

A study of intermediates involved in the folding pathway for recombinant human macrophage colony-stimulating factor (M-CSF): Evidence for two distinct folding pathways

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Abstract

The folding pathway for a 150-amino acid recombinant form of the dimeric cytokine human macrophage colony-stimulating factor (M-CSF) has been studied. All 14 cysteine residues in the biologically active homodimer are involved in disulfide linkages. The structural characteristics of folding intermediates blocked with iodoacetamide reveal a rapid formation of a small amount of a non-native dimeric intermediate species followed by a slow progression via both monomeric and dimeric intermediates to the native dimer. The transition from monomer to fully folded dimer is complete within 25 h at room temperature at pH 9.0. The blocked intermediates are stable under conditions of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and thus represent various dimeric and folded monomeric species of the protein with different numbers of disulfide bridges. Peptide mapping and electrospray ionization mass spectrometry revealed that a folded monomeric species of M-CSF contained three of the four native disulfide bridges, and this folded monomer also showed some biological activity in a cell-based assay. The results presented here strongly suggest that M-CSF can fold via two different pathways, one involving monomeric intermediates and another involving only dimeric intermediates.

Keywords: biological activity; disulfide structure; folding intermediates; mass spectrometry; peptide mapping; protein folding pathways

The study of protein folding is an important area both from the standpoint of understanding fundamental structure–function relationships and from the standpoint of the production of clinically and commercially important therapeutic products (reviewed by Kim & Baldwin, 1990). A particularly difficult aspect in the study of protein folding is the fact that intermediates may be short-lived and therefore hard to isolate and analyze structurally and functionally. Disulfide-containing proteins provide an opportunity to study the molecular structure of covalently blocked, stable intermediate forms. This type of study has been done most intensively on bovine pancreatic trypsin inhibitor (BPTI), for which a detailed folding pathway

has been described by Creighton and his coworkers (e.g., Creighton & Goldenberg, 1984) and more recently by Weissman and Kim (1991). Lu et al. (1992) recently described the folding of a therapeutically important cytokine, human granulocyte colony-stimulating factor (G-CSF), a monomeric protein containing two disulfide bridges in its native form. These workers isolated, refolded, and analyzed different forms of G-CSF with various disulfide structures along with mutant forms of the protein.

The present paper focuses on another cytokine, human macrophage colony-stimulating factor (M-CSF). M-CSF (also known as CSF-1) has the ability to stimulate the growth of macrophage colonies in *in vitro* bone marrow assays (Stanley & Guilbert, 1981) and is known to exert its biological activity through the *c-fms* receptor (Sherr, 1990). Unlike G-CSF, native M-CSF is a homodimer.

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Like G-CSF, the biological activity of M-CSF is sensitive to reduction of internal disulfide bridges. The M-CSF molecule described here was produced as an insoluble 150-amino acid protein in recombinant *Escherichia coli* and was refolded into its native, biologically active form using simple air oxidation at pH 9. We show here that all 14 cysteines in this truncated version of M-CSF are involved in disulfide bridges and that the disulfides appear to form in a stepwise manner. The results presented here suggest that M-CSF can fold via more than one pathway and that intermediates in the folding pathway can exhibit biological activity in the absence of completely formed disulfide structure.

Results

In order to study the mechanism of M-CSF folding, a purified sample of M-CSF dimer was reduced and denatured and allowed to refold as described in the Materials and methods. The progress of the refolding reaction was followed by trapping samples at various times by adding iodoacetamide to a final concentration of 10 or 50 mM

(identical results were obtained at either concentration; see Discussion). As shown in Figure 1, dimer formation (measured by anion exchange high performance liquid chromatography [HPLC]) requires approximately 6–10 h to reach a steady-state level under the conditions used in these experiments. The increase in biological activity paralleled that of dimer formation, as measured by the ability of the solutions to stimulate growth of an M-CSF-dependent cell line (NFS-60; Nakoinz et al., 1990). These results are similar to those found by Halenbeck et al. (1989) for a 218-amino acid version of M-CSF. In another set of samples, the incorporation of ^{14}C from ^{14}C -labeled iodoacetamide into trichloroacetic acid (TCA)-precipitable material was measured in order to assess the availability of free sulfhydryl groups in the protein during refolding. As shown in Figure 1, a rapid loss of available free SH groups occurred (approximately 50% of the initial incorporation of ^{14}C into the monomer) during the first 45 min to 1 h of folding, whereas dimer formation and activity were only 10–20% complete.

Figure 1B shows a 16% SDS gel of selected samples during the refolding reaction. This analysis shows that a

