Selective Biochemical Modification of Functional Residues in Recombinant Human Macrophage Colony-Stimulating Factor β (rhM-CSF β): Identification by Mass Spectrometry†

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ABSTRACT: A rapid method combining classical chemical modification with mass spectrometry was developed to identify amino acids in the recombinant human macrophage colony-stimulating factor (rhM-CSF) protein of potential import to the ligand-receptor interaction. Diethyl pyrocarbonate modification of rhM-CSF β (under nondenaturing conditions) results in a time- and concentration-dependent loss in receptor binding and biological activity. Peptide mapping of the reaction products by mass spectrometry showed that, with low DEP:M-CSF ratios (<50:1), there was selective modification of histidine residues, whereas at higher ratios (>50:1), Tyr and Lys residues were also modified. The loss in rhM-CSF β activity was directly correlated with the extent of carbethoxylation of His9 and His15, as determined by matrix-assisted laser desorption/ionization mass spectrometric molecular weight determinations (MALDI-MS). For these residues mono-modification was observed. By contrast, C-terminal histidine residues His176 and His210 showed bis-modifications, the extent of which had no correlation to losses in biological activity. These data suggest the importance of residues in the A-helix (His9 and His15) to ligand−receptor binding.

Macrophage colony-stimulating factor (M-CSF)[1] is a cytokine that stimulates the survival, proliferation, differentiation, and function of mononuclear phagocytes (Aukerman et al., 1992; Clark & Kamen, 1987; Stanley et al., 1983). Three cDNA clones of human M-CSF have been isolated and expressed as active protein, representing short (α), long (β), and intermediate (γ) mRNA splicing variants of the single M-CSF gene (Ceretti et al., 1988; Wong et al., 1987; Kawasaki et al., 1985).

The recombinant human M-CSF studied here (Figure 1) was cloned and expressed from *Escherichia coli* as a truncated form (aa4−218) of the β-cDNA clone. After renaturation *in vitro*, a fully bioactive, disulfide-linked 49 kDa homodimer is formed (Halenbeck et al., 1989). Deletion studies of rhM-CSF have identified the active core of the molecule as the N-terminal first 150 aa (Takahashi et al., 1989).

Mass spectrometric and X-ray analyses have been used to locate the disulfide linkages in the renatured rhM-CSF

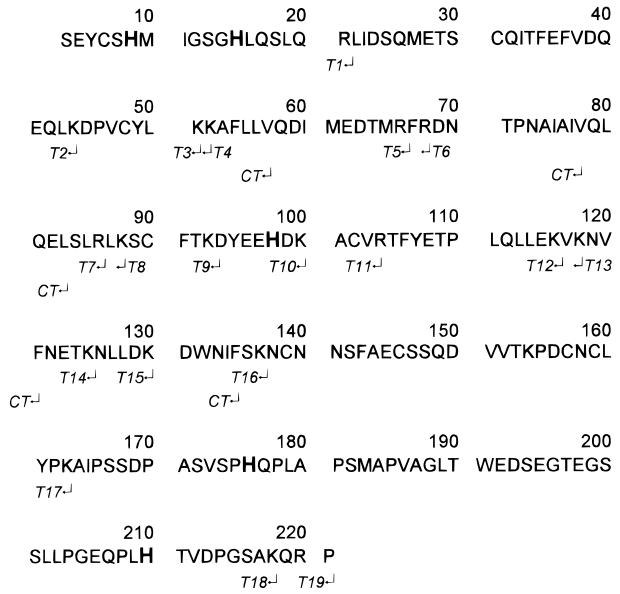

FIGURE 1: rhM-CSF β amino acid sequence. Numbering starts with Ser4 since the first three amino acids from the native M-CSF were deleted (Halenbeck et al., 1989). His residues are depicted in bold letters. Trypsin cleavage sites (T) and cleavages by chymotryptic activity (CT) are indicated.

(Pandit et al., 1992; Glocker et al., 1993) and to follow the time-dependent renaturation of the reduced and unfolded monomer (Glocker et al., 1994a, 1995; Wilkins et al., 1993). X-ray analysis reveals that the dimer is formed by end-to-end disulfide linkage of two four-helical bundles (helices A, B, C, and D), which run up-up-down-down (Pandit et al., 1992). This four-helix bundle structural motif is found in other cytokines such as hGH, prolactin, G-CSF, GM-CSF, IL-2, IL-3, IL-4, and IL-5 playing an important role in

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[1] Abbreviations: rhM-CSF β, recombinant human macrophage colony-stimulating factor β; hGH, human growth hormone; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; HPLC, high performance liquid chromatography; DEP, diethyl pyrocarbonate; HCCA, 4-hydroxy-α-cyanocinnamic acid; E:S, enzyme to substrate ratio; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium chloride; SDS, sodium dodecyl sulfate; MALDI-TOF-MS, matrix-assisted laser desorption/ionization−time-of-flight mass spectrometry; FCS, fetal calf serum.

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receptor recognition and binding (Bazan, 1991, 1992; DeVos et al., 1992; Demchuk et al., 1994).

We sought to develop a rapid and selective means by which we could modify particular amino acid types, alter biological activity, and thereby provide information relevant to the determination of regions structurally important to the ligand−receptor interaction. Previous studies had shown that modification of lysine residues with stereochemically small modifiers, such as *N*-hydroxysuccinimide esters of biotin, had no effects on receptor binding or biological activity even after modification of up to six lysine residues per M-CSF (21.4% of all lysine residues modified; 28 lysines/M-CSF homodimer) (Yamamoto et al., 1993).

In this study we chose to test diethyl pyrocarbonate (DEP), predicated both upon the relative specificity of this chemical modifier and also on the small number of histidines in M-CSF (only 5 histidines in the rhM-CSF β monomer), of which 2 were located in the A-helix of the four-helix bundle. DEP is a commonly used reagent for the specific modification of histidine residues in proteins (Miles, 1977). DEP has been used to demonstrate the importance of histidine residues in enzymes (Gomi & Fujioka, 1983), transport systems (Garcia et al., 1982), muscle proteins (Hegyi et al., 1974), and ligand−receptor interactions (Segal et al., 1971; Nishino et al., 1980; Yamamoto et al., 1989).

We developed a mass spectrometric methodology for analyses of DEP-modified model peptides (Kalkum et al., 1995) and applied that to the M-CSF model. This technique expands our knowledge of protein tertiary structure-selective modification reactions for the characterization of surface topology (Przybylski et al., 1995; Glocker et al., 1994b; Suckau et al., 1992). In this study, we demonstrate that DEP selectively modifies histidines in rhM-CSF, abolishing binding and receptor activation.

