Aminoacylation of *Plasmodium falciparum* tRNA^Asn and Insights in the Synthesis of Asparagine Repeats*

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Background: Proteins from the malaria parasite *Plasmodium* contain extensive asparagine repeats.
Results: *Plasmodium* tRNA^Asn concentration and *in vitro* asparaginylation catalytic parameters were analyzed to explain the frequency of asparagine repeats.
Conclusion: The requirement for high levels of asparaginylated tRNAs hinders efficient protein translation.
Significance: Limiting the availability of asparaginylated tRNAs represents a plausible regulatory mechanism for the production of properly folded proteins.

Genome sequencing revealed an extreme AT-rich genome and a profusion of asparagine repeats associated with low complexity regions (LCRs) in proteins of the malarial parasite *Plasmodium falciparum*. Despite their abundance, the function of these LCRs remains unclear. Because they occur in almost all families of plasmodial proteins, the occurrence of LCRs cannot be associated with any specific metabolic pathway; yet their accumulation must have given selective advantages to the parasite. Translation of these asparagine-rich LCRs demands extraordinarily high amounts of asparaginylated tRNA^Asn. However, unlike other organisms, *Plasmodium* codon bias is not correlated to tRNA gene copy number. Here, we studied tRNA^Asn accumulation as well as the catalytic capacities of the asparaginyl-tRNA synthetase of the parasite *in vitro*. We observed that asparaginylation in this parasite can be considered standard, which is expected to limit the availability of asparaginylated tRNA^Asn in the cell and, in turn, slow down the ribosomal translation rate when decoding asparagine repeats. This observation strengthens our earlier hypothesis considering that asparagine rich sequences act as "tRNA sponges" and help cotranslational folding of parasite proteins. However, it also raises many questions about the mechanistic aspects of the synthesis of asparagine repeats and about their implications in the global control of protein expression throughout *Plasmodium* life cycle.

Malaria is one of the main menaces to human health in developing countries. There were ~220 million cases of malaria (between 154–289 million) and 660,000 deaths estimated (between 610,000–971,000) in 2010 (according to the World Malaria Report of 2012 by the World Health Organization). Most deaths were caused by *Plasmodium falciparum*, which has developed resistance to nearly all anti-malarial treatments.

The sequence of the *P. falciparum* genome was completed in 2002 (1) and led to many fundamental observations about the molecular features of this parasite. Since then, sequences of other *Plasmodium* genomes (*Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium yoelii*, *Plasmodium knowlesi*, and *Plasmodium vivax*) have also been reported. *Plasmodium* genomes are AT-rich, except for a divergent group, including *P. knowlesi* and *P. vivax*, which are characterized by a higher GC content (2). In AT-rich genomes, lysine, asparagine, phenylalanine, and isoleucine should occur in greater numbers than other amino acids. This is indeed the case in *P. falciparum*, *P. berghei*, *P. chabaudi*, and *P. yoelii*, where asparagine, lysine, and isoleucine are predominant in the proteomes of the parasite. These residues comprise 11.3, 10.4, and 10.5% of the *P. falciparum* proteome, respectively; only phenylalanine does not follow this probability trend, occurring at a frequency of only 3.9% (3). Moreover, asparagine residues are characterized by a particular distribution in these proteomes. They concentrate in long single amino acid repeats that are typically associated with regions of low complexity (LCR)[3] (4). LCRs and asparagine repeats are found in 87% of the encoded proteins (5), even in metabolic enzymes and heat shock proteins that are usually resistant to these types of insertions (6). The presence of LCRs makes malarial proteins longer than their homologues in other species and it has been proposed that LCRs will form bulging domains from the core of each protein so that they would not impair structure or function (6). Numerous hypotheses have tried to address the function of plasmodial LCRs. It has been proposed that they would promote mRNA stability (7), protein-protein interactions (8), or may be involved in antigen diversification (9–12). It has also been proposed that LCRs are only neutral spacers between protein structural modules (13) or that

[3] The abbreviations used are: LCR, low complexity region; AsnRS, asparaginyl-tRNA synthetase; aaRS, aminoacyl-tRNA synthetase.

they may be excised by some unknown mechanism (6). Yet, experimental evidence suggesting that LCRs play a functional role is rare. In addition to these hypotheses, we proposed that LCRs in general and asparagine-rich LCRs in particular modulate the rate of translating ribosomes and thus participate in cotranslational folding of plasmodial proteins (14). This proposed process would be driven by limiting access to asparaginylated transfer RNA^{Asn} (tRNA) in the parasite during translation of LCRs.

It is generally accepted that variations in ribosome decoding rates are a consequence of the abundance of tRNAs in the cell; translation is optimal at codons decoded by highly abundant tRNAs and limited at codons read by rare tRNAs, as the ribosome pauses until the cognate tRNA becomes available. Still, it has been shown that tRNA abundance is not the only determinant of elongation rates. Interactions between the mRNA and the 16S ribosomal RNA as well as the presence of positively charged amino acids in the nascent peptide decelerate the ribosomes along transcripts in bacteria and yeast, respectively (15, 16). Among prokaryotic and eukaryotic genomes, genes encoding a given tRNA are usually present in multiple copies. Plasmodia are exceptions to this rule: the plasmodial nuclear genomes display 45 tRNA genes, with only one gene copy per tRNA isoacceptor (1). Thus, this minimal set of tRNA genes leads to the appealing assessment that the product of the unique tRNA^{Asn} gene would decode >10% of the *Plasmodium* proteome. Translation specificity and efficiency depend on the accuracy of tRNA aminoacylation catalyzed by their cognate aminoacyl-tRNA synthetases (17) and on the availability of charged tRNAs, respectively. In the present study, *P. falciparum* cytosolic asparaginyl-tRNA synthetase (AsnRS) and tRNA^{Asn} were cloned and their kinetic parameters were determined *in vitro* to estimate asparaginylation efficiency in this parasite. Moreover, the relative concentrations of each molecular partners involved in *P. falciparum* asparaginylation were compared with other plasmodial aminoacylation systems to deduce the relative accumulation of asparaginyl-tRNA^{Asn} in *Plasmodium* and eventually its impact on the rate of synthesis of asparagine-rich LCRs.

EXPERIMENTAL PROCEDURES

Materials—*Pyrococcus abyssi* asparagine synthetase A was a generous gift from D. Kern. Radioactive L-[^{14}C]tyrosine (483 mCi/mmol) was from Amersham Biosciences (Velizy-Villacoublay, France) and L-[^{14}C]aspartate (200 mCi/mmol) was from PerkinElmer Life Sciences.

Database Screening for AsnRS Sequences—The *P. falciparum* genome encodes two copies of AsnRS, a cytoplasmic version (PlasmoDB ID PF3D7_0211800) and an apicoplastic one (PlasmoDB ID PF3D7_0509600) (14). The cytoplasmic *P. falciparum* AsnRS gene was identified by blasting the sequence of yeast cytoplasmic AsnRS (NP_011883.1) against the PlasmoDB database. Sequence alignments were computed with Tcoffee software.

RNA Seq Data of aaRS mRNA Expression in Sexual and Asexual Stages—The study was performed in the laboratory of Xinzhuan Su (18), and data were retrieved from PlasmoDB for each *P. falciparum* cytosolic aminoacyl-tRNA synthetase gene.

Proteomic Data of P. falciparum Life Cycle Stages—Data were retrieved from Florens *et al.* (19). We considered proteins (i) expressed specifically in a single developmental stage and (ii) with sequences covered by at least three peptides. Thus, we used 27, 18, 31, and 66 protein sequences, corresponding to 50,027, 9629, 24,152, and 55,394 amino acids, identified in the sporozoite, merozoite, trophozoite, and gametocyte stages, respectively.

