Characterization of the Role of the Amino-Terminal Proline in the Enzymatic Activity Catalyzed by Macrophage Migration Inhibitory Factor†

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ABSTRACT: The cytokine macrophage migration inhibitory factor (MIF) mediates several immune and inflammatory processes through unknown or poorly understood mechanisms. The protein shares structural homology with two bacterial isomerases, 4-oxalocrotonate tautomerase (4-OT) and 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI), and catalyzes the enolization of phenylpyruvate and the ketonization of (*p*-hydroxyphenyl)pyruvate. The amino-terminal proline has been identified as the catalytic base in both the 4-OT- and CHMI-catalyzed reactions. MIF also has an amino-terminal proline that has been implicated as a catalytic group in the MIF-catalyzed reaction. To delineate further the role of Pro-1 in the MIF-catalyzed reaction, affinity labeling studies were performed with 3-bromopyruvate (3-BP). The results of this study show that 3-BP acts as an active-site-directed irreversible inhibitor of the enzymatic activity and modifies one site per monomeric subunit. The inhibitor, as its lactyl derivative, is covalently attached to an 11 residue amino-terminal fragment, Pro-1 to Arg-11. The only reasonable site for alkylation within this peptide fragment is the amino-terminal proline. Because the pK_a measured for the pH dependence of k_{inact}/K_I (5.7 ± 0.2) and that measured for the pH dependence of the k_{cat}/K_m for the enolization of phenylpyruvate (6.0 ± 0.1) are comparable and in reasonable agreement with the previously measured pK_a of Pro-1 (5.6 ± 0.1) obtained by its direct titration [Swope, M., Sun H.-W., Blake, P., and Lolis, E. (1998) *EMBO J.* (in press)], it is concluded that Pro-1 acts as the general base catalyst in the MIF-catalyzed reaction. The structural and mechanistic parallels place 4-OT, CHMI, and MIF in a superfamily of enzymes related by their ability to catalyze the keto−enol tautomerization of a pyruvyl moiety.

Macrophage migration inhibitory factor (MIF)[1] was first identified as a soluble protein secreted by sensitized lymphocytes of guinea pigs that inhibited the migration of macrophages (*1*). This observation suggested that MIF concentrated macrophages at the site of an infection so that they could carry out phagocytosis. Since its initial discovery, MIF has been implicated in a host of immune as well as inflammatory processes and may play a role in sepsis, adult respiratory distress syndrome, and rheumatoid arthritis (*2*−*5*). The biochemical basis for many of these biological activities is not well understood.

When the crystal structure of MIF from rat liver was solved in 1996, a tantalizing and potentially useful discovery was made: its three-dimensional structure is nearly superimposable on those of two bacterial isomerases, 4-oxalocrotonate tautomerase (4-OT) and 5-(carboxymethyl)-2-hydroxymuconate isomerase (CHMI) (*6*−*8*). These two enzymes utilize the amino-terminal proline as a general base to catalyze a 1,3-allylic rearrangement in parallel microbial pathways responsible for the degradation of aromatic compounds (*9*−*12*). In both 4-OT and CHMI, the catalytic proline is located in a hydrophobic pocket, which lowers the pK_a value so that it can act as a general base (*9*−*11*). The structural homologies coupled with the fact that MIF has an amino-terminal proline situated within a hydrophobic pocket raised two interesting questions: does MIF catalyze a similar enzymatic reaction and do any of the immunological and/or inflammatory activities attributed to MIF result from the enzymatic reaction (*13*−*16*)?

Rorsman and colleagues attempted to address these questions by identifying a substrate for MIF (*13, 14*). Initially, it was determined that MIF catalyzes a tautomerization of a compound known as dopachrome (**1**, Scheme 1), which is a putative intermediate in the late stages of melanin biosynthesis (*13*). The proposed mechanism involves the MIF-catalyzed abstraction of a proton from C-2 followed by aromatization. More recently, it was found that MIF catalyzes the enolization of phenylpyruvate (**2** → **3**, Scheme 2) and the ketonization of (*p*-hydroxyphenyl)pyruvate (**4** → **5**, Scheme 2) suggesting that MIF and phenylpyruvate

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[1] Abbreviations: Ap, ampicillin; BCA, bicinchoninic acid; 3-BP, 3-bromopyruvate; CHMI, 5-(carboxymethyl)-2-hydroxymuconate isomerase; DEAE, diethylaminoethyl; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-pressure liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; LB, Luria−Bertani medium; MALDI-MS, matrix-assisted laser desorption−ionization mass spectrometry; MIF, macrophage migration inhibitory factor; NMR, nuclear magnetic resonance; 4-OT, 4-oxalocrotonate tautomerase; PPT, phenylpyruvate tautomerase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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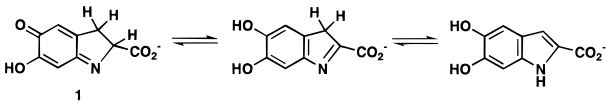
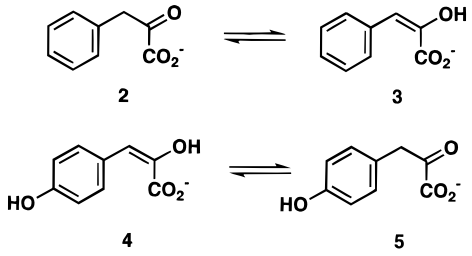
Scheme 1



Scheme 2



tautomerase (PPT) might be the same protein (*14*). Subsequently, it was demonstrated that the amino-terminal 15 amino acid residues of the commercially available PPT (Sigma) are identical to those of MIF. Moreover, western blot analysis of the commercially available PPT using MIF antibody showed a protein identical to that of MIF. On the basis of these experiments, it was concluded that MIF and phenylpyruvate tautomerase are the same protein (*14*).

