A family of cyclophilin-like molecular chaperones in *Plasmodium falciparum*

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Abbreviations: CLD, cyclophilin-like domain; CYP, cyclophilin; CsA, cyclosporin A; FKBP, FK506-binding protein; PPIase, peptidyl-prolyl *cis-trans* isomerase.

Key words: *Plasmodium falciparum*, malaria, cyclophilin, molecular chaperone, protein folding, cyclosporin A.

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Abstract

27 The cyclophilins are a large family of proteins implicated in folding, transport and
28 regulation of other proteins and are potential drug targets in cancer and in some viral and
29 parasitic infections. The functionality of cyclophilins appears to depend on peptidyl-prolyl
30 *cis-trans* isomerase (foldase) and/or molecular chaperone activities. In this study we assessed
31 the peptidyl-prolyl isomerase and chaperone activities of 8 members of the *Plasmodium*
32 *falciparum* cyclophilin family, all produced recombinantly using a common host/vector
33 system. While only two of these proteins had isomerase activity, all of them displayed
34 chaperone function as judged by the ability to prevent the thermal aggregation of model
35 substrates. We suggest that the cyclophilins constitute a family of molecular chaperones in
36 malarial parasites that complement the functions of other chaperones such as the heat-shock
37 proteins.

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39 The cyclophilin family of proteins, which together with the FK506-binding proteins
40 (FKBPs) make up the immunophilins, is considered a potential class of antimalarial drug
41 targets [1]. Cyclophilins have been shown to play roles in such diverse processes as receptor
42 signalling, apoptosis, virion formation in human immunodeficiency virus, replication of
43 hepatitis C virus and growth at high temperature and virulence of *Cryptococcus neoformans*
44 [2; 3], and importantly, are the targets of existing drugs. There are also reports of roles in the
45 survival of parasites and the pathogenesis of parasitic diseases. This family also mediates the
46 immunosuppressive actions of the drug cyclosporin A (CsA) [1], which in addition has
47 antimalarial activity [4]. Since its approval for clinical use in the early 1980s, CsA has
48 played a major role in the prevention of allograft rejection. The mechanism of action of CsA
49 on human T-lymphocytes is well characterized: it impedes the transcription of the
50 interleukin-2 (IL-2) gene after having formed a complex with human cyclophilin 18
51 (hCYP18, hCYPA) that inhibits calcineurin, a crucial enzyme in the regulation of the
52 production of IL-2. Two cyclophilins PfCYP19A and PfCYP19B [5; 6] (see [1] for
53 nomenclature) are major receptors for CsA in *P. falciparum* [7]. Complexes of CsA–
54 PfCYP19A and CsA–PfCYP19B are both inhibitory to calcineurin, whose presence in
55 malarial parasites is known although its function is not [8; 9]. It is still not known how this
56 drug exerts its antiparasitic effects, but some other cyclosporins maintain antimalarial activity
57 even though they are non-immunosuppressive because of lack of affinity for cyclophilins or
58 (as cyclosporin–cyclophilin complexes) for calcineurin [10]. This suggests that the
59 mechanisms of antimalarial and immunosuppressive action may be unrelated [10; 11].
60 The most widely reported activity of immunophilins is their catalysis of protein
61 folding. Spontaneous isomerisation of peptidyl-prolyl bonds constitutes a rate-limiting step
62 in folding of some proteins. Peptidyl-prolyl isomerases (PPIases) stabilize the *cis-trans*
63 transition state of the X-P bond (where X is any amino acid) and accelerate its isomerisation.

64 Most immunophilins display PPIase activity *in vitro* and in some cases this has been shown
65 to be relevant in intact cells as well. However, the influence of immunophilins on the
66 conformations, locations, oligomeric states and activities of various proteins in cells cannot
67 be explained by PPIase activity alone. At least some cyclophilins can act as molecular
68 chaperones in a comparable manner to certain members of stress protein families. Moreover,
69 specific inhibition of PPIase but not chaperone function by immunosuppressant drugs
70 excluded a role for PPIase catalytic activity in chaperone function [1].

71 Besides PfCYP19A and PfCYP19B there are further 9 cyclophilin/cyclophilin-like
72 gene sequences in the *P. falciparum* genome database, all of which are expressed in at least
73 one life-cycle stage [12; 13]. In a recent report [13], eight of the recombinant *P. falciparum*
74 cyclophilins (either full-length or the isolated cyclophilin-like domain [CLD]) and a mutated
75 form of PfCYP19A were produced and purified using the same *Escherichia coli* expression
76 system that adds a hexahistidine tag to the C-terminus of each protein. This allows us to
77 compare members of the *P. falciparum* cyclophilin family of proteins on equal terms for
78 possible PPIase and chaperone functions, the effects if any of CsA on these activities and the
79 relationship of these characteristics to the predicted structural properties of the different
80 proteins.

