Plasmodium Apicoplast Gln-tRNA\textsuperscript{Gln} Biosynthesis Utilizes a Unique GatAB Amidotransferase Essential for Erythrocytic Stage Parasites*

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Background: Plasmodium apicoplast protein synthesis is essential for parasite survival, yet few of the enzymes involved have been biochemically characterized.

Results: Nucleus-encoded apicoplast GatA glutamyl-tRNA amidotransferase forms Gln-tRNA\textsuperscript{Gln} in concert with a non-discriminating glutamyl-tRNA synthetase.

Conclusion: Formation of apicoplast Gln-tRNA\textsuperscript{Gln} is via indirect aminoacylation.

Significance: The apicoplast indirect aminoacylation pathway is a potential drug target.

The malaria parasite Plasmodium falciparum apicoplast indirect aminoacylation pathway utilizes a non-discriminating glutamyl-tRNA synthetase to synthesize Glu-tRNA\textsuperscript{Gln} and a glutamyl-tRNA amidotransferase to convert Glu-tRNA\textsuperscript{Gln} to Gln-tRNA\textsuperscript{Gln}. Here, we show that Plasmodium falciparum and other apicomplexans possess a unique heterodimeric glutamyl-tRNA amidotransferase consisting of GatA and GatB subunits (GatAB). We localized the P. falciparum GatA and GatB subunits to the apicoplast in blood stage parasites and demonstrated that recombinant GatAB converts Glu-tRNA\textsuperscript{Gln} to Gln-tRNA\textsuperscript{Gln} \textit{in vitro}. We demonstrate that the apicoplast GatAB-catalyzed reaction is essential to the parasite blood stages because we could not delete the Plasmodium berghei gene encoding GatA in blood stage parasites \textit{in vivo}. A phylogenetic analysis placed the split between Plasmodium GatB, archaeal GatE, and bacterial GatB pre-phylogenetic to the phylogenetic divide between bacteria and archaea. Moreover, Plasmodium GatA also appears to have emerged prior to the bacterial-archaeal phylogenetic divide. Thus, although GatB is found in Plasmodium, it emerged prior to the phylogenetic separation of archaea and bacteria.

The human malaria parasite Plasmodium falciparum is responsible for 124–283 million cases of malaria and an estimated 0.6 million deaths every year (1). It contains a relict plastid, the remnant of an ancient secondary endosymbiotic event in which the eukaryotic progenitor of the malaria parasite engulfed a photosynthetic eukaryote known as the apicoplast (2). The Plasmodium apicoplast possesses a 35-kb circular genome with 60 genes (3) that encode components of the apicoplast transcriptional and translational apparatus such as RNA polymerase subunits, the elongation factor EF-Tu, several ribosomal proteins, rRNAs, and tRNAs (4–8), as well as the SufB protein thought to play a role in FeS cluster formation (9). Most apicoplast proteins, however, are encoded by the nuclear genome and are imported into the organelle post-translationally (10). Over 500 apicoplast-targeted proteins were identified in P. falciparum (11, 12), revealing apicoplast biosynthetic pathways for fatty acids (13, 14), isoprenoid precursors (15), and heme (16), as well as enzymes for tRNA modification (12) and lipoylation (17). Several of these pathways exhibit prokaryote-like features and are potential drug targets (12, 15, 18). Recent studies have shown that apicoplast isoprenoid precursor biosynthesis is essential in P. falciparum asexual stages (19), indicating that the pathway cannot be bypassed by salvaging lipids from the host and may be a good drug target in asexual stages. The type II fatty acid and heme biosynthetic pathways, however, are not essential in the asexual stages (18), and although not good targets for asexual stage chemotherapy, they may prove to be valuable drug targets in liver stages (2).

Translational accuracy is required to properly decipher the genetic code during protein synthesis. The fidelity of protein synthesis largely depends on the formation of correct aminoacyl-tRNAs by aminoacyl-tRNA synthetases (aaRSs).\textsuperscript{2} In the classic model, each species of aaRS strictly discriminates one amino acid from among the 20 canonical amino acids, as well as

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\textsuperscript{2}The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; ACP, acyl-carrier protein; Glu-AdT, glutamyl-tRNA amidotransferase; GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; Pb, Plasmodium berghei; Pf, Plasmodium falciparum; ND, non-discriminating; AdT, amidotransferase; AsnRS, asparaginyl-tRNA synthetase; AspRS, aspartyl-tRNA synthetase; Ni-NTA, nickel-nitrilotriacetic acid.

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its cognate tRNA isoacceptor from the non-cognate tRNAs. However, genomic and biochemical analyses have revealed that the full complement of 20 aaRSs is used only in the eukaryotic cytoplasm and a minority of bacteria, whereas a majority of bacterial and archaeal genomes lack genes encoding glutamyl-tRNA synthetase (GlnRS) and or asparaginyl-tRNA synthetase (AsnRS) (20). In these organisms, Gln-tRNA<sub>Gln</sub> and/or Asn-tRNA<sub>Asn</sub> are synthesized via an indirect pathway (21). In most bacteria and archaea lacking GlnRS, tRNA<sub>Gln</sub> is first misaminoacylated with Glu in a reaction catalyzed by a non-discriminating GluRS (ND-GluRS) that can glutamylate both tRNA<sub>Glu</sub> and tRNA<sub>Gln</sub> (see Reactions 1 and 2).

\[
\text{L-glutamate} + \text{ATP} + \text{tRNA}^\text{Gln} \rightleftharpoons \text{Glu-tRNA}^\text{Gln} + \text{AMP} + \text{PP}_i
\]

Reaction 1

\[
\text{L-glutamine} + \text{ATP} + \text{Glu-tRNA}^\text{Gln} \rightleftharpoons \text{Gln-tRNA}^\text{Gln}
\]

\[+ \text{L-glutamate} + \text{ADP} + \text{P}_i\]

Reaction 2

The glutamyl residue of Glu-tRNA<sub>Gln</sub> is then transamidated by a glutamyl-tRNA<sub>Gln</sub> amidotransferase (Glu-AdT) in the presence of ATP using Gln as an amide donor, producing Glu-tRNA<sub>Gln</sub>. Similarly, in the case of Asn-tRNA<sub>Asn</sub> formation in organisms lacking AsnRS, Asn-tRNA<sub>Asn</sub> is synthesized by a non-discriminating aspartyl-tRNA synthetase and an aspartyl-tRNA<sub>Asn</sub> amidotransferase (Asp-AdT). Two types of tRNA-dependent amidotransferases are known as follows: the heterotrimeric GatCAB (22) and the heterodimeric GatDE (20). Bacterial GatCAB functions as a Glu-AdT or an Asp-AdT in a species-specific manner. In some bacteria lacking both AsnRS and GlnRS, GatCAB acts as both a Glu-AdT and an Asp-AdT (21). As each subunit of GatCAB is encoded only in archaeal genomes lacking a gene for AsnRS, archaeal GatCAB seems to function as an Asp-AdT. GatDE is only found in archaea and functions as a Glu-AdT (21).

Aminoacyl-tRNA formation is essential for protein synthesis. Despite the central importance of this process in all living organisms, it remains unknown how Plasmodium synthesizes Gln-tRNA<sub>Gln</sub> in the apicoplast. The Plasmodium apicoplast genome does not encode any tRNA synthetases, and the nuclear genome does not contain an apicoplast-targeted GlnRS. We recently reported that a nucleus-encoded non-discriminating GluRS that is imported into the apicoplast is responsible for the formation of misacylated Glu-tRNA<sub>Gln</sub> and is essential in the erythrocytic stages (23). In this study, we aimed to further clarify the formation of Gln-tRNA<sub>Gln</sub> in the Plasmodium apicoplast by a unique Plasmodium signature protein GatAB.

