Electrospray Low Energy CID and MALDI PSD Fragmentations of Protonated Sulfinamide Cross-linked Peptides

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Murine S100A8 (A8) is a major cytoplasmic neutrophil protein and is converted to novel oxidation products containing Cys–ε amino–Lys sulfinamide cross-links and Met–sulfoxide by the neutrophil oxidant HOCl. Seven products were separated using RP-HPLC, with electrospray ionization mass spectrometry (ESI-MS) masses after deconvolution of 10,354, 10,388, ±1, and 20,707, ±3 Da, and all were resistant to reduction by dithiothreitol. The major products with masses of 10,354 Da contained Cys_{41}–$Lys_{34/35}$ intramolecular cross-links. Additional isomeric products with identical masses (10,354 Da) were isolated and peptide mapping and ESI/MS indicated that Cys_{41} forms covalent sulfinamide cross-links with either Lys_6, Lys_{76}, Lys_{83}, or Lys_{87} present in A8. Electrospray low energy collisionally induced (CID) spectra of multiply-charged AspN digest peptides with sulfinamide cross-links contained characteristic fragmentations that corresponded to simple cleavage of the nitrogen–sulfur bond with charge retention on either of the fragment ions, allowing conformation of cross-linked peptides. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) post source decay spectra of $[M + H]^+$ ions of the same sulfinamide-containing cross-linked peptides fragment similarly, but additional facile fragmentation reactions corresponding to formation of a protonated peptide containing de-hydroalanine were attributed to cleavage of the carbon–sulfur bond. In addition, lose of methanesulfenic acid from Met–sulfoxide was observed. A sulfinamide-containing adduct was isolated after incubation of the A8/HOCl reaction mixture with Lys or α N-acetyl Lys with masses of 10,500 or 10,542 Da. ESI/MS/MS and MALDI/ post decay source (PSD) analysis of $A8_{32–57}$–sulfinamide showed the same characteristic fragmentations as those in the sulfinamide cross-linked peptides, confirming the Cys_{41}–Lys sulfinamide cross-link and suggesting that peptide–peptide sulfinamides may all fragment similarly, allowing ready identification of these cross-links in proteins from more complex biological materials. (J Am Soc Mass Spectrom 2002, 13, 709–718) © 2002 American Society for Mass Spectrometry

S100 calcium-binding proteins are a family of 18 highly conserved, small (~10 kDa) acidic proteins with important regulatory functions including regulation of kinases, suppression of tumor progression, embryogenesis, and cell migration [1–5]. S100A8 (A8) has chemotactic activity for leukocytes [6] although other studies indicate additional roles [4, 7], possibly related to regulating inflammation, when present at high levels. S100 proteins form non-covalent homodimers characterized by a symmetric homodimeric fold not found in other Ca^{2+}-binding proteins, in solution. Non-covalent A8 homodimers were also observed using mass spectrometry [8]. Ca^{2+}-binding induces structural changes within helices III and IV, exposing amino acids within the hinge and C-terminal domains that may be involved in binding target proteins [5, 9]. Covalent oxidative modifications may regulate the functions of S100B, A8, and S100A2 [10–12].

Neutrophils (PMN) release reactive oxygen species formed via the NADPH oxidase complex following activation with agents such as phorbol 12-myristate 13-acetate (PMA) and chemoattractants [13]. The respiratory burst produces superoxide anions which dismutate to hydrogen peroxide (H_2O_2) that is converted by myeloperoxidase (MPO) and Cl^- to the powerful two-electron (non-radical) oxidant hypochlorous acid (HOCl), which can chemically modify lipids, proteins and kill invading pathogens [14]. MPO-generated and exogenous HOCl react identically [15–17] and protein thiols and Met are preferred substrates [18]. HOCl also initiates LDL lipid peroxidation and formation of 3-chlorotyrosine and dityrosine [19].

A8 and S100A9 (A9), present constitutively in high concentrations (~40% of cytosolic protein) in PMN,

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may be released from activated or dying cells at inflammatory sites [1, 20, 21], making them likely candidates for oxidation in acute inflammation. High levels of A8 are also released by inflammatory macrophages stimulated by IL-10 [22], suggesting that this protein has an anti-inflammatory role, possibly by scavenging HOCl. A8 is effectively oxidized by low amounts of HOCl (molar ratios of $<\sim 10$) in vitro, with 70–80% conversion to dimer, and neutrophils activated by PMA generate an oxidative burst that efficiently oxidizes exogenous A8 within 10 min, most likely via H_2O_2/MPO released by activated cells [11]. The major A8 HOCl oxidation products isolated by C4 RP-HPLC corresponded to Cys_{41}–Lys_{34} and Cys_{41}–Lys_{35} sulfinamides containing Met(O) which were formed in ~80% yield [23]. Oxidation of the single Cys in A8 by Cu^{2+} to the disulfide-linked homodimer was highly specific and negated its chemotactic properties in vitro and in vivo [11] whereas the major sulfinamide form remained active [23]. Positive chemotactic activity of a Cys_{41} to Ala_{41} mutant (Ala_{41}A8) confirmed that Cys_{41} is not essential for function, implying that covalent dimerization may structurally modify accessibility of the chemotactic hinge domain and may regulate chemotaxis in vivo. The exquisite susceptibility of A8 to oxidation, and the potentially large amounts present at sites of acute inflammation, suggests that it may also have anti-oxidant properties that protect against excessive tissue damage.

Here we further describe the oxidation products of HOCl-oxidised A8 using peptide mapping and mass spectrometry [23]. Intra-molecular sulfinamide bonds between Cys_{41} and 6 of the 7 Lys residues were readily generated, as well as conversion of Met to Met–sulfoxide [Met(O)]. Unusual fragmentations characteristic of sulfinamides were observed in the low energy CID and MALDI/PSD spectra of cross-linked peptides. Additional sulfinamide-containing products, corresponding to addition of Lys forming an ε–amino sulfinamide, were isolated when the amino acid Lys or α N-acetyl Lys was added the reaction mixture.

Experimental

General

Reagents and chemicals were analytical grade (Sigma, St. Louis, MO; BioRad, Hercules, CA) and solvents were HPLC grade (Mallinckrodt, Clayton South, Victoria, Australia). Hypochlorous acid (10–13%) was from Aldrich (Milwaukee, WI) and quantitated spectrophotometrically as described [24]. Recombinant A8/Ala_{41}A8 were produced using the pGEX expression system as detailed [11, 25, 26]. SDS/PAGE/Western blotting were performed using a Mini Protean II apparatus (BioRad, Hercules, CA) with 15% gels and a Tris/Tricene buffer system [27]. Liquid chromatographic separations were performed using a non-metallic LC626 HPLC system (Waters, Bedford, MA) and monitored at A_{214nm} and A_{280nm} with a Waters 996 photodiode array detector or 490 UV/visible detector.

Oxidation Reactions

Oxidation of A8 with hypochlorite. A8 (~100 μg, 10 nmol) was diluted with PBS (100 μl, 25 mM phosphate, 250 mM NaCl, pH 7.5), HOCl (5 μl, 50 nmol), and the solution left at 22 °C for 10 min. Products were separated using C4 RP HPLC (5 μ, 300 Å, 4.6 × 250 mm, Vydac, Separations Group, Hesperia, CA) with a gradient of 35 to 65% CH_3CN, 0.1% TFA, over 30 min. Major peaks (A_{214nm}) were collected and fractions 1 and 1.1 were combined from 10 separate reactions, before partial lyophilization (~500 μl) and reseparation on HPLC using a gradient of 25 to 70% CH_3CN/0.1% TFA over 30 min, before analysis.

