Distinctive features of aspartyl-transfer RNA (tRNA) synthetases (AspRS) from the protozoan Plasmodium genus are described. These apicomplexan AspRSs contain 29–31 amino acid insertions in their anticodon binding domains, a remarkably long N-terminal appendix that varies in size from 110 to 165 amino acids and two potential initiation codons. This article focuses on the atypical functional and structural properties of Plasmodium falciparum cytosolic AspRS, the causative parasite of human malaria. This species encodes a 626 or 577 amino acids AspRS depending on whether initiation starts on the first or second in-frame initiation codon. The longer protein has poor solubility and a propensity to aggregate. Production of the short version was favored as shown by the comparison of the recombinant protein with endogenous AspRS. Comparison of the tRNA aminoacylation activity of wild-type and mutant parasite AspRSs with those of yeast and human AspRSs revealed unique properties. The N-terminal extension contains a motif that provides unexpectedly strong RNA binding to plasmodial AspRS. Furthermore, experiments demonstrated the requirement of the plasmodial insertion for AspRS dimerization and, therefore, tRNA aminoacylation and other putative functions. Implications for the parasite biology are proposed. These data provide a robust background for unraveling the precise functional properties of the parasite AspRS and for developing novel lead compounds against malaria, targeting its idiosyncratic domains.

Aminoacyl-tRNA synthetases (aaRSs) are ubiquitous enzymes that attach amino acids to their cognate transfer RNAs (tRNAs) during protein synthesis. In ensuring high fidelity of tRNA aminoacylation, they maintain the genetic code and consequently contribute to cell viability in all forms of life. aaRSs are divided into two classes depending on the architectural organization of their catalytic domains and the way that they recognize their tRNA substrates. They have modular architectures which exhibit overall evolutionary conservation but also idiosyncratic features that indicate their phylogenetic origins (1). Such features are potential targets for aaRS inhibitors and, thus, for antipathogenic compounds. In addition to their important role in protein synthesis, aaRSs have developed a large panel of new functions during their evolutionary history that impact cell physiology and dysfunctions in ways that are not well understood (2, 3).

Within the aaRS family, aspartyl-tRNA synthetases (AspRS) are among the most thoroughly investigated for both structural and functional aspects (4–11). AspRSs are dimeric proteins that belong to class IIb synthetases. Their subunits have a conserved modular architecture in the three kingdoms of life with a C-terminal catalytic module linked by a short hinge domain to an N-terminal anticodon binding module. An additional domain is found on the N-terminal module of eukaryotic AspRSs that has been shown in the case of the Saccharomyces cerevisiae enzyme to help anchor the tRNA to the enzyme core (12) (see Fig. 1A). However, despite the large amount of data collected, our understanding of AspRSs is incomplete as enzymes originating from entire classes in the tree of life have not been studied. This is the case with eukaryotic AspRSs and, among them, those of the genus Plasmodium.

To date nothing is known about plasmodia AspRSs; in fact, all aaRSs (and translation in general) in the Plasmodium genus remain unstudied despite the acute medical importance of these parasites that cause malaria. Plasmodia belong to the Apicomplexa, a phylum of the alveolates ( unicellular protists) (13) close to fungi and plants (14). Among the several species that infect humans, Plasmodium falciparum causes the most deadly form of malaria. Each year it infects 600 million people and causes three million deaths (see the Malaria Foundation International website). Plasmodia contain three genomes; in their nuclei, in their mitochondria, and in their apicoplasts, a secondary plastid. The three complete genomes of P. falciparum have been sequenced, namely a 23-megabase nuclear genome divided between 14 chromosomes (15), a 6-kilobase mitochondrial genome (16) and a 35-kilobase apicoplast genome (17). Altogether 5300 protein genes were predicted, including ~35 aaRS genes of the highly conserved genes coding for proteins involved in translation (cytosolic and apicoplast), all encoded in the nuclear genome. The nuclear genome also contains 43 tRNA genes (Genomic tRNA Database) (18). Noticeably, the small mitochondrial genome is devoid of both aaRS and tRNA genes, whereas the apicoplast genome codes only tRNAs (19, 20). Thus, for translation to occur in the organelles implies the import of tRNA and aaRSs.
Based on known genomic sequences, this work analyzes the structure of plasmodial AspRSs and demonstrates their similarity to S. cerevisiae and Homo sapiens homologs. Interestingly, it also reveals peculiarities not previously observed in other AspRSs, in particular an insertion in the anticodon binding domain and an extra long N-terminal extension. The AspRS from P. falciparum was chosen for more thorough investigation. The protein was overexpressed in Escherichia coli and its distinctive physicochemical and biochemical properties were established and compared with those of its human host counterpart. The implications of these new findings are discussed.

**EXPERIMENTAL PROCEDURES**

*Data Base Screening for AspRS Sequences*—AspRS genes were identified by blasting the sequence of yeast cytoplasmic AspRS (21) against the PlasmoDB data base. Sequence alignments were computed with T-Coffee software (22).

*Parasite Culture*—3D7 strain parasites were cultured at 37 °C in 1% hematocrit as previously described (23). P. falciparum cultures were harvested with 12–15% parasitemia.

