Biochemical and Mutational Investigations of the Enzymatic Activity of Macrophage Migration Inhibitory Factor†

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ABSTRACT: The protein mediator MIF has been identified as being released from immune cells by glucocorticoid stimulation and to counter-regulate glucocorticoid action. MIF also has been described recently to exhibit dopachrome tautomerase activity and to be structurally homologous to the bacterial enzymes 4-oxalocrotonate tautomerase (4-OT) and 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI). We performed site-directed mutagenesis and biochemical analyses of mouse MIF in order to identify amino acid residues and protein domains that are essential for enzymatic reactivity. Mutant proteins which lacked a free N-terminal proline residue were enzymatically inactive, as was a preparation of native MIF modified covalently at its N terminus by 3-bromopyruvate, suggesting that this proline has a catalytic function. Substitutions of the internal histidine residues 42 and 63 did not affect enzymatic activity, indicating that these basic residues are not involved in dopachrome tautomerization. Carboxy-truncated forms of MIF (residues 1−110 and 1−104) also were inactive, affirming the role of the carboxy terminus in stable trimer formation and the importance of the trimer for enzymatic activity. Additional evidence for the homotrimeric structure of MIF under native solution conditions was obtained by SDS−PAGE analysis of MIF after chemical cross-linking at low protein concentrations. The enzymatic activity of MIF was found to be reversibly inhibited by micromolar concentrations of fatty acids with chain lengths of at least 16 carbon atoms. Of note, molecular modeling of the substrate L-dopachrome methyl ester into the active site of MIF suggests an acid-catalyzed enzymatic mechanism that is different from that deduced from studies of the enzymes 4-OT and CHMI. Finally, *in vitro* analysis of an enzymatically inactive MIF species (P2 → S) indicates that the glucocorticoid counter-regulatory activity of MIF can be functionally dissociated from its tautomerization activity.

Macrophage migration inhibitory factor (MIF)[1] has been identified recently as being both an endocrine hormone and an immunological mediator that counter-regulates glucocorticoid action (*1*, *2*). The potential importance of this protein in a variety of host functions is also underscored by the widespread occurrence of MIF in a variety of tissues and cell types (*1*, *3*−*7*). Recent X-ray crystallographic and 3-dimensional NMR data indicate that both human and rat MIF consist of a donut-shaped homotrimer containing α/β-motifs and a solvent-accessible inner channel (*8*−*11*). Although MIF lacks any significant primary sequence homology with other proteins (*12*), homologous three-dimensional structures exist between MIF and several bacterial enzymes (*13*−*15*). Each of these catabolic enzymes is involved in the degradation of aromatic compounds and catalyzes isomerization reactions. In the case of 4-oxalocrotonate tautomerase (4-OT), inhibition and NMR studies suggest that a conserved N-terminal proline with an unusually low pK_a acts as a general base during catalysis (*15*, *16*). MIF also has been shown recently to catalyze a tautomerization reaction, specifically the conversion of the non-naturally occurring D-isomer of dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) to dihydroxyindole carboxylic acid DHICA (*17*). Significantly, both human and murine MIF also have an N-terminal proline residue that is located at the base of a hydrophobic cavity.

To better characterize the dopachrome tautomerase activity of MIF, we have performed site-directed mutagenesis and biochemical analyses of recombinant mouse MIF. The mutations that were studied include substitutions of the N-terminal proline, internal histidine residues, and deletions of the C terminus, which is involved in important contacts with the other subunits.

MATERIALS AND METHODS

Reagents. All chemicals were from Sigma (St. Louis, MO) and were the highest grade commercially available. [^{35}S]αATP was purchased from New England Nuclear (Boston, MA). Media, oligonucleotide primers, and enzymes were from Gibco-BRL (Gaithersburg, MD) or Boehringer Mannheim (Indianapolis, IN).

Strains and Plasmids. DNA amplification products were cloned and sequenced in the pT7Blue "T-vector" (Novagen, Madison, WI) and propagated in *Escherichia coli* strain DH5α. For protein production in *E. coli* (strain BL21DE), the DNA fragments were cloned into the *Nde*I/*Bam*HI sites of pET11b (Novagen). Bacteria were grown in LB media containing 200 μg/mL ampicillin. Plasmids were prepared using the Plasmid kit from Qiagen (Chatsworth, CA).