Oxidation of A8 with hypochlorite and reaction with lysine or N-acetyl Lys. A8 (~100 μg, 10 nmol) was diluted with PBS (100 μl, 25 mM phosphate, 250 mM NaCl, pH 7.5), HOCl (5 μl, 50 nmol), and the solution left at 22 °C for ~20 s, then Lys, α N-acetyl Lys, or ε N-acetyl Lys (7.5 μl, 340 mM, Aldrich) was added and the solution left for 10 min at 22 °C. Products were separated using C4 RP HPLC (5 μ, 300 Å, 4.6 × 250 mm, Vydac, Separations Group, Hesperia, CA) with a gradient of 25 to 75% CH_3CN, 0.1% TFA, over 30 min. Major fraction (A_{214nm}) were collected and lyophilised before MS analysis.

Peptide Mapping

A8 oxidation products (100 μg) isolated from C4 RP-HPLC were digested in ammonium bicarbonate (400 μl, 50 mM, pH 8.0) using endoprotease AspN (sequencing grade, Roche, Castle Hill, NSW) at an enzyme to substrate ratio of approximately 1:100 at 37 °C for 3 h. The pH of the digest was adjusted to approximately 2 (TFA 1%) and the mixture applied directly to a C18 RP-column (5 μ, 300 Å, 4.6 × 250 mm, Vydac, Separations Group, Hesperia, CA). Peptides were eluted with a gradient of 5 to 75% acetonitrile (0.1% TFA) at 1 ml/min over 30 min. Fractions with major A_{214nm} were collected, then lyophilized and redissolved in H_2O/CH_3CN/acetic acid (50:49:1) for ESI or MALDI MS or further enzymatic digestion.

Both isomers of oxidized $A8_{32–57}$–$A8_{62–83}$ (~5 μg) isolated after AspN cleavage were digested with endoprotease LysC (sequencing grade, Roche) in ammonium bicarbonate (50 μl, 50 mM, pH 8.0) at an enzyme to substrate ratio of approx. 1:100 at 37 °C for 5 h. Peptides were isolated using RP-HPLC as described above before ESI/MALDI MS analysis or the total digest (1 μl) was analyzed by MALDI MS.

Mass Spectrometry

Electrospay ionization mass spectrometry. Masses of proteins and peptides were determined using ESI. Spectra

were acquired using a single quadrupole mass spectrometer equipped with an ESI source (MSD1100, Hewlett Packard, Palo Alto, CA). Samples (~50 pmol, 10 μl) were injected into water:acetonitrile (50:50) 1% acetic acid (10 μl/min) using an LC1100 pump (HP) coupled directly to the electrospray source. Nitrogen was used as the nebulizer and drying gas (7.0 L/min, 150 °C). Sample droplets were ionized at a positive potential of approximately 4000 V and transferred to the mass analyzer with a fragmentor voltage (capillary to skimmer lens voltage) of 75 V. Spectra were acquired over the mass range *m/z* 200 to 1500 in 0.5 s with unit resolution. Low energy CID MS/MS spectra were recorded using a triple quadrupole (TSQ 7000, Finnigan, San Jose CA) where multiply-charged (+3, +4, or +5) precursor ions were selected with Q1, collisionally activated within rf-only Q2 (20–50 eV, argon, manifold pressure ~1.5 mTor). Spectra recorded with unit resolution scanning Q3 over *m/z* 50–2550 in 2 s were accumulated into a single data file for 4–5 min. Ions were formed using an in house nano-ESI device. Borosilicate glass capillaries (75 × 1.5 mm) were drawn to an exit i.d. of <1 μm using a micro-pipette puller (Narishige, Model PN-3, Tokyo, Japan) then tips were broken under a microscope, forming an exit i.d. of ~50 μm. Peptide-containing solutions (~10 μl, $H_2O/CH_3CN/CH_3CO_2H$, 50:49:1) were loaded into the capillary and electrical contact (~1–2 kV) was maintained using a gold electrode protruding into the liquid. The glass capillary was positioned 2–5 mm from the entrance to the heated capillary, which was at 150 °C; stable ion currents were maintained with flow rates of ~100–200 nl/min.

Matrix assisted laser desorption. Peptide solutions (~25 pg/μl) were mixed with matrix [1 μl, 2,5 dihydroxybenzoic acid (DHB, Sigma) 10 mg/ml] and air-dried before analysis. Spectra were acquired in linear mode and positive ions generated using a N_2 laser (337 nm, 3-ns pulse width) and accelerated to 25 keV after an extraction delay of 200 μs (Voyager STR, Perseptive Biosystems, Framingham, MA). Typically, 50 spectra were averaged and calibrated externally using angiotensin I and insulin (ox) B chain (peptides) or insulin (ox) B chain and myoglobin (proteins).

PSD were also acquired using a Voyager STR mass spectrometer, with the timed ion selector set to the precursor mass, a mirror ratio of 1.12, 11 segments with a 75% decrement ratio were acquired and stitched automatically using the instrument software (Data Explorer V3.2, Perseptive). In some experiments collision gas (air) was introduced into a collision cell located between the source and flight tube to enhance fragmentation. A different target position was chosen for each segment and ~200 spectra acquired and averaged. Spectra were calibrated using the metastable fragments of substance P or angiotensin-I (Sigma) and were generally ±1.5 Da of their predicted value for peptides *m/z* ~3000 and ±4 for peptides *m/z* ~6000.

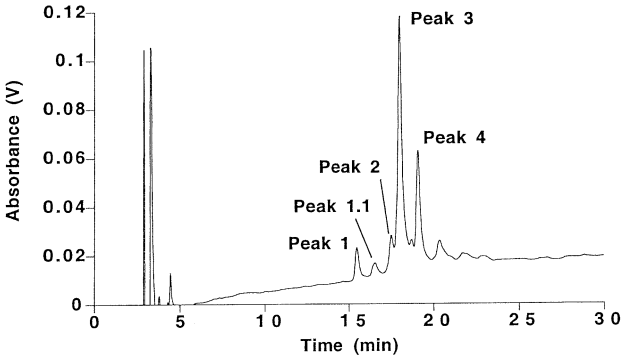


Figure 1. Preparative C4 RP-HPLC of S100A8 HOCl oxidation products. (**a**). Products were separated after incubation of A8 with ~5 molar equivalents of HOCl for 10 min at 22 °C. ESI masses after deconvolution were 10,354 Da (peak 1), 10,354 Da (peak 1.1), 10,388 Da (peak 2, 10,354 Da ±1 (peak 3), and 10,354/20,707 Da ±3 (peak 4).

Results and Discussion

Hypochlorite (OCl^-) is the major oxidant produced by neutrophils in inflammatory responses and murine A8 is exquisitely susceptible to oxidation by HOCl [11, 23]. Low concentrations of hypochlorite (<40 μM) convert A8 to a modified monomer (10,354, +46 Da) corresponding to addition of three oxygens and loss of dihydrogen, and to oxidized dimer (20,707, +93 Da), indicating oxidation of susceptible amino acids (Cys/Met) and formation of intra- and inter-molecular sulfinamide cross-links [11, 23]. The resistance of Ala_{41}A8 to HOCl-mediated dimerisation at all HOCl concentrations confirmed the role of Cys_{41}. Reagent or MPO-generated HOCl form identical reaction products in proteins [16]. Cys and Met are the preferred substrates followed by amines (e.g., Lys, forming chloramines) [18, 28]. Almost all α amino acids form reactive aldehydes after incubation with HOCl formed using the MPO/H_2O_2/Cl^- system [24]. Thus, products generated by reagent HOCl in vitro are likely to reflect those produced by HOCl generated by activated phagocytes.

