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Research Article

Degradation Pathways for Recombinant Human Macrophage Colony-Stimulating Factor in Aqueous Solution

Jay A. Schrier,[1] Richard A. Kenley,[1,2] Rick Williams,[1] Robert J. Corcoran,[1] Yangkil Kim,[1] Richard P. Northey, Jr.,[1] Darren D'Augusta,[1] and Michael Huberty[1]

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Recombinant human macrophage colony-stimulating factor (rhM-CSF) promotes macrophage proliferation and activity. rhM-CSF clinical trials are currently in progress and require a stable, pharmaceutically acceptable dosage form. This report documents pH effects on rhM-CSF degradation profiles in aqueous solution, with an emphasis on identifying degradation products. Thus, highly purified rhM-CSF was maintained at 30 to 50°C in solutions adjusted to pH 2 to 10. Stressed samples were analyzed by SDS-PAGE, reverse-phase HPLC, size exclusion HPLC, scanning microcalorimetry, and murine bone marrow activity. The results show maximal protein stability in the region pH 7 to 8. Degradation product chromatographic and electrophoretic analyses show distinctly different degradation product profiles in acidic versus alkaline solution. For samples stressed in acidic solution, degradation products were isolated chromatographically and electrophoretically. These degradation products were characterized by N-terminal amino acid sequencing, fast-atom bombardment mass spectrometry, and peptide mapping. The results show that the major degradation pathway in acidic solution involves peptide cleavage at two sites: aspartate$_{169}$-proline$_{170}$ and aspartate$_{213}$-proline$_{214}$. A third potential cleavage site (aspartate$_{45}$-proline$_{46}$) remains intact under conditions that cleave Asp$_{169}$-Pro$_{170}$ and Asp$_{213}$-Pro$_{214}$. In alkaline solution, degradation proceeds via parallel cleavage and intramolecular cross-linking reactions. A β-elimination mechanism is proposed to account for the degradation in alkaline solution. Consistent with literature observations, the rhM-CSF N-terminal cleavage products retain biological activity.

KEY WORDS: macrophage colony stimulating factor; rhM-CSF; degradation; products; mass spectrometry; bioactivity; β-elimination; aspartate-proline cleavage.

INTRODUCTION

Macrophage colony-stimulating factor (rhM-CSF) is a polypeptide hormone that promotes the growth, survival, and differentiation of cells in the monocyte-macrophage lineage (1–4). rhM-CSF also stimulates macrophage effector functions, to include microbicidal (5), tumoricidal (6), and cholesterol-lowering (7) activities. rhM-CSF is currently undergoing clinical trials for antineoplastic and other indications.

The human rhM-CSF gene has been cloned (8,9) and expression studies have been undertaken using mammalian cells (8) and *Escherichia coli* (10,11). The predominant rhM-CSF present in human serum and urine is the so-called "long form" (12). The "long form" of rhM-CSF is a 90-kD disulfide-bridged homodimer (8,13). The monomer features 223 amino acids (including 9 cysteines) plus N- and (variable) O-linked glycosylation. The reduced rhM-CSF monomer lacks biological activity, but the deglycosylated dimer is active (13). Various nonglycosylated and truncated rhM-CSF analogues show bioactivity (1,4,10,11). Apparently, only the first approximately 150 N-terminal amino acids are necessary to confer bioactivity to rhM-CSF.

Developing a suitable parenteral dosage form to support clinical studies requires (among other things) demonstrating that the drug under investigation is storage stable. Recombinant DNA technology has now provided several approved protein drugs and the literature addresses both general analytical approaches to (14–16) and specific examples of protein stability testing (17–23). Reviews of common protein and peptide degradation modes are also available (24,25).

With respect to the literature, most protein drug stability studies emphasize analytical and kinetic aspects and usually provide only inferential information concerning protein degradation products. Textbook protein degradation modes can provide useful background information, but protein tertiary structure complicates the overall degradation chemistry. It is therefore essentially impossible to predict confidently degradation products for a specific protein. Understanding protein drug degradation products is important, however, to elucidating degradation mechanisms and to assessing bioactivity of partially degraded samples.

Considering the foregoing, we have undertaken to probe the pH dependence of rhM-CSF degradation in aqueous solution. Although this investigation provides some kinetic in-

[1] Genetics Institute, 1 Burtt Road, Andover, Massachusetts 01810.
[2] To whom correspondence should be addressed.

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formation, the focus is on identifying degradation products and evaluating their bioactivity.

EXPERIMENTAL

Materials

rhM-CSF was produced as the 223-amino acid, glycosylated protein expressed by Chinese hamster ovary cells (Fig. 1). Various analytical methods demonstrated >98% purity for the protein stock solutions employed. rhM-CSF stock solutions and control samples were maintained at −80°C throughout the study. rhM-CSF sample solutions in various buffer systems and at various protein concentrations were prepared by dialysis and ultrafiltration of the stock solutions.

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

HPLC runs used Waters 600E pumps and system controller with WISP Model 700 autoinjector. Detection was by Model 490E detector with quantitation by Model 860 Expert software.

The rhM-CSF degradation product study used three different RP-HPLC methods as summarized in Table I. Method 1 was used to quantitate intact rhM-CSF and to quantitate cleavage product accumulation. With Method 1 the cleavage products elute as a single peak. Method 2 was used to "fingerprint" cleavage products. With this method cleavage products elute at 44 min (so-called "early-eluting fragments") and as a complex pattern of peaks at approximately 110 min. Method 3 was used to separate peptide fragments produced by a lysine-specific proteinase digestion of rhM-CSF and rhM-CSF degradation products.

High-Performance Size Exclusion Chromatography (HP-SEC)

The HP-SEC method for quantitating high molecular weight degradation products used tandem TosoHaas TSK 3000_{SWXL}, 5-μm-particle, 7.8 × 300-mm columns. The flow rate was 1 mL/min and detection was spectrophotometric at 200 nm. The isocratic mobile phase was aqueous 20 m*M* $NaHPO_4$, plus 100 m*M* Na_2SO_4, pH adjusted to 6.8. Under these conditions, intact rhM-CSF eluted at approximately 15 min.

E E V S E Y C S H M I G S G H L Q S L Q R L I D S	25
Q M E T S C Q I T F E F V D Q E Q L K D P V C Y L	50
K K A F L L V Q D I M E D T M R F R D N T P N A I	75
A I V Q L Q E L S L R L K S C F T K D Y E E H D K	100
A C V R T F Y E T P L Q L L G K V K N V F N E T K	125
N L L D K D W N I F S K N C N N S F A E C S S Q D	150
V V T K P D C N C L Y P K A I P S S D P A S V S P	175
H Q P L A P S M A P V A G L T W E D S E G T E G S	200
S L L P G E Q P L H T V D P G S A K Q R P P R	223

Fig. 1. rhM-CSF predicted amino acid sequence showing N-terminal sequence (italics), N-glycosylation sites (underlined), potential variable O-glycosylation sites (shaded), and cysteines (bold).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE used a Hoefer SE600 vertical slab gel unit with a 1000/500 power supply. For characterizing degradation product profiles, separating gels were 8.5% polyacrylamide (0.8% piperazine diacrylamide cross-linking), 160 × 160 × 0.75 mm. Electrophoresis buffer was 0.25 *M* Tris (pH 8.8) plus 0.1% SDS. Sample buffer (nonreduced) was 0.2 *M* Tris (pH 6.8), 0.1% SDS, plus 30% glycerol and 0.01% bromophenol blue. The sample buffer (reduced) was identical to the nonreducing buffer but also contained 0.025 *M* dithiothreitol. Sample load was 1 to 5 μg in 20 μL. Visualization was by silver staining using an ISS Daiichii Silver Stain-II kit.