Polymerase Chain Reaction. PCR Supermix (Gibco BRL) and 0.2 μM primers were used in a Perkin-Elmer 9600

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[1] Abbreviations: CHMI, carboxymethylhydroxymuconate isomerase; DC, dopachrome; DCME, dopachrome methyl ester; DCT, dopachrome tautomerase; DHICA, dihydroxyindolecarboxylic acid; DME, dopa methyl ester; MIF, macrophage migration inhibitory factor; 4-OT, 4-oxalocrotonate tautomerase; PCR, polymerase chain reaction.

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thermocycler. Initial denaturation was for 2 min at 94 °C, and then 30 cycles at 94 °C (30 s), 50 °C (30 s), and 72 °C (30 s), followed by 2 min at 72 °C.

Preparation of L*-Dopachrome Methyl Ester*. Equal volumes of aqueous solutions of L-dopa methyl ester (4 mM) and sodium periodate (8 mM) were mixed and incubated for 3−5 min. The remaining periodate was removed from the deeply colored L-dopachrome methyl ester by chromatography over a C18 reversed-phase column (10 mL). After the column was flushed with 3 volumes of deionized water (30 mL), L-dopachrome methyl ester was eluted with 5 mL of methanol. The methanolic eluate was found to be stable at -70 °C for at least 3 months.

Dopachrome Tautomerase Assay. Enzymatic activity was measured according to the following protocol: buffer A, 0.2% Tween in 25 mM potassium phosphate at pH 6.0; and buffer B, 500 μM EDTA in 25 mM potassium phosphate at pH 6.0. One milliliter of buffer A or B was mixed with 20−30 μL of the substrate DCME concentrate (starting $E_{475\ nm} \approx 1-1.4$). After the background rate was monitored, recombinant mouse MIF was added (typical assays contained 0.05−0.5 μg of MIF). Background and MIF-catalyzed reactions were monitored at 475 nm on a Shimadzu UV160U spectrophotometer.

Chemical Cross-Linking of MIF. Recombinant MIF (2 μg/mL in 50 mM potassium phosphate buffer at pH 6.5) was incubated in the presence of 1% glutaraldehyde for 3 h (*18*). The reaction was stopped, and the cross-linked products were stabilized by addition of $NaBH_4$ (2 M stock solution in 0.1 M NaOH) to a final concentration of 50 mM. After 20 min, sodium deoxycholate was added (final concentration of 0.01%). The precipitated protein was collected by centrifugation (20 min, 13000*g*) after lowering the pH to 2.0 with trichloroacetic acid (100% w/v) and then analyzed by SDS−PAGE under reducing conditions.

MIF Inhibition by 3-Bromopyruvic Acid. Recombinant MIF (200 μg/mL) was preincubated in PBS (0.14 M NaCl in 20 mM sodium phosphate at pH 6.8) for various times in the presence of various concentrations of 3-bromopyruvic acid and assayed for residual tautomerase activity. Five microliters of the preincubated MIF was added to 1 mL of assay mix (buffer A), thus lowering the concentration of the 3-bromopyruvate during the assay by 200-fold.

MIF Inhibition by Fatty Acids. Various concentrations of fatty acids (1 mM stock solutions in methanol) were added to 1 mL of the assay mix (buffer B), and the residual enzymatic MIF activity was assayed. In the preincubation experiments, MIF (0.5−1 μg) was preincubated in assay buffer B containing the inhibitor for 1 min before the reaction was started by adding the substrate.

Glucocorticoid Counter-Regulatory Activity. The capacity of various recombinant MIF proteins to regulate glucocorticoid inhibition of cytokine production was assayed as described previously (*1*). Briefly, human mononuclear cells were isolated from whole blood by Ficoll density gradient centrifugation and plated at 5×10^6 cells/mL (0.75 mL/well) in 24-well plates in RPMI/10% human AB serum. The monocytes were purified by adherence, and approximately 1×10^6 cells/well were preincubated for 1 h with dexamethasone (10^{-9} M) or with dexamethasone (10^{-9} M) plus rMIF (native human, native mouse, and mouse P2 → S species) before the addition of 0.5 μg/mL LPS (*E. coli* O111:B4, Sigma Chemical Co.). Cell culture supernatants were collected after 16 h of stimulation, and secreted TNFα was quantified by ELISA (*19*).

Miscellaneous Methods. Internal site-directed MIF mutants were generated according to Mikaelian and Sergeant (*20*). All recombinant MIF proteins were purified as described previously (*21*). SDS−PAGE analysis of MIF cross-linking products was performed using a PhastSystem (Pharmacia Biotech, Piscataway, NJ). The modeling was performed using the Insight/Discover 2.9.7 program suite (Biosym/MSI, San Diego, CA).

