Macrophage Migration Inhibitory Factor Interactions with Glutathione and S-Hexylglutathione

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Macrophage migration inhibitory factor (MIF) has been reported to interact with glutathione and S-hexylglutathione and to possess glutathione S-transferase activity. However, contrary to these reports, a recent NMR study concluded that MIF shows no affinity for glutathione. Re-examination of the glutathione-MIF interactions indicates that the reported increase in fluorescence upon addition of glutathione is because of pH-induced unfolding of the protein and not to any direct interactions. Circular dichroism shows that MIF remains folded from pH 4.5–7.5 but is 50% unfolded at pH 2.9 ± 0.2. The reported increase in fluorescence can be achieved by acid titration. Under strongly buffered conditions, no fluorescence change is observed upon addition of glutathione. In contrast to the results with glutathione, MIF binds S-hexylglutathione with a $K_d$ of 2.5 ± 0.6 m. Using NMR spectroscopy, a binding site which clusters around the N-terminal proline was identified. These data indicate that the binding site for S-hexylglutathione is the same as the catalytic site for the dopachrome tautomerase activity of MIF. Consequently, the binding of S-hexylglutathione as well as hexanethiol inhibits this catalytic activity.

Macrophage migration inhibitory activity was first described in 1966 as a secretion product from activated T cells and has been associated with delayed-type hypersensitivity reactions ever since (1, 2). MIF is expressed in macrophages, B-cells, and T-cells, and has been implicated in a variety of inflammatory diseases such as sepsis, adult respiratory distress syndrome (ARDS), and rheumatoid arthritis (3–8). MIF is released by glucocorticoids (9). MIF is the only cytokine known to be induced by glucocorticoids and to regulate glucocorticoid activity. MIF is also expressed in cells not normally associated with immune or inflammatory reactions and therefore may have additional activities. MIF is found in testes, ovaries, and early embryos, suggesting a possible role in development (10, 11). It is also found in the islet cells of the pancreas where it plays a role in regulating insulin secretion (12). And perhaps most importantly, MIF has been detected in the corticotropic cells of the anterior pituitary where it may mediate neural-immune system communication (9).

In addition to the proinflammatory activities, MIF has also been reported to possess three catalytic activities: $\alpha$-dopachrome tautomerase, hydroxyphenylpyruvate tautomerase, and glutathione S-transferase (GST) (13–15). $\alpha$-Dopachrome tautomerase, a nonphysiological reaction which involves the conversion of $\alpha$-dopachrome methyl ester to 5,6-dihydroxindole-2-carboxymethylester, was discovered fortuitously during the study of melanin biosynthesis (13). Subsequently, hydroxyphenylpyruvate tautomerase, which catalyzes the keto-enol isomerization of both $p$-hydroxyphenylpyruvate and phenylpyruvate, was discovered in an attempt to identity natural ligands for MIF (14). Glutathione S-transferase activity was identified when a purification scheme intended to isolate novel glutathione S-transferases yielded a protein from rat liver that was identified as a homologue of MIF (15). This protein had 25 of 26 residues identical with the amino terminus of MIF and was reported to catalyze the conjugation of glutathione to 1,2-epoxy-3-($p$-nitrophenoxyl) propane (EPNP) (15). The glutathione S-transferase activity and sequence identity at the N terminus led to the speculation that MIF functioned as a GST. This view was supported by fluorescence titration experiments which showed that human MIF binds glutathione with a dissociation constant that is within the physiological concentration of glutathione (16).

The potential interaction between MIF and glutathione was intriguing because both of these molecules have important functions in the immune system (17, 18). The release of MIF and glutathione by lipopolysaccharide (19), the proinflammatory effects of MIF and glutathione metabolites, and the reported molecular interactions between these two molecules invited speculation that their mechanism of action was linked. However, a number of reports argued against any role for glutathione in MIF activity (20–22). To resolve the discrepancy in the literature with regard to glutathione binding to MIF, we reexamined glutathione-MIF interactions. Using fluorescence spectroscopy and circular dichroism, we find that titration of MIF with unbuffered glutathione induces a change in tryptophan fluorescence due to pH-induced denaturation of MIF rather than a direct interaction with glutathione as previously reported. This is in agreement with a recent NMR study that was unable to detect glutathione binding (22). Moreover, we...
show that MIF displays affinity for S-hexylglutathione. Using NMR spectroscopy and enzymatic assays, we were able to quantify this affinity, locate the binding site, and determine that S-hexylglutathione inhibits MIF dopachrome tautomerase activity. We used hexanethiol to confirm that the basis of this inhibition is the S-hexyl moiety.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant MIF and [15N]MIF—**