Scanning Microcalorimetry

The instrumentation for differential scanning calorimetry (DSC) was a Microcal Model MC2. Samples were started at ambient temperature and run versus a reference standard (buffer without protein added). Scanning rates were typically 53 to 55°C/hr. After each run, samples were returned to ambient temperature at 2°C/min using a circulating bath. Aliquots for chromatographic analysis were withdrawn after return to ambient temperature. The sample and reference cell were pressurized at 35 psi with nitrogen to prevent cavitation during heating.

Fast Atom Bombardment Mass Spectrometry (FAB-MS)

FAB-MS used a JEOL HX110HF/HX110HF tandem (EB/EB) mass spectrometer. Samples were prepared in thioglycerol solution and bombarded with 20-kV Cs^+.

Peptide Mapping

Partially degraded rhM-CSF samples were run on preparative SDS-PAGE. Degradation product bands were isolated and the protein fragments electroeluted into buffer.

The isolated fractions were reduced (2-mercaptoethanol plus triethylamine), alkylated (4-vinyl pyridine), and concentrated to near-dryness by vacuum centrifugation. The dried samples were redissolved in 50 m*M* Tris (pH 9) buffer and a sample of *Achromobacter* protease (a lysine C-specific protease; Waco) was added. The final enzyme:substrate ratio was 1:75. The reaction mixture was sparged with helium and incubated for 5 hr at 30°C. The digestion was stopped by adding trifluoroacetic acid and the peptide fragments separated and analyzed by RP-HPLC (Method 3, see Table I).

N-Terminal Amino Acid Sequencing

N-terminal amino acid sequencing used literature procedures (26,27). The sequence determinations were per-

Table I. Summary of Reverse-Phase HPLC Method Conditions[a]

Method no.	Column	Flow rate (mL/min)	Gradient[b]	Retention time (min)
1	Vydak C4 4.6 × 50 mm 5-μm particle 300-Å pore	2.5	Start (20%B) 2 min (20%B) 2.5 min (40%B) 9 min (40%B) 14 min (70%B) 17 min (20%B)	rhM-CSF (18.5) Deg prod (4)
2	Vydak C18 2 × 250 mm 5-μm particle 300-Å pore	0.4	Start (1%B) 10 min (1%B) 100 min (30%B) 140 min (35%B) 170 min (60%B) 190 min (80%B) 200 min (80%B) 205 min (1%B)	rhM-CSF (185) Deg prod (44) Deg prod (110)
3	BioRad HiPore RP318 4.6 × 250 mm 5-μm particle	1	Start (1%B) 10 min (1%B) 70 min (30%B) 100 min (60%B) 140 min (60%B) 145 min (90%B) 150 min (1%B)	Peptide mapping

[a] All analyses used spectrophotometric detection at 214 nm.
[b] Mobile phase A, 0.1% trifluoroacetic acid in water; mobile phase B, 0.1% trifluoroacetic acid in 90% aqueous acetonitrile.

formed with an ABI Model 477A automated pulsed liquid protein sequencer. PTH-derivatized amino acids were analyzed online with an ABI Model 120A reverse-phase HPLC and Brownlee PTH C18, 2.1 × 200-mm column.

Murine Bone Marrow Assay

Partially degraded rhM-CSF samples were subjected to semipreparative SDS-PAGE. Individual bands were isolated and electroeluted as described above for peptide mapping analysis. Biological activities of isolated species were determined as described elsewhere (8).

RESULTS

Figure 1 shows the rhM-CSF amino acid sequence predicted (8) from the corresponding cDNA sequence. Based on structural information, significant structural features are captured in Fig. 1.

Effect of pH on rhM-CSF Thermal Denaturation and Degradation

rhM-CSF solutions were prepared at 1.2 mg/mL in 20 m*M* "PolyB" buffer (equimolar amounts of citrate, succinate, Tris, HEPES, imidazole, histidine, and glycine) and the pH was adjusted to cover the range pH 2 to 10. Each sample was then admitted to the microcalorimeter and heated over the range 5 to 110°C. In each case, the samples were returned to 5°C and aliquots removed for analysis by scanning microcalorimetry, RP-HPLC (Method 1, Table I) and HP-SEC.

For the samples prepared at pH 6, an endothermic response was observed (Fig. 2), consistent with protein thermal denaturation via a single two-state (native ↔ denatured) transition. The midpoint temperature, T_m, for the endotherm was 86.7°C as measured by scanning microcalorimetry. The corresponding van't Hoff enthalpy (ΔH_{vh}) was 157 kcal/mol and the calorimetric enthalpy (ΔH_{cal}) was 73 kcal/mol. The ratio $\Delta H_{vh}/\Delta H_{cal} = 2.2$ is close to the integer ratio 2, indicating a dimeric protein (28–30). Additional testing revealed that sample T_m varied with sample pH. Figure 3 plots the observed T_m values versus pH and reveals maximal rhM-CSF thermal stability near pH 7.5.

Sample aliquots removed from the heat-stressed solutions at various pH were analyzed by RP-HPLC (Method 1, Table I) and by HP-SEC. The RP-HPLC data (see following section for additional details) indicated C-terminal cleavage product accumulation. The HP-SEC analysis (not shown) revealed accumulation of a high molecular weight aggregation species. SDS-PAGE analysis (not shown) of the isolated aggregation species showed $M_{app} = 180$ kD, consistent with rhM-CSF tetramer. For both the RP-HPLC and the HP-SEC analyses, quantitation (by area normalization relative to intact rhM-CSF) showed a strong pH dependence on the degradation product profile. As shown in Fig. 4, the C-terminal fragment accumulation increases in slightly acidic pH regions, whereas acidic (pH $\leq$ 4) and alkaline (pH $\geq$ 8) conditions favor the aggregation product accumulation. Both the cleavage products and the aggregation products minimally accumulate near pH 7.5, consistent with the thermal denaturation results.