Cloning and Expression of Recombinant P. falciparum and H. sapiens AsnRSs—Genes coding for both the *P. falciparum* and human AsnRSs were amplifed by PCR from either *P. falciparum* (provided by H. Vial, Montpellier, France) or human liver cDNA libraries, respectively. Primers for PCR were designed according to the sequences of the AsnRS genes. In the case of the parasite AsnRS, the PCR reaction was performed with a customized dNTP mixture adapted to the *P. falciparum* genome composition (80% AT-rich): 240 μM dATP, 240 μM dTTP, 80 μM dCTP, 80 μM dGTP, and elongation was performed at 65 °C. Amplified DNA fragments were entirely sequenced and cloned into pQE30 (Qiagen, Courtaboeuf, France) to yield N-terminal His$_6$-tagged recombinant proteins. *Escherichia coli* Top10 strains, separately expressing each construct, were grown at 37 °C in Luria broth with 2% glucose and 50 μg/ml ampicillin until they reached an absorbance of 2.0 (1-cm path length). The medium was then substituted with the same medium lacking glucose and AsnRS expression was induced for 3 h at 37 °C with 1 mM isopropyl-β-D-thiogalactopyranoside. Recombinant proteins were purified on nickel-nitrilotriacetic acid resin according to the manufacturer's instructions (Qiagen). Fractions containing AsnRS were pooled and dialyzed overnight at 4 °C against 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$, pH 7.4, 10 mM β-mercaptoethanol, 150 mM KCl, and 50% glycerol. AsnRS proteins were quantified by Bradford assay (Bio-Rad) and stored at -20 °C. Preparations yielded one band on SDS-polyacrylamide gel and were estimated to be at least 95% pure.

Cloning and Expression of Recombinant P. falciparum AspRS and TyrRS—Purification of recombinant *P. falciparum* AspRS was performed as described in Ref. 20. The gene coding for *P. falciparum* TyrRS was amplified by PCR from *P. falciparum* cDNA, cloned into pQE30, and purified as described for *P. falciparum* AsnRS.

Dynamic Light Scattering—*P. falciparum* and *H. sapiens* AsnRS samples (20 μM) were incubated in HEPES-KOH, pH 7.0, 50 mM NaCl, 10% glycerol and were analyzed with a Zetasizer Nano S (Malvern, Orsay, France). Five intensity measurements were recorded at 20 °C, and the data were processed with the manufacturer's software, assuming that the enzyme particles were spherical. This method measures distributions of particle diffusion coefficients (D) that were transformed via the Einstein equation ($D = \text{kT}/6\text{p}\eta_0 R_h$) into hydrodynamic radii (R_h) and thus apparent particle sizes. Corrections for solvent refractive index and viscosity were applied, and the contribution of the solvent components was subtracted.

Preparation of L-[^{14}C]Asparagine—This procedure was adapted from Roy and collaborators (21). The reaction mixture (200 μl), containing 50 mM HEPES-NaOH, pH 7.2, 10 mM MgCl$_2$, 2 mM NH$_4$Cl, 2 mM KF, 240 μM L-[^{14}C]Asp (300 cpm/

pmol), and 10 mM ATP, was incubated at 70 °C for 2 h in the presence of 2 μM *Pyrococcus abyssi* asparagine synthetase A. Under these conditions, 95% of the initial aspartic acid was amidated into asparagine (as analyzed by thin layer chromatography). The reaction was stopped by phenol extraction, the L-[^{14}C]Asn was further purified on an Illustra NAP-5 column (GE Healthcare Life Sciences) and concentrated by cryo-desiccation. The pellet was recovered in H_2O to reach a final concentration of 200 μM.

Preparation of tRNA Samples—Transfer RNA transcripts were produced *in vitro* using either the classical method for *P. falciparum* tRNA^{Asn} (22) or the "transzyme" system for *H. sapiens* tRNA^{Asn} (23). Briefly, synthetic genes made of overlapping oligonucleotides reconstructed the T7 RNA polymerase promoter, the optional hammerhead ribozyme, the tRNA, and a BstNI site matching the CCA 3′-end of the tRNA sequence. When transcribed, the hammerhead ribozyme self-cleaved and released the mature tRNA that was further purified on a denaturing polyacrylamide gel. Before any assay, tRNA transcripts were heated at 65 °C for 2 min in water and refolded at 37 °C in the presence of $MgCl_2$ (10 mM).

Total RNA from *P. falciparum* 3D7 strain parasites were cultured at 37 °C in 1% hematocrit as described previously (24), and cultures were harvested with 12–15% parasitemia. Infected erythrocytes were washed with incomplete growth medium and lysed with 0.15% saponin, and the parasites were used immediately for total RNA isolation by the Tri°-reagent BD (Sigma) extraction method according to the manufacturer's instructions.

Northern Blots—Samples of *P. falciparum* RNA were resolved on an 8% polyacrylamide gel, with 8 M urea in Tris-borate-EDTA buffer. Molecules were electrotransferred to an N$^+$ nylon membrane (Amersham Biosciences) for 3 h at 250 mA at 4 °C and cross-linked to the membrane by UV irradiation. Specific ^{32}P-labeled oligodeoxyribonucleotide probes were incubated with the membrane at 60 °C in ultra-sensitive hybridization buffer (Ambion, Saint Aubin, France), and the membrane was washed twice for 10 min at room temperature in 2× SSC, 0.1% SDS. Ribosomal 5S RNA was used as an internal loading control when quantifying tRNAs. Two amounts (5 μg and 15 μg) of three different *P. falciparum* RNA samples were tested, and probes were designed to detect tRNA$^{Asn}_{GUU}$ (5′-CCGCACGCTCTAAC-CAACTGAGCTACGGAACC-3′), tRNA$^{Asp}_{GUC}$ (5′-GCGT-GACAGGCGGAAATACTTGCACTATACTATCTCGGA-3′), tRNA$^{Tyr}_{GUA}$ (5′-ATTTCAACTACAGTCTGCCGCTCT-ACCAACTGAGCTATCATCGG-3′), tRNA$^{Lys}_{CUU/UUU}$ (5′-CCAGATTAAGAGTCTGGCGCTCTACCGACTGAGCTA-AGGCAGC), and 5S rRNA (5′-CGAGCGGTCTCCCACCTC-AGTATCT-3′) with melting temperatures ranging between 80 and 83 °C.

ATP/PP$_i$ Exchange and tRNA Aminoacylation Assays—ATP/PP$_i$ exchange assays were performed at 37 °C in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1.5 mM [^{32}P]PP$_i$ (3 cpm/pmol), 2 mM KF, and 250 nM AsnRS. The concentrations of either L-Asn or ATP varied from 0.05 to 1.5 mM. The synthesized [^{32}P]ATP was quantified as described by Campanacci and collaborators (25).

Aminoacylation assays were conducted in 50 mM HEPES-NaOH, pH 7.2, 10 mM $MgCl_2$, 2 mM ATP, 30 mM KCl, and appropriate amounts of tRNA^{Asn} and AsnRSs (diluted in 100 mM HEPES-NaOH, pH 7.4, 1 mM DTT, and 10% glycerol for the plasmodial enzyme and the same buffer containing 5 mg/ml bovine serum albumin for the human enzyme). The mixture was incubated for 15 min at 37 °C and the reaction was initiated by adding 20 *versus* 10 μM L-[^{14}C]asparagine for the plasmodial and the human enzymes, respectively. Apparent kinetic parameters were determined from Lineweaver-Burk plots. Data were expressed as averages of at least three independent experiments.

Aminoacylation plateaus of *Plasmodium* total RNA were performed in the presence of 0.5 μM of the relevant aminoacyl-tRNA synthetases (aaRS; *P. falciparum* recombinant AspRS, TyrRS, and AsnRS). Values were measured after 60-min incubations at 37 °C.

RESULTS

In Silico Identification of Plasmodial AsnRSs

Six plasmodial genomes were screened for cytosolic AsnRS genes. Complete AsnRS genes were found in primate-infecting (*P. falciparum*, *P. vivax*, and *P. knowlesi*) and rodent-infecting (*P. yoelii*, *P. berghei*, and *P. chabaudi*) plasmodia (Fig. 1). The gene coding for the cytosolic *P. falciparum* AsnRS (PF3D7_0211800) is 1833 base pairs, is located on chromosome 2, and codes for a 610-amino acid protein. AsnRS is classified with both the aspartyl- and lysyl-tRNA synthetases as a class IIb aaRSs (26). Plasmodia AsnRSs were compared with homologues from *H. sapiens*, the vertebrate host of *P. falciparum*, and from *Brugia malayi*, for comparison with the crystal structure determined by Crepin and collaborators (27). Structure-based sequence alignments show characteristic eukaryotic AsnRS features, in particular in the C-terminal catalytic domain that contains the three class II aaRS motifs. All AsnRSs exhibit N-terminal appendices, which are predicted to fold as helical modules. The plasmodial modules are shorter (75 to 82 amino acids), and their sequences diverge from the N-terminal appendices of *B. malayi* AsnRS (110 amino acids) (28) and *H. sapiens* AsnRS (103 amino acids) (29). Yet, they all contain a helical lysine-rich motif (KMKKLK) that is similar to the RNA-binding motif identified in eukaryotic AspRSs and LysRSs (30–32). Lastly, the N-terminal module of plasmodia AsnRSs does not contain the amino acid sequence involved in the cytokine activity of *B. malayi* AsnRS (28).

