Novel Intra- and Inter-molecular Sulfinamide Bonds in S100A8 Produced by Hypochlorite Oxidation*

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Hypochlorite is a major oxidant generated when neutrophils and macrophages are activated at inflammatory sites, such as in atherosclerotic lesions. Murine S100A8 (A8) is a major cytoplasmic protein in neutrophils and is secreted by macrophages in response to inflammatory stimuli. After incubation with reagent HOCl (hydrogen peroxide) for 10 min, ~85% of A8 was converted to 4 oxidation products, with electrospray ionization mass spectrometry masses of m/z 10354, 10388, 10354 ± 1, and 20707 ± 3. All were resistant to reduction by diithiothreitol. Initial formation of a reactive Cys sulfenic acid intermediate was demonstrated by the rapid conjugation of 5,5-dimethyl-1,3-cyclohexanediol (dimeadone) to HOCl-treated A8 to form stable adducts. Matrix-assisted laser desorption-reflectron time of flight peptide mass fingerprinting of isolated oxidation products confirmed the mass additions observed in the full-length proteins. Both Met^54675 were converted to Met^54673 sulfoxides. An additional product with an unusual mass addition of m/z 14 (±0.2) was identified and corresponded to the addition of oxygen to Cys^41, conjugation to various e-amines of Lys^6, Lys^43/5, or Lys^87 with loss of dihydrogen and formation of stable intra- or inter-molecular sulfinamide cross-links. Specific fragmentation identified in matrix-assisted laser desorption-post source decay spectra and low energy collisional-induced dissociation tandem mass spectroscopy spectra of sulfinamide-containing digest peptides confirmed Lys^34/35 to Cys^41 sulfinamide bonds. HOCl oxidation of mutants lacking Cys^41 (A41A8, S100A8) or specific Lys residues (e.g. Lys^84/5, Ala^34/35A8, S100A8) did not form sulfinamide cross-links. HOCl generated by myeloperoxidase and H2O2 and by phorbol 12-myristate 13-acetate-activated neutrophils also formed these products. In contrast to the disulfide-linked dimer, oxidized monomer retained normal chemotactic activity for neutrophils. Sulfinamide bond formation represents a novel oxidative cross-linking process between thioles and amines and may be a general consequence of HOCl protein oxidation in inflammation not identified previously. Similar modifications in other proteins could potentially regulate normal and pathological processes during aging, atherogenesis, fibrosis, and neurogenerative diseases.

S100 calcium-binding proteins are highly conserved, small

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1The abbreviations used are: A8, recombinant murine S100A8; PMN, polymorphonuclear leukocytes; PMA, phorbol 12-myristate 13-acetate; A9, S100A9; H2O2, hydrogen peroxide; MPO, myeloperoxidase; HOCl, hypochlorous acid; Ala^41A8, recombinant Cys^41-Ala^41 mutant S100A8; Met(O), Met sulfoxide; Ala^34/35A8, recombinant Lys^34/5-lys^34/5 to Ala^34/35 mutant S100A8; ESI-MS, electrospray ionization mass spectrometry; CID, collision-induced dissociation; m/z, mass to charge ratio; MALDI, matrix-assisted laser desorption; MS, mass spectrometry; TOF, time of flight; PSD, post source decay; OCl, hypochlorite; Cys(O), Cys sulfenic acid; PBS, phosphate-buffered saline; dimedone, 5,5-dimethyl-1,3-cyclohexanedione.
and formation of 3-chlorotyrosine and dityrosine (31).

A8/S100A9 (A9), present constitutively in high concentrations (~40% of cytosolic protein) in PMN, can be released from activated or dying cells at inflammatory sites (1, 32, 33), making these proteins likely candidates for oxidation in acute inflammation. A8 is effectively oxidized in vitro by low amounts of reagent HOCl (molar ratios of ~< 10), with 70–80% conversion to dimer, and PMA activation of neutrophils generated an oxidative burst that efficiently oxidized exogenous A8 within 10 min, most likely via H₂O₂/MPO released by activated cells. (10). Moreover, disulfide-linked A8 homo-, but not A8/A9 heterodimer, is found in lung lavage fluid of mice at the beginning of the resolution phase of LPS-induced pulmonary injury (10), indicating oxidative modifications of A8 in inflammatory responses in vivo.

