The crystal structures of macrophage migration inhibitory factor from *Plasmodium falciparum* and *Plasmodium berghei*

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Abstract: Malaria, caused by *Plasmodium falciparum* and related parasites, is responsible for millions of deaths each year, mainly from complications arising from the blood stages of its life cycle. Macrophage migration inhibitory factor (MIF), a protein expressed by the parasite during these stages, has been characterized in mammals as a cytokine involved in a broad spectrum of immune responses. It also possesses two catalytic activities, a tautomerase and an oxidoreductase, though the physiological significance of neither reaction is known. Here, we have determined the crystal structure of MIF from two malaria parasites, *Plasmodium falciparum* and *Plasmodium berghei* at 2.2 Å and 1.8 Å, respectively. The structures have an α/β fold and each reveals a trimer, in agreement with the results of analytical ultracentrifugation. We observed open and closed active sites, these being distinguished by movements of proline-1, the catalytic base in the tautomerase reaction. These states correlate with the covalent modification of cysteine 2 to form a mercaptoethanol adduct, an observation confirmed by mass spectrometry. The *Plasmodium* MIFs have a different pattern of conserved cysteine residues to the mammalian MIFs and the side chain of Cys58, which is implicated in the oxidoreductase activity, is buried. This observation and the evident redox reactivity of Cys2 suggest quite different oxidoreductase characteristics. Finally, we show in pull-down assays that *Plasmodium* MIF binds to the cell surface receptor CD74, a known mammalian MIF receptor implying that parasite MIF has the ability to interfere with, or modulate, host MIF activity through a competitive binding mechanism.

Keywords: macrophage migration inhibitory factor (MIF); malaria; crystal structure; tautomerase; oxidoreductase

Introduction

*Plasmodium falciparum* is responsible for 300–500 million cases and 1–3 million deaths from malaria each year, mainly in sub-Saharan Africa, making it one of the main causes of mortality in the world. More than 40% of the world’s population are living in areas where malaria is transmitted, meaning around two billion people are at risk of contracting the disease. The main causes of mortality are complications due to
severe anemia and cerebral malaria. The *P. falciparum* life cycle is complicated, spanning both human and mosquito hosts, but the clinical manifestations of the disease are a result of the human blood stage cycle in which the parasite invades red blood cells and proliferates asexually.

Patients with severe malarial anemia demonstrate ineffective erythropoiesis. It has been proposed that some pathogenic manifestations of severe malaria, such as anemia, could be brought on by the release of proinflammatory cytokines by host macrophages in response to infection by the parasite. The proinflammatory cytokine, macrophage migration inhibitory factor (MIF) has been identified as one such host-derived factor that inhibits erythropoiesis and which therefore could contribute to severe malaria.

MIF was one of the first cytokines to be identified. Originally described as an immune mediator isolated from T-lymphocytes that inhibit the random binding protein (Jab-1), the ribosomal toxic shock syndrome toxin 1, and malaria parasites. MIF is notable among cytokines in having two catalytic activities. The first, a keto-enol tautomerase activity, is mediated by a conserved N-terminal prasuble. The second, an oxidoreductase activity, is associated with a thioredoxin-like motif. The physiological substrates for the two enzyme activities remain unknown as does their biological function. In crystals, mammalian MIF, which is secreted via nonclassical pathways, retains both catalytic activities but at about 20% specific activity compared with recombinant human MIF.

The single MIF homologue in *Plasmodium* was identified through the sequencing of the parasite genome. Subsequently, three studies have been published with a more detailed analysis of *Plasmodium* MIF. It is expressed in all life cycle stages and is localized to both the parasite and the host cytoplasm in the intracellular blood stage forms. Transcription levels peak at the late trophozoite blood stage. Like mammalian MIF, which is secreted via nonclassical pathways, *Plasmodium* MIF lacks a secretion signal. It is likely that it is released from infected red blood cells during schizont rupture and merozoite release. Parasite derived MIF has been detected in the serum of patients infected with *P. falciparum*. In vitro experiments showed a decrease in the cell surface markers TLR2, TLR4, and CD86 in response to *Plasmodium* MIF exposure. Although MIF is not essential for parasite survival, there is an increase in the number of circulating reticulocytes in animals infected with *PbMIF* knockout parasites, in agreement with other studies which found that host MIF inhibits erythropoiesis.

In earlier work, we have determined structures of proteins important in redox regulation in *P. falciparum*. To better understand the functional role of macrophage MIF in malaria, including its redox significance, we determined the crystal structures of *P. berghei* and *P. falciparum* MIF (*PbMIF* and *PfMIF*, respectively). Additionally, as an indication of mechanism of action of *Plasmodium* MIF, we determined its ability to bind to one receptor for mammalian MIF, the cell surface determinant CD74.