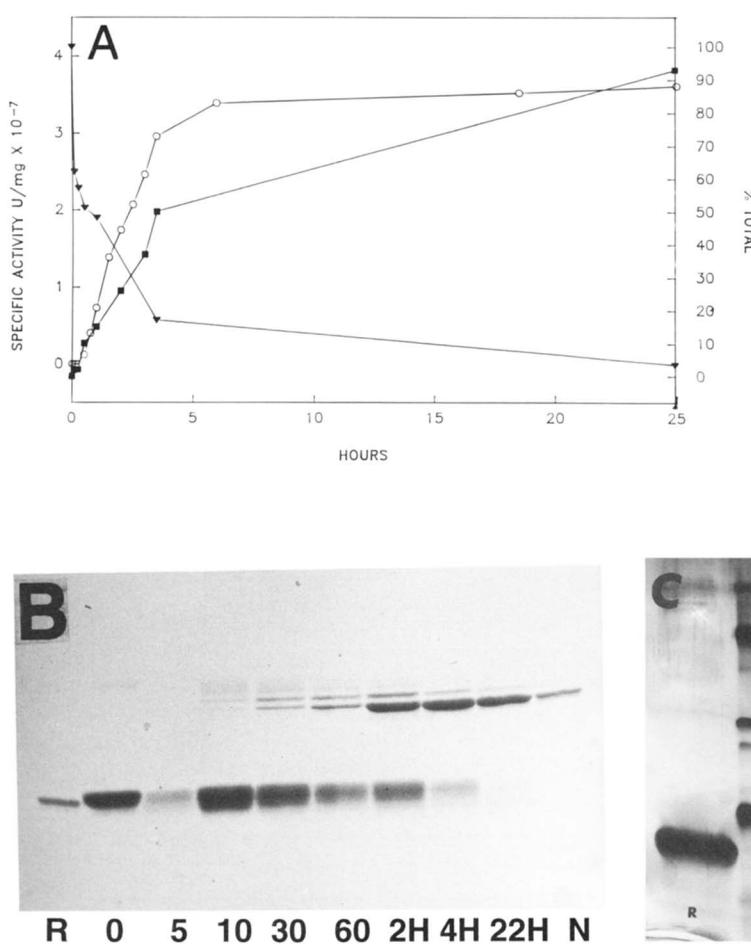


Fig. 1. Analysis of samples from time course of refolding. **A:** Measurement of macrophage colony-stimulating factor (M-CSF) dimer as a percentage of total protein (squares), activity in NFS-60 assay (circles); and disappearance of ^{14}C label from trichloroacetic acid-precipitable protein (triangles) in refolding samples stopped with iodoacetamide as described in the text. **B, C:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of time course incubation samples blocked with iodoacetamide. Samples of a refolding reaction were blocked at various times with iodoacetamide (50 mM final concentration). Equal amounts (4 μg) of each sample were run on 16% SDS gels under non-reducing conditions. Sampling times are indicated beneath appropriate lanes. All times are in minutes unless otherwise specified (S, seconds; H, hours). One microgram each of reduced (R) and nonreduced (N) M-CSF was run as standards. The gel in (B) was stained with Coomassie blue. In (C), samples from early time points (up to 15 min) were run as above and the gel was silver stained to highlight early intermediate dimeric forms (arrow). Molecular weight standards (St; 97, 66, 43, 31, 21, and 14 kDa) were run along with native and reduced M-CSF.

number of intermediate species with apparent molecular weights different from those of native or reduced M-CSF standards were formed during the folding reaction and were trapped by blocking free cysteine residues. At the earliest time point measurable, M-CSF is mostly monomeric; however, a significant amount of a species with an apparent molecular weight greater than that of the native dimer can be seen in the silver-stained gel shown in Figure 1C (arrow). This dimer appeared at the earliest time point in the reaction measurable using iodoacetamide as blocking agent and has also been detected by electrospray ionization mass spectrometry (ESI/MS; Witkowska et al., 1992). With increasing time, other dimeric intermediate species appeared with apparent molecular weights between those of the initially formed dimer and the native dimer, whereas the apparent molecular weights of the monomeric species became smaller than that of fully reduced, denatured M-CSF monomer. After 25 h of folding, most of the monomeric and dimeric intermediates were converted into native dimers.

To characterize the intermediates further, we performed a folding reaction on a large amount of M-CSF and blocked the reaction after 45 min in order to maximize the number of folding intermediates. Intermediates were isolated by anion exchange chromatography on Mono Q. As shown in Figure 2A, the mixture of folding intermediates was separated into several zones when applied to the column at pH 8.0. Figure 2B shows analysis of the fractions on a 16% SDS gel. Under the conditions chosen, monomeric M-CSF was mostly found in the breakthrough fractions, whereas dimeric intermediates, fully folded dimer, and folded monomers (see below) were eluted when the NaCl concentration in the buffer was raised from 0.15 to 0.2 M. Fractions from the column were pooled, and SDS-PAGE analysis of the pooled fractions (Fig. 3) showed that although the dimeric intermediates were not completely separated from each other by this chromatography step, some of them could be separated from the native molecule. The pools made from Mono Q fractions were saved and used for further analysis as described below.

Early-eluting fractions in the 0.2 M NaCl eluate from the Mono Q column contained, in addition to dimeric intermediates, distinct species of monomeric M-CSF that migrated with apparent molecular weights significantly less than that of fully reduced, denatured M-CSF monomer (Fig. 2B). To further purify these monomers, appropriate fractions were applied to a preparative C4 reversed-phase column eluted as described in the Materials and methods. The chromatogram from such a separation is shown in Figure 4A. Several folding intermediates were separated by this procedure including folded monomers (FM), native (N), and dimeric intermediate (DI). Figure 4B shows a comparison of the retention times of several M-CSF species on an analytical reversed-phase HPLC column. As expected, reduced, denatured M-CSF mono-

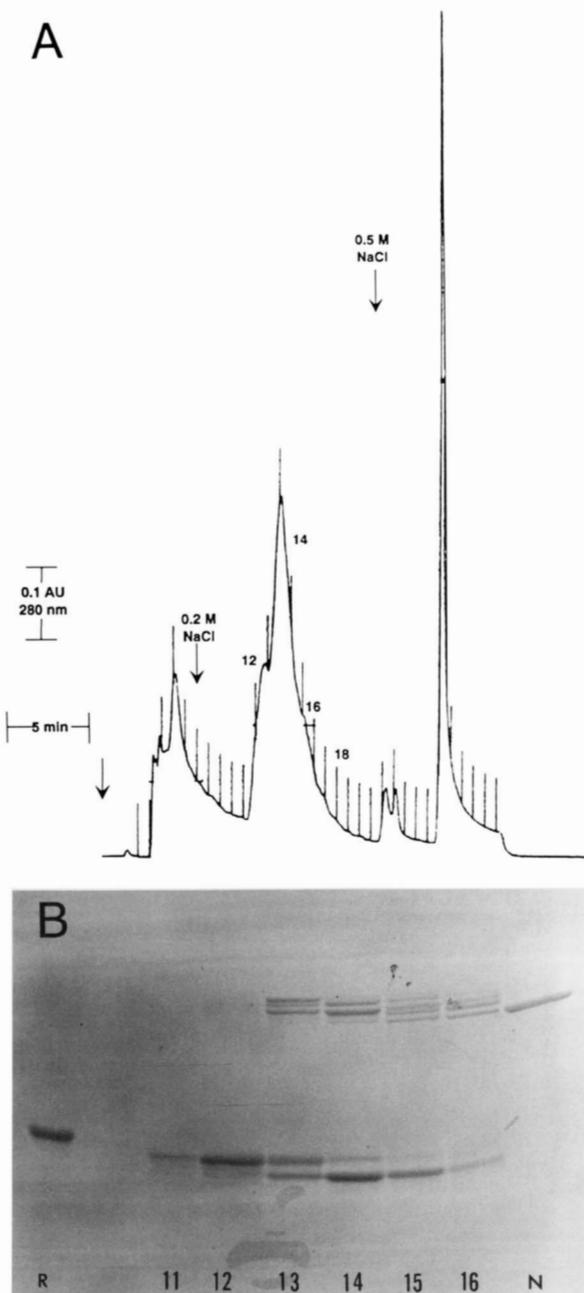


Fig. 2. Anion exchange chromatography of refolding intermediates. **A:** Chromatogram from Mono Q column. A refolding reaction was stopped with iodoacetamide 45 min after its initiation. The solution was applied to a Mono Q HR 10/10 column as described in the Materials and methods. Absorbance was monitored at 280 nm. Fractions (indicated on the chromatogram by vertical marks) collected from the column were pooled according to their apparent molecular weights by SDS-PAGE (see B). **B:** Fractions 11–16 (left to right; 20 μ L each) from the Mono Q column in A were run on 16% SDS gels as described in the Materials and methods. Two micrograms of reduced (R) and native (N) standards were run as standards. The gel was stained with Coomassie blue.