*Cloning and in Vitro Transcription of tRNA*—P. falciparum and human cytoplasmic tRNA_{Asp} were cloned downstream of a hammerhead ribozyme sequence under the control of the T7 RNA polymerase promoter (24), and transcription was performed as previously described (25). To improve self-cleavage activity (initially very low), the hybridization region between ribozyme and tRNA that defines the cleavage site was extended from 4 nucleotides (in human tRNA_{Asp}) to six nucleotides in P. falciparum cytosolic tRNA_{Asp}. Transcription reactions were performed at 60 °C for 1 h. Full-length transcripts with the correct CCA (amino acids 1–577) were purified by preparative 12% PAGE with 8M urea. Alternatively, transcripts were purified by native 12% PAGE (without separation of full-length tRNA and longer molecules). Concentration of transcripts was determined by UV absorbance at 260 nm. Before use, all tRNA transcripts were renatured (heating to 65 °C for 90 s followed by slow cooling to room temperature in Mg²⁺-containing solutions) following established procedures. Aspartylation assays were preferably conducted with H. sapiens tRNA_{Asp} transcripts (see “Results and Discussion” for details).

*Cloning and Production of AspRSs*—Primers for PCR were designed according to the sequence of the retrieved cytoplasmic P. falciparum AspRS gene. The gene was amplified from a cDNA library (provided by H. Vial, Montpellier). The PCR reaction was performed with a customized deoxynucleotide triphosphate (dNTP) mixture adapted to the P. falciparum genome composition (76% AT-rich): 240 μM dATP, 240 μM dTTP, 80 μM dCTP, and 80 μM gDTP. PCR elongation was performed at 65 °C, and the amplified DNA fragment was entirely sequenced and cloned into pGEX-2T (Amersham Biosciences) to yield an N-terminal glutathione S-transferase fusion protein. The pGEX-2T-P. falciparum AspRS plasmid was co-transformed with the pGro7 plasmid (TaKaRa Bio) into the E. coli strain ER2566, which expresses GroEL and GroES chaperones. Cultures were grown at 37 °C in Luria-Bertani medium in the presence of 2% glucose, 50 μg/ml ampicillin, and 50 μg/ml chloramphenicol with 0.25 mg/ml 1-arabinose (for induction of the chaperones) until an A_{600} of 2 (1 cm path length) was reached. The medium was then substituted with a solution lacking glucose, and AspRS expression was induced overnight at 18 °C with 0.25 mM isopropyl-β-D-thiogalactopyranoside. The fusion protein was purified on a glutathione-Sepharose resin according to the manufacturer’s instructions (Amersham Biosciences). AspRS was freed from the resin-bound glutathione S-transferase fusion by thrombin digestion: 50 units/ml of thrombin from bovine plasma (Sigma) for 3 h at 37 °C. The protein was dialyzed overnight at 4 °C against 50 mM KH₂PO₄/K₂HPO₄, pH 7.4, 10 mM β-mercaptoethanol, 150 mM KCl, and 50% glycerol buffer and diluted 3-fold in water before being loaded onto a UnOs ion exchange column (Bio-Rad). Fractions with AspRS were pooled and dialyzed under the same conditions.

Human cytoplasmic AspRS was amplified by PCR from pET28a-hRDs (provided by Dr. S. Kim) (26) and cloned into pGEX-2T. This construct was prepared and handled as previously described (27). Overexpression and purification were as for P. falciparum AspRS. Full-length AspRS from S. cerevisiae (amino acids 1–577) was prepared as previously described (12).

PCR mutagenesis was used to introduce mutations or deletions in the AspRS RNA binding motif (within the lysine-rich 70–78 region) and the Plasmodium-specific insertion (residues 129–159). Constructs were sequenced and subcloned into the pGEX-2T expression vector. The wild-type sequences 7KKKEKKAKK₇₈ (helical part in the RNA binding motif) and 12₉SIMELYNLEDY¹⁴₀ (N-terminal domain in the plasmoidal insertion) were replaced with 7₀QEREEGQM₇₈ and 1₂₉DSGI-IKFIRSYI¹⁴₀, and the entire plasmoidal insertion (129–160) was deleted. Overexpression and purification of mutants were performed as for wild-type AspRS.

AspRS proteins were quantified by the Bradford assay (Bio-Rad) and stored at −20 °C in the dialysis buffer. The preparations yielded only one band on SDS polyacrylamide gel and were estimated to be at least 95% pure.

*3’-Rapid Amplification of cDNA Ends PCR*—The P. falciparum cDNA library was cloned into the Triplex vector (Clontech) with M13 forward primer hybridizing in the vector and a specific primer hybridizing at the 3’-end of the P. falciparum AspRS gene. Other PCR reactions used a second primer hybridizing in the Triplex vector combined with three other primers for positions +180, +330, and +540 in the AspRS sequence. Reactions were run in a volume of 100 μl containing 2 units of Dynazyme EXT (Finzymes) with the corresponding buffer, 1 μM concentrations of each oligonucleotide, 160 μM concentrations of each dNTP, and 1.5 mM MgCl₂.

*Western Blot*—Infected human erythrocytes (12–15% parasitemia) were lysed by 4 freeze-thaw cycles, and membrane debris was removed by centrifugation. Non-infected erythrocytes were prepared in the same way and served as negative controls. Different dilutions of the lysate supernatants were loaded on 12% SDS-polyacrylamide gels. The proteins were separated and transferred to Immobilon-P membrane (Millipore). Primary antibodies raised in rabbit against yeast AspRS
(cross-react efficiently with *P. falciparum* AspRS) were used, and signals were detected via a secondary antibody (Bio-Rad) and the enhanced chemiluminescence (ECL) Western blot kit (Amersham Biosciences).