RESULTS

A Periodate-Free Assay System for MIF Tautomerase Activity. To facilitate the analysis of the enzymatic activity of MIF, we first developed a reliable method for quantifying the tautomerization of D-dopachrome to DHICA. The photometric-based enzymatic assay described in the past relies on freshly prepared dopachrome substrate that is synthesized from dopa (or the methyl esters) by chemical oxidation with excess periodate (*22*). No further purification steps were utilized in these studies, and significant amounts of the oxidizing reagent periodate remain in the assay. This is disadvantageous for several reasons. First, the remaining periodate increases the rate of spontaneous substrate (dopachrome) decay. Second, periodate can react with other components that may be included in the assay mixture, such as potential inhibitors of the MIF enzymatic activity. Finally, periodate can oxidize cysteine residues, chemically modifying and denaturing MIF itself (unpublished observations).

To eliminate these drawbacks, we devised a facile method for purifying the dopachrome substrate from the other components (*i.e.* periodate) of the synthetic reaction. Dopachrome was found to bind with high affinity to C18 silica beads, and periodate and its reaction product iodate are removed by washing the C18 resin with deionized water. Pure dopachrome then is eluted from the column with 100% methanol, concentrating the substrate 50−100-fold and yielding a methanolic solution that is stable at −70 °C for a period of several months. The concentrated methanolic substrate solution is also substantially more stable at room temperature than an aqueous solution of dopachrome, which undergoes spontaneous tautomerization to the dihydroxyindole product at a slow rate.

Cross-Linking of MIF under Native Conditions. Although a homotrimeric structure of MIF has been reported by several groups on the basis of X-ray crystallographic data (*8*−*10*), the possibility remains that MIF may assume other tertiary structures under physiological solution conditions. Size exclusion chromatography and equilibrium density centrifugation studies have suggested that MIF may have an apparent molecular mass of 24−25 kDa, consistent with that of a dimer (ref 23 and unpublished results).

We used chemical cross-linking with glutaraldehyde under native conditions and a dilute MIF concentration (2 μg/mL) to further investigate this question. By "native", we refer to solution conditions in which MIF exhibits full enzymatic activity. As shown in Figure 1, an SDS−PAGE analysis of glutaraldehyde-treated MIF indicated a structure that was clearly consistent with that of a native trimer. MIF cross-linked with glutaraldehyde and fixed by $NaBH_4$ treatment migrated at an apparent molecular mass of 35−40 kDa (Figure 1, lane B). The lack of higher-molecular mass

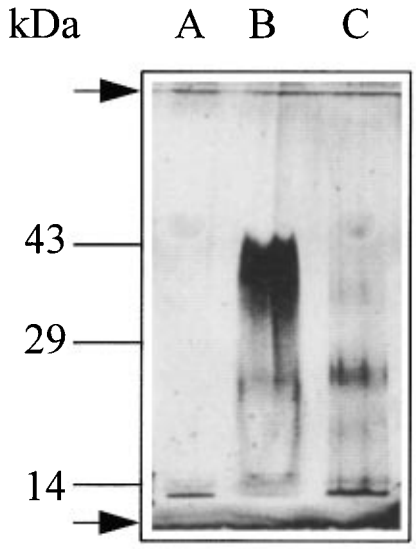


FIGURE 1: Silver-stained 8−25% SDS−PAGE of human rMIF after cross-linking with glutaraldehyde: lane A, rMIF (control) treated as described in Materials and Methods; lane B, rMIF treated with 1% glutaraldehyde followed by $NaBH_4$ fixation; and lane C, MIF treated only with 1% glutaraldehyde. The arrows mark the start of the separation gel (top) and the buffer front (bottom).

oligomers in the range from 40 to 110 kDa further suggests that the trimer is the predominant form present under these cross-linking conditions. A control sample of MIF treated identically but in the absence of glutaraldehyde showed only a monomeric species at 12 kDa (lane A). MIF treated with glutaraldehyde but without the subsequent $NaBH_4$ fixation step showed very limited cross-linking that was predominantly in the dimeric range around 26 kDa (lane C).

Mutations of the N Terminus. Recent investigations of the catabolic bacterial isomerases 4-OT and CHMI have revealed close structural relationships to MIF despite the lack of significant sequence homology (*8*−*10*). Inhibition and labeling data suggest that, in the case of 4-OT and CHMI, an unusually acidic N-terminal proline is the active residue during catalysis (*15*, *16*). To further characterize the tautomerization activity of MIF, we first tested MIF for its ability to isomerize the substrates of the two bacterial enzymes. Recombinant MIF showed very low but detectable activity with the 4-OT substrate, 2-hydroxymuconate, and no activity with the CHMI substrate, 5-carboxymethyl-2-hydroxymuconate (data not shown).