All plasmodial AsnRSs are characterized by the presence of an insertion in the first half of the catalytic domain, between motifs 1 and 2 (Fig. 1), which likely does not disturb the active site architecture. This insertion corresponds to an LCR and has not been previously identified in AsnRSs of other phylogenetic origins. The insertion site of this LCR is strictly conserved among plasmodial AsnRSs, but the size and sequence vary from one species to another. In *P. falciparum*, *P. berghei*, *P. chabaudi*, and *P. yoelii*, this insertion contains several asparagine stretches.

To test the contribution of both the N-terminal extension and the LCR, *P. falciparum* AsnRS variants missing these

N-terminal extension

RNA binding motif

```
P.falciparum   MCEKD----------------DITVNEEILQKAQEFQVENEK------------------DIKMKKLKPITEGLLKPEVDLLQISERGSRGRVKICNVLN
P.berghei   M------------------AIEVDEETLKIAKEFQKKNEE------------------EIKSKKLKPESIKLDNPSSNLIKINEASCRGRIKICNLLN
P.chabaudi   M------------------AIEVDEETLKIAKEFQKNEE-------------------EIKFKKLKPETVKVNEPSSNLLKINEAACRGRIKICNLLN
P.yoelii   M------------------AIEVDEETLKIAKEFQKKNEE------------------EIKSKKLKPETIQLDSPSSNLIKINEASCRGRIKISNLLN
P.knowlesi   M------------------DIQLDDGVIQKAKALQEANEE------------------EIKMKKLKPQSKGLLSPKSNLLEVTDTGCRSRIRICNVLN
P.vivax   M------------------DVQVDEAVLQKAKALQQANEE------------------EIQAKKLKPQSEGLLSPKSDLLQVTDKGCRSRIRICNVLN
H.sapiens   MVLAELYVSDREGSDATGDGTKEKPFKTGLKALMTVGKEPFPTIYVDSQKEN-ERWNV---ISKSQLKNIKKMWHREQMKSESREKKEAEDS---LRREK
B.malayi   MT---VYICPETGDDG-NDGSELKPLRTLYQAM-IITKSSKGDFLIRTKKDGKQIWEA--ASKTALKKSWKHYEQEMLKNEKVAAKMLEKDATEVGVKA
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Anticodon binding domain

```
P.falciparum   VPRSEKEYNNNNSDKV--|-------ENKYIGKIITVCGWSKAIRKQGGGRFCFVNLNDGSCHLNLQIVVNQCIENYEKLLKCGAGCCFRFTGELIISPV
P.berghei      VPISEVEYNNKDKIE---|-------KNKYIDQLVVVCGWSKAVRKQGGGRFCFVNLNDGSCHLNLQIVVNQNIDNYDKLLKCGIGCCFRFTGTLILSPV
P.chabaudi     VPMSENEYNNKDKME---|-------KNKYIDQLVVVCGWSKAVRKQGGGRFCFVNLNDGSCHLNLQIVVNQNIENYDKLLKCGIGCCFRFTGTLILSPV
P.yoelii       APISETEYNNKDKIE---|-------KNKYIDQLVVVCGWSKAVRKQGGGRFCFVNLNDGSCHLNLQIVVNQNIDNYDKLLKCGIGCCFRFTGTLILSPV
P.knowlesi     VPKSEGEFNDTSR-----|-------KNKYIDQIITVCGWSKAVRKQGGGRFCFVNLNDGSCHLNLQIIVDQSIQNYEKLLKCGVGCCFRFTGKLIVSPV
P.vivax        VPKSEAEFNDSSR-----|-------KNEYIDQVITVCGWSKAVRKQGGGRFCFVNLNDGSCHLNLQVIVDQSIDNYEKLLKCGVGCCFRFTGKLIISPV
H.sapiens      NLEEAAKKITIKNDPSLPEYLTGVKIRDLVKHRNERVCIKGWIHRMRRQGKS-LMFFILRDGTGFL-QVLLMDKLCQTYDALT-VNTECTVEIYGAIKEVPE
B.malayi       ALEEAAKKVQIELDTSLSYLTGVKIRDLVKHRNERVCIKGWIHRMRRQGKS-LMFFILRDGTGFL-QVLLMDKLCQTYDALT-VNTECTVEIYGAIKEVPE
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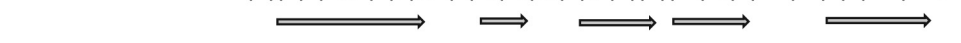
Linker   **Motif I**

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P.falciparum   QNDNNKKGLLKENVELALNNNDIHNFEIYGENLDPQKYPLSKKNH|GKEFLREVAHLRPR|SYFISSVIRIRNSLSIATHLFFQSRGFLYIHTPLITTSDCE
P.berghei      QNT-EKKGLLNENVELTLTDNSIHSFEIYGENLDPQKYPLSKKNH|GKEFLREVAHLRPR|SYFISSVMRIRNALMLSTHLFFQSRGFICIHTPLITASDCE
P.chabaudi     QNT-EKKGLLNENVELTLTDNSIHSFEIYGENLDPQKYPLSKKNH|GKEFLREVAHLRPR|SYFISSVMRIRNALMVSTHLFFQSRGFICIQTPLITTSDCE
P.yoelii       QNT-EKKGLLNQNVELTLTDNSIHSFEIYGENLDPQKYPLSKKNH|GKEFLREVAHLRPR|SYFISSVMRIRNALMLSTHLFFQSRGFICIQTPLITTSDCE
P.knowlesi     QNE-GEKGLIKENVELSLKDNSIHNFEIYGENLDPQKYPLSKKNH|GKEFLREVAHLRPR|SYFISSVIRIRNALAIATHLFFQSRGFLYIHTPLITTSDCE
P.vivax        QNE-EKKGLLKENVELALKDNSIHNFEIYGENLDPQKYPLSKKNH|GKEFLREVAHLRPR|SYFISSVIRVRNALATATHLFFQSRGFLYVHTPLITTSDCE
H.sapiens      GKQ------APGGHELSCDFWELIGLAPAGGA-DNLNEES----|DVDVQLNNRHMMIRG|ENMSKILKARSMVTRCFRDHFFDRGYYEVTPPTLVQTQVE
B.malayi       GKE------APNGHELIADFWKIIGNAPPGGI-DNVNEEAS----|VDKMLDNRHLVIRG|ENAAALLRLRAAATRAMREHFYNAGYLEVAPPTLVQTQVE
                :        ***  :    *   *    *        *        * :    *:    *   *  : : :. *: : .* :. :: *
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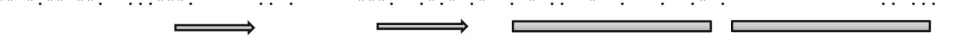
LCR

