The subunit structure of human macrophage migration inhibitory factor: evidence for a trimer

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The subunit structure of human macrophage migration inhibitory factor (MIF) has been studied by preliminary X-ray analysis of wild-type and selenomethionine-MIF and dynamic light scattering. Crystal form I of MIF belongs to space group P2₁₂₁, and is grown from 2 M ammonium sulfate at pH 8.5. A native data set has been collected to 2.4 Å resolution. Self-rotation studies and Vₘ values indicate that three molecules per asymmetric unit are present. A data set to 2.8 Å resolution has been collected for crystal form II, which belongs to space group P3₂₁, and grows from 2 M ammonium sulfate, 2% polyethylene glycol (average molecular mass 400), 0.1 M HEPES, pH 7.5. Three, four, five or six monomers in the asymmetric unit are consistent with Vₘ values for this crystal form. Analysis of crystal form II containing selenomethionine-MIF indicates nine selenium sites are present per asymmetric unit. Dynamic light scattering of MIF suggests that the major form of the protein in solution is a trimer. The results of these studies are in contrast to previous reports indicating that MIF is a monomer or dimer. The subunit arrangement of MIF is similar to that of tumor necrosis factor and suggests that signal transduction might require trimerization of receptor subunits.

Keywords: crystallization/dynamic light scattering/macrophage migration inhibitory factor/protein structure/X-ray diffraction

Introduction

Macrophage migration inhibitory factor (MIF) was originally identified as a lymphocyte-derived protein that inhibits monocyte migration and was implicated in delayed-type hypersensitivity (Bloom and Bennett, 1966). Subsequently, the pituitary (Bernhagen et al., 1993) and macrophages (Calandra et al., 1994) have been found to be the source of most MIF in the serum. MIF is also produced in the brain and the developing eye (Wistow et al., 1993; Galat et al., 1994). The precise physiological role of MIF is unknown, but most of its in vitro activities are associated with immune system function. MIF induces the production and release of tumor necrosis factor-α and nitric oxide in macrophages (Bernhagen et al., 1994; Calandra et al., 1994). MIF secretion is induced by glucocorticoids. In turn, MIF counter-regulates the actions of glucocorticoids and serves to inhibit the protective effects of glucocorticoids against lethal endotoxemia (Calandra et al., 1995). In vivo studies also support a critical role for MIF in sepsis: co-injection of LPS and MIF in mice potentiates lethality; injection of anti-MIF antibody confers significant protection against LPS-induced death (Bernhagen et al., 1993).

In addition to immune system activities, MIF is also reported to have glutathione S-transferase activity, potentially linking the host's chemical and immunological defense systems (Blocki et al., 1992). Whether MIF is structurally related to other glutathione S-transferases (GST) is controversial. Three observations indicate that these proteins may be related. (1) MIF and other glutathione S-transferases share about 28% sequence identity at the amino terminus (seven out of 25 residues) (Blocki et al., 1992). (2) Glutathione S-transferases have a conserved hydroxyl-containing residue (serine for θ-class GST and tyrosine for α-, π- and μ-class GSTs) at the active site which is involved in the protonation of the glutathione thiol group (Liu et al., 1992). MIF also has a hydroxyl-containing residue (threonine) at the equivalent position (Blocki et al., 1993). (3) Antibodies to MIF are reported to show cross-reactivity with θ-class GST (Blocki et al., 1993). Based on these observations, MIF is proposed to be related to certain classes of glutathione S-transferases (Blocki et al., 1992, 1993). The contention that MIF is related to any of the glutathione S-transferases has been challenged based on the weak sequence similarity along the entire length of the proteins (Pearson, 1994). Furthermore, the MIF polypeptide is only half the size of GST. Circular dichroism and NMR experiments as well as secondary structure predictions based on the primary sequence indicate MIF has a low helix content (Bemhagen et al., 1994; Nishihira et al., 1995). In contrast, the three-dimensional structures of a number of glutathione S-transferases indicate they have a high helix content (Ji et al., 1992; Reinemeyer et al., 1991, 1992).

