Research Article

Structural and functional insights into macrophage migration inhibitory factor from *Oncomelania hupensis*, the intermediate host of *Schistosoma japonicum*

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*Oncomelania hupensis* is the unique intermediate host of *Schistosoma japonicum*. As an irreplaceable prerequisite in the transmission and prevalence of schistosomiasis japonica, an in-depth study of this obligate host–parasite interaction can provide glimpse into the molecular events in the competition between schistosome infectivity and snail immune resistance. In previous studies, we identified a macrophage migration inhibitory factor (MIF) from *O. hupensis* (*OhMIF*), and showed that it was involved in the snail host immune response to the parasite *S. japonicum*. Here, we determined the crystal structure of *OhMIF* and revealed that there were distinct structural differences between the mammalian and *O. hupensis* MIFs. Noticeably, there was a projecting and structured C-terminus in *OhMIF*, which not only regulated the MIF’s thermostability but was also critical in the activation of its tautomerase activity. Comparative studies between *OhMIF* and human MIF (hMIF) by analyzing the tautomerase activity, oxidoreductase activity, thermostability, interaction with the receptor CD74 and activation of the ERK signaling pathway demonstrated the functional differences between hMIF and *OhMIF*. Our data shed a species-specific light on structural, functional, and immunological characteristics of *OhMIF* and enrich the knowledge on the MIF family.

**Introduction**

Schistosomiasis is a destructive parasitic zoonosis caused by agents in the genus *Schistosoma*. In spite of considerable efforts invested in its prevention and treatment, schistosomiasis remains a serious public health problem, with an estimated 250 million people infected and over 780 million people at risk worldwide, especially in tropical and subtropical areas [1,2]. The amphibious snail *Oncomelania hupensis* prevalent in regions along the Yangtze River in China is the unique intermediate host for *Schistosoma japonicum* [3,4]. As an irreplaceable prerequisite in the transmission and prevalence of schistosomiasis japonica, it is of great importance to investigate the molecular mechanisms in this obligate host–parasite interaction. While significant progresses have been made in understanding the snail–schistosome interaction by transcriptomics, proteomics, genomics, and functional genetics on a related but different * Biomphalaria–Schistosoma* interaction [5–8] in recent years, little attention has been paid to the *Oncomelania–Schistosoma* interaction.
Macrophage migration inhibitory factor (MIF) is a pleiotropic immunoregulatory cytokine involved in immune and inflammatory responses. MIF can manifest itself as a hormone, a chemokine, and an enzyme [9,10]. By binding to the type II receptor CD74 and the chemokine receptors CXCR2, CXCR4, and CXCR7 [11–13], MIF mediates many important cellular processes including cell proliferation, apoptosis, survival, migration, and regulation of chemotaxis [14–16]. In addition, MIF is implicated in the pathogenesis of many inflammatory and autoimmune diseases including rheumatoid arthritis (RA) [17–20], atherosclerosis [17–20], and systemic lupus erythematosus (SLE) [19,20], acute respiratory distress syndrome [21], cardiovascular disease [22], and cancers [23], suggesting it is a pivotal mediator in acute and chronic inflammatory diseases. MIF also has the unusual ability to be an enzyme to catalyze a variety of biochemical reactions. The most conserved and well studied of these is its tautomerase activity [24], which is dependent on the penultimate Pro2 residue in the N-terminus for catalysis and can be significantly inhibited by its antagonist, (S, R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) [25,26]. However, the MIF’s physiological substrates have not been identified. Generally, the natural (p-hydroxyphenylpyruvate, HPP) and synthetic ligands (p-dopachrome or its analogs) were used as the substrates for assaying the tautomerase activity [37], but shares no structural similarity with any other cytokines. MIF is a homotrimer and also function as a nuclease to mediate cell death [34].

The first crystal structure of MIF was determined in 1996 [35,36]. It was with the similar topology for three microbial enzymes of oxaloacetate tautomerase, chorismate mutase, and 5-carboxymethyl-2-hydroxymucounate isomerase [37], but shares no structural similarity with any other cytokines. MIF is a homotrimer and each monomer is with two α-helices packed against a four-stranded β-sheet, which is extended by two short β-strands from the adjacent subunits. The overall structure of MIF is barrel-shaped and has a solvent channel consisting of three β-sheets running through the center of the trimer. MIF’s tautomerase active site is located between two adjacent monomers of the homotrimer. As an evolutionarily conserved molecule, orthologues of MIF have been found across a wide range of different species from bacteria to mammals [9,38]. In recent years, the crystal structures of several invertebrate MIFs were determined and compared with hMIF, but most of these were MIFs from parasites, such as Plasmodium sp. (including Plasmodium yoelii, Plasmodium falciparum, and Plasmodium berghei) [39,40], Brugia malayi [41], Trichinella spiralis [42], Leishmania Major [43], Ancylostoma ceylanicum [44], Toxoplasma gondii [45], and Giardia lamblia [46]. In addition, the crystal structure of MIF from the marine cyanobacterium Prochlorococcus marinus was also reported [47]. However, no structure of MIF from snails has been determined up to date. Further investigation showed that there were some significant differences in structures and biological activities between vertebrate and invertebrate MIFs. For example, the catalytic pockets in MIF from L. major, A. ceylanicum, and T. gondii seemed to be different, rendering ISO-1 inactive [43–45]. While vertebrate MIFs are generally involved in innate and adaptive immune responses and invertebrate MIFs are more important in innate immunity with the lack of adaptive responses, MIF in parasitic organisms appears to function as a virulence factor to aid the establishment or persistence of infection by modulating the host immune response, indicating MIFs have undergone important structural-functional changes during the evolution from invertebrates to vertebrates [38].

Previously we identified a homolog of mammalian MIF from O. hupensis (OhMIF) and showed its differential expression in snails following challenges with its natural parasite S. japonicum [48]. We also demonstrated that OhMIF had some conserved biological activities and involved in the snail host immune response to its natural parasite S. japonicum as a regulator in the innate immune defense system of O. hupensis [32,48]. However, the structural basis and molecular mechanism of OhMIF remain to be investigated.

