Volume 319, number 3, 233–236 FEBS 12243 March 1993

Purification of macrophage migration inhibitory factor (MIF) from bovine brain cytosol

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Received 13 January 1993

Two isoforms of the macrophage migration inhibitory factor (MIF) have been isolated to homogeneity from bovine brain cytosol. In agreement with the cDNA sequence of their human counterpart, they both have an apparent molecular weight of 12 kDa and are characterized by the following N-terminal amino acid sequence NH_2-PMFVVNTNVPRASVPDGLLSELTQQLAQATGKPPQYIAV-. CD spectra revealed that bovine MIF contains 42% (± 3%) α-helix and 21% (± 3%) β-structure. CD-constrained prediction of the secondary structure assigned MIF to the α/β-class of proteins.

Macrophage migration inhibitory factor; Circular dichroism; Secondary structure prediction

1. INTRODUCTION

The macrophage migration inhibitory factor (MIF) was the first lymphokine to be discovered as a soluble component which was capable of inhibiting in vitro migration of macrophages [1,2]. The physiological effects of MIF were also correlated with delayed hypersensitivity, cellular immunity and the immobilization of macrophages in the sites of chronic inflammation [1–11]. The MIF activity was also detected in various inflammatory processes [3], including allograft rejection [4,5] and rheumatoid polyarthritis [6]. It is believed that MIF is generated by stimulated subsets of T-cells, and its physiological functions are augmented by other early gene products elicited by stimulated T-cells, notably γ-interferon and interleukin-4 [7,11]. MIF in a mixture with mineral oils and an antigen (bovine serum albumin or HIV virus 120 kDa glycoprotein) appeared to be a potent adjuvant of macrophages and T-cells, comparable to that of complete Freund adjuvant [10,11].

Despite the fact that MIF was the first lymphokine to be discovered it has never been isolated to homogeneity from any mammalian tissue. Therefore the cDNA sequence of MIF has been elucidated using functional expression cloning from a cDNA library of lectin-stimulated human T-cell hybridoma cell line [8]. Human MIF is encoded by a 115 amino acid residue long polypeptide chain the sequence of which is unrelated to any other protein [8]. Although the 12 kDa recombinant human MIF possesses in vitro activity in inhibiting the migration of human blood monocytes [10] and acts as a potent adjuvant of different lymphoid cells [10,11], no direct evidence exists to support its cDNA sequence. Here we report on the purification and partial characterization of a 12 kDa MIF-like protein isolated from bovine brain cytosol.

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The sequence of the N-terminal fragment of bovine MIF has been deposited to the Protein Sequence Data Bank (EMBL), accession no. P80177.

2. MATERIALS AND METHODS

2.1. *Tissue sources, chemicals, chromatography materials and gel electrophoresis*

Calf brain was supplied fresh on ice by the Henri Meunier slaughter house (Meaux, 77, France). The fresh tissue was shredded and frozen in liquid nitrogen. Acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate, *N,N,N',N'*-tetramethylenediamine were purchased from Serva. Pharmalyte 8–10.5 and Ampholite 9–11 were from Pharmacia/LKB. Molecular weight markers were purchased from Bio-Rad and Sigma. PBE118 and CM-sepharose were from Pharmacia/LKB, and DE52 from Whatman. All other chemicals were of analytical grade and purchased from either Serva or Sigma. SDS-PAGE gels (12%, 20 × 20 cm) were performed according to standard procedures and visualized by silver staining [12].

2.2. *Preparation of tissue samples and primary processing of soluble proteins*

The frozen tissue was dry-homogenized with a Waring blender and dispersed in buffer containing 0.250 M NaCl, 10% glycerol, 50 mM Tris, pH 7.2 at 4°C, 5 mM β-mercaptoethanol (β-ME), 0.02% NaN_3 and 1 mM PMSF. The mixture was centrifuged for 30 min at 8,000 × *g* and the supernatant was further centrifuged at 37,000 × *g* for 3 h. The resulting supernatant was concentrated from 4 l to about 450 ml in a 3 kDa dialysis bag (Spectrum) using polyethylene glycol (Sigma, mol. wt. approx. 15,000 Da). The final protein solution was dialyzed against 50 mM Tris buffer (pH 6.8) containing 10% (v/v) of ethylene glycol, 5 mM β-ME and 1 mM PMSF.

