Cross-linking and mutational analysis of the oligomerization state of the cytokine macrophage migration inhibitory factor (MIF)

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Received 12 February 1998; revised version received 24 March 1998

Abstract The structure of the cytokine MIF has been investigated by X-ray crystallography, NMR, and biochemical methods with conflicting results regarding the structural and functional oligomerization state of this protein. Determination of the oligomeric state(s) is important for understanding more precisely the molecular mechanism of MIF action. To address this issue, we performed cross-linking of human and mouse MIF and selected mutants by various methods and analyzed the oligomerization by SDS-PAGE and gel filtration. MIF was found to form a mixture of monomeric, dimeric, and trimeric states at physiological concentrations, with the monomer and dimer representing the major species. Similar results were obtained when the carboxy-truncated mutants MIF(1–104) and MIF(1–109) were examined, indicating that the C-terminus of MIF is not critical for trimer stabilization. Cross-linking analysis of the isosteric Cys→Ser mutants C56S and C80S of human MIF resulted in a similar oligomer distribution, whereas substitution of Cys^{59} led to a significant reduction in the dimeric and trimeric forms, indicating that the hydrophobic region around Cys^{59} is important for the oligomerization of MIF. Together, our data argue that physiological MIF solutions contain a mixture of monomers, dimers, and trimers.

Key words: Macrophage migration inhibitory factor; Cytokine; Protein structure; Oligomerization; Cross-linking; Dimer

1. Introduction

The biological role of the cytokine macrophage migration inhibitory factor (MIF) was recently elucidated. MIF is a pituitary hormone, T cell cytokine, and macrophage-derived factor with a pro-inflammatory spectrum of activities [1–4]. MIF was found to be unique in counter-regulating the immuno-suppressive and anti-inflammatory effects of glucocorticoid hormones [5]. A central regulatory role for MIF within the host immune and inflammatory response was demonstrated when MIF was found to play a role in a number of pathologic conditions such as septic shock, physiological stress, or certain immune-mediated diseases [1,5–8]. MIF is exceptional in acting by (additional) enzymatic functions [9–11].

X-ray crystallographic analysis has suggested that MIF is a trimer of identical subunits that are characterized by an extended β-sheet and 2 antiparallel α-helices [12–14]. The subunits interact to form a symmetrical trimer with the β-sheets forming a central channel of unknown function. However, reports about the oligomerization state of MIF have been contradictory and have varied with the techniques applied. In addition to the X-ray analyses which suggested that MIF is a trimer, NMR and biochemical methods have been used. NMR analysis of human MIF was consistent with the formation of a MIF dimer [15]. Analytical ultracentrifugation of rat MIF showed the formation of a dimer [16], while reports applying gel filtration suggested that MIF is a dimer (human MIF) [17] or a monomer (bovine MIF) [18]. A study involving SDS-PAGE of cross-linked mouse MIF indicated that MIF forms a trimer [19] and is contrast to the other reports that have investigated the oligomerization state of MIF under solution conditions. Despite these various reports, no detailed study of the oligomerization state of MIF has been performed.

The C-terminal sequence stretch of MIF has been suggested to be important for trimer stabilization [12–14] and thus would be a good target region for mutational analyses and investigating the oligomerization state of MIF. We recently examined the role of the C-terminal residues for the conformation, conformational stability, and biological activity of MIF [20]. Using two carboxy-truncated mutants of human MIF with five and ten residues deleted, we found that the overall protein structure was not influenced by the C-terminus. Although carboxy-truncated MIF showed a somewhat decreased conformational stability, the enzymatic activity of the mutants was significantly retained. However, the mutants did not show any remaining macrophage-activating effects. Overall, these data were consistent with the notion that the trimer may not be the only biologically active form of MIF. Oligomerization of MIF by covalent disulfide formation has essentially been ruled out by electrophoretic and gel filtration experiments [4], but no mutational analysis has been performed to confirm this notion.

To fully characterize the structural and functional oligomerization state of MIF under physiological conditions, we analyzed the size of native MIF by gel filtration and performed cross-linking of biologically active human MIF (wtMIF) using different cross-linking methods. Various solutions containing physiological and experimental concentrations of MIF were tested and the cross-linked proteins analyzed by SDS-PAGE and gel filtration. Cross-linking analysis then was applied to the carboxy-truncated mutants MIF(1–104) and MIF(1–109) that lack the putative trimer-stabilizing C-terminal region of MIF. Moreover, to confirm the non-covalent character of any inter-subunit interactions and to examine the role of the hydrophobic region around Cys^{59}, various cysteine mutants of human MIF were subjected to the cross-linking analysis.