**Results**

**Protein expression and characterization**

Following purification of the recombinant *Plasmodium* MIFs, MALDI mass spectrometry was performed on each sample. The molecular mass of *PbMIF* was measured as 14,091Da. This compares with the predicted mass of 14,013Da, the difference being satisfactorily accounted for by the covalent attachment of a mole of 78 g mol⁻¹. The presence of the 78 g mol⁻¹ adduct, respectively. The calculated mass of *PfMIF* is 13,924Da and so the two observed molecular mass peaks of 13,931 and 14,007Da correspond to the native protein and a 78 g mol⁻¹ adduct, respectively.
Analytical ultracentrifugation

The oligomeric state of MIF has been the subject of debate with suggestions that the active species is variably a monomer, a dimer, or a trimer. The two Plasmodium MIFs appeared to be trimers in vitro from their gel filtration characteristics. Analytical ultracentrifugation was performed to establish rigorously the oligomerization state of PfMIF. Sedimentation equilibrium analysis showed the average molecular weight to be 40 kDa consistent with a trimer form (calculated molecular weight 41.7 kDa). However, the sample was heterogeneous. Velocity sedimentation analysis revealed a predominant lower molecular weight species with the mass of a trimer, and a three-fold less abundant larger species with a mass five times greater (Fig. 2). The combined results of the equilibrium and velocity sedimentation experiments therefore suggest that Plasmodium MIF consists mainly of trimeric material with about 30% pentamers of the trimer (i.e., 15-mers) in a non-, or very slowly exchanging mixture. The observation of the higher molecular weight species is interesting in the light of an earlier attribution of chaperone-like activities to MIF. Although these results show that purified Plasmodium MIF is predominantly a trimer, this is not necessarily the physiologically relevant oligomeric state of the protein. For example, MIF may be stored in the trimeric form but dissociate on release from the cell. Extracellular concentrations of MIF range from 4 ng mL⁻¹ in normal blood plasma to 150 ng mL⁻¹ in patients with septic shock, values that are much lower than the concentrations used for these experiments. At these 1000-fold lower concentrations, it is possible that the trimer dissociates and that extracellular Plasmodium MIF is monomeric or dimeric.

Three-dimensional structure of Plasmodium MIF

The crystal structures of PbMIF and PfMIF reveal an identical polypeptide chain topology to human MIF. Each protomer consists of a four-stranded β-sheet (β₁-β₂-β₃-β₄) onto one face of which are packed two anti-parallel α-helices [Fig. 3(A,B)]. There is a pseudo two-fold axis of symmetry within the subunits of both MIF
structures, which relates the secondary structure elements $\beta_1$ (residues 2–7), $\beta_2$ (39–44), and $\alpha_1$ (13–31) to $\beta_3$ (58–63), $\beta_4$ (96–100), and $\alpha_2$ (72–88). These elements of the structure can be superposed with a root mean squared displacement (rmsD) in $\text{Ca}$ positions of 1.7 Å. Least squares superposition of the two $P$. berghei protomers and six $P$. falciparum MIF subunits gives average pairwise rmsD values in the range 0.9–1.2 Å [Fig. 3(C)]. The secondary structure elements superimpose closely with the largest differences occurring in the loop regions, residues 31–37 and 64–70, where the electron density is less well defined.

The C-terminal residues (102–114 together with the poly-His tag) are not defined by the electron density maps for either chain of $P$.falciparum and $P$. berghei MIF and these residues are assumed to be disordered. In $P$. berghei, the chain is defined up to residue 114 and it is seen that the C-terminal residues extend away from the core of the subunit to pack onto an adjacent protomer in the trimer [Fig. 3(C,D)]. The secondary structure elements superimpose closely with the largest differences occurring in the loop regions, residues 31–37 and 64–70, where the electron density is less well defined.

Figure 2. Analytical ultracentrifugation of $P$.falciparum MIF. A solution of $P$.falciparum MIF at a concentration of 2.6 mg mL$^{-1}$ was serially diluted in 50 mM Tris, 200 mM NaCl, 5% glycerol to give $P$.falciparum MIF/2 (1.3 mg mL$^{-1}$), $P$.falciparum MIF/4 (0.65 mg mL$^{-1}$), $P$.falciparum MIF/8 (0.33 mg mL$^{-1}$), and $P$.falciparum MIF/16 (0.16 mg mL$^{-1}$) samples. The velocity sedimentation experiment was conducted at 35,000 rpm over 4 h and the absorbance was plotted against the apparent MW. The results show that in solution $P$.falciparum MIF is predominantly trimeric with a small percentage of species at five times this MW.