Herein, the crystal structure of OhMIF was determined and characterized. The overall structures between OhMIF and hMIF were similar despite their sequence identity was low at 27%. The significant differences between the two were in their enzymatic active sites, the electrostatic potential on the protein surfaces and central channels, as well as the C-termini. Unlike hMIF, the N-terminal methionine (Met1) of OhMIF was intact, which partially occupied the substrate-binding site and its tautomerase activity could only be activated in the presence of its substrate or inhibitor with the cleavage of Met1. Further analysis showed that this activation of enzymatic activity might be regulated by the conformational plasticity of the protruding C-terminus of OhMIF, which was in different conformations by binding either the substrate HPP or the inhibitor ISO-1.
The comparative studies between OhMIF and hMIF on tautomerase activity, oxidoreductase activity, thermostability, binding to receptor CD74, and ERK1/2 activation indicated that the tautomerase activity and thermostability of OhMIF were lower, and its oxidoreductase activity might be of different mechanism. The extended C-terminus of OhMIF had a significant influence on its thermostability and tautomerase activity but was not required for the interaction with CD74 and activation of the ERK1/2 signaling pathway.

Materials and methods
Expression and purification of recombinant OhMIF and its mutants
The recombinant plasmid OhMIF-pET22b from a previous study [32] was the template to generate the desired mutants with the primers listed (Supplementary Table S1). The plasmids were transformed into Escherichia coli BL21 (DE3) cells, induced for protein production with the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a concentration of 0.2 mM at 18°C overnight. The cells were harvested by centrifugation, resuspended in buffer A (20 mM Tris–HCl, pH 8.0, 200 mM sodium chloride) containing a complete cocktail protease inhibitor (Roche), and then lysed by sonication. The supernatant was collected by centrifugation at 15 000×g and 4°C for 30 min, and loaded onto the Ni-NTA Resin (Invitrogen) equilibrated with buffer A. After washed by 30 mM imidazole in buffer A, the proteins were eluted with 500 mM imidazole in buffer A. The proteins were collected and identified by SDS–PAGE analysis, and further purified in a Superdex 75 16/60 gel filtration column (GE Healthcare). The fractions containing pure OhMIF were collected, pooled, and concentrated with Amicon Ultra filters (molecular mass cutoff, [MWCO], 10 000) (Millipore).

Dynamic light scattering (DLS)
DLS experiments were carried out with a Malvern NanoZS (Malvern) instrument equipped with a 70 µl cell (UV-Cuvette micro). Samples of OhMIF at concentrations of 2, 5, and 10 mg/ml in buffer A, were centrifuged at 13 000×g and 4°C for at least 20 min before the DLS measurements. The supernatant was passed through 0.22 µm-pore-size membrane filters (Whatman) by injection and then loaded to a 1 cm path length cuvette for measurements at 4°C. After an equilibration time of 120 s, the data from a total of 110 measurements for each sample were averaged and analyzed using Malvern software, which calculated the size distribution of particles, data correlation coefficient, PdI, and the apparent Z-Average (d.nm). DLS data were obtained from three independent measurements for all samples, and the averaged values were reported as the results.

Small-angle x-ray scattering (SAXS)
SAXS data were collected using the synchrotron radiation at beamline BL19U2 of the Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, China. The protein sample was dissolved in a buffer of 20 mM Tris–Cl, pH 8.0, 50 mM NaCl at a concentration of 4 mg/ml. Samples of 60 µl were exposed to X-rays by flowing through the 1.5 mm-diameter quartz capillary using the automated sample loading system. A Pilatus 1 M detector (Dectris) was employed for data collection. The wavelength of X-ray radiation was set at 1.033 Å. The data collection time was 1.0 s at a temperature of 293 K. Software RAW 1.5 was used to process images into data correlation coefficients, p(r), and the apparent Z-Average (d.nm). DAMMIN was initially estimated and then adjusted until the χ² value close to 1, and the random statistical errors were propagated correctly without systematic errors. Automated superposition of shapes and macromolecular models was carried out by using the program SUPCOMB.

Crystallization of OhMIF with and without the substrates
OhMIF was concentrated to ~20 mg/ml with a Centricon centrifugal filter unit (MWCO, 10 000) for crystallization. The crystallization of OhMIF was carried out by sitting-drop vapor diffusion at 16°C. The reservoir solution consisted of 30% (w/v) MPD, 0.1 M sodium acetate (pH 4.6), and 20 mM calcium chloride. About 0.6 µl of the protein solution was mixed with an equal volume of the reservoir solution. Plate-shaped crystals (Supplementary Figure S1A) appeared after 3 days. The crystallization of OhMIF122 is under the reservoir solution consisted of 5% v/v 2-Propanol, and 2.0 M ammonium sulfate. For crystallization of the complexes, the
substrate HPP or inhibitor ISO-1 (Sigma) (Supplementary Figure S2) was prepared and incubated with the purified OhMIF at a molar ratio of 1:3 for 2 h. The proteins were then concentrated to 20 mg/ml for crystallization. Cube-shaped crystals were obtained after 3 days at 16°C (Supplementary Figure S1B).

Data collection, structure determination and refinement
The crystals were transferred into a solution containing 0.1 M sodium acetate (pH 4.6), 20 mM calcium chloride, and 15% glycerol as the cryoprotectant prior to the flash cooling to the liquid nitrogen temperature at −170°C for data collection. The diffraction data were collected at 100 K under the synchrotron radiation at beamline BL19U1 in the SSRF. The diffraction data were integrated and scaled with HKL3000 [51]. The structure of OhMIF was determined by molecular replacement with the program Phaser [52] and the crystal structure of hMIF (Protein Data Bank [PDB] accession code 5BSI) as the search model. Model building was performed with Buccaneer [53] and rebuilt manually using coot [54] iteratively. The structure was refined with Refmac5 [55]. The statistics for data collection and refinement are in Table 1. The presentations were made with the PyMOL Molecular Graphics System, version 2.2.3 (Schrödinger LLC).