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Abbreviations: MIF, macrophage migration inhibitory factor; wt, wild-type; huMIF, human MIF

PII S0014-5793(98)00400-1

2. Materials and methods

2.1. Materials

Miscellaneous chemicals and enzymes were bought from Sigma-Aldrich Chemicals (Deisenhofen, Germany) and were of the highest grade commercially available. All molecular biology reagents including primers for polymerase chain reaction-based cloning of wtMIF and the mutants were acquired from Gibco BRL (Eggenstein, Germany) or New England Biolabs GmbH (Heidelberg, Germany). Bis-[β-(4-azidosalicylamindo)ethyl]disulfide (BASED) was obtained from Pierce (Rockford, IL).

2.2. Site-directed mutagenesis, cloning, bacterial expression, and purification of wtMIF and the mutants

Human MIF was amplified originally from Jurkat H33HJ-JA1 T cell DNA and cloned into the pET11b expression vector [4]. The C-terminal deletion mutants and the isosteric Cys→Ser mutants of human MIF were cloned from huMIF/pET11b by DNA amplification as described elsewhere ([20], Kleemann et al., manuscript submitted).

Bacterial expression and purification of wtMIF and the mutants is described in detail elsewhere ([4], Kleemann et al., manuscript submitted, [20]). Briefly, wtMIF, the carboxy-truncated mutants, and the Cys→Ser mutants (except for mutant C59S) were expressed and purified essentially following the established protocol for wtMIF [4], using isopropyl-1-thio-β-D-galactopyranoside-induced overexpression of proteins in pET11b/*E. coli* BL21(DE3) and purification of recombinant protein by the anion exchange and C8-SepPak reverse-phase two-step chromatography procedure, and renaturation of acetonitrile-denatured protein by controlled dialysis. Purification of mutant C59S was performed by a separate method involving extraction of the protein from inclusion bodies, gel filtration, and C8 reverse-phase chromatography (described in detail elsewhere; Kleemann et al., manuscript submitted).

2.3. Protein cross-linking

Biological activity of the renatured recombinant proteins was confirmed prior to the cross-linking experiments using an enzyme or macrophage-activating bioassay [20].

Cross-linking with glutaraldehyde: Wild-type or mutant MIF protein at the indicated concentrations was dissolved in 20 mM sodium phosphate buffer (pH 7.2) and incubated for 3 h in the presence of 1% glutaraldehyde [21]. The reaction was stopped by adding $NaBH_4$ (2 M in 0.1 M NaOH) to a final concentration of 50 mM and the cross-linked products stabilized. After 20 min, sodium deoxycholate was added (final concentration of 0.01%) and the pH of the mixture lowered to 2.0 with an aqueous 78% trichloroacetic acid (TCA) solution. After centrifugation (20 min, 13 000×*g*, 4°C), products were neutralized with ammonium hydroxide, boiled in Laemmli sample buffer, and analyzed by SDS-PAGE under reducing conditions. For examining concentration dependency of oligomer formation, cross-linking was performed between a concentration range of 0.1 μM and 18 μM MIF protein. Additional lower (<0.1 μM) and higher (>20 μM) concentrations of MIF were not investigated due to experimental difficulties pertaining to the handling of large volumes at low protein concentrations and insolubility of the renatured MIF, respectively. Cross-linking efficiency and electrophoretic behavior of cross-linked oligomers was controlled by using the trimeric cytokine tumor necrosis factor-α (concentration: 1 μM). By this standard, a >95% cross-linking efficiency was achieved.

Cross-linking with Bis-[β-(4-azidosalicylamindo)ethyl]disulfide (BASED): BASED was dissolved in DMSO and added to the MIF-containing solution (final BASED concentration: 0.6 mg/ml). Photoactivation was performed by exposing the sample to long wave UV light (366 nm; room temperature) for 30 min at a distance of 3 cm. Cross-linked samples were TCA-precipitated as described above and electrophoresed under non-reducing conditions.