Oxidation of the single Cys residues in A8 by Cu²⁺ to the disulfide-linked homodimer was highly specific and negated bioactivity in vitro and in vivo (10). Positive chemotactic activity of a Cys⁴¹-Ala⁴¹ mutant (Ala⁴¹A8) confirmed that Cys⁴¹ is not essential for function and implied that covalent dimerization may structurally modify accessibility of the chemotactic hinge domain. Therefore, oxidation-dependent dimerization may also be a physiologically significant regulatory mechanism controlling A8-provoked leukocyte recruitment (10). Moreover, the exquisite susceptibility of A8 to oxidation and the potentially large amounts present may protect against excessive tissue damage at sites of acute inflammation.

Here we describe the oxidation products of reagent HOCl-oxidized A8 using peptide mapping and mass spectrometry. Novel intra-and intermolecular sulfonamide bonds between Cys⁴¹ and Lys residues were readily generated as well as conversion of Met to Met sulfoxide (MetO). Sulfonamide formation apparently occurs via sulfenic acid and/or sulfinyl chloride intermediates, which may decompose upon reaction with amines, yielding stable covalent bonds. Identical products were formed by HOCl generated enzymatically via the MPO/H₂O₂/Cl⁻ system and from PMA-activated human neutrophils. The oxidized A8 monomer retained activity in chemotaxis assays in vitro.

**EXPERIMENTAL PROCEDURES**

**General—**Reagents and chemicals were analytical grade (Sigma, Bio-Rad), and solvents were HPLC grade (Mallinkrodt Chemical Works). Reagent hypochlorous acid (10–13%) was from Aldrich. Recombinant A8/Ala⁴¹A8 was produced using the pgEX expression system as detailed (10, 34). SDS/polyacrylamide gel electrophoresis/Western blotting were performed using a Mini Protean II apparatus (Bio-Rad) with 15% gels and a Tris/Tricine buffer system (35). Liquid chromatographic separations were performed using a non-metallic LC626 HPLC system (Waters, Bedford, MA) and monitored at A₂₅₄ nm and A₂₀₀ nm, with a Waters 996 photodiode array detector or 490 UV-visible detector.

**Oxidation Reactions**

**Oxidation of A8 with Reagent 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 was left at 22 °C for 10 min. Products were separated using C4 RP-HPLC (5 µm, 300 Å, 4.6 × 250 mm, Vydac, Separations Group). Amino acids were identified using a Waters 996 photodiode array detector or 490 UV-visible detector.

**Oxidation of A8 by Myeloperoxidase/H₂O₂/Cl⁻—**Human MPO (40 milliuunits, Sigma) was added to A8 (~25 µg, 2.5 nmol) and H₂O₂ (10 nmol) in PBS (50 µl) were incubated for 5–15 min at 22 °C. Oxidation products were separated using C4 RP-HPLC (as above). The masses of oxidized A8 were determined using electrospray ionization mass spectrometry (ESI-MS) after lyophilization and resuspension of samples in H₂O/CH₃CN/acetic acid (50 µl). Oxidation of A8 by PMA-simulated Neutrophils—Neutrophils were isolated by standard procedures (10), washed twice, and resuspended in Dulbecco’s PBS (Sigma). A8 (~25 µg, 2.5 nmol) was added to 10⁷ cells/ml, activated with PMA (1 µg/ml, Sigma), and incubated for 30 min at 37 °C (~10 milliuunits of MPO). Aliquots (500 µl) removed 10 and 30 min after activation were centrifuged (10,000 g, 30 s), and supernatants were frozen immediately at −80 °C. After thawing, oxidation products were separated using C4 RP HPLC (as above), and masses of oxidation products were determined using ESI-MS (as above). In some experiments sodium azide (50 µM) was included to inhibit HOCl formation by MPO.

