Identification and Location of a Cysteinyl Posttranslational Modification in an Amyloidogenic \( \kappa_1 \) Light Chain Protein by Electrospray Ionization and Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

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Amyloid-deposited light chain (AL) amyloidosis is linked to the overproduction of a monoclonal immunoglobulin light chain protein by a B-lymphocyte clone. Since the amyloid fibril deposits in AL amyloidosis most often consist of the N-terminal fragments of the light chain, the majority of studies have focused on the determination of the primary structure of the protein, and reducing agents have been used routinely in the initial purification process. In this study, two light chain proteins were isolated and purified, without reduction, from the urine of a patient diagnosed with kappa 1 (\( \kappa_1 \)) AL amyloidosis. One protein had a relative molecular mass of 12,000 and the other 24,000. Electrospray ionization and matrix-assisted laser desorption/ionization mass spectrometry, in combination with enzymatic digestions, were used to verify the amino acid sequences and identify and locate posttranslational modifications in these proteins. The 12-kDa protein was confirmed to be the N-terminal \( \kappa_1 \) light chain fragment (variable region) consisting of residues 1–108 or 1–109 and having one disulfide bond. The 24-kDa protein was determined to be the intact \( \kappa_1 \) light chain containing a cysteinyl posttranslational modification at Cys214 and disulfide bonds located at Cys23–Cys88, Cys134–Cys194, and Cys214–Cys. The methods used in this report enable high-sensitivity determination of amino acid sequence and variation in intact and truncated light chains as well as posttranslational modifications. This approach facilitates consideration of the effect of cysteinylation on the native protein structure and the potential involvement of this modification in AL amyloidosis. © 2001 Academic Press

Key Words: amyloidosis; cysteinylation; disulfide bonds; immunoglobulin; peptide mapping.

Amyloidosis is a disease associated with abnormal protein deposition in organs and tissues (1). At least 18 human proteins are known to undergo a conformational change into an abnormal form that assembles into amyloid fibrils (2, 3). This conformational change can be sporadic, dominantly inherited, or induced as a result of infection. Although the sequences and structures of these amyloidogenic proteins are diverse, they all undergo an abnormal conformational change producing partially folded states that give rise to disease-related amyloid fibril deposits that share the same characteristics. All amyloid fibrils are unbranched and approximately 60 to 120 Å in diameter, stain with Congo red and display a green birefringence, and exhibit a cross-\( \beta \) structure.

Amyloid-deposited light chain (AL)\(^2\) amyloidosis is linked to the overproduction of a monoclonal immunoglobulin light chain protein by a particular B-lymphocyte clone (1, 4–6). Although the underlying factors responsible for initiating the deposition are not yet

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2 Abbreviations used: AL, amyloid-deposited light chain; BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; DHB, 2,5-dihydroxybenzoic acid; DTT, dithiothreitol; ESI, electrospray ionization; \( \kappa \), kappa; \( \lambda \), lambda; MALDI, matrix-assisted laser
fully understood, in vitro studies have shown that amino acid substitutions in the variable region of the light chain could cause the protein to undergo a conformational change into amyloid fibrils (7–9). While the fibril deposits most often consist of the N-terminal (variable region) fragments of the light chain (10–13), they may also be composed of the constant region and/or the intact light chain itself (14–20).

Two light chain proteins were isolated and purified, without using any reducing agents, from the urine of a patient (AL-9666) diagnosed with AL amyloidosis of the kappa 1 (κ1) subtype. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), it was determined that one protein had a relative molecular mass of 12,000 and the other 24,000. Both proteins stained positively with polydonal anti-κ light chain antibody on a Western blot. From these analyses, the two proteins were thought most likely to be a light chain fragment and the intact light chain, respectively.

The variable region amino acid sequence of the AL-9666 light chain was deduced from the variable region cDNA, which had been sequenced previously and shown to be derived from a κ1 germline donor (018-08), that had been amplified from bone marrow cells via reverse-transcriptase polymerase chain reaction using variable region-specific primers (sequence J COK1, GenBank Accession Numbers AF124196 and AF054662) (21). This deduced amino acid sequence was tentatively assigned to the 12-kDa protein, which was hypothesized to be a variable region fragment of the light chain. The 24-kDa protein was presumed to be the intact light chain. Since there is only one germline κ constant region, the amino acid sequence of the 24-kDa protein was predicted by combining the single known cDNA-deduced amino acid sequence of the constant region of the κ light chain (22) with the known cDNA-deduced amino acid sequence of the AL-9666 variable region described above, with the assumption that there were no amino acid substitutions in the constant region.