**Experimental Procedures**

**Bioinformatics**—A list of putative apicoplast-targeted proteins conserved in *P. falciparum*, *Theileria parva*, and *Toxoplasma gondii* was obtained from the supplementary information in Gardner et al. (24). Nucleotide or amino acid sequences of *Plasmodium* genes or proteins (11, 25) and those from other species were obtained from PlasmoDB (26), the Wellcome Trust Sanger Institute GeneDB website, or UniProt (27).

**Homology Modeling**—The structure of *Plasmodium* GatA and GatB was modeled according to the crystal structures of *Staphylococcus aureus* GatCAB (2g5h and 3ip4) (28, 29) using the Swiss-Model automated comparative protein-modeling server (30). In the modeled range, the sequence identity of PfGatA and 2g5h was 32.2%, and the sequence identity of PfGatB and 3ip4 was 24.8%. No suitable structural template could be found for amino acid regions 1–180, 425–539 and 708–744 in PfGatA and amino acid regions 1–350 and 461–530 in PfGatB. The spatial arrangement of PfGatA and PfGatB was determined based on the crystal structures of *S. aureus* GatCAB. Attempts to model GatA and GatB according to the crystal structure of *Pyrococcus abyssi* archaeal GatDE (1zq1) (31) resulted in unusable models. All figures were prepared with Chimera (32).

**Phylogenetic Analysis**—The phylogenetic trees were constructed using the tools provided on line (33). The “one-click” mode was used, employing ClustalW (34) for sequence alignment, and Gblocks (35, 36), PhyML (36), and TreeDyn (37) for curation of the multiple sequence alignment, tree construction, and rendering, respectively. The final tree was constructed using 100 bootstraps. P. falciparum Cell Culture, Plasmid Constructs, and Parasite Transfection—*P. falciparum* clone 3D7 parasites (MRA-102, MR4, BEI Resources, Manassas, VA) were grown in human O+ red blood cells at 4% hematocrit in RPMI 1640 medium supplemented with Albumax (Life Technologies, Inc.) to a final concentration of 0.5% and gassed with 5% CO<sub>2</sub> and 0.5% O<sub>2</sub> in N<sub>2</sub> at 37 °C as described previously (38). To generate an episomal transfection vector, the bipartite apicoplast targeting sequences of *PfGatA* (amino acids 1–102) and *PfGatB* (amino acids 1–98) were amplified from *P. falciparum* 3D7 genomic DNA and cloned into the BglII and AvrII sites of a pCHD-GFP vector (39).

**Subcellular Localization**—Subcellular localization of cloned *P. falciparum* transfectants expressing GFP-tagged *PfGatA*, *PfGatB*, or Myc-tagged *PbGatA* was performed as outlined in Ref. 42. Double staining was performed using a rabbit polyclonal anti-acyl carrier protein (ACP) primary antibody (diluted 1:500) (10) as an apicoplast marker and a mouse monoclonal anti-Myc antibody (Santa Cruz Biotechnology, diluted 1:500) to detect PbGluRS-Myc. Fluorescent staining was achieved using Alexa Fluor-conjugated secondary antibodies (Invitrogen) specific to rabbit (Alexa Fluor 594, red) or mouse (Alexa Fluor 488, green) IgGs. DAPI was used to stain nucleic acids, and the mitochondrion was stained by incubating the parasites in culture media for 30 min with 20 nM MitoTracker Red (Invitrogen) and fixing the cells as outlined above. Images were acquired using an Olympus Delta Vision imaging system.
Myc Tagging and Attempted Deletion of the Endogenous Plasmodium berghei GatA Gene—A 4 x myc tag was appended to the 3’ end of the gene encoding the putative apicoplast-targeted P. berghei GatA (PlasmoDB ID PBANKA_071810) as described previously (18). A 3.5-kb fragment of the 3’ end of the gene without the stop codon was amplified from P. berghei ANKA genomic DNA using primers PbGatA_F2 (TACCGCGGATA-TATACAACCAATACATTATAG) and PbGatA_R (ATA-CTAGTACTAGCCTTTATTTCCAAATTGTGAAC); the SacII and SpeI restriction sites are underlined. Polymerase chain reactions (PCR) (50 μl) contained 50 ng of genomic DNA, 0.1 μM of each primer, 5 μl of buffer, and 1 μl of Advantage 2 polymerase (Clontech). The PCR product was digested with SacII and SpeI and cloned into the B3D KO Red vector. The strategy described previously (42) was used to attempt deletion of the PfGatA gene via double-crossover recombination. The primers used to amplify the genomic regions were as follows: PbGatA_KO.3For (GCCCCCCGCTATGTTGTTTAAAA-GTTGCC) and PbGatA_KO.3Rev (AGTCTCATTGCGCCCC- AAATTTAAGCATACAGAAAGTGAC); PbGatA_R (ATA-CTGCTTATTTACC). Primers 1 and 2 were designed to amplify a 942 bp fragment containing the first 62 bp of the PfGatA coding sequence and 840 bp of the 3’-UTR. Primers 3 and 4 were designed to amplify a 734-nucleotide fragment containing the first 54 bp of the PfGatA coding sequence and 840 bp of the 3’-UTR. Primer 1 contained an added 5’-terminal GC dinucleotide and a SacII site. Primer 2 contained an added 5’-terminal GC dinucleotide and a SacII site. Primers 3 and 4 contained an ApaI site flanked by complementary sequences (underlined) for recombinatorial PCR. The two genomic fragments were first amplified in separate reactions using the cycling parameters described above, and the resulting products were combined in a second PCR to form a single product, which was digested with SacII cloned into the B3D KO Red vector. This construct was linearized with ApaI, and P. berghei ANKA parasites were transfected as described (43). The transfection experiments were performed in duplicate and repeated once.

Expression and Purification of the Mature PfGluRS, PfGatA, and PfGatB—PfGluRS, PfGatA, and PfGatB were expressed in Escherichia coli KRX cells (Promega) containing the pRARE2 plasmid (EMD4BioSciences) and the pET-29a vector encoding either the predicted mature PfGluRS coding sequence (PFD7_1357200, amino acids 78–574) (23), PfGatA (PFD7_0416100, amino acids 101–827), and PfGatB (PFD7_0628800, amino acids 96–884). PfGluRS was codon-optimized for wheat (23) and PfGatA and PfGatB for E. coli (GeneArt, Inc.). All constructs included N-terminal His6 tags.

Preparation and Aminoacylation of tRNA Substrates—Synthetic genes encoding P. falciparum apicoplast tRNA Glu and tRNA Glu (both from GenBank TM accession number X95276) were expressed and purified from E. coli and 32P-labeled on their 3’-OH termini using the E. coli CCA-adding enzyme as described (23). The aminoacylation assay using recombinant PfGluRS was performed and quantified as described (23). Glutamylated tRNAs were phenol (Tris-buffered, pH 7.9)/chloroform-extracted, and unincorporated [α-32P]ATP was removed using Bio-Spin 30 columns (Bio-Rad).