**Derivatization A8 Sulfenic acid with 5,5-Dimethyl-1,3-cyclohexanedione (Dimedone)**

A8 (~25 µg, 3 nmol) was diluted with PBS (25 µl), HOCl (1.5 µl, 50 nmol), and the solution was left at 22 °C for 20 s. Dimedone (2 µl, 10 – 6 M, Aldrich) was added, and the mixture was incubated at 22 °C for 10 min. Products were separated using C4 RP HPLC with a gradient of 35–55% CH₃CN, 0.1% trifluoroacetic acid over 30 min. Major A₈₂₄,₄₆ were collected and lyophilized before analysis by ESI-MS (as above).

**Peptide Mapping**

A8/Ala⁴¹A8 (100 µg) isolated from C4 RP-HPLC were digested in ammonium bicarbonate (400 µl, 50 mM, pH 8.0) using endoproteinase Asp-N (sequencing grade, Roche Molecular Biochemicals) at an enzyme to substrate ratio of ~1:100 at 37 °C for 3 h. The pH of the digest was adjusted to ~2 (1% trifluoroacetic acid), and the mixture was applied directly to a C18 RP column (5 µm, 300 Å, 4.6 × 250 mm, Vydac, Separations Group). Peptides were eluted with a gradient of 5–75% acetonitrile (0.1% trifluoroacetic acid) at 1 ml/min over 30 min. Fractions with major absorbances at A₂₁₄ nm were collected then lyophilized and reconstituted in H₂O/CH₃CN/acetic acid (50:49:1) for ESI or MALDI-MS or further enzymatic digestion.

Oxidized or oxidized A₈₈₋₉₅ (~40 µg) isolated after Asp-N treatment were digested with pepsin (100 ng, Roche Molecular Biochemicals) in sodium formate (250 µl, 100 mM, pH 4.0) at 22 °C for 4 h and endoproteinase Lys-C or Glu-C (sequencing grade, Roche Molecular Biochemicals) in ammonium bicarbonate (50 µl, 50 mM, pH 8.0) at an enzyme to substrate ratio of ~1:100 at 37 °C for 5 h. Peptides were isolated as described above before ESI-MALDI-MS analysis.

**Site-directed Mutagenesis**

The coding region of A8 was subcloned from a previously described construct (GST-CP10) (34) into the BamHI site of pbBlueScript (SK) vector (Stratagene, La Jolla, CA). The A8 insert was subcloned from pbBlueScript (SK) into the NotI/EcoB site of pUCOS-2 vector (Novagen, Madison, WI). The point mutations (Lys⁴⁸ and Lys⁶⁵ to Ala⁴⁸ and Ala⁶⁵) were made using a modified version of the whole plasmid polymerase chain reaction technique described by Fisher and Pei (36), omitting the DpnI digestion step. Sequences of the reverse and forward primers used to generate the double point mutations were GAA GAT CTT CTA GAG GGC ATG GTG ATT and GCC GCA ATG ACT GCT AGT CAT. Both primers were phosphorylated using the T4 polynucleotide kinase (Roche Molecular Biochemicals) before polymerase chain reaction. After polymerase chain reaction and gel purification, the linearized plasmid was recircularized using T4 ligase (Promega, Madison, WI), then transformed in Escherichia coli. Plasmid DNA derived from individual colonies was bidirectionally sequenced to confirm that correct base substitutions had been made. The mutated A8-coding sequence was subcloned from pUCOS-2 vector into the BamHI site of pGEX-2T vector (Amrad-Pharmacia, Melbourne, Australia) and transformed into E. coli, and recombinant mutant protein was purified after isopropyl-1-thio-β-D-galactopyranoside induction (34).