The tautomerase active site

The tautomerase active site has been defined in human MIF by the determination of crystal structures of complexes with the substrate p-hydroxyphenylpyruvate (HPP)$^{25}$ and a competitive inhibitor 2-fluoro p-hydroxycinnamamide.$^{45}$ In each structure, three ligand molecules are bound per trimer each located at a subunit interface in hydrophobic cavities that contain the catalytic base, Pro1. Besides the N-terminal proline, the important substrate binding residues are Lys32, Ile64, Tyr95, and Asn97, where the apostrophes signify residues from the neighboring subunit.$^{25}$ These residues are conserved among the mammalian MIFs (Fig. 1). Comparison of the active site region of unliganded huMIF and huMIF bound to HPP with the corresponding region of PfMIF, following least squares superposition of the respective chains, reveals a noticeable difference in the position of Pro1, the catalytic proline. It is shifted $\sim$3 Å further into the active site, partially filling the volume that is occupied by the HPP molecule in the huMIF-substrate complex [Fig. 4(A,B)]. Pro1 in PfMIF is in van der Waals contact with Tyr96, a residue whose side chain in huMIF packs over the aromatic ring of the HPP from the opposite face of the cavity. This Pro1-Tyr96 interaction effectively closes off the active site in PfMIF [Fig. 4(A,B)]. Interestingly, the two protomers in the asymmetric unit in the PfMIF crystal exhibit different conformations of Pro1. In chain B, the electron density maps define Pro1 in a position that superimposes with that in PfMIF, whereas in chain-A they reveal the proline in a location reminiscent of that in huMIF [Fig. 4(B)], giving rise to an open active site cavity as observed for huMIF.
The alternative conformations of Pro1 correlate with other changes that distinguish the "closed" active sites of the two PbMIF trimers and trimer B of PfMIF (PfMIF-B) from the "open" active sites of the huMIF trimer and the trimer formed from the PfMIF A chains (PfMIF-A). First, and as discussed in more detail later, Cys2 is covalently modified in PbMIF and PfMIF-B by disulphide linkage to a molecule of β-mercaptoethanol [Fig. 4(C)]. In PfMIF-A, there is no such covalent modification. The distinguishing feature of this protomer is the presence of two molecules of glycerol located at the entrance to the substrate binding cavity.

Second, Ile65 in PhMIF and PfMIF-B is flipped out of the active site with its main chain amide group displaced by 1.2 Å so that it is out of hydrogen bonding distance of the carboxyl group of the HPP moiety observed in huMIF. Third, the aromatic ring of Tyr95 in huMIF lies perpendicular to that of HPP so as to form favorable π-stacking interactions with the substrate. In PhMIF and PfMIF-B, the corresponding ring of tyrosine 96 is displaced from, and has a parallel orientation with respect to, the aromatic ring of HPP [Fig. 4(B)]. This suggests either that HPP has a different mode of binding in Plasmodium MIF compared

Figure 3. Structure of Plasmodium MIF. A: Topology diagram of the Plasmodium berghei MIF subunit with strands shown as yellow arrows and helices as red cylinders. The secondary structure elements are numbered. The amino (N) and carboxyl (C) termini are indicated. The pseudosymmetry relating β-strands 1 and 2 and α-helix 1 and β-strands 3 and 4 and α-helix 2 is apparent. B: Ribbon diagram of the PbMIF subunit with secondary structure elements colored as in (A). C: Overlay of the P. berghei (blue) and P. falciparum (yellow) MIF subunits showing the high structural similarity between the two proteins. The C-terminal residues of PfMIF are disordered and missing from the model. D: The PbMIF trimer viewed down the molecular three-fold axis with subunits colored individually in yellow, green, and blue. E: Overlay of the MIF subunits from P. berghei (blue) and human (magenta). F: Overlay of the MIF trimers from P. berghei (blue) and human (magenta) showing the close superposition of the β-sheets and the slight variation in the orientation of the α-helices. These and subsequent images were produced in CCP4MG.68
with huMIF, or more likely, that conformational changes take place in PbMIF and PfMIF-B to generate the catalytically active state.

**Receptor interactions**

CD74 has been identified as a cell surface receptor for huMIF. We used ConSurf to determine clusters of conserved residues on the Plasmodium MIF trimer surface that might represent a CD74 binding site. As expected, the highly conserved residues cluster principally around the tautomerase active site (data not shown). To establish whether Plasmodium MIF interacts with CD74 we investigated the protein–protein interaction by GST-pulldown. Since in our hands full length CD74 failed to express in useful amounts, we expressed and purified the mouse CD74 ectodomain