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P.falciparum   GGGEMFTVTTLLNENGDIRSIPRINLKNKKKEKREDILNEKNGKKDHMNDSLNNNTCNNNNNNGNSSSSNIVSSPQYED---------NYLIDYKKDFFS
P.berghei      GGGEMFTVTTLFNEHGDISSIPKTKKKINNNTGKREDKNANEPINTNELANTTNNTNTNELETNNTN--------------------QYIVDFKKDFFS
P.chabaudi     GGGEMFTVTTLLNENGDISAIPKTKKKTSNNQTVKREDQTTNNNTNANEPINTNELANTTNNTN------------------EYVIDYKKDFFS
P.yoelii       GGGEMFTVTTLFNEEGDINSIPKTKKKINNNQIVKREDQEATHAADVGGDESRSGNAHESSAQTNNNTN------------------QYIVDFKKDFFS
P.knowlesi     GGGEMFTVTTLLGEDADYSAIPRVKKLAKEGKKREDIQQSAGQSAGQSASVGADTHGAPPAPTATHAADVGGDESRSGNAHESSAQPTQYLIDYKKDFFS
P.vivax        GGGEMFTVTTLLGEDADYGAIPRVRKAVKGGQKREDNVQSAGQSAGQSASVGADTHGAPPA-----------------------------QYLIDYKKDFFN
H.sapiens      GGATLF------------------------------------------------------------------------------------------KLDYFG
B.malayi       GGSTLF------------------------------------------------------------------------------------------NLDYFG
                **. :*                                                                                      : *:*.
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Motif II

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P.falciparum   KQAFLTVSGQLSLENLCSSMGDVYTFGPTFRAENSHTSRHLAEFWMIEPEIAFADLYDNMELAEAYIKYCIDYVLNNNFHDIYYFEENVETNLIKRLKNI
P.berghei      KQAFLTVSGQLSLENLCSSMGDVYTFGPTFRAENSHTSRHLAEFWMIEPEMAFADIYDNMELAEAYIKYCIRYVLNNNFHDIYYFEENVEKGLIERLKNI
P.chabaudi     KQAFLTVSGQLSLENLCSSMGDVYTFGPTFRAESSHTSRHLAEFWMIEPEMAFADIYDNMEVAEAYIKYCIRYVLDNHFHDIYYFEENVEKGLIDRLKNI
P.yoelii       KQAFLTVSGQLSLENLCSSMGDVYTFGPTFRAENSHTSRHLAEFWMIEPEMAFADIYDNMEVAEAYIKYCIRYVLNNNFHDIYYFEENVEKGLIDRLKNI
P.knowlesi     KQAFLTVSGQLSLENLCSSMGDVYTFGPTFRAENSHTSRHLAEFWMIEPEIAFADLYDNMELAESYIKYCIGYVLSNHFDDIYYFEKNVENGLISRLKNV
P.vivax        KQAFLTVSGQLSLENLCSSMGDVYTFGPTFRAENSHTSRHLAEFWMIEPEIAFADLYDNMELAESYIKYCIGYVLKNHFDDIYYFEENVEKGLISRLRNI
H.sapiens      EEAFLTQSSQLYLETCLPALGDVFCIAQSYRAEQSRTRRHLAEYTHVEAECPFLTFDDLLNRLEDLVCDVVDRILKSPA-----------GSIVHELNPN
B.malayi       EQSFLTQSSQLYLETCIPTLGDVFLHCSVLQGGKISHSSTLAEYAHVEAECPFITLDDLMEKIEELVCDTVDRLLADEE----------AKKLLEHINPK
                :::***  *.** **.  .: :***:         :. .      ***: :*.* .*  : * ::  *   :    : :*.  .    :: .:.
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P.falciparum   L---NEDFAKITYTNAIEILQNYSDSFE--VKVEWGMDLQSEHERFIAEKIFKKPVIVYNYPKDLKAFYMK-LNEDNKTVAAMDVLVPKIGEVIGGSQRE
P.berghei      L---NDDFAKITYTNAIELLTKYSNNFD--VKVEWGMDLQSEHERFIAEQIFKKPVIVYNYPKDLKAFYMK-LNDDNKTVAAMDVLVPKIGEVIGGSQRE
P.chabaudi     L---NDDFAKITYTNAIDLLTKYSTNFD--IPVKWGMDLQSEHERFIAEQIFKKPVIVYNYPKDLKAFYMK-LNDDQKTVAAMDVLVPKIGEVIGGSQRE
P.yoelii       L---NDNFAKITYTNAIDLLTKYSNNFE--VPVKWGMDLQSEHERFISEQIFKKPVIVYNYPKDLKAFYMK-LNDDNKTVAAMDVLVPKIGEVIGGSQRE
P.knowlesi     L---DEKFAKITYTNVIDLLLPYSEKFE--VPIKWGMDLQSEHERFVAEQIFKKPVIVYNYPKDLKAFYMK-LNEDQKTVAAMDVLVPKIGEVIGGSQRE
P.vivax        L---DENFAKITYTNVIDLLMPFSNQFE--VPVKWGMDLQSEHERFVAEQIFKKPVVVYNYPKDLKAFYMK-LNEDQKTVAAMDVLVPKIGEVIGGSQRE
H.sapiens      FQPPKRPFKRMNYSDAIVWLKEHDVKKEDGTFYEFGEDIPEAPERLMTDTI-NEPILLCRFPVEIKSFYMQRCPEDSRLTESVDVLMPNVGEIVGGSMRI
B.malayi       FQPPERPFLRMEYKDAIKWLQEHNVENEFGNTFFTYGEDIAEAAERFMTDTI-NKPILLNRFPSEIKAFYMQRDAQDNTLTESVDLLMPGVGEIVGGSMRI
                .  * ::  *.:* *  ..     :*  *:.      :*.  *::  .: *.*::*:*:* :**::*** *
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Motif III