**Mass Spectrometry**

**ESI-MS—**Masses of proteins and peptides were determined using ESI. Spectra were acquired using a single quadrupole mass spectrometer equipped with an ESI probe (MSSD1100, 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 (Hewlett Packard) coupled directly to the electrospray source. Nitrogen was used as the nebulizer and drying gas (7.0 liter/min, 150 °C). Sample droplets were ionized at a positive potential of ~4000 V and transferred to the mass analyzer with a fragmentor voltage (capillary to skimmer lens voltage) of ~250 V. Spectra were acquired in rf-only Q2 (20 ms, 2000–7000 V) mode. Spectra were acquired in rf-only Q2 (20–100 eV, argon 1.5 millitorr), and spectra were recorded with unit resolution using Q3 (m/z 2500–2500, 2 s) and accumulated into...
a single file for 4–5 min. Ions were formed using an “in-house” nano-ESI device consisting of a bora-silicate glass capillary (50 × 2 mm, exit inner diameter <50 μm) containing peptide solutions (~10 μL, H2O/CH3CN/CH3CO2H, 50:49:1). Electrical contact (~1.5–2 kV) was maintained using a platinum 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 <200 nL/min.

MALDI—Protein or peptide solutions (~25 pg/μL) were mixed with matrix (1 μL of sinapinic acid or 2,5-dihydroxybenzoic acid (Sigma) 10 mg/ml) and air-dried before analysis using a Voyager STR TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA). Spectra were acquired in linear mode, and positive ions were generated using an N2 laser (337 nm, 3-ns pulse-width, 20-Hz repetition rate) and accelerated to 25 kV after an extraction delay of 100–250 μs. Typically, 25–50 spectra were averaged and calibrated externally using angiotensin I and insulin (oxidized) B chain (peptides) or insulin (oxidized) B chain and myoglobin (proteins).

Post source decay spectra (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 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.

MALDI peptide mass fingerprinting of lyophilized proteins (~100 ng) were determined after resuspension in H2O/CH3CN (85:15, 12.5 μL, 10 mM NH4HCO3, pH 8) with endoprotease Asp-N (~2 ng). After digestion for 14 h at 37 °C, digests (1 μL) were analyzed directly after the addition of matrix (2.5-dihydroxybenzoic acid, 1 μL, 10 mg/ml) by MALDI over a mass range of m/z 500–7,000. Approximately 100 spectra were acquired in reflectron mode (Voyager) with an accelerating voltage of 20 kV and an extraction delay of 250 ns, and spectra were calibrated either externally using angiotensin I and insulin (oxidized) B chain or internally using protonated A8 and myoglobin (proteins).

Chemotaxis Assay

A8 analogues (10 μg) lyophilized (Speedvac, Savant, Farmingdale, NY) with gynecol (1 μL) were stored at ~80 °C. Reconstitution to a 10–6 M stock solution was with PBS (10 μL) and RPMI 1640 (990 μL) and serial dilutions were made in RPMI, bovine serum albumin (0.2%) immediately before use. Thiglycollate-elicited murine neutrophils were used as responding cells (15) and were suspended in HEPES-buffered saline solution, bovine serum albumin (0.2%) at 5 × 107/ml, then incubated with calcine AM (5 μM, Molecular Probes, Inc., Eugene, OR) for 15 min at 37 °C in 5% CO2 in air, washed twice with HEPES-buffered saline solution, and resuspended at 0.5 × 106/ml in RPMI, bovine serum albumin (0.2%). Assays were performed using M6A9 well chambers fitted with 5-μm polycarbonate membranes (Neuro Probe Inc., Bethesda, MD). Proteins (10–13–10–9 M) diluted in RPMI, bovine serum albumin (0.2%, 410 ng) were placed in the lower compartment, and cells (300 μL) were placed in the upper compartment. To determine the effects of random migration, equivalent concentrations of protein were included in the upper chamber. The chamber was incubated at 37 °C in 5% CO2 in air for 90 min. Cells collected in the lower chamber were measured by fluorescence (λex = 485 nm, λem = 530 nm) using a multi-well plate reader (Cytoflour, Perceptive Biosystems), and numbers were extrapolated using a standard curve obtained using the fluorescence readings of a known number of labeled cells. Complement C5a (10–9 M) was used as positive control in all experiments. Data from at least three experiments were analyzed using Student’s t test.