2.4. Gel filtration HPLC

Native size exclusion chromatography was performed on a Tosohaas TSK G2000SWXL HPLC column (300×7.8 mm, Tosohaas, Stuttgart, Germany) at a flow rate of 0.25 ml/min using a 50 mM sodium phosphate buffer (pH 7.2). For denaturing gel filtration, a 50 mM sodium phosphate buffer (pH 7.2) containing 7 M guanidine hydrochloride (GdnHCl) was used.

2.5. SDS-PAGE

For SDS-PAGE, cross-linked proteins were electrophoresed in 20% homogenous polyacrylamide gels using the Pharmacia PhastGel electrophoresis system (Pharmacia, Upsala, Sweden). The gels were silver-stained according to the manufacturer's protocol, scanned, and the relative quantities of the monomeric and oligomeric forms estimated by densitometry analysis with the program ScionImageNT (Scion Corp., Frederick, MD). It should be noted that in normal SDS-PAGE analyses of MIF, a faint non-covalent dimer band is frequently observed and is likely to result from non-covalent hydrophobic interactions between subunits.

3. Results and discussion

To begin to characterize the oligomerization state(s) of MIF, biologically active MIF was subjected to gel filtration chromatography under non-denaturing conditions. Confirming previous reports using native methods for size determination [16,17], we found that native recombinant human or mouse MIF chromatographed as a dimer (apparent molecular mass = 27 000 Da; data not shown). By contrast, acetonitrile-denatured MIF eluted at a volume that corresponded to the monomeric species (apparent molecular mass = 13 000 Da). Of note, analysis of the renatured recombinant proteins is likely to fully represent the behavior of the natural eukaryotic MIF as MIF is not secreted by endoplasmatic reticulum-mediated

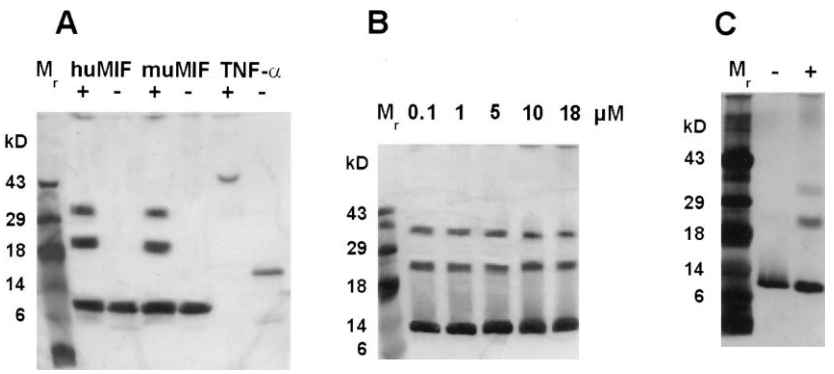

Fig. 1. SDS-PAGE and silver staining of wtMIF after cross-linking. A: Comparison of wild-type human (huMIF) and mouse MIF (muMIF) with TNF-α. Cross-linking was performed with glutaraldehyde at a protein concentration of 1 μM. Control reactions without cross-linker were analyzed for comparison. B: Dependence of oligomerization on the protein concentration during cross-linking with glutaraldehyde. Wild-type MIF at the indicated concentrations was cross-linked. C: Cross-linking with the photo-cross-linker BASED. Wild-type human MIF at a concentration of 1 μM was cross-linked with BASED and electrophoresed. Molecular weight markers (M_r) are indicated. +, sample cross-linked prior to electrophoresis; −, non-cross-linked control.

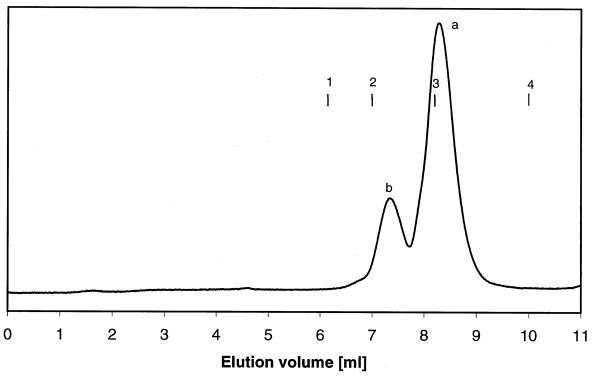

Fig. 2. Gel filtration HPLC analysis of cross-linked wild-type human MIF. The column was calibrated with standard proteins as described in Section 2. After cross-linking with glutaraldehyde, MIF was chromatographed under denaturing conditions. The elution volumes of the marker proteins (1, bovine serum albumin; 2, carbonic anhydrase; 3, cytochrom c; 4, aprotinin) are indicated. a, peak eluted at ca. 12 400 Da; b, peak eluted at ca. 27 000 Da.