Deducing amino acid sequences of proteins from their corresponding cDNAs does not provide any information on posttranslational modifications of the proteins. Obtaining the primary structure of a protein by N-terminal Edman and/or C-terminal amino acid sequencing is time consuming and may require an order of magnitude more material than mass spectrometry (MS). In addition, each method works reliably only for pure sample. Furthermore, there are complications associated with each method. For instance, the α-amino group of the N-terminus must be free in N-terminal Edman sequencing, and side reactions involving certain amino acid residues decrease the efficiency of C-terminal amino acid sequencing.

In contrast, MS provides an abundance of information about the protein of interest in a shorter period of time, requires only femtomoles to low picomoles of sample, and can tolerate the presence of secondary components. It has been proven to be a very powerful technique for verifying cDNA-deduced amino acid sequences and determining posttranslational modifications (23–27).

Electrospray ionization (ESI) (28) and matrix-assisted laser desorption/ionization (MALDI) (29) MS, in combination with enzymatic digestions, were used to verify the amino acid sequences and to identify and locate posttranslational modifications in the 12- and 24-kDa proteins. Complete sequence coverage was obtained for both proteins. The 12-kDa protein was confirmed to be the N-terminal κ1 light chain fragment (variable region) consisting of residues 1–108 or 1–109 and having one disulfide bond. The 24-kDa protein was identified as the intact κ1 light chain containing a cysteinyl posttranslational modification at Cys214. Three disulfide bonds were located in the 24-kDa protein: Cys23–Cys88, Cys134–Cys194, and Cys214–Cys.

MATERIALS AND METHODS

Initial purification and characterization of the urinary proteins. Two proteins were purified from the urine of a patient (AL-9666) diagnosed with AL amyloidosis of the κ1 subtype (sequence J COK1, GenBank Accession Numbers AF124196 and AF054662) (21). The patient has had amyloid for 10 years and suffered from severe limitations due to extensive soft tissue involvement of the tongue, synovial membranes, and muscles, with sparing of the heart. The urine was dialyzed in a Spectra/Per dialysis tubing (molecular weight cutoff 12 to 14 kDa, Spectrum Laboratories, Inc., Rancho Dominguez, CA) exhaustively against double-distilled water and then lyophilized. A portion of the lyophilized product was combined with Affi-Gel blue gel (Bio-Rad Laboratories, Hercules, CA) in 20 mM sodium phosphate buffer to remove albumin, allowed to stand at 4°C overnight, and filtered through a Whatman grade No. 4 filter paper (Maidstone, Kent, England). The post-Affi-Gel filtrate was dialyzed exhaustively, lyophilized, stored at –20°C, and prior to gel filtration, solubilized in 20 mM Tris buffer at pH 7.5.

This lyophilized Affi-Gel blue gel-treated product was fractionated by gel filtration on a Sephacryl S-200 HR column (2.6 × 100 cm, Amersham Pharmacia Bio-tech, Buckinghamshire, England), equilibrated, and eluted with 20 mM Tris buffer at pH 7.5 in the ascending mode. The major peak was dialyzed exhaustively.
against double-distilled water before lyophilization. SDS-PAGE analysis on a 20% homogeneous PhastSystem PhastGel (Amersham Pharmacia Biotech) suggested that this major peak contained a protein having a relative molecular mass of 24,000. Corresponding Western blot analysis (20% SDS-PAGE on the PhastSystem with electrophoresis to nitrocellulose) utilizing polyclonal goat anti-human κ light chain primary antibody (DiaSorin, Stillwater MN), rabbit anti-goat IgG secondary antibody conjugated to alkaline phosphatase (Promega Corporation, Madison, WI), and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) detection (Promega Corporation) confirmed this 24-kDa protein as being of the κ type. Repeating this procedure for the minor peak that eluted after the major peak described above suggested that the minor peak contained a protein having a relative molecular mass of 12,000. No reducing agent [e.g., dithiothreitol (DTT), β-mercaptoethanol] was used in the purification process.

