Structural comparison of recombinant human macrophage colony stimulating factor β and a partially reduced derivative using hydrogen deuterium exchange and electrospray ionization mass spectrometry

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Abstract

Hydrogen deuterium exchange, monitored by electrospray ionization mass spectrometry, has been employed to characterize structural features of a derivative of recombinant human macrophage colony stimulating factor beta (rh-m-CSF β) in which two of the nine disulfide bridges (Cys157/Cys159–Cys'157/Cys'159) were selectively reduced and alkylated. Removal of these two disulfide bridges did not affect the biological activity of the protein. Similarities between CD and fluorescence spectra for rh-m-CSF β and its derivative indicate that removing the disulfide bonds did not strongly alter the overall three-dimensional structure of rh-m-CSF β . However, differences between deuterium exchange data of the intact proteins indicate that more NHs underwent fast deuterium exchange in the derivative than in rh-m-CSF β . Regions located near the disulfide bond removal site were shown to exhibit faster deuterium exchange behavior in the derivative than in rh-m-CSF β .

Keywords: Macrophage colony stimulating factor β ; disulfide bond; hydrogen deuterium exchange; electrospray ionization mass spectrometry

A correctly folded protein is fundamental in the mechanism and control of a wide range of cellular processes. A thorough understanding of the complex processes of protein folding requires the identification of cooperative interac-

tions among various folding events. This is intrinsically difficult, because these events take place quickly and the transient intermediates cannot be readily obtained. However, in disulfide-coupled folding processes folding intermediates can be isolated by selective reduction of disulfide bonds followed by alkylation of free cysteine residues. In this way, intermediates can be structurally characterized using a variety of physical techniques such as X-ray crystallography, NMR, CD, and fluorescence spectroscopy. The recent development combining hydrogen deuterium exchange and electrospray ionization mass spectrometry has provided an additional tool for the characterization of protein-folding intermediates (Zhang and Smith 1993). It is a nonperturbing, sensitive method that offers sufficient structural and temporal resolution for identifying conformational changes resulting from ligand binding (Kragelund et al. 1995; Wang et al. 1997) and chemical modifications (Resing and Ahn 1998).

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Abbreviations: aa, amino acid; CD, circular dichroism; CDAP, 1-cyano-4-dimethylaminopyridinium fluoroborate; CID, collision-induced dissociation; CN, cyanylation; rh-m-CSF β , recombinant human macrophage stimulating factor β ; cDNA, complementary DNA; *E. coli*, *Escherichia coli*; H/D-ESI-MS, hydrogen deuterium exchange electrospray ionization mass spectrometry; kD, kilodaltons; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; TCEP, Tris(2-carboxyethyl)phosphine.

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Macrophage colony stimulating factor is the principle regulator of monocyte and macrophage development (Stanley et al. 1997). The interaction of m-CSF and its receptor, m-CSFR, induces proliferation of fibroblasts, or of hematopoietic cells, which results in differentiation towards the monocyte/macrophage lineage of myeloid progenitor cell lines (Bourette and Rohrschneider 2000). The recombinant human m-CSF β was expressed from *Escherichia coli* as a truncated form (aa 4–218, 49 kD) of one of the three human cDNA clones (Pandit et al. 1992), missing 340 aa from the precursor. Another form of m-CSF, named m-CSF α , can be obtained by cDNA cloning. In the membrane-bound form, m-CSF α and β are identical in their N- and C-terminal regions, but m-CSF β contains inserts at residue 149 as a result of alternative mRNA processing (Pandit et al. 1992). The N-terminus (aa 1–149) contains the receptor-binding domain, and the C-terminus contains a transmembrane region that is believed to anchor m-CSF in an active, cell-associated form. It has been proposed that m-CSF is expressed in variable lengths from a single gene to provide membrane-associated m-CSF with variable length spacers to allow direct contact with receptors on neighboring cells (Cerretti et al. 1988). In the membrane-bound form of m-CSF β , aa 150–221 is part of the spacer that connects the receptor-binding domain (N-terminus) to the transmembrane domain (C-terminus). This agrees with previous experiments that indicated that the C-terminal region in m-CSF β (aa 150–221) is not important for the protein's biological activity (Pandit et al. 1992).