**Light Scattering—**AspRS samples (25 and 15 μM for wild-type and mutated AspRSs, respectively) were incubated overnight in 35 mM phosphate buffer, pH 7.5, 100 mM KCl, 350 mM ammonium sulfate, 270 μM poly(dT) (~10-fold excess) in the presence of 0.2 mM dodecyl maltoside (at least twice the critical micellization concentration). Analyses were performed with a Zetasizer Nano S (Malvern). Five intensity measurements were recorded at 20 °C, and the data were processed with the manufacturer’s software (the particles were assumed to be spheres). Corrections for solvent refractive index and viscosity were applied, and the contribution of the solvent components (essentially micelles) was subtracted.

**ATP/PPi Exchange and tRNA Aminoacylation Assays—**ATP/PPi exchange was done in 100 mM Hepes-KOH, pH 7.4, 3 mM MgCl₂, 2 mM [³²P]PPi (1.5 cpm/pmol), 0.5 mM ATP, 2 mM aspartic acid, 2 mM KF, and 1.5 μg of AspRS. Incubations were at 37 °C, and the synthesized [³²P]ATP was measured as described (28). Aminoacylation of tRNA⁻Asp species was conducted at 37 °C in 100 mM Hepes-KOH, pH 7.4, 3 mM MgCl₂, 0.5 mM ATP, 15 mM KCl, 25 μM L-¹³⁴Aspartate (Amersham Biosciences) and appropriate amounts of tRNA and aaRS (25). Concentration of tRNA transcripts varied from 1- to 10-fold that of the Kₘ value. tRNA aspartrylation was determined by incubating 1 μM pure tRNA (wild type or transcript) or 2 μg of total RNA in the presence of 10 nM AspRS. Data were expressed as the averages of at least two independent experiments. Values of kₖcat/Kₘ ratios for replicate experiments varied at most by 20%. For rapid estimation of AspRS activity, tRNA aminoacylation of tRNA⁻Asp transcripts from *H. sapiens* was conducted with a large excess of aaRS. Values of aminoacylation plateaus were measured after 10, 20, and 30 min of incubation at 37 °C. Determination of Kₘ of small ligands (aspartate and ATP) for tRNA⁻Asp charging by AspRSs and determination of inhibition type and constant (Kᵢ) of the reaction by adenylate analogs were performed as previously described (11, 29).

**RESULTS AND DISCUSSION**

In *Silico* Identification of Plasmodial AspRSs—Eight plasmodial genomes have been completely or partially sequenced to date (PlasmoDB) and screened for AspRS genes. Sequences corresponding to complete genes were found in primate-infecting plasmodia (*e.g.* *Plasmodium* (*P.* reichenowi, *P. vivax*, *P. knowlesi*) and rodent-infecting plasmodia (*e.g.* *P. yoelii, P. berghei*). The identified AspRS genes did not appear to be spliced and coded for characteristic eukaryotic AspRS architectures (Fig. 1A).

Structure-based sequence alignments show highly conserved sequences (Fig. 1B), in particular in the C-terminal catalytic domain containing the signature motifs of class II aaRSs (30, 31). The strictly conserved residues in motif 1 and motif 2 involved in dimerization and binding of the small ligands are present (*e.g.* Pro⁷⁳⁹ in motif 1 is important for dimerization (32) and Arg⁹⁳¹ in motif 2 is important for adenylate binding (33)). Motif 3 and the flipping loop exhibit eukaryotic characteristics (8, 34) and the large eubacterial insertion (~150 amino acids (aa)) downstream motif 2 is missing. The hinge domain between anticodon-binding domain and catalytic domain comprises ~30 aa.

Interestingly, one notices the repeated presence of an insertion in the first half of the anticodon binding domain (in the first loop of its oligonucleotide/oligosaccharide binding fold architecture (35)). This insertion was not identified so far in the AspRSs of other phylogenetic origins and likely does not disturb the oligonucleotide/oligosaccharide binding fold architecture. It varies in sequence in the different *Plasmodium* species and can be divided into two distinct elements, the first one (amino acids 129–140 in *P. falciparum* AspRS) being more conserved than the second one. Structure prediction (see the Predict Protein website) suggests the presence of helices in the conserved domain.

All plasmodial AspRSs exhibit N-terminal appendices that are predicted to fold as helical modules (Fig. 1B) and are longer than the well characterized appendix of yeast AspRS (12). Their sizes vary from 110 to 165 aa (110 in *Plasmodium* (*P.* reichenowi, 111 in *P. falciparum*, 114 in *P. knowlesi*, 116 in *P. vivax*, 165 in *P. berghei*, and 165 in *P. yoelii*, compared with 70 aa in *S. cerevisiae*). All contain a strictly conserved lysine-rich RNA binding motif (⁷⁰KKKEKKAKK⁷⁸) that resembles the yeast RNA binding helix (12). The absence of the appendix in *Plasmodium gallinaceum* and *Plasmodium chabaudi* AspRSs likely reflects incomplete sequencing of their genomes.