Isolation and Characterization of HOCl Oxidation Products of A8

Murine A8 treated with ~4–5 equivalents of HOCl for 10 min at 20 °C yielded 5 separate peaks after C4 RP-HPLC (Figure 1). The major product (Figure 1, peak 3) had a mass of 10,354 ±1 Da, 46 Da greater than the unmodified protein. Three additional components of mass 10,354, 10,354, 10,388, and 10,354/20,707 (Figure 1, peaks 1, 1.1, 2, and 4) were separated and isolated using a HPLC gradient of 35 to 65% acetonitrile. We previously showed that the major product is a mixture of oxidized Cys_{41} covalently linked to $Lys_{34/35}$ via the ε–amine forming a sulfinamide bond, and oxidation of Met to Met(O) [23]. Characterization of the novel sulfinamide oxidation product was accomplished using peptide mapping and mass spectrometry [23]. Other

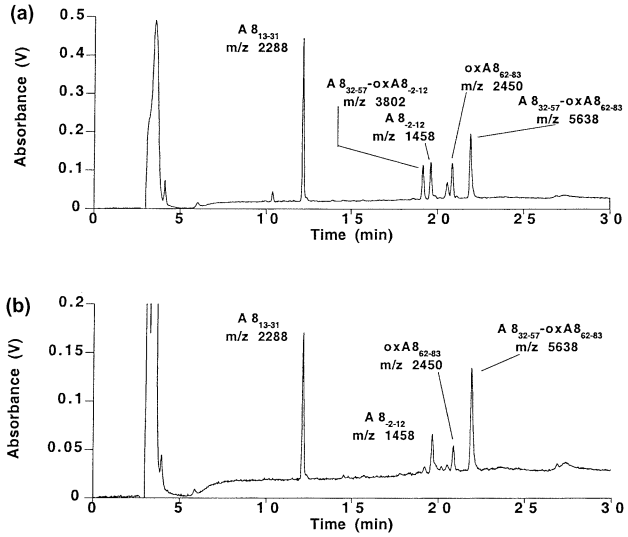


Figure 2. AspN digestion of S100A8 HOCl oxidation products [peak 1 and 1.1 (Figure 1)] separated by C18 RP-HPLC. Masses and predicted identities of digest peptides are indicted for digestion of peak 1 (**a**) and peak 1.1 (**b**). Sulfinamide cross-linked peptides have *m/z* 3802, 5638 (peak 1), and 5638 (peak 1.1).

products identified in the previous study were peak 1 (Cys_{41}–Lys_{87} sulfinamide) peak 2 (Cys–sulfonic acid), and peak 4 (Cys_{41}–Lys_6 sulfinamide dimer) [23]. Formation of a reactive Cys–sulfonic acid intermediate that subsequently underwent nucleophilic substitution by the ε–amine (from Lys) was proposed. Early publications suggest that protein sulfenic acids may undergo nucleophilic substitution with amines (e.g., benzylamine) to yield stable covalent bonds although none have been isolated or rigorously characterized [29]. Our results indicate that seven A8 oxidation products form after reaction of specific amino acids with a Cys(O) intermediate and that six of the seven ε–amines readily react with this intermediate.

The isomeric HOCl-modified proteins (peaks 1 and 1.1, Figure 1) not fully characterized previously were digested with AspN and peptides separated by C18 RP-HPLC (Figure 2). The identities of the major digest peptides were determined based on comparisons of theoretical and experimental masses. Probable sulfinamide-containing peptides had masses of *m/z* 3802 and 5638. Previously, we showed that the peptide with *m/z* 3802 contained a sulfinamide between Cys_{41} and Lys_{87} [23]. A peptide with an identical experimental and theoretical mass corresponding to oxidized $A8_{32–57}$ linked to $A8_{62–83}$ (*m/z* 5638) via a sulfinamide was also isolated (Figure 2a). This peptide ion may not have been observed in the previous study because of suppression of ionization in MALDI [30]. This indicates that peak 1 (Figure 1) is a mixture of A8 oxidation products containing sulfinamides between Cys_{41}–Lys_6 and Cys_{41} and a Lys within peptide $A8_{62–83}$. The C18 RP-HPLC chromatogram of the AspN digest of peak 1.1 shows one peak with a mass (*m/z* 5638) also indicating a probable sulfinamide (Figure 2b). The mass (±1 Da) and retention time (±10 s) were identical to the peptide with sulfinamide between Cys_{41} and $A8_{62–83}$ isolated after AspN digestion of peak 1 (Figure 2a and b). C4 RP-HPLC of peaks 1 and 1.1 revealed that each component was >95% pure, indicating that there was little cross contamination of the 2 components and suggesting that they contain structural isomers. $A8_{62–83}$ has two Lys residues (Lys_{76} and Lys_{83}) and the precise identity of the isomers with *m/z* 5638 was determined after digestion of each cross-linked peptide with Lys C.

Endoprotease Lys C cleaves on the C-terminal side of Lys residues and formation of sulfinamides would likely inhibit digestion, allowing differentiation of free and covalently-linked Lys resides. Figure 3 shows the structures and masses, determined by MALDI and ESI MS, of isomeric peptides (*m/z* 5638) digested with LysC (Figure 3a and b) together with the digest products of Cys_{41}–Lys_6 sulfinamide (Figure 3c) for comparison. Digestion of sulfinamide $oxA8_{32–57}$–$oxA8_{62–83}$ from peak 1 gave peptides with masses of *m/z* 5120 and 408 which correspond to $oxA8_{37–57}$–$oxA8_{62–83}$ and $A8_{32–36}$ indicating that Lys_{76} was not susceptible to Lys C digestion and therefore most likely contains the sulfinamide bond between Cys_{41} and Lys_{76}. In comparison, digestion of sulfinamide $oxA8_{32–57}$–$oxA8_{62–83}$ from peak 1.1 yielded peptides with masses of *m/z* 3385, 1770 and 408 which correspond to sulfinamide $A8_{37–57}$–$oxA8_{77–83}$, $oxA8_{62–76}$ and $A8_{32–36}$ indicating that Lys_{76} was susceptible to Lys C digestion and therefore the most likely sulfinamide bond would be between Cys_{41} and Lys_{83}. The expected Lys C digest products of Cys_{41}–Lys_6 are *m/z* 3285 and 408 corresponding to sulfinamide $oxA8_{37–57}$–$A8_{-2–12}$ and are identical to the predicted masses of digestion of this peptide (Figure 3c).

Peak 4 (Figure 1) contains sulfinamide-bonded dimer of A8, with sulfinamide bonds between Cys_{41} and Lys_6 and Cys_{41} and $Lys_{34/35}$ [23]. This peak also contained a variable proportion of monomer (up to ~50%) (mass 10,354 Da) that was not resolved from the dimer using C4 RP HPLC. A sulfinamide cross-linked peptide ($oxA8_{32–57}$–$A8_{-2–12}$) of mass *m/z* 4647 was isolated from peak 4 (Figure 1) after AspN digestion and C18 RP-HPLC and was also used in the MS structural studies. The amino acid sequence of A8, together with the sequence and position of modified Cys and Lys residues and the structures of the major HOCl oxidation products of A8, are shown in Figure 4.