M-CSF β is biologically active only in the homo-dimeric form, and contains three inter- and six intramolecular disulfide bridges (Fig. 1). The systematic reduction of the disulfide bonds in rhm-CSF β can reveal the specific role that

each disulfide bond plays in maintaining the overall protein structural integrity as well as the biological activity. The disulfide bridges were assigned using enzymatic digestion and fast atom bombardment mass spectrometry (Pandit et al. 1992; Glocker et al. 1993). Two intermolecular disulfide bonds in the C-terminal region were found between Cys157/159 and Cys'157/159. It is unclear whether Cys157 forms a disulfide bond with Cys'157 or Cys'159. Nonetheless, it is evident that these cysteine residues form symmetrical disulfide bonds that are in close proximity in three-dimensional space. The present study focuses on the effect of removing Cys157/159–Cys'157/159 on the three-dimensional structure of rhm-CSF β using the H/D-ESI-MS approach.

Results

Selective reduction of Cys157/159–Cys'157/159 in rhM-CSF β was achieved at pH 3.5. Low pH condition was necessary to avoid disulfide bond scrambling. The reduced cysteine thiols were then alkylated with CDAP at pH 3.5. In contrast to what was reported in the literature (Wu and Watson 1997), TCEP was found to interfere with cyanylation, and must be removed prior to adding CDAP to the partially reduced protein. Chromatographic separation by RP-HPLC provided a homogenous species whose molecular weight was determined to be 49,130 Daltons (Fig. 2). The partially reduced cyanylated protein has an MW of 100.6 Daltons greater than rhM-CSF β (49,029.4 Daltons), a mass increase that corresponds to an incorporation of four cyano groups. The charge state distributions in ESI-MS for the native and the CN^{157,159}-modified rhm-CSF β are different (Fig. 2). The native protein shows a distribution of charges ranging from 31+ to 20+, with 21+ being the most intense peak. In contrast, the modified protein gives a distribution of higher charges with a maximum at 32+.

To identify the sites of cyanylation, the modified protein was subjected to base hydrolysis (Wu and Watson 1997), which cleaved the N-terminal peptide bonds of the cyanylated cysteinyl residues and formed a cyclic ring structure. After cleavage at the cyanylated cysteinyl residues, the truncated peptide chains still linked by the remaining disulfide bonds were reduced with excess TCEP. Mass spectra indicated that polypeptides with masses of 6517.5 and 17,840.8 Daltons were present (Fig. 3). After complete reduction with TCEP, the nascent thio groups were alkylated with iodoacetamide. The mass spectra indicated there were species with molecular weights of 6518 and 18,239.4 Daltons (Fig. 3). By comparing the predicted and experimental masses, the polypeptide with mass of 6517 Daltons was identified as aa 159–221 with Cys¹⁵⁹ cyanylated. The polypeptide with a mass of 17,840.8 Daltons was identified as aa 4–156 with no alkylation, and the polypeptide with a mass of 18,239.4 was determined to be aa 4–156 with seven cysteinyl residues carboxyamidomethylated. Peptide frag-

Fig. 1. Schematic representation of rhm-CSF β based on X-ray crystal structure of m-CSF α (Pandit et al. 1992). The C-terminal strands represented by the dotted lines have been drawn in. No X-ray data is available because no crystals of rhm-CSF β have been produced.

Fig. 2. Mass spectrum of rhm-CSF β (A) MW rhm-CSF β = 49,029.4 Daltons and CN^{157,159}-modified rhm-CSF β (B) MW CN^{157,159}-modified rhm-CSF β = 49,130.0 Daltons determined by ESI-MS.

ment aa 157–158 was not found; however, cyanylation must have taken place at Cys 157 based on the MW of the intact, modified protein. These results indicated that Cys 157 and 159 had been cyanylated, and that the disulfide bonds linking these cysteinyl residues in the dimer had been reduced. The tryptic digestion of the completely reduced and carboxyamidomethylated protein further verified this conclusion. CID MS/MS experiments confirmed that Cys 157 and Cys 159 were the only cyanylated cysteinyl residues, and all other cysteines were carboxyamidomethylated.

The m-CSF-dependent NFS-60 assay was used to compare the specific biological activity of the modified protein to that of rhm-CSF β . The doubling time of cell growth stimulated by CN^{157,159}-modified rhm-CSF β was identical to that of cell culture stimulated by rhm-CSF β (Fig. 4A). Thus, the removal of Cys157/159–Cys'157/159 did not affect the biological activity of rhm-CSF β .