RESULTS AND DISCUSSION

Hypochlorite (OCl−) is the major oxidant produced by neutrophils in inflammatory responses, and murine A8 is highly susceptible to oxidation by reagent HOCl (10). Low concentrations of hypochlorite (~40 μM) converted A8 to a modified monomer (10,354, +46 Da), corresponding to the addition of 3 oxygens and loss of dihydrogen and to oxidized dimer (20,707, +93 Da), indicating oxidation of susceptible amino acids (Cys/Met) (10). SDS-polyacrylamide gel electrophoresis separation with silver staining showed that when A8 was treated with as little as 10 μM HOCl, ~20% was converted to dimer and yields increased to 70–80% with 40 μM HOCl, whereas levels >100 μM caused loss of detectable protein, possibly due to aggregation. The resistance of Ala41A8 to HOCl-mediated dimerization at all HOCl concentrations confirmed the role of Cys41 (10). Reagent or MPO-generated HOCl (28) form identical reaction products in proteins with reactive aldehydes from α-amino acids (37), and Cys and Met, followed by amines (e.g. Lys, forming chloramines), are substrates (30, 38, 39). 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 reagent HOCl for 10 min at 20 °C yielded 4 major products after C4 RP-HPLC (Fig. 1A; compare with the single peak of unmodified A8, Fig. 7A). The major product (Fig. 1A, peak 1) had a m/z of 10,354 ± 1, 46 greater than the unmodified protein. Three additional components of m/z 10,354, 10,388, and 20,707 (Fig. 1A, peaks 1, 2, and 4) were also isolated.

A8 has three potentially readily oxidizable residues, Met36, Cys41, and Met73. The expected hypochlorite oxidation products of Met and Cys are Met sulfoxide and cysteic acid, respectively (30), and mass additions of 20,444 (peak 3a) and 10,477 (peak 3b) were isolated after incubation of the A8/HOCl reaction mixture with dimedone, indicating initial formation of a reactive Cys(O).

FIG. 1. Preparative C4 RP-HPLC of S100A8 HOCl oxidation products. A, products were separated after incubation of protein with ~5 mol eq of reagent HOCl for 10 min at 22 °C. ESI masses were m/z 10,354 (peak 1), 10,388 (peak 2), 10,354 ± 1 (peak 3), and 20,707 ± 3 (peak 4). After deconvolution, B, additional products, with masses of 10,444 (peak 3a) and 10,477 (peak 3b) were isolated after incubation of the A8/HOCl reaction mixture with dimedone, indicating initial formation a reactive Cys(O).
confirming conversion to Met(O) (not shown).

The minor peak (Fig. 1A, peak 2), eluting as a partially resolved shoulder immediately preceding peak 3, had an m/z of 10,388 (i.e. +80, or the addition of 5 oxygens) and corresponds to fully oxidized A8 (i.e. Met$^{36773}$ to Met$^{36778}$O) and Cys$^{41}$ to cysteic acid. MS of Met/Cys-containing Asp-N digest peptides confirmed the Met(O) and cysteic acid products (not shown).