Amidotransferase Assay—Transamidation assays were carried out in 1 x AdT buffer (100 mM Hepes-KOH, pH 7.2, 30 mM KCl, 12 mM MgCl2, and 5 mM DTT) with 2.6 mM 1-glutamine, 4 mM ATP, 500 nM 32P-labeled Glu-tRNA Glu, and 50 nM each of GatA and GatB. Reactions were carried out at 37 °C for 5 min. Aliquots (4 μl) were quenched on ice with 4 μl of 100 mM sodium citrate, pH 4.7, and 0.66 mg/ml of nuclease P1 (Sigma) and incubated at room temperature for 35 min. To separate glutamyl-AMP (Gln-AMP) from Glu-AMP and AMP, 2.0 μl of the digested samples were separated on 20 x 20-cm PEI cellulose TLC plates. The plates were then developed in 100 mM ammonium acetate, 5% acetic acid and air-dried. Spot positions and intensities were measured by phosphorimaging, as described (44). To test whether PfGluRS and PfGluAdT together could sequentially form Gln-tRNA Glu from precursors in a single reaction, we initiated some reactions by adding pre-mixed PfGluRS, PfGatA, and GatB (50 nm each) in the presence of 2.6 mM 1-Glu and 1-Gln at 37 °C for 5 min and quantified as described above. To test whether PfGatAB could use an alternative amide donor, 2.6 mM L-asparagine was used instead of 1-glutamine.

Results

Subunit Composition of the Apicoplast Glu-tRNA Glu Amidotransferase—The formation of aminoacyl-tRNAs is a crucial step in protein synthesis. Despite the central importance of this process in all living organisms, it has been unclear how Plasmodium synthesizes Gln-tRNA Glu in the apicoplast. We recently reported that Plasmodium apicoplast glutamyl-tRNA synthetase is a non-discriminating enzyme that forms both Glu-tRNA Glu and Glu-tRNA Glu and is essential in erythrocytic stages of the parasite life cycle (23). Amidation of Glu-tRNA Glu to Gln-tRNA Glu requires a tRNA-dependent amidotransferase (AdT). The P. falciparum genome contains two single-exon genes that encode putative orthologs of the GatA and GatB subunits of the bacterial glutamyl-tRNA amidotransferase (Glu-AdT) as follows: GatA (PFD7_0416100, 96 kDa, 826 amino acids) and GatB (PFD7_0628800, 102 kDa, 882 amino acids) (Table 1). Both P. falciparum proteins possess predicted N-terminal bipartite apicoplast targeting sequences, suggesting that they are the subunits of an amidotransferase that participates in an apicoplast indirect aminoacylation pathway. Orthologs of PfGatA and PfGatB are conserved in apicomplexans that possess an apicoplast (e.g. Theileria (24, 45)) but not in apicomplexans that lack an apicoplast (e.g. Cryptosporidium)
**TABLE 1**

Components of the apicoplast indirect aminoacylation pathway in *P. falciparum* and *P. berghei*

The PlasmoDB identifiers for the nucleus-encoded proteins are indicated.

| Component | *P. falciparum* | *P. berghei* |
|-----------|----------------|-------------|
| tRNA^Glu | Apicoplast* | Apicoplast* |
| tRNA^Gln | Apicoplast* | Apicoplast* |
| GluRS | PF3D7_1357200 | PBANKA_112350 |
| Glu-ATT GatA subunit | PF3D7_0416100 | PBANKA_071810 |
| Glu-ATT GatB subunit | PF3D7_0628800 | PBANKA_112750 |

*S GenBank accession number X95276 (3).
*The *P. berghei* apicoplast genome sequence was obtained from the Wellcome Trust Sanger Institute FTP site.

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**P. falciparum Apicoplast Glutamyl-tRNA Amidotransferase**

**Figure 1.** PfGatAB tertiary structure reveals conserved functional features. To predict the tertiary structure and identify conserved and divergent features of PfGatAB, homology modeling was performed using the *S. aureus* GatCAB crystal structure as a template (28, 29).

Bacterial GatCAB includes three subunits as follows: the glutaminase GatA; the transamidase GatB; and GatC, which is a small protein (12 kDa) that appears to perform the role of a structural stabilizer at the interface between the GatA and GatB subunits (28, 29). GatB contains a transamidase domain called the "cradle" and a helical domain that binds to tRNA.

**Plasmodium** GatB (Fig. 1) includes an N-terminal apicoplast targeting sequence (residues 1–183), two unique inserts (residues 184–362 and 461–524) of unknown functions, and two domains typical for bacterial GatB, the cradle domain (residues 350–460 and 526–700), and a helical domain (residues 704–880). Instead of GatB, archaea use GatE, which contains an AspRS-like insertion in its cradle domain (31). No similarity could be found between the *Plasmodium* and archaeal insertions. Furthermore, we could not find a structural template for the *Plasmodium* insertions, which is the reason why they were not modeled. Most of the amino acids that are important for substrate recognition, Mg^2+ /Mn^2+ coordination, and the ammonia channel are either conserved in PfGatB, bacterial GatB (28, 29), and archaeal GatE (31) proteins or replaced by conservative substitutions (Fig. 1).

In *S. aureus* GatB (28, 29), the conserved residues His^{12}, Glu^{124}, and Glu^{550} and three water molecules coordinate the Mg^{2+} ion; the equivalent residues in PfGatB are Glu^{591} and Glu^{595}. The corresponding residue to His^{12} of *S. aureus* is probably situated in the histidine-rich unmodeled N-terminal region of PfGatB. Most likely residues Asp^{579} and Glu^{595} are part of the second transient binding site of Mg^{2+}/Mn^{2+} ions; the corresponding residues in *S. aureus* GatB are Glu^{10}, Asp^{192}, and Glu^{210} (28, 29). Furthermore, all residues forming the ADP-binding site are conserved or functionally replaced in *Plasmodium*. In *P. falciparum*, the phosphate moiety could interact with the conserved residues Asn^{581}, Ser^{583}, and Arg^{595}, which is also an arginine in archaeal GatE (Arg^{239}, *P. abyssi*) (31) but a lysine in bacterial GatB (*Lys^{208}, S. aureus*) (28, 29). The hydrophobic adenosine binding pocket is less conserved between the species; in *S. aureus* it is formed by Val^{152}, Pro^{155}, and Phe^{205} (28, 29) and in *P. abyssi* by Ser^{191}, Pro^{194}, and Gly^{236} (31). The corresponding residues in *Plasmodium* are Val^{341} and two charged residues Lys^{544} and Lys^{590}. However, the lysine residues interact with their hydrophobic side chains as well as with the adenosine, so that the plasmodial adenosine binding pocket is also hydrophobic. Inspection of the different GatB structures revealed that the C-terminal region in PfGatB (amino acids 805–880, Fig. 1) is highly mobile and could be involved in tRNA recognition, as seen in bacterial GatBs (28, 29).

Bacterial GatA consists of a single domain (28, 29), which is homologous to other amidases in its catalytic core (22, 28, 29, 48), whereas archaeal GatD consists of three domains as follows: an N-terminal domain and two AnsA-like domains (31). The predicted PfGatA (Fig. 1) contains an apicoplast targeting sequence (amino acids 1–110) and two inserts of unknown function, which are 115 and 37 amino acids long and found only in *Plasmodium* (residues 425–539 and 708–744, pale orange) or for the insert in PfGatB (461–525, pale green). Furthermore, no template was found for the first 180 N-terminal residues in PfGatA and the first 349 N-terminal residues in PfGatB.
anion hole (28, 29) that stabilizes the tetrahedral covalent inter-
mediate (Thr343, Gly344, Gly345, and Ser346) are conserved in
PfGatA (Fig. 1). In contrast to PfGatB, which has only 21%
sequence identity to a potential human analog, PfGatA exhib-
ited 40% identity to the human homolog, primarily due to the
conservation of residues in the vicinity of the glutaminase
domain (Fig. 1).