The CD spectrum for CN^{157,159}-modified rhm-CSF β closely resembles that of rhm-CSF β , which exhibits a positive maximum near 190 nm and a negative minimum near 208 nm (Fig. 4B). In contrast, the denatured rhm-CSF β has a spectrum reflecting primarily a random coiled structure. These CD spectra indicate the secondary structure of the α -helical bundle is maintained in the CN^{157,159}-modified rhm-CSF β .

The fluorescence emission spectrum for CN^{157,159}-rhm-CSF β shows the same maximum at 353 nm as the native

protein, while the emission spectrum of the denatured protein exhibits an increase in intensity and a slight red shift to 355 nm (Fig. 4C). A shoulder at 390 nm is also observed, with the CN^{157,159}-rhm-CSF β having an intensity between that of the native and denatured proteins. It is thus concluded that little structural change resulted from the chemical modification of the disulfide bonds in the modified protein.

To probe detailed structural changes caused by removing Cys157/159–Cys'157/159, both CN^{157,159}-modified rhm-CSF β and rhm-CSF β were incubated in D₂O for 4 sec to 70 h. Deuterium exchange in both proteins increased with incubation time until approximately 320 of the NHs had been replaced with deuterium (Fig. 5). The increase in deuterium incorporation with incubation time was fitted to Equation 2 to estimate rate constants for isotope exchange at peptide amide linkages (Table 1). After rounding off to whole numbers, these data reveal that the removal of Cys157/159–Cys'157/159 resulted in an increase of 19 very fast exchanging NHs ($k > 10 \text{ min}^{-1}$), a decrease of 10 fast exchanging NHs ($k \sim 1 \text{ min}^{-1}$), a decrease of five slow exchanging NHs ($k \sim 0.01 \text{ min}^{-1}$), and a decrease of four very slow exchanging NHs ($k < 0.001 \text{ min}^{-1}$). The shift in population in these rate categories indicates a general increase in the rate of deuterium exchange as the disulfide bonds are removed, resulting in a looser three-dimensional structure of the protein.

Fig. 3. (A) Mass spectrum of polypeptide aa 4–156 (MW 17,840.8 Daltons) after base hydrolysis and reduction. (B) Mass spectrum of polypeptide aa 4–156 after complete reduction and carboxyamidomethylation (MW 18,239.4 Daltons, CAM^{7.31,48,90,102,139,146}). (C) Amino acid sequence of the monomeric unit of rhm-CSF β including fragments covering aa 4–156 and aa 159–221 (MW 6517.5 Daltons, CN¹⁵⁹). Basic amino acid residues are highlighted in red.

To locate the specific regions where H/D exchange was affected after the removal of Cys157/159–Cys'157/159, deuterium-labeled CN^{157,159}-modified rhm-CSF β and rhm-CSF β were digested by pepsin prior to mass spectrometric analysis. Peptic digestion produced 45 and 38 overlapping peptides which cover >90% of the backbone in CN^{157,159}-modified rhm-CSF β and rhm-CSF β , respectively. The deuterium incorporation in each peptide was determined by analyzing the doubly charged ions. The change in deuterium incorporation with incubation time was fitted to Equation 2 to estimate rate constants for individual peptides (Table 2).

Local structural differences between the two proteins can be identified by comparing the extent and rate of deuterium incorporation for corresponding peptides. As seen in the intact proteins, most of the peptides from the modified pro-

tein exhibit similar deuterium exchange behavior as those in the native protein. This is particularly true in the N-terminal region (aa 4–149) where exchange kinetics of peptides CN^{157,159}-modified rhm-CSF β are identical to those in rhm-CSF β . For example, deuterium exchange in the segment covering aa 4–19 (Fig. 6A) was essentially the same for both native and modified rhm-CSF β (Table 2). Therefore, the deuterium exchange rates of NHs in this segment are independent of whether the disulfide bonds linking Cys157/159–Cys'157/159 are present. Similar analyses were done for other segments covering aa 4–143 derived from both proteins. Because no significant difference in amide hydrogen exchange rates was detected in this region, the results suggest that removing the disulfide bonds had little effect on the structure in the N-terminal region.