Two investigations of the enzymatic activity of MIF suggested that Pro-1 plays a role in these enzyme-catalyzed reactions. Bendrat et al. (*15*) found that the incubation of MIF with 3-bromopyruvate (3-BP) resulted in a loss of the enzymatic activity. Although the modified group was not identified, it was argued that Pro-1 is the site of modification by analogy to the studies of 4-OT with the affinity label 3-BP. Modeling studies, however, showed that the methyl ester of **1** could not be positioned into a presumed active site such that Pro-1 could abstract the proton at C-2. Thus, it was concluded that Pro-1 acts as a general acid catalyst (*15*). In contrast, Swope et al. (*16*) proposed that Pro-1 functions as a general base catalyst and provided two lines of evidence. First, a pK_a for Pro-1 was determined as 5.6 ($\pm$ 0.1) by titration of ^{15}N-labeled MIF (by using ^{15}N NMR spectroscopy), suggesting that Pro-1 can act as a catalytic base at physiological pH. Second, the ^{15}N chemical shift of Pro-1 shifts, broadens, and finally disappears as the concentration of a mixture of **4** and **5** increases, suggesting that these isomers bind in the N-terminal region.

To resolve these conflicting reports, we reinvestigated the inactivation of MIF by 3-BP. Our data clearly demonstrate that 3-BP is an active-site-directed irreversible inhibitor of the enzymatic activity of MIF and that Pro-1 is the site of modification. It was also found that the pH dependence of k_{cat}/K_m for **2** is comparable to the pH dependence of k_{inact}/K_I for 3-BP, suggesting that the same group is involved in both catalysis and inactivation. These results strongly implicate Pro-1 as the catalytic base in the enzymatic activity of MIF. These findings set the stage for an in-depth characterization of the active site of MIF that may result in a better understanding of the biological roles of MIF.

MATERIALS AND METHODS

Materials. All biochemicals were purchased from Sigma Chemical Co. Centricon (10 000 MW cutoff) centrifugal microconcentrators and ultrafiltration membranes were purchased from Amicon. The clone containing recombinant mouse MIF was obtained from Dr. Richard Bucala (The Picower Institute for Medical Research, Manhasset, NY). The composition of LB medium is described elsewhere (*17*).

General Methods. HPLC separations were performed on a Waters system using either a Waters Protein Pak (DEAE−5PW) anion-exchange column, a Pharmacia Superose 12 (HR 10/30) gel-filtration column, or an Alltech Econosil C_{18} reverse-phase column, as noted in the text. Protein concentrations were determined with the commercially available bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL). Protein was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under denaturing conditions on 17% gels (*18*). Kinetic data were obtained on a Hewlett-Packard 8452A diode array spectrophotometer. The cuvettes were mixed by a stir/add cuvette mixer. The kinetic data were fitted by nonlinear regression data analysis using the Grafit program (Erithacus Software Ltd., Staines, U.K.) obtained from Sigma Chemical Co. Mass spectra of MIF, modified MIF, and the tryptic peptide fragments were obtained on either a Sciex API-III quadrupole electrospray mass spectrometer (ESI-MS) or a Thermo BioAnalysis Dynamo system (MALDI-MS) as noted in the text.

Overexpression and Purification of Recombinant MIF. MIF was purified to homogeneity as assessed by SDS−PAGE in a modification of literature procedures (*3*, *19*, *20*). Recombinant mouse MIF cloned into a pET11b plasmid was expressed in *Escherichia coli* BL21(DE3)pLysS with the T7 expression system (pET system, Novagen) as described elsewhere (*3*). A single colony of the expression strain was used to inoculate 50 mL of LB/Ap (50 μg/mL) medium. After overnight growth at 37 °C, a sufficient quantity of the culture was used to inoculate 500 mL of LB/Ap (50 μg/mL) medium in a 2 L Erlenmeyer flask so that the initial A_{600} was 0.05. Cultures were grown to an A_{600} of 0.6−0.8 at 37 °C with vigorous shaking and then induced with IPTG (1 mM final concentration). Incubation was continued for 3−3.5 h at 37 °C. Cells were harvested by centrifugation (6000*g*, 15 min) and stored at −20 °C. Typically, 3 L of culture grown under these conditions yields ~9 g of cells.

In a typical procedure, the cells (9 g) were thawed and suspended in 20 mM Tris buffer (15 mL, pH 7.4) made 20 mM in NaCl. The cells were disrupted at 4 °C by sonication with 1 pulse (1 min) from a Heat Systems W-385 sonicator equipped with a 0.5 in. tapped horn delivering approximately 330 W/pulse. After the solution is made 2 mM in phenylmethanesulfonyl fluoride, 1 mM in 6-aminocaproate, 50 μM in leupeptin, and 0.067 μM in aprotonin, sonication was continued for 10 min using 5-s pulses spaced at 5-s intervals. After the solution was allowed to cool for 5 min, it was sonicated for an additional 10 min as described above and centrifuged (30000*g*, 30 min). The pellet was discarded and the supernatant was centrifuged (150000*g*) for 1.5 h.

The supernatant was injected in six portions (~4 mL each) onto a Waters Protein Pak (DEAE−5PW) anion-exchange column (15 × 2.15 cm) that had been equilibrated with the Tris buffer described above at 5 mL/min. MIF was not retained on the column so it was eluted by washing the column with the equilibrating buffer for 20 min. The eluent was monitored at 280 nm, collected in 5-mL fractions, and assayed for activity with substrate **2**. Typically, the protein eluted at 8−15 min after injection. Active fractions were pooled and concentrated to about 12 mL by ultrafiltration

in a 50-mL Amicon filtration cell with a YM-10 membrane. The concentrate was reinjected in six portions (~2 mL each) onto the Waters Protein Pak, eluted, and collected as described above. This procedure yielded ~50 mg of MIF/L of culture that is greater than 95% pure as judged by SDS−PAGE.

For greater purity, the protein was injected in 5 mg portions (in ~0.5 mL) onto a Superose 12 (Pharmacia) gel-filtration column equilibrated with 20 mM Tris buffer (30 mL, pH 7.4) made 20 mM in NaCl at a flow rate of 0.4 mL/min. The eluent was collected in 0.4 mL fractions. Typically, activity eluted at ~50 min. Active fractions were pooled and concentrated. Passage of the protein through the anion-exchange column twice followed by the Superose column yields ~20−25 mg of MIF/L of culture.