The mass addition (m/z +46) of the major A8 oxidation product (Fig. 1A, peak 3) was not totally accounted for by Met oxidation, suggesting that Cys$^{41}$ was modified by the addition of 14 Da. This would numerically correspond to the addition of a single oxygen (m/z +16) to Cys to form Cys sulfenic acid (Cys(O)) followed by loss of dihydrogen (m/z -2). Mechanistically, HOCl oxidation of Cys to cysteic acid (CysSO$_2$H) proceeds stepwise via Cys sulfenic and Cys sulfonic acid (CysSO$_2$O) intermediates (40). Sulfenic acids are transient intermediates that readily undergo further oxidation or substitution reactions but may be stabilized within proteins by hydrogen bonding to carboxyl or amino groups (40). Protein-containing Cys(O) are proposed as important biochemical intermediates located at active site Cys residues and possibly responsible for oxidative inactivation of enzymes such as papain and glyceraldehyde-3-phosphate dehydrogenase (40). Importantly, redox regulation of the transcription factors Fos, Jun, and OxyR and the reversible inactivation of protein-tyrosine phosphatase 1B and CD45 may also involve Cys to Cys(O) conversion (40). Intermediates of bacterial flavoprotein thioredoxin reductase may also contain Cys(O) (43). Because of their instability, Cys(O)-protein intermediates have been difficult to isolate. Cys(O) in the enterococcal flavoprotein NADH peroxidase was identified and characterized using x-ray crystallography under cryogenic conditions (40, 41), and the Cys(O) adduct of alkyl hydroperoxidase reductase was identified after prior trapping with the electrophilic reagent 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))Cl (44).

Sulfenic acids undergo substitution reactions with nucleophilic reagents such as dimedone (44, 45) to form thiol adducts that would yield a calculated mass increase of +138 Da. After incubation of the A8/HOCl reaction mixture with dimedone and C4 RP-HPLC separation of the products, two additional components with masses of m/z 10,477 and 10,444 (Fig. 1B, peaks 3a and 3b) indicated dimedone adducts. Asp-N peptide mapping confirmed dimedone substitution on A8$^{32-57}$ and low energy CID of the [M + 3H]$^{+3}$ ion showed fragmentations consistent with the addition to Cys$^{41}$ (not shown). The observed mass difference (m/z -32) found between peaks 3a and 3b (Fig. 1B) was due to incomplete Met oxidation. Interestingly, A8-dimedone adducts were evident only if excess dimedone was added within ~20 s of initiation of HOCl oxidation, suggesting that conversion of A8 sulfenic acid to the stable products isolated here (Fig. 1A) occurred almost immediately. Early publications suggest that protein sulfenic acids may undergo nucleophilic substitution with amines (e.g. benzylamine) to yield stable covalent bonds (45), although none have been isolated or rigorously characterized. Our results suggest that A8 oxidation products form after reaction of specific amino acids with a Cys(O) intermediate.

MALDI Reflection TOF Peptide Mapping of A8 Oxidation Products—Definitive characterization of the mass additions of all A8 HOCl oxidation products was facilitated by peptide mapping-MALDI reflection TOF MS, which allows mass accuracies of < ±0.2 Da and resolution of isotopic envelopes in peptides of m/z < ±7000, and tandem MS (46). The complete peptide mass profiles of oxidized A8 adducts isolated from C4 RP-HPLC (Fig. 1A) were determined after Asp-N digestion (without separation of peptides by RP-HPLC). Comparison of experimental and predicted masses of covalently linked oxidized Asp-N peptides (Fig. 2) confirmed the proposed oxidative additions and mass assignments determined using ESI-MS of the full-length proteins (Fig. 1).

The spectrum of the A8 Asp-N digest (Fig. 2A) gave the expected digest peptides (47), and experimental masses of the most intense ions (m/z 615.16, 1457.79, 2287.98, 2433.64) corresponded to the protonated peptides A8$^{84-88}$, A8$^{2-12}$, A8$^{13-31}$, and A8$^{62-83}$ and differed by < m/z -0.2 of predicted values. The calculated (m/z 3156.5862) and experimental monoisotopic masses (m/z 3156.8195) of protonated A8$^{32-57}$ (formula C$_{145}$H$_{223}$N$_{36}$O$_{42}$S$_{2}$) also differed by m/z -0.2, and the expanded mass scale showed resolved isotopic peaks (Fig. 2A, inset, resolution M/ΔM ~ 5000, 50% peak height), which also agreed well with the calculated isotopic pattern (not shown). Reflectron TOF-MS of Asp-N digest products confirmed the identity of fully oxidized A8 (Fig. 1A, peak 2). Intense ions with m/z 1457.41, 2288.40, 2449.67, identical to the protonated peptides A8$^{2-12}$, A8$^{13-31}$, and Met(O)-A8$^{62-83}$, and an ion at m/z 3222.2092, corresponding to A8$^{32-57}$, containing Met(O) and cysteic acid (Fig. 2B), were evident. Theoretical and experimental monoisotopic masses of the fully oxidized A8$^{32-57}$ peptide differed by m/z < 0.4. Two additional peptides at m/z 1114.41 and 2144.98 had masses identical to the oxidized protonated peptides A8$^{32-40}$ and A8$^{41-57}$ that form after Asp-N digestion at cysteic acid, which supported assignment of the oxidative modifications.