mer migrated with the longest retention time due to its hydrophobic nature. Folded monomer species (see below) migrated with the shortest retention time in this system,

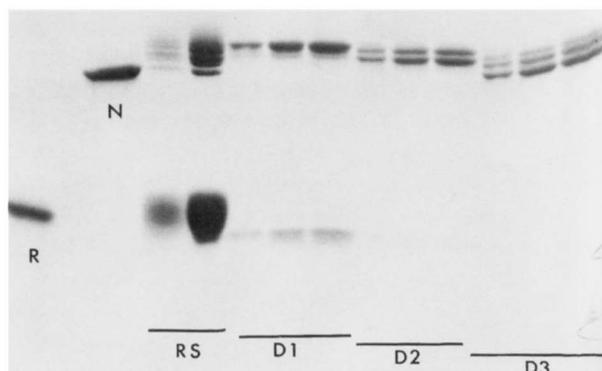


Fig. 3. SDS-PAGE of pooled fractions from Mono Q. Early (D1), middle (D2), and late (D3)-eluting intermediate fractions from the Mono Q column were pooled in order to obtain populations of dimers and monomers. Five, 10, and 20 μ g each of the above populations were run under nonreducing conditions. In addition, two samples of the refolding solution (RS; 10 and 20 μ g) were run. M-CSF standards were run as in Figure 2.

a finding consistent with their more nativelike structures. Dimeric intermediates migrated in this system with retention times between those of native dimer and reduced denatured monomer.

Monomeric M-CSF species from the C4 column were lyophilized and analyzed by SDS-PAGE and compared with dimeric pools from the Mono Q column (Fig. 5). Two different folded monomer species were isolated from Mono Q pools by reversed-phase HPLC. One species, designated FM1, had a less compact structure than the other, which was designated FM2. The retention times for these two species on reversed-phase HPLC (e.g., Fig. 4B) could be converted to that of fully reduced M-CSF monomer by incubation with 50 mM dithiothreitol (data not shown).

The disulfide structures of M-CSF species were probed by peptide mapping and amino acid sequence analysis as described in the Materials and methods. Figure 6 compares the peptide maps of the monomeric FM2 intermediate, a dimeric M-CSF intermediate (D2), and native M-CSF dimer. Table 1 lists the identities, retention times, and amounts of peptides found in samples from the peptide maps of the FM2 and dimer intermediate species. Although all peaks from the peptide maps were subjected to sequence analysis, only cysteine-containing peptides are listed here. The disulfide linkages found were Cys³¹-Cys³¹, Cys⁷-Cys⁹⁰, Cys⁴⁸-Cys¹³⁹, and Cys¹⁰²-Cys¹⁴⁶. All of the native disulfide bridges were detected in the FM2 intermediate map with the exception of the Cys³¹-Cys³¹ bridge, i.e., the *interchain* bridge. In addition, we consistently detected a peptide containing Cys¹⁰² (fraction 3A in the dimer intermediate map) that was found *not* to be paired with another peptide containing Cys¹⁴⁶. Sequence analysis of this peptide yielded a phenylthiohydantoin (PTH)-amino acid peak migrating near the position

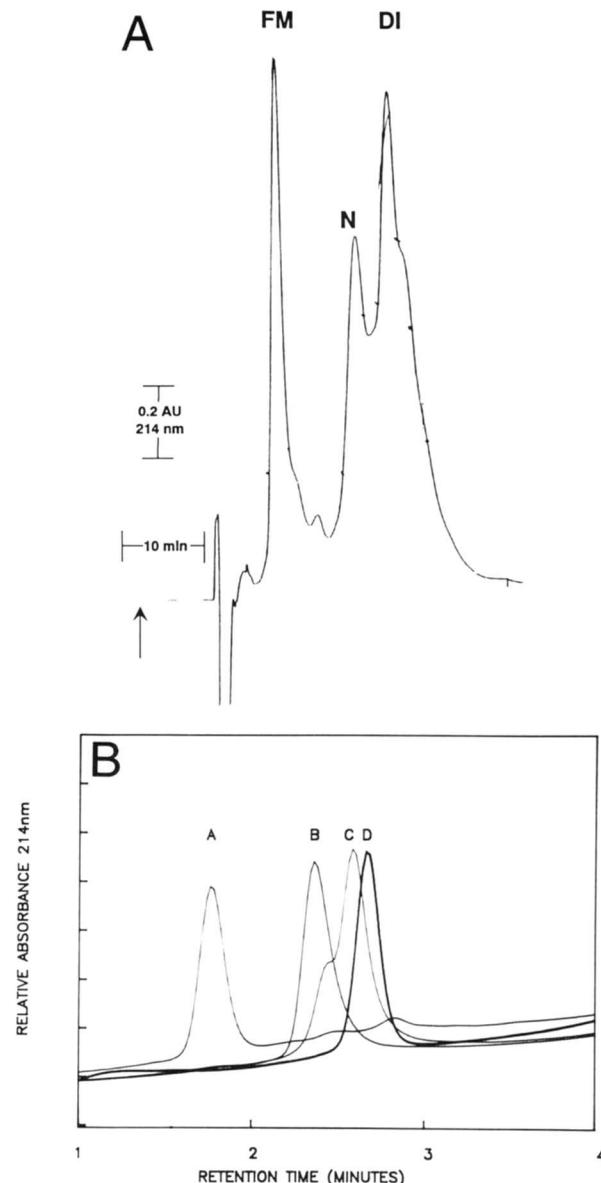


Fig. 4. Reversed-phase HPLC on pooled fractions from Mono Q column. **A:** Separation of folded monomer from dimeric species, including native M-CSF. One milligram of the D2 fraction was applied to a C4 column (Vydac). The column was equilibrated and proteins were eluted as described in the text. Elution positions of species are noted on the chromatogram as follows: FM, folded monomer; N, native M-CSF; D2, dimeric intermediates. **B:** Comparison of retention times of 5- μ g aliquots each of folded monomer (A); native M-CSF (B); D2 intermediate (C); and reduced M-CSF (D) on reversed-phase HPLC (Poros R).

of glutamic acid in the cycle where cysteine would have been expected. This is the retention time expected for carbamidomethyl (CAM) cysteine. Therefore, at least a fraction of the molecules in this dimer intermediate pool appeared to contain an open Cys¹⁰²-Cys¹⁴⁶ disulfide bridge, although some of the species in this pool clearly had the Cys¹⁰²-Cys¹⁴⁶ disulfide bridge intact. Figure 7 presents models for the disulfide structure of native