Tautomerase assay with hydroxyphenylpyruvate as the substrate
Phenylpyruvate tautomerase activity of OhMIF was assessed and measured using p-hydroxyphenylpyruvate (HPP) tautomerase assay as described previously [25,32]. Briefly, HPP was dissolved in 50 mM ammonium acetate (pH 6.0), equilibrated at room temperature overnight, and stored at 4°C. Catalytic activity was initiated at 25°C by adding 7.5 μl (∼0.7 μM) HPP to ELISA plate strips (JET BIOFIL) containing MIF and 0.435 M boric acid (pH 6.2) to a total volume of 150 μl. The activity was measured by following the increase in absorbance at 306 nm per 10 s due to the formation of the borate-enol complex using Tecan Spark (Tecan). The concentration of OhMIF used for the catalytic assay was 50 nM. The inhibition of ISO-1 was determined by preincubating OhMIF or hMIF with 100 μM ISO-1 dissolved in DMSO prior to the addition of the HPP. The steady-state kinetic values for the HPP tautomerase activity of OhMIF and hMIF were obtained using the Lineweaver–Burk plot [32]. Data presented were the average of at least three measurements.

Oxidoreductase assay
The oxidoreductase activity of OhMIF and its mutants (C64A and C107A) were measured using an insulin disulfide reduction assay as described previously [29,32]. Briefly, a solution of 100 mM potassium phosphate (pH 7.0), 1 mM EDTA (pH 8.0), 240 μM insulin (MCE), and 8.3 μM of either OhMIF or OhMIF mutants or equal volume of the medium as the control, was freshly prepared and added to ELISA plate strips (JET BIOFIL). The assay was initiated at 25°C by the addition of 1 mM dithiothreitol (DTT) to a total volume of 100 μl and the absorbance at 630 nm was measured at 5 min interval for 90 min by using Tecan Spark (Tecan). Quantification of the absorbance values was carried out by taking the average of three independent experiments with the absorbance from the medium subtracted.

Isothermal titration calorimetry (ITC)
ITC experiments were used to determine the enthalpy (ΔH), entropy (−TΔS), and change in the binding free energy (ΔG) to determine several thermodynamic parameters including the dissociation constant (Kd) and stoichiometry (N) of the interactions between protein and ligands [56]. The interactions between OhMIF or hMIF and ISO-1 were detected on a Malvern ITC-200 instrument (Malvern). All the samples were degassed thoroughly prior to the ITC experiments. The MIF protein (40 μM), which was dissolved in an assay buffer (20 mM Tris–HCl, pH 8.0, 200 mM sodium chloride), was added to the sample cell and reference cell, respectively. Calorimetric titration of the ligand solution (400 μM ISO-1 in the syringe; 2 μl aliquots at 120 s intervals) to MIF (200 μl) was performed with stirring at 300 rpm at 25°C. The initial titration before the first injection was 0.2 μl. The ITC experiments were conducted with 20 injections. After subtracting the heat from dilution, the ITC data were analyzed using the software installed in the ITC instrument. All data points were fitted to a single-site binding model.

Thermal shift assay
The thermostability of the purified proteins was determined by differential scanning fluorimetry (DSF) [57]. The purified protein was mixed with SYPRO orange fluorescent dye (Life Technologies S6650) in a 96-well optical quantitative PCR plate. The optimal dye and protein concentration were determined experimentally.
All protein dilutions were performed in 200 mM sodium chloride in 20 mM Tris–HCl (pH 8.0) buffer, and a negative control sample containing only the dye was used as a reference. The measurement was performed in a quantitative PCR instrument (BIO RAD CFX96) with a temperature ramp from 25 to 90°C at a rate of 0.3/0.5°C per 5 s. Data were collected continuously. Melting temperatures were calculated at 50% of the fluorescence maximum using a non-linear EC50 shift equation.

## Western blot detection of ERK1/2 phosphorylation

Hela cells were cultured to a density of 2 × 10⁶ cells/well in a 6-well cell culture plate using DMEM containing 10% FBS medium for 24 h. The cells were starved for 24 h with DMEM medium without FBS. After starvation, a solution of MIF (1 μg) in PBS was added to 2 ml of the same media to an ascribed well. PBS was used as the negative control. The cells were incubated for 0.5, 1, and 2 h, before washing with D-PBS, and lysing in 100 μl of ice-cold lysis buffer containing D-PBS (pH 7.2), 5 mM EDTA, 1% Nonidet P-40, 0.2% SDS, 1% mammalian protease inhibitor cocktail P8340 (Sigma), and 1% phosphatase inhibitor cocktail P5726 (Sigma). Protein extracts were centrifuged to remove cell debris, separated on 12% acrylamide gels, and electrotransferred to a PVDF membrane with 0.45 μm pore size. Blocking was done in 5% (wt/vol) BSA prepared in TBS-T buffer for 4 h at room temperature before probing with the primary antibodies including anti-phosphop44/42 MAPK (Erk1/2) (Thr202/Tyr204) or anti-p44/42 MAPK (Erk1/2) (CST) at 1 : 2000 dilution at 4°C overnight. After washing three times with TBS-T buffer, the membrane was stained with the horseradish peroxidase

**Table 1. Crystallographic data and refinement statistics**

|                          | Apo-OhMIF | OhMIF with ISO-1 | OhMIF122 |
|--------------------------|-----------|------------------|----------|
| **Data collection**       |           |                  |          |
| Space group              | P3 2 1    | P4 3 2           | P2₁      |
| **Unit cell**            |           |                  |          |
| a (Å)                    | 110.85    | 172.84           | 49.3     |
| b (Å)                    | 110.85    | 172.84           | 139.48   |
| c (Å)                    | 97.65     | 172.84           | 105.67   |
| α (%)                    | 90.00     | 90.00            | 90.00    |
| β (%)                    | 90.00     | 90.00            | 89.81    |
| γ (%)                    | 120.00    | 90.00            | 90.00    |
| Rmerge (%)               | 6.7 (47.3)| 10 (59.6)        | 7.4 (24.2)|
| **Total observations**   | 711 251   | 1 148 561        | 602 220  |
| **Unique reflections**   | 35 642    | 15 176           | 135 295  |
| **Resolution range (Å)** | 50.0E–2.20 (2.20–2.24) | 50.0–3.2 (3.26–3.2) | 50–1.77 (1.80–1.77) |
| **Completeness (%)**     | 99.9      | 100              | 96.2     |
| **Average I/σ(I)**       | 55.25 (9.0) | 55.33 (10.0)  | 19 (4.6) |
| **Redundancy**           | 20.0 (20.7)| 75.7 (65.2)     | 4.5 (4.0) |
| **Refinement**           |           |                  |          |
| No. of protein atoms     | 4400      | 4262             | 10 813   |
| No. of water molecules   | 424       | 114              | 2091     |
| No. of other atoms       | 7         | 2                | 40       |
| Rwork/Rfree              | 0.1795 (0.2263) | 0.2278 (0.3212) | 0.1503 (0.1797) |
| **R.m.s deviation**      |           |                  |          |
| Bond lengths (Å)         | 0.0097    | 0.007            | 0.0128   |
| Bond angles (°)          | 1.5092    | 1.5898           | 1.5965   |
| Dihedral angles (°)      | 0.0722    | 0.0673           | 0.0829   |
| Ramachandran analysis    | 98.7% in allowed regions | 95.4% in allowed regions | 99.0% in allowed regions |
(HRP)-conjugated goat anti-rabbit IgG antibody (Santa Cruz) at a 1:2000 dilution for 2 h at room temperature. The detection was carried out by using the ECL Western blotting system (Bio-Rad) according to the manufacturer’s recommendations.