Materials. All reagents were of the highest purity. DTT, iodoacetamide, sodium phosphate (dibasic), human angiotensin I, porcine renin substrate tetradecapeptide, and bovine oxidized insulin chain B were purchased from Sigma Chemical Co. (St. Louis, MO); trifluoroacetic acid (TFA) from Fluka Chemical Corp. (Milwaukee, WI); modified trypsin, endoproteinase Asp-N, and endoproteinase Glu-C (all sequencing grade) from Roche Molecular Biochemicals (Indianapolis, IN); and 2,5-dihydroxybenzoic acid (DHB) from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). All solvents were prepared with the in-house reverse osmosis-purified water, which had been further purified by passing through a Hydro Picotech 2 water purification system (Research Triangle Park, NC).

Reduction and S-carboxyamidomethylation. Reduction and S-carboxyamidomethylation of the purified proteins were used to probe disulfide linkages. Reduction of the intact protein was performed with a 10-fold molar excess of DTT over expected disulfides in 100 mM ammonium bicarbonate at pH 8 for 30 min at 37°C. S-Carboxyamidomethylation was carried out with a 5-fold molar excess of iodoacetamide over total thiols in the dark for 1 h. The S-carboxyamidomethylated protein solution was then desalted by passing through a Vydac (5-µm particle size, 214GD54, Hesperia, CA) high-performance guard column. The salts were washed away by running 95% mobile phase A (0.1% TFA in water) and 5% mobile phase B (acetonitrile containing 0.1% TFA) isocratically for 5 min at 0.75 mL/min. The eluent was monitored at 214 nm. The protein was eluted with 40% mobile phase A and 60% mobile phase B. The acetonitrile/water/TFA mixture was removed from the protein using a Savant Instruments SC110 Speedvac concentrator (Holbrook, NY).

Enzymatic digestions. Asp-N digestion of the 12-kDa protein (enzyme:substrate, 1:300), with and without reduction and S-carboxyamidomethylation, was carried out in 50 mM sodium phosphate buffer at pH 8 at 37°C for 15 h. Each Asp-N digestion was quenched by drying the reaction mixture in a Savant Instruments SC110 Speedvac concentrator. Each peptide mixture was dissolved in 0.1% TFA and analyzed by MALDI MS without further purification.

Trypsin (1:100) digestion of the native 24-kDa protein was performed in either 100 mM ammonium bicarbonate at pH 8 or 100 mM ammonium acetate at pH 6 at 37°C for 15 h. Trypsin (1:300) digestion was carried out in 100 mM ammonium bicarbonate at pH 8 at 37°C for 5 h. Lys-C (1:100) digestion was performed in 100 mM ammonium bicarbonate at pH 8 at 37°C for 7 h; to investigate the presence of disulfide-linked peptides, 20 mM DTT was added to an aliquot of the Lys-C digest mixture. Glu-C (1:60) digestion was carried out in either 100 mM ammonium bicarbonate at pH 8 or 100 mM ammonium acetate at pH 4 at room temperature for 22 h. Each enzymatic digestion was quenched by drying the reaction mixture in a Savant Instruments SC110 Speedvac concentrator. Each peptide mixture was then dissolved in 0.1% TFA and analyzed by MALDI MS without further purification.

Electrospray ionization mass spectrometry. ESI mass spectra of the intact proteins were obtained in the positive-ion mode with a Micromass Quattro II triple quadrupole mass spectrometer (Beverly, MA) during scans over the m/z range 700–2100. The instrument was calibrated using sodium trifluoroacetate ion clusters (30). After calibration, this mass spectrometer was capable of achieving at least 0.007% mass accuracy for the deconvoluted mass spectrum. A 2-µM protein solution in 50:50:0.1 (v/v/v) of methanol:water:acetic acid was infused into the ESI source by a Harvard Apparatus syringe pump (South Natick, MA) at a flow rate of 2 µL/min. The capillary potential and cone voltage were held at 3.6 kV and 30 V, respectively. Each resulting mass spectrum, which contains a distribution of charge states for each protein species in the sample, was obtained by summing a minimum of 10 scans. This ESI mass spectrum was then deconvoluted using the Micromass MassLynx (version 3.4) software to obtain the molecular mass of each protein species.