MATERIALS AND METHODS

DEP Modification and Purification of rhM-CSF β-Derivatives. Initial experiments evaluated the consequences of DEP chemical modification (Miles, 1977) on the bioactivity of M-CSF; varied ratios of DEP:M-CSF were used in the first three reactions, prior to establishing a final protocol (see below). The stock concentration of rhM-CSF β (lot no. FAP 007) was 15 mg/mL. In the first reaction, rhM-CSF β was diluted to 150 μg/mL in 0.1 M Na_2HPO_4, pH 6.5, and DEP was added to a final concentration of 340 mM with a resulting molar ratio of DEP to rhM-CSF β (DEP:M-CSF) of 110000:1 (with 10 His per M-CSF β homodimer). The reaction was terminated with a 1/100 dilution into 1% BSA, 0.2 M imidazole, and 0.01 M Na_2HPO_4, pH 6.5 (Stop Buffer I), at preselected time points (minutes after initiation of reaction). In the second reaction, rhM-CSF β was at 10 μg/mL in 0.01 M ammonium acetate, pH 6.0, and the DEP concentration was 6.9 mM (DEP:M-CSF = 33 000:1). In the third reaction, rhM-CSF β was at 1 mg/mL in 0.01 M ammonium acetate, pH 6.0, and the DEP concentration was 100 mM (DEP:M-CSF = 5000:1). In both the second and third experiments, the reaction was terminated by a 1/1000 dilution into 0.2 M imidazole/0.01 M Na_2HPO_4, pH 6.5 (Stop Buffer II).

A final protocol for the modification of rhM-CSF β was developed to be compatible with the mass spectrometry methodology. To 150 μL of rhM-CSF β solutions (1.6 μg/μL, 50 mM ammonium acetate, pH 6), DEP solutions in dried acetonitrile were added. For 10-fold and 20-fold molar excess per rhM-CSF β 4.9 μL and 9.8 μL of a 0.01 M DEP solution were used, respectively. For 50-fold, 100-fold, and 200-fold molar excess, 2.4 μL, 4.9 μL, and 9.8 μL of a 0.1 M DEP solution were used, respectively. For 500-fold excess 2.4 μL of a 1 M DEP solution was applied. After 1 h of reaction time, protein derivatives were purified using a microconcentrator device (AMICON, microcon, cutoff: 3000 Da). Retentates were washed with 300 μL of 50 mM ammonium acetate and collected to result in a final protein concentration of 1 μg/μL. Aliquots (1 μL) were used for MALDI-MS molecular weight determinations without further purification.

Tryptic Digestion of rhM-CSF β and DEP-Modified Derivatives and Reduction of Disulfide Bonds. To 100 μL of solutions of unmodified rhM-CSF β and DEP-modified derivatives (1 μg/μL, 50 mM ammonium acetate, pH 6) 20 μL of 0.1 M ammonium bicarbonate (pH 8.5), 43 μL of methanol, and 2 μL of trypsin solution (1 μg/μL, 0.1 M ammonium bicarbonate, pH 8.5) were added to yield a final pH of 7.5; methanol content was 30%, E:S = 1:50. Samples were incubated at 37 °C for 4 h. The peptide mixtures (10 μL) were mixed with 10 μL of 0.1 M β-mercaptoethanol solutions (0.1 M ammonium bicarbonate, pH 8.5), yielding a 10-fold molar excess of reducing agent over thiol groups of the protein. Samples were incubated at 37 °C for 20 min. Aliquots (1 μL) were used for MALDI-MS peptide mapping experiments without further purification.

HPLC Separation of Tryptic Peptides from rhM-CSF β. A Waters Millipore solvent delivery system, consisting of two HPLC pumps (Waters M510 and Waters M45), was used. Separations were carried out using a 250 × 4.6 mm Vydac reversed phase C-18 column (300 Å, 10 μm) equipped with a Vydac precolumn. In all cases, solvent A was 0.1% TFA in H_2O and solvent B was 0.07% TFA in acetonitrile. The flow rate was adjusted to 1 mL/min. After sample injection, the solvent mixture was kept constant at 5% B for 5 min and then was raised to 70% B over a time period of 60 min. Peptide-containing samples were collected and used for subsequent MALDI-MS or Edman sequencing analysis after lyophilization.

Mass Spectrometric Molecular Weight Determination and Peptide Mapping. Matrix-assisted laser desorption/ionization−time-of-flight mass spectrometric (MALDI-TOF-MS) analyses were carried out using a Bruker Biflex time-of-flight spectrometer (Bruker-Franzen, Bremen, Germany), equipped with a UV−nitrogen laser (337 nm) and a dual microchannel plate detector. For the molecular weight determinations acceleration voltage was set to 25 kV and spectra were calibrated with cytochrome *c* or myoglobin as internal standard. For peptide mapping experiments acceleration voltage was set to 10 kV and insulin was used for internal mass calibration. Protein- or peptide-containing solutions (1 μL) were mixed with 1 μL of matrix solution (10 μg/μL 4-hydroxy-α-cyanocinnamic acid, HCCA) dissolved in acetonitrile/0.1% trifluoroacetic acid (2:1), directly on the target. Spectra were recorded after evaporation of the solvent and processed using the X-MASS data system.

Bioactivity Studies of DEP-Modified rhM-CSF β Derivatives. Biological activity of the modified rhM-CSF β derivatives was studied by proliferation assay, using the mouse M-CSF-dependent cell line M-NFS 60 (Bauer et al., 1994; Nakoinz et al., 1990). rhM-CSF β derivatives (50 μL, 1 ng/μL) were mixed with 100 μL of RPMI 1640 growth

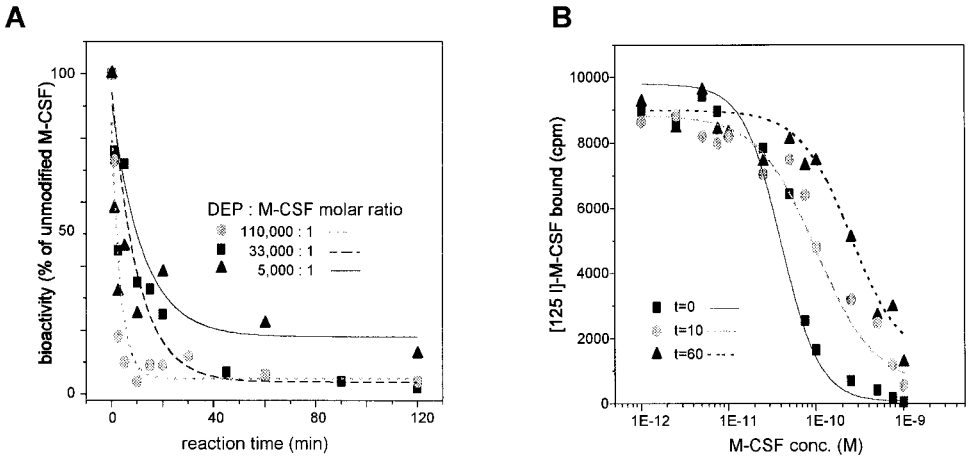

FIGURE 2: Studies of DEP modification of M-CSF. (A) Time-dependent loss in M-CSF biological activity. In experiment 1, the ratio of DEP:M-CSF was 110000:1 (10 His per M-CSF homodimer). In experiment 2, DEP:M-CSF = 33 000:1. In the third reaction, DEP:M-CSF = 5000:1. The reactions were terminated by dilution into 0.2 M imidazole/0.01 M Na_2HPO_4, pH 6.5 (Stop Buffer II), and biological activity was measured by the M-NFS 60 proliferation assay. (B) Time-dependent loss in M-CSF binding affinity to receptor.