pathways and thus is not glycosylated. The latter modification is known otherwise to interfere with protein-protein interactions and association of monomers. Moreover, it is widely established that the recombinant MIF protein exhibits full natural-like biological activity [1–5,20]. In native gel filtration, interference of the supporting matrix with subunit interactions is not excluded. Therefore, we used a principle covalent cross-linking approach in conjunction with denaturing conditions to circumvent this potential problem. First, biologically active wtMIF was dissolved at physiological concentrations and the protein subjected to chemical cross-linking by glutaraldehyde. MIF was used at a concentration of 1 μM in 20 mM sodium phosphate buffer (pH 7.2). Fig. 1A is a SDS-PAGE of cross-linked human wtMIF in comparison to the non-cross-linked protein. Cross-linked wtMIF was found to consist of a mixture of monomeric, dimeric, and trimeric forms, whereas only monomeric MIF was detected when MIF was electrophoresed without prior cross-linking. Analysis of mouse MIF resulted in an identical oligomer distribution. Estimation of the relative densities of the protein bands of human MIF showed that 44% migrated as monomer and 33% of the protein formed dimers, whereas only 23% trimers were seen. Under the conditions used, MIF exhibits full cytokine and enzymatic activity [2,4,20]. Of note, baseline serum concentrations of MIF readily rise into the low micromolar range upon immune stimulation of experimental animals and similar concentrations have been measured in the synovium of mice that were manipulated to develop rheumatoid arthritis [22], suggesting together that the applied low micromolar concentrations, although unusual for a cytokine, represent physiological conditions.

The observed detection of MIF monomers may represent correctly the physiological oligomer distribution, but may also be due to incomplete cross-linking. Thus to better control for a maximal cross-linking efficiency, the cytokine TNF-α which has a similar molecular mass as MIF and which is known to form non-covalently linked homo-trimers in solution [23], was treated under the same conditions and used as a control. As expected, cross-linked TNF-α was found to form >95% trimers and non-cross-linked TNF-α migrated as a monomer (Fig. 1A). It would thus appear that detection of monomeric MIF in the cross-linked reactions represents a true association state.

One factor that influences subunit association of oligomeric proteins is the protein concentration and frequently, structural studies are not performed at physiological concentrations. In particular, X-ray crystallography and NMR require much higher protein concentrations (about 1 mM) than those physiologically found for MIF, i.e. in the low and submicromolar range [2,4,20]. To address this potential problem, we performed cross-linking of MIF over a broad range of protein concentrations spanning both physiological concentrations (0.1 to 1 μM) and higher experimental concentrations (1 to 18 μM). Renatured recombinant MIF exhibiting biological activity is not soluble above a concentration of 20–25 μM and therefore, no higher concentrations were tried to avoid non-specific subunit interaction. No concentration-dependent differences were observed over the concentration range tested when cross-linking of these preparations was performed with 1% glutaraldehyde and all reactions were found to contain an identical oligomer distribution (Fig. 1B). This indicated that MIF exists in the same assembly state within the tested concentration range. As additional lower and higher MIF concentrations could not be tested in this experiment due to experimental difficulties (see Section 2.3), we cannot rule out that concentration-dependent effects would be observed at these concentrations. In fact, one would expect that further dilution of the MIF solution may result in the ultimate dissociation of oligomers into the monomeric species.