Fig. 4. (A) Biological activity of CN^{157,159}-modified rhm-CSFβ and rhm-CSFβ determined by M-NFS-60 cell assay. Doubling time was determined to be 14.9 h for rhm-CSFβ and 15.08 h for CN^{157,159}-modified rhm-CSFβ. (B) CD spectra of rhm-CSFβ, CN^{157,159}-modified rhm-CSFβ and denatured rhm-CSFβ in 10 mM ammonium phosphate buffer at pH 6.8. (C) Fluorescence emission spectra of rhm-CSFβ, CN^{157,159}-modified rhm-CSFβ, and denatured rhm-CSFβ in 10 mM ammonium phosphate buffer at pH 6.8 and excitation at 290 nm.

Although peptides from the N-terminus exhibit similar deuterium exchange profile, peptides from the C-terminus (aa 151–221) exhibit different exchange behavior in the modified protein relative to the native one. This is not surprising, because Cys157 and Cys159 are located in this region. The deuterium exchange in peptic peptides upstream of Cys157/159–Cys'157/159 (aa 151–160) in CN^{157,159}-rhm-CSFβ was fitted to Equation 2. This exact peptide was not found in the peptic digest of rhm-CSFβ. However, information on deuterium incorporation in this segment can be deduced by analyzing two segments covering aa 151–189 and aa 160–189. The difference between deuterium incorporation for these two segments yields information regarding deuterium exchange of NHs in the peptide covering aa 152–160 in rhm-CSFβ (Table 3). The number for slow

exchanging NHs in the hypothetical peptide was assigned 0 because the actual difference is –0.2, which has no meaning. It is significant that the deuterium exchange pattern in this segment of rhm-CSFβ is different from the same segment derived from CN^{157,159}-rhm-CSFβ. Most importantly, about three amide hydrogens that exchanged slowly in rhm-CSFβ became fast exchanging in the modified protein, thereby showing important local structural changes resulted from removing Cys157/159–Cys'157/159.

In the region downstream of Cys157/159–Cys'157/159, nearly all NHs underwent fast isotope exchange in both proteins, suggesting very little protection against deuterium exchange. Also, deuterium content did not change much over time, indicating that penetration of solvent was relatively unhindered. In addition, peptides derived from the

Fig. 5. Deuterium content in rhm-CSF β and CN^{157,159}-modified rhm-CSF β are shown as a function of the exchange time (4 sec to 70 h).

CN^{157,159}-modified rhm-CSF β showed only a slightly higher population of very fast and fast exchanging NHs than in rhm-CSF β (Table 2). For instance, the deuterium levels in peptide 190–221 of the modified protein were found to be greater than the same segment found in rhm-CSF (Fig. 6B), suggesting that at least some part of the structure of the former is more open. However, the difference is quite small, indicating that the overall three-dimensional structure in this area is not significantly different for the two proteins.

Discussion

Clearly, the overall three-dimensional integrity of the native and modified rhm-CSF β has not been altered by reducing Cys157/159–Cys'157/159, as suggested by the unchanged biological activity, and the similarities in CD and fluorescence spectra in the two protein forms. However, structural differences between the native and the modified rhm-CSF β were revealed by the charge state envelopes in the ESI-MS spectra of the intact protein forms (Fig. 2). The distribution of the charge-state centers on 21+ in native rhm-CSF β and 32+ in the modified protein. There are 11 extra charges on the modified protein relative to the native protein. The difference in charge states between the two protein forms suggests that five more basic sites became accessible in each unit of the dimer upon reduction of the disulfide bond. There are four basic residues (K¹⁶³, H¹⁷⁶, H²¹⁰, and K²¹⁸) in the C-terminus downstream of Cys157 and Cys159 (Fig. 3C). The H/D exchange data in peptides aa 160–189 and aa 190–221 (Table 2) indicate that this region is accessible to solvent in both proteins forms. However, according to the numbers of hydrogens with very fast rates of exchange, there appears to be somewhat greater solvent penetration in

the modified protein in this region, suggesting that some residues become more accessible to protonation. Thus, for example, K¹⁶³ and even H¹⁷⁶ are located close enough to Cys157/159–Cys'157/159 to perhaps be relatively inaccessible to protonation in the native form, but not after reduction of the disulfide bond. An additional basic site, i.e., K¹⁵⁵, immediately upstream of Cys157 and Cys159, can reasonably be expected to become much more available for protonation after reduction of the disulfide linkage. X-ray data of the rhm-CSF α (same as the N-terminal region of rhm-CSF β ; Pandit et al. 1992) indicate also that R¹⁰⁴, located in the β_2 strand (Fig. 1), may become exposed to solvent as the C-terminal region becomes unstructured. Thus, the differences in charge states between the two protein forms could easily result from greater accessibility of additional basic sites in the modified protein as the local structure in the region downstream of Cys157/159–Cys'157/159 is removed. It is also possible that under the conditions of the LC-MS experiment (H₂O/ACN, pH 2), the modified protein is further destabilized, resulting in greater solvent accessibility and a higher degree of charging.