Nakamura et al. (28) propose that a hydrophilic tunnel chan-
nels NH3 from the glutaminase site in GatA to the transamidase
site of GatB. Almost all residues that line this tunnel in
S. aureus
GatCAB (21 residues) (28) are strictly conserved in
Plasmo-
dium (17 residues), including a conserved Thr (343PfGatA and
175
S. aureus
, GatA) at the entrance and a conserved Lys (410PfGatB
and 79
S. aureus
, GatB) at the tunnel exit.

GatC, a small protein, is the third subunit in bacterial Glu-
AdTs (28, 29) and performs a structural stabilizer role at the
interface between the GatA and GatB subunits. A homolog of
the gatC
gene could not be identified in the
P. falciparum
genome, but components of the insertions found in GatA (res-
ides 425–539 and 708–744) and in GatB (residues 1–350 and
461–525) could perform the task of bacterial GatC. This
hypothesis is supported by the presence of two conserved resi-
dues (Asn52, S. aureus
, and Arg268, S. aureus
), which form hydrogen
bonds between the bacterial GatC and GatB subunits are also
present in PfGatB (Asn863, PfGatB and Asn669, PfGatB) (Fig. 1).

Phylogenetic Analysis of PfGatB and PfGatA—To determine
the evolutionary origin of plasmodial GatB and GatA proteins,
we constructed phylogenetic trees with bacterial GatB and
archaeal GatE proteins and with bacterial GatA and archaeal
GatD proteins, respectively. If
P. falciparum
GatB or GatA
evolved from either the bacterial GatB/GatA or archaeal GatE/
GatD lineages, one would expect
P. falciparum
GatB/GatA to
appear in either the GatB/GatA or GatE/GatD clades, much like
GlnRS enzymes grouping with the eukaryotic glutamyl-tRNA
synthetases (GluRS) (50, 51). Instead, our phylogenetic trees
had three branches representing three distinct subfamilies with
Plasmodium GatA placed on a separate branch away from bacterial
GatA, archaeb GatD, or the plastid GatA clades. Scale bar, 0.9 changes/site.


**FIGURE 3. PfGatA and PfGatB localize to the apicoplast.** Transgenic PfGatA-GFP and PfGatB-GFP parasites were generated in which an episomal construct contained the bipartite apicoplast targeting sequences from PfGatA and PfGatB were cloned in front of a GFP, and the expression was controlled by the *P. falciparum* calmodulin S′ promoter (35). The transfection experiments were performed in duplicate and repeated once. Differential interference contrast and fluorescent images were captured and processed using deconvolution microscopy; a merge of the images is presented on the far right column (overlay). A and B, PfGatA-GFP and PfGatB-GFP apicoplast localization was monitored via immunofluorescence assay using an anti-GFP antibody (green), and the apicoplast was detected by staining it with anti-ACP antibody (red). Nucleic acid was stained with DAPI (blue). PfGatA-GFP and PfGatB-GFP form characteristically small and round compartments early in the infection cycle (A, panel i, and B, panel i), which then elongate and develop into complex and multiply branched forms at the trophozoite stage prior to splitting into individual spots, one for each merozoite, in the schizont stage (A, panel ii, and B, panel ii). PfGatA-GFP and PfGatB-GFP co-localized with ACP (α-GFP/α-ACP overlay), confirming localization to the plastid. C and D, PfGatA-GFP and PfGatB-GFP do not localize to the mitochondrion in erythrocytic stages. The mitochondrion was labeled using MitoTracker Red, whereas PfGatA-GFP and PfGatB-GFP were detected via immunofluorescence assay using α-GFP antibody (green). Nucleic acid was stained with DAPI. Panels Cii and Dii show rings and early trophozoites, respectively, expressing PfGatA-GFP. In rings (C, panel i), the mitochondrion and apicoplast are clearly distinct organelles that enlarge and elongate during trophozoite development (C, panel ii). Panels Di and Dii show early and late schizonts, respectively. Nuclear division is underway in early schizonts (panel Dii), and the apicoplast and mitochondrion are beginning to elongate. In late schizonts (panel Dii), daughter merozoites have formed, and the apicoplast (green) and the mitochondrion (red) have segregated to daughter merozoites. (53); the close affinity of *Plasmodium vivax* with *Plasmodium cynomolgi* and *Plasmodium knowlesi*, each with bootstrap values > 0.79; and the separate clustering of *P. falciparum* from the clades containing the rodent plasmodial parasites or the clade that contains *P. vivax* (53, 54). Furthermore, the GatA and GatB sequences from two other Apicomplexa, the piroplasms *Theileria* (24, 45) and *Babesia* (55), which like *Plasmodium* lack *gatC* or *gatF* in their nuclear genomes, were placed in the same clade as the *Plasmodium* spp. (Fig. 2, A and B), indicating that the placement of *Plasmodium* GatA and GatB proteins on a separate branch was unlikely to be an artifact.

These results indicate that *Plasmodium* GatA and GatB belong to subfamilies that are distinctly different from those of known GatA, GatB, GatD, or GatE subunits. These findings imply that *Plasmodium* GatA and GatB co-evolved and that *Plasmodium* GatAB is a paralog of GatCAB, GatFAB, and GatDE.

**Subcellular Localization of PfGatA and PfGatB by Tagging the Bipartite Apicoplast Targeting Sequence—**The *P. falciparum* GatA (PF3D7_0416100) and GatB (PF3D7_0628800) contain a predicted apicoplast bipartite targeting sequence (11, 25, 56), but their subcellular localization has never been established experimentally. To determine whether the enzymes are targeted to the apicoplast, *P. falciparum* parasites were transfected with episomal constructs in which the predicted apicoplast targeting sequences of either PfGatA and PfGatB were fused to the N terminus of green fluorescent protein (GFP) and expressed in transfected parasites under the control of the *P. falciparum* calmodulin promoter (39). Fixed blood stage parasites expressing PfGatA-GFP or PfGatB-GFP were stained with anti-GFP and anti-ACP antibodies (14) to detect the fusion proteins and mark the apicoplast, respectively. Deconvolution fluorescence microscopic examination revealed distinct subcellular GFP localization for all constructs. As with other cell lines expressing GFP in the apicoplast (39), PfGatA-GFP and PfGatB-GFP were immunolocalized within a characteristically small and round compartment in ring stage parasites (Fig. 3, A, panel i, and B, panel i), which then elongated and developed into a complex branched form at the trophozoite stage (Fig. 3, A, panel ii, and B, panel ii) prior to splitting into numerous individual structures in schizonts, one for each daughter merozoite. Furthermore, the anti-GFP and anti-ACP signals were colocalized, confirming that the GatA- and GatB-GFP fusion proteins were present in the apicoplast.

To investigate potential dual localization to the mitochondrion, the same transgenic PfGatA- and PfGatB-GFP *P. falciparum* clones were incubated with MitoTracker Red and then fixed, stained with anti-GFP monoclonal antibody, and examined via deconvolution fluorescence microscopy. In ring stages transfected with PfGatA-GFP, the anti-GFP antibody and MitoTracker Red marked closely apposed organelles that were clearly distinct (Fig. 3C, panel i) and which began to enlarge in early trophozoites (Fig. 3C, panel ii). Little co-localization between the mitochondrial and PfGatB-GFP was observed in late trophozoites (Fig. 3D, panel i) or late schizont stages with
the mitochondrial staining largely distinct from that of PfGatB-GFP (Fig. 3D, panel ii).