2.3. *Primary sorting of proteins: anion exchange chromatography*

The concentrated supernatant (usually about 450 ml) was applied onto a DE-52 column (5 × 100 cm) equilibrated with 50 mM Tris, pH 6.85, 10% glycerol, 2 mM β-ME, 1 mM PMSF. The proteins were eluted with the equilibration buffer (on average 70 fractions, 12 ml each). Fractions were collected at a speed of about 40 ml/h.

2.4. *Fractionation of basic proteins· chromatofocusing on PBE118*

PBE118 was loaded onto a 1.6 × 20 cm column and equilibrated with 0.025 M triethylamine-HCl (pH 11). The mixture of basic proteins was concentrated using a PM10 membrane in a stirred cell, followed by extensive dialysis against 0.025 M triethylamine-HCl, pH 11. The equilibrated mixture of proteins was applied onto the column (about 20 ml volume). The column was washed with 5 void vols. of the equilibrating buffer, and proteins were eluted with the Ampholite 8–10.5 (1:45 v/v in water, pH 8.0). The fractions (5 ml each) were collected at a speed of about 20 ml/h. The pH of each fraction was measured with a pH electrode.

2.5. *Final purification of bovine MIF on a weak cation exchanger*

The fractions containing the mixture of proteins with pI's from 10.5 to 9.2 were applied directly onto a CM-Sepharose column equilibrated with 10 mM phosphate, pH 7.2, 2 mM β-ME and 1 mM PMSF. The MIF isoforms were eluted in a gradient of salt from 10 mM to 1 M NaCl.