We next used site-directed mutagenesis to alter the N terminus of MIF and tested the mutant proteins for dopachrome tautomerase activity. Three mutants of mouse MIF were generated with respect to the N-terminal proline (Figure 2). In a deletion mutant (ΔP2), methionine 1 and proline 2 were removed, leaving a protein with methionine 3 in the N-terminal position. In a substitution mutant (P2 → S), proline 2 was replaced by a serine. We also generated a blocked N terminus by "masking" proline 2 with the N-terminal sequence Met-Asp-Ser-Met (mutant MDSM). All mutants lacking a free N-terminal proline residue were found to lack activity in the dopachrome tautomerase assay ($\Delta E_{475}\text{min}^{-1} < 0.01$).

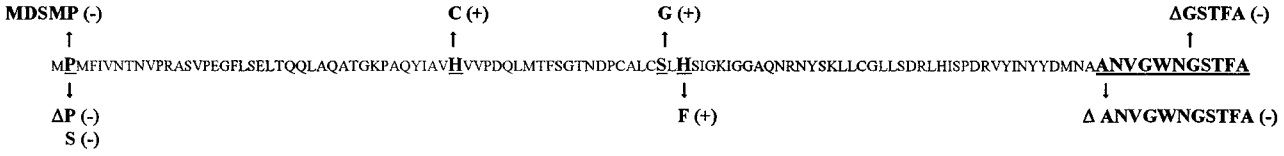
Substitutions of His41 and His63. Because of the possibility that other basic residues such as histidine might participate in the tautomerization reaction, we also generated MIF mutants in which two internal histidine residues were altered. Substitutions of histidine residues 41 and 63 with cysteine (H41 → C) and phenylalanine (H63 → F), respectively, had no effect on the catalytic activity of MIF. Thus, neither of these residues is involved directly in catalysis or is necessary for maintaining a native, enzymatically active MIF structure (Figure 2). A S61 → G mutant that was inadvertently cloned also displayed full enzymatic activity.

Truncations of the C Terminus. The three-dimensional structure of the C-terminal region of rat MIF was not determined from the crystal data, most likely due to the inherent flexibility of this domain in the crystal form (*9*). The C-terminal domain of human MIF, by contrast, was well visualized and participates in extensive intersubunit interactions through stable β-sheet structures (*8*). To investigate the influence of alterations in the C-terminal domain on enzymatic activity, two MIF mutants were generated in which the polypeptide chain ended with the residues asparagine 110 (ΔGSTFA) or alanine 104 (ΔANVGWNGSTFA). Both C-terminal truncations were completely inactive in the dopachrome tautomerase assay.

N-Terminal Modification with 3-Bromopyruvic Acid. 3-Bromopyruvic acid (3-BP) has been shown to be an irreversible inhibitor of 4-OT (*17*), acting by covalent modification of the active N-terminal proline residue. Preincubation of mouse MIF with 3-BP also resulted in a complete and irreversible loss of dopachrome tautomerase activity with concentration-dependent half-lives of approximately 30 min (1 mM 3-BP) and 4 min (10 mM 3-BP) (Figure 3).

Inhibition of MIF by Long-Chain Fatty Acids. MIF resembles the bacterial enzymes 4-OT and CHMI with respect to three-dimensional structure and the presence of a catalytically important N-terminal proline. 4-OT and CHMI also act as isomerases on catabolic substrates that have previously incorporated molecular oxygen in oxygenase reactions. Interestingly, the MIF substrate, D-dopachrome, also fits this pattern since MIF rearranges an electronic system created by the oxidation of D-dopa. Assuming that MIF has a similar role *in vivo*, we looked for similar oxygen-dependent reactions in immunological or cell-signaling pathways. Intermediates within these pathways could act as potential substrates for MIF or, alternatively, as regulatory effectors of its enzymatic activity. Candidate pathways that were considered included the synthetic routes for steroids and eicosanoids, such as the prostaglandins, thromboxanes, and leukotrienes.