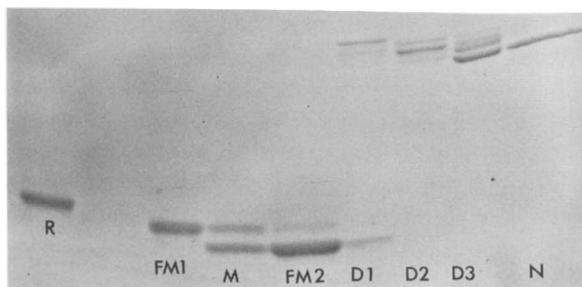


Fig. 5. SDS-PAGE of monomeric and dimeric folding intermediate pools. The molecular weights of monomeric (FM1 and FM2: 5 µg each; M: mixture of 2.5 µg each of FM1 and FM2) and dimeric (D1, D2, and D3: 2 µg each) M-CSF folding intermediates were compared on 16% gels under nonreducing conditions. Monomeric intermediates were analyzed after separation from dimeric intermediates as in Figure 4. Dimeric pools from Mono Q (e.g., Fig. 3) were run for comparison. Standards are labeled as in Figure 2.

M-CSF along with the proposed structures of two folded monomer species and a dimeric folding intermediate.

The monomer intermediates, FM1 and FM2, were analyzed by ESI/MS (Fig. 8). ESI-MS measurements on the two monomer species were consistent with the results obtained from peptide mapping of FM2 (for a review of the technique, see Smith et al. [1990]). The molecular weight of the FM2 molecule (Fig. 8A) was 17,546, a value within 2 Da of the molecular weight expected if the molecule contained a single CAM group. The molecular weight measured for the FM1 species (Fig. 8B), 17,655, is consistent with the presence of three CAM groups on this species. No evidence for the presence of covalent M-CSF dimer was found in either folded monomer sample by mass spectrometry or by gel permeation chromatography (data not shown). In addition, the retention time of folded monomer was vastly different than that of covalent dimer on reversed-phase chromatography (Fig. 4B).

The pools from the Mono Q column (Fig. 3) were assayed for biological activity using the NFS-60 assay described previously (Nakoinz et al., 1990). As shown in Table 2, although the intermediates clearly have not yet reached their final, native conformation (see, e.g., Fig. 3), they appear to have at least 50% of the biological activity found for the native dimer. This result suggests that not all of the disulfide bridges present in the native M-CSF dimer are necessary for its biological activity. The monomer species were also assayed for activity using the NFS-60 assay. FM1 had no detectable activity in the assay, whereas FM2 consistently exhibited activity (Fig. 9). FM2 exhibited a steeper slope in the linear portion of the curve than the native dimer. The specific activity of FM2 was approximately 1/30 that of the native dimer based on measurements done at an FM2 concentration of 0.125 µg/mL. FM2 was also found to stimulate the growth of macrophage colonies from mouse bone marrow, confirming

Table 1. Peptide sequence data from peptic maps

| Peptide map | Peak number | Sequence | Amount ^a (pmol) |
|--------------------|-------------|-----------------------------|----------------------------|
| FM-2 | 2 | FAEXSSQ YEEHDKAXVR | 45] 33] |
| | 3 | FAEXSSQ YEEHDKAXT | 33] 26] |
| | 4 | SEYXSHMIGSG LKSXFTKD | 112] 207] |
| | 5 | SKNXNNNSF FVDQEQLKDPVXYL | 95] 119] |
| | | | |
| Dimer intermediate | 1 | ETSXQ | 183 |
| | 2 | YEEHDKAXVRT FAEXSSQD | 183] 186] |
| | 3 | YEEHDKAXVRT AEXSSQD | 324] 410] |
| | 3A | YEEHDKAXVRT | 186 |
| | 4 | SEYXSHMIGSG RLKSXFTK | 93] 143] |
| | 5 | LKDGVXY SKNXNN | 60] 60] |
| | 6 | KSXFTK SEYXSHM8GSG | 92] 77] |

^a The amount shown represents quantity of the first amino acid in each sequence measured.

^b Phenylthiohydantoin-amino acid in this position migrated with the retention time of glutamic acid.

that the activity observed is not an artifact of the NFS-60 assay system (data not shown).

Discussion

This report examines the folding pathway and disulfide bridge placement of a 150-amino acid form of the dimeric cytokine M-CSF. The molecule described here is a truncated version (150 amino acids) of the full-length molecule. Biologically active M-CSF dimer was obtained by solubilization of M-CSF from a recombinant strain of