The subunit structure of MIF has been the subject of interest and may be important for understanding the mechanism of receptor-induced signal transduction. Gel filtration experiments indicate that MIF exists as a monomer (Blocki et al., 1992; Galat et al., 1994) or dimer (Zeng et al., 1993). Analytical ultracentrifugation of rat liver MIF indicates that the protein is a dimer (Nishihira et al., 1995). In contrast, the three-dimensional structures of a number of glutathione S-transferases indicate they have a high helix content (Ji et al., 1992; Reinemeyer et al., 1991, 1992).

Materials and methods

Expression and purification of recombinant MIF

The cloning of human MIF into the IPTG-inducible expression plasmid pET11b has already been described (Bernhagen et al., 1994). BL21(DE3) cells grown to an OD₆₀₀ of 0.5–1.0 were induced by addition of IPTG to a final concentration of 0.4 mM. After a 4 h growth, the cells were harvested and resuspended in 20 mM NaCl, 20 mM Tris, pH 7.5, at 4% of the original volume of the growth media. Cells were lysed using a French Press and centrifuged at 100 000 g for 30 min to remove cell debris. The supernatant was sequentially applied over 5/10 Mono Q (Pharmacia) anion-exchange and Mono S
Amino acid analysis and mass spectrometry

Amino acid analysis was performed after hydrolysis of 3 µg of MIF in 6 M HCl with 0.2% phenol at 115 °C in vacuum-sealed tubes for 16 h. Amino acids were analyzed on a Beckman (Model 7300) automatic amino acid analyzer. N-Terminal amino acid sequences were determined by automated Edman degradation using an Applied Biosystems Model 470A gas-phase sequencer. For mass spectrometry, MIF and [Se-Met]-MIF were further purified by HPLC using a Vydac protein C4 column and eluted with a gradient of 30-80% acetonitrile in 0.1% trifluoroacetic acid. Samples were diluted into 2-propanol and water (1:1, v/v) with 0.1% formic acid and analyzed on a VG Quattro (Fisons Instruments) triple quadrupole mass spectrometer equipped with an electrospray ion source. The mass spectra of MIF and [Se-Met]-MIF were further purified by HPLC using a Vydec protein C4 column and eluted with a gradient of 30-80% acetonitrile in 0.1% trifluoroacetic acid. 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Macrophage migration inhibitory factor is a trimer.

Fig. 2. Mass spectra of (A) recombinant MIF and (B) [Se-Met]-MIF.

belong to $P3_121$ (or its enantiomorph) with unit cell dimensions $a = 96.70 \, \text{Å}$, $c = 106.14 \, \text{Å}$. A 2.8 Å resolution data set was collected from three crystals of this crystal form. The $R_{\text{merge}}$ for 35 726 measured reflections of 13 512 unique reflections (92% complete) is 7.1%. Three, four, five or six monomers per asymmetric unit result in $V_m$ values that are within the typical range for protein crystals.

Crystals of [Se-Met]-MIF could only be grown in the
Table I. Individual dynamic light scattering measurements

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<th>Measurement No.</th>
<th>Amplitude (nm)</th>
<th>Diffusion coefficient (10^{-13} \text{ m}^2/\text{s})</th>
<th>Radius (nm)</th>
<th>Polydispersity (nm)</th>
<th>Temp. (°C)</th>
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<tr>
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<td>0.797</td>
<td>25.2</td>
<td>1.003</td>
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</table>

KAPPA =120

Fig. 3. The self-rotation function (ϕ = 120°) showing the threefold non-crystallographic axis at φ = 55, ψ = 135 (or any of its symmetry mates). The self-rotation function is calculated using the program MERLOT (Fitzgerald, 1988) with data from 10 to 4 Å having intensities greater than 2σ and a Patterson cut-off radius of 23.0 Å. The map is contoured starting at 0.5σ with 0.5σ increments, σ being the root mean square value of the rotation function.