Interestingly, sulfinamide bonds in the cross-linked peptides appeared stable at pH >7 for extended times (>7 days), whereas the bond was hydrolysed within a few days storage at pH ~2.5 (4 °C) forming a peptide containing Cys–sulfinic acid ($CysSO_2H$) and a peptide with unmodified Lys. These products were readily separated by RP-HPLC and masses determined by ESI (not shown). A possible hydrolysis mechanism is outlined in Scheme **1** and is similar to acid-catalysed hydrolysis of ester/amide bonds [31]. Protein containing sulfinamides were stable at pH ~2 for >6 months at −80 °C.

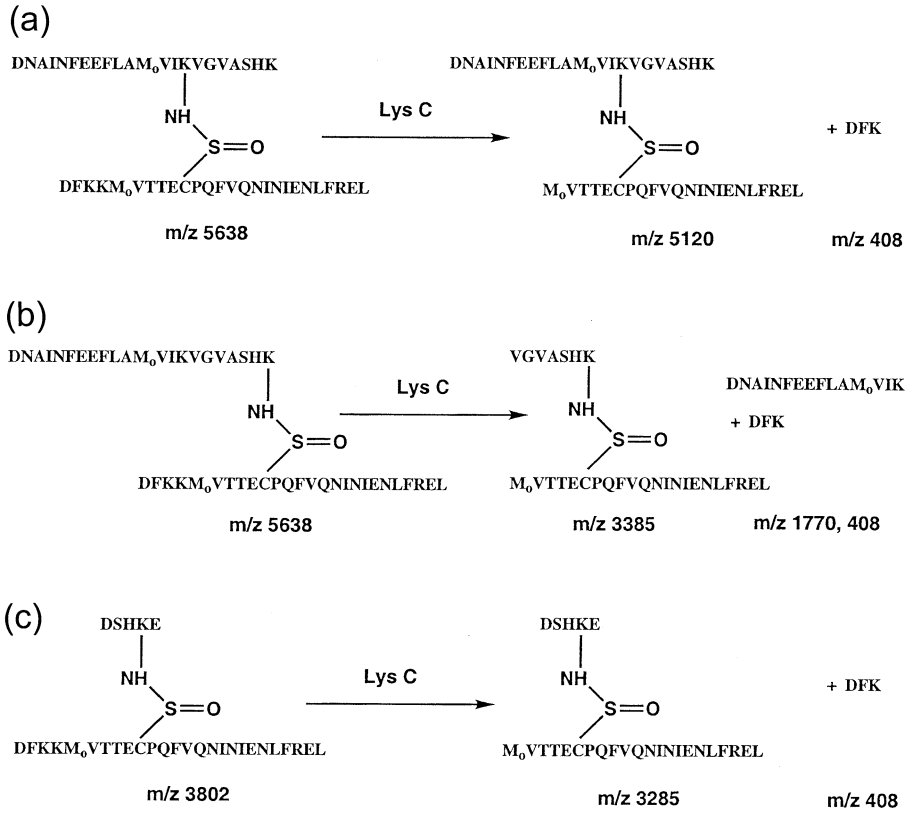


Figure 3. Products of endoprotease Lys C digestion of sulfinamide cross-linked peptides *m/z* 5638 and 3802. The specific Lys residues on isomeric peptides (*m/z* 5638) derived from AspN digestion of peaks 1 (**a**) and 1.1 (**b**) were determined after digestion with endoprotease LysC because sulfinamide-linked Lys resides were resistant to protease digestion.

Low Energy Collision Induced Dissociation of Multiply-Charged Sulfinamide Containing Peptides

Four sulfinamide cross-linked peptides were analyzed by ESI low energy CID tandem MS isolated after Asp N digestion and C18 RP-HPLC of peaks 1, 1.1, and 4. These corresponded to oxidized $A8_{37-57}$–$A8_{84-88}$ (*m/z* 3802, Cys_{41}–Lys_{87} sulfinamide), $A8_{37-57}$–$A8_{62-83}$ (*m/z* 5638, Cys_{41}–Lys_{76} sulfinamide), $A8_{37-57}$–$A8_{62-83}$ (*m/z* 5638, Cys_{41}–Lys_{83} sulfinamide), and $A8_{37-57}$–$A8_{-2-12}$ (*m/z* 4647, Cys_{41}–Lys_6 sulfinamide). The most intense multiply-charged ion for each peptide was selected with MS1 and a collision energy chosen that showed the most intense product ions (Figure 5a, b, c, d). The collision gas pressure was constant for all MS/MS spectra at ~1.5 mTorr Ar. MS/MS CID spectra of each multiply-charged ion contained very similar fragmentation patterns for all the sulfinamide cross-linked peptides. Two sets of intense multiply charged product ions that corresponded to protonated peptides derived after cleavage of the nitrogen sulfur sulfinamide bond, with charge retention on either fragment were generally observed. These products are similar to those expected after y- or b-type cleavage of peptide bonds after CID and are designated F_N (charge retention on Lys containing peptide) and F_S (charge retention on Cys containing peptide) fragment ions in Figure 5. F_N fragment ions have experimental masses corresponding to theoretical masses of protonated peptides containing no modifications (*m/z* ±0.5). The theoretical average mass of the $[M + H]^+$ ion (F_S fragments Figure 5) formed after simple cleavage of the sulfinamide bond was *m/z* 3187.7. The average experimental mass, derived from the multiply charged fragment ions, is *m/z* 3188 (±0.6). Charge remote fragmentation mechanism have been proposed for elimination reactions of protonated peptides containing alkylated oxidized Cys resides after low energy CID with a Q-star mass spectrometer [32]. More accurate products ion mass measurement using a Q-TOF or MS^3 (ion trap) may resolve the precise mechanism and the products formed during low energy CID of sulfinamide bonded peptides. Additional ions, some corresponding to the expected sequence ions of both peptides were also observed and were enhanced by increasing the collision energy by 50–100% (not shown).

MALDI-Post Source Decay Analysis of Protonated Sulfinamide Containing Peptides

The four sulfinamide cross-linked peptides from C18 RP-HPLC were analyzed by MALDI/PSD using DHB

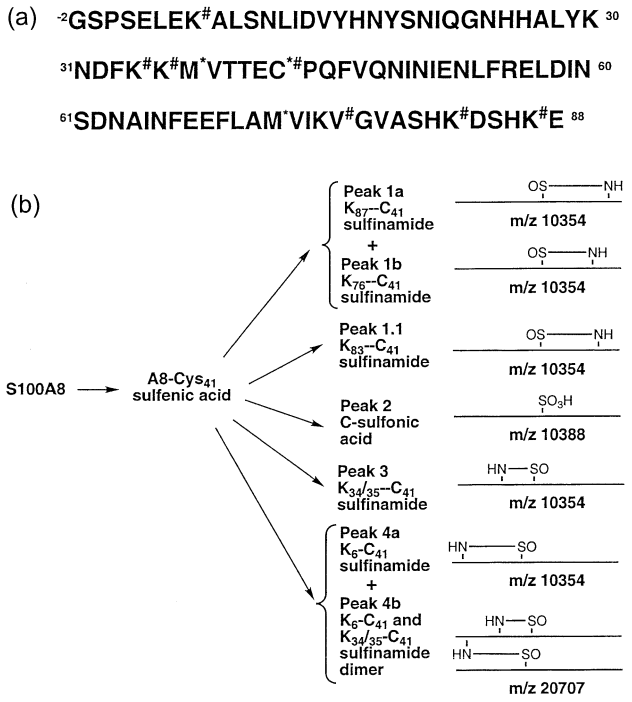
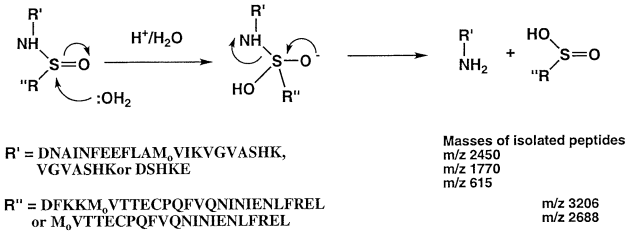