**Cell culture**

Hela cells (ATCC CCL-2) were propagated in the DMEM media (GIBCO-BRL, Invitrogen), supplemented with 10% FBS and penicillin/streptomycin (GIBCO-BRL, Invitrogen). Hela cells were incubated at 37°C in 5% CO₂ atmosphere. For routine passage, cells were split from 1:3 to 1:6 when they reached confluence, generally in 1–3 days.

**Results**

**The structures of OhMIF**

The sequence alignment showed that OhMIF and hMIF had a sequence identity of 27% (Figure 1A). The evidences from size-exclusion chromatography (SEC), DLS, and SAXS indicated that OhMIF was in a similar dimension to homotrimeric hMIF and the higher-order aggregates in the solution were low (Supplementary Figure S3). The crystal structure of OhMIF was determined by molecular replacement to 2.20 Å resolution with an R_free of 0.2263 (Figure 1 and Table 1). The crystallographic data indicated that the structure of OhMIF was consisted of four protomers in the asymmetric unit. Three of the protomers formed a homotrimer, and the fourth monomer formed a trimer with two other protomers from the adjacent asymmetric units. The monomeric OhMIF and hMIF were of the same fold (Figure 1B), and each monomer was with two α-helices (α1 and α2) stacking against a four-stranded β-sheet to form a β-α-β fold. There were two additional short β-strands (β3 and β6) from the adjacent subunits. OhMIF has a long C-terminal region that formed an extra-long helix (α3), which was not seen in any other MIF structures (Figure 1A,B). The center of the OhMIF trimer is like a cylinder with a solvent channel along the molecular 3-fold symmetric axis with a possible chloride ion in the central pore with positive charges (Figure 1C and Supplementary Figure S4).

**Substrate and inhibitor activate the tautomerase activity of OhMIF with the cleavage of n-terminal Met1**

Tautomerase activity is a distinctive feature of the MIF superfamily and both d-dopachrome and HPP were generally used as the substrates in the assay for its enzymatic activity [9,10,25]. The substrate-binding pocket for the tautomerase activity in OhMIF seemed to be too constricted to accommodate the substrate (Figure 2A) despite our previous evidence showed that OhMIF was with the tautomerase activity using d-dopachrome as the substrate and could be inhibited by the MIF antagonist ISO-1 [32]. Here, we further demonstrated that OhMIF could also use HPP as the substrate for its tautomerase activity (Figure 2B), which was partially inhibited by ISO-1 (Supplementary Figure S5). The constricted substrate-binding pocket at the tautomerase active site in OhMIF was due to the presence of N-terminal Met1. Met1 in hMIF was cleaved in the crystal structures so Pro2 could be properly positioned for the catalysis. Like hMIF, OhMIF’s tautomerase activity required the presence of the penultimate residue Pro2 (Figure 2B). It raised the question on the origin of OhMIF’s tautomerase activity as the presence of Met1 would prevent the binding of the substrate. To address this issue, OhMIF was cocryrstallized with either HPP or ISO-1 for structural characterization. While no electron density could be linked to the binding of either HPP or ISO-1 in the structures (Supplementary Figure S6B), there was no electron density for a Met1 in one of the protomers either. As this N-terminal Met1 seemed to have been cleaved, the substrate-binding pocket in OhMIF was enlarged to the dimension to a volume of ~795.17 Å³ and 10.04 Å in the largest dimension, which was larger than that in hMIF with a volume of ~193.87 Å³ and 6.29 Å in the largest dimension, to accommodate the substrate (Figures 2A and 3). These results indicated that the tautomerase activity of OhMIF was activated in the presence of either its substrates or its inhibitors with the cleavage of the N-terminal Met1.

**Analysis of the enzymatic active sites in OhMIF**

Data from enzyme kinetics indicated that the catalytic rate constant (k_cat) of OhMIF was 38.64-fold lower than that of hMIF, and the substrate specificity index (k_cat/K_m) of OhMIF was 12.74-fold lower than that of hMIF, indicating that HPP might not be a specific substrate for OhMIF’s tautomerase activity (Supplementary Figure S7 and Table 2). We compared the Met1-free structure of OhMIF and the structure of hMIF in complex
with HPP by superimposing their tautomerase active sites (Figure 3A,B). There were seven residues (Pro2, Lys33, Ile65, Val107, Asn98, Tyr96, and His63) in the active site of hMIF [9,58]. Three of these residues (Pro2, Lys33, and Ile65) were conservative in OhMIF and they superimposed well in structure to form the left half of the substrate-binding pocket and interact with the pyruvate moiety of HPP (Figure 3B). His63, Val107, Asn98, and Tyr96 in hMIF were replaced with Glu63, Cys107, Arg98, and Val96, respectively, in OhMIF. These residues formed the right half of the substrate-binding pocket and the changes altered the enzyme interaction with the phenyl group of HPP. In hMIF, the phenyl ring of HPP could form a ‘Burley’ bond with the aromatic ring of Tyr96 with aromatic rings stacking perpendicular to each other to yield favorable charge–charge interactions due to the polarization of the aromatic rings [25], while Val96 in OhMIF did not have this interaction, so a Tyr96 to Val96 change would have a profound impact on ligand binding. The replacement of Asn98 in hMIF
with Arg98 in OhMIF led to the reorientation of the side chain with a loss of two hydrogen bonds. The change of His63 in hMIF to Glu63 in OhMIF was also significant as the His residue formed a hydrophobic interaction with the phenol moiety from HPP favorably while Glu63 would weaken this interaction. With the difference in the substrate-binding pocket, it is quite possible that OhMIF is with a different substrate specificity over hMIF, which was supported by the kinetic data (Table 2). We made a double mutation of V96Y/R98N in OhMIF but this replacement of hMIF residues for OhMIF residues only led to the loss of OhMIF’s tautomerase activity for HPP. Single mutations of V96Y and R98N was also made, both of which resulted in significantly reduced enzymatic activity (Supplementary Figure S8). So, it seemed that the change of these residues would lead to the change of substrate specificity and the enzyme could no longer bind HPP.