Enzymatic Activity Assays. The enzymatic activity of MIF was monitored by following the formation of the borate complex of **3** at either the λ_{max} (300 nm, $\epsilon = 8664\ M^{-1}\ cm^{-1}$) or a shoulder at 320 nm ($\epsilon = 4320\ M^{-1}\ cm^{-1}$ for the pH range 5.4−6.5, 3980 $M^{-1}\ cm^{-1}$ at pH 6.8, and 3560 $M^{-1}\ cm^{-1}$ at pH 7.2) (*21*). All assays were carried out at 24 °C. For the quantitation of enzymatic activity in the purification process and in the kinetic studies of irreversible inhibition, the reaction was monitored at 300 nm. The assay mixture contained 0.5 mL of a 1 M boric acid solution (pH 6.2), 0.2 mL of a 1 M NaH_2PO_4 buffer solution (pH 6.2), MIF (0.2−0.5 μM) in 20 mM Tris buffer made 20 mM in NaCl (pH 7.4), and a sufficient quantity of water to make a final volume of 1 mL. The assay was initiated by the addition of **2** (2−60 μL) from a 100 mM stock solution made up in 50 mM sodium acetate buffer (pH 6.0). The final pH of the assay mixture was 6.5. Different quantities of MIF were used in these assays in order to obtain reliable initial rates at all concentrations of substrate. The initial rates were determined from plots of absorbance vs time at 300 nm. Stock solutions of **2** were made up fresh daily. In the studies of the pH dependence of enzymatic activity and inactivation, the competitive inhibition studies, and the protection studies, the reaction was monitored at 320 nm. The initial rates were determined from plots of absorbance vs time at 320 nm. In the pH studies, the rapid nonenzymatic enolization of **2** to **3** at higher pH values precludes accurate measurements of the enzymatic activity at 300 nm when higher substrate concentrations are used. In the studies with the competitive inhibitor (*E*)-*p*-hydroxycinnamic acid (**6**), the strong UV absorbance of **6** at 300 nm precluded accurate measurements at this wavelength.

*pH Dependence of the Enzymatic Activity of MIF with Substrate **2***. The dependence of the rate of enolization of **2** on pH was determined for the pH range of 5.4−7.2. At pH values > 7.2, the rapid nonenzymatic enolization of **2** and the instability of the enol−borate complex (as indicated by a variable molar absorptivity coefficient) precluded the accurate measurement of the enzymatic reaction. The boric acid solution and the NaH_2PO_4 buffer were adjusted to the desired pH. A constant ionic strength was maintained. MIF (0.2−0.7 μM) was allowed to incubate in the assay mixture and the reaction was initiated by the addition of a quantity of **2** (2−60 μL) from a 100 mM stock solution made up in 50 mM sodium acetate, pH 6.0. The reported pH was measured after the reaction was complete. The pH dependences of the kinetic parameters were fitted by nonlinear regression data analysis the Grafit program (Erithacus Software Ltd., Staines, U.K.).

*Determination of K_I for (E)-p-Hydroxycinnamic Acid (**6**)*. To determine whether **6** was a reversible inhibitor of the enzymatic activity of MIF, the inhibition was examined at increasing concentrations of **6** (50, 100, and 250 μM) in the presence of six concentrations of **2** (0.4, 0.6, 1.0, 2.0, 3.0, and 6.0 mM). Stock solutions of **6** (25 mM) were made up in 100 mM NaH_2PO_4 buffer (final pH 7.3). The kinetic data were fitted by nonlinear regression data analysis using the equation for competitive inhibition provided with the Grafit program.

Kinetics of Irreversible Inhibition. The inactivation of MIF by 3-BP was determined by the incubation of varying amounts of inhibitor (0−40 mM) with enzyme (54 μM) in a solution containing Tris buffer (13.5 mM), sodium phosphate buffer (32.5 mM), and NaCl (13.5 mM) and adjusted to pH 7.35 at 24 °C (*10*). The incubation mixtures (total volume 100 μL) were made up in 1.5 mL Eppendorf micro test tubes. Aliquots (10 μL) from these solutions were removed at various time intervals and diluted into the assay buffer, and the residual MIF activity was measured at 300 nm. The assay was initiated by the addition of **2** to give a final concentration of 2 mM. Two stock solutions of 3-BP were made up fresh daily to either 200 mM (for the inhibitor concentrations of 5−40 mM) or 25 mM (for the inhibitor concentrations of 0.5−1 mM) in 100 mM NaH_2PO_4 buffer (pH = 6.7). The pH of the stock solution was adjusted to pH 6.7 with small amounts of a solution of NaOH. Stock solutions of **2** were made fresh daily.

The observed rate constant for inactivation (k_{obsd}) at each inhibitor concentration was determined from a nonlinear least-squares fit of the data for loss in enzyme activity as a function of incubation time to the equation for first-order decay. At all concentrations of 3-BP used, the decrease in activity was pseudo-first-order in enzymatic activity for at least 6 half-lives. The values of K_I, k_{inact}, and k_{inact}/K_I were determined by fitting the data to a rectangular hyperbola by nonlinear least-squares analysis (*22*).

Protection against the inactivation of the enzymatic activity of MIF by 3-BP was carried out using (*E*)-*p*-hydroxycinnamic acid (**6**) as described above with the following modifications. After incubation of MIF with varying concentrations of **6** (0−15 mM) for 5 min, a fixed concentration of 3-BP (40 mM) was added to the buffered solution. Aliquots (10 μL) were removed at various times over a 5 min period and assayed for residual activity (see above). The data were plotted and analyzed as described above and elsewhere (*22*).