Figure 4. Structures of sulfinamide cross-linked S100A8. (**a**) A8 amino acid sequence indicating locations of Lys, Met, and Cys residues modified by HOCl oxidation (labeled with a pound sign or an asterisk). Six of the seven Lys resides in S100A8 formed sulfinamide cross-links with the reactive intermediate Cys_{41} sulfenic acid. (**b**) Proposed structures of Cys_{41}–Lys_X sulfinamide-bonded products identified after oxidation of A8 with HOCl. The structures of the major products (peaks 2, 3, and 4) were determined in a previous study [23].

matrix (Figure 6a, b, c, d) after selecting the precursor ion using the timed ion selector. Apart from an increase in the intensities of low-mass immonium ions, identical spectra were obtained employing CID (not shown). Mass accuracy of product ions were generally within *m/z* ±1.5 of theoretical values for precursor ions at ~3000 and *m/z* ~±4 for precursor ions at ~6000. Although product ion mass accuracy was modest, fragmentations of sulfinamides, which were only evident by MALDI, were observed and are probably high-energy or photo-dissociation processes not normally observed in low energy CID MS/MS spectra using triple quadrupoles. Fragmentation of the sulfinamide bond forming two protonated product ions, similar to those observed in the ESI/MS/MS spectra, were also



Scheme 1 Acid catalysed hydrolysis of sulfinamide cross-linked peptides forms Cys–sulfinic acid and Lys containing peptides.

evident in the PDS spectra (i.e., ions at *m/z* ~3190 in Figure 6a, b, c, d and 2449 in b and c). However additional fragmentations characteristic of sulfinamides and Met(O) that corresponded to elimination reactions forming dehydro-alanine were also observed. Eliminations of this type in CID MS/MS spectra were recently reported for alkylated oxidized Cys residues [32] and Met(O) readily eliminates methanesulfenic acid [33]. Scheme **2** proposes a similar charge remote fragmentation mechanism to account for the formation of a protonated peptide containing dehydro-alanine after CID of protonated sulfinamides. Loss of *m/z* ~64 from the precursor ion, and from other major ions, corresponds to the loss of methansulfenic acid from Met(O) would occur as described [32, 33]. More accurate product ion mass measure, possibly using high energy CID with a TOF-TOF MS, would allow products ions and fragmentation mechanism to be more precisely defined [34]. The ion at *m/z* ~1974 in all PSD spectra corresponds to the sequence $A8_{42–57}$ (PQFVQNINIENLFREL) formed after y-type peptide bond cleavage of the Cys_{41}–Pro_{42} bond of the precursor ion.

Low Energy CID/MALDI PSD Analysis of A8–Lys Sulfinamide Adduct

The reactivity of the initially-formed A8–Cys sulfenic acid was tested by addition of lysine, α N-acetyl lysine or ε N-acetyl lysine to the reaction mixture. The C4 RP-HPLC chromatograms were virtually unchanged except for a minor peak eluting as a partially resolved shoulder before peak 2 (Figure 1) after addition of Lys, which was isolated (not shown). The mass of this adduct was 10,500 Da and corresponds to the theoretical mass (10,499.5 Da) of Met-oxidized A8 covalently linked to Lys via a sulfinamide bond. No additional adducts were isolated after addition of ε N-acetyl Lys, but an adduct with 10542 Da was isolated after incubation with α N-acetyl Lys, corresponding to ε-substituted A8 α N-acetyl Lys sulfinamide (theoretical mass 10542 Da), indicating preferential substitution of the ε–amine versus α–amine of Lys. The most likely protein containing sulfinamides would also be via the ε–amine of Lys.

Digestion of the A8–Lys sulfinamide adduct with AspN and separation by C18 RP-HPLC revealed the expected A8 digest peptides together with a peptide corresponding to $A8_{32–57}$–Lys sulfinamide (*m/z* 3336) (not shown). Fragmentations consistent with a Cys_{41}–Lys sulfinamide were identified using ESI low energy CID MS/MS and MALDI PSD analysis of isolated peptides. Electrospray low energy CID MS/MS spectra of $[M + 4H]^{4+}$ and $[M + 3H]^{3+}$ precursor ions of $A8_{32–57}$–Lys sulfinamide are shown in Figure 7a and b. Similar to the fragmentations observed in the spectra of the sulfinamide cross-linked peptides (Figure 5), the major fragmentation in both spectra is cleavage of the sulfinamide bond forming protonted Lys (*m/z* 147) and

