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Biochemical characterization of the apicoplast-targeted AAA+ ATPase ClpB from *Plasmodium falciparum*

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ABSTRACT

ClpB is a molecular chaperone from the AAA+ superfamily of ATPases, which reactivates aggregated proteins in cooperation with the DnaK chaperone system. ClpB is essential for infectivity and in-host survival of a number of pathogenic microorganisms, but systematic studies on ClpB from pathogens have not been reported yet. We purified and characterized one of the two ClpB isoforms from the malaria parasite *Plasmodium falciparum*, PfClpB1. PfClpB1 is targeted to the apicoplast, an essential plastid organelle that is a promising anti-malaria drug target. PfClpB1 contains all characteristic AAA+ sequence motifs, but the middle domain of PfClpB1 includes a 52-residue long non-conserved insert. Like in most AAA+ ATPases, ATP induces self-association of PfClpB1 into hexamers. PfClpB1 catalyzes the hydrolysis of ATP and its ATPase activity is activated in the presence of casein and poly-lysine. Similar to *Escherichia coli* ClpB, PfClpB1 reactivates aggregated firefly luciferase, but the PfClpB1-mediated aggregate reactivation is inhibited in the presence of *E. coli* DnaK, DnaJ, and GrpE. The lack of effective cooperation between PfClpB1 and the bacterial DnaK system may arise from the *Plasmodium*-specific sequence of the ClpB middle domain. Our results indicate that the chaperone activity of PfClpB1 may support survival of *Plasmodium falciparum* by maintaining the folding status and activity of apicoplast proteins.

1. Introduction

Plasmodium falciparum is an obligate intracellular parasite responsible for the most severe form of malaria, a disease that kills over 1 million people every year [1,2]. The fatal outcome of malaria is linked to a large-scale infection of erythrocytes. Since the parasite is becoming increasingly resistant to several existing anti-malaria drugs, it is essential to develop new strategies aimed at inhibiting the *Plasmodium* infections and combating the deadly disease. Intracellular survival and growth of parasites may critically depend on their ability to evade the defense mechanisms of the host and to protect themselves from the stressful environment of the infected cells. Molecular chaperones are used by all forms of life as factors protecting the quality of proteins under conditions of stress. However, the role of chaperones in supporting the infectivity and survival of *Plasmodium* has not been explored yet.

ClpB is an ATP-dependent chaperone from the Hsp100 sub-family of AAA+ ATPases (ATPases associated with different activities) [3]. ClpB is essential for survival of bacteria, yeast, and plants under severe heat shock [4–6]. Importantly, ClpB supports infectivity and survival of a number of pathogenic microorganisms [7–9]. Under conditions of stress, ClpB specifically targets aggregated proteins and induces their resolubilization and reactivation [10,11]. The unique disaggregase activity involves cooperation between ClpB and the DnaK (Hsp70) chaperone system [12]. Unlike most chaperones, which have orthologs in all forms of life, ClpB is not found in animal and human proteomes. Thus, understanding the biological role and the biochemical mechanism of ClpB in pathogens offers an opportunity to design novel antimicrobial strategies.

There are two putative ClpB isoforms in *P. falciparum* with PlasmoDB gene IDs PF08_0063 and PF11_0175 and protein products designated as PfClpB1 and PfClpB2, respectively [13]. Interestingly, both PfClpB isoforms contain the N-terminal secretion signal [13], which suggests that there is no ClpB in the *Plasmodium* cytoplasm. PfClpB2 (also known as *Plasmodium* Hsp101) becomes secreted to the parasitophorous vacuole, which envelops the parasite inside an infected erythrocyte [13]. In the parasitophorous vacuole, PfClpB2 participates in the export of *Plasmodium* proteins to the erythrocyte cytosol [14]. In contrast, PfClpB1 localizes to the apicoplast [13], a plastid organelle that is

Abbreviations: IPTG, isopropyl β-D-1-thiogalactopyranoside; ATPγS, adenosine-5′-(γ-thio)-triphosphate.

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essential for survival of *Plasmodium* and other *Apicomplexa* parasites [15,16]. Since the apicoplast is uniquely related to plant and algal plastids, it appears as a promising target for chemical interventions against *Plasmodium* [17]. The apicoplast-targeting of PfClpB1 suggests that the plastid proteins may require a chaperone protection from misfolding and aggregation during the *Plasmodium* life cycle. Indeed, the PfClpB1 mRNA is strongly upregulated during the first 20 h post-infection of erythrocytes (according to the PlasmoDB database, plasmodb.org), which suggests that PfClpB1 participates in the pathogen response to the host-induced stress. However, no studies linking PfClpB1 to the protein quality control in the *Plasmodium* apicoplast have been reported yet.