One criticism that is often leveled upon chemical cross-linking analyses using glutaraldehyde is the occurrence of artefactual protein subunit linkage due to glutaraldehyde autopolymerization. The above examination of the concentration dependency of the cross-linking of MIF already indicated that the observed oligomer distribution pattern was specific. Moreover, occurrence of a mixture of monomeric, dimeric, and trimeric forms in physiological MIF solutions was confirmed using another cross-linking agent, BASED. Cross-linking with BASED and the subsequent electrophoretic analysis resulted in a similar distribution of oligomers as seen in the glutaraldehyde cross-linking reactions, with 59% monomer, 29% dimer, and 12% trimer (Fig. 1C). Cross-linking yields with photo-reactive reagents such as BASED have been found to be low for many proteins [24]. Thus, it was not unexpected that the oligomer distribution was shifted somewhat towards the monomer in the BASED cross-linking experiment. Despite

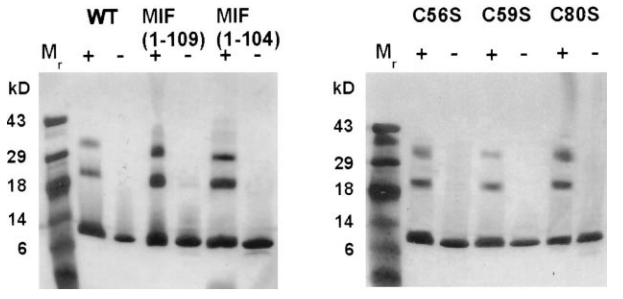

Fig. 3. SDS-PAGE and silver staining of the carboxy-truncated and Cys→Ser mutants of MIF after cross-linking with glutaraldehyde. Mutant proteins at a concentration of 1 μM were cross-linked with glutaraldehyde and samples electrophoresed as indicated. Wild-type MIF and non-cross-linked samples were analyzed for comparison. Molecular weight markers (M_r) are indicated. +, sample cross-linked prior to electrophoresis; −, non-cross-linked control.

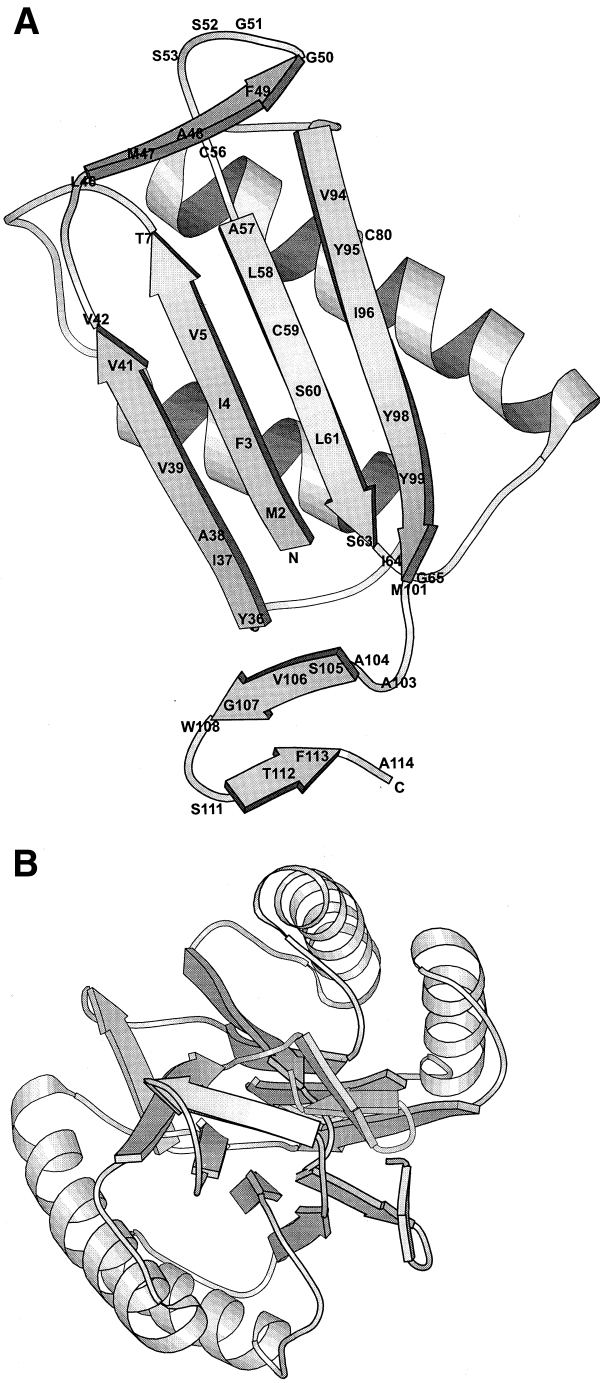

Fig. 4. Three-dimensional structure of human MIF. A: Ribbon structure of the MIF monomer. Hydrophobic residues on the β-sheet side are marked. The hydrophobic character of the residues was determined following to the scale of Wolfenden et al. [43]. The positions of the cysteines are indicated by the notations C56, C59, and C80. B: Proposed model of the MIF dimer. Figures were prepared using the program MOLSCRIPT [44].

