The macrophage migration inhibitory factor MIF is a phenylpyruvate tautomerase

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Abstract A macrophage migration inhibitory factor (MIF), originally described as a product of activated lymphocytes, has been defined as a 12 kDa protein, expressed in a wide variety of tissues. Here MIF is identified as a phenylpyruvate tautomerase (EC 5.3.2.1) having *p*-hydroxyphenylpyruvate and phenylpyruvate as its natural substrates. The definition of MIF as an enzyme may yield insight into the mechanism of action of this proinflammatory and immunomodulating cytokine.

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Key words: Macrophage migration inhibitory factor; Phenylpyruvate tautomerase; D-Dopachrome tautomerase; Cytokine

1. Introduction

A macrophage migration inhibitory factor (MIF) was first described as a soluble material released from sensitized lymphocytes when stimulated by antigen [1,2]. Since the cloning of its cDNA [3], MIF has been found to be expressed in a large number of tissues [4–12]. More recently MIF has been reported to be released from macrophages exposed to low concentrations of glucocorticoids. Once secreted, MIF acts to counter-regulate the immunosuppressive effects of the glucocorticoids [13,14]. MIF has also been found to potentiate endotoxemia, and anti-MIF antibodies are protective in this condition [7]. An unexpected activity of MIF was recently discovered in studies of the enzymatic conversion of iminochromes to indoles [15]. A 12 kDa protein, which catalyzes the conversion of the non-natural D-isomer of 2-carboxy-2,3-dihydroindole-5,6-quinone (D-dopachrome) to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by a tautomerization reaction, was isolated from the cytosol of the bovine optic lens. The first 27 N-terminal amino acids were found to be identical with those of bovine MIF. The catalytic activity of isolated native MIF was confirmed by studies of purified recombinant human MIF which manifested the same tautomerase activity. The observation that MIF has a tautomerase activity suggested that it may exert its biological effects by an enzymatic reaction. Here we identify MIF as a phenylpyruvate tautomerase (EC 5.3.2.1.) with *p*-hydroxyphenylpyruvate and phenylpyruvate as natural substrates.

2. Materials and methods

2.1. Isolation and definition of proteins

MIF was isolated from the bovine lens as previously described [15]. SDS-PAGE, immunoblotting, and detection of proteins were performed as described by Finke et al. [16]. MIF-specific monoclonal antibody was purchased from R&D Systems. Mass spectrometry of recombinant MIF was performed by Professor Åke Engström, Department of Medical and Physiological Chemistry, Uppsala, Sweden. Total amino acid content was determined at the amino acid analysis unit of the BMC, Uppsala, Sweden.

2.1.1. Protein blotting and amino acid sequence analysis. After SDS-PAGE, using a semi-dry electroblotting apparatus (JKA Biotech, Denmark) the proteins were electroblotted to a PVDF membrane (Millipore, Bedford, MA, USA) as described by Matsudaira [17]. The protein band was cut out with a razor and sequenced at the Biomolecular Resource Facility (Lund, Sweden), using an ABI 477 protein/peptide sequencer.

2.1.2. Chromatofocusing. The proteins were isolated with the Pharmacia FPLC system and a prepacked Mono P HR 5/20 column. The column was equilibrated with starting buffer (75 mM Tris-HAc, pH 9.3). After sample application, the proteins were eluted, using a 50 ml superloop, with polybuffer 96 (diluted 1:10, pH 6.0) at a flow rate of 1.0 ml/min.

Isoelectric focusing was performed according to a previously described method [18].

2.2. Human recombinant MIF

The coding region of the MIF was specifically PCR-amplified from a human liver cDNA library, and the PCR fragment was cloned into the pKK223-3 prokaryotic expression vector (Pharmacia) and expressed in IPTG-induced transformed *E. coli* bacteria. The human recombinant MIF used in our studies has the nucleotide sequence described by Paralkar and Wistow [19].

2.3. Determination of enzymatic activity

Tautomerase activity was measured in two ways: (1) by ascertaining the rate of DHICA formation from D-dopachrome, as described by Aroca et al. [20]; and (2) by measuring the rate of the formation of the enol form from the keto form of *p*-hydroxyphenylpyruvate and of phenylpyruvate as described by Knox [21,22]. The substrates were purchased from Sigma. The rate of the reaction was determined in the first 60 s.