To develop an overview of rhM-CSF degradation patterns, we stressed samples prepared at various solution

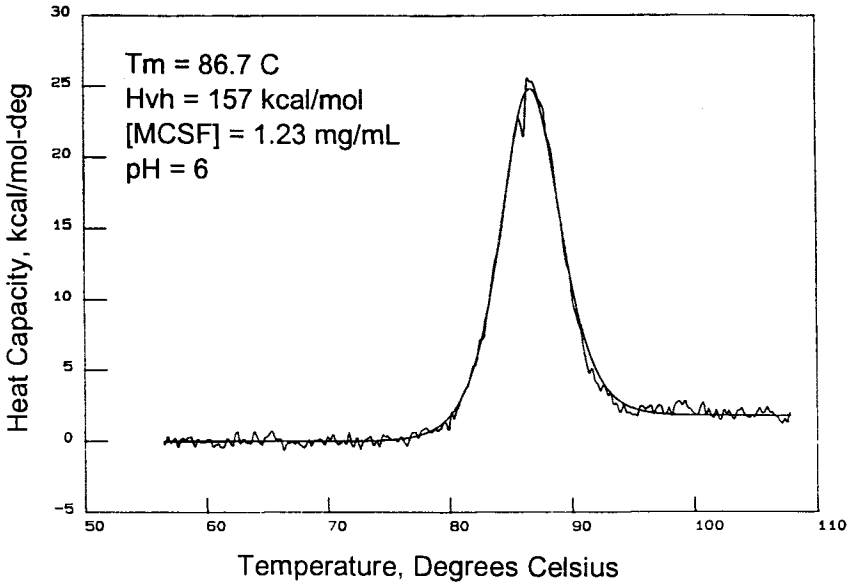

Fig. 2. Differential scanning microcalorimetry of M-CSF at pH 6. Observed (points) and calculated (solid line) data for a single two-state (native ↔ denatured) transition.

pH values. RP-HPLC (Method 2, Table I) analyses then provided characteristic rhM-CSF degradation product profiles. RP-HPLC (Method 1, Table I) analysis also provided quantitative information on degradation product accumulation.

rhM-CSF samples were prepared at 1.2 mg/mL in acidic (pH 2) and alkaline (pH 9.5) buffers. These samples were stressed at 40°C and aliquots removed for RP-HPLC (Method 2) profiling. Figure 5 shows the degradation product profile under acidic conditions, and Figure 6 demonstrates the corresponding profile under alkaline conditions. Figure 6 shows intact rhM-CSF eluting at approximately 185 min, an early-eluting degradation product peak eluting at approximately 44 min, and a complex series of degradation product peaks eluting at approximately 110 min.

The relatively low RP-HPLC capacity factors for the degradation product peaks indicate hydrophilic species (relative to intact rhM-CSF). The complex peak pattern for the species eluting near 110 min indicates a high degree of microheterogeneity. Figure 1 shows that the variably O-linked glycosylation sites in rhM-CSF reside C-terminal to Val_{152}. The heterogeneous degradation product shown in Fig. 5 is therefore consistent with a highly glycosylated C-terminal peptide cleavage product.

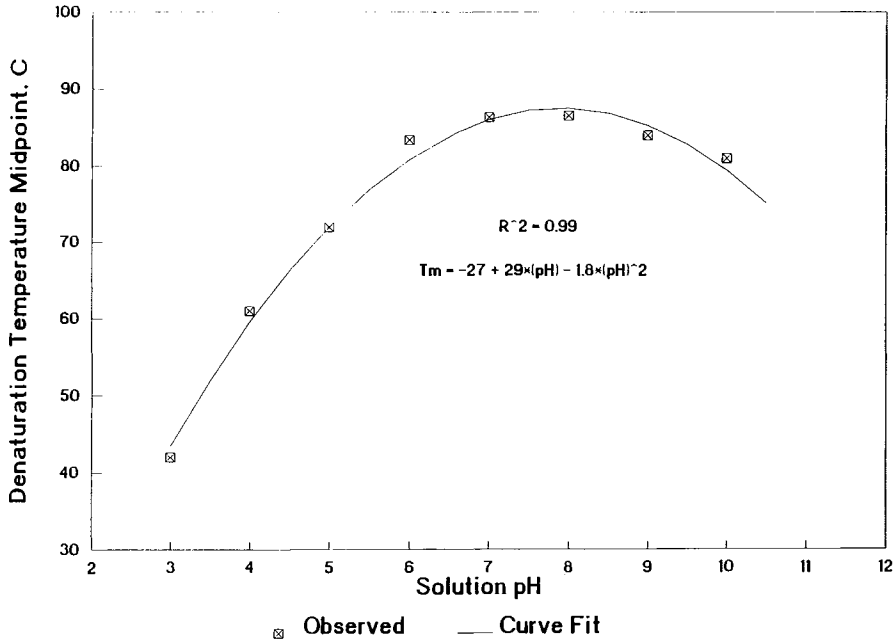

Fig. 3. M-CSF thermal denaturation midpoint temperature (T_m) values as a function of pH in PolyB buffer. Observed (points) data and calculated (solid line) best fit to quadratic equation.

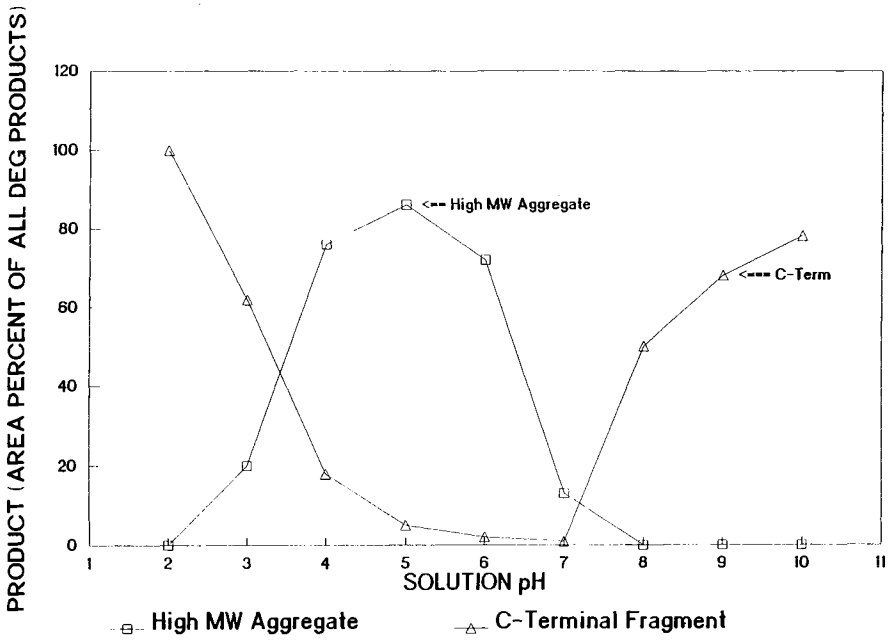

Fig. 4. Degradation product profile for M-CSF after thermal scanning to 95°C. RP-HPLC (Method 1) was used to assay the "C-terminal" fragment. HP-SEC was used to assay the aggregation product.

Under alkaline conditions, a distinctly different degradation profile emerges. As shown in Fig. 6, there is essentially no accumulation of an early-eluting (44-min retention) peak in the pH 9.5 samples. Furthermore, the major degradation product accumulating under alkaline conditions (elution near 110 min) lacks the detailed fingerprint pattern characteristic of the major degradation product formed in acidic solution. As evidenced by rather similar elution times, the major alkaline and acidic degradation products may share some structural features, but the detailed elution patterns indicate that, at least qualitatively, the two product species must be different.