Hydrogen/deuterium exchange experiments also indicated observable structural changes that had taken place upon the removal of Cys157/159–Cys'157/159. As a result of eliminating the disulfide bonds, nearly 10 fast exchanging NHs ($k \sim 1 \text{ min}^{-1}$), five slow exchanging NHs ($k \sim 0.01 \text{ min}^{-1}$), and four very slow exchanging NHs ($k < 0.001 \text{ min}^{-1}$) in the native protein became very fast exchanging in the modified protein. This shift indicates a general increase in the accessibility of exchangeable sites in a looser regional structure in the modified rhm-CSF β . Detailed structural features of the C-terminal region of rhm-CSF β are not known because no crystals of rhm-CSF β have been produced. The present study provides a significant structural description of this region. The similarities of CD spectra indicate that the α -helical bundle core is not disturbed by the removal of Cys157/159–Cys'157/159. The fluorescence emission spectra for the CN^{157,159}-modified rhm-CSF β and the native protein are similar, indicating that the environment of the Trp residues was similar in the two proteins. Interestingly, Trp¹⁹¹ is located on the C-terminus of Cys157/159–Cys'157/159. Apparently, the modification had a negligible effect on the fluorescence of this residue, suggesting Trp¹⁹¹ may already be exposed to solvent in the native protein. In support of this conclusion, there is only a slight increase in the number of very fast and fast exchanging NHs in peptides covering aa 160–189 and aa 190–221 in the modified protein relative to the native one (Table 2). However, significant differences in deuterium exchange behavior are observed for the peptide aa 151–160 where three slow exchanging NHs in native rhm-CSF β became fast exchanging in the modified protein. These data suggest that portions of the C-terminal region (aa 151–221) are affected to various degrees by removing Cys157/159–Cys'157/159. As

Table 1. Distribution of rate constants for isotope exchange at peptide amide linkages in rhm-CSFβ and CN^{157,159}-modified rhm-CSFβ

	NHs	Number of amide hydrogen			
		k > 10 min ⁻¹	k ~1 min ⁻¹	k ~ 0.01 min ⁻¹	k < 0.001 min ⁻¹
rhm-CSFβ	404	153.3 ± 5.4	57.3 ± 5.9	50.7 ± 4.6	142.7 ± 15.9
CN ^{157,159} -rhm-CSFβ	404	172.6 ± 4.0	47.4 ± 3.9	45.9 ± 2.7	138.1 ± 10.6

Cys157/159–Cys'157/159 linkages are eliminated, the amino acids upstream of this site (aa 151–160) exhibit more significant exchange behavior than those downstream (aa 160–221). It is possible that Cys157/159–Cys'157/159 are placed relatively close to the protein α-helical core (Fig. 1). Upon the removal of these disulfide bonds, the region (aa 151–160) whose structure is maintained by Cys157/159–Cys'157/159 becomes flexible, and exhibits a different deuterium exchange behavior. The region that is already flexible (aa 160–221) prior to the chemical modification is not strongly affected by the modification as supported by the unchanged fluorescence property of Trp¹⁹¹ as well as the peptide deuterium exchange data. Deuterium exchange data in the region of aa 142–151 was not available because the peptides(s) involving these NHs were not detected in the digest of either protein from. It is likely that this region of the protein exhibits significantly different deuterium exchange behavior in the two forms as the local structure

Table 2. Distribution of rate constants for isotope exchange at peptide amide bonds in rhm-CSFβ (N) and CN^{157,159}-modified rhm-CSFβ (M) (k, min⁻¹)