medium (Biochrom, Berlin, Germany) and pipetted in 1:3 dilution steps on a microtiter plate. M-NFS 60 cells (2×10^4) were added to each well. After 48 h incubation at 37 °C (4% CO_2, 100% moisture) 25 μL of MTT solution (4 μg/μL) was added, and incubation was extended for an additional 4 h. Cell lysis was achieved by addition of 20% SDS solution in 20 mM HCl. After 9 h, the optical density of the product was recorded at 560 nm. M-CSF units are determined as the reciprocal dilution of M-CSF which attains the half-maximum signal for cell proliferation.

Iodination of M-CSF and Receptor Binding Assays. rhM-CSF β was dialyzed overnight against phosphate buffered saline (PBS), pH 7.4, and iodinated using Iodogen (Pierce). ^{125}I-Labeled rhM-CSF β was purified from unincorporated label by chromatography on GF-5 columns. The binding constants for ^{125}I-M-CSF calculated by saturation binding vs competitive "cold" displacement assays were comparable, giving dissociation constants of 50 pM vs 37 pM, respectively. This indicates that no substantial loss in binding activity occurred as a consequence of radiolabeling.

Competitive displacement experiments were utilized to determine the extent of loss of binding activity after DEP derivatization. Isotherms were generated by incubating 3×10^5 M-NFS 60 cells with ^{125}I-M-CSF in a 100 μL final volume of RPMI-1640, 10% FCS, and 50 mM HEPES, pH 7.2. As competitor, the unlabeled rhM-CSF and chemically modified rhM-CSF were added at increasing concentrations in a 2-fold serial manner and coincubated with the radiolabeled M-CSF. Incubation of duplicate samples proceeded in 96-well tissue culture plates for 20 h at 4 °C using an orbital shaker. Samples were applied onto a phthalate−oil layer in polyethylene centrifugation tubes. ^{125}I-M-CSF bound to cellular receptors was separated from unbound ^{125}I-M-CSF by centrifugation. The radioactivity in cell pellets containing bound ^{125}I-M-CSF was measured by γ counting for 1 min. Nonspecific background was determined by the amount of radioactivity bound in the presence of an excess of unlabeled rhM-CSF β (1.6 mg/100 mL) per data point.

Computer-Assisted Data Analysis and Curve Fitting. The data were analyzed by a nonlinear least squares fit to a noncooperative single-site model (LIGAND; Munson, 1983).

Calculation of Static Surface Accessibilities of Imidazolyl Nitrogen Atoms from rhM-CSF β. The calculation of the contact area (CA), reentrant area (RA), and total area (TA) of imidazolyl nitrogen atoms of histidine residues 9, 15, and 98 was performed on a Silicon Graphics workstation with the Insight-II/Discover software (Version 3.2.5; Biosym), using the algorithm from Connolly (1983). Standard probe radius was set to 1.4 Å, and standard van der Waals radii of the Insight-II/Discover program were used.

RESULTS

Initial Studies of DEP Modification. Effects on Biological Activity and Binding to Receptor. Initial studies ascertained the effect of DEP treatment on the biological activity of rhM-CSF β. In three independent experiments, rhM-CSF β was treated with an excess of DEP, ranging from a 5000- to a 110000-fold molar excess of DEP to M-CSF (10 histidines per M-CSF homodimer). Treatments were for varying lengths of time, and reactions were stopped with an excess of imidazole. Samples were assayed for biological activity by proliferation assays and by radioligand binding competition assays. There was a clear, time-dependent loss of biological activity after modification by DEP (Figure 2A). The reaction was rapid, with activity losses of >50% within 2−10 min after addition of DEP. A maximal reduction in rhM-CSF β activity of between 80 and 90% was observed.

Studies of the receptor binding properties of the DEP-modified rhM-CSF β showed similar results. Samples from the third DEP modification experiment were analyzed in binding competition experiments. Unmodified rhM-CSF had an IC_{50} of 35 pM, i.e., the concentration at which it displaced 50% of the ^{125}I-M-CSF binding (Figure 2B). After DEP treatment the IC_{50} increased to 250−350 pM, indicating that the M-CSF derivatives were 8- to 10-fold less able to bind to the receptor. The loss in binding activity was time-dependent and was correlated with the decreased activity observed in the proliferation assay (Table 1). Thus, after a 10 min treatment with DEP, there was a 7-fold decrease in binding potency and a 4-fold reduction in biological activity. After 60 min treatment, there was a 10-fold decrease in binding potency and a 9-fold reduction in biological activity.

Molecular Weight Determination of Carbethoxylated rhM-CSF β. Given that a 5000-fold DEP excess resulted in dramatic changes in M-CSF behavior, further studies of the DEP modification reaction utilized a range of DEP concentrations from 10- to 500-fold molar excess in an attempt to ensure specificity in the amino acid modification. The

Table 1: Comparison of Proliferation Assays and Radioligand Binding Competition Assays of DEP-Modified rhM-CSF β[a]

sample[b]	binding competition assay [IC_{50} (pM)]	potency ratio	proliferation assay (% act.)
T0[c]	34	1.0	100
T2	250	0.14	58
T10	250	0.14	24
T60	350	0.097	12

[a] Molar ratio DEP:M-CSF 5000:1. [b] T refers to minutes of DEP reaction time. [c] Refers to unmodified M-CSF.

reaction conditions utilized for the carbethoxylation were made compatible with the mass spectrometry requirements (see Materials and Methods) and yielded stable products for mass spectrometric analyses. In our hands, DEP-modified model peptides (angiotensin II and insulin), which were incubated in aqueous buffers at pH 6−7 for 48 h at 20 °C, showed ion signals for the modified peptides only. However, at pH 8.5, DEP-modified peptides hydrolyzed to some extent, exhibiting ions for both DEP-modified and the unmodified peptide,[2] which is in agreement with previous results (Miles, 1977; Vangrysperre et al., 1989). Bioactivity studies of DEP-modified rhM-CSF β derivatives were carried out for 20 h (radioligand binding competition assays) and 48 h (proliferation assays). Due to the minimal amounts of M-CSF and DEP-modified derivatives used (approximately 1 fmol each), protein derivatives were not recovered after the bioassays. Therefore, potential loss of modification during assaying for biological activity (at pH 7.5) cannot be ruled out entirely.