Comparing Fig. 5 with Fig. 6 also suggests that total degradation product accumulation proceeds more rapidly under acidic conditions than at alkaline pH. To establish more quantitatively the pH dependence of rhM-CSF degradation product accumulation, we assayed stressed samples by RP-HPLC (Method 1). Chromatograms (not shown) obtained with RP-HPLC Method 1 do not provide detailed degradation product "fingerprinting" useful for qualitative structural assessment. Rather RP-HPLC Method 1 yields relatively narrow, unresolved degradation product peaks suitable for quantitation by area normalization. Figure 7 shows the C-terminal degradation product accumulation for stressed (40°C for 0 to 4 weeks) rhM-CSF solutions (0.5 mg/mL in 20 m*M* PolyB buffer) under acidic, neutral, and alkaline conditions.

Figure 7 reveals that a C-terminal cleavage product(s) does accumulate somewhat more rapidly in acidic solution compared with alkaline solution. Furthermore, degradation to a C-terminal cleavage product(s) proceeds very slowly at neutral pH. Thus, degradation product studies for isothermal stressing of rhM-CSF solution confirm the results of thermal scanning experiments wherein neutral pH appears optimal for rhM-CSF stability.

rhM-CSF Degradation Product Characterization

Although the predicted rhM-CSF monomeric polypeptide molecular weight is approximately 25 kD, the extensive glycosylation significantly increases the actual molecular weight. Consequently, dimeric rhM-CSF migrates on SDS-PAGE with an apparent molecular weight, M_{app}, of approximately 90 kD. To reconcile observed bands on SDS-PAGE analysis with degradation product structure, it is convenient to adopt the convention that the molecular weight is given by the M_{app} value of 90 kD. Thus, rhM-CSF is known (1) to be a disulfide-bridged homodimeric protein and electrophoreses with M_{app} = 45 kD on a reducing SDS-PAGE gel.

SDS-PAGE analysis (with and without sample reduction) provides structural information regarding apparent molecular weight and disulfide bridging architecture for proteins and breakdown products. Accordingly, we profiled stressed rhM-CSF solutions electrophoretically to supplement the information gained by chromatographic fingerprinting studies noted in the preceding section. In the first study, rhM-CSF samples were prepared at 0.5 mg/mL in pH 3, 5, and 10 (20 m*M* PolyB buffer) and stressed for 2 days at 40°C. Aliquots of these stressed samples were electrophoresed (reduced and nonreduced) versus −80°C control samples. Figure 8 shows the electropherograms for control and stressed samples. At pH 3, degradation is extensive after only 2 days at 40°C and the nonreduced gel shows a large band corresponding to a 50-kD species. At pH 5 and pH 10, the nonreduced gel shows that a 70-kD species begins to accumulate after 2 days of exposure to 40°C. Both the 90-kD intact rhM-CSF and the 70-kD bands appear at somewhat lower apparent molecular weights for the pH 10 samples than for the pH 5 samples, suggesting different degradation pathways for acidic versus alkaline conditions.

The differences between acidic and alkaline degradation

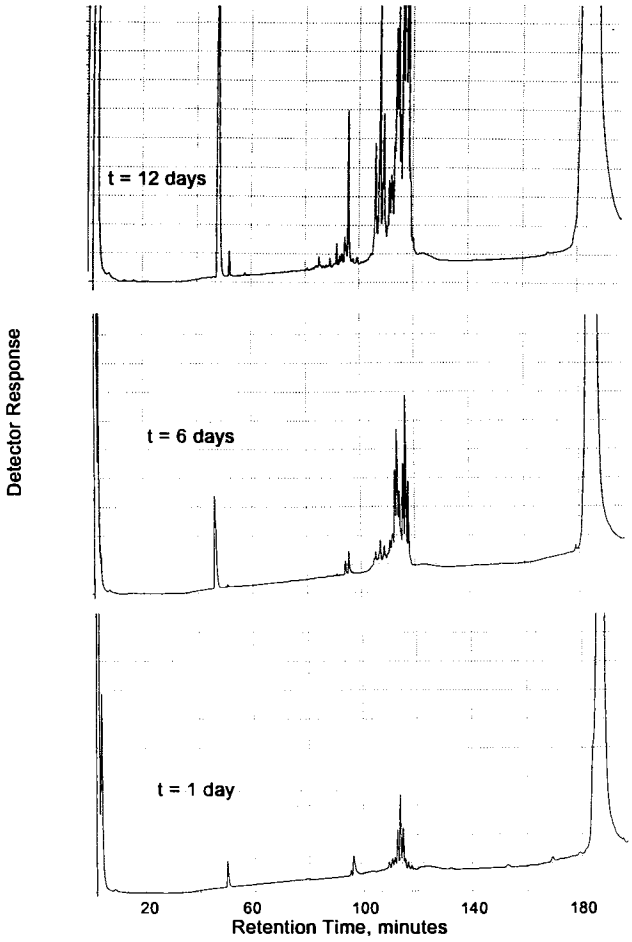

Fig. 5. RP-HPLC (Method 2, Table I) analyses of 1.2 mg/mL MCSF in acidic solution (pH 2) maintained for timed intervals at 40°C.

pathways are also evident in the gels run under reducing conditions. Here the pH 3 sample shows only a 25-kD species. At pH 5, 45-kD monomer (derived from intact rhM-CSF reduction) is the predominant species. For the pH 10 sample, two products are evident, namely, 45-kD monomer (from intact 90-kD rhM-CSF) and a 90-kD species that does not reduce in the presence of dithiothreitol. This nonreducible (NR) 90-kD species apparently arises from intermonomeric, nondisulfide covalent bond formation during storage at elevated temperature and alkaline pH.

Figure 9 shows the results of more extensive 40°C exposure for pH 5 and pH 10 samples. The nonreducing (lower set) gels in Fig. 9 show the time-dependent accumulation of 70-kD species and 50-kD species in the pH 5 samples. It is also apparent that intact 90-kD rhM-CSF gradually disappears and migrates at somewhat lower M_{app} at increasing exposure times for the pH 5 samples. The nonreducing gel for the pH 10 sample similarly shows 70- and 50-kD species formation concomitant with decreases in intact rhM-CSF bands. The reducing (upper set) gels in Fig. 9 show that the pH 5 samples contain tract amounts of NR 90-kD species at the initial timepoint. At 40°C, the pH 5 sample shows 45-kD monomer as the predominant species and a detectable 25-kD species at longer ($t \geq 4$ days) exposure intervals. The pH 10 samples also show 45- and 25-kD bands. Here again, a NR 90-kD band is evident and appears to break down further with increasing exposure interval.

rhM-CSF solutions (1.9 mg/mL in pH 6 citrate/saline buffer) were maintained for 6 weeks at 40°C followed by 2 weeks at 50°C. HP-SEC traces (not shown) of degraded samples demonstrated the accumulation of a low molecular weight (M_{app} = 20 kD by SDS-PAGE) species. This 20-kD species was isolated and structurally characterized by FAB-MS and N-terminal AA sequencing. The N-terminal AA sequence determined for the 20-kD species was PASVSPHQ-PLAPSMAPVA. Referring to Fig. 1, the demonstrated sequence begins at Pro_{170} and indicates that the peptide cleavage at the Asp_{169}-Pro_{170} position produces the 20-kD fragment. Although the N-terminal sequence identifies the cleavage position, the isolated 20-kD fragment structure remains ambiguous in the absence of complete or C-terminal sequence data. To conclusively establish the 20-kD fragment structure, FAB-MS was used to assign the mass to the isolated 20-kD fragment. Table II shows the mass spectral results and confirms that the isolated 20-kD fragment corresponds to the C-terminal Pro_{170}-Arg_{223} fragment with variable glycosylation. The additional mass assignments for methionine sulfoxide homologues could represent additional