hMIF is with a TPOR activity and can catalyze the reduction of disulfides. This oxidoreductase activity is associated with a conserved C\textsuperscript{57}XX\textsuperscript{60} motif and Cys60 in hMIF as the indispensable residue for its enzymatic activity [29,31,59]. Cys60 was substituted by an Ala in OhMIF (Figure 1A). However, this lack of Cys60 did not impair OhMIF’s ability to reduce insulin [32]. In hMIF, the distance between the thiol groups of Cys57 and Cys60 was 9.7 Å. Analysis of the OhMIF structure showed that its Cys57 was separated as far as 24.5 Å from Cys64, the closest Cys residue to Ala60. However, there was a different pair of thiol groups, from Cys64 and Cys107, were with the similar separation of 9.5 Å as that between Cys57 and Cys60 in hMIF, but in a different orientation (Figure 3C). It is possible that OhMIF may also be associated with the TPOR activity by a cysteine thiol-mediated mechanism. It could be showed that mutations of either C64A or C107A resulted in a
Figure 3. Comparative views at the substrate-binding pockets from OhMIF and hMIF.

(A) The tautomerase substrate-binding pockets of OhMIF (left) and hMIF (right). hMIF was with the substrate HPP (sticks) bound. As this N-terminal Met1 seemed to have been cleaved, the substrate-binding pocket in OhMIF was enlarged to a volume of ∼795.17 Å³ and 10.04 Å in the largest dimension, which was larger than that in hMIF with a volume of ∼193.87 Å³ and 6.29 Å in the largest dimension. (B) Superimposition of the tautomerase substrate-binding pockets of OhMIF and hMIF in complex with HPP. Residues within hydrogen bonding distance to HPP are colored in either magenta (OhMIF) or cyan (hMIF),
significantly reduced oxidoreductase activity in the insulin activity assay (Supplementary Figure S9), supporting the notion that Cys64 and Cys107 provided the thiol groups to mediate the oxidoreductase activity in OhMIF.

The thermostability of OhMIF trimer

Despite the overall similarity in structure, the electrostatic potential on the surface of OhMIF are distinctly different from those of hMIF, especially in the interface between subunits of the OhMIF trimer (Figure 4A). The interface of OhMIF is more hydrophilic and highly charged compared with that of hMIF, suggesting that the interacting force between monomers in the MIF trimer might be different in solution. It could be showed that OhMIF was with a lower melting temperature ($T_m$) of 64.3 ± 0.6°C compared with that of hMIF (74.5 ± 0.6°C) by thermal shift assay (Figure 4B and Supplementary Table S2), indicating that the hMIF trimer was more stable than OhMIF in solution.

The central pore of MIF trimer was believed to have a unique solvent channel, and it was proposed that its positively charged electrostatic surface might either involve in the transportation of negatively charged ions or provide a binding site for negatively charged ligands. Its biological relevance though is still to be substantiated [9,28,58]. In this study, we observed that the central pore of OhMIF trimer was even more positively charged than that of hMIF (Supplementary Figure S4), and the radius of the cavity in OhMIF was from 1.3 to 4.4 Å, which was slightly smaller than those in hMIF (from 1.8 to 4.6 Å). Three symmetry-related Arg residues at position 98 formed positively charged electrostatic surface and bound a possible chloride ion in the pore of OhMIF structure (Figure 4C). In addition, three symmetric Arg residues at position 43 formed another positively charged electrostatic surface (Supplementary Figure S10). There was also hydrogen bonding between Arg98 and Glu63 from the adjacent subunits (Figure 4C). These features suggested that Arg98 might play an important role in the thermostability of the OhMIF trimer. Indeed, a R98N mutation significantly reduced the $T_m$ value by 17.55 ± 0.15°C (Figure 4D and Supplementary Table S2), although it was still trimeric in solution based on the SEC data (Supplementary Figure S11). The correlation between this reduced thermostability and the reduced tautomerase activity awaited further investigation.

The c-terminus of OhMIF played a role in its thermostability and tautomerase activity

In contrast with all other MIF structures determined up to date, the C-terminal residues 122–132 of OhMIF formed a distinct helix (Hα3) projecting outwards in a fixed conformation (Figure 1B,C). From the sequence alignments, this extended C-terminus is a unique feature to snail MIF [48]. Previous studies showed that a

| Table 2. Steady state kinetic values for HPP tautomerase activity of MIF |
|------------------|------------------|
| MIF        | OhMIF | hMIF |
| $K_m$ (mM)    | 0.96 ± 0.08 | 2.90 ± 0.20 |
| $k_{cat}$ (min⁻¹) | 5.24 ± 0.37 | 202.46 ± 8.34 |
| $k_{cat}/K_m$ (mM⁻¹ min⁻¹) | 5.48 ± 0.11 | 69.79 ± 2.1 |
| $k_{cat}/k_{cat}$ (OhMIF/hMIF) | 1 | 38.64 |
| $(k_{cat}/K_m)/(k_{cat}/K_m)$ (hMIF/OhMIF) | 1 | 12.74 |
deletion at the C-terminal residues 105–114 of hMIF would inactivate the tautomerase activity [60], despite the appearance that the C-terminal residues did not participate in the direct interactions with its substrate. Our truncations at the C-terminus showed that the thermostability of the truncated OhMIFs was gradually reduced and the $T_m$ values of OhMIF were lowered from 64.3 ± 0.6°C for the full-length protein (amino acid residues 1–132) to 53.05 ± 0.6°C for an OhMIF truncated with 10 residues at the C-terminus (OhMIF of amino acid residues 1–122, or OhMIF122), to 50.5 ± 1.05°C for the OhMIF of 17 residues shorter (OhMIF115), and to 50.05 ± 1.05°C for the OhMIF of 27 residues shorter (OhMIF105) (Figure 5A,B; Supplementary Table S2). SEC analyses showed that OhMIF122, OhMIF115, and OhMIF105 were still trimeric in solution (Supplementary Figure S12). That the OhMIF became gradually more labile to heat as its C-terminus became shorter implicated that the protruding C-terminus played a role in maintaining the stability of OhMIF in solution.