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P.falciparum   DNLERLDKMIKEKKLNIDSYWWYRQLRQYGSHPHAGFGLGFERLIMLVTGVDNIKDTIPFPRYPGHAEF
P.berghei      DNLELLDKMIIEKKLNIESYWWYRQLRKYGTHPHSGFGLGFERLIMLVTGVDNIKDTIPFPRYHGHAEF
P.chabaudi     DNLELLDKMIVEKKLNIESYWWYRQLRFGTHPHSGFGLGFERLIMLVTGVDNIKDTIPFPRYHGHAEF
P.yoelii       DNLELLDKMINEKKLNIESYWWYRQLRKYGTHPHSGFGLGFERLIMLVTGVDNIKDTIPFPRYHGHAEF
P.knowlesi     DNLERLDKMILEKKLNMESYWWYRQLRKFGSHPHAGFGLGFERLIMLVTGVDNIKDTIPFPRYPGHAEF
P.vivax        DNLELLDKMLNMESYWWYRQLRKFGSHPHAGFGLGFERLIMLVTGVDNIKDTIPFPRYPGHAEF
H.sapiens      FDSEEILAGYKREGIDPTPYYWYTDQRKYGTCPHGGYGLGLERFLTWILNRYHIRDVCLYPRFVQRCTP
B.malayi       WKFDELSKAFKNVEIDPKPYYWYLDQRLYGTCPHGGYGLGLERFICWLTNTNHIRDVCLYPRFVGRCVP
                . :  . . :: .*:** : * *: **.*:***:**::   :  .*:*. :**: :.
```

Catalytic domain

TABLE 1

Kinetic parameters of *P. falciparum* and *H. sapiens* AsnRSs the ATP/PP$_i$ exchange reaction

Results represent averages of at least three independent experiments.

AsnRS	ATP			Asn		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	$s^{-1} \mu M^{-1} 10^{-3}$	μM	s^{-1}	$s^{-1} \mu M^{-1} 10^{-3}$
P. falciparum	740 ± 240	10.1 ± 3.37	13.6	40 ± 40	4.2 ± 2.63	105
H. sapiens	970 ± 320	5.0 ± 1.58	5.2	460 ± 150	5.7 ± 1.09	12.4
E. coli (34)	500	2.2	4.4	40	2.8	70
CHO cells (35)				60		

domains were designed and constructed. However, they were not tested because recombinant mutant proteins were not soluble (data not shown).

Physicochemical Analysis of *P. falciparum* and *H. sapiens* AsnRSs by Dynamic Light Scattering

Dynamic light scattering spectroscopy is a convenient and non-invasive method for protein sizing (33) and has been applied to measure the aggregation states of aaRSs (*e.g.* Ref. 20). Measured diffusion coefficients of *P. falciparum* and *H. sapiens* AsnRS samples showed that both recombinant enzymes are dispersed at the concentrations used (20 μM monomeric protein). Only a small portion of the human AsnRS (13%) was aggregated (particle diameter, 110 nm; data not shown). Analysis of dynamic light scattering data allowed the determination of the size of AsnRS particles. Distributions of scattered intensities indicated that the average diameter of the *P. falciparum* and the *H. sapiens* AsnRSs were 13.8 and 12.5 nm, respectively, which correspond to spherical particles of 295 and 210 kDa (*versus* 142 and 127 kDa, theoretically). Because the crystallographic structure of the *B. malayi* AsnRS is more elongated than spherical (27), these data indicate that, as expected, both AsnRSs organize as dimers.

Catalytic Constants for the ATP/PP$_i$ Exchange Activity

The apparent K_m and k_{cat} values for asparagine activation were determined under identical conditions for the *P. falciparum* and *H. sapiens* AsnRSs (Table 1). The kinetic parameters for both enzyme orthologues are similar to the values determined for the AsnRSs from *E. coli* and CHO cells (34, 35). However, the K_m for asparagine is 11-fold higher for the human AsnRS (460 μM) than for the other enzymes (40 μM for *P. falciparum* and *E. coli* AsnRS and 60 μM for CHO AsnRS).

Kinetic Analyses of tRNA Aminoacylation by *P. falciparum* and *H. sapiens* AsnRSs

Both recombinant *P. falciparum* and *H. sapiens* AsnRSs were tested for tRNA charging under the same conditions (Fig. 2). They aminoacylated their cognate tRNA^{Asn} transcripts at plateau levels of 65 and 35%, respectively (Fig. 2*C*). The apparent K_m for their cognate tRNA transcripts were the same, but

the velocity (apparent k_{cat}) was 13-fold lower for *P. falciparum* AsnRS than for *H. sapiens* AsnRS, significantly decreasing (13-fold) the catalytic efficiency of the parasite AsnRS (Table 2).

Despite the similarity between the *P. falciparum* and *H. sapiens* tRNA^{Asn} sequences (Fig. 2, *A* and *B*), cross-species aminoacylation was not efficient (Fig. 2*C*). The human enzyme did not recognize the plasmodial tRNA^{Asn} transcript, and the extent of asparaginylation of the human tRNA^{Asn} transcript by the *P. falciparum* AsnRS was reduced 8-fold compared with its cognate tRNA^{Asn}. The only remarkable feature distinguishing these two tRNAs is the presence of a UU mismatch at base pairs 4–69 in the acceptor arm of the *P. falciparum* tRNA^{Asn}; it is conserved in all plasmodial tRNA^{Asn} sequences (Fig. 2*B*), which is an indication that the acceptor stem might play a role in this species-specific barrier. However, a human tRNA^{Asn} mutant, where the UG base pair was replaced by UU did not show any improvement in its aminoacylation capacity with *P. falciparum* AsnRS (data not shown).

Expression of Asparaginylation Molecular Partners and Comparison with Other Aminoacylation Systems in *P. falciparum*

tRNA Concentrations—We investigated the expression levels of tRNA^{Asn}, tRNA^{Tyr}, tRNA^{Asp}, and tRNA^{Lys} in non-synchronized *in vitro* cultures of *P. falciparum*. Transfer RNA^{Tyr} and tRNA^{Asp} are unique isoacceptors, which are each encoded by a single tRNA gene similar to tRNA^{Asn}, but they decode fewer codons (4.4 and 6.4%, respectively) than tRNA^{Asn} (11.2%) (Codon Usage Database). On the contrary, frequencies of asparagine and lysine codons are comparable (11.2 and 10.4%, respectively), but tRNA^{Lys} is encoded by two tRNA genes (tRNA$^{Lys}_{AAA}$ and tRNA$^{Lys}_{AAG}$). Northern blots and aminoacylation detection showed that two of the four selected tRNAs, tRNA^{Asn} and tRNA^{Tyr}, are globally expressed at the same level, independent of their use in translation (Fig. 3). Logically, the two tRNA^{Lys} genes result in twice as much tRNA^{Lys} compared with either tRNA^{Asn} or tRNA^{Tyr}. Surprisingly, tRNA^{Asp} appeared ~3-fold more concentrated than the others, despite only one copy of the tRNA^{Asp} gene.

aaRS Expression—RNA-Seq coverages from different *P. falciparum* development stages (18) were used to estimate the