Subcellular Localization in P. berghei by Myc Tagging the Endogenous GatA Gene—To confirm apicoplast localization that was determined via transfection of the episomal constructs in P. falciparum, we tagged the endogenous gene encoding the P. berghei ortholog of PfGatA (PBANKA_071810) with a quadruple Myc tag (Fig. 4). We tagged the endogenous PfGatA coding sequence to reduce the possibility that the fusion protein would be mistargeted due to inappropriate timing or intensity of expression. Fixed blood stage parasites were stained with anti-Myc antibody to detect PfGatA and ACP antisera (14) to detect the apicoplast, and the samples were observed using Del-tavision deconvolution fluorescence microscopy. Structures containing the Myc-tagged PfGatA (PfGatA-myc) exhibited a typical apicoplast appearance (Fig. 4C) similar to that observed using the episomal PfGatA-GFP and PfGatB-GFP constructs in P. falciparum in vitro.

Attempted Deletion of PfGatA Gene—Bioinformatic analyses strongly suggest that Plasmodium lacks an apicoplast-targeted GlnRS (57, 58), implying that indirect aminoacylation is probably the sole route for Gln-tRNA\textsubscript{Gln} formation in the apicoplast and that GatA and GatB are essential components of the protein biosynthetic pathway in Plasmodium. To test whether PfGatA was required for blood stage growth, we transfected P. berghei parasites with a construct (Fig. 5) designed to delete the endogenous PfGatA gene via double-crossover recombination. As a control, we transfected parasites from the same batch with the construct used previously to generate the PfGatA-myc transgenic parasites (Fig. 4). In three independent experiments, the PfGatA genomic locus was refractory to gene deletion via double-crossover recombination. Transgenic PfGatA-myc parasites, however, were readily obtained (data not shown). These two experiments indicate that although the PfGatA locus is accessible to recombination, the PfGatA gene is refractory to deletion, strongly suggesting that the apicoplast-targeted GatA is essential in blood stage parasites.

PfGatAB Is a Glutamyl-tRNA Amidotransferase—To show biochemically that PfGatAB encodes the apicoplast glutamyl-tRNA amidotransferase, we independently expressed PfGatA and PfGatB in E. coli. A two-step purification procedure combining Ni-NTA affinity and size exclusion chromatography allowed purification of 10 mg of PfGatA and PfGatB per liter of culture. SDS-PAGE analysis of the purified enzymes corroborated the predicted molecular masses of the two open reading frames, GatA and GatB (85.0 and 94.5 kDa, respectively, Fig. 6, A and B), indicating that both P. falciparum subunits could be expressed and purified independently. This was also the case with Helicobacter pylori GatCAB (22), but not with Bacillus subtilis GatCAB, in which the GatA subunit could not be expressed in E. coli in the absence of GatB (59).

Before assaying PfGatAB for amidotransferase activity, we first performed control reactions to verify that it would not glutamylate \textsuperscript{32}P-labeled apicoplast tRNA\textsubscript{Gln} or tRNA\textsubscript{Glu}. The control reactions showed that PfGatAB did not exhibit any glutamylation activity (Fig. 6C, lanes 1 and 2). We then tested
FIGURE 5. **Attempted knock-out of the PbGatA gene via double-crossover recombination.** Two fragments containing 5'- and 3'-UTRs of the PbGluRS gene, with ~100 nucleotides of the start and end, respectively, of the PbGatA coding sequence were cloned into the B3D KO Red vector (38). After linearization with Apal, the construct was transfected into *P. berghei* ANKA parasites (39). In three independent experiments, we were unable to delete the endogenous PbGatA gene by integrating the deletion construct via double-crossover recombination.

FIGURE 6. **PfGatAB purification and amidotransferase assay.** 

A, purification of PfGatA assessed by 7.5% SDS-PAGE. Lanes 1 and 2, eluate from Ni-NTA column; lane 3, eluate from gel filtration chromatography. 

B, purification of the PfGatB assessed by 7.5% SDS-PAGE. Lanes 1–3, eluate from Ni-NTA column; lane 4, eluate from gel filtration purification. 

C, representative phosphorimage of the separation of Gln-[32P]AMP, Glu-[32P]AMP, and [32P]AMP by PEI-cellulose TLC after PfGatAB-catalyzed amidotransferase reactions. The amidotransferase assays were performed as described under "Experimental Procedures" with the following modifications. Lanes 1 and 2 contained PfGatA, PfGatB, and either 32P-labeled apicoplast tRNAGln or tRNAGlu substrates in the presence of L-glutamate. No Glu-tRNAGln or Glu-tRNAGlu was observed, implying that PfGatAB does not possess aminoacylation activity. Lanes 3 and 4 contained PfGatA, PfGatB, and either 32P-labeled Glu-tRNAGlu or Glu-tRNAGln in the presence of L-glutamate. No reaction was observed, implying that PfGatAB does not utilize L-Glu as a substrate. Lanes 5 and 6 contained PfGatA, PfGatB, and either 32P-labeled Glu-tRNAGlu or Glu-tRNAGln in the presence of L-glutamine. PfGatAB converted Glu-tRNAGln to Gln-tRNAGln but did not utilize Glu-tRNAGlu as a substrate, indicating that PfGatAB has a high specificity for Gln-tRNAGln. 

D, representative phosphorimage of the effect of different amide donors on amidation activity. Lane 1 contained 50 nM each of PfGluRS, PfGatA, PfGatB, and 32P-labeled apicoplast tRNAGln. Lane 2 contained 50 nM each of PfGluRS, PfGatA, PfGatB, and 32P-labeled apicoplast tRNAGln. Lane 2 contained 50 nM each of PfGluRS, PfGatA, PfGatB, and 32P-labeled apicoplast tRNAGln. Lane 3 contained 50 nM each of PfGluRS, PfGatA, PfGatB, and 32P-labeled apicoplast tRNAGln, and L-glutamate, plus either L-glutamine or L-asparagine as amide donors. PfGatAB utilizes both L-glutamine and L-asparagine as amide donors.
PfGatAB for transamidation activity in a reaction containing glutamate as the amide donor and either \textit{P. falciparum} \textsuperscript{32P}-labeled Glu-tRNA\textsuperscript{Gln} or Glu-tRNA\textsuperscript{Glu} that had been produced using recombinant PfGluRS (23). PfGatAB transamidated neither of these glutamylated tRNA substrates (Fig. 6C, lanes 3 and 4), implying that PfGatAB does not utilize glutamate as an amide donor. However, PfGatAB transamidated Glu-tRNA\textsuperscript{Gln} to form Gln-tRNA\textsuperscript{Gln} in the presence of glutamine (Fig. 6C, lane 6) but as expected did not utilize Glu-tRNA\textsuperscript{Glu} as an amide acceptor (Fig. 6C, lane 5). These data show that PfGatAB has a high specificity for Glu-tRNA\textsuperscript{Gln} and utilizes glutamate as the amide donor in the transamidation reaction.

Next, we explored the possibility that PfGluRS and PfGlu-AdT could act together in a single reaction to sequentially form Gln-tRNA\textsuperscript{Gln} from precursors. We performed an experiment that allowed the transamidation reaction to take place immediately after aminoclaylation by premixing equimolar amounts of PfGluRS, PfGatA, and PfGatB for 3 min on ice and then incubating them at 37°C with all substrates required for the glutamylation and amidation reactions. Formation of Glu-tRNA\textsuperscript{Gln} and its conversion to Gln-tRNA\textsuperscript{Gln} was observed (Fig. 6D, lane 3). Similar results were obtained when we used l-asparagine as the amide donor for the transamination reaction (Fig. 6D, lane 4), implying that PfGatAB can utilize both l-asparagine and l-glutamate as amide donors. We also noted that the Glu-AMP spot in the TLC assay was less intense than the Gln-AMP (Fig. 6D, lanes 3 and 4) and control Glu-AMP spots (Fig. 6D, lane 2), indicating that the sequential glutamylation and amidotransferase reactions proceeded rapidly. This could be due to the sequestration and immediate substrate channeling (60) of the misacylated Glu-tRNA\textsuperscript{Gln} from the ND-GluRS to GatAB to prevent the release of misacylated Glu-tRNA\textsuperscript{Gln} and subsequent misincorporation of l-Glu in place of l-Gln during protein synthesis (61). The different amidotransferase assay approaches used here and the results obtained demonstrate that PfGatAB does not require the GatC subunit for transamidation reaction.