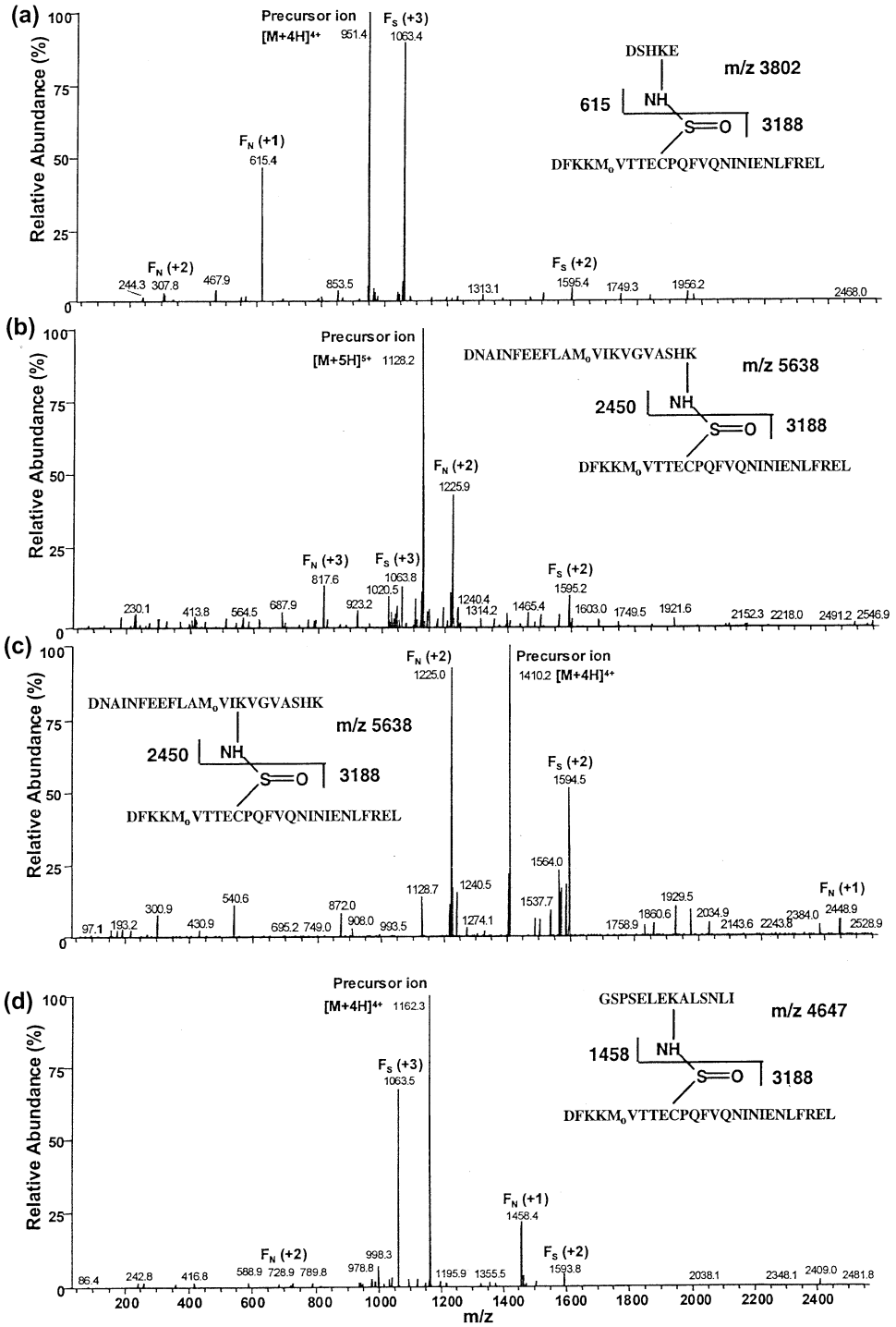


Figure 5. Low energy CID MS/MS spectra of protonated sulfinamide containing peptides. Collisional activation of multiply-charged sulfinamide cross-linked peptides caused simple cleavage of the nitrogen–sulfur bond forming two peptide fragments which share the charges originally located on the precursor ion. Precursor ions are indicated together with major fragment ions, these are designated F_N or F_S and charge states are shown in parenthesis (the insert in each spectrum shows the structure of precursor ion and experimentally derived *m/z* of singly protonated precursor and product ions). (**a**) $[M + 4H]^{4+}$ ion (*m/z* 951.4) of $A8_{32-57}$–$A8_{84-88}$ (Cys_{41}–Lys_{87} sulfinamide) was fragmented at 25 eV, forming major product ions at *m/z* 1063.4 and 615.4; similarly, (**b**) $[M + 5H]^{5+}$ ion (*m/z* 1128.2) of $A8_{32-57}$–$oxA8_{62-83}$ (Cys_{41}–Lys_{83} sulfinamide) was fragmented at 40 eV, forming major product ions at *m/z* 1595.2, 1063.4, 1225.9, and 817.6; (**c**) $[M + 4H]^{4+}$ ion (*m/z* 1410.2) of $A8_{32-57}$–$oxA8_{62-83}$ (Lys_{76}–Cys_{41} sulfinamide) was fragmented at 50 eV, forming major product ions at *m/z* 1594.5 and 1225.0; and (**d**) $[M + 4H]^{4+}$ ion (*m/z* 1162.3) of $A8_{-2-12}$–$A8_{32-57}$ (Cys_{41}–Lys_6 sulfinamide) was fragmented at 50 eV, forming major product ions at *m/z* 1063.5 and 1458.4.

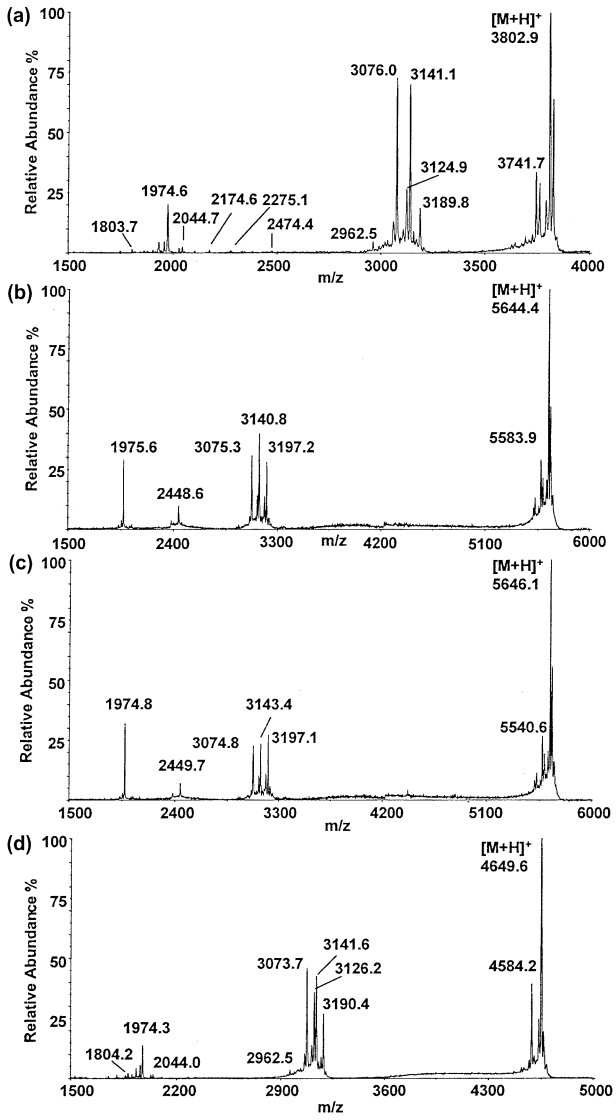
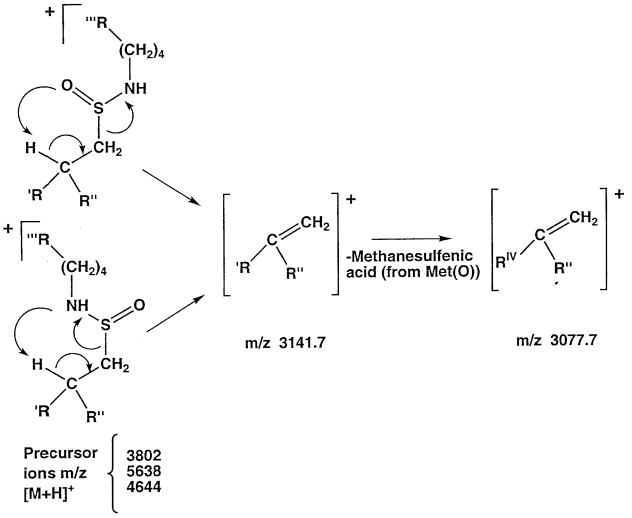


Figure 6. MALDI/PSD spectra of protonated sulfinamide containing peptides. Singly protonated sulfinamide cross-linked peptides all fragment similarly, by fragmentation of the sulfinamide bond (similar to those observed in the low energy CID spectra). Major losses corresponding to fragmentation of sulfur methylene bond, forming protonated peptides containing dehydroalanine also occur (see Scheme **2**). Intense losses of methanesulfenic acid [*m/z* 64 from Met(O)] are also evident, as is y''_{16} forming the ion at *m/z* 1975. (**a**) $A8_{32-57}$–$A8_{84-88}$ (Cys_{41}–Lys_{87} sulfinamide) *m/z* 3802; (**b**) $A8_{32-57}$–$oxA8_{62-83}$ (Cys_{41}–Lys_{83} sulfinamide); (**c**) $A8_{32-57}$–$oxA8_{62-83}$ (Cys_{41}–Lys_{76} sulfinamide); (**d**) $A8_{32-57}$–$A8_{-2-12}$ (Lys_6–Cys_{41} sulfinamide).