FIGURE 1. Multiple alignments between plasmodia, *H. sapiens*, and *B. malayi* AsnRS sequences. Sequence alignments between *P. falciparum* (PF3D7_0211800), *P. berghei* (PBANKA_030860), *P. chabaudi* (PCHAS_031080), *P. yoelii* (PY05639), *P. knowlesi* (PKH_040840), *P. vivax* (PVX_002940), *H. sapiens* (NP_004530.1), and *B. malayi* (XP_001898693) AsnRSs were performed with Tcoffee and were further refined by comparisons between the crystal structure of the *B. malayi* AsnRS (27), and the secondary structure prediction of *P. falciparum* AsnRS (on the PredictProtein website) (data not shown). Secondary structures related to the *B. malayi* AsnRS crystal structure are indicated *below* the alignment. *Cylinders* correspond to α-helices, and *arrows* indicate to β-strands. *Dashed lines* delineate the N-terminal extensions containing the putative RNA-binding motif, the tRNA anticodon recognition domain, the linker, and the C-terminal catalytic domains. The class II-specific motifs are *boxed* in *gray*, and the plasmodia-specific insertions are *boxed* in *black*. The chemokine-like activity of *B. malayi* AsnRS involves the *underlined* and *boldface* residues located in its 80-amino acid N-terminal domain (28).
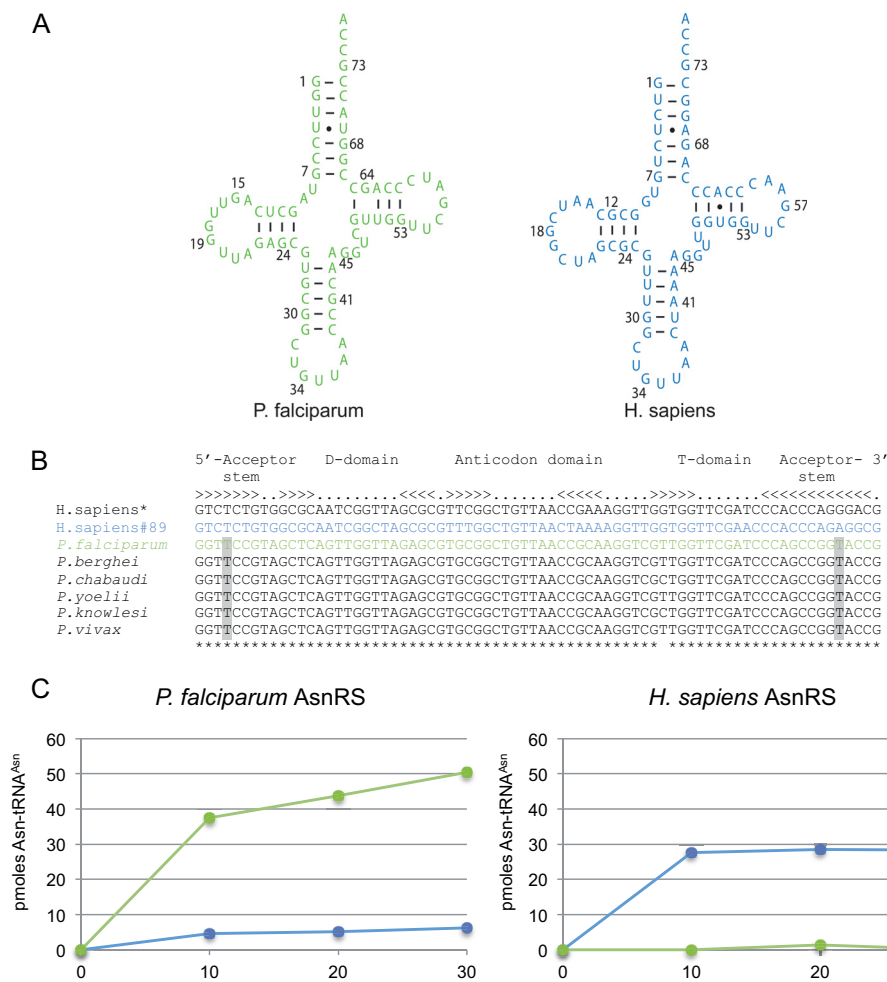
FIGURE 2. **Sequence, secondary structure, and aminoacylation plateaus of *Plasmodium* and *H. sapiens* tRNA^Asn transcripts.** *A*, cloverleaf structure of tRNA^Asn transcripts: the motifs involved in tRNA folding are present in both the plasmodial (*green*) and the human (*blue*) tRNA^Asn sequences. The few differences are spread across their sequences and do not involve residues strategic to the structure and are presumed to be harmless for folding and aminoacylation. *B*, sequence alignments of human and plasmodial tRNA^Asn sequences: the human genome codes for 35 tRNA^Asn genes partitioned into two isoaccepting families either with an AUU anticodon (two genes coding for a unique isodecoder tRNA sequence) or a GUU anticodon (GtRNAdb, Genomic tRNA Database). The GUU isoacceptor tRNA family contains 14 different isodecoders encoded by the 33 other genes. Among them, the sequence of the major tRNA^Asn isodecoder (*asterisk*) is represented 11 times. This sequence was first chosen to produce the human tRNA^Asn transcript *in vitro* but was a poor aminoacylation substrate. Another sequence was thus chosen, tRNA^Asn-89, whose transcript is a better substrate for the human AsnRS. The sequences of plasmodial tRNA^Asn are highly conserved, except for a U-G base pair that becomes the C-G in the T-stem of *P. chabaudi* and *P. knowlesi* tRNA^Asn. The $U_4 \cdot U_{69}$ mismatch conserved in the acceptor stem is highlighted in *gray*. *C*, aminoacylation plateaus of tRNA^Asn transcripts. Reactions were performed in the presence of 80 pmol of transcript and 25 pmol pf of *P. falciparum* AsnRS or 3.5 pmol of *H. sapiens* AsnRS. The color code is the same as described in *A*.

TABLE 2

Kinetic parameters of *P. falciparum* and *H. sapiens* AsnRSs for tRNA transcript aminoacylation

Results represent averages of at least three independent experiments. nd indicates not detectable.

	P. falciparum AsnRS			*H. sapiens* AsnRS		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
	μM	min^{-1}	$min^{-1}\ \mu M^{-1}\ 10^{-3}$	μM	min^{-1}	$min^{-1}\ \mu M^{-1}\ 10^{-3}$
tRNA^Asn transcripts						
P. falciparum WT	2.0 ± 0.82	0.5 ± 0.17	252	nd	nd	nd
H. sapiens WT	6.0 ± 0.28	0.2 ± 0.06	31	2.0 ± 1.12	6.7 ± 3.73	3350

relative expression levels of AsnRS compared with other aaRSs in *P. falciparum* (Fig. 4*A*). Concentrations of mRNAs varied from one aaRS to another. For example, trophozoites had 300-fold less ValRS mRNA than TyrRS mRNA. They also differed from one developmental stage to another: the mRNA encoding AsnRS was 4-fold higher in trophozoites than it was in gametocytes. This mRNA belongs to a group of three aaRS mRNAs (AsnRS, SerRS, and TyrRS) that are, on average, more concentrated than the remaining 18 aaRS mRNAs (Fig. 4*A*). Thus, the AsnRS mRNA has a propensity to accumulate noticeably; however, it is not the most abundant mRNA at any stage growth.

Amino Acid Availability—Based on genome analysis, *Plasmodium* possesses biosynthetic pathways for Asn, Glu, Asp, Gly, Pro, and Gln (36). However, its growth depends on a supply of a complete set of exogenous amino acids. Thus, during the blood stage, *Plasmodium* development relies mostly on hemo-
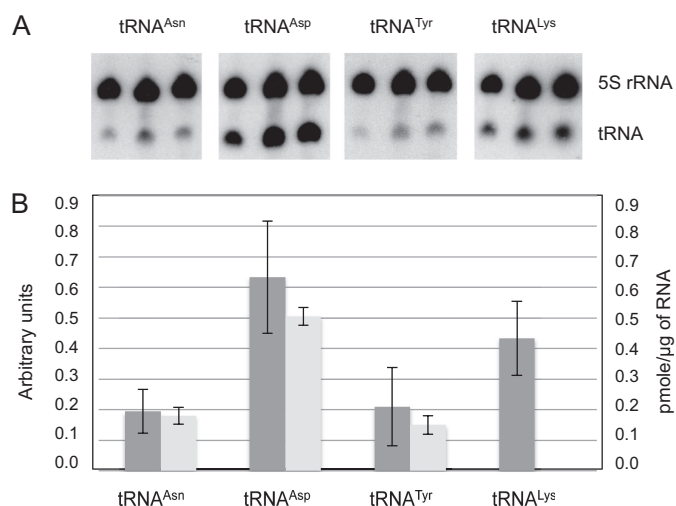
FIGURE 3. **Quantification of *P. falciparum* selected tRNAs.** *A*, total RNA was purified from blood stage *P. falciparum* and tRNA^{Asn} (one isoacceptor), tRNA^{Asp} (one isoacceptor), tRNA^{Tyr} (one isoacceptor), and tRNA^{Lys} (two isoacceptors) were detected by Northern blot. *B*, their relative concentrations were estimated by (i) Northern blot quantification (*dark gray*) and (ii) by determining aminoacylation plateaus in presence of the corresponding recombinant AsnRS, TyrRS, and AspRS (*light gray*).

globin catabolism and circulating amino acids (37). Based on the amino acid concentrations measured in human blood, the amount of asparagine available for translation in *Plasmodium* corresponds to 3.5% of all amino acids (Fig. 4*B*).

Asparagine Content of P. falciparum Stage-specifically Expressed Proteins

Data from stage-specific proteomes were retrieved from Ref. 19. They relate to four distinct stages of the parasite: sporozoites (the infectious form injected by the mosquito), merozoites (the invasive stage of the erythrocytes), trophozoites (the form multiplying in erythrocytes), and gametocytes (the sexual stage). For each stage, amino acid contents and amino acid repeats were determined. Global proteome asparagine content is not strictly conserved among the different stages; for example, there is 1.8-fold more asparagine in sporozoite proteins than in trophozoites proteins (Fig. 5*A*). This observation is strengthened when looking at the frequency of asparagine repeats (Fig. 5*B*): asparagine repeats are more abundant in sporozoites (2.7- and 4.2-fold) and in gametocytes (2- and 3-fold) than in merozoites and trophozoites, respectively, and longer. Glutamate and valine contents are also stage-specific, but unlike asparagine, these amino acids do not cluster in long repeats (data not shown).

DISCUSSION

AsnRS Activity

aaRSs are diverse in sequence, size, and structure but perform the same function: they catalyze specific tRNA aminoacylation (17). In turn, the efficiency and accuracy of these reactions govern the efficiency and accuracy of protein synthesis. Crystal structures of *Thermus thermophilus* and *B. malayi* AsnRSs have been reported (27, 38). Yet, biochemical and enzymatic studies are rare. The only exception relates to the *T. thermophilus* asparaginylation system (39).

