuperscript{5} cells) were resuspended in 300 μl of 1 x SBB (20 mM glucose, 0.15 mM NaCl, 20 mM NaF, pH 7.9) containing 100 μg/ml cycloheximide and 20–40 μCi of \textsuperscript{[35S]methionine/\textsuperscript{[35S]cysteine mixture (1000 Ci/mmol). The labeled \textsuperscript{[35S]cysteine (25% of total) was competed out by the addition of 0.5 μM unlabeled cysteine. The reaction was incubated for 15 min at 37 °C. Intact cells were then centrifuged (3000 x g, for 3 min at 4 °C), and the RNA was isolated from the resulting pellet as described above.

**In Vitro Aminocaylation Assay**—The assays were done in 100 μl of acylation buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 8 mM MgCl\(_2\), 10 mM dithiothreitol, 10% glycerol) containing 20–40 μCi of a \textsuperscript{[35S]methionine/\textsuperscript{[35S]cysteine mixture, 0.5 μM unlabeled cysteine, 2 mM ATP, RNA isolated from 10\textsuperscript{7} T. gondii tachyzoites or from the digitonin fractions (2 x 10\textsuperscript{6} cells equivalents each), and 30–60 μg of RNA-free cytosolic protein extract of either T. gondii or T. brucei, as sources of methionyl-tRNA synthetase activity (22). To be able to test externally added substrate tRNAs, it was essential to use protein extracts that were free of endogenous RNAs. Removal of endogenous RNA from the cytosolic fractions was achieved through binding to a DEAE-Sepharose column in the presence of 150 mM NaCl (23). Incubation was for 15 min at 37 °C, and other conditions as described above.

The \textsuperscript{[35S]labeled tRNAs were either to the in vivo and the in vitro assays were analyzed on 3% urea/10% polyacrylamide gels and visualized by fluorography. To prevent deacylation of the labeled tRNAs during electrophoresis, the samples were subjected to a deamination step results in a pellet that essentially only contains mitochondrial RNA fractions of T. brucei or from the digitonin fractions and RNAs were re-isolated as described above.
extracted with 0.05 and 0.1% of digitonin and subjected to RNase digestion. The resulting RNA fractions together with total T. gondii RNA were then analyzed by specific Northern hybridizations. To identify the different organellar fractions oligonucleotide probes recognizing specific marker RNAs were used. These RNAs were the 5.8 S rRNA for the cytosol and the plastid-encoded tRNA\textsubscript{Met} for the plastid, both of which can easily be detected on Northern blots. To choose a mitochondrial marker was more difficult, because the mitochondrial genome of T. gondii, unlike that of many other apicomplexans, has not been sequenced yet. However, fragments apparently derived from the mitochondrial DNA have been detected in the nuclear genome of T. gondii (21). These sequences appear to code for typical mitochondrially encoded proteins and show a high homology to the mitochondrial genomes of other apicomplexans but are not expressed. Southern blot hybridization furthermore showed that in addition to the nucleus these sequences also occur in an extrachromosomal, tandemly repeated element of ~6 kb, suggesting that they are also present within the bona fide mitochondrial genome of T. gondii (8). We therefore chose a segment of the nuclear mitochondrial-like sequences that is identical to a region coding for a fragment of the mitochondrial LSU rRNA in Plasmodium to design an oligonucleotide expected to hybridize to mitochondrial RNA only. It has been shown that specific mutations in the cytchrome b gene lead to atovaquone-resistant T. gondii cells, indicating that mitochondrial cytchrome b is expressed (6). Thus, in addition to the putative LSU rRNA we were using the mRNA of cytosome b as a mitochondrial marker. The results of a digitonin-based organellar fractionation of T. gondii cells are shown in Fig. 1. Total RNA and RNA isolated from the RNase-treated pellet fractions obtained from 0.05 and 0.1% digitonin extractions were tested for the presence of cytosolic and organellar RNAs. The top panel shows that as expected the cytosolic marker, 5.8 S rRNA, is detected in the total RNA fraction only. The next two panels show the intracellular distribution of the mitochondrial LSU rRNA fragment and cytosome b mRNA, respectively. Both RNAs are recovered in the 0.05% digitonin fraction but are accessible to added nucleases in the presence of 0.1% of detergent. Thus, mitochondria with an intact membrane barrier are specifically recovered in the 0.05% digitonin fraction. The last panel shows that the plastid marker, the tRNA\textsubscript{Met}, remains protected from added RNase in both pellet fractions, indicating that the plastid membranes (at least the innermost), unlike the mitochondrial ones, remain intact even after the 0.1% digitonin extraction. Hence, 0.05% digitonin extraction and subsequent RNase digestion yield a fraction containing mitochondrial and plastid RNAs, which is essentially free of intact cytosolic RNAs. Extraction with 0.1% digitonin, in contrast, results in a fraction containing plastid RNAs only.