a protonated peptide with *m/z* 3188. Some additional ions corresponding to peptide bond cleavages of protonated $oxA8_{32-57}$ are also evident at higher collision energies (not shown). Fragmentations in the MALDI PSD spectra were also consistent with $A8_{32-57}$–Lys sulfinamide (Figure 7c). Major losses forming protonated dehydroalanine$_{41}$–$oxA8_{32-57}$ (*m/z* 3139) with subsequent loss of methanesulfenic acid (*m/z* 3077.4) were the most intense peaks observed. Loss of methanesulfenic acid from the precursor the ion at *m/z* 3273.1



Scheme 2 Proposed structures and fragmentation mechanism of Cys_{41}–Lys sulfinamide cross-linked peptides forming de–hydroalanine containing peptides by MALDI-PSD.

was evident. Peptide bond fragmentations (y-type) are also shown; $y_{17-19,21}$ indicate the presence of dehydroalanine, suggesting that formation of this product ion is very favorable in MALDI (high energy) processes.

Conclusions

Sulfinamide bond formation is a novel oxidative cross-linking process resulting in covalent bonds between thiols and amines, which may stabilise/modify protein function and would be resistant to regeneration by conventional pathways involving reductases. In the inflammatory milieu, sulfinamide bond formation between Lys and Cys residues in proteins, or S100 cross-linking with other proteins, could potentially regulate normal and pathological processes. HOCl generated by activated macrophages in inflammatory lesions modifies Apo B 100 in LDL without significant lipid oxidation. Apo B becomes aggregated and cross-linked, with loss of amines producing a high uptake form that accelerates foam cell formation in atherosclerosis [16]. The protein may be cross-linked/aggregated by sulfinamide bonds. Further experiments are required to define the generality of this oxidative modification and its importance in regulating protein function and disease processes, particularly relevant to aging, atherogenesis, fibrosis, and neurogenerative diseases. The sensitivity and specificity of mass spectrometry will allow characterization of sulfinamides isolated from inflammatory lesions, or from normal tissue, where they may form only at low levels.

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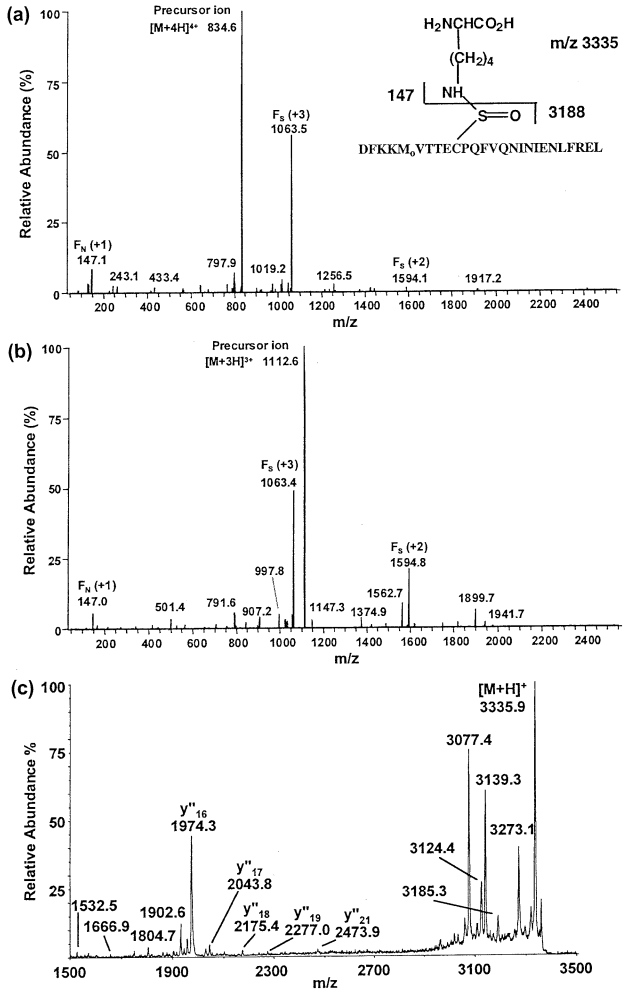

Figure 7. ESI low energy CID MS/MS and MALDI/PSD spectra of protonated $A8_{32–57}$ Cys_{41}–Lys sulfinamide derivative. $A8_{32–57}$ Cys_{41}–Lys sulfinamide (*m/z* 3336) isolated by C18 RP-HPLC after AspN digestion of the $S100A8_{32–57}$–Lys sulfinamide derivative (mass 10500 Da). (**a**) ESI MS/MS spectrum (40 eV) of $[M + 4H]^{4+}$ ion (*m/z* 834.6) of $A8_{32–57}$–Lys (Cys_{41}–Lys sulfinamide) with formation of multiply charged $A8_{32–57}$ ions (designated F_S ions, *m/z* 1063.5 +3 charge state, 1594.1 +2 charge state) and protonated Lys (F_N ion, *m/z* 147). The insert shows the structure of the precursor ion and experimentally derived *m/z* of singly protonated product ions); (**b**) similar fragmentations and product ions are observed after CID (50 eV) of the $[M + 3H]^{3+}$ ion (*m/z* 1112.6) of $A8_{32–57}$–Lys sulfinamide. (**c**) MALDI/PSD spectrum of $A8_{32–57}$ Cys_{41}–Lys sulfinamide. Formation of protonated dehydroalanine$_{41}A8_{32–57}$ (*m/z* 3139) followed by loss of methansulfenic acid (*m/z* 3077) are evident. Additional sequence-specific y-type ions are also indicated in C.