Segment	Possible NHs	Very fast k > 10	Fast 1 > k > 0.1	Slow 0.1 > k > 0.01	Very slow k < 0.001
4–19 (N)	15	10.3	0.4	0.7	3.6
(M)		10.3	0.6	0.7	3.4
37–55	17	5.8	2.4	3.2	5.6
		5.8	3.3	2.6	5.3
56–62	6	0.7	1.3	1.2	2.8
		0.4	1.5	1.2	2.9
63–76	12	5.2	1.2	3.0	2.6
		4.8	1.4	3.5	2.3
77–82	5	0.9	0.4	1.2	2.5
		0.9	0.6	1.2	2.3
83–105	22	5.3	5.0	5.0	6.7
		5.2	5.8	5.3	5.7
106–113	6	1.7	0.8	1.2	2.3
		1.5	0.9	0.8	2.8
114–143	29	11.7	6.0	2.9	8.4
		11.7	5.8	4.1	7.4
160–189	22	21.1	0.3	0.4	0.2
		21.8	0.2	0	0
190–221	27	24.6	0.2	0.5	1.7
		25.9	1.1	0	0

becomes exposed to solvent. As demonstrated in this study, structural changes that are not readily detected by optical methods can be identified by ESI charge state distributions and localized by hydrogen/deuterium exchange experiments.

Fig. 6. (A) Deuterium content found in segment covering aa 4–19 rhm-CSFβ and CN^{157,159}-modified rhm-CSFβ are shown as a function of the exchange time (4 sec to 40 h). (B) Deuterium content found in segment covering aa 190–221 in rhm-CSFβ and CN^{157,159}-modified rhm-CSFβ are shown as a function of the exchange time (4 sec to 40 h).

Table 3. Distribution of rate constants for isotope exchange at peptide amide bonds in aa151–160 in rhm-CSFβ and hypothetical peptide aa152–160 in CN^{157,159}-modified rhm-CSFβ (*k*, min^{−1})

Segment	Possible NHs	Very fast <i>k</i> > 10	Fast 1 > <i>k</i> > 0.1	Slow 0.1 > <i>k</i> > 0.01	Very slow <i>k</i> < 0.001
rhm-CSFβ 151–189	30	25.1	0.3	0.2	4.4
rhm-CSFβ 160–189	22	21.1	0.3	0.4	0.2
rhm-CSFβ ^a 152–160	8	4.0	0	0 ^b	4.2
CN ^{157,159} -151–160	8	6.30	0.4	0.5	0.8

^a This hypothetical peptide includes the exact same amide hydrogens as in CN^{157,159}-aa151–160 due to the loss of N-terminal NH in the latter. The number of NHs in each category is deduced by subtracting the number of NHs in aa160–189 (row 2) from the number of NHs in aa151–189 (row 1) within the same exchange rate categories.

^b The number given by subtraction is −0.2 and has no meaning. It has been assigned 0.

Conclusions

The present study suggests that disulfide bonds linking Cys157/159 and Cys'157/159 play only a minor role in maintaining the three-dimensional structure in the C-terminus and no role at all in preserving biological activity. An important question then arises: because these residues are in a spacer that connects the receptor-binding domain to the transmembrane domain, do the disulfide bonds play any role in the folding pathway of rhm-CSFβ? They might, of course, help direct proper folding, but most likely they help to stabilize the folded structure rather than introduce structure into the polypeptide chain as the protein folds. It is possible that they may provide additional stability for the proper protein conformation in the final stage of folding.

A previous study reported the characterization of a stable dimeric intermediate isolated from the *in vitro* folding reaction of rhm-CSFβ (Maier et al. 1999). This refolding intermediate contains seven disulfide bonds and four carboxyamidomethylated cysteinyl residues. In addition, its charge state distribution closely resembles that of the CN^{157,159}-modified rhm-CSFβ, indicating they may be the same intermediate that exists in both the folding and unfolding pathways. Thus, during folding these intermolecular disulfides are the last to form and the three-dimensional structural development is not dependent on their being in place early in the folding process.

Materials and methods

Materials

Purified, biologically active rhm-CSFβ was provided by Dr. C. Cogwill (Chiron Corp.). The NFS-60 cell line was provided by Dr. J. Weaver (Chiron Corp.). Tris(2-carboxyethyl)phosphine (TCEP) and immobilized pepsin were obtained from Pierce. 1-Cyano-4-dimethylaminopyridinium fluoroborate (CDAP) was obtained from Sigma Chemicals. Modified trypsin was purchased from Promega. Deuterium oxide and urea-d₄ were obtained from Aldrich Chemical Co. All other chemicals and reagents were of the highest grade commercially available.