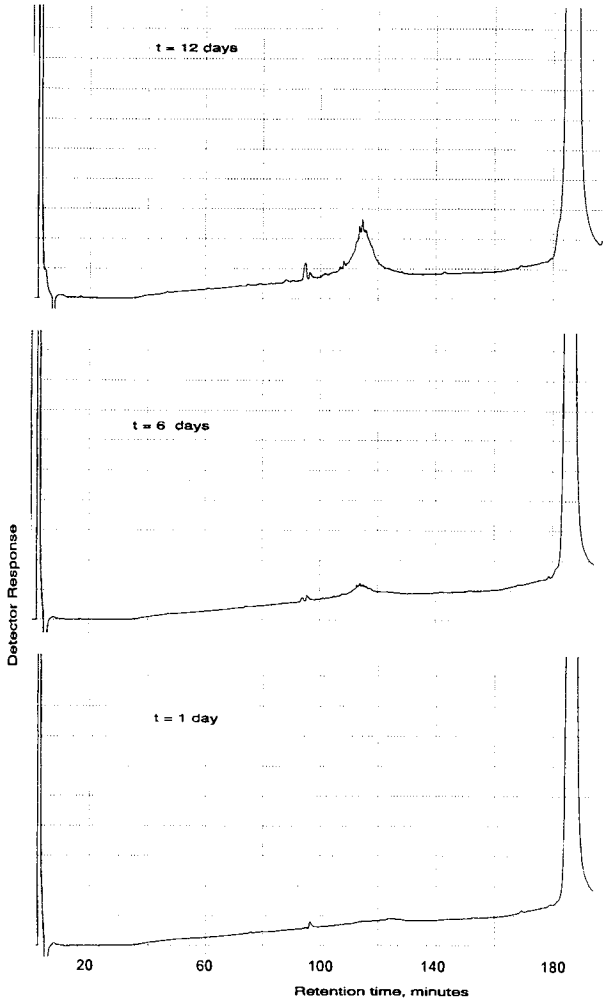

Fig. 6. RP-HPLC (Method 2, Table I) analyses of 1.2 mg/mL MCSF in alkaline solution (pH 9.5) maintained for timed intervals at 40°C.

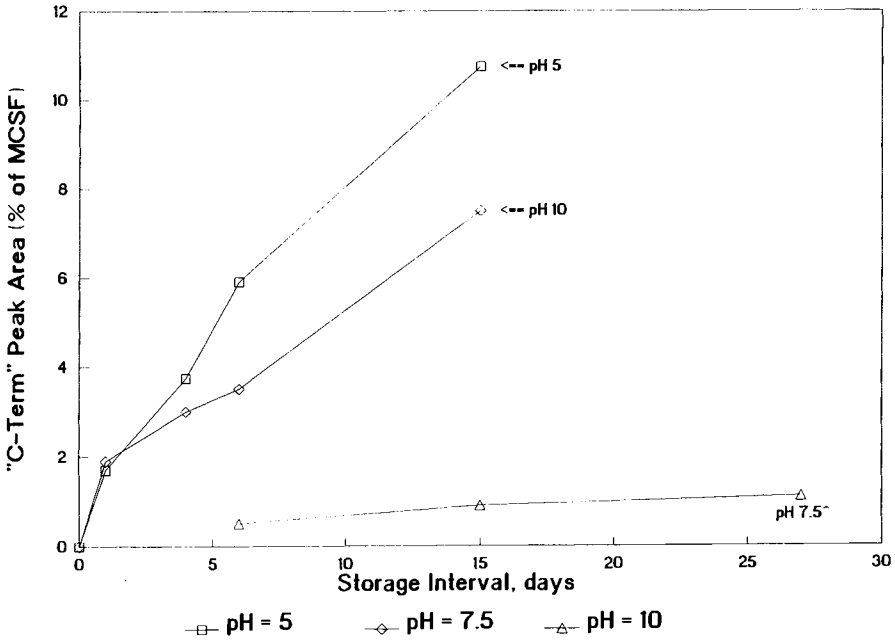

Fig. 7. RP-HPLC (Method 1, Table I) analysis of C-terminal degradation product in M-CSF solutions maintained for timed intervals at pH 5, 7.5, and 10 at 40°C. Results by area normalization relative to M-CSF peak.

oxidative conversions during stress testing or sample handling.

The multiple glycosylation pattern and AA sequence of the 20-kD fragment suggest that the 20-kD fragment corresponds to the multiple peaks eluting near 110 min in RP-HPLC (Method 2, Table I) assays (see Fig. 5). Figure 5 also shows an early-eluting degradation product peak at 44-min retention time. To establish the structure of this early-eluting peak, a 2.5 mg/mL rhM-CSF sample was prepared in pH 2 HCl buffer and stressed at 40°C for 2 weeks. The early-eluting peak was isolated by fraction collection and characterized by N-terminal AA sequencing. The amino acid determination showed the following sequence: PGSAKQRPPR.

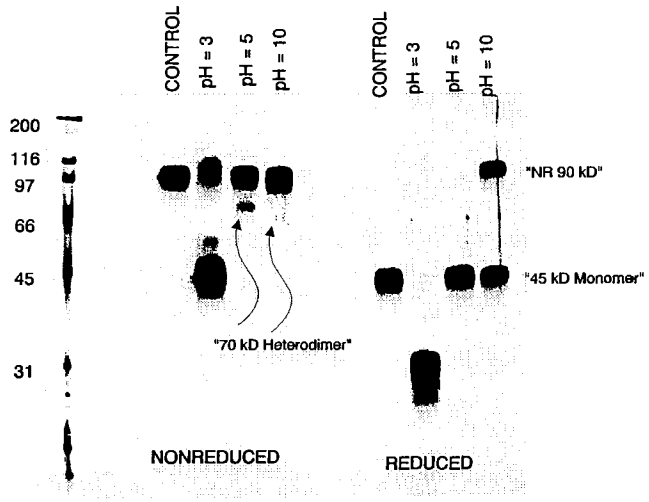

Fig. 8. SDS-PAGE analysis (reducing and nonreducing conditions) of M-CSF samples stressed at pH 3, 5, or 10 and maintained 2 days at 40°C. "NR 90 kD" represents a nonreducible 90-kD species.

Referring to Fig. 1 reveals that the observed AA sequence corresponds to the Pro_{214}-Arg_{223} decapeptide (produced by cleavage at Asp_{231}-Pro_{214}).