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Figure 4. The tautomerase active site. A: Comparison of the human (left), *P. berghei* (center) and *P. falciparum* chain-A (right) MIF active site cavities. In the PbMIF and PfMIF images, the HPP substrate is taken from the huMIF coordinate set following superposition with the ligand-bound form of this protein. In huMIF and PfMIF chain-A, the active site is “open,” whereas in PbMIF, it is “closed” and there is a steric clash between the protein and the HPP moiety. The proteins are shown as electrostatic surfaces highlighting the fact that in Plasmodium MIF the active sites are more negatively charged than that of huMIF. B: Stereo view of the active site of MIF. The human (magenta), *P. berghei* (blue), and *P. falciparum* chain-A (yellow) MIFs have been superposed and the active site residues are shown in the context of the tautomerase substrate, HPP (colored by atom type C, green; O, red) bound to huMIF. Pro1 of human-MIF and PfMIF-A are in equivalent positions but Pro1 of PbMIF is markedly displaced. In both the Plasmodium MIFs, the aromatic ring of Tyr96 is in a perpendicular plane to that of this tyrosine in the huMIF active site. C, D: Electron density maps displayed in the vicinity of residue 2 of PbMIF chain C and PfMIF chain-A, respectively. The position 2 side chain was truncated to Ala and cycles of REFMAC refinement were carried out prior to the calculation of *2Fo-Fc* (blue) and *Fo-Fc* (green) maps which are displayed at the 1.0 and 3.0 σ levels respectively and displayed on the final refined models. The covalent modification of Cys2 in the PbMIF chain and the dual conformation of the Cys2 side chain in PfMIF chain-A are evident.

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Phe59, Leu48, Asn41 and Glu46 and Ile6 are in the hydrophobic core of the molecule [Fig. 6(B)]. Cys58 of PfMIF is the first residue of strand and contributes to the subunit interface. In the crystal structures, this motif is not conserved and the corresponding residues are C58FVR61. In the crystal structures, this motif is not conserved and the corresponding residues are C58FVR61. A C56S mutant retains 55 and 68% wild-type activity for insulin and 2-hydroxyethyldisulphide (HED) reduction, respectively, whereas the C59S mutation completely abolishes these activities.26 In the Plasmodium MIFs, the motif is conserved and the corresponding residues are C56FVR64. In the crystal structures, this motif is quite differently structured [Fig. 6(B)]. Cys58 of Plasmodium MIF is the first residue of strand β3 and its side chain together with that of Val60 are buried in the hydrophobic core of the molecule [Fig. 6(B)]. Phe59 and Arg61 are on the opposite face of this β-strand and contribute to the subunit interface. In huMIF, Cys56 lies in the loop region preceding β3 and the closure of the active site.

**The C-terminus**

It was recently reported that deletion of residues 105–114 at the C-terminus of MIF results in abolition of the tautomerase activity.51 Although the C-terminal residues are important for tautomerase substrate binding, they form no direct interactions with the HPP moiety and it is logical to conclude that the importance of the C-terminus, for the tautomerase activity, is through its effect on trimer stability. The sequence of this segment is reasonably well conserved (Fig. 1) and in the structure these residues are involved in interactions between subunits in forming the trimer. The crystal structures of human and rat MIF25,46,52 are closely similar except that the C-terminal residues of rat MIF are disordered. This difference recurs in the Plasmodium structures in which the C-terminus is fully ordered in the PbMIF protomers, but disordered in PfMIF beyond residues Asp101 and Asn106 of chains A and B, respectively.

The intersubunit interactions in the Plasmodium MIFs were analyzed using the program PISA.53 In PbMIF, a total of 2674 Å² of solvent accessible surface becomes buried on formation of the trimer compared with only 1539 Å² in PfMIF, where the C-terminal residues are missing. Although the PnMIF trimer appears to be less thermodynamically stable than the PbMIF trimer, both molecules are evidently trimeric. In PbMIF, residues 107–112 interact with residues 92–99 of the adjacent subunit. Residues spanning Phe107 to Ser112 of one subunit form main chain hydrogen bonds with β4 of the adjacent protomer to make the additional β-strand [Fig. 6(A)].

**The oxidoreductase active site**

In huMIF, the oxidoreductase activity is associated with a conserved motif, C56ALC59. A C56S mutant retains 55 and 68% wild-type activity for insulin and 2-hydroxyethyldisulphide (HED) reduction, respectively, whereas the C59S mutation completely abolishes these activities.26 In the Plasmodium MIFs, the motif is not conserved and the corresponding residues are C56FVR64. In the crystal structures, this motif is quite differently structured [Fig. 6(B)]. Cys58 of Plasmodium MIF is the first residue of strand β3 and its side chain together with that of Val60 are buried in the hydrophobic core of the molecule [Fig. 6(B)]. Phe59 and Arg61 are on the opposite face of this β-strand and contribute to the subunit interface. In huMIF, Cys56 lies in the loop region preceding β3 and

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**Figure 5.** GST-pulldown showing CD74–PbMIF interaction. Left hand lane: 1% input PbMIF; Lanes 1–3: background binding to GST loaded glutathione sepharose beads with 1, 10, and 25 mM HPP, respectively; Lanes 4–6: PbMIF binding to GST-CD74 loaded glutathione sepharose beads with 1, 10, and 25 mM HPP, respectively. The masses in kDa of molecular markers are indicated at the left.
it is hypothesized that a disulphide-stabilized β-turn may form in the CALC region on oxidization. 26