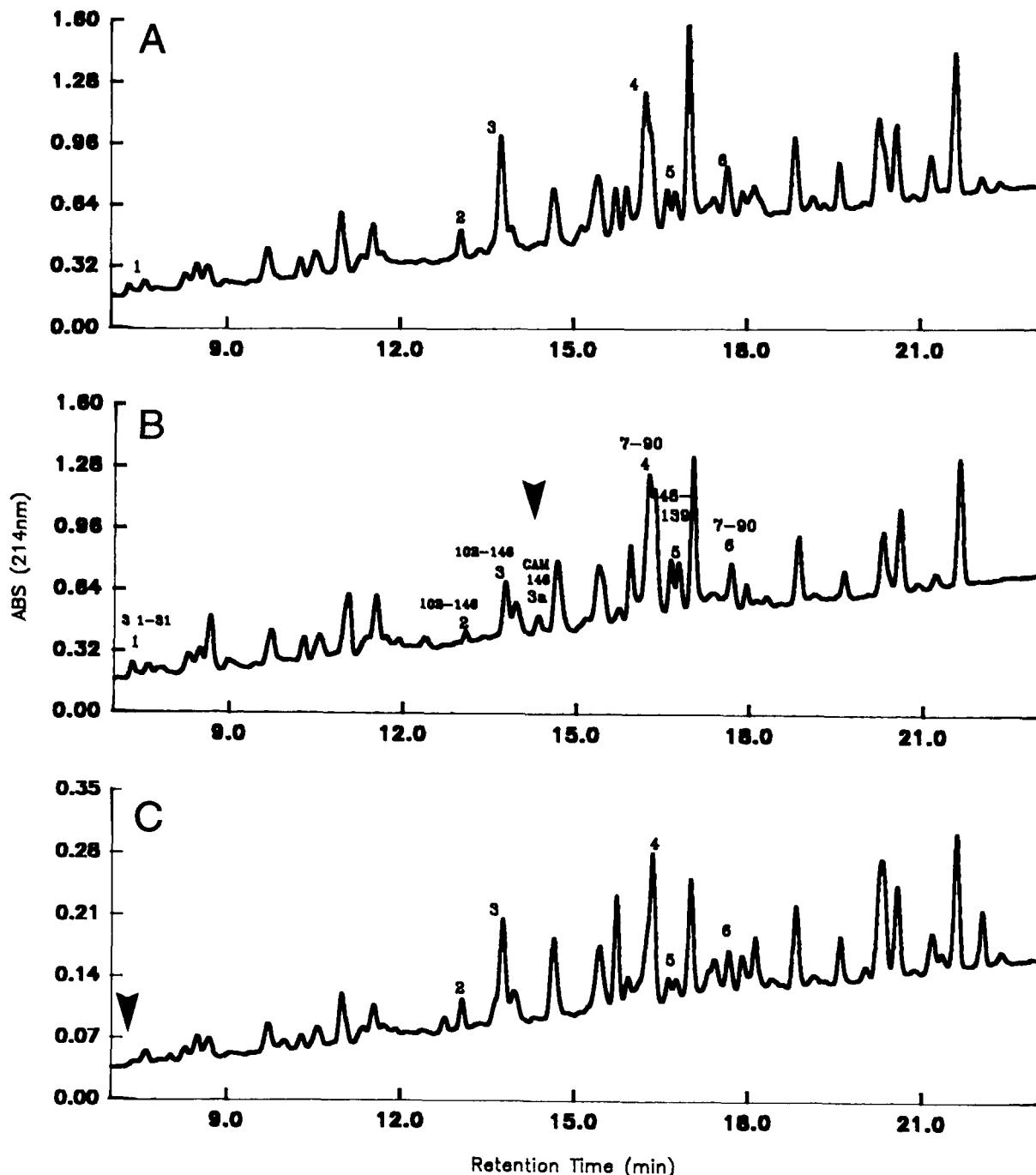


Fig. 6. Peptide maps of native M-CSF (A), dimeric intermediate D2 (B), and folded monomer FM2 (C). The above M-CSF species were subjected to pepsin hydrolysis and reversed-phase HPLC as described in the text. Fractions were collected and analyzed by amino acid sequence analysis (Table I). The positions of disulfide-bridged peptides are numbered: 1, Cys³¹-Cys³¹; 2, Cys¹⁰²-Cys¹⁴⁶; 3, Cys¹⁰²-Cys¹⁴⁶; 4, Cys⁷-Cys⁹⁰; 5, Cys⁴⁸-Cys¹³⁹; 6, Cys⁷-Cys⁹⁰. A peak was identified in the dimeric intermediate (panel B, arrow) that contained carbamidomethyl (CAM) cysteine¹⁰². The FM2 map (panel C) lacked the Cys³¹-Cys³¹-containing peak 1 (panel C, arrow).

E. coli followed by refolding and purification. The procedures used were similar to those outlined previously (Yamanishi et al., 1991). After purification, the active protein was found to be a homodimer of two 17,500-Da subunits.

The dimeric molecule, in contrast to the reduced, carboxymethylated monomer, was found to be quite resistant to digestion with proteases including trypsin and staphylococcal V8 protease. Pepsin digestion was used to attain cleavage between all 14 cysteine residues in the molecule,

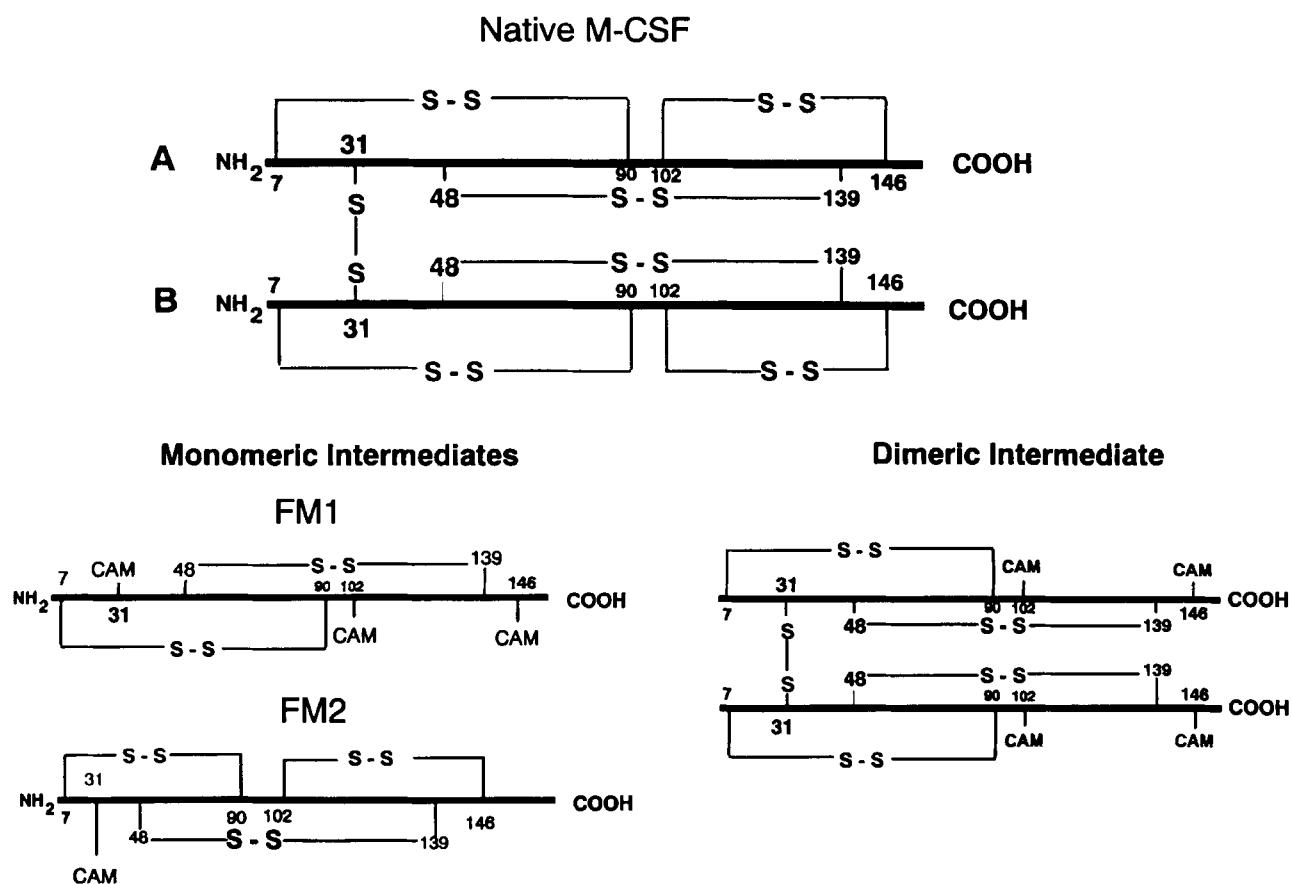


Fig. 7. Models of the structures of native M-CSF, folded monomers, and a dimeric intermediate. The model presents the structures of the folding intermediates elucidated by peptide mapping and electrospray ionization mass spectrometry (ESI/MS) measurements.