Interestingly, OhMIF122 with a deletion of 10 residues from the C-terminus was with a greatly enhanced tautomerase activity using HPP as the substrate. Yet, the tautomerase activities of OhMIF115 and OhMIF105 were abolished (Figure 5C). It seems that the truncation of C-terminal Hx3 in OhMIF122 enhanced the tautomerase activity.

Figure 4. Analysis of subunit interface in OhMIF.
(A) Comparison of the electrostatic potential at the interface between subunits of OhMIF and hMIF. Red is for the negative charges and blue is for the positive charges. The interface between subunits of the MIF trimers are in circles (left). Right: one OhMIF subunit was in surface rendering while two others were in ribbon diagrams. (B) Melting temperature ($T_m$) values of OhMIF and hMIF trimer in the presence or absence of the antagonist ISO-1 by thermal shift assay. The $T_m$ values of hMIF in the presence or absence of ISO-1 are 74.5 ± 0.6°C, and the $T_m$ values of OhMIF in the presence or absence of ISO-1 are 64.3 ± 0.6°C. The data obtained were the means ± S.E.M. of three independent experiments. (C) Arg98 is implicated in maintaining the structural stability of OhMIF trimer. Arg98 trimer not only bound to a possible chloride ion, but also formed the hydrogen bond interaction with residue Glu63 of the adjacent subunit. The interacting distances are shown. (D) Melting temperature ($T_m$) values of OhMIF and its mutants. The $T_m$ values of OhMIF, V96Y, R98N, and V96Y + R98N are 64.3 ± 0.6°C, 58.7 ± 0.4°C, 46.75 ± 0.75°C, and 46.6 ± 2.7°C, respectively. The data obtained were the means ± S.E.M. of three independent experiments.
activity, but further truncation would destabilize OhMIF to the detriment of its tautomerase activity. Moreover, the crystal structure of OhMIF122 showed no electron density for a Met1 in all the promoters (Supplementary Figure S6C), which is likely to be associated with the enhanced tautomerase activity of OhMIF122. These results indicated that the C-terminus of OhMIF played an important role in its tautomerase activity.

To better understand the activation of tautomerase activity, the crystal structures of OhMIF in the absence and presence of ligands were superimposed and analyzed. With the presence of substrate HPP or inhibitor ISO-1 for crystallization, the C-terminal Hα3 of OhMIF could rotate 36.3° with residue Arg120 as the pivot (Figure 5D). Residue Arg120 is the first residue before the rotatable C-terminal Hα3. Before this rotation, Asp123 as part of the N-terminal Hα3 was in interaction with Arg67, so Lys33 could interact with the sulfur atom from Met1 and the carbonyl oxygen atom from Ile65 (Figure 5E). As Hα3 rotated, Asp123 was pulled away from Arg67. Without the constraint of Asp123, the side chain of Arg67 flipped to replace Met1 for the interaction with Lys33 and Ile65. In this shuffle of interactions, Met1 was cleaved. The helix was re-positioned...
by the formation of two hydrogen-bonds between Arg67 and Asp121 and between Glu119 and Ala104 (Figure 5F). The enzyme was subsequently activated with an opened substrate-binding pocket for the interaction with substrates, possibly by a conformational selection mechanism. The cleavage of Met1 would also allow Pro2 to function as the catalytic residue. That a mutation of D123A was associated with a significantly enhanced tautomerase activity was a support to the notion, that the rotation of the C-terminal helix involving Asp123 was important to regulate the tautomerase activity of OhMIF (Supplementary Figure S13).

C-terminus of OhMIF is not required for activating the ERK1/2 pathway

MIF can effectively phosphorylate ERK1/2 to activate the signaling pathway by binding to the type II receptor CD74 as a pro-inflammatory cytokine [11,14]. We previously showed that OhMIF was also able to phosphorylate ERK1/2 to activate the pathway [32]. Since the extended C-terminus of OhMIF was a distinct feature, one would wonder whether it was involved in the CD74 binding to activate the ERK1/2 MAPK pathway. Our ERK1/2 phosphorylation assay showed that OhMIF_{122} would still effectively phosphorylate ERK1/2 (Figure 6A, B), indicating that the C-terminus of OhMIF is not required for the interaction with CD74.

Discussion

MIF is a multifunctional pro-inflammatory cytokine involved in both acquired and innate immunity. It mediates many inflammatory processes by interacting with CD74-CD44 receptor complex or chemokine receptor CXCR to activate the ERK1/2 MAPK signaling pathway [9,11–14]. As an evolutionarily conserved protein, orthologues of MIF have also been identified and characterized in many invertebrate species [38]. In our previous studies, OhMIF was shown to be involved in the snail host immune response to the parasite S. japonicum [32,48], but the structural basis and molecular mechanism for the OhMIF function was unclear. Here, we reported the crystal structural and functional characterizations of OhMIF. The unraveling of OhMIF’s distinct features in structure, function and immunological properties provides insights into the evolution and adaptation of MIF and it lays a foundation for further structural research of MIF in molluscs.

Figure 6. C-terminus of OhMIF is not required for the phosphorylation of ERK1/2 and activation of the signaling pathway.