Discussion

The cytokine macrophage MIF has a widespread distribution with homologues in *Plasmodium, Leishmania, Toxoplasma*, and parasitic nematodes. The absence of MIF in other protozoans indicates that the expression of MIF in unicellular eukaryotes is confined to parasites that engage in interactions with host cells in the blood. Given the established role of MIF in the immune system, it may be that differences between the parasite and host MIF proteins are important for progression of parasitemia and evasion of the host immune response. MIFs from protozoan parasites have previously been structurally and functionally characterized for the parasitic nematodes *Brugia malayi*, 54 *Ancylostoma ceylanicum*, 55 and the apicomplexan *Leishmania major*. 56

The tertiary and quaternary structures of the *P. falciparum* and *P. berghei* MIFs determined here are similar to one another and to the mammalian and parasite MIFs. Electrostatic surface analysis reveals that the tautomerase active site of *Plasmodium* MIF has more negatively charged character than huMIF [Fig. 4(A)], a phenomenon also observed in the *L. major*, 56 and hookworm structures. The extra negative charge is attributable to Glu98; which replaces Asn97 in the human homologue. The differences observed in the active site, in terms of electrostatic potential and the conformation of catalytic residues, may account for why *Plasmodium* MIF has a lower tautomerase specific activity toward HPP than huMIF. 29,32 It may also point to different substrate specificity, given that the physiological substrate has yet to be identified.

Receptor interactions

CD74 has been identified as a cell surface receptor for huMIF 49 and our results suggest this interaction is...
conserved in the *Plasmodium* MIFs. Although better defined in its intracellular form, it is known that 2–5% of the CD74 protein localizes to the cell surface.\(^5\) The NMR structure of the CD74 ectodomain is trimeric,\(^5\) sharing the three-fold rotational symmetry of MIF. The programme ConSurf\(^5\) shows that highly conserved residues on the *Plasmodium* MIF trimer surface cluster around the tautomerase active site. A possible coincidence/overlap of the CD74 binding surface and the tautomerase active site has recently been inferred for huMIF following the observation that antagonists of the huMIF-CD74 interaction also inhibit the tautomerase activity of the enzyme.\(^5\) This prompted us to investigate the influence of the HPP substrate on *Plasmodium* MIF-CD74 interaction. Although the mouse CD74 ectodomain was able to pull down recombinant histidine tagged *Plasmodium* MIF, we found no significant effect of the presence of HPP, even at concentrations up to 10 times the *K*\(_{M}\) for HPP of huMIF, on the interaction with CD74. The fact that saturating concentrations of the tautomerase substrate do not inhibit CD74 binding leads to the tentative suggestion that the tautomerase active site and the CD74 binding epitope are nonoverlapping.

**Implications for oxidoreductase mechanism**

One of the most interesting aspects of the structures described here is the modification of Cys2, where the presence of disulphide-linked mercaptoethanol adducts is clearly indicated by the electron density maps. The modified residue *S*,*S*-(2-hydroxyethyl)thiocysteine (CME) is present in the six crystallographically independent subunits of PfMIF and in one of the two crystallographically independent subunits of PfMIF. There is no evidence of covalent modification of the three other cysteine residues (Cys3, Cys58, or Cys102) in either structure, indicating that Cys2 is uniquely reactive. 

Oxidoreductase activity in mammalian MIFs was first considered when the existence of the well-conserved CALC motif was correlated with the presence of CXXC motifs at the catalytic centers of thiol-protein oxidoreductases such as thioredoxin.\(^2\) The reaction cycles of thioredoxin-type oxidoreductases feature intermolecular disulphide bonds with substrate proteins and intramolecular disulphide bonds between the adjacent cysteines of the motif.\(^6\) HuMIF was subsequently shown to exhibit oxidoreductase activity toward insulin and 2-hydroxyethyl disulphide (HED) substrates.\(^2\) In huMIF, site-directed mutagenesis showed that the second cysteine (Cys59) of the CALC motif is essential for oxidoreductase activity, whereas serine substitution of the first cysteine led to quite modest reductions in rate. The location of the CALC motif in the huMIF structure suggests that the oxidoreductase reaction could only plausibly be mediated by the monomeric form. In the trimer, neither of the CALC motif cysteine residues (at positions 56 and 59) nor cysteine 80 is surface accessible. In the monomer, Cys59 and 80 would be on the surface of the protein [Fig. 6(B)].