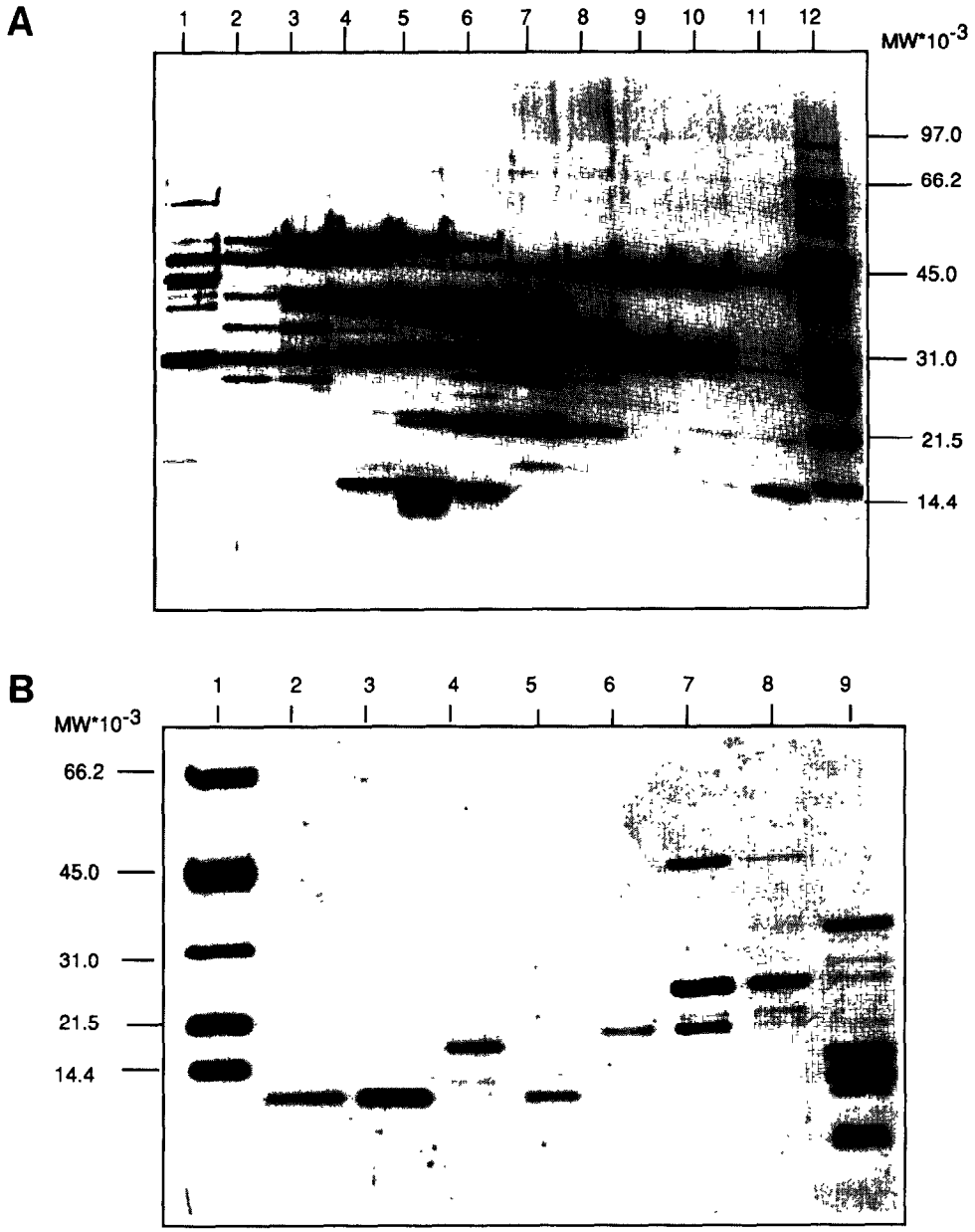

Fig. 1. SDS-PAGE analysis of the two last purification steps of MIF from calf brain cytosol. (A) Aliquots from 11 fractions from chromatofocusing were fractionated on a 12% SDS-polyacrylamide gel and visualized by silver staining. Lane 1–11 show proteins in fractions with the following pHs; (1) 8.7, (2) 8.9, (3) 9.1, (4) 9.2, (5) 9.3, (6) 9.5, (7) 9.7, (8) 9.8, (9) 10.0, (11) 10.20; lane 12 shows molecular weight markers (Bio-Rad). (B) Aliquots of selected fractions from a CM-Sepharose column were applied on a 12% SDS-polyacrylamide gel and visualized by silver staining. Lane 1, Bio-Rad molecular weight markers; lanes 2 and 3, two isoforms of bovine MIF eluted at 0.30 and 0.25 M NaCl; lanes 4–9, other proteins eluted from CM-Sepharose. Salt concentration decreases from 2 to 9.

2.6 NEPHGE/SDS-PAGE (2D gels)

About 5 μg of proteins were mixed with the lysis buffer (9.6 M urea, 1% NP-40, 2% carrier Ampholite and 2 mM DTT) and applied immediately to NEPHGE which was carried out at 400 V (2.4 mm × 18 cm) in a Protean II apparatus (Bio-Rad). The gels were excised from the tubes, soaked for 15 min in the SDS-PAGE buffer and reloaded onto a 12% SDS-polyacrylamide gel (20 × 20 cm).

2.7. *Amino-terminal peptide sequence analysis*

Samples of both MIF isoforms were dialysed extensively against 10 mM ammonium carbonate. Each sample was directly applied to a polybrene precycled glass fiber filter. An Applied Biosystems Model 477A protein sequencer was used.

2.8. *Circular dichroism (CD) spectroscopy and analysis of CD spectra*

The CD spectra were recorded with a Jobin-Yvon Mark VI dichrograph. The spectra were measured with quartz squared cuvettes (0.2 cm Hellma) in 20 mM phosphate, pH 7.3, at 20°C. Data were averaged over 10 repetitive scans. Protein concentrations were estimated by means of molar extinction coefficient at λ 278 nm, $\varepsilon = 12{,}600$ $M^{-1}\cdot cm^{-1}$. The circular dichroism ($\Delta\varepsilon$) was calculated using a mean residual weight of 108 and expressed as $M^{-1}\cdot cm^{-1}$ units.