The average extent of modification was determined by MALDI-MS molecular weight analyses. Unmodified and modified proteins gave a series of singly and multiply charged ions, which allowed precise molecular weight determinations (Figure 3). Incorporation of one carbethoxy group (CEt) from DEP yields a molecular weight increase of 72 amu (single modification). By contrast to other nucleophilic amino acid side chains, histidine imidazolyl groups can also be modified by DEP at the second nitrogen atom (bis-modification), leading to a ring-opened urethane carbethoxy derivative (UCEt) with a mass increase of 134 amu. The mass difference between two single modifications and one bis-modification (Δm 10 amu) could not be separated by MALDI-MS molecular weight determinations of intact protein derivatives; thus, protein molecular ions were not resolved for the various rhM-CSF β derivatives, and degree of modification is expressed as the average number of introduced carbethoxy groups. However, the comparable molecular ion peak widths of carbethoxylated protein derivatives and unmodified rhM-CSF β suggested quite homogeneous degrees of modification. With increasing concentrations of DEP, a gradual increase with up to approximately 16 carbethoxy groups/rhM-CSF β dimer was detected (the 10 histidine residues of the rhM-CSF β-homodimer would allow 20 carbethoxylation groups).

Determination of Carbethoxylation Sites and Relative Reactivities. The sites of carbethoxylation were identified by MALDI-MS peptide mapping analyses of the reduced protein derivatives after tryptic digestion in solution (see Materials and Methods). rhM-CSF β is resistant to proteolytic digestion (Glocker et al., 1993). Therefore, tryptic

[2] M. Kalkum, M. Przybylski, and M. O. Glocker, manuscript in preparation.

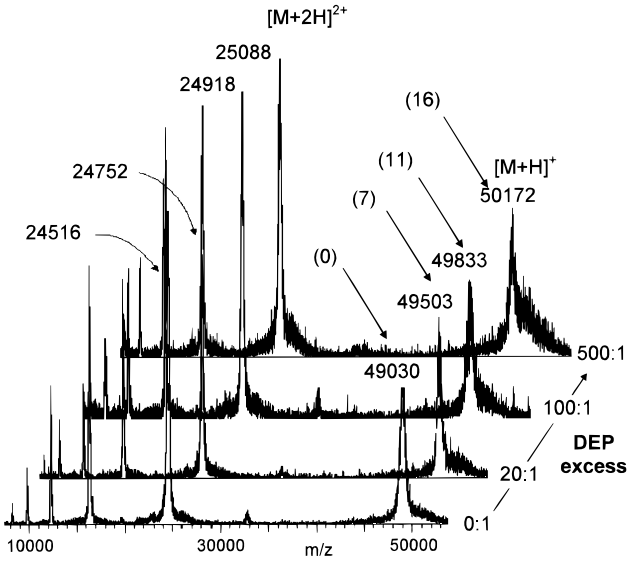

FIGURE 3: MALDI-MS molecular weight determinations of rhM-CSF β and modified derivatives with increasing DEP molar excess. Series of singly and multiply charged ions are recorded, and *m/z* values for the singly ($[M + H]^+$) and doubly ($[M + 2H]^{2+}$) charged ions are given. Incorporation of one carbethoxy group (CEt) from DEP yields in a molecular weight increase of 72 amu (single modification). Histidine imidazolyl groups can be modified by DEP also at the second nitrogen atom (bis-modification), leading to a ring-opened urethane carbethoxy derivative (UCEt) with a mass increase of 134 amu. The mass difference between two single modifications and one bis-modification (Δm 10 amu) is not separated by MALDI-MS molecular weight determinations. Thus, protein molecular ions are not resolved for the individual rhM-CSF derivatives, and degree of modification is expressed in average numbers of introduced carbethoxy groups (numbers in parentheses). The comparable molecular ion peak widths of carbethoxylated protein derivatives and unmodified rhM-CSF suggest quite homogeneous degrees of modification. Spectra were recorded using HCCA as matrix.

digestion of rhM-CSF β and DEP-modified derivatives was carried out in the presence of methanol. Under these conditions, we showed that rhM-CSF β was completely digestible; however, trypsin had some chymotryptic activity leading to additional cleavages at Phe121/135, Leu83, and Val58/78 (Figure 1). All peptides were mapped by mass spectrometry using mixtures (Figure 4). In addition, peptide assignments were verified by HPLC isolation of tryptic peptides and separate mass spectrometric identification and Edman sequencing (Tables 2 and 3).

The corresponding ions of modified peptides were found with increasing relative abundances as the concentration of DEP was increased. At low reagent excess (up to 50-fold molar excess of DEP:M-CSF), mainly singly modifed (CEt) histidines were found (Table 2). For example, with a 50-fold molar excess of DEP (Figure 4B), the N-terminal peptide (aa4−21; *m/z* 2034) was found together with the singly modified CEt derivative (His9 or His15; *m/z* 2107) and the doubly modified CEt derivatives (His9 and His15; *m/z* 2179). However, when using a larger excess of DEP, bis-modifications of His residues occurred, with the formation of a ring-cleaved urethane product (UCEt). For example, the C-terminal peptide (aa164−218; *m/z* 5506) was present together with the singly CEt-modified peptide (His176 or His210; *m/z* 5578) and the bis-modified UCEt-derivative (*m/z* 5640). Moreover, nonspecific modification of other nucleophilic amino acids (Lys52, Lys116, Lys118, Lys130, Lys218, Y107, and Y161; Table 3) also occurred when using larger excess of DEP (DEP:M-CSF > 1:100). These nonspecific

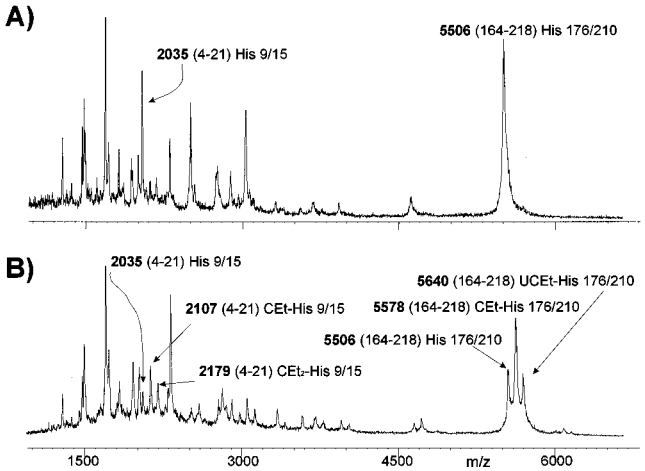

FIGURE 4: MALDI-MS peptide mapping analyses of rhM-CSF β and DEP-modified derivatives, after tryptic digestion and reduction. (A) Unmodified rhM-CSF β; (B) modified derivative using 50-fold molar excess DEP. All peaks were identified (see Tables 2 and 3) and His-containing peptides are labeled. Numbers in parentheses correspond to amino acid positions in the peptides.

modifications were observed as minor components in the mass spectra (Table 3).