Finally, all *Plasmodium* AspRS genes present the same organization (Fig. 1C) with two potential initiation codons. A second ATG codon is located 144 nucleotides (nt) (*P. falciparum*, *P. reichenowi, P. knowlesi*), 150 nt (*P. vivax*), 315 nt (*P. yoelii*), and 330 nt (*P. berghei*) downstream of the first ATG. The use of the second ATG as initiator codon would lead to the synthesis of AspRSs with shorter N-terminal extensions (shortened by 48, 50, 109, or 105 aa at their N terminus, respectively). This would create 60–66 aa extensions (depending on *Plasmodium* genus) and would make them look more like the 70-aa appendix of yeast AspRS (12).

Idiosyncrasies in *P. falciparum* AspRS and Comparison with Yeast and Human AspRSs—Based on the structure of *S. cerevisiae* AspRS, two genes could be identified in the three genomes of *P. falciparum* (15–17). One corresponds to the cytosolic AspRS and is labeled PFA0145c in PlasmoDB. The second one was annotated as an AsnRS gene (PFE0715w) but corresponds to an AspRS gene with a rather degenerate sequence (AspRSs and AsnRSs belonging to class IIb aaRSs are both evolutionary and structurally related (1)). This AspRS gene was predicted to code for the apicoplast enzyme, whereas a gene that could code for the mitochondrial synthetase was not found.

The gene of cytosolic *P. falciparum* AspRS located on chromosome 1 codes for a 626-aa-long protein. Because the parasite infects man and is evolutionarily closely related to yeast, its cytosolic AspRS was compared with homologs from *S. cerevisiae* and *H. sapiens*. Structure-based comparison of the three AspRSs shows important sequence homology (33% of amino acids strictly conserved in the 116–626 AspRS core, excluding the *Plasmodium* insertion) (Fig. 2). The hinge region (43% conservation) and the C-terminal catalytic domain (40% conservation) are even more conserved. The three class II aaRS motifs as...
well as the AspRS-specific flipping loop are well conserved (34% in motif 1, 56% in motif 2, 60% in motif 3, and 100% in flipping loop) and display all AspRS-specific signature residues (highlighted by stars in Fig. 2). Overall sequence conservation (40%) is similar when the core of the parasitic AspRS is compared with either yeast or human AspRS.

Apparent parasite idiosyncrasies are found in the N-terminal half of the AspRS molecule (Fig. 2). The N-terminal extension of 110 aa (that varies in length in the Plasmodium genus, see Fig. 1B) differs significantly from the shorter appendices in yeast (70 aa) and even more so from human (24 aa) AspRS. The putative RNA binding motif within the appendix (amino acids 70–78) is absent in human AspRS (as well as in other mammals and in birds) and differs in sequence with that of yeast AspRS. Nevertheless, they are both lysine-rich and are predicted to adopt a helical conformation. These structural, sequence, and folding

**FIGURE 2.** Comparison of *P. falciparum*, *S. cerevisiae*, and *H. sapiens* cytosolic AspRS sequences (numbering is according to *P. falciparum* protein sequence). The sequence alignment perfectly agrees with the crystal structure of *S. cerevisiae* AspRS (35). The AspRS structural modules are highlighted by backgrounds alternatively white and gray. Characteristic class II motifs as well as the flipping loop (f.l.) are boxed. The RNA binding motif, absent in *H. sapiens* AspRS, and the insertion in the anticodon binding domain of *P. falciparum* are boxed. Stars indicate amino acids strictly conserved in all known AspRSs (8); potential amino acids involved in catalysis (for review and discussion, see Refs. 8 and 11) and interactions with tRNA<sup>Asp</sup> (58) are indicated by circles and arrows, respectively. The locations of mutations and deletions in *P. falciparum* AspRS are shown.
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similarities in the N-terminal extension of *P. falciparum* and *S. cerevisiae* AspRSs are in agreement with their close evolutionary relatedness.

The most typical feature is the plasmodial insertion in the anticodon binding domain that differentiates *P. falciparum* AspRS from its yeast and human homologs (as well as from other known AspRSs, Fig. 1B). This plasmodial insertion is 29–31 aa in length.

Production and Activity of Different *P. falciparum* AspRS Forms—The cytosolic AspRS gene was amplified by PCR using a *P. falciparum* cDNA library (36). This step was delicate because of the high proportion of A and T residues in the *Plasmodium* genome. Both forms (long and short) of *P. falciparum* AspRS gene and *H. sapiens* AspRS gene were cloned and fused to the 3′-end of the glutathione S-transferase gene. This strategy was chosen to facilitate rapid purification of the enzymes. To favor homogeneous folding, the corresponding fusion proteins were overexpressed in an *E. coli* strain co-expressing chaperones. Purified AspRS samples from *P. falciparum* (626 and 577 aa) and *H. sapiens* (500 aa) expressed in *E. coli* were characterized by SDS-PAGE together with AspRS from *S. cerevisiae* (557 aa) as a control (Fig. 3A). Although similar in overall architecture and sequence, the four AspRSs display different solubility properties. Although the short version of the parasite AspRS (starting at methionine 49) was easily recovered and concentrated, severe solubility problems were encountered with the long version (starting at methionine 1). This AspRS, when over-expressed in *E. coli* mainly remains in the pellets during the purification procedure, and only small amounts are present in the supernatants.