In the present work, we studied *P. falciparum* AsnRS and its host *H. sapiens* AsnRSs to directly compare both enzymes. Neither the plasmodial nor the human enzymes are optimal in charging their cognate tRNA^{Asn} transcripts *in vitro*, as compared with the asparaginylation kinetics measured for the *T. thermophilus* system with native tRNA^{Asn}. The kinetic parameters determined for the prokaryotic system are 2000- and 150-fold more efficient than the parasite and the human systems, respectively. Similarly, the K_m of the CHO AsnRS for its cognate tRNA^{Asn} (35) is ~30-fold lower (0.06 μM) than the apparent K_m values determined in the present study (2 μM). These differences could be explained by the use of *in vitro* transcripts in this study, which are deprived of post-transcriptional modifications. Occasionally, these modifications have been shown to be involved in tRNA aminoacylation (*i.e.* Ref. 40). In particular, the absence of a queuosine at position 34 in both the *Plasmodium* and the human tRNA^{Asn} could explain the reduced K_m and k_{cat} values. The presence of two genes coding for putative queuosine tRNA ribosyltransferases in the *P. falciparum* genome (PF3D7_1242200, probably targeted to the apicoplast, and PF3D7_0717400, the putative cytosolic enzyme) support the existence of this particular post-transcriptional modification in *P. falciparum* tRNA^{Asn}, as it is the case in all organisms except yeast (41).

tRNA^{Asn} Availability

The genomes of most organisms display multiple copies of tRNA genes; the number of copies for each tRNA isoacceptor diverges from species to species (42). It is accepted that (i) tRNA gene content determines the relative amount of each tRNA in the cell, (ii) the most abundant codons are decoded by the most abundant tRNAs, and (iii) translation efficiency is governed by the relative abundance of each tRNA (reviewed in Ref. 43). In this context, the fact that *P. falciparum* but also all other plasmodia genomes encodes only one gene copy per tRNA isoacceptor is confusing. Assuming that each gene is transcribed to similar extents, one can calculate the theoretical availability of *Plasmodium* tRNA species compared with their codon usage in *P. falciparum* protein synthesis (Fig. 4*C*). Strikingly, unlike other organisms, there is no correlation between the number of tRNA genes and its codon usage in this parasite (or the related amino acid frequency). For example, the ratios of tRNA gene copy number to amino acid frequency varies at most by 4-fold among all *H. sapiens* tRNA/amino acid pairs. In contrast, in *P. falciparum*, these ratios vary over a 20-fold range, with the lowest value assigned to tRNA^{Asn} and asparagine (Fig. 4*C*). Thus, tRNA^{Asn} is the most limiting tRNA in the translation of *P. falciparum*. This hypothesis was confirmed by Northern blots and aminoacylation tests on total RNA isolated from *in vitro* cultures of different *P. falciparum* blood stages (Fig. 3). In these experiments, tRNA^{Asn} did not accumulate more than any of the three other tRNAs tested (tRNA^{Asp}, tRNA^{Lys}, and tRNA^{Tyr}). Because the codons that are decoded by tRNA^{Asn} are highly represented in the *Plasmodium* genome, translation efficiency from that particular tRNA is expected to be compromised. This scenario might be partially compensated for by an increased local concentration of this specific tRNA near the
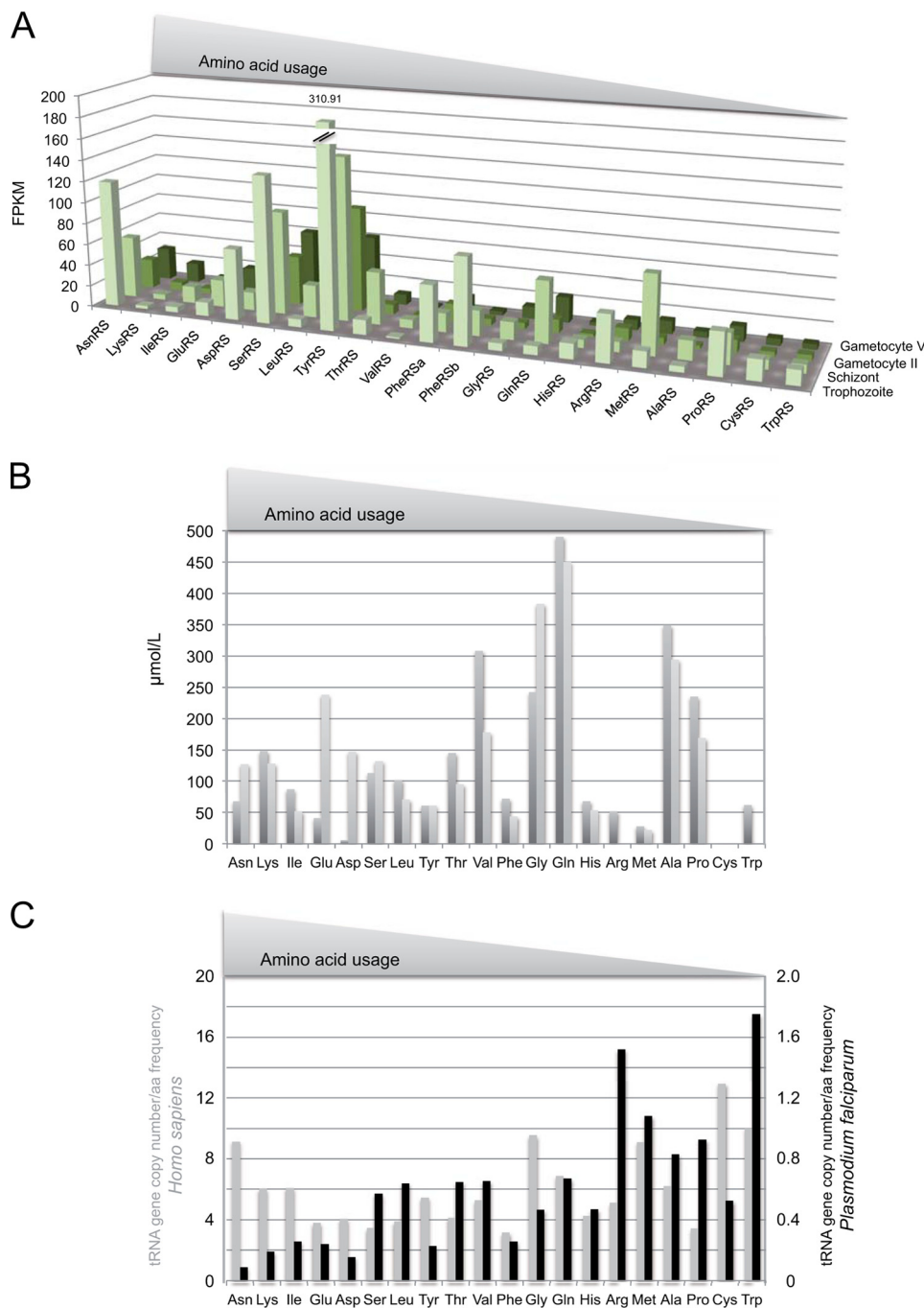
FIGURE 4. **Theoretical availability of aaRS mRNAs, amino acids, and tRNAs in *P. falciparum*.** *A*, RNA Seq data of aaRSs mRNA expression in sexual and asexual stages. Sequencing data of strand-specific cDNA libraries are from the laboratory of Xin-zhuan Su (18). The *y* axis corresponds to fragments per kilobase of exon model per million mapped reads (*FPKM*). Data were retrieved from the PlasmoDB website and map four different developmental stages: late trophozoite, schizont, gametocyte II, and gametocyte V. *B*, amino acid availability in the host blood. During the blood stage, the *Plasmodium* translational machinery uses a small portion of blood cell amino acids produced by the degradation of the hemoglobin of erythrocyte (*light gray*) in particular; most of the exogenous amino acids come from the host plasma (*dark gray*). Amino acids concentrations in human blood are derived from Ref. 57. *C*, comparison of tRNA availability in *H. sapiens* (*gray*) and *P. falciparum* (*black*). *Bars* (arbitrary units) represent the ratio between the number of tRNA gene copies encoded by each genome (tRNA isoacceptor genes were combined, GtRNAdb, Genomic tRNA Database) and the corresponding amino acid frequency (Codon Usage Database). *Lower bars* correspond to the rarest tRNAs. Note that scales on the *y* axes are different for *H. sapiens* (*left*) and *P. falciparum* (*right*). *L*, liter.

translation site of asparagine stretches, as has been suggested for other organisms (44).

Asn-tRNA^{Asn} Availability

The regulating action of tRNA^{Asn} depends above all on the proportion of asparagine-charged tRNA^{Asn} available for

translation. Based on the RNA seq data, the mRNA encoding AsnRS is highly expressed in the cell, suggesting that, in the absence of specific post-transcriptional inhibitory mechanism, AsnRS would substantially accumulate in the parasite. Moreover, *P. falciparum* AsnRS shows an optimal affinity for asparagine (Table 1), indicating that even if the aspara-

gine concentration is getting low, it should not affect significantly the rate of the first step of the asparaginylation reaction. These observations favor efficient asparaginylation of the tRNA^{Asn} pool in *Plasmodium*.

Open Questions

Slippery Sequences?—The relative scarcity of tRNA^{Asn} in plasmodia could sequentially decrease the rate of Asn-tRNA^{Asn} selection at the ribosome A-site, trigger ribosomal frameshifting, and finally lead to a reading frameshift when translating