A comparison of peptides containing the five different histidine residues showed some differences in reactivities. Thus, for His9 and His15, principally CEt-His residues were obtained at low excess DEP (<50-fold; DEP:M-CSF). Only at >100-fold molar excess of DEP were UCEt derivatives observed in the mass spectra, and then as minor peaks. Similarly, CEt-His derivatives were found for His98, however, only at higher DEP excess (>50-fold; DEP:M-CSF), and there was no observed formation of UCEt derivatives. By contrast, a difference in chemical reactivity was observed for the C-terminal His176 and His210 residues. Here, the UCEt derivative of C-terminal peptide (aa164−218), containing the bis-modified histidine residues (His176 and His210), was observed as the dominant peak in the mass spectra at concentrations of DEP:M-CSF > 200:1 (Table 2).

Biological Activity of rhM-CSF Derivatives. The relationship between the DEP concentration and residual rhM-CSF β biological activity was determined using concentrations of DEP:M-CSF of 10:1, 20:1, 50:1, 100:1, 200:1, and 500:1. The activity of the unmodified rhM-CSF β was 18 440 (±2870) units. The activity of the derivatized rhM-CSF β decreased incrementally as the concentration of DEP was increased, with an apparent plateau in the loss of bioactivity occurring after treatment with a 500-fold molar excess of DEP. At this concentration of DEP, rhM-CSF β had lost >60% of its activity. The largest fractional loss in bioactivity occurred at DEP concentrations of <200-fold molar excess (Figure 5).

The relationship between the loss in biological activity and modification of specific histidine residues was further investigated. Of the five His residues in rhM-CSF β, only His9, His15, and His98 are found in the bioactive core. His98 was found mainly unmodified in peptide aa94−104. Only when using DEP in large molar excess (>200:1) did the CEt-modified His98 derivative become the dominant peptide (Table 2). By contrast, modification of His9 and His15 occurred more rapidly, and modified peptides were detected at a low 10-fold molar excess of DEP (Table 2). These observations suggested that modification of His9 and His15 might be more critical to the loss in bioactivity than His98.

The degree of His9 and His15 modifications was semiquantitatively determined by ascertaining the residual content of the unmodified N-terminal peptide (aa4−21; *m/z* 2034). The relative peak intensity of peptide 4−21 in the mass spectra was determined by comparison to that of the internal standard, i.e., peptide 69−86 (*m/z* 1998), which gave comparable peak intensities in all spectra, and has no possible modification sites for DEP. As the concentration of DEP increased, there was a parallel decrease in the percentage of unmodified N-terminal peptide (aa4−21) and rhM-CSF β bioactivity (Figure 5).

A semiquantitative evaluation of the DEP modification was also performed for His98 using peptide 94−104 (*m/z* 1365). The relative peak intensity of peptide 94−104 in the mass spectra was compared to that of another internal standard, i.e., peptide 105−116 (*m/z* 1483), which has no possible modification sites for DEP and also gave comparable peak intensities in all spectra. By contrast to the data for the His9- and His15-containing peptide, the decrease in the percentage of unmodified peptide 94−104 did not parallel the decrease in bioactivity. Thus, the percentage of unmodified peptide was only first found to decline at concentrations of DEP:M-CSF molar ratios >50:1. At this point bioactivity was already reduced to 75% (Figure 5).

Separation of proteolytic peptide fragments from rhM-CSF β by reversed phase HPLC gave a complex chromatogram with numerous fractions. Fractions were analyzed by MALDI-MS and showed that in some cases separation was incomplete. For example, the HPLC-fraction that contained peptide 164−218 (bearing His176 and 210; *m/z* 5506) coeluted with a peptide with *m/z* 2481 (data not shown). Thus, quantitation of DEP modification by HPLC analysis and/or amino acid analysis (Vangrysperre et al., 1989) was not applied.

Comparison of Relative Reactivities and Surface Accessibilities of Protein Amino Groups. Possible structural parameters for correlation with the relative reactivities (Glocker et al., 1994b) of imidazolyl carbethoxylation are relative (static) surface accessibilities (SA values) defined at a 1.4 Å van der Waals sphere of solvent accessibility in proteins, which can be obtained from the crystal structure data. SA values are compared with the relative reactivities for the histidine residues 9, 15, and 98. For monoderivatization (CEt) only one nitrogen atom of the imidazolyl group has to be exposed on the surface, as is the case for His9, His15, and His98 as deduced from the X-ray data (Table 4). The total area accessible for NE2 of His9 and His15 is comparable, i.e., 12.1 Å^2, whereas for His98, it is half of that (6.5 Å^2). The lower calculated accessibility of NE2 from His98 reflects the experimental observation that His98 requires >50-fold molar (DEP:M-CSF) excess before it is carbethoxylated, whereas modification of His9 and His15 occurs at 5-fold lower DEP concentration, i.e., a 10-fold molar (DEP:M-CSF) excess. No X-ray structural information is available for His176 and His210. However, it is readily apparent that the chemical reactivitiy of His176 and His210 differs from the three aforementioned histidines in that UCEt derivatives are formed more readily (Table 2; Figure 4). This implies that the surface accessibility of histidine nitrogen atoms in the C-terminal tail region is more similar to that of nonstructured peptides in solution than to His residues participating in the N-terminal globular cytokine structure.