81 In order to know the degree of conservation of the amino acids that contact CsA and
82 are related to the PPIase activity, the 8 *P. falciparum* cyclophilin and cyclophilin-like
83 sequences were aligned by the ClustalW2 program (**Fig. 1**) with the 'archetypal' hCYP18
84 and its orthologue in *Saccharomyces cerevisiae,* ScCYP17. There are blocks of amino acids
85 that, if not identical, are well conserved along the cyclophilin domains, supporting the idea
86 that most of these sequences are closely related. Thirteen amino acids contact CsA but not all
87 of them participate in the PPIase active site [14; 15]. All of these amino acids are present in
88 hCYP18 and ScCYP17, but among the 8 *P. falciparum* sequences, they are fully conserved

89 only in PfCYP19A and PfCYP19B. There is a gradient of conservation of these residues
90 among the different sequences and only six of the cyclophilin domains contain the W121
91 residue that is supposed to be crucial for CsA inhibition. For instance, PfCYP25 exhibits two
92 changes relative to PfCYP19A and PfCYP19B –namely A103T and H126N (hCYP18
93 numbering) – that involve loss of a positive charge but no major predicted secondary-
94 structural divergences (data not shown). At the other extreme, PfCYP32CLD only preserves
95 three of the catalytic/CsA-binding residues, having for example an I at position 121.
96 We assessed the molecular chaperone ability of the recombinant cyclophilins using
97 two model substrates: rhodanese from bovine liver mitochondria and citrate synthase from
98 porcine heart mitochondria, which are the substrates of choice to measure temperature-
99 induced aggregation and its suppression by molecular chaperones. The FKBP-type *P.*
100 *falciparum* immunophilin PfFKBP35 was previously shown to prevent the aggregation of
101 both of these substrates [16]. Recombinant cyclophilins or cyclophilin-like domains (CLD)
102 were produced and purified as described before [13]. The chaperone assay was carried out as
103 described elsewhere [16]. When citrate synthase was used as the model substrate, 6 out of
104 the 8 proteins (PfCYP19A, PfCYP19B, PfCYP25, PfCYP26, PfCYP32CLD and
105 PfCYP52CLD; data not shown) reduced aggregation. When the assay was performed using
106 rhodanese, all of the recombinant proteins prevented aggregation to varying degrees (**Fig. 2**).
107 The inhibition of thermal aggregation of rhodanese by the cyclophilins was then assessed in
108 the presence of equimolar concentrations of CsA. This drug did not interfere with the
109 chaperone action of any of the cyclophilins (**Fig. 2**); we had shown in a previous study that it
110 did not bind to most of them (**Table 1**) [13].
111 Next, the PPIase activity of the recombinant proteins was assessed by the standard
112 isomer-specific proteolysis method as described previously [16] using the tetrapeptide
113 substrate succinyl-Ala-Leu-Pro-Phe-*p*-nitroanilide. Both PfCYP19A and PfCYP19B showed

114 PPIase activity under the assay conditions, and this activity was inhibited by CsA (**Table 1**)
115 confirming previous reports (k_{cat}/K_M for PfCYP19B of 2.3 x 10^6 $s^{-1}M^{-1}$, IC_{50} for CsA of 10
116 nM: [5]; k_{cat}/K_M for PfCYP19A of 1.7 x 10^7 $s^{-1}M^{-1}$, K_i for CsA of 6.9 nM: [6]). Furthermore,
117 a single-amino acid mutant from one of them ($PfCYP19A_{W128F}$) that was generated in the
118 same manner showed PPIase activity but this was ~8-fold less susceptible to CsA (data not
119 shown), confirming a previous finding using a thermal-melt assay [13]. No PPIase activity of
120 any of the other recombinant proteins at up to 1 μM was detected. This may be related to a
121 lack of key catalytic residues as discussed above. The lack of activity was not due to
122 proteolytic degradation by the helper protease (α-chymotrypsin) as none of the proteins was
123 significantly degraded under the assay conditions (data not shown).

124 In view of the somewhat unphysiological nature of the peptide-based assay, PPIase
125 activity was also measured by monitoring the refolding of chemically-denatured RNase T1,
126 which is also dependent on peptidyl-prolyl isomerisation [17]. Unfolded RNase T1 was
127 produced by a 3-h incubation at 25°C of 50 μM RNase T1 in 50 mM Tris-HCl (pH 8.0), 1
128 mM EDTA and 6 M guanidine hydrochloride. Refolding conditions were 1 μM RNase T1 in
129 50 mM Tris-HCl (pH 8.0) and 1 mM EDTA, containing a range of concentrations of the
130 corresponding recombinant protein. The constituents of the cuvette were pre-equilibrated at
131 10°C for 5 min. before addition of RNase. Refolding was monitored by the increase in
132 tryptophan fluorescence at 330 nm (10-nm band width) with excitation at 280 nm (2.5-nm
133 band width) in a Perkin Elmer LS50B luminescence spectrometer at 10°C. Again, only
134 PfCYP19A and PfCYP19B showed PPIase activity (**Table 1**) and the results were
135 comparable in magnitude and timing to published data for cyclophilins of other organisms
136 such as *E. coli* [17].