The irreversibility of the inactivation of the enzymatic activity of MIF by 3-BP was established as follows. MIF (25.5 nmol) was incubated with a large excess of 3-BP (25 μmol) in 500 μL of 40 mM sodium phosphate buffer (pH 7.15) for 1 h at 4 °C. In a separate control experiment, the same quantity of MIF was incubated without 3-BP under identical conditions. Both samples were dialyzed exhaustively against 40 mM sodium phosphate buffer (pH 7.15) for several days. The samples were monitored periodically for activity by the removal of an aliquot (10 μL) that was assayed as described above. After 6 days, the inactivated MIF regained about 3% of its original activity when compared to the control sample.

pH Dependence of Inactivation. The pH dependence of the kinetic parameters for inactivation by 3-BP was determined in 50 mM NaH_2PO_4 buffer over a pH range of 5.4−7.1 at 24 °C. At each pH, the inhibition mixture contained 50 mM NaH_2PO_4 buffer, MIF (54 μM), and 3-BP (0−40 mM) in a final volume of 100 μL. The inactivation at each pH was determined with six different concentrations of inhibitor (0.5, 1.0, 5.0, 10, 20, and 40 mM). Two stock solutions of 3-BP were made up fresh daily to either 400 mM (for the inhibitor concentrations of 5−40 mM) or 20 mM (for the inhibitor concentrations of 0.5−1 mM) in 100 mM NaH_2PO_4 buffer (pH = 6.7). The pH of each solution was checked before and after the reaction with 3-BP. In separate control experiments it was shown that the enzyme retains ≥95% of its initial activity when preincubated at all pH values studied prior to assaying for activity at pH 6.2. The assay was initiated by the addition of **2** to give a final concentration of 1.5 mM. The kinetic parameters (k_{inact}/K_I, k_{inact}, and K_I) were obtained as described above.

Preparation and Matrix-Assisted Laser Desorption−Ionization Mass Spectrometry of the Covalently Modified MIF. A quantity of MIF (4 mg in 0.5 mL of 20 mM Tris buffer containing 20 mM NaCl, pH 7.4; 0.65 mM) and 3-BP (4 mg in 1.2 mL of 100 mM NaH_2PO_4 buffer, pH 6.7; 20 mM) were incubated at 24 °C for 1 h. A control sample was made by incubating a quantity of MIF (4 mg in 0.5 mL of 20 mM Tris buffer containing 20 mM NaCl, pH 7.4) with buffer (1.2 mL of 100 mM NaH_2PO_4 buffer, pH 6.7) at 24 °C for 1 h. The pH of both solutions was 6.7. Subsequently, the two samples were treated with a large excess of $NaBH_4$ (40 mg in 400 μL of H_2O), added dropwise, and allowed to stand for 1 h at 24 °C. The pyruvyl adduct on MIF was reduced to its lactyl derivative because previous experience with similarly modified amino-terminal proteins has shown that the pyruvyl adducts decompose when exposed to the acidic HPLC buffers (*10*). The pH was adjusted to 7 by the addition of small amounts of HCl. Subsequently, a portion of the modified and unmodified MIF sample was analyzed by MALDI mass spectrometry in order to verify the presence of a singly labeled residue per monomer. Accordingly, the samples were diluted in H_2O/0.1% TFA to a final concentration of approximately 10 pmol/μL. The buffer contaminants were less than 1 mM. The modified or unmodified diluted sample (1 μL) was mixed with an equal volume of the saturated matrix solution on the sample plate, air-dried, and introduced into the spectrometer. The matrix solution consisted of α-cyano-4-hydroxycinnamic acid in acetonitrile/water (1:1) made 0.1% in TFA. Positive-ion, MALDI mass spectra were collected using a Thermo BioAnalysis Dynamo system equipped with a nitrogen laser at 337 nm. Typically, the ion signals generated from 20−30 laser pulses were summed to yield a single mass spectrum.

Peptide Mapping. The lactyl-modified MIF and the control sample were purified (in separate runs) on a Waters HPLC system using an Econosil C_{18} reverse-phase HPLC column (250 mm × 22 mm, 10 μ) in 500 μL portions. After the sample was loaded onto the column, it was washed with H_2O/0.05% trifluoroacetic acid for 15 min and eluted with a linear gradient (0−80% acetonitrile/0.05% TFA) over 50 min at a flow rate of 5 mL/min. The effluent was monitored at 214 nm, and the fractions were collected at 1 min intervals. The lactyl-modified MIF and the control sample eluted as broad peaks at ∼53−54 min after the time of injection. The appropriate fractions were collected and concentrated to dryness in vacuo.

Each purified sample (estimated to contain 1−1.5 mg of protein) was dissolved in 25 μL of 400 mM ammonium bicarbonate buffer (pH 8.0) containing 3 mM $CaCl_2$, 8 M urea, and 5 mM DTT and incubated at 24 °C for 1 h. Subsequently, the solution was diluted to 100 μL (with H_2O) and combined with 4 μL of a trypsin solution (10 μg/μL) made up in 0.5 mM HCl (*23*). After the solution was incubated for 2 h at 37 °C, 3.5 μL of the trypsin solution and 2 drops of toluene were added, and the mixture was incubated for 22 h at 37 °C. The peptide fragments in the two incubation mixtures were separated on the Waters HPLC system using an Econosil C_{18} reverse-phase HPLC column as described above. The major peaks were collected and analyzed by electrospray ionization (ESI) mass spectrometry using a Sciex API-III quadrupole electrospray mass spectrometer. The lactyl-modified amino-terminal peptide fragment ($C_3H_5O_3$-PMFIVNTNVPR) and the unmodified amino-terminal peptide fragment (PMFIVNTNVPR) elute as broad peaks at ∼43−52 min after the time of injection. Samples were analyzed in a solution of 30% acetonitrile/H_2O/0.1% TFA at concentrations of approximately 10 μM.

The lactyl-modified MIF and unmodified sample were also treated with trypsin and the products were analyzed directly by MALDI mass spectrometry. In this procedure, each sample (∼200 μg) was combined with trypsin (10 μg) and dissolved in 1 mL of 100 mM Tris buffer (pH 8.5) containing 5 mM $CaCl_2$ and 2 M guanidine hydrochloride. The samples were incubated at 37 °C overnight, concentrated, and desalted using a procedure described elsewhere (*24*). Subsequently, the concentrated and desalted sample was taken up in 10 μL of 70% acetonitrile/H_2O/0.1% TFA and analyzed by MALDI mass spectrometry as described above.