Since HED reduction produces two molecules of β-mercaptoethanol, the Cys2 adducts observed here could represent intermediates in the *Plasmodium* MIF-catalyzed reduction of this substrate. This would imply a quite different mechanism of action from that proposed for human MIF consistent with the strikingly different pattern of cysteine residues in the *Plasmodium* MIF sequences and the absence of the CXXC motif. In the *Plasmodium* proteins, cysteines are conserved at positions 2, 3, 58, and 102 (Fig. 1), in contrast to the mammalian MIFs where the conserved cysteines occur at 56, 59, and 80. Although, Cys58 in *Plasmodium* MIF and Cys56 in huMIF are corresponding residues in the sequence alignment (Fig. 1), the structures reveal quite different environments. In contrast to Cys56 in huMIF, which would be surface-exposed in the monomeric form, the Cys58 side chain in *Plasmodium* MIF is buried in the hydrophobic core of the subunit and clearly incapable of participating in redox reactions in the absence of major conformational changes in the protein [Fig. 6(B)].

The *Plasmodium* MIF oxidoreductase activity was shown in a previous study to be very low. In this work, we found that the activity of wild-type *Pf*MIF against HED was scarcely above background levels, and much lower than that of recombinant human glutaredoxin 1, which was used as a positive control (data not shown). This very low activity prevented us from evaluating the catalytic contribution of cysteine-2 by site-directed mutagenesis. The uniquely reactive character of Cys2 is unexplained and may be associated with uncharacterized chemistry in MIF possibly involving the adjacent active site proline, Pro1.

**The cytokine activity of MIF**

The CC motif at the N-terminus of *Plasmodium* MIF (Cys2-Cys3) is a recognized motif in one of the four chemokine families. As *Plasmodium* MIF has been characterized as a cytokine, it throws up the possibility that it could mediate some of its activity via this CC motif. Although, there is no evidence of disulphide bonds in the *Plasmodium* MIF crystal structures, the subunit does exhibit a high degree of structural similarity to the dimeric form of interleukin-8 (IL-8), a CXC chemokine. There is also evidence that MIF is a noncognate ligand of the CXC chemokine receptor CXCR2 that is known to bind IL-8.\(^2\) In the same study, it was pointed out that huMIF may qualify as a pseudo-CXC or ΔC chemokine, given its lack of C-terminal cysteines.\(^2\) *Plasmodium* MIF has a C-terminal cysteine (Cys102) that is absent in huMIF, which may indicate that it has added chemokine functionality that could differentiate the two homologues during parasite invasion. An added complexity is that activation of CXCR2 by IL-8 requires an N-terminal ELR motif, of
which there is a pseudo motif in huMIF composed of two nonadjacent but adequately spaced Asp and Arg residues. In Plasmodium, MIF Glu55 and Arg93 are surface exposed and spaced such that they could mimic the ELR motif of IL-8.

**Conclusion**

The role of Plasmodium MIF in the human host is little understood. Human MIF is known to have a role in multiple immune pathways and its function/dysfunction is associated with a multitude of diseases. The malaria parasite releases MIF during the blood stage cycle of infection when it would seem counter-productive to release a protein that could elicit a potentially lethal host immune response. In presenting the crystal structure of MIF from *P. berghei* and *P. falciparum*, it is hoped that the important differences, and indeed similarities, between Plasmodium and human MIF may provide an indication of how the parasite-derived protein contributes to the progression of parasitemia. There is a possibility that Plasmodium MIF could be a potential drug target in malaria and it would therefore be important to target the parasite protein selectively relative to the host protein. MIF clearly has potential for inhibitor design with huMIF having been the subject of a virtual screen for MIF-CD74 antagonists, and the Plasmodium MIF structures presented here provide a basis for extending this approach.

**Materials and Methods**

**Plasmid preparation**

Protein expression constructs were prepared by previously published protocols. Briefly, the coding sequences for *P. falciparum* (PlasmoDB entry: PFL1420w) and *P. berghei* (GeneDB entry: PB000372.0) MIF were amplified from mixed blood stage cDNA using the primers 5’-AAAAATTCGCCAGCTGCTGTAAGTAA TAAC-3’ and 5’-ATGGATCCCGCGAAAAGAGAAC CACTGAGGC-3’ for *P. falciparum* and 5’-AAATTT CGGATCGCTGTAAGTAA TAAC-3’ and 5’- ATGGATCCACCATTGAGCCACTAAAGCC-3’ for *P. berghei* (italicized bases indicate restriction sites). The PCR products were digested with SphI and BamHI and ligated into the similarly digested pQE-70 (QIAGEN) expression vector such that recombinant protein products were expressed with a C-terminal His6-tag. Constructs were sequenced to confirm MIF sequence identity.