Table 2: Mass Assignments of His-Containing Peptides after Tryptic Digestion from Unmodified and DEP-Modified rhM-CSF β

peptide	no. and type of modification(s)[a]	modified residue(s)	*m/z* (calcd)	*m/z* (obsd); DEP:M-CSF excess[b] 0:1	10:1	20:1	50:1	100:1	200:1	500:1
4−21	/	/	2034	**2034**	**2034**	**2034**	2035	2036	d	d
	CEt	H9 or H15	2106	d	**2108**	**2108**	**2106**	**2107**	2106	2106
	2CEt	H9, H15	2178	d	d	d	2179	**2181**	**2179**	**2180**
	CEt + UCEt	H9, H15	2240	d	d	d	d	2240	2242	2241
	3CEt	H9, H15, α	2250	d	d	d	d	d	2248	2252
	2CEt + UCEt	H9, H15, α	2314	d	d	d	d	d	2314	2314
94−104	/	/	1365	**1366**	**1367**	**1366**	**1365**	**1367**	**1367**	1365
	CEt	H98	1439	d	d	d	1439	**1439**	**1439**	**1438**
164−193[c]	/	/	3026	**3026**	**3025**	**3026**	**3025**	**3025**	**3025**	**3024**
	CEt	H176	3098	d	3098	3096	3097	**3097**	**3098**	**3098**
	UCEt	H176	3160	d	d	d	d	3162	3162	**3161**
164−218[c]	/	/	5506	**5506**	**5506**	**5506**	5506	5506	5506	5506
	CEt	H176 or H210	5578	d	5577	5578	**5578**	**5577**	5578	5579
	UCEt	H176 or H210	5640	d	d	d	5640	**5640**	**5641**	**5641**
	CEt + UCEt	H176, H210	5712	d	d	d	d	5711	5713	5712
164−221[c]	2CEt	H176, H210	5961	d	d	d	**5960**	**5960**	d	d
	3CEt	H176, H210, K218	6033	d	d	d	6032	6032	6032	6032
	2CEt + UCEt	H176, H210, K218	6095	d	d	d	d	**6093**	**6094**	**6094**
	CEt + 2UCEt	H176, H210, K218	6157	d	d	d	d	d	6155	6156

[a] CEt = carbethoxy; UCEt = ring-opened urethane bis-modified derivative. [b] Numbers printed in bold letters represent dominant peaks. [c] Assignment confirmed by gas-phase Edman sequencing. [d] Mass not detected.

Table 3: Mass Assignments of Peptides without His Residues after Tryptic Digestion from Unmodified and DEP-Modified rhM-CSF β

peptide	type of modification[a]	modified residue(s)	*m/z* (calcd)	*m/z* (obsd); DEP:M-CSF excess[b] 0:1	10:1	20:1	50:1	100:1	200:1	500:1
22−49	/	/	3309	d	d	3310	3308	3310	3310	3309
22−51	/	/	3553	3552	3552	3552	3554	3553	3552	3552
22−52	/	/	3681	3680	3680	3681	3682	3680	3680	3680
52−66	/	/	1811	**1812**	**1811**	**1811**	**1812**	**1812**	**1811**	**1812**
	CEt	K52	1884	d	d	d	d	1883	1884	1884
53−66	/	/	1683	1683	1683	1683	1683	1683	1683	1683
55−66	/	/	1466	1465	1466	1466	1465	1466	1466	1466
58−68	/	/	1442	1442	1441	1441	1441	1442	1442	1442
67−83	/	/	1943	1943	1943	1942	1943	1943	1943	1943
67−86	/	/	2300	2300	2300	2299	2299	2299	2300	2300
69−86	/	/	1996	1996	1996	1996	1996	1997	1997	1997
79−93	/	/	1790	1791	1791	1790	1790	1789	1791	1790
94−100	/	/	936	936	937	937	937	937	937	937
101−104	/	/	446	445	445	446	445	445	445	445
101−121	/	/	2498	**2498**	**2499**	**2499**	**2500**	**2500**	**2499**	**2499**
	CEt	K116 or K118 or Y107	2570	d	d	d	2570	2570	2570	2570
105−116	/	/	1483	1483	1483	1483	1483	1483	1483	1483
105−118	/	/	1710	d	d	1711	1711	1711	1711	1711
105−125	/	/	2543	d	d	d	d	**2543**	**2542**	**2542**
	CEt	K116 or K118 or Y107	2615	d	d	d	d	2615	2615	2615
117−125	/	/	1079	**1079**	**1080**	**1079**	**1080**	**1080**	**1080**	**1080**
	CEt	K118	1153	d	d	d	1151	1152	**1152**	**1152**
119−125	/	/	851	852	852	852	852	852	852	852
119−135	/	/	2110	2111	2109	2110	2110	2109	2110	2109
126−135[c]	/	/	1278	1279	1279	1278	1278	1278	1279	1279
126−163	/	/	4351	d	d	d	d	**4352**	**4352**	**4352**
	CEt	K130 or K137 or Y161	4423	d	d	d	d	d	4423	4424
131−137	/	/	910	911	911	910	910	910	911	910
138−163	/	/	2880	**2880**	**2879**	**2879**	**2881**	**2881**	**2881**	**2879**
	CEt	Y161	2952	d	d	d	2955	2954	2955	2995
219−221	/	/	400	400	400	401	400	401	400	400

[a] CEt = carbethoxy derivative. [b] Numbers printed in bold letters represent dominant peaks. [c] Assignment confirmed by gas-phase Edman sequencing. [d] Mass not detected.

DISCUSSION

In the present study, classical DEP chemical modification paired with mass spectrometric peptide mapping has been used as an efficient and sensitive approach for the direct, molecular assignment of His carbethoxylation modification sites and relative reactivities in rhM-CSF β. The DEP chemical modification of His9 and His15 was shown to have a significant effect on the interaction between cytokine and receptor.

Stability of DEP-modified imidazole and His derivatives was shown to be pH-dependent (Miles, 1977) and exhibited maximum stability in a pH range of 5.5−7.5. A half-life of 55 h for DEP-imidazole in phosphate buffer at pH 7.25 and 20 °C was reported (Melchior & Fahrney, 1970), and stability of DEP-His modification seemed unaffected over 50 h in borate buffer at pH 7.6 (Mühlrád et al., 1967). In our experiments, bioactivity studies were carried out in time intervals of 20 h (radioligand binding competition assays)

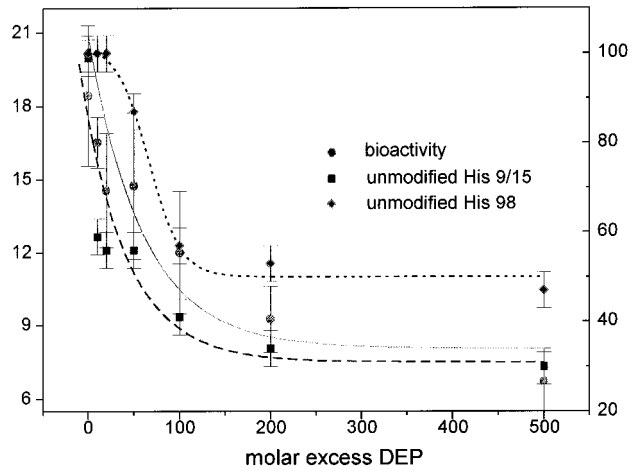

FIGURE 5: Correlation of bioactivity and degree of DEP modification of His9/15 and His98 residues as a function of DEP excess. The bioactivity of modified rhM-CSF β is given in M-CSF units as determined by the M-NFS 60 cell assay. The amount of unmodified His9/15 was determined by semiquantitative analyses using the relative peak intensities of unmodified, His-containing peptide 4−21 (*m/z* 2034) compared to peptide 69−86 (*m/z* 1996). The relative peak intensity of His98 containing peptide 94−104 (*m/z* 1365) in the mass spectra was compared to that of another internal standard, i.e., peptide 105−116 (*m/z* 1483). The amount of unmodified N-terminal peptide (aa4−21) shows a decrease in intensity that parallels the bioactivity curve. ●, bioactivity; ■, % unmodified His9/15; ◆, % unmodified His98.