We did not test whether PfGatAB possesses Asp-AdT activity as bacterial GatCABs do because the \textit{Plasmodium} genome possesses two predicted AspRSs, one cytoplasmic and the other apicoplast-targeted (11, 25, 56, 62), as well as an apicoplast-targeted AsnRS. Thus, the apicoplast appears to possess all components required for asparaginylation of apicoplast tRNA\textsuperscript{Asn} via direct aminoclaylation. Together, these data are consistent with the conclusion that PfGatAB is a Glu-AdT.

\textbf{Discussion}

To date, two different tRNA-dependent AdTs are known: the heterotrimeric GatCAB (22) and the heterodimeric GatDE (20) enzymes. The latter is an archaeal signature enzyme and serves as the Glu-AdT for Gln-tRNA\textsuperscript{Gln} biosynthesis in archaea (20). GatCAB is found in both bacteria and archaea (20, 59). In archaeal genomes, GatCAB is encoded only when an AsnRS is not (63). All bacterial GatCAB enzymes studied to date are able to serve as both a Glu-AdT and an Asp-AdT \textit{in vitro} (64–69). The activity/ies actually performed by bacterial GatCABs \textit{in vivo} is/are determined by the non-discriminating aaRS (ND-GluRS and/or ND-AspRS) possessed by each organism. For example, bacteria such as \textit{B. subtilis} (70) that have an ND-GluRS but lack an ND-AspRS use their GatCAB solely as a Glu-AdT (22). In bacteria possessing an ND-AspRS but lacking an ND-GluRS (e.g. \textit{Pseudomonas aeruginosa}, \textit{Neisseria meningitidis}, \textit{Thermus thermophilus}, and \textit{Deinococcus radiodurans}), GatCAB serves only as an Asp-AdT (64, 65, 71–76). In bacteria carrying both non-discriminating aaRSs (ND-GluRS and ND-AspRS) such as \textit{Chlamydia trachomatis} (66) and \textit{H. pylori} (77–79), GatCAB serves as a Glu/Asp-AdT (66, 68, 69). The \textit{P. falciparum} genome (11) encodes two putative d-AspRS enzymes, one of which (PF3D7_0514300) possesses a predicted N-terminal apicoplast-targeting sequence, suggesting that it is imported into the apicoplast (56, 62). The other AspRS (PF3D7_0102900) lacks an apicoplast-targeting signal and is probably cytoplasmic (57, 58, 80). Similarly, \textit{Plasmodium} contains two putative AsnRSs as follows: one apicoplast-targeted (PF3D7_0509600) and the other cytoplasmic (PF3D7_0211800) (11, 24, 25). Thus, in the cytoplasm and apicoplast of \textit{Plasmodium}, Asn-tRNA\textsuperscript{Asn} is formed via direct aminoclaylation.

We previously demonstrated that the \textit{P. falciparum} nuclear genome (23) encodes two putative GluRS enzymes. One, PF3D7_1349200, appears to be cytoplasmic (58). The other, PF3D7_1357200, possesses a predicted N-terminal apicoplast-targeting sequence (56, 62), was localized to the apicoplast, and exhibits non-discriminating glutamylation activity \textit{in vitro}, producing both Glu-tRNA\textsuperscript{Glu} and Glu-tRNA\textsuperscript{Gln}. It is the first enzyme in the apicoplast’s indirect aminoclaylation pathway (23). In this study we have further dissected the apicoplast’s indirect aminoclaylation pathway by identifying the PfGatA and PfGatB subunits of the apicoplast aminoacyl-tRNA AdT. Using episomal constructs in which GFP was fused to the predicted PfGatA and GatB apicoplast targeting sequences, we demonstrated that GFP was trafficked to the apicoplast in erythrocytic stage parasites. Minor overlaps between the anti-GFP and MitoTracker Red signals were observed where the apicoplast and mitochondrion appeared to contact one another (Fig. 3, C, panel ii, and D, panel ii), a phenomenon observed with other apicoplast-targeted proteins (81, 82). Furthermore, we myc-tagged the endogenous \textit{P. berghei} chromosomal \textit{gatA} gene, and we observed that the tagged protein was trafficked to the apicoplast, confirming the results obtained in \textit{P. falciparum} with episomal constructs. Localization of \textit{P. falciparum} GatAB solely in the apicoplast differs from the situation in \textit{Arabidopsis}, where the GatCAB amidotransferase is targeted to both the plastid and the mitochondrion (83). Dual targeting of PfGatAB to the plastid and mitochondrion is also unlikely because, as in the related parasite \textit{T. gondii}, the \textit{Plasmodium} mitochondrion probably imports aminoclaylated tRNAs from the cytoplasm (58).

We expressed recombinant PfGatA and PfGatB independently in \textit{E. coli} (Fig. 6, A and B), and we combined them \textit{in vitro} to examine their ability to transamidate apicoplast Glu-tRNA\textsuperscript{Gln} or Glu-tRNA\textsuperscript{Glu} that had been previously glutamylated using recombinant PfGluRS (23). PfGatAB demonstrated a remarkable tRNA substrate specificity by converting Glu-tRNA\textsuperscript{Gln} to Gln-tRNA\textsuperscript{Gln} but did not transamidate Glu-tRNA\textsuperscript{Glu} (Fig. 6C). The \textit{Plasmodium} apicoplast possesses an ND-GluRS (23) but lacks an ND-AspRS (57, 58), and therefore it almost certainly utilizes the PfGatAB as a Glu-AdT. As in \textit{Plasmodium}, bacteria such as \textit{B. subtilis} (70) that
pose an ND-GluRS but lack an ND-AspRS use their GatCAB only as a Glu-AdT (64). Because genes encoding GatA and GatB are present in all known Plasmodium genomes but not in any known organism in the other two domains, we concluded that GatAB is the Plasmodium glutamyl-tRNA\(^{Gln}\) amidotransferase (GatAB/Glu-AdT).

We also showed that PfGluRS, PfGatA, and PfGatB, when briefly pre-mixed, can glutamylate apicoplast tRNA\(^{Gln}\) and transamidicate it to form Gln-tRNA\(^{Gln}\) \textit{in vitro} (Fig. 6D). This suggests the Plasmodium apicoplast may contain a tRNA\(^{Gln}\)-ND-GluRS-GatA-GatB complex, akin to the transamidosomes described in archaea (61) and the eubacterium \textit{T. thermophilus} (84). Such a complex would prevent challenging the genetic code integrity as demonstrated for tRNA-dependent Asn formation (61).

We used different approaches to test for the PfGatAB-catalyzed amidotransferase reaction. Our findings consistently showed that the \textit{Plasmodium} parasite has established an evolved glutamyl-tRNA amidotransferase reaction that takes into account the absence of the \textit{gatC} gene in the parasite genome and therefore does not require it for the transamidation reaction, contrary to the amidotransferase activity of the GatCAB paralog where the three subunits are required for enzyme activity (22). This is the first biochemical evidence for Glu-tRNA\(^{Gln}\) transamidation by a GatAB in the absence of a GatC subunit.