**Protein expression and purification**

*P* MJ and *Pb*MIF were expressed in *E. coli* BL21 star (Novagen) cells grown on Luria Broth (10 g tryptone, 5 g yeast extract, 10 g NaCl per liter). Bacteria were grown to an optical density at 600 nm of 0.6 before addition of isopropyl-D-thiogalactoside to 1 mM to induce expression of recombinant protein. Growth was allowed to continue at 37°C for 4 h. Cells were harvested by centrifugation and resuspended and lysed in 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% v/v glycerol, 10 mM β-mercaptoethanol, 0.01% Nonidet P-40, and complete cocktail protease inhibitor (Roche). The lysate was cleared by centrifugation at 15, 000 rpm for 40 min at 4°C and loaded onto a 5 mL HisTrap™ HP chelating column (GE Healthcare) equilibrated in buffer A (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 10% v/v glycerol, 10 mM β-mercaptoethanol, 20 mM imidazole). After a washing step, the bound proteins were eluted over 10 column volumes with a 20–500 mM linear imidazole gradient in buffer A. The MIF-containing fractions were identified by SDS-PAGE analysis, concentrated, and loaded onto a Superdex 200 gel filtration column (Amersham Pharmacia) equilibrated in 50 mM Tris pH 8.0, 200 mM NaCl, 5% v/v glycerol, 5 mM β-mercaptoethanol. Following size exclusion chromatography, the MIF proteins were judged to be pure from the Coomassie blue staining pattern following denaturing gel electrophoresis.

**GST pull-downs**

*Pf* and *Pb*MIF-His6 were purified as described previously. The ORF of the CD74 ectodomain (residues 51-279) was amplified from mouse cDNA using primers (L2809: 5’-TGGCCAGGCTCATA TGCTTACTCTTGATCCGCAAG-3’ and L2808: 5’-GCTTGAATCTTACAGGGGACTTGACCCAGTTCC-3’) and inserted (NdeI, BamHI, italicized in sequence) into pRP265NB (described previously). The empty vector pRP265NB (for control GST expression) and the GST-CD74 expression plasmid were transformed into BL21 (DE3) pLysS, bacteria were grown at 30°C on Luria Broth (LB, Qiogene) containing 30 μg/mL chloramphenicol and 0.05 μg/mL ampicillin. Protein expression was induced at OD₆₀₀ of 0.6 by the addition of isopropyl-D-thiogalactoside to a final concentration of 1 mM. After 5 h, the cells were harvested and lysed in 50 mM Tris (pH 8.0), 500 mM NaCl, 5% (vol/vol) glycerol, 0.01% Tween in the presence of complete protease inhibitor cocktail (Roche) and 1 μg/mL lysozyme. Genomic DNA was fragmented by sonication, after which the lysate was cleared by centrifugation at 30,000 × g for 1 h at 4°C. Approximately 1 μg of GST or GST-CD74 in bacterial lysate was loaded on glutathione sepharose 4B beads (GE Healthcare) by rotating at 4°C for 1 h. After washing with PBS, *Pf* or *Pb*MIF-His6 was added to a final concentration of roughly 4 μM in 500 μL binding buffer (50 mM K₂HPO₄ (pH 7.2), 50 mM NaCl, 5% glycerol, 0.01% Tween) in presence or absence of substrate (p-hydroxyphenylpyruv acid, Sigma), which was added dissolved in DMSO. After 1 h binding at 4°C, the glutathione beads were washed three times with binding buffer, supplemented with the appropriate concentration of HPP, before loading on a 15% polyacrylamide gel. MIF-His6 was detected by western blotting with
Analytical ultracentrifugation
Sedimentation equilibrium experiments were performed on PfMIF with Dr. Andrew Leech (Molecular Interactions Laboratory, Technology Facility, Department of Biology, University of York) to determine the oligomeric state of the protein. A Beckman Optima XL/A analytical ultracentrifuge with an AN-50Ti rotor was used to conduct the experiment using Beckman cells. PfMIF was dialyzed overnight into 50 mM Tris-HCl pH 8.0, 200 mM NaCl, 5% glycerol. Sample concentrations ranged from 2.6 to 0.16 mg/mL and were prepared by serial two-fold dilutions. Initial absorbance scans were taken at 3,000 rpm to verify that the cell contents were evenly distributed before the speed was increased and sedimentation equilibrium was reached. Absorbance scans were taken at 3 h intervals and all measurements were recorded at 293 K.

The distribution of material between species of different oligomeric state was determined by velocity sedimentation. Experiments were conducted using the same material as for the sedimentation equilibrium analysis. The cell contents were agitated so as to destroy the concentration gradient and ensure even distribution of material. A velocity run was then conducted over 4 h at 35,000 rpm. The data from both experiments were analyzed using HETANAL.