Electrophoretic migration of the short form of the parasite AspRS shows a unique band of ~66 kDa (lane M49 in Fig. 3A) that migrates slightly slower than yeast AspRS (64 kDa) and much slower than the human homolog (50 kDa). In contrast, recombinant full-length parasite AspRS migrates as a faint band (at most 10%) compatible with a 68-kDa protein (lane M1 in Fig. 3A). However, the presence of a major band, migrating like the short AspRS (M49), indicates a strong proteolysis of the full-length *P. falciparum* AspRS during purification. Thus, for straightforward *in vitro* investigations, all experiments were conducted with the short form of the parasite AspRS.

With a view to exploring structure-function peculiarities in *P. falciparum* AspRS, three recombinant variants were prepared with alterations in their eukaryotic and plasmodial specific regions (Fig. 2). Two variants were mutated, one in its putative RNA binding motif in the N-terminal appendix (70KKKEKKAKK78 replaced by 70QEEREEGQM78) and the second one in its parasite-specific insertion (the conserved part of the insertion 129SIMELYNLEDIY140 replaced by 129DSGIKFRSYIF140). In a third variant the complete insertion was deleted (residues 129–159). The three proteins could be over-expressed in *E. coli* and purified to homogeneity with the expected lengths.

Aminoacylation capacities of the *P. falciparum* AspRS samples, expressed as plateau aspartylation levels of purified tRNAAsp transcripts, are summarized in Table 1. The wild-type *Plasmodium* AspRS shows significant activity and charges *H. sapiens* tRNAAsp to ~70% (the value below 100% reflects the presence in the tRNA samples of inactive molecules that are misfolded or have heterogeneity in their 3′-terminal CCA sequences). The mutated AspRSs have poor activity or are com-
pletely inactive. Residual activity is only found for the AspRS mutated in its RNA binding motif. This incomplete aminoacylation does not exceed 7% and is explained by the fact that plateaus reflect the equilibrium between slow charging and the various deacylation reactions (37). When the plasmoidal specific insertion is mutated or deleted, the resulting proteins are completely inactive not only in aminoacylation (Table 1) but also in aspartyl-adenylate formation (not shown).

Identification of the Initiation Codon in the mRNA of *P. falciparum* AspRS and Size of Endogenous AspRS—Among the two in-frame ATG initiation codons present in all *Plasmodium* AspRS genes, only the second one fulfills the Kozak requirements. A purine at position −3 and a G residue at position +4 (−3RccATGG^+4) are important for efficient translation initiation in eukaryotes (38) (Fig. 1C). This suggests that endogenous synthesis of the parasite AspRS starts preferentially at the second in-frame initiation codon but does not exclude alternate possibilities. 3'-Rapid amplification of cDNA ends PCR experiments were designed to check whether the cDNA pool corresponding to the parasite mRNAs contains one or several AspRS mRNAs (with one or two AUG codons or both forms together). Sequencing of the amplified fragments clearly shows the existence of a unique AspRS mRNA containing the two in-frame AUG codons (Fig. 3B). This result leaves open the question about whether initiation of the parasite AspRS commences at either the first or second initiation codon or whether it commences at both.

To clarify this point, Western blot analyses were conducted to detect the endogenous AspRS in infected human erythrocytes. Because of sequence conservation it was anticipated that *P. falciparum* AspRS shares epitopes with *H. sapiens* and *S. cerevisiae* AspRSs and that anti-yeast AspRS antibodies would, therefore, detect the parasite AspRS. This proposition was confirmed in control experiments with recombinant *P. falciparum* AspRSs, and the Western blot results show that the endogenous form of the parasite AspRS migrates like the short recombinant protein synthesized in *E. coli* cells (Fig. 3C). However, a faint band is systematically detected and represents less than 5% that of the major band. It migrates as a slightly larger protein and, thus, likely corresponds to the AspRS initiated at the first AUG codon. Altogether, these experiments strongly suggest that the second AUG, fulfilling Kozak rules, contributes to the synthesis of the preponderant isoform of the synthetase.

**Physicochemical Characterization of *P. falciparum* AspRS by Laser Light Scattering**—Dynamic light-scattering spectroscopy is a convenient and non-invasive method for protein sizing under different solvent conditions (39) and has already been used to measure the aggregation state of aaRSs (e.g. Refs. 40 and 41). The technique measures distributions of particle diffusion coefficients (D) that can be easily transformed via the Einstein equation (D = kT/6πηR_p) into hydrodynamic radii (R_p) and, thus, apparent particle size. Measured diffusion coefficients of AspRS samples showed first that both *P. falciparum* AspRSs, wild-type (49–626) or displaying a mutated plasmodial insertion (within amino acids 129–140 in the most conserved part of the insertion where 129SIMELYNLEDIY^140 replaced by 129DSGIIFRYSYI^140), were aggregated at the protein concentrations necessary for these tests (15–20 μM) and that no detectable single AspRS particles were present in the samples under these conditions (Fig. 4A). The fact that wild-type AspRS displays aminoacylation activity (see Table 1) is explained by enzyme concentrations ~500-fold lower in aminoacylation assays than in dynamic light-scattering measurements, leading to the dissociation of aggregates. Similar behavior, although much less pronounced, was observed with yeast AspRS that has a tendency to aggregate and precipitate when concentration increases (42).