(A) Immunoblotting of cell lysates pre-incubated with OhMIF, OhMIF_{122}, hMIF, and PBS using an anti-phosphorylated ERK1/2 antibody (p-ERK) and an anti-ERK1/2 antibody as the control for the total ERK content (Total ERK) in each sample. Cells in different groups were treated for 0.5, 1, and 2 h. (B) The optical density of migrating bands was quantified using Image J software. The mean values of relative intensity are 1.53 (OhMIF), 1.23 (OhMIF_{122}), 0.98 (hMIF), and 0.41 (PBS), respectively. Results shown are the means ± S.E.M. of three independent measurements.
The MIF family is a group of conserved proteins with a β–α–β fold [61] and a conserved catalytic N-terminal proline (Pro2) for its tautomerase activity [25]. The crystal structure revealed that OhMIF was with the same fold as for hMIF (Figure 1). A distinct feature in OhMIF was its protruding and structured C-terminus. It was also the first view of an uncleaved Met1 that blocked the active site for its tautomerase activity (Figure 2A). Our previous [32] and present data (Figure 2B) indicated that OhMIF was with the Pro2-dependent tautomerase activity suggesting that Met1 would have to be removed. Our structural studies showed that Met1 was cleaved with the presence of either its substrate (HPP) or antagonist (ISO-1) to enlager the substrate-binding pocket and free Pro2 for catalysis (Figure 2A). Despite the fact that the cleavage of M1 could only occur with the presence of either HPP or ISO-1, the co-crystallization experiments resulted in no electron density that could be linked to the binding of either the substrate or the antagonist in the structures of OhMIF (Supplementary Figure S6). One possible explanation is that the binding pocket of OhMIF is so large that the balance between association and dissociation in binding its substrate (HPP) or antagonist (ISO-1) would favor the dissociation, thereby leading to a rapid dissociation of its ligands from the binding pocket. This explanation was supported by our enzyme kinetics data and ITC data (Table 2 and Supplementary Figure S4).

In this study, DLS and SAXS measurements were carried out to understand the oligomeric state and homogeneity of OhMIF, which were done at pH 8.0. The results showed that OhMIF was in a similar dimension to hMIF as a homotrimer, and its homogeneity was sufficient for crystallization. Unfortunately, the crystals could only be obtained at pH 4.6 and the crystal structure showed that the protein was still a trimer under this completely different pH value. It was reported that hMIF was stable and enzymatically active at lower pH [25]. It is quite likely that the active OhMIF at higher pH would still adopts the conformation as was observed at pH 4.6.

Analysis of the OhMIF structure revealed significant differences around the tautomerase active site compared with hMIF. The differences in the tautomerase activity and inhibition by ISO-1 of OhMIF compared with hMIF (Table 2 and Supplementary Figure S5) are likely to be the results of four different residues in the substrate-binding pocket leading to different substrate specificity (Figure 3A,B). It is reasonable to suggest that OhMIF can have different physiological ligands. TPOR activity is another important function in the MIF family, especially in the mammalian MIFs. This enzymatic activity was reported to be dependent on the CALC motif in the center of the molecule and use GSH as its reductant [29,30]. Recently, several studies, including ours, demonstrated that some invertebrate MIFs could also display the oxidoreductase activity without the CALC motif. These enzymes though used DTT as the reductant instead of GSH [31-33], implicating a different mechanism from that of the canonical MIF. Structural analysis showed that catalytically important cysteines in OhMIF were at positions 57, 64, and 107, in contrast with hMIF where the conserved cysteines were at positions 57, 60, and 81. Despite the absence of the conserved C57ALC60 motif, the distance between Cys64 and Cys107 in OhMIF (9.50 Å) was similar to that of C57ALC60 catalytic motif in hMIF (9.70 Å) (Figure 3C). Our further mutational analysis also demonstrated Cys64 and Cys107 could be the key residues in the oxidoreductase activity of OhMIF (Supplementary Figure S9). As none of the reported X-ray crystallographic data demonstrated the existence of an intramolecular disulfide bridge in MIF structures for reducing the physiological substrates [35,36], the mechanism for MIF's oxidoreductase activity awaits further investigation, which could be facilitated by the availability of this OhMIF structure with different arrangements of the Cys residues. MIF is a conserved cytokine and has been found in a wide range of species, from bacteria to mammals [9,38]. In OhMIF, the interface between subunits is more hydrophilic and highly charged compared with that of hMIF (Figure 4A), which would be the origin of the lower Tm value and weakened stability (Figure 4B). MIF trimer has a solvent channel that runs through the center of the protein along the molecular 3-fold axis. It was suggested that the positively charged electrostatic surface of this channel could be involved in the transportation of negatively charged ions or provide a binding site for negatively charged ligands such as dopachrome or sialic acid-containing glycolipids for MIF’s function in the human monocyte response to MIF [35,58]. OhMIF is with different electrostatic potential distributions on the surface and channel. Trimeric Arg98 and Arg43 formed two positively charged electrostatic constrictions along the channel (Supplementary Figure S10B). The trimeric Arg98 layer not only bound with an ion (possibly chloride ion), but also formed a hydrogen bond with Glu63 from the neighboring subunit (Figure 4C). This inter-subunit interaction was important for maintaining the thermostability of OhMIF trimer as was demonstrated by the mutation experiments (Figure 4D). Besides the constriction formed by Arg98, 3-fold related Arg43 could also form an ion binding site along the channel as was observed in the crystal structure of OhMIF122 (Supplementary Figure S10C). Judged by the size, the density surrounding by Arg43 residues could be a sulfate ion. The possible existence of an anion in
interaction with positive charges in the central pore of OhMIF trimer bestowed a support to the argument that OhMIF could be an anion transporter [62].

The C-terminal region of MIF was shown to participate in the induced-fit mechanism for inhibitor binding and be responsible for the formation of the trimer [60,63]. A unique feature in the OhMIF structure is the projecting C-terminal Hx3 of ten amino acids from residue 123 to 132. Our data showed that the C-terminus of OhMIF played a significant role in its thermostability in solution. A truncation of 10 residues in this C-terminus would result in a decrease of 10°C in its $T_m$ value (Figure 5B). There seemed to be two reasons that the truncations at the C-terminus would reduce the thermostability of the protein. First, residues 115–120 formed a short helix (3$_{10}$ helix) in the full-length OhMIF, which seemed to be important for the inter-subunit interaction among β6 strands from the neighboring subunits. After the residues after this 3$_{10}$ helix were removed as in OhMIF$_{122}$, these residues adopted a loop conformation with the loss of a hydrogen bond between the carbonyl oxygen atom from Glu119 and the amide nitrogen atom from Ala104. As a consequence, the inter-subunit interaction among β6 strands was disrupted to the detriment of the thermostability of OhMIF. Second, the truncation also eliminated an ionic interaction between Arg67 and Asp123, which significantly attenuated the thermostability of OhMIF. The proof for this came from D123A mutation which resulted in a decrease of the OhMIF $T_m$ value by 9.4 ± 0.9°C due to the loss of the ionic interaction (Supplementary Figure S14). Therefore, despite the fact that the C-terminus protruded outwards, the interactions from residues near the end of the extended helix are still vitally important to the structure and function of the protein.