Nucleus-encoded T. gondii tRNAs Are Imported into Mitochondria—Fig. 1B shows the intracellular distribution of four nucleus-encoded T. gondii tRNAs. All were detected in the total RNA fraction as well as in the 0.05% digitonin pellet but were absent from the 0.1% pellet. These results show that, although the largest fraction of each of the tRNAs is found in the cytosol (note that the total RNA fraction essentially corresponds to the cytosol), a small but significant part is recovered in the mitochondria. Quantitatively between 1.5 and 2% of the total cellular content of each tRNA is localized within the mitochondrion (Fig. 1C). These numbers are in the same range as those for T. brucei where 1–7% of a given tRNA is localized within mitochondria (13).

Localization of T. gondii tRNAs\textsubscript{Met}—A global analysis of mitochondrial tRNA import in T. brucei has shown that, with a single exception, all trypanosomal tRNAs are in part imported into mitochondria (13). The only tRNA behaving differently is the tRNA\textsubscript{Met}\textsuperscript{Met}, which is exclusively found in the cytosol. It was therefore of great interest to analyze the intracellular localization of the nucleus-encoded T. gondii tRNAs\textsubscript{Met}. As expected BLAST searches of the T. gondii genomic sequences identified...
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In vitro aminoacylation using [35S]methionine. Aminoacylation assays were performed by using either cytosolic extract of T. brucei or cytosolic extract of T. gondii as source of methionyl-tRNA synthetase activities. T. gondii RNAs isolated from total cells (Tot.), and from 0.05% or 0.1% digitonin fractions were used as substrates. Positions of tRNA\textsubscript{Met}\textsuperscript{i} and tRNA\textsubscript{Met}\textsuperscript{e} are indicated by arrows.

In a further analysis we subjected aliquots of total and digitonin-extracted organellar RNA fractions to in vivo aminoacylation assays using radioactive [35S]methionine and either RNA-free cytosolic extract of T. brucei or T. gondii as a source of enzyme. As expected and in agreement with the in vivo results shown in Fig. 2B, for both extracts two labeled bands, corresponding to tRNA\textsubscript{Met}\textsuperscript{i} and tRNA\textsubscript{Met}\textsuperscript{e}, were detected in the total RNA fraction (Fig. 3, left lanes). In the 0.05% digitonin pellet containing the mitochondrial RNAs, only the lower band corresponding to the tRNA\textsubscript{Met}\textsuperscript{e} was observed (Fig. 3, middle lanes). Thus, these results confirm the cytosol-specific localization of the tRNA\textsubscript{Met}\textsuperscript{i}. Furthermore, they suggest that the only tRNA\textsubscript{Met} present in mitochondria of T. gondii, corresponds to the tRNA\textsubscript{Met}\textsuperscript{e} identified in the genomic database. The methionyl-tRNA synthetase activity in the T. gondii cytosolic extract was lower than the one in the T. brucei extract. This is explained by the fact that it was difficult to prepare a sufficiently concentrated RNA-free cytosolic fraction from T. gondii due to the limited amount of material that was available. However, this cannot account for the fact that the T. gondii methionyl-tRNA synthetase preferentially charges the tRNA\textsubscript{Met}\textsuperscript{e} when tested in vitro (Fig. 3, bottom panel left lane), whereas the T. brucei extract preferentially charges the tRNA\textsubscript{Met}\textsuperscript{i} (Fig. 3, top panel left lane).