In this study, we performed the first biochemical characterization of PfClpB1 from *Plasmodium falciparum*. We found that the recombinant PfClpB1 forms ATP-induced oligomers, displays the ATPase and chaperone activities, but does not cooperate *in vitro* with bacterial DnaK or human Hsp70 during the aggregate reactivation. This work sets the stage for further characterization of the role of ClpB in supporting the life cycle of the malaria parasite.

2. Materials and methods

2.1. Proteins

The DNA construct containing the entire protein coding sequence of PfClpB1 was obtained as a gift from Dr. Raymond Hui, University of Toronto, Canada. A DNA fragment encoding the residues Ser145-Ser1070, i.e., downstream from the secretion signal and the apicoplast-targeting sequence, was amplified using Pfu DNA polymerase (Promega) and subcloned into pET28a vector (Novagen) between the NheI and XhoI cloning sites. Recombinant PfClpB1 was produced from this plasmid construct with the N-terminal His-tag in the *Escherichia coli* strain Rosetta™ 2(DE3) (EMD Millipore). The *E. coli* cells were grown at 37 °C until the optical density at 600 nm reached ~0.5 and then induced with 0.4 mM IPTG for 2 h. The cells were then collected, disrupted by sonication and centrifuged to collect the soluble extract. After centrifugation (14,000 g, 1 h), the supernatant was incubated with Ni–NTA resin (Invitrogen) by gentle mixing at 4 °C overnight and the bound proteins were eluted with 250 mM imidazole. Fractions containing PfClpB1 were identified with SDS–PAGE with Coomassie Blue stain (molecular weight 107 kDa), combined, and further purified by gel filtration on Superdex®200 (GE LifeSciences). The identity of the purified PfClpB1 was confirmed with an MS analysis of tryptic peptides, performed at the Biotechnology/Proteomics Core Facility at KSU.

E. coli ClpB was produced as previously described [18]. DnaK, DnaJ, and GrpE from *E. coli* as well as human Hsp70 and Hsp40 (Hdj1) were obtained from Enzo Life Sciences. Firefly luciferase was obtained from Promega, κ-casein and poly-lysine from Sigma. Protein concentration was determined spectrophotometrically and reported in monomer units.

2.2. Analytical ultracentrifugation

Beckman XL-I analytical ultracentrifuge was used in sedimentation velocity experiments with two-channel analytical cells. PfClpB1 was dialyzed in 50 mM Tris/HCl pH 7.5, 0.2 M KCl, 20 mM $MgCl_2$, 1 mM EDTA, 2 mM β-mercaptoethanol. Ultracentrifugation of the nucleotide-free 0.7-mg/ml protein sample was performed at 49,000 rpm and 20 °C with absorption profiles measured at 242 nm. Subsequently, 2 mM ATPγS [adenosine-5′-(γ-thio)-triphosphate] was added to the sample and ultracentrifugation was performed at 42,000 rpm with absorption profiles measured at 291 nm. The sedimentation data were analyzed using the time-derivative method [19] and the software distributed with the instrument.

2.3. ClpB ATPase assay

PfClpB1 and *E. coli* ClpB were incubated in the assay buffer (100 mM Tris/HCl pH 8, 1 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, and 5 mM ATP) at 37 °C for 15 min without or with 0.2 mg/ml κ-casein or 0.04 mg/ml poly-lysine. The concentration of ClpB was 0.1 mg/ml for the basal activity determination and 0.01 mg/ml in the presence of κ-casein or poly-lysine. The concentration of phosphate generated from ATP by ClpB was measured as described before [11].

2.4. Aggregate reactivation assay

To produce aggregates of firefly luciferase, 226 μM luciferase stock was diluted 300-fold with 50 mM Tris pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT and then incubated at 45 °C for 10 min. Aggregated luciferase (0.75 μM) was diluted 20-fold in the same buffer containing 6 mM ATP, 2 μM PfClpB1 or *E. coli* ClpB, 1 μM DnaK or human Hsp70, 1 μM DnaJ or human Hdj1, and 0.5 μM GrpE. The mixture was incubated at room temperature and aliquots were withdrawn to test the recovery of the luciferase activity using the luminescence assay system (Promega) and Turner Designs microplate luminometer.