Matrix-assisted laser desorption/ionization mass spectrometry. MALDI mass spectra were acquired in the positive-ion mode with a Finnigan MAT Vision
2000 MALDI time-of-flight (TOF) reflectron mass spectrometer (Thermo BioAnalysis Corp., Santa Fe, NM) equipped with a Laser Science nitrogen laser (Franklin, MA) having a 3-ns pulse width at 337 nm. All data shown in the figures were obtained in the linear mode with delayed extraction (31, 32). An acceleration voltage of 20,000 V was applied to the target probe and the delay time was 450 ns. The instrument was calibrated with a standard peptide mixture consisting of human angiotensin I [M + H]\(^+\) m/z 1297.5, porcine renin substrate tetradecapeptide [M + H]\(^+\) m/z 1760.0, and bovine oxidized insulin chain B [M + H]\(^+\) m/z 3496.9. After this external calibration, this MALDI-TOF mass spectrometer was capable of achieving approximately 0.1% mass accuracy in the linear mode with delayed extraction. However, where greater mass accuracy was needed, the instrument was internally calibrated or operated in the reflectron mode so that a 0.01% mass accuracy could be obtained. Typically, 0.5 µL of a 10 pmol/µL solution of a peptide digest was mixed on the sample target with 0.5 µL of a 15 mg/mL solution of DHB dissolved in 30:70:0.1 (v/v/v) acetonitrile:water:TFA, and the mixture was allowed to air dry. Each MALDI-TOF mass spectrum was obtained by accumulating the signals resulting from 50 laser shots.

RESULTS AND DISCUSSION

Mass spectrometric peptide mapping of the 12-kDa protein. ESI mass analysis of the 12-kDa protein indicated that it had a deconvoluted molecular mass 11,688 ± 1 Da (Fig. 1A), which is in agreement with the mass 11,689 Da, calculated from the cDNA-deduced amino acid sequence of the AL-9666 variable region (residues 1–108) containing one disulfide bond (Fig. 2). To probe whether the native protein contained any free Cys residues, the 12-kDa protein was subjected to S-carboxyamidomethylation with iodoacetamide. After S-carboxyamidomethylation, the deconvoluted molecular mass 11,688 ± 1 Da (Fig. 1B) was detected, which is (within experimental error) the same mass observed for the native protein shown in Fig. 1A. Therefore, it was concluded that the native protein does not contain any free Cys residues. Moreover, ESI mass analysis of the native protein after reduction with DTT followed by S-carboxyamidomethylation with iodoacetamide showed a product having the deconvoluted mass 11,804 ± 1 Da (Fig. 1C), which is 116 ± 2 Da higher than the initial mass 11,688 ± 1 Da (Fig. 1A). Reduction and S-carboxyamidomethylation result in a 57-Da mass increase at each liberated Cys residue. Thus, the observed 116-Da mass increase establishes the presence of two available Cys residues in the reduced 12-kDa protein.

Similarly, the small peak observed at 11,788 ± 1 Da (Fig. 1A) may be assigned to a protein containing the variable region (residues 1–108, Figs. 2 and 5) plus one residue (residue 109) of the constant region (Fig. 5), having a calculated mass of 11,790 Da (with one disulfide bond). As above, it was concluded that this minor component did not have any free Cys residues since its mass did not change after S-carboxyamidomethylation.

![FIG. 1. Deconvoluted ESI mass spectra of the 12-kDa protein before (A) and after treatment with (B) iodoacetamide and (C) DTT and iodoacetamide.](image)

**FIG. 2.** Deduced amino acid sequence tentatively assigned to the 12-kDa protein purified from the urine of a patient (AL-9666) diagnosed with AL amyloidosis of the k1 subtype. The 12-kDa protein was hypothesized to be a light chain fragment containing the variable region, whose cDNA had previously been sequenced via reverse-transcriptase polymerase chain reaction using light chain variable region specific primers (sequence) [sequence] COK1, GenBank Accession Numbers AF124196 and AF054662 (21). The amino acid sequence contains two Cys (C) residues, at positions 23 and 88.

DIQMTQSPLSLASVGVDRVTITCQA 25
SQDISOYLNYYQKPGKAPKLIDG 50
ASNLLETGVPSRFSGSGTDFTPF 75
SSLPEDIAHYCQQYDNLPTFGG 100
GTKVEIKK 108

![FIG. 2. Deduced amino acid sequence tentatively assigned to the 12-kDa protein purified from the urine of a patient (AL-9666) diagnosed with AL amyloidosis of the k1 subtype.](image)
Furthermore, the 116 ± 2 Da mass increase [11,904 ± 1 Da (Fig. 1C)] after reduction and S-carboxyamidomethylation confirmed the presence of the two Cys residues in the reduced protein. Taken together, these observations suggest that the native 12-kDa protein is a fragment of the light chain, consisting of residues 1–108 or 1–109. Each protein contains two disulfide-linked Cys residues and no other posttranslational modifications.