RESULTS

Kinetic Properties of MIF. The kinetic properties of the enzymatic activity of MIF with substrate **2** were measured and compared to those measured previously. The steady-state kinetic parameters (K_m = 4.2 mM; k_{cat} = 360 s^{-1}, and $k_{cat}/K_m = 8.6 \times 10^4$ $M^{-1}s^{-1}$) are comparable to the previously reported values (*14*, *25*). In addition, (*E*)-*p*-hydroxycinnamate (**6**) was examined as a potential competitive inhibitor of the enzymatic activity of MIF because it was previously reported to be a competitive inhibitor of phenylpyruvate tautomerase with a K_I = 0.21 mM (*25*). Compound **6** is found to be a competitive inhibitor of the enzymatic activity of MIF with a K_I = 0.3 mM.

*pH Dependence of the Enzymatic Activity of MIF with Substrate **2***. The pH-rate profile for k_{cat}/K_m vs pH shows a single ascending limb with a slope of 1 (○, Figure 1). A nonlinear least-squares fit of the pH dependence of k_{cat}/K_m to

$$k_{cat}/K_m = (k_{cat}/K_m)^{max}/1 + [H^+]/K_{HE} \qquad (1)$$

where K_{HE} corresponds to the ionization constant for the free enzyme gives a pK_a value of 6.0 ± 0.1. The plot of k_{cat} vs pH also shows a single ascending limb with a slope of 1 (●, Figure 1). A nonlinear least-squares fit of the pH dependence of k_{cat} to

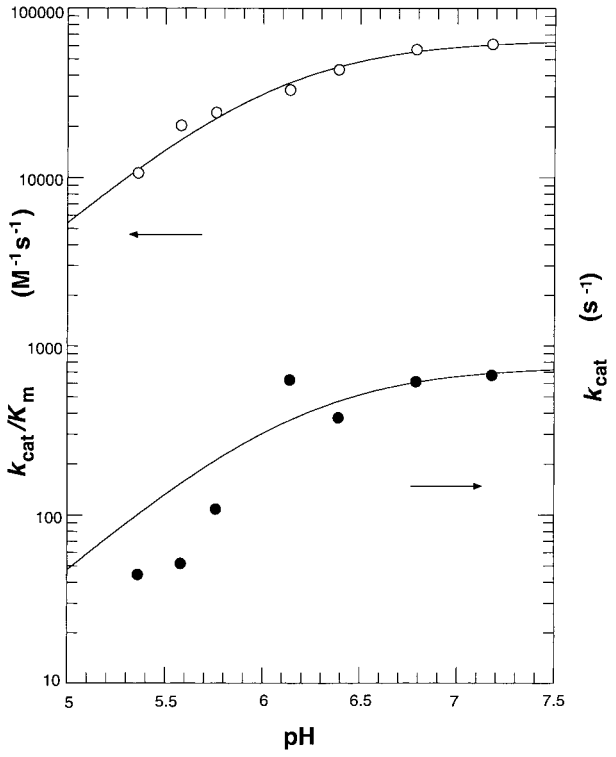
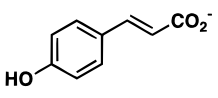


FIGURE 1: pH dependence of the kinetic parameters for the MIF-catalyzed enolization of **2**. The pH dependence of k_{cat}/K_m (○) and the pH dependence of k_{cat} (●) are shown. The curves were generated by a nonlinear least-squares fit of the data to eqs 1 and 2, respectively. The pK_a values are given in the text.

$$k_{cat} = (k_{cat})^{max}/1 + [H^+]/K_{HES} \quad (2)$$

where K_{HES} corresponds to the ionization constant for the enzyme−substrate complex gives a pK_a value of 6.2 ± 0.3. The pH dependence of the enzymatic activity of MIF could only be studied in the pH range of 5.4−7.2 because a rapid nonenzymatic enolization of **2** occurs at pH values greater than 7.2 and the enol−borate complex of **2** is either unstable or not formed in quantitative yields.

Inactivation of the Enzymatic Activity of MIF by 3-BP. Incubation of MIF with 3-BP resulted in a time-dependent, irreversible loss of the enzymatic activity of MIF in a pseudo-first-order process (Figure 2A). The k_{obsd} values measured in six experiments were plotted vs the inhibitor concentration and fit to a rectangular hyperbola (Figure 2B). The values of k_{inact} and K_I obtained from this plot are 0.025 ± 0.002 s^{-1} and 36.3 ± 4.1 mM, respectively. The hyperbolic inactivation is a hallmark of saturation kinetics and indicates that MIF and 3-BP form a dissociable complex at the active site prior to covalent modification and inactivation (*22*). Binding at the active site of MIF is also indicated by the observation that the presence of a competitive inhibitor, (*E*)-*p*-hydroxycinnamate (**6**), prevents the loss of enzymatic activity (Figure 3). Finally, the enzymatic activity of MIF is not regenerated by exhaustive dialysis which is consistent with the formation of a covalent bond between 3-BP and a residue of MIF.



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pH Dependence of the Inactivation of the Enzymatic Activity of MIF. The pH dependence of the inactivation of