3. Results and discussion

3.1. Sequence analysis of ClpB from P. falciparum

Members of the Hsp100 sub-family of AAA+ ATPases contain two highly-conserved ATP-binding modules (called D1 and D2) and two variable regions: the N-terminal domain (ND) and the middle domain (MD) [20]. The sequence alignment of ClpB from *E. coli* and *P. falciparum* (Supplementary Fig. 1) shows that the domain organization of PfClpB1 and PfClpB2 is similar to that of *E. coli* ClpB and that the nucleotide-binding Walker A/B and sensor-1/-2 motifs as well as the substrate-binding pore motifs are present in the sequence of PfClpB1 and PfClpB2. Unlike *E. coli* ClpB, both *Plasmodium* ClpBs contain the N-terminal leader sequences that specify their cellular localization. Both PfClpB1 and PfClpB2 contain the ER-targeting signal sequence, according to the SignalP algorithm [21], with the predicted signal peptide cleavage between Ser23 and Lys24 in PfClpB1. Unlike PfClpB2, PfClpB1 also contains a 120-residue long predominantly basic, K- and N-rich segment, which is a predictor of an apicoplast-targeting sequence [22].

Since the mechanism of cleavage of the apicoplast-targeting sequences is poorly understood, it is difficult to predict the N-termini of the mature variants of apicoplast proteins. The sequence of PfClpB1 switches from a predominantly basic to acidic in the vicinity of Phe143 and a sequence similarity between *E. coli* ClpB, PfClpB1 and PfClpB2 is apparent in the region following that residue (see Supplementary Fig. 1). Thus, we propose that the N-terminus of the mature PfClpB1 after the protein's translocation to the apicoplast might be located in the vicinity of Phe143.

The sequence similarity and the conservation of the domain structure (see Supplementary Fig. 1) suggest that mature PfClpB1 might fold into a structure similar to that of bacterial ClpB (Fig. 1). The single major divergence between the sequences of PfClpB1 and both *E. coli* ClpB and PfClpB2 occurs within the sequence of the middle domain (see Supplementary Fig. 1), which in PfClpB1 contains an inserted segment of 52 residues. Interestingly, the non-conserved region inserted into the PfClpB1 middle domain occurs at the junction of two helices (L1 and L2) in *E. coli* ClpB, which form a tip of the coiled-coil structure (see Fig. 1).

Fig. 1. Structure of the ClpB monomer from *Thermus thermophilus* [25]. The N-terminal domain is on the top, the C-terminal D2 module is on the bottom. The helical segments in the middle domain are indicated with colors: L1 (blue), L2 (magenta), L3/L4 (orange). The predicted position of the 52-residue insert in the sequence of PfClpB1 is indicated with an arrowhead (see Supplementary Fig. 1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The ClpB middle-domain coiled-coil supports the chaperone activity by mediating interactions with DnaK and regulating the disaggregase activity of ClpB in the DnaK-dependent manner [12,23,24]. Thus, the 52-residue segment inserted at the tip of the coiled-coil in PfClpB1 might influence the cooperation with the Hsp70 co-chaperone.

3.2. Oligomeric structure of PfClpB1

The biologically active form of bacterial ClpB is a cylinder-shaped homohexamer [25]. The self-association of ClpB into hexamers is induced by nucleotide binding [26,27]. The ATP-hydrolysis-driven reactivation of aggregates mediated by ClpB is linked to substrate translocation through the narrow central pore in the hexameric assembly [28].

We produced and purified a recombinant form of mature PfClpB1 (without the bipartite N-terminal targeting sequence) in Rosetta BL21(DE3) strain of *E. coli*, which contains tRNAs for the rare codons found in the PfClpB1 mRNA (see Fig. 2A). We asked if PfClpB1 forms nucleotide-induced oligomers, which is a prerequisite for the Hsp100-like biological activity. In sedimentation velocity experiments in the absence of nucleotides (Fig. 2B), PfClpB1 sedimented as a single predominant species with the apparent sedimentation coefficient of ~4.7 S, which agrees with the previously determined sedimentation coefficient of the monomeric *E. coli* ClpB [18]. The addition of saturating non-hydrolyzable ATP analog, ATPγS produced a loss of the monomeric species and induced self-association of PfClpB1 into a mixed population of ~9.5-S and ~16.7-S particles (Fig. 2C). The 16.7 S sedimentation coefficient approximates that of the hexameric *E. coli* ClpB [27]. We conclude that PfClpB1 binds the ATP analog, which induces the protein self-association into hexamers with a significant population of intermediate-size oligomers.