137 In the case of PfCYP25 we were unable to replicate the original finding of PPIase
138 activity on a peptide substrate by Reddy [18], although this could conceivably be due to the

139 tag attached to our protein. It should be noted that we purified and assayed the full-length
140 recombinant protein while in that previous report [18] a crude, soluble *E. coli* extract was
141 assayed. It had previously been stated that all the amino acids involved in CsA binding are
142 conserved in the sequence with just one exception (H126N, hCYP18 numbering); but in
143 strain 3D7 there is a second change relative to PfCYP19A and PfCYP19B (A103T).
144 Moreover, the H126 residue has been reported to be essential for PPIase activity in hCYP18
145 [15].

146 Most of the human cyclophilins have been characterized [19] and it was concluded
147 that there are cyclophilin family members that, while sharing overall conservation with active
148 members of the family, do not possess isomerase activity. In the same study, it was
149 postulated that there is some flexibility in the active site with regard to the W121 position: a
150 tryptophan is clearly optimal at this position but tyrosine is somewhat permissive for activity,
151 as is histidine. Such a residue is present in that position in PfCYP23 but in this sequence
152 there are several changes apart from that to W121 that might affect the PPIase activity.

153 In conclusion, some of the sequences annotated as cyclophilins did not have the
154 complete set of the amino acids believed to be required for substrate and CsA binding and
155 this was reflected in the lack of detectable PPIase activity of all except two of them.
156 Molecular chaperone function, by contrast, is known to be independent of PPIase activity at
157 least in some cases [20]. Although many immunophilins have additional protein–interacting
158 domains (e.g. the tetratricopeptide repeat domain of PfFKBP35 [16]), it has been
159 demonstrated that even small immunophilins containing very little sequence besides the
160 PPIase domain may have a chaperone function distinct from their PPIase activity. The
161 binding surfaces of immunophilin chaperones must presumably be flexible enough to
162 accommodate the large variety of client proteins in the cell and perhaps also recognize
163 strategic hydrophobic residues. The lack of effect of CsA was therefore not unexpected, but

164 it contrasts with the findings using PfFKBP35, whose chaperone ability was reduced by
165 certain compounds related to FK506 [21].

166 Our results, showing chaperone function of all the proteins studied on at least one
167 model substrate, are therefore suggestive of a family of at least 8 cyclophilin/cyclophilin-like
168 molecular chaperones in malarial parasites. Molecular chaperones are likely to be crucial to
169 the survival and development of *Plasmodium*, e.g. in the export of proteins to host
170 erythrocytes [22] and the response of the parasite to stresses such as temperature shifts [23].
171 The question then arises of why the parasite has such a large quota of cyclophilins, which
172 although not the largest found in biology is for example far in excess of the single FKBP-type
173 immunophilin present. One possibility, that the different cyclophilins are expressed in
174 different developmental stages, seems not to be supported by the detection of multiple
175 cyclophilins in asexual and presexual blood stages, and in some insect stages, in
176 transcriptomic and proteomic studies. Another suggestion could be that in common with
177 what is found in other eukaryotes, different cyclophilins are present in different subcellular
178 compartments. There is some evidence for this idea from the detection of PfCYP19A and
179 PfCYP19B in the cytosol [7] and PfCYP19B at the infected erythrocyte surface [24], in
180 addition to the predicted location of PfCYP32 in the mitochondrion [12; 13]. A third
181 hypothesis, perhaps related to the first two, is that some of the cyclophilins may be
182 specialised for the folding and assembly of individual protein substrates, as in the case of
183 tubulin folding co-factors [25].

184 One should be cautious not to overinterpret results obtained from the aggregation
185 experiments. Even if there is a clear indication that a protein *might* function physiologically
186 as a chaperone, a positive result from an aggregation assay *in vitro* alone is not sufficient, as
187 is commonly stated, to classify the protein under investigation as a molecular chaperone.

More studies should be carried out to find out the natural substrates of these proteins and then clearly show whether or not they possess chaperone function in the intact cell.

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Figure legends

Fig. 1. Alignment of hCYP18, ScCYP17, cyclophilins and cyclophilin-like domains (CLD) in *P. falciparum*. Only residues 53–132 (hCYP18 numbering) are shown. For accession numbers see [13]. Cyclosporin A binding residues (13 residues) from hCYP18 are indicated with # above [14]. The W with † above refers to the sole amino acid supposed to be critical for CsA inhibition. ϕ above indicates other residues that are believed to be important for the PPIase activity [15]. Blocks of conserved residues present in at least 5 out of the 10 sequences are shaded in black. In brackets the % sequence identities shared with the archetypal hCYP18 are indicated.

Fig. 2. Inhibition of thermal aggregation of rhodanese by cyclophilin and cyclophilin-like proteins (A–H) and lysozyme as negative control (I) (all at 4.5 μM) and the effect of CsA (4.5 μM). In all cases the filled circles (●) show rhodanese alone, the open squares (□) show rhodanese in the presence of CYP, and the crosses (×) rhodanese+CYP+CsA. The points represent the average values of at least three replicates and the bars show SEM.