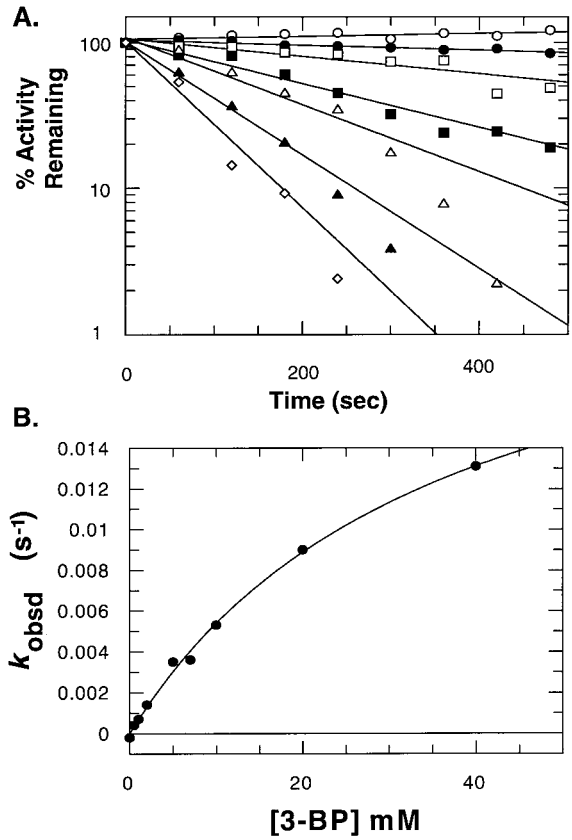


FIGURE 2: Inactivation of the enzymatic activity of MIF by 3-BP. (A) Logarithmic plot of the percent of remaining activity as a function of the concentration of 3-BP (○, 0 mM; ●, 0.5 mM; □, 2 mM; ■, 5 mM; △, 10 mM; ▲, 20 mM; ◇, 40 mM). (B) Plot of k_{obsd} as a function of the varying concentrations of 3-BP. The data were fitted to a rectangular hyperbola as described in the text. The values of k_{inact} and K_I obtained from this plot are given in the text.

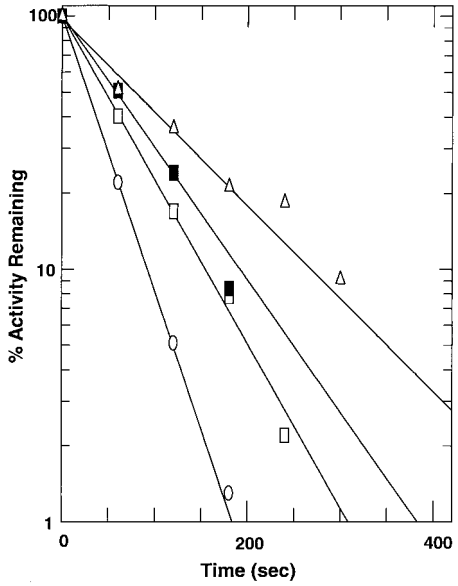


FIGURE 3: Protection against the 3-BP inactivation of the enzymatic activity of MIF by the competitive inhibitor (*E*)-*p*-hydroxycinnimate (**6**). MIF was incubated with different concentrations of **6** (○, 0 mM; □, 4 mM; ■, 10 mM; △, 15 mM) for 5 min prior to the addition of 3-BP (40 mM). Aliquots were removed and assayed for residual activity as described in the text.

the enzymatic activity of MIF by 3-BP was analyzed by a previously described model (*10*). Both k_{inact}/K_I and k_{inact} show a pH dependence over the pH range 5.4−7.1. A nonlinear

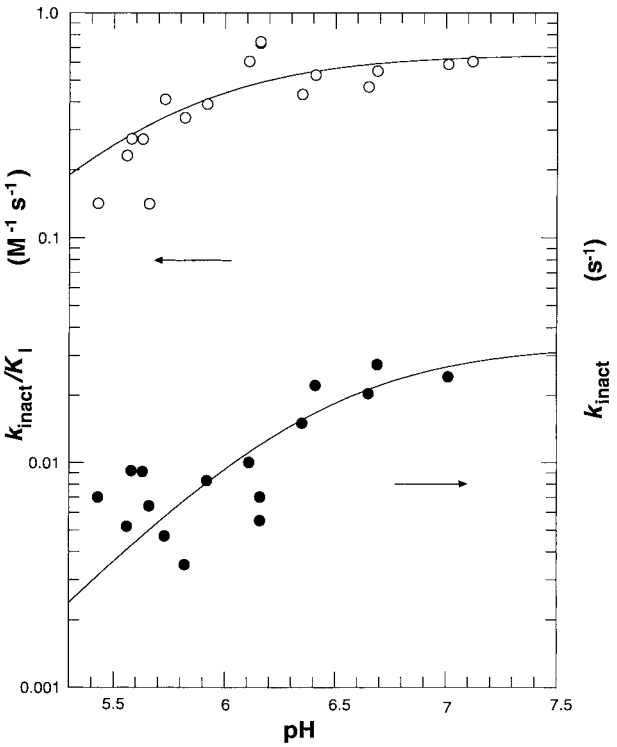


FIGURE 4: pH dependence of the inactivation of the enzymatic activity of MIF by 3-BP. The pH dependence of k_{inact}/K_I (○) and k_{inact} (●) are shown. The curves were generated by a nonlinear least-squares fit of the data to eqs 3 and 4, respectively.

least-squares fit of the pH dependence of k_{inact}/K_I to

$$k_{inact}/K_I = (k_{inact}/K_I)^{max}/1 + [H^+]/K_{HE} \quad (3)$$

where K_{HE} is the dissociation constant for the protonated form of the enzyme gives a pK_a value of 5.7 ± 0.2 (○, Figure 4). The pK_a corresponds to free enzyme. This pK_a is comparable to the pK_a measured for the pH dependence of k_{cat}/K_m (6.0 ± 0.1) as well as the pK_a (5.6 ± 0.1) reported for Pro-1 by Swope et al. (*16*). The latter pK_a was obtained by the titration of Pro-1 in a sample of uniformly labeled [^{15}N]MIF by ^{15}N NMR spectroscopy (*16*).

The maximal rate constant for inactivation by 3-BP (k_{inact}) also showed a pH dependence. A nonlinear least-squares fit of the pH dependence of k_{inact} to

$$k_{inact} = (k_{inact})^{max}/1 + [H^+]/K_{HEI} \quad (4)$$

where K_{HEI} is the ionization constant for the enzyme−inhibitor complex gives a pK_a value of 6.4 ± 0.2 (●, Figure 4). The pK_a for the EI complex is slightly higher than that determined for free enzyme.