which allowed determination of the disulfide linkages in the native dimer. All of the cysteines in the native dimer were found to be involved in disulfide bonds with the following linkages: 7–90, 31–31, 48–139, and 102–146 (see Yamanishi et al. [1991] for the M-CSF sequence). The determination of whether these bridges were inter- or intra-chain was obvious in the case of the 31–31 linkage, but

not directly obvious in the case of the other bridges. The fact that a monomeric species was isolated with all of the other disulfide bridges intact (i.e., FM2) strongly suggests that the remaining linkages in the native molecule are *intrachain* because these monomers disappear during folding and presumably go on to form native dimers.

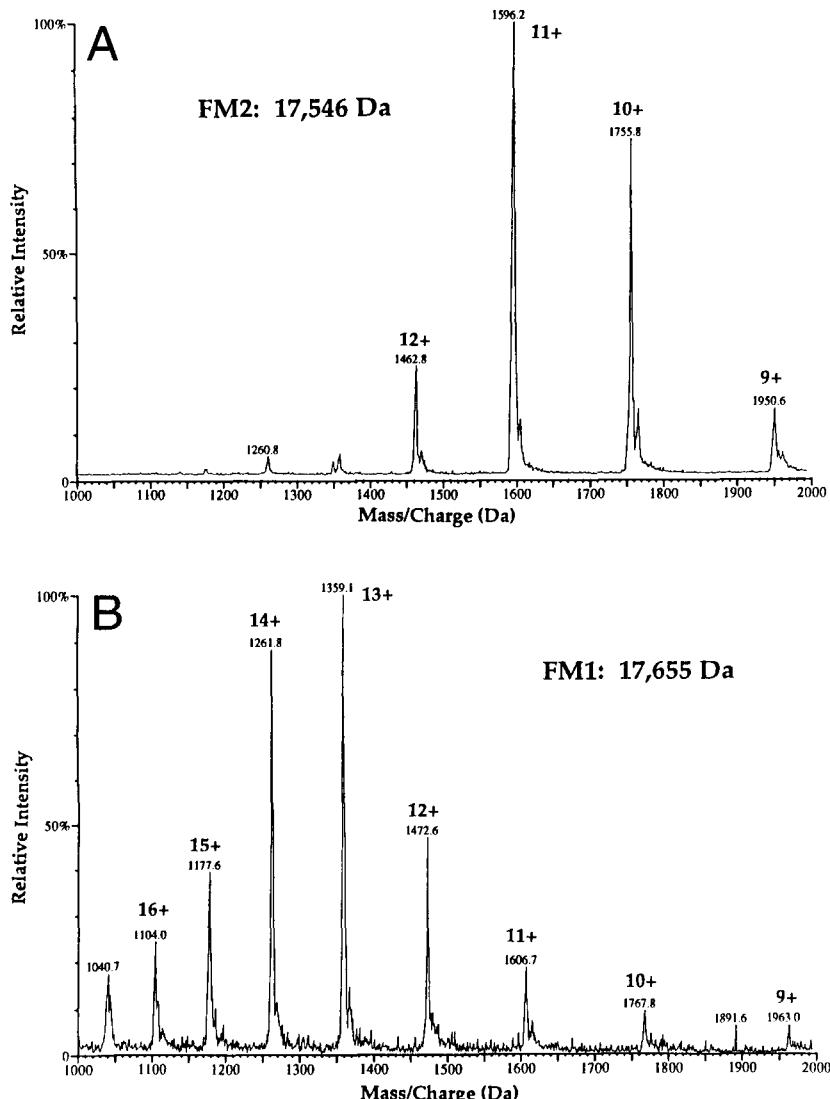
An examination of the time course of folding revealed that the M-CSF molecule goes through a number of intermediate species on its way from a fully reduced, denatured polypeptide to its native, dimeric state. The ability to block the progression of the molecule through the folding pathway using the sulphydryl reagent iodoacetamide has led to the identification of a number of stable intermediate species, based upon their migration on nonreduced SDS-PAGE. Various concentrations (10–100 mM) of iodoacetamide were tested for their ability to block disulfide bond formation during M-CSF folding. In addition, iodoacetate, *N*-ethylmaleimide (NEM), and para-chloromercuribenzoate (PCMB) were used as quenching agents. All of these agents produced similar profiles of intermediates on SDS-PAGE or on reversed-phase HPLC analysis (data not shown). We found no evidence for sig-

Table 2. Biological activity of dimeric M-CSF folding intermediate pools from Mono Q^a

| Sample | Specific activity ^b (units/mg protein) |
|--------|--|
| Native | 5.7 × 10 ⁷ |
| D1 | 3.3 × 10 ⁷ |
| D2 | 3.1 × 10 ⁷ |
| D3 | 2.9 × 10 ⁷ |

^a The ability of each of three dimeric intermediate pools (Fig. 3) to stimulate growth of the NFS-60 cell line was measured and compared to native M-CSF.

^b Values are the means of two separate experiments.



nificant levels of non-native disulfide-linked peptides, even at the lowest concentrations of iodoacetamide used. This result is in contrast to that of Weissman and Kim (1991), who showed significant differences in iodoacetate quenching of the folding of BPTI even at iodoacetate concentrations in excess of 100 mM.

The intermediates fell into two categories: monomeric and dimeric. Some of the monomeric species migrated with apparent molecular weights significantly smaller than that of the reduced, denatured M-CSF monomer, suggesting a compact, folded structure. These intermediate forms were also reported by Halenbeck et al. (1989) for a longer version of M-CSF. In addition, these "folded monomers" moved with a decreased retention time on reversed-phase HPLC when compared to the reduced monomer or the native dimer. Indeed, when two of these species (FM1 and FM2) were isolated, they were found to contain some native disulfide bridges. In the case of FM2,

all of the native disulfide bridges were present except the interchain bridge, 31–31.