We investigated the evolution of \textit{Plasmodium} GatA and GatB subunits in comparison with the human GatA and GatB, the \textit{S. aureus} GatA and GatB subunits of bacterial GatCABs, the \textit{S. aureus} and \textit{G. gallus} subunits of other plastids, and the \textit{G. gallus} and \textit{E. coli} subunits of archaeal GatDE. In the unrooted phylogeny of GatB and GatE proteins, \textit{Plasmodium} GatBs were not placed within the bacterial GatB, archaeal GatE, or the plastid GatB clades but were placed on a separate branch (Fig. 3A). The divide is well supported with a bootstrap value of 100. In a similar fashion, the unrooted phylogeny of GatA and GatD proteins, \textit{Plasmodium} GatA was not placed within the bacterial GatA, the plastid GatA, or the archaeal GatD clades but was placed on a separate branch (bootstrap value of 100) (Fig. 3B). Additionally, the GatB and GatA sequences of \textit{T. parva} and \textit{Babesia bovis}, which like \textit{Plasmodium} lack \textit{gatC} in their genomes, were placed in the same clade as the \textit{Plasmodium} orthologs. Taken in total, the results strongly suggest that GatAB, which is uniquely found in Apicomplexa that possess an apicoplast, is a paralog to GatCAB, GatFAB, and GatDE.

To identify conserved and divergent features of PfGatB and PfGatA, homology modeling was performed in comparison with the known \textit{S. aureus} GatCAB structure (Fig. 1) (28, 29). All functional features of bacterial GatCAB and archaeal GatDE are conserved in PfGatAB, but we found that PfGatAB had unique inserts that could not be fitted into the GatCAB model (Fig. 1). In archaeal GatDE, the unstructured N-terminal insert of GatD has been reported to play a structural role that gives it the ability to associate to GatE (31). GatF found only in fungal genomes (46, 47) and GatC found in bacterial, plant, and mammalian AdTs have similar unstructured characteristics and play a similar structural role where they reinforce the interaction between the GatA and GatB subunits by encircling their inter-

**Author Contributions**—M. J. G. conceived and directed the project. M. J. G. and B. M. designed the research; B. M. M., K. F. W., K. B., G. R., L. L., J. A., and T. M. N. performed the experiments; and M. J. G. and B. M. designed the research; B. M. M., K. F. W., K. B., G. R., L. L., J. A., and T. M. N. performed the experiments; and M. J. G. and B. M. M. wrote the manuscript. All authors approved the final version of the manuscript.

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References

1. World Health Organization (2014) World Malaria Report 2014. World Health Organization, Geneva, Switzerland.

2. Goodman, C. D., and McFadden, G. I. (2013) Targeting apicoplasts in malaria parasites. Expert Opin. Ther. Targets 17, 167–177.

3. Wilson, R. J., Denny, P. W., Preiser, P. R., Rangachari, K., Roberts, K., Roy, A., Whyte, A., Strath, M., Moore, D. J., Moore, P. W., and Williamson, D. H. (1996) Complete gene map of the plastid-like DNA of the malaria parasite Plasmodium falciparum. J. Mol. Biol. 261, 155–172.

4. Gardner, M. J., Feagin, J. E., Moore, D. J., Spencer, D. F., Gray, M. W., Williamson, D. H., and Wilson, R. J. (1991) Organisation and expression of small subunit ribosomal RNA genes encoded by a 35-kilobase circular DNA in Plasmodium falciparum. Mol. Biochem. Parasitol. 48, 77–88.

5. Gardner, M. J., Williamson, D. H., and Wilson, R. J. (1991) A circular DNA in malaria parasites encodes an RNA polymerase like that of prokaryotes and chloroplasts. Mol. Biochem. Parasitol. 44, 115–123.

6. Feagin, J. E., Werner, E., Gardner, M. J., Williamson, D. H., and Wilson, R. J. (1992) Homologies between the contiguous and fragmented tRNAs of the two Plasmodium falciparum extrachromosomal DNAs are limited to core sequences. Nucleic Acids Res. 20, 879–887.

7. Gardner, M. J., Preiser, P., Rangachari, K., Moore, D. J., Feagin, J. E., Williamson, D. H., and Wilson, R. J. (1994) Nine duplicated tRNA genes on the plastid-like DNA of the malaria parasite Plasmodium falciparum. Gene 144, 307–308.

8. Preiser, P., Williamson, D. H., and Wilson, R. J. (1995) tRNA genes transcribed from the plastid-like DNA of Plasmodium falciparum. Nucleic Acids Res. 23, 4329–4336.

9. Seeber, F. (2002) Biogenesis of iron-sulphur clusters in amitochondriate apicomplexan protists. Int. J. Parasitol. 32, 1207–1217.

10. Waller, R. F., Reed, M. B., Cowman, A. F., and McFadden, G. I. (2000) Protein trafficking to the plastid of Plasmodium falciparum is via the secretory pathway. EMBO J. 19, 1794–1802.

11. Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., et al. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 419, 498–511.

12. Ralph, S. A., van Dooren, G. G., Waller, R. F., Crawford, M. J., Fraunholz, M. J., Foth, B. J., Tonkin, C. J., Lai, Z., Koonin, E. V., Shallom, S., Mason, T., Yu, K., Fujii, C., Pederson, J., Shen, R. J. (1992) Homologies between the contiguous and fragmented rRNA sets of Theileria parva, a bovine pathogen that transforms lymphocytes. Science 293, 80–88.

13. Nakamura, A., Yao, M., Chirmaronak, S., Sakai, N., and Tanaka, I. (2006) Ammonia channel couples glutamine synthetase with transaminase reactions in GtCAB. Science 312, 1954–1958.

14. Nakamura, A., Sheppard, K., Yamane, J., Yao, M., Söll, D., and Tanaka, I. (2010) Two distinct regions in T. cruzi pyrophosphorylase correlate with accurate tRNA recognition. Nucleic Acids Res. 38, 672–682.

15. Schmitt, E., Panvert, M., Blanquet, S., and Mechulam, Y. (2005) Structural basis for tRNA-dependent amidotransferase function. Structure 13, 1421–1433.

16. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612.

17. Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M., Claverie, J. M., and Gascuel, O. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 36, W465–W469.

18. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

19. Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.

20. Guindon, S., and Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

21. Chevenet, F., Brun, C., Bafuls, A. L., Jacq, B., and Christen, R. (2006) TreeDyn: toward dynamic graphics and annotations for analyses of trees. BMC Bioinformatics 7, 439.