this disadvantage, cross-linking with BASED represented an important control, because unlike most protein cross-linking agents, the BASED reaction does not rely upon the amino side chains of lysine and arginine. Cross-linking by BASED is based upon non-specific photo-activation and thus, is not restricted by amino side chain distances and steric hindrances. In fact, we were unable to achieve any cross-linking of MIF with the prominent cross-linking agent disuccinimidyl suberate (DSS) (data not shown). Inspection of the inter-amino side chain distances of lysine and arginine residues in both monomeric MIF and the dimeric and trimeric forms showed that the spacer length of this chemical was considerably smaller than the distances between the amino groups (Fig. 4).

Gel filtration analysis under denaturing conditions, i.e. in the presence of 7 M guanidine hydrochloride, of cross-linked MIF was then used to confirm the molecular masses and relative portions of the MIF oligomers. Fig. 2 shows the elution profile of a 25 μg MIF sample that was cross-linked with 1% glutaraldehyde at a protein concentration of 1 μM prior to chromatography. Two protein fractions were detected and found to have apparent molecular masses of 27 000 and 12 400, thus corresponding to dimeric and monomeric MIF, respectively. Of note, significant portions of trimeric MIF were not detected by the gel filtration analysis. The failure of the denaturing gel filtration analysis to detect trimers while this species was readily seen in the SDS-PAGE method is not obvious. However, it may be speculated that the trimeric species tends to aggregate more easily than dimeric or monomeric MIF and that aggregates would then be lost by non-specific binding to the resin. Although unusual, such a behavior has been reported for amyloidogenic proteins/peptides and may also occur under denaturing chromatography conditions [25,26].

Together, electrophoretic and gel filtration analysis of the various cross-linked wtMIF preparations suggested that physiological MIF solutions consist of a mixture of monomeric, dimeric, and trimeric association states. Surprisingly, the trimer, which was determined by X-ray crystallography to be the predominant (or only) species, was found to represent only a minor portion in physiological MIF solutions.

The carboxy-terminal region of MIF has been implicated in the stabilization of trimer formation [13,14]. To examine the contribution of the C-terminal region for oligomer formation and to further investigate the physiological occurrence of MIF trimers, we applied the described glutaraldehyde cross-linking procedure to the C-terminal deletion mutants MIF(1–104) and MIF(1–109). Overall, oligomerization of MIF was found to be altered only slightly by the deletion of either the five or ten C-terminal residues (Fig. 3). However, a more detailed analysis by densitometric scanning showed that MIF(1–109) and MIF(1–104) formed 33% and 32% dimers, and 27% and 29% trimers, respectively, with the remainder consisting of monomers. Thus, contrary to the predicted trimer-stabilizing effect of the C-terminal region, trimer formation was found to be elevated in the mutant preparations. The observed failure of both deletion mutants to activate macrophages in the *Leishmania* killing assay [20] may therefore not be due to reduced trimer formation of these mutants. Consequently, the prior conclusion that the immunological effects of MIF were mediated by MIF trimers and that the trimer may be the receptor-active species may not be correct. Based on data measured by X-ray crystallography, it had been suggested that two or three C-terminal β-strands were important for the stabilization of the MIF trimer [12,14]. We previously found that deletion of the C-terminus led to a decrease in the conformational stability of MIF [20], an observation that was consistent with the trimer-stabilizing role of the C-terminus. However, the present results would indicate that

conformational stabilization of MIF is not critically dependent on the formation of trimers.