Although the prostaglandins PGD_2 and PGE_2 were found not to affect MIF tautomerization activity (up to 50 μM), long-chain fatty acids were effective inhibitors (Figure 4A). When added directly into the assay mixture, each of the fatty acids that was tested inhibited MIF activity by 50% at concentrations of 3−8 μM. Unsaturated fatty acids such as oleic acid (18:1) and arachidonic acid (20:4) showed more than 95% inhibition at concentrations of 20 μM, whereas saturated fatty acids such as palmitic (16:0) and stearic (18:0) acid reached a plateau of inhibition of approximately 60% at concentrations of greater than 10 μM. This limited effect of long-chain saturated fatty acids was due most likely to their lower solubilities in aqueous solutions compared to those of unsaturated fatty acids. Both isomers of the 18:1 unsaturated fatty acid, oleic (*cis*) and elaidic acid (*trans*), inhibited MIF with the same efficiency (data not shown), indicating that the conformation of the carbon backbone is unimportant in affecting MIF activity. Oleyl alcohol, lacking



Mutant MIF	DCT Activity
N-terminal modifications	
ΔP2	-
P2→S	-
MDSM tag	-
C-terminal deletions	
Δ GSTFA	-
Δ ANVGWNGSTFA	-
Internal substitutions	
H41→C	+
H63→F	+
S61→G	+

FIGURE 2: Schematic representation of mouse MIF and the mutant species that were generated. Altered residues are underlined, and the mutant names are printed in bold letters. All enzymatic assays were performed with recombinant protein over the range of 0.1−10 μg. Active (+) species showed an enzymatic rate that was equivalent to that of native rMIF (d*E*/min = −0.5), and inactive (−) species showed a rate that was equivalent to that of spontaneous dopachrome decay (d*E*/min = −0.03). All assays were repeated five times.

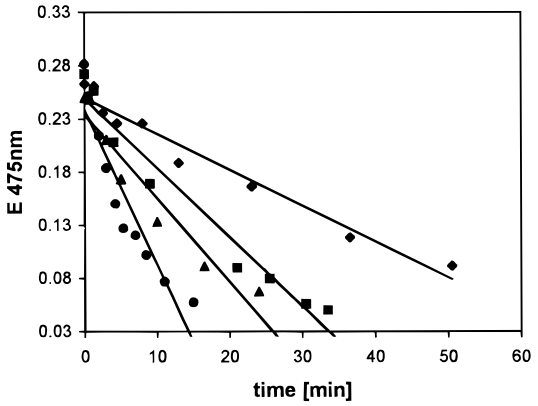


FIGURE 3: Inactivation of rMIF with 3-bromopyruvic acid (3-BP). Mouse rMIF was preincubated with various concentrations of 3-BP and then assayed for dopachrome tautomerase activity at the indicated times: (●) 1 mM 3-BP, (▲) 2 mM 3-BP, (■) 5 mM 3-BP, and (◆) 10 mM 3-BP.

the carboxyl function, also did not significantly inhibit MIF. Efficient inactivation of MIF was critically dependent on the carbon chain length, however. At least 14 carbon atoms were required to affect MIF activity, and short organic acids such as acetic acid (up to 50 μM) were completely inactive as inhibitors (Figure 4B).

The inhibitory effect of fatty acids on dopachrome tautomerization was even more pronounced when MIF was preincubated with fatty acids. Incubation of MIF with 5 μM oleic acid for 1 min resulted in more than 95% inhibition compared to the untreated control (Figure 4C, columns 3 and 4). The inhibition of MIF by fatty acids also could be prevented completely by the addition of a detergent. If MIF was preincubated in a mixture of 5 μM oleic acid and 0.2% Tween, no inhibitory effect was observed (column 2). Moreover, MIF that had previously been inactivated by incubation with 5−10 μM oleic acid could be completely reactivated by adding 0.2% Tween for 1 min (column 1).

It was necessary to rule out the possibility that fatty acids simply cause a reversible-phase transition of MIF, resulting in precipitation or adsorption of the protein to the surface of the sample tube. For this purpose, MIF was preincubated with 5 μM elaidic acid in a polystyrene cuvette and then centrifuged in a polypropylene tube for 30 min at 13000*g*. The (inactive) supernatant then was transferred to a new cuvette and assayed for MIF activity after the addition of 0.2% Tween. All of the MIF activity that was present in the original solution was fully recovered. By contrast, no MIF activity could be identified in a 0.2% Tween wash of the walls of the sample cuvette that had been used for preincubation and centrifugation.

Relationship between Tautomerization Activity and Glucocorticoid Counter-Regulatory Activity. Recent studies *in vivo* have established that MIF exerts profound regulatory effects on the development of both inflammatory and antigen-dependent immune responses (*1*−*3*, *6*, *24*−*26*). It has been proposed that many if not all of these effects may be attributed to the unique ability of this mediator to "override" or counter-regulate the immunosuppressive effects of glucocorticoids on immune cell activation and inflammatory cytokine production (*1*, *3*).