It was postulated that the structural versatility of the N-terminal extension was the cause of *P. falciparum* AspRS aggregation (*H. sapiens* AspRS with a short extension is monodispersed under the same conditions; data not shown). Consequently, salts, polyanions (poly(dT)), and a non-ionic detergent were added to try to reduce aggregation. Polyanions were used to specifically help the N-terminal extension to fold (43), whereas salts and detergent are commonly used to dissociate protein aggregates. Under these conditions the aggregates dissociate (Fig. 4B).

Analysis of the dynamic light-scattering data permitted the size of isolated AspRS particles to be determined. The distributions of scattered intensities indicate that the average diameter of the wild-type AspRS and the AspRS mutated in the plasmodial insertion were 11.7 and 8.7 nm, respectively, which correspond to spherical particles of 210 and 100 kDa (Fig. 4). Because in the crystal structure yeast AspRS appears to be more elongated than spherical (35), these data indicate that wild-type
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### TABLE 2

Comparison of kinetic parameters for aspartylation of human tRNA<sup>Asp</sup> transcripts by *P. falciparum* and *H. sapiens* AspRSs

| AspRS          | ATP/PP<sub>i</sub> | tRNA aspartylation | Kinetic constants for tRNA | Kinetic constants of small ligands |
|----------------|-------------------|-------------------|---------------------------|-----------------------------------|
|                | <i>K<sub>m</i></i> | <i>k<sub>cat</sub></i> | <i>k<sub>cat</sub>/K<sub>m</sub></i> | Asp | ATP | Asp-AMP | Asp-AMS |
| *H. sapiens* wild type (1–500) | 6.7 ± 0.4 | 160 ± 23 | 0.22 ± 0.03 | 1.2 | 16 ± 3 | 515 ± 110 | 330 ± 70 | 79 ± 15 |
| *P. falciparum* wild type (49–626) | 5.9 ± 0.5 | 18 ± 4 | 0.20 ± 0.05 | 15.3 | 210 ± 20 | 140 ± 30 | 800 ± 200 | 260 ± 70 |
| *P. falciparum* mutated RNA binding motif (K<sup>70</sup>KKKEKKK<sup>78</sup>→K<sup>70</sup>QEREEGAQM<sup>78</sup>) | 8.5 ± 1 | 2400 ± 400 | 0.01 ± 0.004 | 0.006 |

*P. falciparum* AspRS organizes as dimers (132 kDa), as is the case for all known AspRSs (8). In contrast, the mutated AspRS with an altered plasmodial insertion in the anticodon-binding domain is monomeric (66 kDa). Functional assays indicated that this mutant is inactive (see Table 1), in agreement with former studies on *E. coli* AspRS, which demonstrated that the monomeric state does not allow aspartylation (32).

**Kinetic Analyses of tRNA Aminoacylation by *P. falciparum* AspRS**—Both recombinant enzymes, short *P. falciparum* and *H. sapiens* AspRSs, have been tested for tRNA charging under the exact same conditions. They charge to high plateau levels transcripts of human tRNA<sup>Asp</sup> (see Table 1) as well as total parasite RNA (not shown). The similar charging behavior of homologous and heterologous tRNA<sup>Asp</sup> is explained by the high similarity of the *P. falciparum* and *H. sapiens* tRNA<sup>Asp</sup> sequences (see tRNA gene data base (44)). The structural motifs in tRNA governing conformation are conserved (45) (e.g. the α3 and β3 stretches around conserved G18G19 in the D-loop and the variable region with ν = 4). Furthermore, the aspartate identity set as determined in yeast tRNA<sup>Asp</sup>, namely G34, U35, C36, and C38 in the anticodon loop, G73 in the acceptor stem, and G10–U25 base pair in the D-arm (46, 47), is conserved in the plasmodial and human tRNA<sup>Asp</sup> species. The few differences spread across their sequences do not concern functionally relevant residues and are presumed to not affect aminoacylation. However, despite various attempts at renaturation, a large proportion of the *P. falciparum* transcript does not fold correctly as reflected by incomplete aminoacylation plateaus (below 25%) (data not shown). As observed in other systems (for review, see Ref. 10), this suggests that post-transcriptional modifications play a role in the folding of parasite tRNA<sup>Asp</sup>. As a consequence, *H. sapiens* tRNA<sup>Asp</sup> transcripts that fold better (more than 70% of the transcript is chargeable, see Table 1) were chosen as the preferred substrates for kinetics studies.

Interestingly, despite the use of the same tRNA substrate, the parasite and human AspRSs behave differently in *vitro* (Table 2). The velocity (k<sub>cat</sub>) stays the same, but the K<sub>m</sub> for the transcript is ~10-fold lower with *P. falciparum* AspRS than with *H. sapiens* AspRS, significantly increasing (13-fold) the aminoacylation efficiency of parasite AspRS (as estimated by k<sub>cat</sub>/K<sub>m</sub>). Compared with what has been measured with other aspartylation systems, the K<sub>m</sub> for charging tRNA<sup>Asp</sup> transcripts by *P. falciparum* AspRS was surprisingly low. Indeed, until now, 10 nM-range K<sub>m</sub> values have been observed with fully modified tRNA<sup>Asp</sup> (25). Although it is tempting to propose that the unusual kinetics of tRNA<sup>Asp</sup> charging by *P. falciparum* AspRS are due to the four sequence peculiarities (Asp<sup>188</sup>, Ser<sup>191</sup>, Lys<sup>192</sup>, Asp<sup>249</sup> for RNA binding, see Fig. 2) that differentiate it from the human and yeast AspRSs, these data could also be explained by the presence of a lysine-rich motif in the parasite AspRS N-terminal extension.