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References

1. Hessian, P. A.; Edgeworth, J.; Hogg, N. MRP-8 and MRP-14, Two Abundant Ca(2+)-Binding Proteins of Neutrophils and Monocytes. *J. Leukoc. Biol.* **1993,** *53,* 197–204.
2. Fano, G.; Biocca, S.; Fulle, S.; Mariggio, M. A.; Belia, S. The A-100—A Protein Family in Search of a Function. *Prog. Neurobiol.* **1995,** *46,* 71–82.
3. Schäfer, B. W.; Heizmann, C. W. The S100 Family of EF-Hand Calcium-Binding Proteins: Functions and Pathology. *TIBS* **1996,** *21,* 134–140.
4. Passey, R. J.; Xu, K.; Hume, D. A.; Geczy, C. L. S100A8: Emerging Functions and Regulation. *J. Leukoc. Biol.* **1999,** *66,* 549–556.
5. Donato, R. Functional Roles of S100 Proteins, Calcium-Binding Proteins of the EF-Hand Type. *Biochim. Biophys. Acta* **1999,** *1450,* 191–231.
6. Lackmann, M.; Cornish, C. J.; Simpson, R. J.; Moritz, R. L.; Geczy, C. L. Purification and Structural Analysis of a Murine Chemotactic Cytokine (CP-10) with Sequence Homology to S-100 Proteins. *J. Biol. Chem.* **1992,** *267,* 7499–7504.
7. Harrison, C. A.; Raftery, M. J.; Alewood, P. F.; Geczy, C. L. Structure/Function Studies of S100A8/A9. *Lett. Peptide Sci.* **2000,** *6,* 359–369.
8. Raftery, M. J.; Geczy, C. L. Identification of Noncovalent Dimeric Complexes of the Recombinant Murine S100 Protein CP10 by Electrospray Ionization Mass Spectrometry and Chemical Cross-Linking. *J. Am. Soc. Mass Spectrom.* **1998,** *9,* 533–539.
9. Kligman, D.; Hilt, D. C. The S100 Protein Family. *TIBS* **1988,** *13,* 437–443.
10. Scotto, C.; Mely, Y.; Ohshima, H.; Garin, J.; Cochet, C.; Chambaz, E.; Baudier, J. Cysteine Oxidation in the Mitogenic S100B Protein Leads to Changes in Phosphorylation by Catalytic CKII-α Subunit. *J. Biol. Chem.* **1998,** *273,* 3901–3908.
11. Harrison, C. A.; Raftery, M. J.; Walsh, J.; Alewood, P.; Iismaa, S. E.; Thliveris, S.; Geczy, C. L. Oxidation Regulates the Inflammatory Properties of the Murine S100 Protein S100A8. *J. Biol. Chem.* **1999,** *274,* 8561–8569.
12. Deshpande, R.; Woods, T. L.; Fu, J.; Zhang, T.; Stoll, S. W.; Elder, J. T. Biochemical Characterization of S100A2 in Human Keratinocytes: Subcellular Localization, Dimerization, and Oxidative Cross-Linking. *J. Invest. Dermatol.* **2000,** *115,* 477–485.
13. Weiss, S. J. Tissue Destruction by Neutrophils. *N. Engl. J. Med.* **1989,** *320,* 365–376.
14. Winterbourn, C. C.; Kettle, A. J. Biomarkers of Myeloperoxidase-Derived Hypochlorous Acid. *Free Radic. Biol. Med.* **2000,** *29,* 403–409.
15. Drozdz, R.; Naskalski, J. W.; Sznajd, J. Oxidation of Amino Acids and Peptides in Reaction with Myeloperoxidase, Chloride, and Hydrogen Peroxidase. *Biochim. Biophys. Acta* **1988,** *957,* 47–52.
16. Hazell, L. J.; van der Berg, J. J. M.; Stocker, R. Oxidative Aggregation of Isolated LDL with Hypochlorite Involves Lysine Residues but not Lipid Oxidation. *Biochem. J.* **1994,** *302,* 297–304.
17. Anderson, M. M.; Requena, J. R.; Crowley, J. R.; Thorpe, S. R.; Heinecke, J. W. The Myeloperoxidase System of Human Phagocytes Generates N^{ε}-(Carboxymethyl)Lysine on Proteins: A Mechanism for Producing Advanced Glycation End Products at Sites of Inflammation. *J. Clin. Invest.* **1999,** *104,* 103–113.
18. Winterbourn, C. C. Comparative Reactivities of Various Biological Compounds with Myeloperoxidase–Hydrogen Peroxide–Chloride, and Similarity of the Oxidant to Hypochlorite. *Biochim. Biophys. Acta* **1985,** *840,* 204–10.
19. Hazen, S. L.; Heinecke, J. W. 3-Chlorotyrosine, a Specific Marker of Myeloperoxidase-Catalysed Oxidation, is Markedly Elevated in Low Density Lipoprotein Isolated from Human Atherosclerotic Intima. *J. Clin. Invest.* **1997,** *99,* 2075–2081.

20. Kerkhoff, C.; Klempt, M.; Sorg, C. Novel Insights into Structure and Function of MRP8 (S100A8) and MRP14 (S100A9). *Biochim. Biophys. Acta* **1998,** *1448,* 200–211.
21. Hetland, G.; Talgö, G. J.; Fagerhol, M. K. Chemotaxins C5a and fMLP Induce Release of Calprotectin (Leucocyte L1 Protein) from Polymorphonuclear Cells in Vitro. *Mol. Pathol.* **1998,** *51,* 143–148.
22. Xu, K.; Yen, T.; Geczy, C. Il-10 Up-Regulates Macrophage Expression of the S100 Protein S100A8. *J. Immunol.* **2001,** *166,* 6358–66.
23. Raftery, M. J.; Yang, Z.; Valenzuela, S. M.; Geczy, C. L. Novel Intra- and Inter-Molecular Sulfinamide Bonds in S100A8 Produced by Hypochlorite Oxidation. *J. Biol. Chem.* **2001,** *276,* 33393–33401.
24. Hazen, S. L.; Hsu, F. F. ; d'Avignon, A.; Heinecke, J. W. Human Neutrophils Employ Myeloperoxidase to Convert α Amino Acids to a Battery of Reactive Aldehydes: A Pathway for Aldehyde Generation at Sites of Inflammation. *Biochem.* **1998,** *37,* 6864–6873.
25. Iismaa, S. E.; Hu, S. P.; Kocher, M.; Lackmann, M.; Harrison, C. A.; Thliveris, S.; Geczy, C. L. Recombinant and Cellular Expression of the Murine Chemotactic Protein, CP-10. *DNA Cell Biol.* **1994,** *13,* 183–192.
26. Raftery, M. J.; Harrison, C. H.; Geczy, C. L. Characterization of a Mutant Recombinant S100 Protein Using Electrospray Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **1997,** *11,* 405–409.
27. Schägger, H. von; Jagow, G. Tricine-Sodium Dodecyl Sulphate-Polyacrylamide Electrophoresis for the Separation of Peptides in the Range 1 to 100 kDa. *Anal. Biochem.* **1987,** *166,* 368–379.
28. Carr, A. C.; Tijerina, T.; Balz, F. Vitamin C Protects Against and Reverses Specific Hypochlorous Acid and Chloramine-Dependent Modifications of Low-Density Lipoprotein. *Biochem. J.* **2000,** *346,* 491–499.
29. Allison, W. S. Formation and Reactions of Sulfenic Acids in Proteins. *Acc. Chem. Res.* **1976,** *9,* 293–299.
30. Wang, M. Z.; Fitzgerald, M. C. A Solid Sample Preparation Method that Reduces Signal Suppression Effects in the MALDI Analysis of Peptides. *Anal. Chem.* **2001,** *73,* 625–631.
31. March, J. *Advanced Organic Chemistry.* John Wiley and Sons: New York, 1985, p 334.
32. Steen, H.; Mann, M. Similarity Between Condensed Phase and Gas Phase Chemistry: Fragmentation of Peptides Containing Oxidized Cysteine Residues and Implications for Oroteomics. *J. Am. Soc. Mass Spectrom.* **2001,** *12,* 228–232.
33. Mo, W.; Yuliang, M.; Toshifumi, T.; Neubert, T. A. Sequencing of Oxidized Methionine Containing Peptides for Protein Identification. *Rapid Commun. Mass Spectrom.* **2000,** *14,* 2080–2081.
34. Medzihradszky, K. F.; Campbell, J. M.; Baldwin, M. A.; Falick, A. M.; Juasz, P.; Vestal, M. L.; Burlingame, A. L. The Characteristics of Peptide Collision-Induced Dissociation Using a High-Performance MALDI-TOF/TOF Tandem Mass Spectrometer. *Anal. Chem.* **2001,** *72,* 552–558.