To assess the potential relationship between the enzymatic activity of MIF and its immunological activity, we measured the capacity of an enzymatically inactive, mutant MIF protein to override the inhibitory effect of dexamethasone on TNFα production by human monocytes. Consistent with prior studies (*21*), there was no apparent difference between the activity of native mouse or native human MIF in this human cell-based assay. Importantly, the enzymatically inactive, mutant MIF (P2 → S) showed glucocorticoid counter-regulatory activity that was similar to that of the native, enzymatically active, mouse and human MIF proteins (Figure 5).

DISCUSSION

We provide evidence that MIF forms stable trimers at low concentrations (2 μg/mL) and under solution conditions

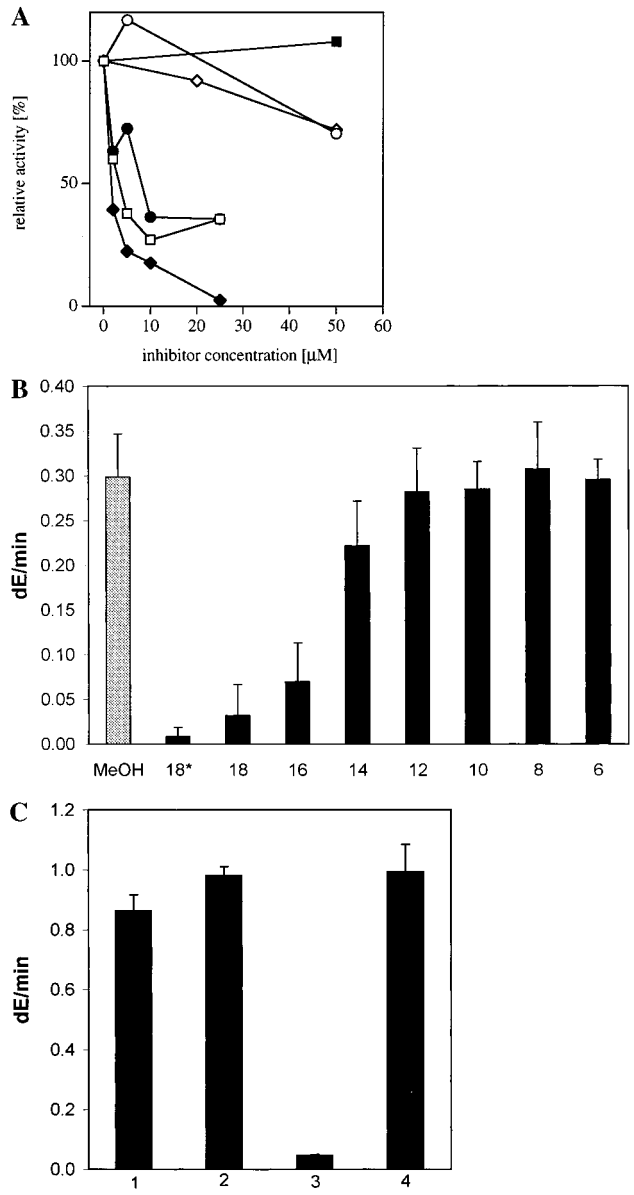


FIGURE 4: Effect of long-chain fatty acids on MIF tautomerase activity. (A) Identical quantities of mouse rMIF were assayed for residual activity in the presence of various concentrations of different fatty acids. The assays were started by adding rMIF to the reaction mixture containing the inhibitors together with the substrate DCME: (■) acetic acid, (○) oleyl alcohol, (◇) SDS, (□) palmitic acid, (●) stearic acid, and (◆) oleic acid. (B) Effect of fatty acid carbon chain length on MIF activity. All fatty acids (saturated and unbranched) were tested without preincubation at a final concentration of 20 μM. Me denotes the methanol control (20 μL of solvent added, no fatty acid). The numbers indicate the carbon atom chain lengths of the unbranched, saturated fatty acids. The asterisk marks the unsaturated (elaidic) fatty acid. All activities were standardized against a sample which contained solvent (20 μL) but no fatty acids. The mean ± SD of triplicate experiments are shown. (C) Identical amounts of mouse rMIF (preincubated for 1 min) were assayed by adding the reaction substrate: column 1, MIF preincubated with 5 μM oleate followed by 1 min of incubation with 0.2% Tween 20; column 2, MIF preincubated in a mixture of 5 μM oleate and 0.2% Tween 20; column 3, MIF preincubated with 5 μM oleate; and column 4, MIF preincubated with 0.2% Tween 20. Each column represents the mean ± SD.