Cross-linking of the isosteric cysteine mutants C56S and C80S resulted in the same oligomer distribution as that seen for wtMIF (26 and 29% dimer, and 18 and 24% trimer, respectively) (Fig. 3). This finding confirmed earlier data which had indicated that subunit association of MIF is not mediated by covalent, intermolecular disulfide bridging [4,11]. Moreover, these data are consistent with recent findings that the measured disulfides of MIF [11,27] represent intramolecular disulfide bonding and that these disulfides are of functional rather than structural character [11]. By contrast, mutant C59S behaved differently and was found to form a significantly decreased portion of trimers (27% dimer and 10% trimer). X-ray crystallography of MIF shows that both cysteines of the Cys^{56}-Ala-Leu-Cys^{59} motif are located in a highly hydrophobic environment. Cys^{59} is closely surrounded by Ile^{4}, Leu^{58}, Tyr^{95} and Ile^{96} with additional hydrophobic residues in near proximity [14] (Fig. 4A). Substitution of Cys^{59} by Ser is likely to lower the hydrophobicity of this region, thus providing a smaller area for hydrophobic interactions. Interestingly, disturbance of the hydrophobic character of this region by modification of Cys^{59} ([28], R. Kleemann, unpublished observations) has been proposed to be responsible for a local conformational change together with a significant change in the biological activity of MIF.

Our results provide evidence that MIF forms a mixture of monomers, dimers, and trimers in physiological solutions, with the monomer and the dimer representing the predominant species. The trimer is only a minor species. This finding was confirmed by our mutational analysis of the C-terminal region of MIF. This region had been suggested by the X-ray crystallographic analyses to stabilize trimer formation, but cross-linking analysis of our C-terminal deletion mutants showed increased rather than decreased trimer formation. Thus, the present data are not consistent with the X-ray crystallographic results. X-ray crystallography is surely the most prominent method for determining protein structures. However, although no simple packing due to mass action of subunits was observed [14] with this method for MIF, one may argue that the high protein concentrations used in crystallography, i.e. approximately 1 mM, do not represent physiological concentrations. At high protein concentrations, a given equilibrium mixture of different oligomeric states could then be shifted towards the larger association state, resulting in crystallization of this form. Dependence of the association state of oligomeric cytokines on protein concentration has been shown for the proteins interleukin-8 (IL-8), monocyte chemoattractant protein (MCP-1), I-309, and neutrophil-activating peptide 2 [29–31]. Also, IL-8 has been shown to form different oligomeric states at physiological concentrations versus the crystallographic or NMR conditions. Under physiological solution conditions, IL-8 is monomeric and this form is biologically active [29,32–34]. In contrast, the available crystal and NMR structures of IL-8 show that this protein is a dimer [35,36]. Incidentally, the MIF monomer is somewhat reminiscent of the IL-8 dimer [14]. MCP-1 was shown to form both monomeric and dimeric crystals and it has not been resolved finally whether it functions as a monomer or dimer in solution [29,30,37,38]. For other cytokines, it has been shown that their oligomeric state in solution and in the crystal are identical. For example, TNF-α and interferon-γ form a trimer and dimer, respectively [39–41], regardless of the method applied. Furthermore, the oligomeric state relates directly to the mode of activation of the receptor of these cytokines. The TNF-α trimer was found to induce receptor trimerization and the interferon-γ dimer leads to receptor dimerization [39,42]. To date, no receptor has been identified for MIF.

We suggest that in solution MIF oligomer formation may be determined by the protein concentration or by signaling events such as receptor activation or substrate binding that may favor one oligomeric state. Our data indicate that the favored oligomer might well be the dimer or the monomer. A possible arrangement of subunits in the dimer is depicted in Fig. 4B. In this model, several hydrophobic residues located in the four-stranded β-sheet of each subunit plus in the interconnecting loops, together forming a hydrophobic area (Fig. 4A), would provide for inter-subunit interactions within the dimer (Fig. 4B).

However, from the data it may not be fully excluded that putative receptor activation by MIF occurs from the minor trimeric state. Further mutational analyses evaluating the contact sites between subunits in the dimer and trimer are in progress and should assist in obtaining the specific oligomers in soluble form to selectively measure their biological activities.

Acknowledgements: We are thankful to G. Tolle and O. Flieger for technical assistance with the expression and purification of wtMIF and the mutants. We thank A. Kapurniotu, J. Pleiß, and R. Bucala for helpful discussions. J.B. is supported by the Deutsche Forschungsgemeinschaft (DFG) grant number BE 1977/1-1.

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