2.4. NMR spectroscopy

^{1}H nuclear magnetic resonance (NMR) spectra were recorded using a Bruker DRX-400 spectrometer. The chemical shifts are given in parts per million relative to methanol as internal standard ($\delta = 3.31$ ppm). The coupling constants are given in Hertz. First-order chemical shifts and coupling constants were obtained from one-dimensional spectra, and proton resonances were assigned from COSY experiments. *p*-Hydroxyphenylpyruvate (3.5 μmol) was dissolved in 0.7 ml 50 mM acetate/D_2O buffer (pH 6.2).

2.4.1. ^{1}H NMR (400 MHz, 50 mM acetate/D_2O at pH 6.2) of p-hydroxyphenylpyruvate. Enol form: δ 6.32 (s, H), 6.91 (d, J = 8.6 Hz, 2H), 7.66 (d, J = 8.6 Hz, 2H). Keto form: δ 3.98 (s,H), 6.88 (d, J = 8.6 Hz, 2H), 7.13 (d, J = 8.7 Hz, 2H).

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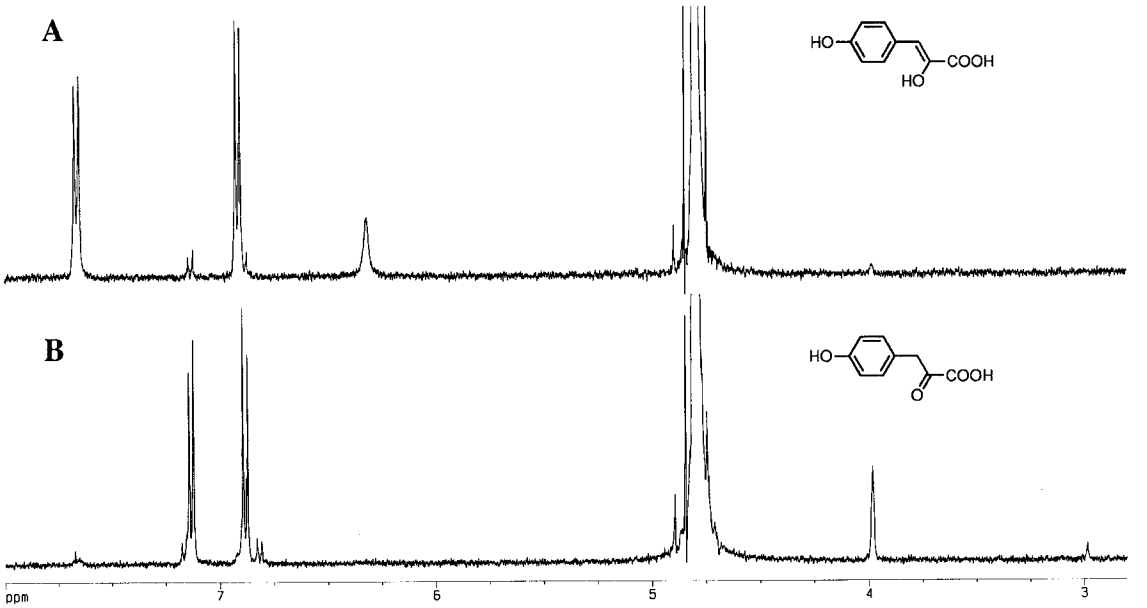


Fig. 1. The keto-enol tautomerism of *p*-hydroxyphenylpyruvate studied by ^{1}H NMR. A: Spectrum recorded immediately after dissolving 3.5 μmol *p*-hydroxyphenylpyruvate in 0.7 ml 50 mM acetate/D_2O buffer (pH 6.2). B: Spectrum of the *p*-hydroxyphenylpyruvate solution 15 min after the addition of 1 μg of human recombinant MIF [36].

3. Results

A series of compounds were examined as potential substrates of MIF by analyzing their competitive inhibition of the tautomerization of D-dopachrome to DHICA. One of the substances tested, *p*-hydroxyphenylpyruvate, at a concentration of 1 mM, was found to completely inhibit the formation of DHICA from D-dopachrome. Assuming that *p*-hydroxyphenylpyruvate might be a substrate, we examined its UV spectrum after the addition of MIF and found a rapid and pronounced decrease in UV absorption at 300 nm, consistent with tautomerization of the enol form to the keto form. The enol form is characterized by strong absorption at about 300 nm, a wavelength at which absorption of the keto form is more than 10 times lower [21]. The MIF-catalyzed tautomerization was demonstrated by means of NMR studies (Fig. 1). The spectrum recorded immediately after dissolving *p*-hydroxyphenylpyruvate in acetate buffer at pH 6.2 (Fig. 1A) revealed two doublets in the aromatic region and a sharp singlet representing an olefinic proton at $\delta = 6.32$ ppm consistent with the enol form. Minor changes were noted after the solution was left standing at room temperature for 1 h. Fig. 1B shows the ^{1}H NMR spectrum of the *p*-hydroxyphenylpyruvate solution 15 min after the addition of 1 μg of human recombinant MIF. Conversion of the enol to the keto form was demonstrated by changes in the chemical shifts of the aromatic protons and by registration of a singlet corresponding to methylene protons at $\delta = 3.98$ ppm. A small amount of the 2,2-dihydroxy derivative of the keto form was indicated by the presence of a weak singlet at $\delta = 2.98$ ppm.