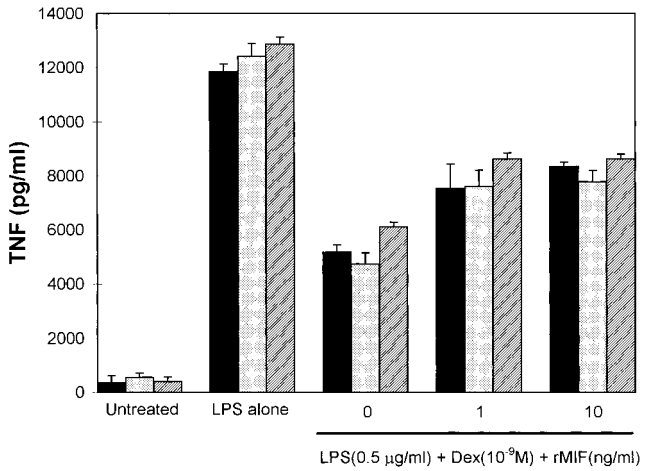


FIGURE 5: Effect of MIF on glucocorticoid-mediated inhibition of TNFα production by LPS-stimulated human monocytes. Cells were purified by adherence from the peripheral blood of normal human volunteers and incubated sequentially with dexamethasone (10^{-9} M) or dexamethasone (10^{-9} M) plus native recombinant human (solid), native recombinant mouse (stippled), or mutant recombinant mouse (P2 → S) (cross-hatched) proteins (1 or 10 ng/mL) prior to the addition of LPS (0.5 μg/mL). Sixteen hour-conditioned medium was assayed for TNFα by ELISA. The values shown are the means ± SD of quadruplicate wells. $P < 0.002$ for each of the recombinant MIF species tested at 1 or 10 ng/mL versus the corresponding recombinant MIF species tested at 0 ng/mL (Student's *t* test statistic, independent variable).

where MIF is known to have enzymatic activity. We also show that truncation of the C-terminal loop, which is believed to provide critical stabilization of the trimeric structure, results in a complete loss of enzymatic activity. Chemical modification and mutational analysis of the N terminus of MIF demonstrate that, in addition to significant structural homology, MIF shares a functionally important N-terminal proline residue with the bacterial isomerases 4-OT and CHMI.

While these data suggest that MIF catalysis follows a mechanism similar to that proposed for 4-OT and CHMI (*15*), molecular modeling of the substrate L-DCME into the active site of MIF reveals potentially significant steric restrictions with respect to substrate binding. Analysis of both the crystal data (*8*) and a computer-generated, conformationally more relaxed version of MIF suggest that it is impossible, without incurring a major conformational change, to fit L-DCME into an active site so that the imino group of proline 2 could abstract a proton from the C2 of the indole ring. However, good binding scores for L-DCME were achieved by assuming entry of the substrate into the active site with the indole ring first. This mechanism for substrate binding suggests that the very acidic imino group of the N-terminal proline ($pK_a = 6.2 \pm 0.1$, R. M. Czerwinski and C. P. Whitman, personal communication) might act catalytically as an acid rather than as a base, forming a hydrogen bond to the C5-carbonyl oxygen of the indole ring (Figure 6). Isomerization then would occur by the protonation of the indole carbonyl oxygen, followed by aromatization and subsequent elimination of the proton at C2.

Fatty acids and lipids in general are involved in a variety of biochemical and cellular regulatory processes. One well-established example is the signaling cascade involving arachidonic acid. Fatty acids also are known to interfere with receptor−ligand interaction by influencing proteolytic events and intracellular signaling (*27*). Additionally, they have been shown to be important modulators of enzymatic activity, potentiating the allosteric inhibition by regulatory effectors (*28*). In certain cases of solvent entropy-driven reactions, they can even serve catalyst-like functions by