Functional impacts of the *P. falciparum* AspRS-specific domains within N-terminal and anticodon binding domains were tested by mutagenesis. The similarity in size and structure between the N-terminal extensions in yeast and parasite AspRSs suggest that these extra domains have the same or similar functions. Indeed, replacement of the lysine-rich RNA binding motif present in the plasmodial enzyme (K<sup>70</sup>KKKEKKK<sup>78</sup> replaced by K<sup>70</sup>QEREEGAQM<sup>78</sup>) leads to an AspRS with strongly affected kinetics. Contrary to the yeast enzyme where only the K<sub>m</sub> is changed (12), here both K<sub>m</sub> and k<sub>cat</sub> are drastically affected. The K<sub>m</sub> is increased more than 130-fold (100-fold in yeast AspRS), and the k<sub>cat</sub> is reduced by a factor of 20. As anticipated, mutations in this extra domain do not affect the conformation or activity of the catalytic site, as the rate of ATP/PP<sub>i</sub> exchange remains unchanged (5.9 versus 8.5 s<sup>−1</sup>, Table 2). This result indicates that the RNA binding motif present in the N-terminal extension of the *Plasmodium* AspRS plays a crucial role in anchoring the tRNA to the enzyme properly.

Finally, and in the perspective of seeking specific inhibitors of *P. falciparum* AspRS, preliminary comparative experiments were conducted to target the binding site of aspartyl-adenylate in parasite and human AspRSs. The apparent K<sub>m</sub> values of both AspRSs for aspartate and ATP binding in aspartylation of human tRNA<sup>Asp</sup> transcripts were determined in strictly identical conditions. Surprisingly, *H. sapiens* AspRS has a low K<sub>m</sub> for aspartate, close to values determined for prokaryotic AspRSs (i.e. 30 and 5 μM for AspRS 1 and 2 from *Thermus thermophilus* (48). This low K<sub>m</sub> for aspartate (16 μM) contrasts with a remarkably high K<sub>m</sub> for ATP (515 μM). Interestingly, the K<sub>m</sub> for aspartate is 13-fold higher (210 μM) for parasite AspRS (Table 2) and resembles the value for the yeast enzyme (400 μM) (49).

Although the K<sub>m</sub> for aspartate is considerably reduced in the *H. sapiens* AspRS compared with the *P. falciparum* enzyme, aspartyl-adenylate analogs (aspartol-AMP and Asp-AMS (50)) were used in inhibition tests. In both cases no significant difference between the synthetases could be detected (Table 2). With
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FIGURE 5. Model of the three-dimensional organization of dimeric P. falciparum AspRS in interaction with tRNA. The model is based on the crystal structure of the complex between yeast AspRS and cognate tRNAAsp (59). The overall AspRS structure is colored in gray, dark for catalytic domains (Cat1 and Cat2) and light for anticodon-binding domain (Ac1 and Ac2) and the bound tRNAAsp in backbone representation is colored in yellow. The location of the Plasmodium-specific insertion, protruding from the anticodon-binding domain, is highlighted in red.

leads to inactive, monomeric proteins. Despite conservation of strategic residues in motif 1, in particular Pro339, known to govern dimerization in functional yeast AspRS (32), this insertion is essential for AspRS dimerization and would act as molecular glue to assemble the two monomers. Modeling of the dimeric structure, showing localization of the plasmodial insertion between the two monomers, supports this interpretation (Fig. 5).

On the other hand, the presence of the RNA binding motif in the N-terminal extension is necessary for optimal tRNA aspartyla-
tion activity, as shown by a 4000-fold decrease of the catalytic efficiency of the mutant lacking the RNA binding motif, mainly due to a strong effect on $K_m$. Thus, this motif provides tighter binding of tRNAAsp on P. falciparum AspRS and its correct positioning in the catalytic site (as anticipated, the motif does not influence ATP/PP exchange). This effect is much stronger than in yeast AspRS, where deletion of the N-terminal extension affects catalytic efficiency by a factor of, at most, 100 (12, 25). Taken together, these results provide a robust background for answering challenging questions raised by the peculiarities of the P. falciparum AspRSs. Several perspectives will now be outlined.

Toward the Biology of P. falciparum AspRS—Understanding the global biology of the parasite AspRS implies knowledge of its structural organization and functionality that likely go beyond tRNA aminoacylation (2, 3). For instance, many protozoan parasites, more particularly plasmodia, are characterized by numerous functional and structural oddities. Besides complex life cycles (51), their genomes code for proteins of an eukaryotic type but decorated with fragments of sequences (extensions and insertions) not found elsewhere in the tree of life (52). These atypical sequences have been referred to as low complexity segments, as they are built up by a limited number of amino acids (e.g. large runs of asparagines in plasmodia) (53). This low complexity is not found in either the N-terminal extension or the insertion of the P. falciparum AspRS that show diversity in amino acids composition.