There are at least two possible explanations for this observation. It could be that, although the methionyl-tRNA synthetase activities in T. gondii and T. brucei can in principle charge both T. gondii tRNAs\textsubscript{Met}, their preferred substrate tRNAs\textsubscript{Met} are different. Alternatively, it is possible that the T. brucei extract aminoacylates (with methionine) a T. gondii tRNA species other than tRNA\textsubscript{Met}.

Expression of T. gondii tRNAs\textsubscript{Met} in T. brucei—Expression of tRNAs\textsubscript{Met} variants in transgenic T. brucei has successfully been used to identify the determinants responsible for their intracellular localization (14). In principle the same approach should be feasible for T. gondii. However, despite repeated attempts, we were not able to express tRNAs\textsubscript{Met} variants in transgenic T. gondii. We therefore took the converse approach and expressed T. gondii tRNAs\textsubscript{Met} in T. brucei. However, this was also problematic, because, of the many variants of T. gondii tRNA\textsubscript{Met} that were assessed, only the wild-type tRNA\textsubscript{Met}\textsuperscript{i} and a variant thereof carrying the T-arm of the tRNA\textsubscript{Met}\textsuperscript{e} could be expressed. This was surprising, because we have previously successfully expressed many variants of trypanosomal (14) as well as heterologous tRNAs (26), and this suggests that for unknown reasons expression of most T. gondii tRNA\textsubscript{Met} variants is harmful for T. brucei. The results of the two T. gondii tRNAs\textsubscript{Met} that could be expressed are shown in

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**Fig. 2.** Expression and intracellular localization of tRNAs\textsubscript{Met}.

A, predicted secondary structures of T. gondii tRNA\textsubscript{Met}\textsuperscript{i} and tRNA\textsubscript{Met}\textsuperscript{e}. Nucleotides that are different between the two molecules are shown in gray in the tRNA\textsubscript{Met}\textsuperscript{i}. B, two tRNAs\textsubscript{Met} are expressed in vivo. Left panel: in vivo aminoacylation assays using [35S]methionine. Right panel: Northern blot containing total cellular RNAs probed for the predicted tRNA\textsubscript{Met}\textsuperscript{i} and tRNA\textsubscript{Met}\textsuperscript{e}, respectively. The left and the right parts of this panel represent two stripes from the same preparative lane. C, intracellular localization of T. gondii tRNAs\textsubscript{Met}. RNA isolated from total cells (Tot.), and from the 0.05% or 0.1% digitonin fractions were probed for tRNA\textsubscript{Met}\textsuperscript{i} and tRNA\textsubscript{Met}\textsuperscript{e}, respectively. Loadings are the same as in Fig. 1A and 1B. D, quantification of import using the hybridization signals from Fig. 2C. Calculations were done as for Fig. 1C.
tRNA\text{Met-i} and the imported wild-type tRNA\text{Met-e} of tRNA\text{Glu} and tRNA\text{Gln} and which act as anti-import determinants (15). To see whether this might also be the case in T. gondii, we analyzed the localization determinants.

DISCUSSION

The mitochondrial genome of apicomplexans lacks tRNA genes (7). It has therefore been postulated that these organisms import all their tRNAs from the cytosol. Using defined organellar RNA fractions, obtained by a newly established fractionation procedure (Fig. 1A), we have tested this prediction for the apicomplexan parasite T. gondii. Six out of six nucleus-encoded elongator-type tRNAs (tRNA\text{Ala}, tRNA\text{Ile}, tRNA\text{Ser}, tRNA\text{Trp}, tRNA\text{Gln}, and tRNA\text{Met-i}) tested were in part recovered in the mitochondrial fraction (Figs. 1B, 2C, and 5) and therefore imported from the cytosol. This suggests that in T. gondii a small fraction of any given cytosolic elongator-type tRNA is imported into mitochondria. Interestingly, however, the nucleus-encoded eukaryotic-type tRNA\text{Met-i} only occurs in the cytosol. tRNA\text{Met-i} and tRNA\text{Met-e} differ only by 31 nucleotides (Fig. 2A) indicating that these differences or a subset thereof must be responsible for their differential localization. This situation is reminiscent of the one in the trypanosomatid T. brucei, which also imports all mitochondrial tRNAs (13). Furthermore, the only trypanosomal cytosol-specific tRNA, just as in T. gondii, corresponds to the cytosolic tRNA\text{Met-i}. It differs from the imported tRNA\text{Met-e} by 26 nucleotides, and a recent study (14) has shown that two nucleotide pairs in the T-stem region are both necessary and sufficient to specify the localization of the trypanosomal tRNAs\text{Met-i}.