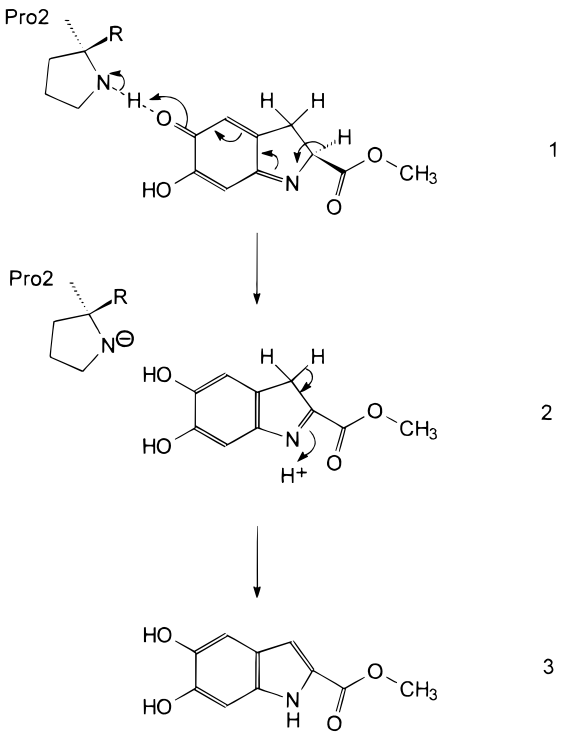


FIGURE 6: Proposed mechanism for the MIF-catalyzed isomerization of L-dopachrome methyl ester (DCME) to 5,6-dihydroxyindolecarboxylic acid methyl ester as implied by modeling the substrate **1** into the active site of human MIF (*8*). The unusually acidic imino proton of proline 2 [p$K_a \approx 6.5$ (*15*)] initiates the rearrangement by forming a hydrogen bond to the carbonyl oxygen of C5: **1**, L-dopachrome methyl ester; **2**, 5,6-dihydroxy-3-dihydroindole-2-carboxylic acid methyl ester; and **3**, 5,6-dihydroxyindole-2-carboxylic acid methyl ester.

providing a water-free, hydrophobic compartment (*29*). We observed a high-affinity, reversible interaction between MIF and various fatty acids that was reflected in a concentration-dependent loss of tautomerase activity. The apparent high efficiency and reversibility of the MIF interaction with fatty acids make it conceivable that these or structurally related compounds may play a role in the regulation of MIF activity *in vivo*. Phospholipases, for instance, release fatty acids during the course of the inflammatory cascade and may act to modulate the immunological properties of MIF. Oleic acid comprises approximately one-third of the total free fatty acids in blood plasma, reaching concentrations of 80−130 μM (*30*), and could serve as a negative effector of MIF action *in vivo*.

The identity of the *in vivo* substrate for MIF is unknown at present. If the natural substrate is structurally similar to dopachrome, it remains striking that MIF accepts the nonphysiological D-isomer dopachrome as a substrate, but not its abundant, naturally occurring counterpart, L-dopachrome (*17*). The reason for this apparent rejection of the "natural" isomer may reflect a requirement to specifically avoid interference with the normal pathway of melanin biosynthesis. That the methyl ester derivatives of dopachrome are almost equally active with MIF regardless of the stereo conformation may indicate that the natural MIF substrate is similar to L-dopachrome but with a carboxyl group that has been modified to an ester, thioester, or amide. Conceivably, certain peptides bearing an N-terminal tyrosine may serve as substrates for MIF *in vivo*. N-Terminal tyrosines exist in many biologically active neuropeptides that exhibit both neuroendocrine and immunological activities. Furthermore, the enzyme tyrosinase is known to convert N-terminal tyrosine-containing peptides into the corresponding dopachrome peptides (*31*). The recent demonstration of widespread MIF expression in the CNS (*32*) is consistent with the possibility that MIF may act to convert dopachrome peptides into their DHICA homologs, potentially modulating their bioactivity.

At the structural level, MIF and the bacterial isomerases 4-OT and CHMI have no significant homologies in their primary sequences. However, two features that are likely to be paramount for enzymatic activity are strictly conserved: the overall tertiary/quarternary structures and the catalytic, N-terminal prolines located at the base of a hydrophobic pocket. The few amino acid differences in the composition of the hydrophobic pocket may reflect necessary adjustments for the entry and orientation of different substrates. Thus, it would appear unlikely that the specific structure of MIF would either be incidentally conserved or evolve independently without serving a similar enzymatic function.

At the present time, there is no direct data linking the enzymatic activity of MIF with its profound immunological effects *in vivo* (*1*, *3*, *4*, *24*−*26*). The enzymatically inactive, P2 → S MIF mutant showed full activity in the glucocorticoid counter-regulatory assay. Similar data also have been obtained recently in a study of T cell activation, where an enzymatically inactive, mutant MIF species was found to override glucocorticoid inhibition of IL-2 expression in a IL-2 promoter/luciferase reporter plasmid (M. Wuttke and R. Bucala, unpublished observations). Although these data do not rule out a potentially important role for MIF tautomerization activity in certain immunological functions, enzymatic catalysis does not appear to play a role in the glucocorticoid counter-regulatory activity of MIF. Further insight into the *in vivo* significance of the enzymatic activity of MIF must naturally await the identification of native, biological substrate(s), the evaluation of mutant MIF proteins in additional immunological phenomena, and the creation of a transgenic mouse strain that expresses an enzymatically inactive form of the protein.

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