Identification of the Lactyl-Modified MIF by Mass Spectrometry. To determine whether the incubation of MIF and 3-BP led to inactivation by the modification of a single or multiple sites, MIF was treated with a large excess of 3-BP, reduced by $NaBH_4$, and analyzed by MALDI mass spectrometry. As a control, an unmodified sample of MIF was also treated with $NaBH_4$ and subjected to MALDI mass spectrometry. MALDI-MS analysis of the untreated MIF is shown in Figure 5A. The observed mass of the monomer (12 373 Da, MH^+) is in excellent agreement with the expected molecular mass, 12374 Da (MH^+, average isotopic composition). MALDI-MS analysis of the modified MIF is shown in Figure 5B. The observed mass of the monomer (12 463 Da, MH^+) is in good agreement with the expected molecular mass, 12 464 Da (MH^+, average isotopic composition). The difference between these masses is 90 mass units so that the mass of the bound species is 89, which is consistent with the mass of a lactyl group. No other significant signals were observed in the mass spectrum consistent with the modification of a single residue.

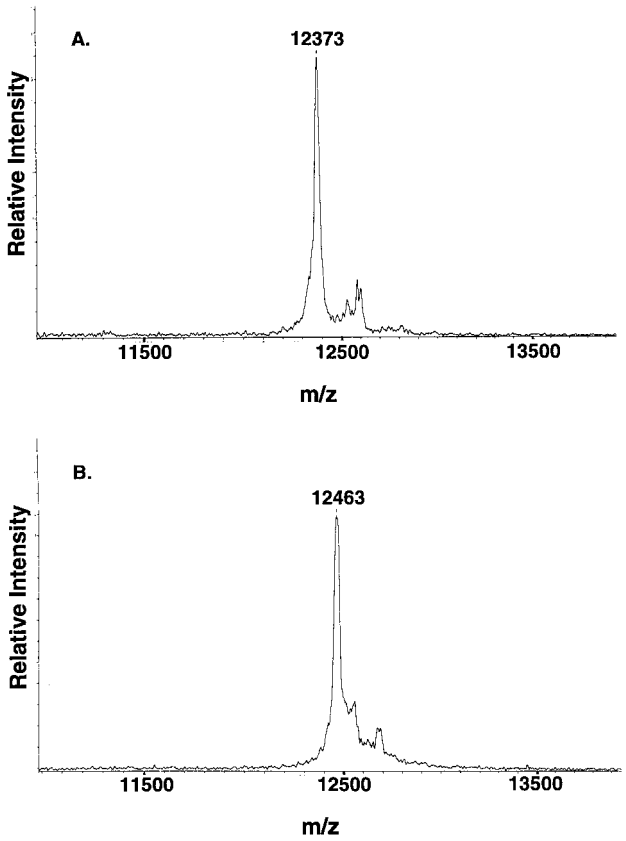


FIGURE 5: MALDI mass spectra of (A) MIF incubated without 3-BP, reduced with $NaBH_4$, and purified by reverse-phase HPLC and (B) MIF incubated with 3-BP, reduced with $NaBH_4$, and purified by reverse-phase HPLC as described in the text. The major signal corresponds to the expected masses for the monomer of MIF (12 373 Da, MH^+) and the monomer of MIF modified by a lactyl derivative (12 463 Da, MH^+).

Isolation of the Modified Peptide and Identification by Mass Spectrometry. To identify the site of modification on MIF by 3-BP, the inactivated and reduced MIF was purified by reverse-phase HPLC and treated with trypsin. An unmodified sample of MIF was also treated with $NaBH_4$ and treated with trypsin. Trypsin cleaves predominantly at the carboxyl side of arginine and lysine residues (*23*). There are four arginine residues and three lysine residues in mouse MIF so a complete tryptic digestion should result in eight fragments (*3*). Because Lys−Pro peptide bonds are cleaved very slowly by trypsin, it is likely that only seven fragments will be generated as Lys-32 is followed in the sequence by a proline (*23*).

The identity of the modified fragment was established by two methods. In the first method, the fragment was purified from a trypsin digestion mixture and identified by ESI mass spectrometry. In the second method, the trypsin digestion mixture was analyzed directly by MALDI mass spectrometry.

Chromatographic separation of the trypsin digestion mixture of the modified and unmodified MIF resulted in the recovery of a peptide fragment eluting as a broad peak ~43 min after injection. The peak recovered from the tryptic digest of the unmodified MIF was identified as the amino-terminal fragment Pro-1 to Arg-11 (PMFIVNTNVPR) by ESI-MS because the observed molecular mass of 1287 Da agrees with the expected molecular mass of 1287.7 Da. The peak recovered from the tryptic digest of the modified (and reduced) MIF was identified as the lactyl-modified amino-terminal fragment Pro-1 to Arg-11 ($C_3H_5O_3$-PMFIVNTNVPR) by ESI-MS because the observed molecular mass of 1375 Da agrees with the expected molecular mass of 1375.7 Da. MALDI mass spectral analysis of the trypsin digestion mixture of the inactivated and reduced MIF identified the same 11 residue lactyl-modified amino-terminal fragment. The only possible target for alkylation within this sequence is Pro-1. Thus, it is concluded that Pro-1 is the site of modification.

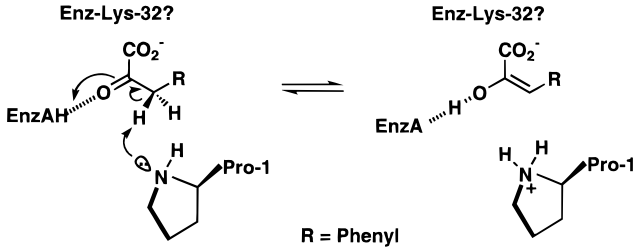
DISCUSSION

Our findings demonstrate that 3-bromopyruvate, a well-known affinity labeling reagent (*22*, *26*), is an active-site-directed irreversible inhibitor of the enzymatic activity of MIF. Several observations were used to characterize the nature of this inhibition. First, the inactivation of the enzymatic activity of MIF by 3-BP showed saturation kinetics, indicating that a complex forms between 3-BP and MIF at the active site prior to inactivation. Second, the active site is protected from inactivation by the presence of a competitive inhibitor, (*E*)-*p*-hydroxycinnamate. Finally, the irreversibility of inactivation was confirmed by two results: the enzymatic activity was not regenerated by exhaustive dialysis, and 3-BP (as its lactyl derivative) was shown to be covalently bound to MIF.