Imported tRNAs are always of the eukaryotic type (10). Thus, we have the extraordinary situation in T. gondii and
The import of tRNAs into mitochondria of a bacterial-type translation system that has to function with imported eukaryotic type tRNAs only. Although most eukaryotic type tRNAs can be expected to function in the context of a bacterial type translation system, this is not the case for tRNAsMet. All organisms have two classes of tRNAsMet, an tRNAsMet-i, which is used for initiation of protein synthesis, and a tRNAsMet-e, which functions in the insertion of methionine into internal peptidic linkages. Whereas elongator tRNAsMet-e of all organisms look similar, there are two distinct groups of tRNAsMet-i. The tRNAsMet-i of the eukaryotic cytosol carries an A1:T72 base pair, which is not found in any other tRNA. Bacterial and organellar tRNAsMet-i, on the other hand, are characterized by a mismatch at the top of the acceptor stem and carry a formylated methionine (28, 29). Thus, it makes sense that the only cytosol-specific tRNA detected in T. gondii as well as T. brucei corresponds to the eukaryotic-type tRNAsMet-i, because this tRNA could not possibly function in the context of the bacterial type translation systems of mitochondria. However, the fact that the imported tRNAsMet-e appears to be the only tRNAsMet present in mitochondria raises the question of how mitochondrial translation initiation in apicomplexans and trypanosomatids, known to require a formylated bacterial type tRNAsMet-i, can function with an imported tRNAsMet-e. Mitochondrial translation initiation has been studied in detail in T. brucei, and it was shown that a fraction of the imported tRNAsMet-i becomes formylated after import into mitochondria and subsequently can act in translation initiation (22, 30). Formylation of a tRNAsMet-i is very unusual and requires a distinct tRNAsMet formyl-transferase (MTF). The enzyme has been identified in T. brucei and was shown to have a diametrically opposed substrate specificity compared with all other known bacterial and organellar MTFs, in that it selectively recognizes tRNAsMet-e. Furthermore, the trypanosomal protein is approximately twice the size of all other MTFs (22).

Do apicomplexan mitochondria also use formylated tRNAsMet-e to initiate mitochondrial translation? Due to the limited amount of mitochondrial RNA available it was not possible to analyze the formylation state of the imported tRNAsMet-i in T. gondii. However, BLAST searches of the two complete and annotated genomes of P. falciparum and Plasmodium yoelii show that these two apicomplexans possess a single MTF, each of which has approximately twice the size of MTFs from other organisms. Preliminary analysis of the as yet unannotated genome suggests that also T. gondii has an unusually large MTF. Thus, it is possible that, just as in T. brucei, the apicomplexan MTF formylates imported tRNAsMet-i and that the large molecular weight of the proteins is linked to their unusual substrate specificity. However, the problem with this explanation is that apicomplexans appear to have a single MTF gene only. This is unexpected, because both mitochondria and the plastid are expected to require formylated tRNAsMet for organellar translation initiation. It is therefore possible that the identified apicomplexan MTF genes encode the plastid enzymes or that the proteins are targeted to both organelles. Examples for dual targeting of proteins to the plastid and to mitochondria have been described before (31). Finally, taking into account that only three proteins are synthesized in the mitochondria of apicomplexans (8), it is also conceivable that the need for a formylated tRNAsMet for translation initiation has been bypassed altogether. Thus, the question of which tRNA is used for mitochondrial translation initiation at present remains open.

In summary, we present for the first time experimental evidence for mitochondrial tRNA import in apicomplexans and show that the specificity of the process is identical to the one in trypanosomatids. Apicomplexans and trypanosomatids are not closely related, which raises the question, whether the uncovered similarities of the two systems are due to a remote common ancestor or arose independently through convergent evolution. Answering this question will require a detailed knowledge of the tRNA import mechanisms in both organisms.

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