Although the foregoing conclusively identifies low molecular weight cleavage products as Pro_{170}-Arg_{223} and Pro_{214}-Arg_{223}, it remains to assign structures to the higher molecular weight (70- and 50-kD) fragments that also obtain on the basis of mass balance. To gain structural information, rhM-CSF solutions were prepared at 1.9 mg/mL in pH 6 citrate/saline and stressed at 40°C. The stressed samples were applied to semipreparative SDS-PAGE gels and the bands corresponding to 70- and 50-kD species (see Fig. 9) collected by electroelution.

The isolated species were then digested by *Achromobacter K*, a lysine C-specific endoproteinase (cleaves C terminal to each lysine). Referring to Fig. 1 shows 14 lysine residues in rhM-CSF (AA positions 44, 51, 52, 88, 93, 100, 116, 118, 125, 130, 137, 154, 163, and 218). Therefore, peptide maps of samples digested by *Achromobacter K* should show 15 fragments (K1, K2, and so forth, in sequence order beginning from the N terminus).

Figure 10 shows the peptide maps of intact rhM-CSF and the isolated 70-kD fragment. Figure 11 shows the corresponding map for the 50-kD species. In each map, individual RP-HPLC peaks have peptide assignments corresponding to the notations K1, K2, and so forth, as noted above. These assignments were made independently through isolating and sequencing peptides in the maps of intact rhM-CSF.

Note that the K14 peptide (Ala_{164}-Lys_{218}) in the intact rhM-CSF map demonstrates considerably heterogeneity, consistent with multiple and variable glycosylation sites (see Fig. 1). It is also noteworthy that the K14 fragment contains both the Asp_{169}-Pro_{170} and the Asp_{213}-Pro_{214} cleavage sites implicated in 20-kD and early-eluting degradation product

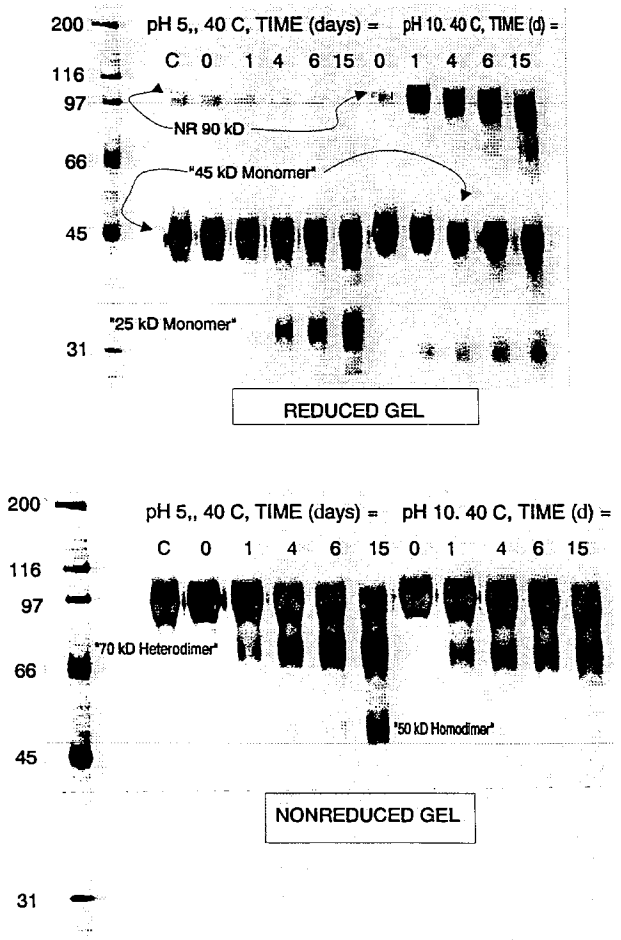

Fig. 9. SDS-PAGE analysis (reducing and nonreducing conditions) of M-CSF samples stressed at pH 5 or 10 and maintained at 40°C for 0 to 15 days. "NR 90 kD" represents a nonreducible 90-kD species.

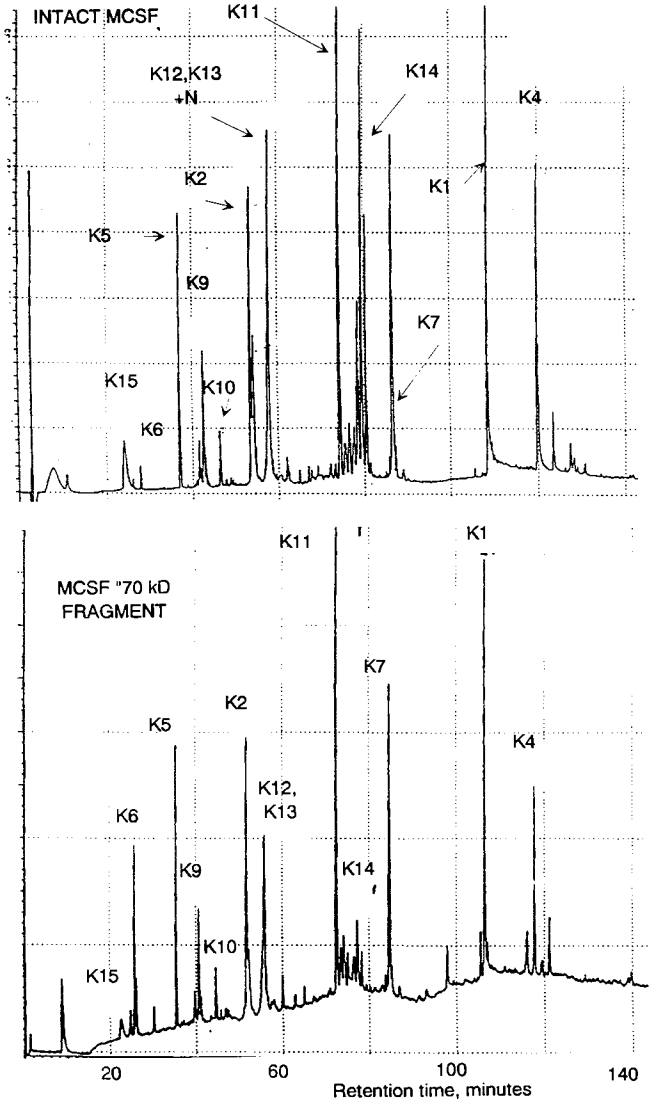

Fig. 10. Lysine C-specific proteinase peptide map of intact M-CSF and 70-kD fragment isolated from stressed sample (50°C, pH 6). Notice loss of K14 peptide in 70-kD map.

Table II. FAB-MS Analysis of 20-kD Fragment Isolated by HP-SEC from rhM-CSF, pH 6, Solution Maintained 6 Weeks at 40°C Plus 2 Weeks at 50°C

Exact mass of protonated molecular ion		
Theoretical	Observed	Assignment[a]
6300.9	6301.5	Pro_{170}-Ala-. . .Arg_{223} $(Gal\text{-}GalNAc)_2$
6316.9	6317.3	Pro_{170}-Ala-. . .Arg_{223} $(Gal\text{-}GalNAc)_2$ methionine sulfoxide
6666.2	6666.48	Pro_{170}-Ala-. . .Arg_{223} $(Gal\text{-}GalNAc)_3$
6682.2	6681.8	Pro_{170}-Ala-. . .Arg_{223} $(Gal\text{-}GalNAc)_3$ methionine sulfoxide

[a] $(Gal\text{-}GalNAc)_n$ represents galactose *N*-acetyl 1,4-galactosamine dimer occurring at $n = 2$ or 3 sites as indicated by the subscript.

formation. By inspection, it is clear that the 70- and 50-kD fragment peptide maps differ from the intact rhM-CSF map only insofar as the K14 peptide is present in the latter case, but greatly reduced in the first two cases. Thus, the 70- and 50-kD fragments do represent the N-terminal residues produced by cleavage at the Asp_{169}-Pro_{170} site.