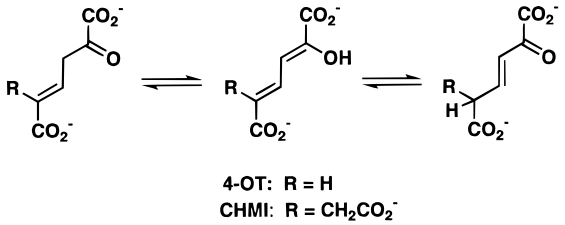
Mass spectral analysis of the modified MIF monomer coupled with the isolation and analysis of a singly modified peptide fragment (Pro-1 to Arg-11) show that 3-BP alkylates only one site per monomer of MIF even though a high concentration of inhibitor (~30:1 molar ratio of 3-BP to MIF monomer) was used in the study. The only chemically reasonable (and thus probable) site of modification within the amino-terminal fragment is Pro-1. Identification of the modified residue is significant because the pH dependence of inactivation (k_{inact}/K_I) of the enzymatic activity of MIF by 3-BP indicates that a group with a pK_a of 5.7 ± 0.2 is involved. This value is comparable to that obtained for the pH dependence of k_{cat}/K_m (6.0 ± 0.1) as well as the pK_a measured directly for Pro-1 (5.6 ± 0.1) by Swope et al. (*16*) using ^{15}N NMR spectroscopy. The combination of these findings indicates that Pro-1 can act as a general base catalyst and that the residue being modified by 3-BP is the same residue as that involved in catalysis.

A previous study also reported that 3-BP inactivates the enzymatic activity of MIF, but the nature of the inactivation process was not characterized and the site of modification was not identified (*15*). Moreover, modeling studies of MIF and the methyl ester of **1** suggested that Pro-1 acts as a general acid catalyst in the reaction and not as the general base catalyst (*15*). One possible explanation for the discrepancy between our conclusion and these modeling studies is that the steric bulk of the dopachrome substrate precludes an accurate portrayal of substrate binding.

Scheme 3



Scheme 4



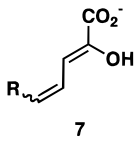
Because MIF catalyzes a tautomerization reaction, it is anticipated that a general acid catalyst will be required in addition to the general base catalyst (*11*). The identity of this residue has not yet been determined. The crystal structure of MIF (solved in the absence of a ligand) shows that there are four tyrosine residues and two lysines in the vicinity of Pro-1 (*7*, *27*).[2] The role of these residues in the tautomerization reaction has not yet been determined although some recent work by Suzuki et al. (*27*) suggests that one of the lysine residues, Lys-32, might interact with the carboxylate group of **1** (*27*).

Suzuki's study (*27*), previous studies on PPT (*25*, *28*), and the study described herein provide a picture of a minimal catalytic mechanism for the MIF-catalyzed enolization of **2** (Scheme 3). In this scenario, Pro-1 abstracts the *pro-R* hydrogen of **2** and the developing negative charge is stabilized by a general acid catalyst. Lysine-32 interacts with the carboxylate group. The stereospecificity of the PPT-catalyzed reaction was demonstrated by Retey et al. (*28*), who showed that PPT removes the *pro-R* hydrogen of **2**. Pirrung et al. (*25*) examined the PPT-catalyzed reaction using a series of *E* and *Z* vinyl fluoride analogues of **3** and **4** as potential inhibitors. It was determined that the *E* isomers were better competitive inhibitors than the corresponding *Z* isomers. On this basis, it was concluded that the PPT-catalyzed enolization of **2** and **5** will generate an enol having the *E* stereochemistry (*25*).

The structural similarities between MIF and the bacterial isomerases, 4-OT and CHMI, coupled with these mechanistic parallels raise questions about the extent to which these three proteins are related. One question concerns whether MIF catalyzes an isomerization reaction analogous to those catalyzed by 4-OT and CHMI in addition to its tautomerization reactions. Kinetic and stereochemical studies indicate that both 4-OT and CHMI convert a β,γ-unsaturated ketone to its α,β-isomer through a dienol intermediate (Scheme 4) (*12*, *29*, *30*). Inspection of the substrates for 4-OT and CHMI

[2] Other possible candidates for a general acid catalyst include an ordered water molecule or main-chain amide groups.

suggests that corresponding substrate for MIF might be 2-hydroxy-2,4-pentadienoate (**7**) or one of its derivatives.



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Addressing this question is of both practical and intellectual significance. While it has been shown that MIF catalyzes the enolization of **2** (and the ketonization of **3**), it is not yet clear how either of these reactions relate to any of the biological activities of MIF. Thus, the identification of other reactions catalyzed by MIF may shed light on the relationship between the enzymatic activity and the biological activity. If, however, MIF functions strictly as a tautomerase (interconverting a β,γ-unsaturated ketone and its dienol) it will be of considerable interest to compare the three active sites in order to identify the features that control the regiochemistry.

A second more global question concerns the nature of the evolutionary relationship among these three structurally homologous proteins that utilize the same general base to catalyze a chemical reaction involving the keto−enol tautomerization of a pyruvyl moiety. While there is little sequence homology, the structural and mechanistic similarities suggest that these proteins diverged from a common ancestral protein that might have involved a similar keto−enol tautomerization step. The function of such a protein is not clear although it appears to be a ubiquitous one. 4-OT and CHMI are found in bacteria [soil and enteric bacteria, respectively (*31*, *32*)], while homologues of MIF are found in parasites, nematodes, plants, and mammals (*16*). On the basis of the common structures, Murzin (*8*) classified 4-OT, CHMI, and MIF as being members of a superfamily of enzymes. Our results are consistent with this categorization. Further work on the three proteins is underway in order to fully define the active-site template for this superfamily.

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