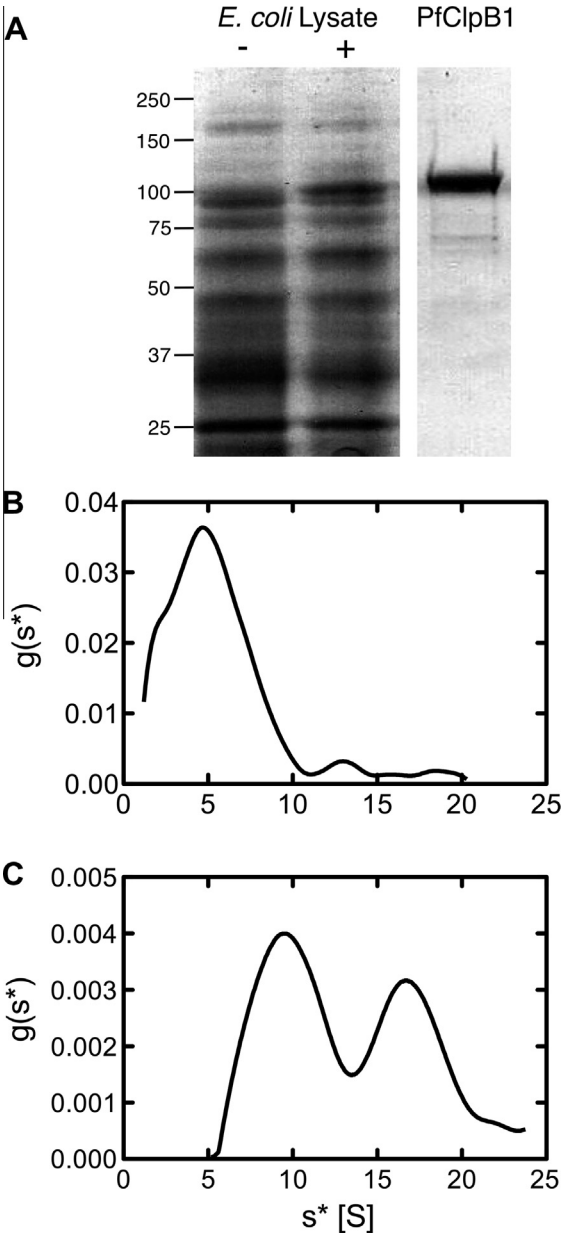

Fig. 2. Purification and nucleotide-induced oligomerization of PfClpB1. (A) SDS–PAGE with Coomassie Blue stain of the lysates from *E. coli* cells transformed with pET28a carrying the sequence of PfClpB1 incubated without (−) and with (+) IPTG and of the purified PfClpB1. (B, C) Sedimentation velocity of PfClpB1. Shown are the apparent distributions of the sedimentation coefficient measured at 20 °C for 0.7 mg/ml PfClpB1 in the absence of nucleotides (B) and in the presence of 2 mM ATPγS (C).

3.3. ATPase activity of PfClpB1

We next investigated the ATPase activity of the recombinant PfClpB1. As shown in Fig. 3, the basal ATPase of PfClpB1 was similar to that of *E. coli* ClpB (EcClpB). Moreover, the ATPases of both EcClpB and PfClpB1 were activated in the presence of the previously described pseudo-substrates: casein and poly-lysine [29].

3.4. Chaperone activity of PfClpB1

As has been shown before, thermally-inactivated or urea-denatured firefly luciferase does not refold efficiently and instead forms inactive aggregates that can be reactivated by the ClpB/DnaK

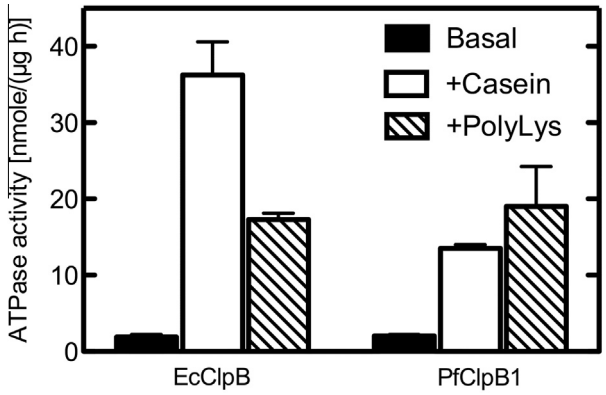

Fig. 3. ATPase activity of PfClpB1 and *E. coli* ClpB (EcClpB). The initial rate of hydrolysis of ATP catalyzed by PfClpB or EcClpB was determined at 37 °C in the absence of other proteins (basal activity), with 0.2 mg/ml κ-casein, or 0.04 mg/ml poly-lysine. The average values from four separate experiments are shown with the standard deviations.