The kinetic properties of the enzyme were studied using spectrophotometry [22]. The tautomerization of *p*-hydroxyphenylpyruvate was catalyzed by MIF in borate buffer with a K_m of 2.4 mM, and V_{max} of 1.1 mmol/mg·min for the keto to enol form reaction, k_{cat}/K_m 0.92×10^5 M^{-1} s^{-1}. Phenylpyruvate also functioned as a substrate with a K_m of 6.0 mM and V_{max} of 3.9 mmol/mg·min for tautomerization of the keto form to the enol form, k_{cat}/K_m 1.3×10^5 M^{-1} s^{-1}.

Our MIF preparation purified from the bovine lens, examined by chromatofocusing, was found to occur in two forms with p*I*s of 8.0 and 7.7, as determined by isoelectric focusing. Determination of total amino acid content showed the two forms to have the same amino acid composition (Table 1). Western blot analysis with mouse anti-MIF antibody identified both proteins as MIF with molecular weights of about 12 kDa (Fig. 2). Both forms manifested the same enzymatic activity with D-dopachrome, *p*-hydroxyphenylpyruvate and phenylpyruvate as substrates. We have at present no data permitting interpretation or conclusions regarding the observed two p*I* forms of MIF.

The finding that MIF isolated from bovine lens is a phenylpyruvate tautomerase was confirmed in studies of human re-

Table 1
Amino acid composition (mol %) of two forms of bovine MIF with different p*I* values

	p*I* 8.0	p*I* 7.7
Asx	9.81	9.73
Thr	5.31	5.39
Ser	8.97	9.07
Glx	8.17	8.48
Pro	6.04	6.19
Gly	9.32	9.27
Ala	9.14	8.85
Cys	2.99	2.91
Val	6.51	6.32
Met	2.52	2.66
Ile	5.42	5.40
Leu	10.94	10.69
Tyr	2.55	2.68
Phe	3.57	3.65
His	1.49	1.58
Lys	2.57	2.55
Arg	4.69	4.58

Tryptophan not determined.

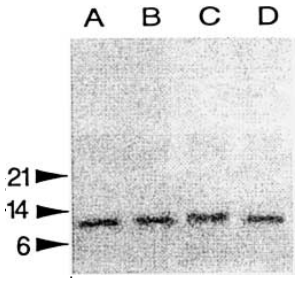


Fig. 2. Western blot analysis of isolated proteins with tautomerase activity on *p*-hydroxyphenylpyruvate and phenylpyruvate. The antibody used was a monoclonal anti-human MIF antibody (R&D Systems). Positions of size markers are indicated and sizes given in kDa. A: Tautomerase of bovine lens, p*I* 8.5. B: Tautomerase of bovine lens, p*I* 8.3. C: Human recombinant tautomerase, p*I* 8.7. D: Phenylpyruvate tautomerase of bovine kidney (Sigma T-6004).

combinant MIF. Chromatofocusing of human recombinant MIF showed the presence of a single protein with p*I* of 8.1 as determined by isoelectric focusing. Mass spectrometry of the human recombinant MIF protein gave a molecular mass of 12 353 ± 0.1%, consistent with the mass of 12 345 calculated from the amino acid sequence of human MIF (Fig. 3). Amino acid sequencing showed that the N-terminal residue was proline confirming the removal of the starting formylmethionine of the recombinant protein. The enzymatic activity of recombinant human MIF was the same as that of bovine MIF isolated from the lens. Western blot analysis with anti-MIF antibody showed the presence of a protein with molecular weight of about 12 kDa (Fig. 2).

A commercially available bovine phenylpyruvate tautomerase (Sigma T-6004) was examined for the possible presence of MIF protein. SDS-PAGE of the preparation yielded several protein bands. The protein with *p*-hydroxyphenylpyruvate tautomerase activity was isolated as previously described for MIF [15]. The enzymatic activity of the isolated protein was the same as that isolated from the bovine lens. The purity and homogeneity of the protein with tautomerase activity was established by SDS-PAGE, which showed the presence of a single protein of molecular weight of about 12 kDa. The N-terminal 15 amino acid residues of the protein were identical to those of bovine MIF [9]. Western blot analysis of the protein isolated from the commercial phenylpyruvate tautomerase preparation and of MIF from the bovine lens yielded identical results (Fig. 2).