The protruding C-terminus of OhMIF seemed also playing an important role in its tautomerase activity. The initial truncation as in OhMIF$_{122}$ led a significantly increased tautomerase activity. However, OhMIFs with additional truncations, be it OhMIF$_{115}$ or OhMIF$_{105}$, lost the tautomerase activity (Figure 5C). This phenomenon could be explained by way of OhMIF’s activation mechanism and thermostability. OhMIF was activated through interruption of the ionic interaction between Arg67 and Asp123 so that C-terminal Hx3 of OhMIF could rotate as much as 36.3° with the formation of two hydrogen-bonds between Arg67 and Asp121 and between Glu119 and Ala104 (Figure 5D,F). This conformational change in the C-terminus of OhMIF led to the shift of interactions among Arg67, Lys33, and Ile65 and the cleavage of Met1 (Figure 5E,F) to activate its tautomerase activity. The truncation of 10 residues in OhMIF$_{122}$ would enhance the enzymatic activity before it eliminated Asp123 to nullify the interaction between Asp123 and Arg67. Further truncations as in OhMIF$_{115}$ or OhMIF$_{105}$, however, would further reduce the thermostability of the protein to the degree that they lost the tautomerase activity.

The first amino acid residue in a nascent protein is a methionine. About 80% of these methionines are removed by methionine aminopeptidases (MetAP1 and MetAP2). MetAP2 contains a single Cys228–Cys448 disulfide bond that has the features of an allosteric disulfide, which controls the substrate specificity and catalytic efficiency by influencing its nearby catalytic His231 [64]. In this study, the removal of the N-terminal Met1 in OhMIF is intriguing. In our structure, residues Cys64 and Cys107 at the rim of the active site of OhMIF could mediate the oxidoreductase activity. In addition, there was a His114 close to one of the Cys residues, Cys107 (Supplementary Figure S15B). It could be hypothesized that OhMIF is associated with the enzymatic activity of MetAP2 for the removal of its N-terminal methionine. The hypothesis could be augmented by a data that the mutation of H114A significantly reduced the tautomerase activity of OhMIF (Supplementary Figure S15C).

The salient feather of OhMIF is its protruding C-terminal helix. The crystal structures also showed that this C-terminal helix was in a rigid conformation, which might be influenced by the crystal contact. There were four protomers in one asymmetric unit in OhMIF crystals. Since each protomer was in a different environment, it is possible that these 4 C-terminal helices were in the same fixed orientation in solution before they contacted the neighboring subunits to form the crystals. In addition, the C-terminal helix underwent a large movement during the activation. The fact that the C-termini adopted rigid conformations both before and after the activation, albeit at different angles, indicated that the full OhMIF was a 3-fold symmetrical molecule.

MIF could either be a monomer, dimer, or trimer. Yet the active form that binds and activates the MIF receptor (CD74) is still subjected to debate [65]. Our current knowledge is that the ectodomain of CD74 is also a symmetric trimer hence the argument that the interaction in MIF-CD74 might be along the 3-fold axes [66–68]. With the extended C-terminus in OhMIF, its role in the interaction with the receptor CD74 was investigated. Our results in this study showed that the C-terminus of OhMIF was not required for the phosphorylation of ERK1/2 and activation of the downstream signaling pathway (Figure 6).
In summary, this study investigated the structure and functions of MIF from the freshwater snail *O. hupensis*, the intermediate host for *S. japonicum*. Overall, OhMIF is with a conserved fold, but also with a distinct C-terminal helix (Hα3). This Hα3 can adopt multiple conformations in association with the activation of the tautomerase activity of OhMIF. OhMIF was initially with an intact Met1 that occupied the tautomerase substrate-binding pocket. The intact Met1 also prevented Pro2 to function as the catalytic residue. With the presence of interacting compounds, be it the substrate or inhibitor, there was a shift of the interaction network around the tautomerase active site, which resulted in breaking the interaction between R67 and Asp123 in Hα3. The free Arg67 would substitute the interactions of Met1 with Lys33 and Ile65. The result was the cleavage of Met1 and the activation of the tautomerase activity. As the Hα3 only existed in snail MIF and was directly linked to the enzymatic activity, this could be a species-specific regulatory mechanism for the tautomerase activity on one hand, but could also provide a system to understanding the tautomerase activation on the other.

Data Deposition
The atomic co-ordinates of the structure and the structure factors were deposited in Protein Data Bank with PDB ID codes 6LKV, 6LK, and 6LR3.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions
S.H. and Z.S. conceived and designed the study. Z.S. and X.T. codesigned the study and performed crystallization, biochemical assays and 3D structure analyses. H.L. and Z.W. carried out mutagenesis, expression and purification. L.C. and S.W. carried out related structural analyses, and wrote the related section. H.R. and W.P. analyzed the data and approved the manuscript. C.T. approved the manuscript. T.L. and S.H. co-ordinated the work and wrote the manuscript.

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Abbreviations
DLS, dynamic light scattering; DSF, differential scanning fluorimetry; DTT, dithiothreitol; hMIF, human MIF; HPP, p-hydroxyphenylpyruvate; Hα3, helix α3; IPTG, isopropyl β-D-1-thiogalactopyranoside; ISO-1, (S, R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester; ITC, isothermal titration calorimetry; MIF, macrophage migration inhibitory factor; OhMIF, *Oncomelania hupensis* MIF; PDB, Protein Data Bank; SAXS, small-angle X-ray scattering; SEC, size-exclusion chromatography; SSRF, Shanghai Synchrotron Radiation Facility; Tm, melting temperature; TPOR, thiol-protein oxidoreductase.

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