Table 4: Static Accessible Surfaces of Nitrogen Atoms from rhM-CSF α His Residues

His residue (sequence position)[a]	atom[b]	contact area (Å²)	reentrant area (Å²)	total area (Å²)
9	ND1	0.1	1.1	1.2
	NE2	7.8	4.4	12.1
15	ND1	0.0	0.0	0.0
	NE2	3.4	8.7	12.1
98	ND1	0.0	0.0	0.0
	NE2	2.1	4.3	6.5

[a] Areas are calculated only from histidine residues of one monomer. [b] Nomenclature according to X-ray data set.

and 48 h (proliferation assays) in Tris buffer. Due to the minimal amounts of M-CSF and DEP-modified derivatives used (approximately 1 fmol each), protein derivatives were not recovered after the bioassays. Therefore, potential loss of modification during assaying for biological activity (at pH 7.5) cannot be ruled out entirely. In our hands, DEP modification of model peptides was stable in aqueous buffers at pH 6−7 for the observed time interval (48 h), which is in agreement with previous results (Miles, 1977; Vangrysperre et al., 1989).

DEP modification resulted in carbethoxylation of the five histidines in M-CSF. At higher concentrations, various lysine and tyrosine residues were also modified. However, in our studies with lysines and tyrosines (Table 3), at concentrations of DEP which had major effects on biological properties, we found only minimal carbethoxylation products. Moreover, previous studies had shown that chemical modification of lysine residues using a series of activated *p*-nitrophenyl carbonates and *N*-hydroxysuccinimide esters of biotin had little effect on rhM-CSF binding or biological activity at modifications of up to 6 lysines per rhM-CSF homodimer (Yamamoto et al., 1993). Together, these data suggest that the DEP modification of lysine and tyrosine modifications is nonspecific and has only a minimal impact on receptor binding and biological activity.

The interpretation that His modifications, rather than nonspecific Lys or Tyr modifications, were associated with the loss in M-CSF''s biological activity led us to investigate differences among the five histidines of rhM-CSF β. The topologic rendering and space-filling models of the rhM-CSF homodimer (Figure 6A,B,C,D) show the locations of the three relevant histidines (His9, His15, His98) on the A-, B-, C-, and D-helices in the aa4−158 M-CSF (α) protein. The C-terminal histidines (His176 and -210) have been shown not to be important for biological activity (Takahashi et al., 1989). His98 is not present in murine M-CSF, but species' cross-reactivity in binding is retained (murine M-CSF contains a Gln residue at this position; Kawasaki & Ladner, 1990). A semiquantitative measurement of the His9/15 modification showed an increase in the percentage of modified His9/15 that paralleled the loss in M-CSF bioactivity. This was not the case for His98 (Figure 5). These results are in agreement with those of Taylor et al. (1994), where significant loss of binding was observed when His residues of the A-helix and Val78 of the C-helix were exchanged to Ala. In our studies we did not observe any modification of amino acids in the C-helix.

His9 and His15 are located in the A-helix of M-CSF. In the family of four antiparallel α-helical bundle cytokines, it has been shown that residues in the A-helix are part of the hormone−receptor interface. For GM-CSF, IL-3, and IL-5, it has been ascertained that a conserved glutamate residue in helix A is critical to the binding of ligand to the common receptor b-chain (Shanafelt et al., 1991; Lopez et al., 1992). For IL-2, a solvent-accessible acidic (Asp20) residue in the A-helix is functionally important for binding to the intermediate affinity (b-chain) (Berndt et al., 1994). Solvent-exposed residues in helix 1 (helix A) of hGH interact with both binding sites of the hGHbp (De Vos et al., 1992). Our data are consistent with the A-helix of M-CSF being involved in the receptor interface.

The loss in biological activity as a consequence of the DEP modification differs from that obtained by site-directed mutagenesis (Taylor et al., 1994). A trivial explanation might be that DEP modification is not absolute, or uniform. However, even after treatment with a DEP molar excess of 110 000 (DEP:M-CSF), the maximum loss in biological activity was 80−90%. By contrast, losses after Ala substitution were 6-fold for the H9A mutation, 1200-fold for the H15A mutation, and 9100-fold for the double H9A, H15A mutations. It is thus possible that the effect of DEP modification is more subtle than the conversion of His to Ala. The carbethoxylation of His might be less disruptive of His-dependent hydrogen bonding, or Zn^{2+} chelation, a His-dependent chemistry of importance to the prolactin and hGH ligand−receptor interactions (Rowlinson et al., 1994; Cunningham et al., 1990; Fukushima et al., 1987). Further investigation of the chemical differences between histidine and carbethoxylated histidine might provide further insight into the mechanism by which His9 and His15 interact with the receptor.

MALDI-MS is a rapid and very sensitive method for the analysis of complex peptide mixtures that derive from either proteolytic degradation or fractional chemical modification. In model studies with angiotensin II, it was shown that degrees of His modification, determined by UV-absorption measurements ($\lambda = 240$ nm; $\Delta\epsilon_{240} = 3200$ cm^{-1} M^{-1}; Ovadi & Keleti, 1967) at pH 6 in phosphate buffer, were in good agreement with modification degrees determined by MALDI-