system [11,30]. To test the intrinsic chaperone activity of PfClpB1, we applied mild aggregation conditions for luciferase (short incubation at 45 °C). As shown in Fig. 4, the luciferase reactivation rate was similar with PfClpB1 and *E. coli* ClpB in the absence of the co-chaperones DnaK, DnaJ, and GrpE (KJE). As has been shown before [31], the efficiency of aggregate reactivation increased significantly when *E. coli* ClpB was supplied with *E. coli* KJE (see Fig. 4). In contrast, the reactivation of luciferase was inhibited in the presence of PfClpB1 with KJE. Similar results were obtained with the urea-denatured aggregated luciferase or when human Hsp70 (1A/1B) and Hsp40 (Hdj1) were included instead of the bacterial DnaK and DnaJ (data not shown).

The results in Fig. 4 demonstrate that the recombinant PfClpB1 interacts with and reactivates aggregated luciferase even in the absence of the co-chaperones. As has been reported before [10], the cooperation between ClpB and the co-chaperones appears to be species-specific, i.e., the bacterial DnaK system does not activate PfClpB1. Previously, an efficient cooperation was observed between the yeast ClpB variant, Hsp104 and human Hsp70/40 [32]. However, human co-chaperones did not cooperate with PfClpB1 (data not shown), which suggests that the inter-chaperone specificity may be more stringent in *Plasmodium* than in yeast. On the other hand, inhibition of the PfClpB1-mediated aggregate reactivation in the presence of *E. coli* KJE (see Fig. 4) suggests that the bacterial co-chaperones do interact with PfClpB1, but the KJE–PfClpB1 interaction is not productive and inhibits PfClpB1. The lack of cooperation with other chaperones shown by PfClpB1 may be due to the non-conserved sequence of the middle domain (see Supplementary Fig. 1), which mediates the ClpB interactions with DnaK [24].

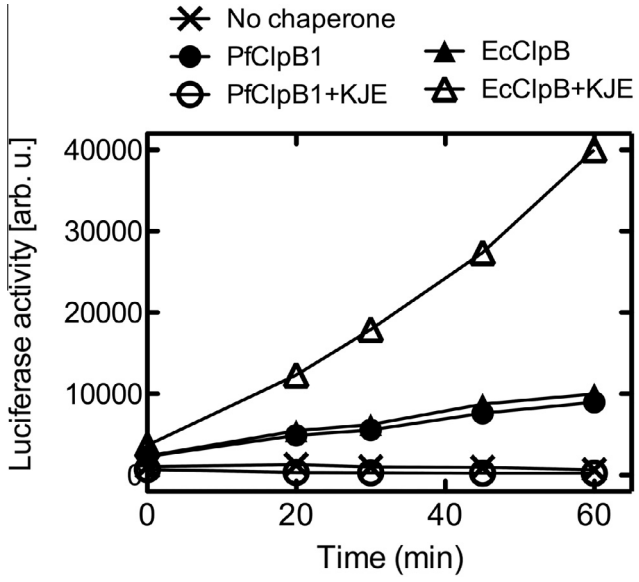

Fig. 4. Reactivation of aggregated firefly luciferase in the presence of PfClpB1 and *E. coli* ClpB (EcClpB). Shown are representative time-courses of the reactivation of aggregated luciferase in the absence and presence of DnaK/DnaJ/GrpE (KJE) from *E. coli*.

Our results indicate that the apicoplast of the malaria parasite *Plasmodium falciparum* contains an ATP-dependent, aggregate-reactivating chaperone PfClpB1 whose activity may depend on the yet-uncharacterized apicoplast-targeted co-chaperones from the Hsp70 and Hsp40 families. The PfClpB1 chaperone activity may be needed to maintain folding and activity of the apicoplast proteome, which may support the survival of *Plasmodium* under environmental stress. Ultimately, the apicoplast chaperones, including PfClpB1 may become targets for pharmacological treatment aimed at eradicating malaria and other *Apicomplexa*-borne diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.064.

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