The cloning and expression of human MIF into the IPTG-inducible expression plasmid pET11b has already been described (23). The cloning and expression of human MIF into the IPTG-inducible expression plasmid pET11b has already been described (23). BL21(DE3) cells were grown to an expression plasmid pET11b has already been described (23). The cloning and expression of human MIF into the IPTG-inducible plasmid pET11b has already been described (23).

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**RESULTS**

**Fluorescence Titration of Glutathione—**

Glutathione was reported to bind MIF with a \( K_d \) of 500 \( \mu \)M based on fluorescence titration experiments (16). We repeated these experiments in our laboratory with recombinant human MIF and were able to observe similar changes in the fluorescence spectrum (Fig. 1A). When the change of intrinsic Trp-108 fluorescence was measured upon addition of increasing concentrations of glutathione, saturation could be achieved with 6 mM glutathione (Fig. 1B). This increased fluorescence was assumed to be due to a conformational change of the protein upon glutathione binding. However, the high concentration of glutathione relative to the MIF concentration was inconsistent with specific binding.

Upon further investigation, we found that addition of increasing amounts of unbuffered glutathione resulted in a pH decrease of the MIF solution. When the glutathione solution was buffered with phosphate at pH 6, addition of glutathione to MIF resulted in little or no change in fluorescence (Fig. 2A). Similarly, when MIF was strongly buffered at pH 6, little fluorescence change was observed upon addition of unbuffered glutathione. A change was observed only when MIF was weakly buffered (20 mM Tris, 20 mM NaCl, pH 7.5) and unbuffered glutathione was added. Under these conditions, the pH of the MIF solution decreases as the glutathione concentration is increased resulting in a reproducible increase in fluorescence (Fig. 2A, inset). Acid titration of MIF revealed the same change in fluorescence as titration with unbuffered glutathione (Fig. 2B).

**pH-induced Unfolding of MIF—**

The high glutathione concentration required to induce a fluorescence change and the change in pH upon addition of glutathione were consistent with pH-induced unfolding of the protein. To address this question, circular dichroism spectroscopy was utilized to characterize the folding of MIF at different pH values (Fig. 3A). Due to a strong circular dichroism signal by glutathione, the CD spectrum of MIF in response to increasing amounts of glutathione could not be measured.

**Circular Dichroism Spectroscopy—** The CD spectrum of 20 \( \mu \)M MIF in 20 mM sodium phosphate, pH 7.4 was measured in an Aviv CD spectrometer at 22 °C in a 2-mm pathlength quartz cuvette. A blank was used to subtract buffer contributions to the CD signal of the protein. The pH of each MIF sample was adjusted with 1 M HCl to the desired pH, and the spectrum was measured from 200 to 270 nm.

**Dopachrome Tautomerase Activity—** Dopachrome methyl ester was prepared by mixing 4 mM 1,3,4-dihydroxypentylmethyl ester (Sigma) with 8 mM sodium periodate (Sigma) for 5 min at room temperature and then placed directly on ice. Hexanethiol was purchased from Acros Organics and diluted in MeSO to a final concentration of 10 mM. Inhibition of dopachrome tautomerase activity by S-hexylglutathione or hexanethiol was determined by mixing 100 \( \mu \)M MIF with varying concentrations of inhibitor, adding 200 \( \mu \)M dopachrome methyl ester in 25 mM potassium phosphate buffer, pH 6, 0.5 mM EDTA, and measuring the decrease in absorbance at 475 nm on a Hewlett Packard 8452 diode array spectrophotometer. MeSO alone was used as a solvent control when measuring the inhibitory activity of hexanethiol.