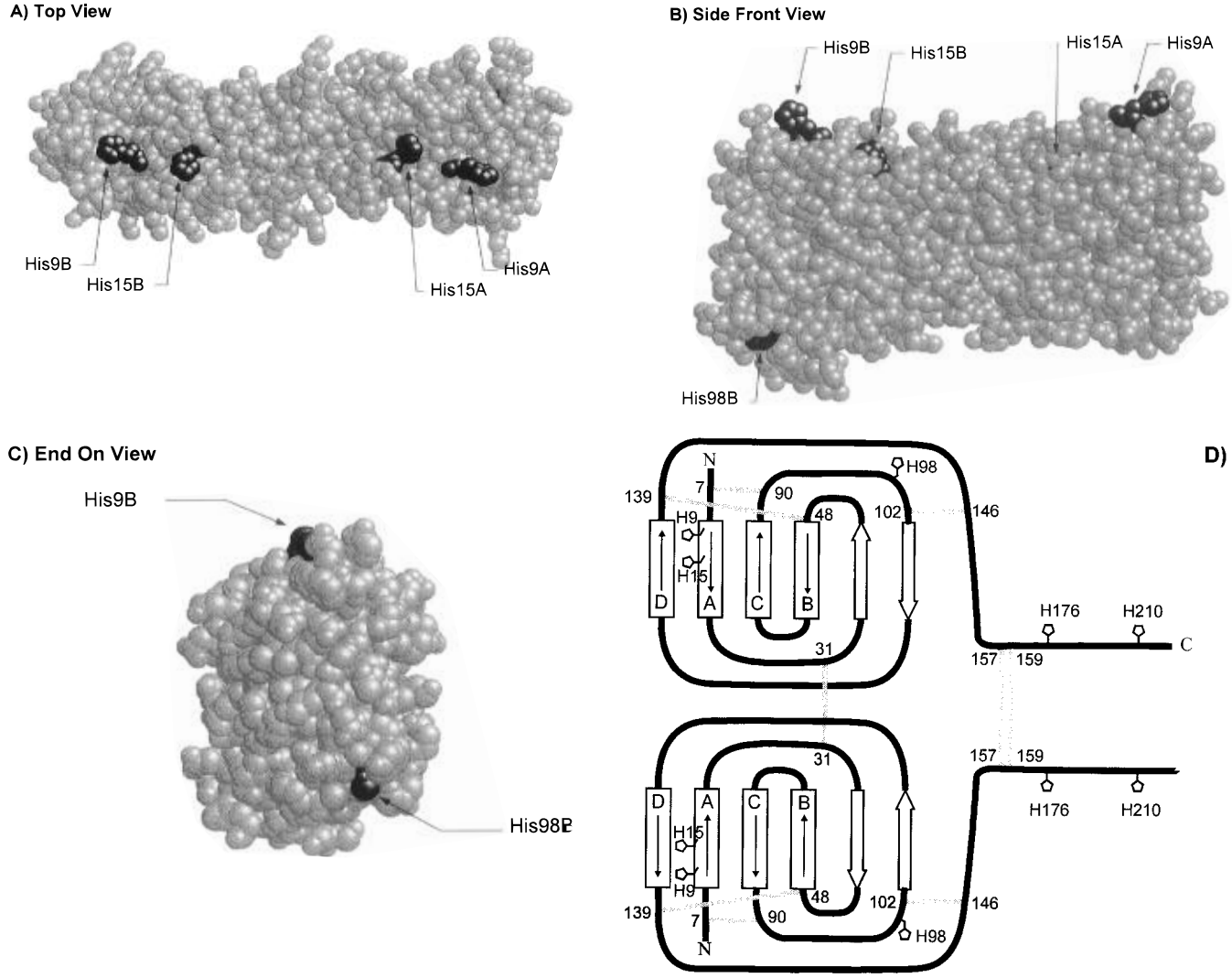

FIGURE 6: Topologic and space-filling models of the rhM-CSF homodimer. (A, B, and C) Space-filling models. The space-filling models of the rhM-CSF homodimer show the locations of the six relevant histidines (three in each monomer), (His9A, His15A, His98A) and (His9B, His15B, His98B), in the aa4−158 M-CSF (α) homodimeric protein using the X-ray coordinates obtained from Pandit et al. (1994). (A) The top view examines the face of M-CSF opposite the COOH-terminal tail, which is thought to act as the anchor or tether for the biologically active transmembrane form of M-CSF. This view clearly shows that the four histidine residues, His9A, His9B and His15A, His 15B of the homodimeric M-CSF molecule, are solvent-accessible residues on the same planar surface. Of all the DEP-modified amino acid residues observed, only His9 and His15 are located in the A-helix. (B) "Front-side view". By contrast, the "front-side" view (COOH-terminal tether is on the bottom of the picture) shows few or no His surface features, indicating that this face of the cytokine is not involved in the His-mediated interaction with the receptor. (C) End-on view. The "end-on" view shows the solvent-accessible His98 residue on the elongated end of the cylindrical cytokine, which is located on the opposite side to His9. As modifications in His98 were not correlated with biological changes, this end of the molecule is not thought to be important to the receptor interaction. (D) Topographic representation. The structure motif of the four-helix bundle (up-up-down-down) is shown, and the locations of His residues (His9, His15, His98, His176, and His210) are indicated. Disulfide bonds are shown as gray bars. β-Sheets are symbolized by arrows.

MS (Kalkum, 1995). However, MALDI-MS seems not generally applicable for absolute quantitation, as ion yields may be varying for different analytes. This can be overcome by using internal calibrants with similar chemical properties, thus providing that signal levels become independent of experimental variables such as inhomogeneous sample distribution and dependence of laser power for analyte desorption (Tang et al., 1993). Relative quantitations show linear relationships over at least 1 order of magnitude (Nelson et al., 1994). Since desorption properties of peptides may be altered upon DEP modification, we compared the decrease of signal intensity of His-containing *unmodified* peptides with the ion abundance of peptides that were found unmodified throughout the course of modification experiments, thereby not changing the chemical relationships, i.e., ionization efficiencies, of the semiquantitatively analyzed peptides of interest.

Selective chemical reactivity reflects both the protein's surface topology and amino acid microenvironment. In recent investigations, the surface topology of proteins has been characterized by tertiary structure-selective modification reactions and subsequent mass spectrometric analyses (Przybylski et al., 1996; Glocker et al., 1994b; Suckau et al., 1992). For mono-derivatization of histidine residues, only one nitrogen atom of the imidazolyl group needs to be exposed on the surface, as is the case for His9, His15, and His98 as deduced from the X-ray data of rhM-CSF α (Table 4). No X-ray structure information is available for His176 and His210 from rhM-CSF β. The chemical reactivities of these last two His residues differ from those of the first three histidine groups in that both nitrogen atoms of the respective imidazolyl groups seem readily accessible, leading to UCEt derivatization. Apparently, these histidines behave in a more "peptide-like" fashion, i.e., the formation of UCEt has been

observed more readily in His modification reactions of peptides (Kalkum et al., 1995; Foti et al., 1991). This indicates that, for His176 and His210, there is little or no apparent restriction imposed by the tertiary structural features of rhM-CSF.

Mass spectrometric peptide mapping is a generally applicable and powerful tool for the molecular identification of *partially modified* protein derivatives, which are relevant for structure−reactivity correlations (Bosshard, 1979). The unequivocal identification of modification sites by direct mass spectrometric peptide mapping may be limited in the case of partial sequences with closely spaced His residues, which may require additional HPLC separation and separate mass spectrometric analysis. In the case of the DEP-modified M-CSF, a simple characteristic proteolytic pattern enabled the direct detection of carbethoxylated residues within the A-helix. Quantitative analyses paired with bioactivity assessments lead us to conclude that this helix is important as a receptor interacting region.

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