Biological Activity Assessment of rhM-CSF And Degradation Products

To probe the consequences of rhM-CSF partial degradation on biological activity, we stressed rhM-CSF samples (1.9 mg/mL at pH 6) for 3.5 weeks at 50°C and isolated individual species by electroelution from semipreparative SDS-PAGE gels. The recovered samples were then assayed spectrophotometrically (280 nm) for total protein concentra-

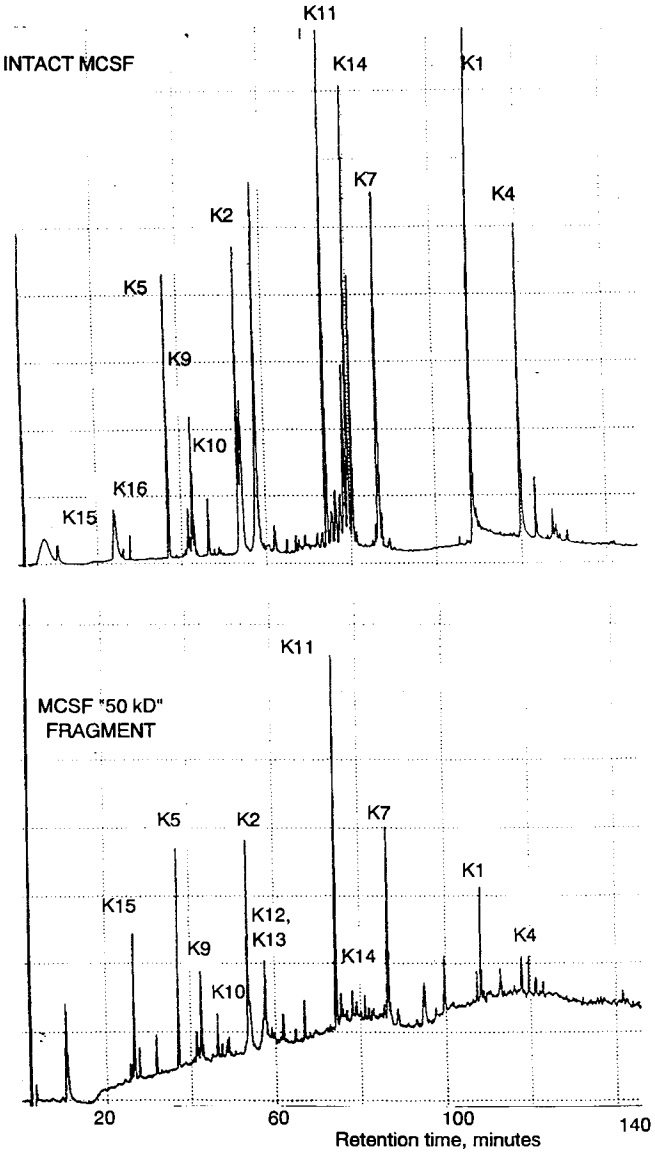

Fig. 11. Lysine C-specific proteinase peptide map of intact M-CSF and 50-kD fragment isolated from stressed sample (50°C, pH 6). Notice loss of K14 peptide in 50-kD map.

tion and screened for biological activity in the murine bone marrow colony formation assay (8). Table III shows the study results and demonstrates that both the 70- and the 50-kD species retain biological activity relative to intact 90 kD (after recovery by electroelution). The activities shown for these samples are equivalent within the limits of experimental uncertainty (approximately ±50%). As noted elsewhere (13), the 45-kD monomer (obtained by electroelution from a reduced gel) is inactive. Our findings are therefore consistent with literature observations (1, 4, 10, 11) that the C-terminal region (after approximately 150 amino acids) is not necessary to confer biological activity on rhM-CSF. Whether or not the C-terminal region is necessary to confer maximal specific activity, however, remains a subject for additional investigation.

Table III. Biological Activity Determinations of Intact rhM-CSF and Degradation Products

Species	Treatment[a,b]	Activity,[c] $\times 10^6$ U/ABS
Intact 90 kD[d]	Control	1.2
Intact 90 kD	Control	0.39
Intact 90 kD	Stressed	0.30
70 kD	Stressed	0.1
50 kD	Stressed	0.1
Monomer (45 kD)[e]	Stressed	0.0014

[a] Control samples stored at −80°C. Stressed samples were stored 3.5 weeks at 50°C.
[b] All samples electroeluted from nonreducing SDS-PAGE gels unless otherwise noted.
[c] Murine bone marrow colony assay; see Ref. 8. Activity values are normalized on the basis of spectrophotometric (280-nm) determinations of protein concentration in the tested samples.
[d] This sample was not electroeluted from SDS-PAGE.
[e] Electroeluted from reduced SDS-PAGE preparation.

DISCUSSION

rhM-CSF Structure and Nomenclature

Understanding rhM-CSF structural characteristics is a prerequisite for elucidating the protein degradation mechanisms. Using information gained from structural studies, we have developed a shorthand notation for the intact molecule and its putative degradation products.

Figure 12 is a schematic representation of intact rhM-CSF homodimer and putative degradation products as observed on reducing and non-reducing SDS-PAGE. As a convention, Figure 12 arbitrarily divides the rhM-CSF monomer at $AA_{159\text{-}160}$ to indicate two fragments with M_{app} = 25 kD (N-terminal fragment) and M_{app} = 20 kD (C-terminal fragment). Comparing Fig. 1 with Fig. 12 shows that all nine cysteines and six of the seven O-linked glycosylation sites reside N-terminal to Cys_{159}. Note also that the rhM-CSF amino acid sequence features three different Asp-Pro ("D-P" using single character abbreviations) couples at amino acid positions $AA_{45\text{-}46}$, $AA_{169\text{-}170}$, and $AA_{213\text{-}214}$.

According to Fig. 12, intact rhM-CSF migrates as a 90-kD homodimer on a nonreducing gel and as a 45-kD monomer under reducing conditions that cleave an intermonomeric disulfide bridge(s). Assuming cleavage under stress conditions between the 25-kD N-terminal and the C-terminal segments in one monomeric protein chain, nonreducing SDS-PAGE should show a heterodimeric 70-kD species and a C-terminal piece. Under reducing conditions, the C-terminal piece, a 25-kD N-terminal fragment, and a 45-kD monomer should be evident.

Additional stress yielding cleavage between the 25-kD N-terminal and the C-terminal segments of both monomeric protein chains should reveal a band with M_{app} = 50 kD on nonreducing SDS-PAGE and smaller fragments under reducing conditions.

The following paragraphs specifically reconcile the putative degradation mechanism outlined in Fig. 12 with profiling and structural characterization data presented under Results.