Partial reduction of rhM-CSFβ

The freshly dissolved rhM-CSFβ (12 nmol) was solubilized in 300 μL of 20 mM ammonium acetate buffer (pH 3.5) containing 1 mM EDTA. Partial reduction of rhM-CSFβ was initiated by adding 0.05 equivalent of TCEP of total cysteine content and followed by incubation at room temperature for 5 h. After TCEP was removed by centrifugal filtration using ultrafree MC filters with a 5000-Dalton molecular mass cutoff (Millipore), a 25-fold molar excess of CDAP solution over the cysteine content was added to the partially reduced protein mixture. Cyanylation of the sulfhydryl groups was done at room temperature for 10 min. CDAP was then removed by centrifugal filtration. The reduced, cyanylated protein was purified by reversed-phase high-performance liquid chromatography (HPLC) on a Vydac C4 TP214 column (300 Å, 5 μm, 4.6 × 250 mm, 1-mL/min flow rate) employing a binary linear gradient elution with 0.1% trifluoroacetic acid (TFA) as solvent A and acetonitrile (ACN) containing 0.09% trifluoroacetic acid as solvent B. The proteins were eluted using a 60-min 44–54% B gradient. Protein fractions were collected manually and lyophilized.

Identification of reduced disulfide bonds

The lyophilized modified rhM-CSFβ was dissolved in 30 μL of 6 M guanidine-HCl in 1 M NH₄OH (pH 11.3). Cleavage of the peptide chain was performed by adding 30 μL of 1 M NH₄OH (pH 11.5) followed by incubation at room temperature for 1 h. Excess ammonia was removed in a vacuum centrifuge. Truncated peptides containing residual disulfide bonds were completely reduced by reacting with 250 μL of 7.2 mM TCEP solution at 37°C for 30 min (pH 8.5). To the completely reduced protein mixture, 8 μL of iodoacetamide (20 mg/mL) in 0.1 M Tris-Cl at pH 8.5 was added, and the mixture was incubated for 1 h at room temperature. The reaction mixture was subjected to mass spectrometric analysis utilizing a Shimadzu HPLC equipped with two LC-10AD pumps coupled to a PE-Sciex API III triple quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray source. A capillary column packed with Vydac C4 TP214 material (5 μm particle size, 300 Å pore size, 20-μL/min flow rate) was used. The binary gradient elution included 0.05% TFA as solvent A and ACN containing 0.05% TFA as solvent B. The proteins were eluted using an 8-min 10–90% B gradient. MS spectra were analyzed using PE Sciex software (MacSpec and BioSpec).

The carboxyamidomethylated protein mixture was subjected to tryptic digestion by reacting with 2 μg of trypsin in 100 μL of 0.1 M Tris buffer (pH 7) containing 1 mM CaCl₂. The tryptic digest mixture was analyzed using a Waters HPLC equipped with two

pumps coupled to a Finnigan quadrupole ion-trap mass spectrometer. A capillary column packed with C18 Luna material (5 μ m particle size, 300 Å pore size, 5- μ L/min flow rate) was used. The identities of tryptic peptides were determined by collision-induced dissociation (CID) experiments. The biological activity of the modified protein was determined using m-CSF-dependent NSF-60 cell culture assay (Nakoinz et al. 1990).

Circular dichroism and fluorescence spectroscopy

CD spectra were obtained using a Jasco J720 spectropolarimeter (Jasco Inc.) over the range of 185–310 nm in a cylindrical 1-cm path-length quartz cuvette and with a bandwidth of 2 nm and a scan speed of 10 nm/min. Fluorescence data was acquired on a 8100C fluorescence spectrometer (SLM Instruments Inc.) with 8 nm emission and excitation slit widths using a square 0.5-cm path-length quartz cuvette.

Fluorescence was measured at excitation wavelengths of 280 and 290 nm, and expressed as a ratio of emission intensity to reference emission intensity.