presence of 2% PEG 400 and belong to the trigonal crystal form. These crystals are usually smaller (0.4×0.3×0.3 mm) than wild-type crystals but diffract to higher resolution. A full data set to 2.6 Å resolution with an R_{merge} of 5.1% was collected. The mean isomorphous difference of these data was 11%. Difference Patterson maps were calculated using the PHASIT package (W.Furey) but could not be used to solve the positions of the selenium atoms. Direct methods using SHELXS (Sheldrick, 1990) with data from 10 to 5 Å revealed nine sites having peak heights that gradually decreased to 68% of the maximum. The peak height of the tenth potential site decreased dramatically to 43% of the maximum, suggesting that nine selenium atoms are present per asymmetric unit. These sites were refined and are shown in Table II. The validity of the positions was confirmed by checking the predicted self- and cross-vectors against the Patterson map. Difference maps calculated with single isomorphous replacement (SIR) were also utilized. Sets of two or three selenium sites were deleted prior to calculating SIR phases and difference maps. Peaks always appeared corresponding to the positions of the deleted atoms for all nine sites. Based on amino acid analysis showing that three methionine residues are present in a single polypeptide chain of purified MIF, these data indicate that three monomers are present per asymmetric unit of the trigonal crystal form.

Table II. Fractional coordinates of the selenium atoms in the trigonal crystal form of MIF

<table>
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<th>Site</th>
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<th>Y</th>
<th>Z</th>
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<td>0.127</td>
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<tr>
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<td>0.178</td>
<td>0.152</td>
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<tr>
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<td>0.099</td>
<td>0.072</td>
</tr>
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<td>0.184</td>
<td>0.094</td>
</tr>
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<td>9</td>
<td>0.407</td>
<td>0.571</td>
<td>0.136</td>
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Dynamic light scattering

In light of the presence of three monomers in the asymmetric unit of both space groups we sought to determine the subunit structure of MIF in solution. Based on the standard size-weight relationship typical of globular proteins, the hydrodynamic radius indicates that the size of MIF is 30-40 kDa, consistent with a trimeric structure for MIF (Table I).

Discussion

The oligomeric structure of cytokines can provide clues as to the mechanism of receptor activation at the cell surface. It is
important to note, however, that the functional form of a cytokine may not correspond to the experimentally observed form, as structure determinations are done at higher concentrations than those required for biological activity. It can be argued that at these high concentrations, mass action is responsible for subunit–subunit interactions which would normally not occur at biologically effective concentrations. Notwithstanding this caution, tumor necrosis factor (TNF) and interferon-γ (IFN-γ) are two prime examples which illustrate that the oligomeric state of the uncomplexed protein is directly related to the mechanism of receptor activation. Tumor necrosis factor was found to be a trimer in solution and in the crystal (Smith and Baglioni, 1987). The determination of the threedimensional structure of the TNF–TNF receptor complex clearly demonstrates that the TNF trimer induces trimerization of its receptor (Banner et al., 1993). Likewise, uncomplexed interferon-γ exists in solution and in the crystal as a dimer (Samudi et al., 1991; Grzesieki et al., 1992). The structure of the IFN-γ–IFN receptor complex shows that the IFN-γ dimer leads to receptor dimerization and activation at the cell surface (Walter et al., 1995).

Previous studies using either gel filtration chromatography or equilibrium sedimentation experiments of rat, bovine, and human MIF demonstrate that the protein exists as a monomer or a dimer (Blocki et al., 1992; Zeng et al., 1993; Galat et al., 1994; Nishihiara et al., 1995). In a preliminary crystallization study, MIF is assumed to form a dimer and it is concluded that two or three dimers are present in the trigonal crystal form and two dimers are present in the orthorhombic crystal form (Suzuki et al., 1994). Our analysis based on dynamic light scattering, self-rotation studies of the orthorhobic crystal form and determination of selenium sites in the trigonal crystal of [Se-Met]-MIF indicates that human MIF exists as a trimer. It is possible that the high protein concentrations involved in crystallization and dynamic light scattering relative to the reported gel filtration experiments may provide an explanation for the different conclusions. However, it is difficult to rationalize the discrepancy between our conclusions with those of the analytical ultracentrifugation study (Nishihiara et al., 1995). The determination of the solution and crystal structure of MIF should resolve questions regarding its subunit arrangement and address structure–function relationships of the protein considered to be the first lymphokine ever characterized. The determination of the crystal structure by isomorphous replacement is in progress. The presence of a non-crystallographic threefold axis will allow molecular averaging to be used for structure determination.

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References


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