**NMR Experiments—** All NMR measurements were made on a DMAX-600 NMR spectrometer operating at 600.144 MHz. Two-dimensional 1H-15N-HSQC spectra were collected on a 1 mm sample of 15N-MIF in 20 mM phosphate, pH 6.8, using a Bruker 5-mm triple resonance probe with 3-mm gradients. S-Hexylglutathione (Sigma) was dissolved in 1 M ammonium hydroxide and the pH adjusted to 6.8 with HCl prior to addition to 15N-MIF. Acquisition parameters for 1H-15N-HSQC spectra were as follows: spectrometer frequency, 600.144 MHz; spectral width, 5388 Hz; acquisition time, 95 msec; recycle delay, 1 s; transients per block, 32; complex blocks, 128; temperature 298 K. To facilitate processing off line, data were transferred to a PC using Felix 95.0 (Biosym). NMRView 2.1 (Bruce Johnson) was employed for peak picking and chemical shift measurements.

**Kd Determination—** Chemical shifts for backbone amides (1H and 15N) were compiled, and the ten residues with the greatest changes in chemical shift upon addition of S-hexylglutathione were identified. The chemical shifts for these residues were then plotted versus S-hexylglutathione concentration and fitted to a hyperbolic curve using Micraical Origin (Microcal Software, Inc., Northampton, MA). These ten individual dissociation constants were then averaged to yield a dissociation constant for S-hexylglutathione binding to MIF.

**S-Hexylglutathione Binding—** In an attempt to identify novel isoforms of glutathione S-transferases, S-hexylglutathione affinity chromatography was used to purify proteins from liver cell extracts. MIF was found to be purified by this method and, based on catalytic assays, was proposed to possess GST activity (15). We have been unable to reproduce the GST activity (data not shown) but find that MIF does bind to S-hexylglutathione resin and can be eluted specifically by S-hexylglutathione (data not shown).
To further elucidate the interaction of MIF and S-hexylglutathione, NMR-based titration experiments were used to quantify the affinity for this ligand and determine its binding site. Backbone $^1$H and $^{15}$N chemical shifts were measured as a function of increasing ligand concentration. Shown in Fig. 4 are representative binding curves obtained from the titration experiment. Averaging these curves along with additional NMR data (not shown), a dissociation constant of 2.5 $\pm$ 0.6 mM was determined for S-hexylglutathione.

$^1$H-$^{15}$N HSQC spectra in the presence of S-hexylglutathione identified many residues with changes in chemical shift (Fig. 5). Many of these are the same residues affected by $p$-hydroxyphenylpyruvate, a substrate for a MIF-catalyzed tautomerization reaction (27). The residues perturbed by addition of S-hexylglutathione include Met-2, Phe-3, Lys-32, Ala-38, Ile-64, Gly-65, Trp-108, and Phe-113. Of these residues, Lys-32, Ile-64, and Gly-65 are invariant among all known eleven MIF homologues (27). Although perturbations do not necessarily indicate direct contact with the ligand, they are extremely useful in localizing the region of the protein involved in ligand binding. These chemical shift perturbations do not appear randomly over the protein surface, but instead occur only in a localized region near the N-terminal proline (Fig. 5). Based on structural similarities with 5-carboxymethyl-2-hydroxymuconate isomer-
MIF possesses dopachrome tautomerase activity and can utilize l-dopachrome methyl ester as a substrate in this assay (13). If S-hexylglutathione binds to the catalytic site, it should be able to inhibit the dopachrome tautomerase activity of MIF.

Using l-dopachrome methyl ester as a substrate, we measured an IC_{50} of 3.3 ± 1.6 mM for S-hexylglutathione (Fig. 6A). To address the source of S-hexylglutathione binding to MIF relative to the inability of glutathione to bind, we measured inhibition of dopachrome tautomerase activity by hexanethiol and found it to exhibit an IC_{50} of 17.4 ± 4.9 μM (Fig. 6B). Because MIF does not catalyze this reaction using typical Michaelis-Menten kinetics, it was not possible to determine a K_{i} for each...
These kinetic and NMR studies indicate that S-hexylglutathione is binding to the catalytic active site and that the source of this binding is the alkyl group.

**DISCUSSION**

The report that glutathione interacted with MIF was intriguing in light of the increased levels of these molecules at inflammatory sites (17). Glutathione serves two general roles in cells: (1) it contributes to the redox state of the cell, and (2) it is involved in the detoxification of xenobiotics via conjugation by glutathione S-transferases. In addition, glutathione has other roles in the immune system. Reduced and oxidized glutathione levels are important for NFκB activation and subsequent transcription of several immunologically important genes such as TNFα and the interleukin-2 receptor α-chain (17). In leukotriene biosynthesis, glutathione is conjugated to LTA₄ by leukotriene C₄ synthase to produce LTC₄ (18). LTC₄ and its metabolites are released from lung tissue of asthma patients upon stimulation by allergens and induces proinflammatory reactions (18). Therefore, it was tempting to speculate that the effects of glutathione in the immune system were mediated by MIF.