4. Discussion

The results presented demonstrate MIF and a phenylpyruvate tautomerase to be identical proteins. Enzymatic catalysis of tautomerization of phenylpyruvate and *p*-hydroxyphenylpyruvate was observed in studies of the degradation of phenylalanine and tyrosine more than 40 years ago [21,22]. Mechanistic and stereochemical studies have been performed, and inhibitors are known [23,24], but the phenylpyruvate tautomerase has not previously been isolated and sequenced and its biological significance has remained unknown. In conjunction with the abundant information already available about the structure of MIF [25–28] and its biologic effects [8–14], the present findings should prove to be of relevance in defining the structure and biologic importance of phenylpyruvate tautomerase. Thus the phenylpyruvate tautomerase isolated is a protein consisting of 114 amino acid residues with an N-terminal proline. It contains cysteines at positions 56, 59 and 80. The cDNAs of man and mouse encode a protein of 115 amino acid residues, and homology between the species is pronounced. The human and murine genes are small, consisting of three exons contained within 1 kbp of genomic DNA [19,29].

A second protein homologous to MIF has recently been described. An enzyme which catalyzes the conversion of D-dopachrome to 5,6-dihydroxyindole was isolated from rat liver [17], and shown to share 27% of its amino acids with rat MIF [30]. A human cDNA encoding a protein with high homology to the rat protein was used to produce a recombinant human protein which had 34% identity with human MIF (Fig. 3). The rat protein as well as the recombinant human protein have been shown to possess phenylpyruvate tautomerase activity with *p*-hydroxyphenylpyruvate and phenylpyruvate as substrates (Thelin et al., unpublished observations). We suggest phenylpyruvate tautomerase I as the enzyme name for MIF, and the name phenylpyruvate tautomerase II for the second protein homologous to MIF. Phenylpyruvate tautomerase II has been found in all tissues examined in several species [17], and the protein has recently been isolated from human erythrocytes [31].

The monomer of MIF has a tertiary structure similar to that of another enzyme, *Escherichia coli* 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) which has a low sequence homology to MIF [25,26]. The quaternary architecture of the MIF crystal is strikingly similar to the crystal of CHMI, and also to the crystal of 4-oxalocrotonate tautomerase (4-OT) isolated from *Pseudomonas putida* [25,26,32–34]. CHMI and 4-OT are involved in the catabolism of products formed by the cleavage of aromatic rings and catalyze the tautomerization of such products in the degradation pathway to the intermediates in the citric acid cycle. The N-terminal proline of 4-OT is responsible for the catalytic function of this enzyme [33], and the N-terminal proline of CHMI seems also to be of catalytic importance [32]. MIF and phenylpyruvate tautomerase II both have proline as N-terminal amino acid residue, though the functional significance of this proline has yet to be demonstrated.

In this study the macrophage migration inhibitory factor MIF has been identified as a phenylpyruvate tautomerase with phenylpyruvate and *p*-hydroxyphenylpyruvate as substrates. These substrates are directly involved in the metabolism of phenylalanine and tyrosine. The relationship between the enzymatic function of MIF and its previously reported biological activities may yield insight into the mechanism of action of this extensively studied cytokine. However, the measured K_m values are high in comparison with reported

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MIF      1 PMFIVNTNVPRASVPDGFLSELTQQLAQATGKPPQYIAVHVVPDQLMAFG 50
           |    || |  || |   |   |  |||    | | |  ||
PPT II   1 PFLELDTNLPANRVPAGLEKRLCAAAASILGKPADRVNVIVRPGLAMALS 50

         51 GSSEPCALCSLHSIGKIGGA.QNRSYSKLLCGLLAERLRISPDRVYINYY 99
              || ||||  |  |||  | |  |||  |      |   |    ||  |
         51 GSTEPCAQLSISSIGVVGTAEDNRSHSAHFFEFLTKELALGQDRILIRFF 100

        100 DMNAASVGANNSTFA 114
                  |
        101 PLESWQIGKIGIVMTFL 117
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Fig. 3. Alignment of the amino acid sequences of human MIF and of human recombinant phenylpyruvate tautomerase II (PPT II), using the BestFit program [37].

normal concentrations of *p*-hydroxyphenylpyruvate [35] and it is possible that the catalytic effect on tautomerization of phenylpyruvates is unrelated to other biological effects of MIF.

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