Deuterium exchange-in experiments

The lyophilized CN^{157,159}-rhM-CSF β (250 μ g) was dissolved in 10 μ L of 10 mM ammonium phosphate buffer (pH 6.8) containing 1 mM EDTA. In-exchange was initiated by diluting the protein solution 20-fold with 10 mM ammonium phosphate/D₂O buffer (pH 6.8) containing 1 mM EDTA. At each time point, 10 μ g of protein sample was removed from the labeling solution and diluted 1:1 with 0.1 M ammonium phosphate buffer (pH 2.3, 0°C, 1:1 D₂O/H₂O) to quench isotopic exchange. The samples were stored in liquid N₂ until analysis. Unlabeled protein (0% reference) was prepared by dissolving the modified protein in 0.1 M ammonium phosphate buffer (pH 2.3, 0°C, 1:1 D₂O/H₂O). The completely deuterated protein (100% reference) was prepared by incubating the modified protein in 8 M urea-d₄/D₂O at 37°C for 6 h. The same exchange procedure was applied to rhM-CSF β .

The deuterated protein samples were analyzed by LC-ESI triple quadrupole MS (PE-Sciex API III⁺) using a self-packed C4 column (5- μ m particle size, 300 Å pore size, 20 μ L/min flow rate) and a binary gradient elution involving 0.05% TFA as solvent A and ACN containing 0.05% TFA as solvent B. After desalting at 10%B for 2 min, the protein was eluted using a 3-min 10–80% B program. The HPLC injector and the column were submerged in ice/water slurry, and the transfer syringe was rinsed with D₂O and precooled on ice before use. MS spectra were analyzed by BioSpec software.

The deuterated CN^{157,159}-rhM-CSF β was digested by immobilized pepsin (protein/enzyme = 1/2 [w/v]) in 0.1 M ammonium phosphate buffer (pH 2.3, 0°C, 1:1 D₂O/H₂O) for 8 min. The digest mixture was analyzed using LCQ-ESI ion trap mass spectrometer using a C18 Luna column (5- μ m particle size, 100 Å pore size, 15- μ L/min flow rate) and 0.05% TFA as solvent A and ACN containing 0.05% TFA as solvent B. After being desalted for 2 min, peptides were eluted using the following program: 10–20%B for 1 min, 20–30%B for 1 min, 30–45%B for 2 min, 45–60%B for 1 min, 60–90%B for 3 min. To minimize deuterium back exchange, the injector and the column were immersed in an ice/water bath, and the transfer syringe was rinsed with D₂O and cooled on ice before use. Due to the compactness of the modified protein, this digest procedure resulted in only 57% coverage of the protein backbone. Consequently, the complete digestion of the deuterated modified protein was obtained by incubating deuterated protein with 0.1 M

ammonium phosphate buffer (pH 2.3, 0°C, 1:1 D₂O/H₂O) containing 8 M urea-d₄ and 1 M TCEP at 0°C for 5 min prior to pepsin digest. This procedure yielded 90% coverage of the entire backbone but did not provide cyanylated peptides because TCEP reduced cyanylated cysteine residues. The peptides were identified by CID experiments. The deuterated rhM-CSF β was subjected to pepsin digestion using the same procedure as described above.

Data analysis

Because the HPLC elution was performed with protiated solvents, a small number of deuteriums at the amide positions are lost. Corrections were made for this deuterium loss using Equation 1 (Zhang and Smith 1993):

$$D = [(m - m_{0\%}) / (m_{100\%} - m_{0\%})] \times N \quad (1)$$

where D is the number of deuteriums present in a particular peptide segment or protein after incubation in deuterated solvent, and m , $m_{0\%}$, and $m_{100\%}$ represent the average molecular weight of a peptide or protein obtained for nondeuterated, partially deuterated and completely deuterated samples, respectively. N is the total number of exchangeable amide hydrogens in the protein or the peptide of interest. The present experiments yielded 10–20% back exchange in both proteins and peptides.

Amide hydrogens undergo deuterium exchange at different rates, depending on whether the hydrogens participate in intramolecular hydrogen bonding, as well as on the extent to which the hydrogens are shielded from the solvent. Exchange-in data for proteins and peptides can be analyzed using Equation 2 (Zhang and Smith 1993), which distinguishes amides according to whether they are very fast, fast, slow, or very slow:

$$D = N - \sum_{i=1}^n \exp(-k_i t) \quad (2)$$

where D is the deuterium level found in a protein or peptide with N amide linkages following incubation of the intact protein in the deuterated solvent for time t , and k_i are the first order rate constants for isotopic exchange at every amide linkage. Thus, higher order structure, structural changes, and structural dynamics of the protein that control exchange rate behavior can be defined. Data fitting for deuterium exchange was accomplished using Origin6.1 (Microcal Software, Inc.).

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