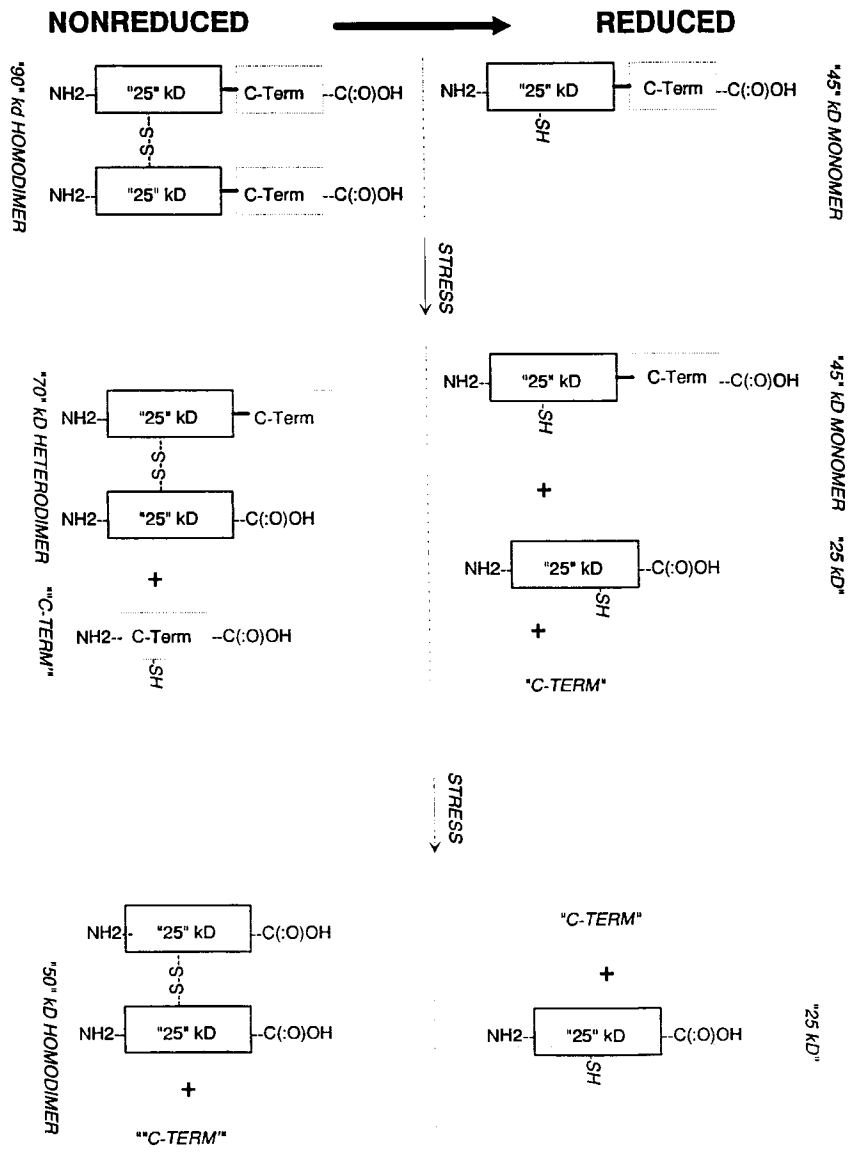

Fig. 12. Shorthand notation for putative M-CSF degradation species showing apparent molecular weights under reducing and nonreducing SDS-PAGE.

Proposed Degradation Mechanism(s)

Electrophoretic profiling and the chromatographic fingerprints establish three general characteristics of the rhM-CSF degradation mechanism. First, rhM-CSF degradation proceeds faster under acidic conditions than under alkaline conditions (see, e.g., Figs. 4 and 7).

Second, rhM-CSF degradation products follow similar, but distinctly different pathways under acidic versus alkaline conditions. At both pH extremes, initial cleavage occurs near Cys_{159} in a single monomeric segment to produce 70-kD heterodimer (see Fig. 8) and 20-kD monomer. Extended exposure to stress conditions cleaves at the second monomeric segment to yield a 50-kD homodimer in addition to a 20-kD monomer (see Fig. 9). Although the general degradation patterns are similar at pH extremes, detailed fingerprints and apparent molecular weights of cleavage products differ for acidic versus alkaline conditions (e.g., compare Figs. 5 and 6). These results suggest that cleavage occurs at different peptide bonds and via different mechanisms in acidic versus alkaline conditions.

Third, alkaline conditions contribute to a degradation pathway not available under acidic conditions, namely, nonreducible 90-kD species accumulation via covalent, non-disulfide intermonomeric bridge formation (Figs. 9 and 10).

Specifically, under acidic conditions and at temperatures below T_m, rhM-CSF degrades via hydrolytic cleavage at Asp_{169}-Pro_{170} and Asp_{213}-Pro_{214} bonds. As shown in Fig. 12, a single Asp_{169}-Pro_{170} cleavage per rhM-CSF dimer yields the 20-kD, highly glycosylated, Pro_{170}-Arg_{223} fragment and the 70-kD heterodimer. A second Asp_{169}-Pro_{170} cleavage per rhM-CSF dimer yields a second Pro_{170}-Arg_{223} fragment and the 50-kD homodimer. Acid hydrolysis of rhM-CSF also produces the decapeptide, Pro_{214}-Arg_{223}. The

available data, however, cannot define whether Asp_{213}-Pro_{214} heterolysis proceeds via intact rhM-CSF or via the Pro_{170}-Arg_{223} primary degradation product (or both).

The literature documents (31–34) that the Asp-Pro sequence is particularly sensitive to hydrolytic scission under acidic conditions. Interestingly, our studies do not reveal cleavage at the third Asp-Pro sequence in rhM-CSF (Asp_{45}-Pro_{46}) under conditions that extensively cleave the other Asp-Pro couples. Presumably the Asp_{45}-Pro_{46} sequence is buried in a tightly folded protein region, whereas the Asp_{169}-Pro_{170} and Asp_{213}-Pro_{214} pairs reside in exposed topographical locations.

Concerning rhM-CSF degradation under alkaline conditions, our understanding of underlying mechanisms is incomplete. Certainly, markedly different degradation pathways are available to rhM-CSF under alkaline versus acidic conditions. Compared with degradation under acidic conditions, degradation under alkaline conditions gives (i) slower degradation rates, (ii) different (albeit uncharacterized) "C-terminal" degradation products, and (iii) significant accumulation of nonreducible 90-kD protein. Although it remains speculative, we propose that degradation under alkaline conditions predominantly follows a β-elimination pathway.

Disulfide-bridged cysteines, for example, are known (34–36) to undergo base-catalyzed elimination. The initial product appears to be an unsaturated dehydroalanine that subsequently either cleaves via hydrolysis or cross-links via condensation with ε-amino groups (e.g., on lysine). Specifically for rhM-CSF, β-elimination from disulfide bridged Cys_{157}-Cys_{159} could yield an approximately 20-kD C-terminal fragment (different from the Pro_{170}-Arg_{223} observed under acidic hydrolysis) *and* nonreducible 90-kD rhM-CSF subsequent to intermonomeric reactions with ε-amino groups.

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