To verify the cDNA-deduced amino acid sequence of the 12-kDa protein, the native protein was digested with Asp-N. The digest mixture was analyzed by MALDI-TOF MS (Fig. 3A). As can be deduced from Fig. 3A, Asp-N digestion of the 12-kDa protein gave 100% sequence coverage. The ion at m/z 1947.2 may be assigned to a peptide containing residues 92–109 (calculated [M + H]+ m/z 1947.2). This peptide would originate from the 109-residue light chain fragment whose deconvoluted mass appeared at 11,788 ± 1 Da (Figs. 1A and 1B). The ion at m/z 2490.9 corresponds to a peptide having residues 28–48 (calculated [M + H]+ m/z 2490.9) and a disulfide-linked peptide containing residues 17–27/82–91 (calculated [M + H]+ m/z 2487.7). To confirm the presence of this disulfide-linked peptide, the 12-kDa protein was reduced with DTT, S-carboxyamidomethylated with iodoacetamide, digested with Asp-N, and analyzed by MALDI-TOF MS (Fig. 3B). The peak corresponding to the disulfide-linked peptide mentioned above disappeared (Fig. 3B). Furthermore, increased relative abundances were observed for the ions at m/z 1279.5 and 1324.8, corresponding to peptides having residues 17–27 (calculated [M + H]+ m/z 1279.4) and 82–91 (calculated [M + H]+ m/z 1325.4), respectively, each S-carboxyamidomethylated at its Cys residue (Fig. 3B). The increase in abundance of each peptide was generated when the disulfide-linked peptide was reduced by DTT. The low abundance ion at m/z 2490.9 corresponds to the peptide containing residues 28–48. Table 1 summarizes the results from these mass spectra. Since these assigned sequences coincide with the amino acid residues of the variable region plus the first residue of the constant region of the k1 light chain, the 12-kDa protein could be confirmed as the N-terminal k1 light chain fragment consisting of residues 1–108 or 1–109.

ESI mass spectrometric analysis of the 24-kDa protein. Using ESI mass analysis, the deconvoluted molecular mass 23,395 ± 2 Da was determined for the 24-kDa protein (Fig. 4A). This observed mass does not agree with the calculated mass 23,278 Da derived from its cDNA-predicted amino acid sequence, allowing for the presence of two disulfide bonds at Cys23–Cys88.
and Cys134–Cys194 (Fig. 5) (33). After attempted S-carboxyamidomethylation of the native protein with iodoacetamide, the deconvoluted mass 23,396 ± 2 Da was observed (Fig. 4B); because the mass of the protein did not change upon S-carboxyamidomethylation, it was concluded that there are no free Cys residues in the native protein. However, after the native protein was reduced with DTT, the deconvoluted molecular mass 23,282 ± 2 Da was determined (Fig. 4C). This mass agrees with the calculated mass 23,282 Da, corresponding to the amino acid sequence of the gene product containing no disulfide bonds (Fig. 5). The 113 ± 4 Da mass decrease [23,282 ± 2 Da (Fig. 4C) minus 23,395 ± 2 Da (Fig. 4A)] after reduction with DTT is consistent with loss of a disulfide-linked cysteine and reduction of two to three disulfide bonds in the native protein. Moreover, ESI mass analysis of the native protein after reduction with DTT and S-carboxyamidomethylation with iodoacetamide shows a mass decrease of 113 Da.

### Table 1: Complete Sequence Coverage of the 12-kDa Protein as Shown by Selected Peptides from Asp-N Digestions (Fig. 3) Observed by MALDI-TOF MS

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<th>Observed [M + H]⁺ m/z</th>
<th>Calculated [M + H]⁺ m/z</th>
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Note. Cysteine and cystine residues are shown as C and C, respectively.

* Denotes a disulfide-linked peptide.