22. Trager, W., and Jensen, J. B. (1976) Human malaria parasites in continuous culture. Science 193, 673–675.

23. Tonkin, C. J., van Dooren, G. G., Spurck, T. P., Struck, N. S., Good, R. T.,
targeting in the malaria parasite *Plasmodium falciparum*. Science 299, 705–708
57. Bhatt, T. K., Kapil, C., Khan, S., Jairajpuri, M. A., Sharma, V., Santoni, D., Silvestrini, F., Pizzi, E., and Sharma, A. (2009) A genomic glimpse of amionic-tRNA synthetases in malaria parasite *Plasmodium falciparum*. BMC Genomics 10, 644
58. Pino, P., Abe, E., Foth, B. J., Sheiner, L., Soldati, T., Schneider, A., and Soldati-Favre, D. (2010) Mitochondrial translation in absence of local tRNA aminoacylation and methionyl tRNA Met formulation in Apicomplexa. Microbiol. 76, 706–718
59. Feng, L., Sheppard, K., Tumbula-Hansen, D., and Söll, D. (2005) Gln-tRNAAsn formation from Glu-tRNA(Gln) requires cooperation of an asparaginase and a Gln–tRNA(Gln) kinase. J. Biol. Chem. 280, 8150–8155
60. Srivastava, D. K., and Bernhard, S. A. (1986) Metabolite transfer via enzyme–enzyme complexes. Science 234, 1081–1086
61. Rampias, T., Sheppard, K., and Söll, D. (2010) The archaeal transamidase for RNA-dependent glutamine biosynthesis. Nucleic Acids Res. 38, 5749–5763
62. Zuege, J., Ralph, S., Schmuker, M., McFadden, G. I., and Schneider, G. (2001) Deciphering apicoplast targeting signals—feature extraction from nuclear-encoded precursors of *Plasmodium falciparum* apicoplast proteins. Gene 280, 19–26
63. Roy, H., Becker, H. D., Reinbolt, J., and Kern, D. (2003) When contemporary aminoacyl-tRNA synthetases invent their cognate amino acid metabolism. Proc. Natl. Acad. Sci. U.S.A. 100, 9837–9842
64. Curnow, A. W., Tumbula, D. L., Pelaschier, J. T., Min, B., and Söll, D. (1998) Glutaminyl-tRNA(Gln) amidotransferase in Deinococcus radiodurans may be confined to asparagine biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 95, 12838–12843
65. Becker, H. D., Min, B., Jacobi, C., Raczniak, G., Pelaschier, J., Roy, H., Klein, S., Kern, D., and Söll, D. (2000) The heterotrimeric *Thermus thermophilus* Asp-tRNA(Asn) amidotransferase can also generate Gln–tRNA(Gln). FEMS Lett. 140, 144–146
66. Raczniak, G., Becker, H. D., Min, B., and Söll, D. (2001) A single amidotransferase forms asparaginyl-tRNA and glutaminyl-tRNA in *Chlamydia trachomatis*. J. Biol. Chem. 276, 45862–45867
67. Salazar, J. C., Zúñiga, R., Raczniak, G., Becker, H. D., Söll, D., and Orellana, O. (2001) A dual-specific Glu–tRNA(Gln) and Asp–tRNA(Asn) amidotransferase is involved in decoding glutamine and asparagine codons in *Acidi-baccillus ferrooxidans*. FEMS Lett. 129, 131–138
68. Cathopoulis, T. I., Chua Wong, P., and Hendrickson, T. L. (2007) A thinline electrophoretic assay for Asp–tRNAAsn/Glu–tRNA(Gln) amidotransferase. Anal. Biochem. 360, 151–153
69. Sheppard, K., Akochy, P. M., Salazar, J. C., and Söll, D. (2007) The *Helicobacter pylori* amidotransferase GatCAB is equally efficient in glutamine-dependent transamidation of Asp–tRNAAsn and Glu–tRNA(Gln). J. Biol. Chem. 282, 11866–11873
70. Lapointe, J., Duplain, L., and Proulx, M. (1986) A single glutaminyl-tRNA synthetase aminoacylates tRNA(Glu) and tRNA(Gln) in *Bacillus subtilis* and efficiently misacylates *Escherichia coli* tRNA(Gln1 in vitro. J. Bacteriol. 165, 88–93
71. Becker, H. D., Reinbolt, J., Kreutzer, R., Giegé, R., and Kern, D. (1997) Existence of two distinct aspartyl-tRNA synthetases in *Thermus thermophilus*. Structural and biochemical properties of the two enzymes. Biochemistry 36, 8785–8797
72. Becker, H. D., and Kern, D. (1998) *Thermus thermophilus*: a link in evolution of the tRNA-dependent amino acid amidation pathways. Proc. Natl. Acad. Sci. U.S.A. 95, 12832–12837
73. Becker, H. D., Roy, H., Moulinier, L., Mazaucier, M. H., Keith, G., and Kern, D. (2000) *Thermus thermophilus*: contains an eubacterial and an archaeal
74. Berends, S. J., Bidwell, S. L., Brown, W. C., Crabtree, J., Fadrosh, D., Feldrod, D., Feldt, M., Forberger, H., Haas, B. J., Howell, J. M., Khouri, H., et al. (2007) Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLoS Pathog. 3, 1401–1413
75. Foth, B. J., Ralph, S. A., Tonkin, C. J., Struck, N. S., Fraunholz, M., Roos, D. S., Cowman, A. F., and McFadden, G. I. (2003) Dissecting apicoplast
A single tRNA base pair mediates bacterial tRNA-dependent biosynthesis of asparagine. *Nucleic Acids Res.* **34**, 6083–6094

77. Salazar, J. C., Ahel, I., Orellana, O., Tumbula-Hansen, D., Krieger, R., Daniels, L., and Söll, D. (2003) Coevolution of an aminoacyl-tRNA synthetase with its tRNA substrates. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13863–13868

78. Skouloubris, S., Ribas de Pouplana, L., De Reuse, H., and Hendrickson, T. L. (2003) A noncognate aminoacyl-tRNA synthetase that may resolve a missing link in protein evolution. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11297–11302

79. Chuawong, P., and Hendrickson, T. L. (2006) The nondiscriminating aspartyl-tRNA synthetase from *Helicobacter pylori*: anticodon-binding domain mutations that impact tRNA specificity and heterologous toxicity. *Biochemistry* **45**, 8079–8087

80. Bour, T., Akaddar, A., Lorber, B., Blais, S., Balg, C., Candolfi, E., and Frugier, M. (2009) Plasmoidal aspartyl-tRNA synthetases and peculiarities in *Plasmodium falciparum*. *J. Biol. Chem.* **284**, 18893–18903

81. Hopkins, J., Fowler, R., Krishna, S., Wilson, I., Mitchell, G., and Bannister, L. (1999) The plastid in *Plasmodium falciparum* asexual blood stages: a three-dimensional ultrastructural analysis. *Protist* **150**, 283–295

82. van Dooren, G. G., Marti, M., Tonkin, C. J., Stimmmer, L. M., Cowman, A. F., and McFadden, G. I. (2005) Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of *Plasmodium falciparum*. *Mol. Microbiol.* **57**, 405–419

83. Duchène, A. M., Giritch, A., Hoffmann, B., Cognat, V., Lancelin, D., Peeters, N. M., Zaepfel, M., Maréchal-Drouard, L., and Small, I. D. (2005) Dual targeting is the rule for organellar aminoacyl-tRNA synthetases in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 16484–16489

84. Bailly, M., Blaise, M., Lorber, B., Becker, H. D., and Kern, D. (2007) The transamidosome: a dynamic ribonucleoprotein particle dedicated to prokaryotic tRNA-dependent asparagine biosynthesis. *Mol. Cell.* **28**, 228–239

85. Jackson, K. E., Habib, S., Frugier, M., Hoen, R., Khan, S., Pham, J. S., Ribas de Pouplana, L., Royo, M., Santos, M. A., Sharma, A., and Ralph, S. A. (2011) Protein translation in *Plasmodium* parasites. *Trends Parasitol.* **27**, 467–476

86. Plaimas, K., Wang, Y., Rotimi, S. O., Olasehinde, G., Fatumo, S., Lanzer, M., Adebiyi, E., and König, R. (2013) Computational and experimental analysis identified 6-diazo-5-oxonorleucine as a potential agent for treating infection by *Plasmodium falciparum*. *Infect. Genet. Evol.* **20**, 389–395