This addresses a wealth of questions that need to be explored experimentally. A first question concerns the structural and functional role of the long N-terminal extension. Because two isoforms of the parasite AspRS can be produced, it suggests a dual role for the extensions. Given the intricate biology of P. fal-
ciparum with its multifaceted lifecycle (51), where it alternates between a mosquito vector (Anopheles gambiae) and a vertebrate host (H. sapiens), this could imply variations in relative expression levels of the two forms of AspRS. In the present study only the erythrocytic stage was explored, which displays an abundant short form and a rare long form. This balance between long and short forms may be different at other stages of the parasite lifecycle. Following this view, both the extension and the insertion could serve to anchor the parasite AspRS in both analogs, we obtained the expected results where the molecules had about the same binding behavior as aspartate, meaning that these inhibitory analogs bind H. sapiens AspRS more efficiently than P. falciparum AspRS.

Altogether, these kinetic data provide the second example, after yeast (49), of a eukaryotic AspRS with an RNA binding motif in its N-terminal extension that has a high $K_m$ for aspartate. It is tempting to propose that both properties are correlated. This contrasts with the kinetic properties of the human and prokaryotic AspRSs, all deprived of the RNA binding motif, which have a low $K_m$ for aspartate. Thus, strong tRNA binding could compensate weak amino acid binding to allow efficient tRNA aspartyla-
tion.

Diversity of AspRSs and the Uniqueness of Cytosolic AspRS from P. falciparum—The structure-function relationship of AspRSs is well understood overall, based on robust molecular biology and crystallographic data on a few model organisms (8). They are homodimeric proteins with the same modular architectural organization (Fig. 1A) and catalytic properties, as sustained by a large ensemble of strictly conserved amino acids. However, AspRSs also exhibit large diversity, with the presence of additional domains idiosyncratic to phylogenetic classes and significant variations in the way they recognize and aminoacy-
late tRNA. In this respect the cytosolic AspRSs from the genus Plasmodium are of particular interest. Although they are of a eukaryotic-type with sequence similarities to yeast and human AspRSs, they differentiate themselves from all known AspRSs by distinctive long N-terminal extensions, 29–31-aa-long insertions in their anticodon binding domain and 2 in-frame initiation AUG codons in their mRNAs. These structural idiosyncrasies have functional consequences as shown with P. fal-
ciparum AspRS. AspRS can be expressed in two versions, short and long, depending on whether translation starts at the first or second initiation codon. Our experiments indicate that the short version is the most abundant (Fig. 3).

An additional finding is that aminoacylation activity is strictly dependent on the presence of the plasmodial insertion. The reason for this dependence was a surprise considering that the insertions are located in the anticodon binding domains of the dimeric AspRS (Fig. 5). The explanation came from mutational and dynamic light-scattering studies, which have unambiguously demonstrated that mutation of the insertion...
supramolecular associations with other cellular components (to be identified) with implications in regulation and/or in cellular traffic. For example, the distal part of the extension might be involved in mitochondrial import. The biology of the P. falciparum mitochondria is not well documented, and despite the lack of explicit proof for protein synthesis in this organelle, it is tempting to suggest that the longer version of AspRS is dedicated to mitochondrial translation. If so, the high affinity of the P. falciparum AspRS for tRNAAsp (via its RNA binding motif present in the proximal part of the extension) could provide an efficient way to import the tRNA into the mitochondria as may occur in yeast and plants (54). Thus, the relative amount of the two AspRS forms could be a way to regulate tRNAAsp concentrations in the cytosol or mitochondria. Notice, however, that no canonical signal sequence for mitochondrial import was found at the N terminus (within residues 1–49) of the parasite AspRS. As to the plasmodial insertion present in the anticodon binding domain of the synthetase, its interaction with other cellular components could be to switch on and off tRNA aspar- tylolation and, hence, the efficiency of translation in the parasite by controlling the formation of the inactive monomeric versus the active dimeric AspRS.

Toward a Strategy to Specifically Inhibit P. falciparum AspRS—Known inhibitors of aaRSs are mimics of amino acids, ATP, or of the enzyme-bound reaction intermediates, aminocyl-adenylates, that interrupt cognate substrate binding (55). If pharmacological applications are the aim, such compounds should specifically target a selected aaRS of a given pathogen and be safe for the human counterparts. As far as AspRSs are concerned, several synthetic or natural aspartyl-adenylate analogs competitively inhibit tRNA charging (11, 50, 56) and translation (57). Considering the close structural similarity of the catalytic site of P. falciparum and H. sapiens AspRSs with conservation of the amino acids in contact with the aspartyl-adenylate, it is not surprising that the inhibition produced by aspartol-AMP and Asp-AMS (Table 2) are similar for the two AspRSs. However, based on the structural peculiarities of P. falciparum AspRS revealed in the present study, molecules that would target these features could be potential antimalarial compounds. Among them, tRNA itself could be an immediate candidate as it can be contacted by the RNA binding motif of the N-terminal extension present in the parasite but absent in the human AspRS. Preliminary experiments exploring this possibility are promising as crude yeast tRNA strongly and specifically inhibits tRNAAsp aspartylation by P. falciparum AspRS, whereas human AspRS is not affected. Efforts are under way to isolate the tRNA species responsible for this behavior and to identify the structural features in tRNA that trigger inhibition of the parasite AspRS.

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