2.9. *Computer analyses of protein sequences and protein database*

The secondary structure of MIF was predicted with the SEQPRO program using the CD-constrained procedure [13]. The predicted α-helical segments were analyzed for potential amphipathic character using the Fourier transform procedure [14]. Hydrophobicity profiles were calculated with SEQPRO using a nine-residue window and the Kyte–Doolittle hydrophobicity scale [15]. Hydrophobicity index (H_i) was calculated as a ratio of the amino acid residues being in the hydrophobic segments with respect to their total. The current edition of the MIPSX protein database (rel. 33) containing 61,811 sequences was analyzed with SEQPRO.

3. RESULTS AND DISCUSSION

The isolation procedure of bovine MIF comprises the following three steps. First, there was a general sorting of proteins on a weak anion exchanger. Second, the basic proteins isolated from the first step were fractionated according to their pI values on a chromatofocusing column (see Fig. 1A). Third, the proteins with pI's in the range 10.5–9.2 were further fractionated on a weak cation exchanger to which a salt gradient was applied (see Fig. 1B). We focused our interest on the two proteins that eluted at salt concentrations equal to 0.25 and 0.30 M NaCl (0.3 mg of each protein was purified from 1 kg of dry brain matter). The two proteins are characterized by pI values equal to 9.5 and 9.4, as derived from 2D gel electrophoresis experiments. Both the basic pI and the molecular weights of the two proteins are compatible with those of the 12 kDa recombinant human MIF [8]. The amino acid sequences of the first 39 N-terminal residues are identical in both proteins, and these N-terminal sequences share nearly 95% identity with that of the 12 kDa human recombinant MIF. Only the following three differences were noted between the three sequences. The initiation methionine found in the 12 kDa human recombinant MIF lacks the two bovine proteins which both start by a proline residue. The two

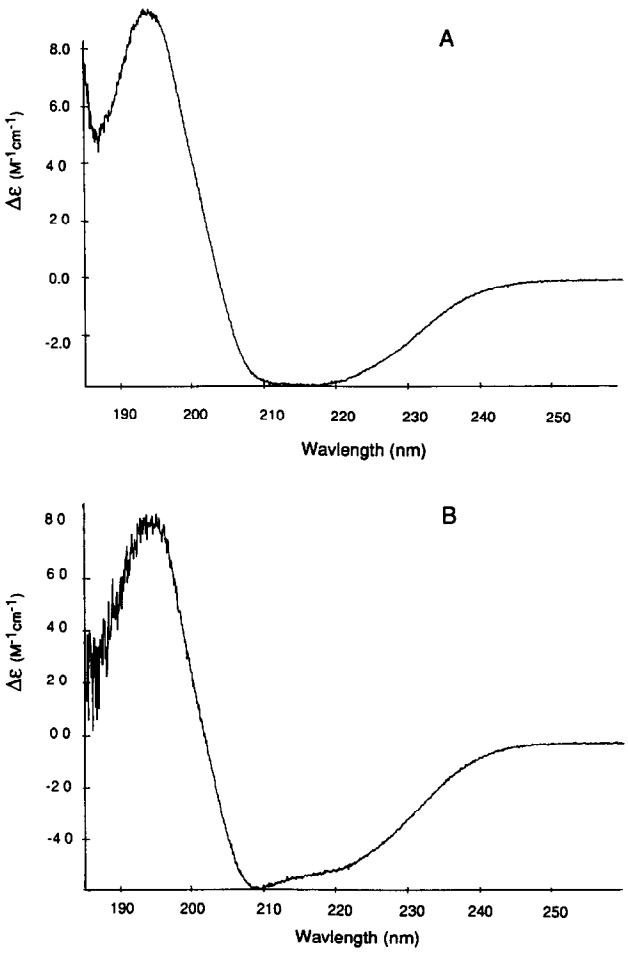

Fig. 3. The far-UV CD spectra of bovine MIF. (A) Isoform with pI 9.5. (B) Isoform with pI 9.4.