MIF was also proposed to be structurally related to the glutathione S-transferase family of proteins based on several observations. First, MIF shares 28% sequence identity at the amino terminus with μ-class glutathione S-transferases (15). The amino-terminal region of glutathione S-transferases plays a major role in glutathione binding. Second, polyclonal antibodies to MIF cross react with θ class glutathione S-transferases (25). Third, glutathione S-transferases have a conserved hy-

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droxyl group from a serine or tyrosine at the active site, which is implicated in glutathione deprotonation. MIF contains an invariant threonine at the equivalent position. Finally, MIF can be purified by S-hexylglutathione affinity chromatography, a method that is routinely used to purify glutathione S-transferases. These observations led to the suggestion that MIF was a distant ancestor of glutathione S-transferases that linked the immune and detoxifying systems (25).

However, several reports argued against any role for glutathione in MIF activity. Sequence analysis showed a statistically insignificant similarity between MIF and glutathione S-transferases along the length of the entire polypeptide (20). The crystal structure for human MIF revealed similar secondary structure between MIF and GST along the first thirty residues, but no similarity in the tertiary or quaternary structure for these two proteins (21). NMR experiments contradicted the results of earlier fluorescence and catalytic studies and showed that the interaction between glutathione and MIF was weak (22). Moreover, we were unable to reproduce the GST activity of MIF using EPNP as a substrate (data not shown) and have shown that the reported interactions between MIF and glutathione are artifacts of the experimental method. Fluorescence and circular dichroism spectroscopy are common methods used to measure protein conformational changes upon ligand binding. The fluorescence spectra of MIF when glutathione was added showed an increase in fluorescence with a shift in emission from 344 to 350 nm. This type of shift is often seen when proteins become unfolded. Creatine kinase is an example of a protein that, like MIF, exhibits an increase in fluorescence and a shift of the emission maximum upon protein unfolding (26). In the crystal structure of MIF, the tryptophan is present on the C-terminal loop which is involved in subunit interactions of the trimer. The fluorescence data suggest that the carboxyl-terminal loop of MIF undergoes a conformational change such as disruption in the subunit interface or random coil formation leading to a concomitant increase in tryptophan fluorescence. In addition to fluorescence spectroscopy, circular dichroism is a sensitive method to assess large changes in protein conformation. Circular dichroism studies of MIF indicate increased unfolding as the pH is decreased from 4.5, with a stable conformation above pH 4.5. Based on these data, it is now evident that there is little, if any, binding of glutathione to MIF, consistent with results from a recent NMR report (22).

In contrast to the results with glutathione, MIF binds to S-hexylglutathione affinity resin and is specifically eluted with S-hexylglutathione (data not shown). In addition, S-hexylglutathione can inhibit MIF dopachrome tautomerase activity. The binding of S-hexylglutathione to MIF is due to the alkyl moiety as hexanethiol inhibits the tautomerase activity of MIF with an IC\textsubscript{50} of 17.4 ± 4.9 μM. HSQC NMR experiments show that several MIF residues are perturbed upon addition of S-hexylglutathione. These residues map to a pocket surrounding the N-terminal proline on the three-dimensional structure of MIF (Fig. 5). This site consists of a hydrophobic region that can provide the binding interactions for the S-hexyl moiety. The structural similarity between this site and the active sites of the bacterial enzymes CHMI and OT, which both use an N-terminal proline as a catalytic base, indicates that the catalytic site of MIF is near the N-terminal proline. The designation of this region as an active site is consistent with inhibition of MIF...
dopachrome tautomerase activity by S-hexylglutathione and hexanethiol. Moreover, this site has been identified as the catalytic site for $p$-hydroxyphenylpyruvate tautomerase activity (27). It therefore appears that this site is a promiscuous binding site for hydrophobic small molecules. The concept of small molecules binding to cytokines is both novel and interesting. If this site is important for biological activity, our studies provide a lead compound for development of a specific high affinity inhibitor for MIF. Based on the importance of this cytokine in the immune response, the design of small molecule inhibitors of MIF may have therapeutic value.

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