**FIG. 4.** Deconvoluted ESI mass spectra of the 24-kDa protein before (A) and after treatment with (B) iodoacetamide, (C) DTT, and (D) DTT and iodoacetamide.
Boxyamidomethylation with iodoacetamide yielded the deconvoluted molecular mass 23,568 ± 2 Da (Fig. 4D). The 286 ± 4-Da mass increase [23,568 ± 2 Da (Fig. 4D) minus 23,282 ± 2 Da (Fig. 4C)] verifies the presence of the five Cys residues (5 × 57 Da) in the main chain of the protein. The sixth cysteine, which is not part of the main chain, is lost upon reduction with DTT. The presence of the sixth cysteine indicates that the 24-kDa urinary protein contains a further posttranslational modification in addition to the disulfide bonds. The low abundance peak corresponding to the deconvoluted molecular mass 23,508 ± 2 Da in this spectrum (Fig. 4D) is due to a reduction followed by an incomplete S-carboxyamidomethylation. The resulting 226 ± 4-Da mass increase [23,508 ± 2 Da (Fig. 4D) minus 23,282 ± 2 Da (Fig. 4C)] designates that only four of the five Cys residues (4 × 57 Da) in the main chain of the protein were S-carboxyamidomethylated.

Asp-N digestion of the 24-kDa protein. To verify the amino acid sequence of the 24-kDa protein and to locate the positions of the disulfide bonds and the cysteinyl posttranslational modification, the native protein was digested with Asp-N. The resulting peptide mixture was then analyzed by MALDI-TOF MS. A large set of peptide ions were detected (Fig. 6A). The assignment of each [M + H]+ ion to a specific peptide is summarized in Table 2. Asp-N digestion of the 24-kDa protein produced two disulfide-linked peptides. The ion at m/z 2487.2 corresponds to a peptide containing residues 17–27/82–91 (calculated [M + H]+ m/z 2487.7) and that at m/z 3852.6 to a peptide having residues 17–27/70–91 (calculated [M + H]+ m/z 3854.2). To confirm that these were disulfide-linked peptides, an aliquot of the Asp-N digest peptide mixture was treated with DTT and then analyzed again by MALDI-TOF MS.
Complete Sequence Coverage of the 24-kDa Protein as Shown by Selected Peptides from Asp-N, Lys-C, and Trypsin Digestions (Figs. 6–8) Observed by MALDI-TOF MS

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<td>127–142/189–207</td>
<td>3824.3*</td>
<td>3824.4*</td>
<td>Trypsin, pH 6 and 8</td>
<td>SGTASVQCLNNFYPREAK/HKVTACEVTHQLSSPVTKSFRNGEC</td>
</tr>
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Note. Three disulfide-linked peptides were located: Cys23–Cys88, Cys134–Cys194, and Cys214–Cys. Main chain cysteine and cystine residues are shown as C and C, respectively. Cys represents the cysteinyl posttranslational modification. * Denotes disulfide-linked peptides.

MALDI-TOF MS (Fig. 6B). Not only did the two peaks corresponding to the disulfide-linked peptides disappear, but increased relative abundances were observed for the ions at m/z 1222.9 and 1269.1 (Fig. 6B). These ions represent peptides having residues 17–27 (calculated [M + H]+ m/z 1222.4) and 82–91 (calculated [M + H]+ m/z 1268.4). The increases in abundance of these peptides were generated when the disulfide-linked peptides were reduced by DTT. Furthermore, the appearance of the peak at m/z 2491.5 (Fig. 6B), which was not seen in Fig. 6A, suggests that the peak centered at m/z 2487.2 in Fig. 6A contained a contribution from the peptide having residues 28–48 (calculated [M + H]+ m/z 2490.9) in addition to the disulfide-linked peptide mentioned above. Thus, the peak observed at m/z 2487.2 (Fig. 6A) contains unresolved signals originating from two peptides: residues 28–48 and 17–27/82–91.