FIGURE 5. **Asparagine content of *P. falciparum* stage-specific proteins.** Stage-specific proteome data were retrieved from the study by Florens and collaborators (19). They correspond to four distinct stages of the parasite: sporozoites, merozoites, trophozoites, and gametocytes. *A*, asparagine frequencies were compared in stage specific proteins; *B*, asparagine repeats were sorted according to their length: five to nine consecutive residues (*light gray*), 10 to 19 residues (*dark gray*), and >20 residues (*black*).

repeated asparagine codons. Moreover, one can wonder whether the post-transcriptionally added queuosine modification in the tRNA^{Asn} anticodon would also influence the fidelity of asparagine insertion in asparagine repeats. It was previously shown that queuosine does not influence frameshifting in bacteria (45) or when decoding a specific sequence of the infectious bronchitis virus (46). However, this hypothesis has not been tested on the long AU-rich sequences that characterize *Plasmodium* mRNAs or in the presence of limiting concentrations of Asn-tRNA^{Asn}.

Asparagine Repeats and Protein Aggregation?—In *P. falciparum*, the presence of asparagine repeats could be deleterious because it leads to the expression of a large number of prion-like domains (~25% of the proteome) (47), which in turn make the corresponding proteins suitable for aggregation. Recently, it was shown that *P. falciparum* Hsp110c, with other chaperones, plays an essential role in overcoming the aggregation tendencies of asparagine-rich proteins (48). Furthermore, we propose that accumulation of such prion-like sequences could be permitted through the presence of charged amino acids, lysine, glutamate, and aspartate, which also cover >25% of the proteome. The presence of so many charged amino acids could, in theory, improve the production of more soluble proteins (49, 50).

Potential Regulatory Activities?—According to the models discussed above, the tRNA pool should remain the same not only in different cell types but also throughout their life cycle. As a matter of fact, recent measurements in yeast and human tissues show that the abundance of different tRNAs deviates from the copy number of the corresponding genes (51, 52). As a consequence, variations in tRNA levels during the parasite life cycle would offer an opportunity to regulate and induce wide variations in translation efficiency and could modulate the expression of stage-specific proteins depending on their content in asparagine repeats. Our analysis of *P. falciparum* stage-specific proteomes (19) supports this idea by revealing an increase in asparagine repeats in proteins expressed exclusively in sporozoites and gametocytes compared with erythrocytic stages. These differences suggest that translation in sporozoites and to a lesser extent in gametocytes requires more asparaginylated-tRNA^{Asn}. When looking at trophozoite and gametocyte stages, there is no apparent correlation between the concentra-
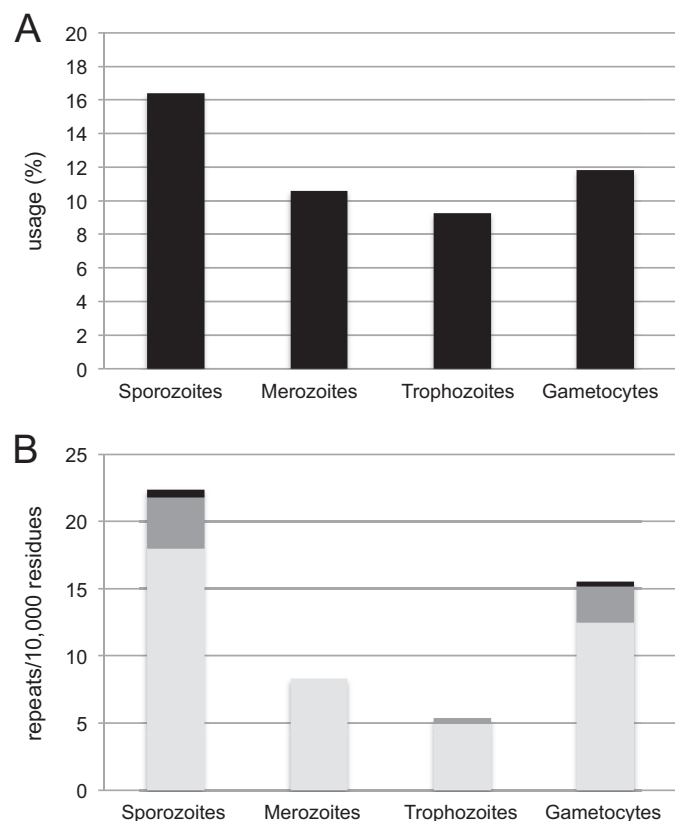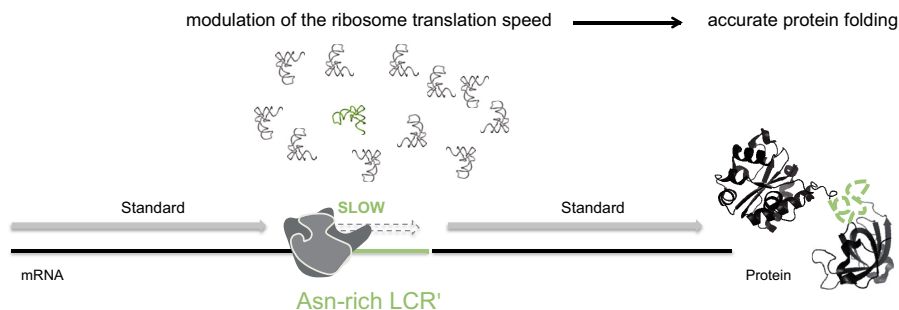

FIGURE 6. **Model for cotranslational folding of proteins in *Plasmodium*.** Asparagine-rich LCRs interspersed in plasmodial proteins likely act as intrinsic chaperones by delaying ribosome translation. Under limiting Asn-tRNA^{Asn} concentrations, translation of asparagine-rich sequences force ribosomes to slow down and allow cotranslational folding of proteins.

tions of mRNA for AsnRS (Fig. 4*A*) and the presence of long single amino acid repeats (Fig. 5). However, one should be aware that mRNA concentrations do not consider post-transcriptional regulatory mechanisms, which control the final cellular concentrations of these enzymes, at least in other organisms (53, 54).

tRNA^{Asn} Limitation and Cotranslational Folding of Proteins?— Our favorite hypothesis is that LCRs and asparagine repeats are intrinsic chaperones. Indeed, the low availability of Asn-tRNA^{Asn} should decrease the rate of translating ribosomes when decoding asparagine-rich LCRs and, in consequence, govern cotranslational folding of the proteins of the parasite: ribosome pauses would give enough time to the newly synthesized N-terminal domain to fold correctly, independently from the protein C-terminal sequence not yet translated. This scenario is consistent with our previous observation that LCRs exist in boundaries between independent folding modules in the aaRSs themselves (14). LCRs are inserted in almost all *Plasmodium* proteins. Their own translation spatially separates the synthesis of defined portions in these proteins (Fig. 6), allowing sequential folding of individual protein domains with translation pauses or slow downs in between. Furthermore, the presence of asparagine repeats in LCRs would increase local Asn-tRNA^{Asn} demand and further postpone translating ribosomes.

Conclusions

Impact on Protein Synthesis versus a Specific Function for Asparagine-rich LCRs—Single amino acid repeats have been found for all 20 amino acids in all three domains of life. Yet, plasmodia are distinct because of the number of amino acid repeats and because they are found in all classes of proteins. The AT content of the *P. falciparum*, *P. yoelii*, *P. chabaudi*, and *P. berghei* genomes definitely plays a part in the accumulation of asparagine repeats in their proteomes; it does not explain why these codons tend to cluster together in LCRs (6). However, the predilection for alanine repeats in *P. knowlesi* and *P. vivax* suggests that the presence of amino acids stretches is important, but the nature of the incorporated amino acid is not. This species-specific usage of amino acid repeats is a further indication that repeats (and LCRs) might not have acquired functions *per se* but that their presence is important for the proper function of the rest of the protein. We propose that differential accumulation of asparagine repeats could direct the correct folding and/or the stage specific expression of nascent *Plasmodium* proteins by controlling the rate of the translating ribosome. This could replace what exists in most organisms, where the tightness of secondary and tertiary structures in mRNAs controls ribosome binding and decoding rate (*i.e.* Refs. 55 and 56). One may indeed wonder whether AU-rich *Plasmodium* mRNAs can adopt such structures and use the same post-transcriptional regulatory strategies.

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