The fact that two types of intermediate species were found, both monomeric and dimeric, and that a fully folded monomer species was also found argue that M-CSF can fold via either of two pathways, perhaps with "crossing-over" between the two pathways. In other words, all of the S-S bridges with the exception of 31–31 can form in the monomer itself prior to the formation of the interchain bridge. Similarly, the data suggest that an unfolded, covalent dimer can also rapidly form during the refolding reaction (see Fig. 2), in which the monomers are presumably linked via the 31–31 bridge because this dimer is stable in SDS under nonreducing conditions. The other bridges would then be formed in this "dimer pathway." The idea that a protein molecule might use more than one pathway in folding into its final, native conformation has been discussed (Harrison & Durbin, 1985).

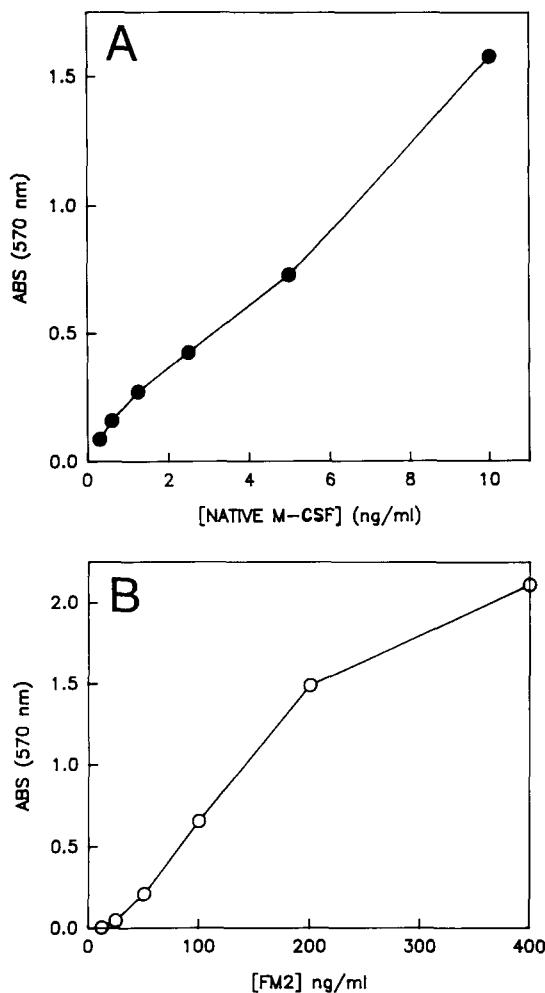


Fig. 9. Comparison of NFS-60 assays on native M-CSF and on FM2. Various amounts of native M-CSF (**A**) or FM2 (**B**) were assayed for their ability to stimulate the growth of the NFS-60 cell line. Activities are expressed as absorbance units at 570 nm, the wavelength of MTT absorbance.

More recently, the folding pathway of hen lysozyme has been studied using stopped-flow CD in the far and near UV regions and NMR spectroscopy (Radford et al., 1992). The results from the above studies suggest that lysozyme also folds along more than one pathway. The proof of this hypothesis in the case of M-CSF awaits further purification and characterization of the partially folded dimers. Clearly, the intermediates isolated in the present study represent an initial step in the full characterization of the M-CSF folding pathways.

The finding that FM2 showed biological activity in that it was able to support the growth of an M-CSF-dependent cell line (NFS-60) and the growth of macrophage colonies in bone marrow assays represents the first time, to our knowledge, that biological activity has been demonstrated for a purified monomeric form of M-CSF. Fully reduced M-CSF has no biological activity (Stanley & Guilbert, 1981). We do not know at present, however, whether the

folded monomer species can form noncovalent dimers in solution, for instance. In addition, dimeric M-CSF folding intermediates blocked with iodoacetamide were found to have substantial biological activity (~50% of the native dimer; Table 2), even though they clearly do not have fully native structures based on their migration on SDS gels, their retention times on reversed-phase HPLC and peptide mapping profiles. It is apparent that several of the partially folded forms of M-CSF have structures closely resembling that of the native molecule. Their differences may provide important clues into the structure and function of the M-CSF molecule and thus into the nature of the interaction of M-CSF and its receptor.

Materials and methods

Isolation and refolding of M-CSF

Human M-CSF (amino acids 4–153) was expressed as an insoluble protein in recombinant *E. coli* using a temperature-sensitive promoter system in a protease-deficient strain. These procedures will be described elsewhere (P. Arthur et al., in prep.). *Escherichia coli* was lysed in a Rannie homogenizer followed by centrifugation to obtain insoluble proteins including M-CSF. Solubilization and denaturation of M-CSF was done by resuspension of the pelleted material in five volumes of a buffer containing 5 M urea/20 mM Tris-Cl/50 mM 2-mercaptoethanol (BME), pH 8.0 (solubilization buffer). Following a 2-h incubation of this suspension at room temperature with mixing, a flocculating agent (BPA 1000, Tosohhaas) was added at a final concentration of 1,000 ppm to facilitate removal of insoluble contaminants and nucleic acids. The suspension was centrifuged at 5,000 × g for 15 min, and the supernatant fraction was decanted directly into refolding buffer (50 mM glycine, pH 9.0) at a final M-CSF monomer concentration of 200 µg/mL, based on analytical reversed-phase HPLC measurements (see below). Following a 48-h incubation at room temperature, dimeric M-CSF was purified using a protocol similar to one previously described (Yamanishi et al., 1991), except that dimeric M-CSF was further purified by gel permeation chromatography on Sephadryl S-200HR (Pharmacia). The refolding efficiency using this protocol ranged between 30 and 50% of the initial monomer added to the refolding solution. The final product (M-CSF dimer) was >95% pure as assessed by SDS-PAGE and by N-terminal amino acid sequence analysis (see below).

To analyze the time course of refolding, a sample of purified M-CSF dimer (7 mg/mL in phosphate-buffered saline, pH 7.5) was dialyzed overnight at 4 °C against at least 500 volumes of 5 M urea/20 mM Tris-Cl, pH 8.0. The protein concentration of the sample was redetermined and the sample was reduced by adding BME to a final concentration of 50 mM. After a 2-h incubation at room temperature, the M-CSF was completely converted

to monomer. Refolding was initiated by diluting the M-CSF monomer to a final concentration of 200 µg/mL in 50 mM glycine, pH 9.0, at room temperature. The concentration of urea did not exceed 5 mM in the refolding reaction.

The refolding reaction was analyzed in detail as follows. At specified time points, 1-mL aliquots of the reaction were added directly to 50- or 10-µL aliquots of a 1 M solution of iodoacetamide in H₂O to block free sulfhydryl groups. The pH of the samples remained at 9.0 after iodoacetamide addition. In some experiments, [¹⁴C] iodoacetamide (25 mCi/mmol; New England Nuclear) was used to block the refolding reaction in order to quantitate the relative number of free cysteines remaining in TCA-precipitable material. M-CSF was precipitated from samples by the addition of an equal volume of ice-cold 20% TCA followed by centrifugation. Pellet fractions from precipitated samples were solubilized and radioactivity was determined by liquid scintillation counting.