Lys-C digestion of the 24-kDa protein. Asp-N digestion of the 24-kDa protein did not provide any information on the cysteinyl posttranslational modification and only gave 85% sequence coverage (182 out of 214 amino acid residues). In an attempt to obtain peptides corresponding to the amino acid residues covering the remaining 15% of the sequence, the 24-kDa protein was next digested with Lys-C, and the resulting peptide mixture was analyzed by MALDI-TOF MS (Fig. 7A). Two disulfide-linked peptides were observed. The ion at m/z 3886.6 agrees with the calculated m/z value of 3887.4 for the [M + H]+ ion of a disulfide-linked peptide containing residues 127–145/191–207. The ion at m/z 932.0 corresponds to a peptide having residues 208–214 containing the cysteinyl posttranslational modification disulfide linked to Cys214 (calculated [M + H]+ m/z 932.0). The facile loss of this cysteinyl posttranslational modification due to prompt fragmentation in the MALDI ion source (34, 35) produced an ion detected at m/z 812.6 (calculated [M + H]+ m/z 812.9) (Fig. 7A). To verify that the ions at m/z 932.0 and m/z 812.6 originate from the peptides containing the disulfide linkage, the Lys-C digest mixture was reduced with DTT. The MALDI-TOF mass spectrum of this DTT-treated mixture evidences the disappearance of these ions (Fig. 7B). In addition, the relative abundance of the ion at m/z 812.9 increased (Fig. 7B). Similarly, increased signals were observed for the ions at m/z 1820.2 and m/z 2070.2 (Fig. 7B), corresponding to peptides having residues 191–207 (calculated [M + H]+ m/z 1820.1) and 127–145 (calculated [M + H]+ m/z 2070.4), respectively. Evidently, the bulk amount of these peptides was generated when the disulfide-linked peptides were reduced by DTT. Lys-C digestion of the 24-kDa protein thus revealed the location of the cysteinyl posttranslational modification and gave sequence coverage for the remaining amino acid residues that were not covered by Asp-N digestion (Table 2). Taken together, the results of the Asp-N and the Lys-C digestions gave 100% sequence coverage of the 24-kDa protein.
24-kDa protein. Our initial hypothesis that there were no amino acid substitutions in the constant region of the \(k_1\) light chain was thus shown to be correct. The 24-kDa protein was identified as the cysteinylated \(k_1\) light chain containing three disulfide bonds: Cys23-Cys88, Cys134-Cys194, and Cys214-Cys.

Trypsin and Glu-C digestions of the 24-kDa protein. Two disulfide-linked peptides were observed in the Asp-N digest (Fig. 6A), each one containing a disulfide bond between Cys23 and Cys88. In addition, the Lys-C digestion also gave two disulfide-linked peptides, one containing a disulfide bond between Cys134 and Cys194 and the other between the main chain Cys214 residue and the cysteinyl posttranslational modification (Fig. 7A). Both digestions were performed at pH 8.

Since disulfide bonds are not very stable at pH \(\approx 7\), thiols and disulfides can undergo rapid exchange (23, 36). As a result, disulfide bond rearrangement or scrambling can occur at neutral to alkaline pH. In contrast, the rate of disulfide interchange is slower at lower pH.

To verify that disulfide bond scrambling did not occur in our experiments, the 24-kDa protein was digested with trypsin at pH 6 and pH 8, and the digest mixtures were then analyzed by MALDI-TOF MS. The MALDI-TOF mass spectrum of the tryptic digest of the 24-kDa protein at pH 8 shows numerous peptide ions (Fig. 8A). Three \([M + H]^+\) ions corresponding to disulfide-linked peptides were detected at \(m/z\) 3559.0 (residues 127–142/191–207; calculated \([M + H]^+\) \(m/z\) 3559.1), \(m/z\) 3824.3 (residues 127–142/189–207; calculated \([M + H]^+\) \(m/z\) 3824.4), and \(m/z\) 931.7 (residues 208–214 containing the cysteinyl posttranslational modification at Cys214; calculated \([M + H]^+\) \(m/z\) 932.0). The ion at \(m/z\) 812.2 correlates with a peptide containing residues 208–214 that had lost the cysteinyl posttranslational modification from Cys214 due to prompt fragmentation in the MALDI ion source (Fig. 8A). These three disulfide-linked peptides were also observed when the protein was digested with trypsin at pH 6 (Fig. 8B).

To support the results of the tryptic digests, the 24-kDa protein was digested with Glu-C at pH 8 and pH 4 and analyzed by MALDI-TOF MS. Ions observed at \(m/z\) 2167.8 and \(m/z\) 2168.5 in the two MALDI-TOF mass spectra (data not shown) correspond to a disulfide-linked peptide having residues 196–214 containing the cysteinyl posttranslational modification at Cys214 (calculated \([M + H]^+\) \(m/z\) 2167.4). The loss of the cysteinyl posttranslational modification from the main chain Cys214 residue due to prompt fragmentation in the MALDI ion source was seen in the two MALDI-TOF mass spectra at \(m/z\) 2047.4 and \(m/z\) 2048.3 (calculated \([M + H]^+\) \(m/z\) 2048.3).