Protein crystallization
PfMIF and PfMIF were concentrated to 5 and 25 mg/mL, respectively, for crystallization experiments. Suitable crystallization conditions were initially screened in 96-well MRC Wilden crystallization plates using a mosquito® (TTP LabTech) liquid handling robot to set up nanoliter drops. Larger crystals were grown via the hanging drop vapor diffusion method, where equal volumes (1 μL) of protein and mother liquor were mixed and allowed to equilibrate at room temperature. PfMIF crystals grew from 0.2M KSCN, 10% PEG 8K, 10% PEG 1K, 100 mM Tris pH 8.0 and belong to space group P2₁ with unit cell dimensions \( a = b = c = 83.29 \) Å. PfMIF crystals grew from 0.2M KSCN, 13% PEG 6K, 100 mM Tris pH 8.5 and belong to space group P2₁, where \( a = 78.70 \) Å, \( b = 69.17 \) Å, \( c = 80.22 \) Å, and \( \beta = 118.20^\circ \).

Crystals were soaked in mother liquor containing 25% glycerol as a cryoprotectant and rapidly cooled in liquid N₂ (110 K) for data collection.

Data collection and processing
Data for PfMIF and PfMIF were collected on beamlines ID14-2 and ID14-4, respectively, at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) and were processed using the DENZO/SCALEPACK® and CCP4 packages.\(^1\)

| Table I. Data collection and refinement statistics |
|-----------------------------------------------|
| Data collection | P. berghei | P. falciparum |
| Space group | Pb₂ | Pb₂\(,\) |
| Cell dimensions | \(a, b, c (\text{Å}) \) | 83.29, 83.29, |
| | | 83.29 |
| | \(\alpha, \beta, \gamma (\text{°}) \) | 90, 90, 90 |
| | Wavelength (Å) | 0.94 |
| | Resolution (Å) | 50–1.8 |
| | No. unique reflections | 72, 962 |
| | \(R_{\text{merge}} \) | 0.084 (0.610) |
| | \(I/\sigma(I) \) | 17.35 (1.36) |
| | Completeness (%) | 99.6 (99.5) |
| | Redundancy | 4.0 (2.7) |
| | Refinement | 12.045 |
| | Resolution (Å) | 58.90–2.05 |
| | No. reflections | 68,494 |
| | \(R_{\text{work}}/R_{\text{free}} \) | 0.25/0.32 |
| | No. atoms | 5419 (including CME) |
| | Protein | 1604 (including CME) |
| | Ligand | GOL 48 |
| | Water | 498 |
| | B-factors | 83 |
| | Proteins | 28.30 |
| | Ligand | 43.86 |
| | Water | 38.05 |
| | Rms deviation | 14.2 (12.1) |
| | Bond lengths (Å) | 0.025 |
| | Bond angles (°) | 2.11 |

Determination of the structure of PbMIF
The structure proved difficult to solve by molecular replacement (MR) with the P₂₁ data with calculations producing solutions that were inconclusive. All of the MR programs tested aligned the trimeric three-fold axis correctly but the rotation about this axis was very uncertain (i.e., the translation function was good but the subsequent rotation function was poor). In addition to the three-fold symmetry axis in the trimer, each subunit has pseudo two-fold symmetry. Self-rotation shows the crystallographic two-fold to be parallel to the molecular three-fold, and perpendicular to the pseudo two-fold axes, generating pseudo 222 symmetry. Additionally, the Patterson map showed noncrystallographic translation with a peak that was 43% of the height of the origin peak at 0.50, 0.45, and 0.00. This suggested that there were six molecules in the

anti-his and sheep-anti-mouse HRP conjugate (GE Healthcare).
asymmetric unit correlating with the Matthews coefficient of 2.24 Å³ Da⁻¹ (relating to a solvent content of 45%). All of this may have contributed to the difficulty in finding an MR solution for the P₂₃ data.

A partial solution was found using the automated molecular replacement pipeline Balbés⁶⁴ following reprocessing of the data in the space group P₃. Sixteen monomers were found using chain-A of coordinate set 20S5⁵⁹ as the MR model. Of these, four protomers, related in pairs by 120°/C₁₄, satisfied the symmetry operations for P₂₃, with the expected pseudo translation. The third chain of each trimer was generated and the subsequent model was good enough to start refinement. ARP/wARP was used to build in the P. berghei sequence, refinement was performed with REFMAC,⁶⁵ and Coot⁶⁶ was used to finalize the model.

**Determination of the structure of PfMIF**

Data collected for PfMIF were processed in the cubic space group P₃₂₁. An ice ring forced the exclusion of some data during processing. Initial molecular replacement calculations using the human MIF coordinate set (pdb entry 1GD0⁵⁵) as the search model were unsuccessful most likely due to the low-sequence identity between PfMIF and the human homologue (~29%). A solution was obtained when the PbMIF structure, which had not been available initially, was used as the model for molecular replacement with MolRep.⁵⁷ Two protomers in the asymmetric unit were sought based on the Matthews coefficient (1.73 Å³ Da⁻¹ with 29% solvent content)⁶⁸ with the biologically relevant trimers being generated via the crystallographic three-fold axis. Data and refinement statistics for both structures are presented in Table I. PDB accession codes are 2WKB and 2WKF for PbMIF and PfMIF, respectively.

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