SDS-PAGE

Samples were run on 16% polyacrylamide gels (Novex) and stained with Coomassie blue according to the manufacturer's instructions. Samples run under reducing conditions were reduced in sample buffer containing 50 mM dithiothreitol. Some gels were silver stained by the procedure of Morrissey (1981).

Quantitation of M-CSF species

M-CSF dimer formed during refolding was measured by analytical HPLC on a 5 × 50-mm POROS Q column (PerSeptive Biosystems, Cambridge, Massachusetts). The buffers used were (A) 20 mM Tris-Cl, pH 7.4, and (B) 20 mM Tris-Cl, pH 7.4, + 1 M NaCl. The column was eluted at 5 mL/min with a linear gradient from 20 to 35% buffer B over 5 min. Dimeric M-CSF eluted with a retention time of approximately 3 min in this system. M-CSF was quantitated by comparing peak heights of unknown samples to those of known standards.

Fully reduced M-CSF monomer was quantitated by reversed-phase HPLC using a 5 × 50-mm POROS R column (PerSeptive Biosystems). The buffers used were (A) 0.1% trifluoroacetic acid (TFA) in H₂O, and (B) 0.1% TFA in CH₃CN. M-CSF monomer was eluted at a flow rate of 5 mL/min with a linear gradient going from 37 to 55% B over 5 min. The retention time of M-CSF monomer was approximately 4 min in this system. Samples were always prepared by diluting either knowns or unknowns into a buffer containing 7 M guanidiniumhydrochloride/20 mM Tris-Cl, pH 8.0, 50 mM dithiothreitol followed by filtration through a 0.22-µm Millex (Millipore) filter. M-CSF monomer was determined by comparing the peak heights of unknown samples to those of known standards.

Isolation of folding intermediates

Folding intermediates of M-CSF were isolated in the following way. M-CSF monomer was prepared and refolded as described above. Following the desired time (usually 45 min), folding was stopped by adding solid iodoacetamide (Fluka) to the refolding solution to achieve a final concentration of 10 mM. After a 30-min incubation at room temperature, the refolding solution was concentrated using a Millipore Minitan apparatus equipped with 600 cm² of PTGC, 10,000 molecular weight cutoff polysulfone membrane to a final protein concentration of 15 mg/mL. The concentrated solution was desalted by diafiltration in the same apparatus. Six volumes of 20 mM Tris-Cl, pH 8.0, were used in the diafiltration.

Folding intermediates were separated on a 1 × 10-cm Mono Q column (Pharmacia) as follows. The column was first equilibrated in a buffer containing 20 mM Tris-Cl, pH 8.0/10% (v/v) ethanol/0.15 M NaCl. An aliquot (100 mg) of the concentrated refolding solution from above was loaded onto the column at a flow rate of 4 mL/min. The absorbance of the column effluent was monitored continuously at 280 nm with a Waters Model 490 dual-wavelength UV detector. Fractions were collected and the absorbance was allowed to stabilize before raising the NaCl concentration to 0.2 M. At 0.2 M NaCl, dimeric intermediates and folded monomeric intermediates were eluted (see below; Fig. 2). Fractions were analyzed by SDS-PAGE as described above and intermediate pools were made. Fractions containing a folded M-CSF monomer were pooled and purified further as described below.

Purification of folded monomer

Folded monomer species of M-CSF were purified from the Mono-Q pools above by reversed-phase HPLC. Approximately 1 mg of a pool containing dimeric and monomeric M-CSF folding intermediates was loaded onto a 1.0 × 25-cm C4 reversed-phase column (Vydac, 5-µm particle size) equilibrated in 50% CH₃CN/50% H₂O/0.1% TFA. The column was eluted at 4 mL/min with a linear gradient of CH₃CN from 50% to 60% over 20 min. In this system, folded monomers eluted with a retention time of 14 min. Fractions were collected and aliquots were subjected to SDS-PAGE after freeze drying in a Speed-Vac (Savant).

Peptic hydrolysis and mapping of disulfide structure of native M-CSF

The disulfide structures of M-CSF species were determined by pepsin hydrolysis followed by reversed-phase HPLC. Samples were exchanged into 0.01 N HCl on a PD10 column (Pharmacia). After adjustment of the protein concentration to 1 mg/mL, pepsin (Boehringer) was added at a ratio of 1:50 (mg pepsin:mg M-CSF). Hydro-

lysis was allowed to proceed for 24 h at room temperature and was stopped by addition of enough 0.1 N NaOH to raise the pH of the reaction to 8.0. Peptides from the reaction were separated on a Vydac C18 column (0.4 × 25 mm, 5-μm particle size), which was run at a flow rate of 1 mL/min. The column was equilibrated in H₂O containing 0.1% TFA and was eluted with a linear gradient of acetonitrile containing 0.1% TFA going from 0% to 40% over 40 min (1%/min). Fractions containing disulfide-linked peptides were identified by performic acid oxidation followed by rechromatography under the conditions described above (data not shown). Fractions were collected and analyzed by amino acid sequence analysis.

Amino acid sequence analysis

N-terminal amino acid sequence analysis of isolated peptides was performed on an Applied Biosystems Model 477A automated sequencer.

NFS-60 assay for M-CSF biological activity

The ability of various M-CSF samples to stimulate the growth of the NFS-60 cell line (Nakoinz et al., 1990) was used as a measure of biological activity. Cell proliferation was quantitated using the tetrazolium salt MTT, which is converted to a colored product by viable cells. The absorbance of the product was measured at 570 nm.

Mass spectrometry

ESI/MS was performed on a VG Bio-Q quadrupole mass spectrometer (VG BioTech/Fisons, Altrincham, UK). The instrument was controlled and data analyzed using a Lab-Base software (VG BioTech/Fisons, Altrincham, UK). The electrostatic-spray ion source was operated at 3.2 kV. The nozzle-to-skimmer bias value was typically 75 V. The instrument was scanned from *m/z* 1,000 to 2,000 in 10 s, and several scans were summed to obtain the final spectrum. Mass scale calibration employed the multiply charged ions from a separate introduction of bovine trypsinogen (Sigma T1143; average molecular mass 23,980.9 Da). Typically, samples of intact recombinant M-CSF were dissolved in 40% formic acid/40% methanol and di-

luted with 50% MeOH/1% acetic acid to a final concentration of 10–50 pmol/μL.

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