Taken together, these results indicate that there was no disulfide bond scrambling in our experiments. The
two disulfide linkages at Cys134–Cys194 and Cys214–Cys were observed in the tryptic digests at both pH 8 and pH 6, and the disulfide linkage at Cys214–Cys was observed in the Glu-C digests at both pH 8 and pH 4. Posttranslational modifications and amyloidosis. The experimentally determined molecular mass 23,395 ± 2 Da (Fig. 4A) of the 24-kDa protein agrees with the mass 23,397 Da, calculated from the predicted amino acid sequence of the intact light chain containing a cysteinylation posttranslational modification disulfide linked to Cys214 of the main chain of the protein. Mass spectrometric peptide mapping identified three disulfide bonds and their locations in the 24-kDa protein: Cys23–Cys88, Cys134–Cys194, and Cys214–Cys. The existence of the Cys23–Cys88 and Cys134–Cys194 disulfide bonds in an immunoglobulin light chain is well known (33). Cysteinylation at Cys214 of a k1 light chain isolated from a patient who had multiple myeloma has been reported (37, 38), although the significance of this posttranslational modification has not been determined. Prior to the results reported herein, cysteinylation of a k1 light chain isolated from a patient diagnosed with AL amyloidosis has not been reported.

While both AL amyloidosis and multiple myeloma are diseases that are associated with the overproduction of the light chain by a particular B-lymphocyte clone, the former is correlated with multimerization of the light chain into amyloid fibrils and the latter into amorphous aggregates (39–41). Since the deposits in AL amyloidosis most often consist of the N-terminal fragments of the light chain (10–13), the majority of studies have focused on the determination of the primary structure of the light chain, and reducing agents such as DTT or β-mercaptoethanol have been used routinely in the initial purification (10–20). Attention was therefore drawn away from consideration of the effect of cysteinylation on the native protein structure. In an immunoglobulin molecule, the Cys214 residue of the k1 light chain normally forms an interchain disulfide bond with the heavy chain (42). This phenomenon is also true for the Cys214 residue of the lambda (λ) light chain, which has 215 amino acid residues. However, in AL amyloidosis and multiple myeloma, free light chains are secreted excessively into the serum without having a heavy chain available for disulfide bond formation. The circumstance of the cysteinylation of Cys214 in the k1 light chain is not known and might occur as an intracellular or extracellular process. More importantly, cysteinylation of the Cys214 residue of the λ light chain has not been reported.

Kappa light chains are known to exist primarily as monomers, and λ light chains are found mainly as covalent dimers (43, 44). Furthermore, λ light chains are known to be more amyloidogenic than k light chains (45–47). It has been hypothesized that the
dimerization of the light chain is the first step in the polymerization of the protein to form amyloid fibrils (48, 49). Furthermore, it has been speculated that cysteinylation of Cys214 of a k1 light chain may prevent the light chain monomer from forming a dimer (38). Since light chains are found mainly as dimers, they may be prone to form amyloid fibrils.

The underlying factors responsible for initiating the overproduction of a monodonal light chain by a particular B-lymphocyte clone are not yet fully understood. In addition, the mechanism of amyloid fibril formation in AL amyloidosis is not known. Although in vitro studies have shown that amino acid substitutions in the variable region of the light chain could cause the protein to undergo a conformational change into amyloid fibrils (7–9), posttranslational modification may also be involved. For instance, glycosylation of both the k and l light chains has been reported (13, 17, 50–53). Moreover, reduction of disulfide bonds of an amyloidogenic l light chain, under nonphysiological conditions, has been shown to strongly enhance the probability that the protein will form amyloid fibrils (54).

CONCLUSIONS

In this paper, we have described the detection and characterization of the cysteinylation of a k1 light chain from an amyloid patient (AL-9666), a modification which has not previously been reported in this context. Many of the previous studies have focused on determination of the primary structure of the light chain, and reducing agents such as DTT or -mercaptoethanol have been used routinely in the initial purification process. As a result, posttranslational modifications that contribute to fibril formation by immunoglobulin light chains may have been altered. Since posttranslational modifications can affect the structure and folding of a protein, they may play an important role in determining the stabilization/destabilization of a protein. In future studies that explore the phenomena leading to AL amyloidosis, we recommend that posttranslational modifications be protected and documented.

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