(A)

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                *               *
Human    MPMFIVNTNVP RASVPDGFLS ELTQQLAQAT GKPPQYIAV
Bovine    PMFVVNTNVP RASVPDGLLS ELTQQLAQAT GKPPQYIAV
                    10        20        30
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(B)

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         10        20        30        40        50        60
PMFIVNTNVPRASVPDGFLSELTQQLAQATGKPPQYIAVHVVPDQLMAFGGSSEPCALCS
 β<—1—>β   ττττ  α<———1———>α ττττ  β<———2———>βα<2>α  ττττ  ααα
 <——H——>                          <————H————>

         70        80        90        100       110
LHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAASVGWNNSTFA
α<—3—>αττττ   α<———4———>α   β<—3—>β α<-5->α
<——H——> ▲     <————H————>                ▲
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Fig. 2. (A) Comparison of the N-terminal sequences of human [8] and bovine MIF. * Indicates the mutation in the amino acid sequence. (B) The predicted secondary structure of human MIF. Hydrophobic peptide segments are designated by ←H→; α, α-helix; β, β-structure; τ, β-turn; predicted glycosylation sites are indicated by an arrow. The hydrophobic peptide segments correlate well with the two predicted β-strands (β1 and β2) and the α-helices (α2–α4). It is likely that the main feature of the folded protein is its hydrophobic core (β1, β2, α3 and α4) the segments of which are connected by a long hydrophilic loop comprising β-turn–α-helix–β-turn (13–34) and the β-turns (13–16), (52–55), and (72–75).

other differences are clearly conservative substitutions (see Fig. 2A). A search of the current edition of the MIPSX protein database revealed no sequence homology with any of 61,811 proteins sequences except the 12 kDa human recombinant MIF [8]. On the basis of the above evidence, we concluded therefore that the two isolated proteins are the MIF isoforms of bovine brain cytosol.

The far-UV CD spectra of both isoforms of bovine MIF exhibit strong CD bands with minima at about λ 222 nm and λ 208 nm and a maximum at about λ 197 nm (see Fig. 3). The CD-estimated values of α-helix and β-structure were 43% (± 3%) and 21% (± 3%) for the MIF isoform with a pI of 9.5, and 41% (± 3%) and 20% (± 3%) for another MIF isoform with a pI of 9.4. There is a difference in the CD profiles of the MIF isoforms (see Fig. 3) but it remains to be established if this difference is due to a chemical modification of bovine MIF or other factors. The secondary structure of human MIF was predicted using its full amino acid sequence [8] and the CD-constrained algorithm [13] (see Fig. 2B). Human MIF has a substantial hydrophobicity index (H_i = 44.4%) and according to our prediction consists of two hydrophobic β-strands (β1 (2–10) and β2 (36–46)), two amphipathic α-helices (α3 (58–71) and α4 (78–91)), and a hydrophilic α-helix (α4) at the C-terminal end. There are also two consensus sequences for asparagine-linked glycosylation which are in the solvent-exposed parts of the protein, namely (72)-NRS, which forms a β-turn, and (109)-NNS, which lies on the edge of the C-terminal α-helix.

The spectrum of physiological actions of MIF has been partially characterized [1–11] but little is known about its molecular interaction with cellular receptors and its involvement in T-cell activation and other signalling pathways of the immune system. MIF is one of the lymphokines secreted by activated T-cells yet its role in the augmentation of macrophage-dependent killing of tumor cells [10] and the intracellular parasite *Leishmania donovani* [9] remains obscure. Likewise its involvment in the process of chronic inflammation [3,6] and graft vs. host diseases [4,5] remains to be elucidated. The availability of MIF isolated from various mammalian sources should allow for a better illumination of the physiological actions exerted by this lymphokine.

Acknowledgements: We are indebted to Dr. André Menez for encouragement and stimulating discussions.

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