The major oxidation product (Fig. 1A, peak 3, m/z 10,354) yielded two peptides with masses distinct from those derived from native A8 (Fig. 2C), A8$^{62-83}$ (m/z +16) indicates conversion of Met$^{73}$ to Met$^{73}$O. A8$^{32-57}$ had an experimental monoisotopic mass of m/z 3187.1149, corresponding to the addition of m/z 20. Conversion of the single Met to Met(O) in this peptide would account for a mass addition of m/z 16. The additional mass (m/z 14 ± 0.2) confirmed the unusual mass addition found in the full-length protein and substantiated its location within A8$^{32-57}$. The mass of the minor early eluting oxidation product (Fig. 1A, peak 1) was identical to that of the major component above, suggesting an alternative isomeric reaction products form. Reflectron TOF-MS of Asp-N digest products indicated intense ions with m/z 1457.61, 2287.98, 2448.30 (Fig. 2D), identical to the protonated peptides A8$^{2-12}$, A8$^{13-31}$, and Met(O)-A8$^{62-83}$. No peptide corresponding to A8$^{32-57}$ was evident, although there was an additional ion at m/z 3802.7562. Calculated masses of possible A8 Asp-N products indicated that this would correspond to the average mass of A8$^{32-57}$ if it were covalently linked to A8$^{84-88}$ (with the addition of m/z 30). The isotopic distribution pattern of the protonated peptide showed an almost identical profile to the calculated isotopic pattern for A8$^{32-57}$-84-88 containing 2 oxygens (~2 hydrogens; formula: C$_{145}$H$_{225}$N$_{34}$O$_{43}$S$_{2}$) (not shown). The monoisotopic masses differed by < ±0.2 Da, confirming that a minor component formed after HOCl oxidation of A8 contained a covalent bond between A8$^{32-57}$ and A8$^{84-88}$, with the addition of m/z 14.

One oxidized component (peak 4, Fig. 1A) had a numerical mass corresponding to a dimer between 2 oxidized monomeric chains. This was 28 Da greater than predicted for the disulfide-linked dimer containing Met(O) (20,707 Da). Cys(O) normally dimerizes to form thiol sulfinites, with loss of water, and the calculated mass of this product would be (2x + 16) (48). This was excluded because the calculated mass of dimeric A8 linked via a thiol sulfinate would be 20,694 Da, 13 Da less than the experimental mass. Dimerization via di-tyrosine, a well characterized product of HOCl cross-linking formed by one-electron oxidation of l-tyrosine to generate the tyrosyl radical (49), was also excluded, based on the differences in calculated (2M − 2)
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Fig. 2. MALDI reflectron TOF spectra of Asp-N digests of S100A8 and HOCl oxidation products. A, digest products analyzed directly by MALDI after the addition of matrix of A8 corresponded to A8<sup>41–80</sup> (m/z 615.16), A8<sup>2–12</sup> (m/z 1457.79), A8<sup>31–31</sup> (m/z, 2287.98), A8<sup>30–31</sup> (m/z 2433.64), and A8<sup>29–30</sup> (m/z 3158.87). Oxidation products (Fig. 1A, peaks 2, 3, 1, and 4) were digested with Asp-N and analyzed directly by MALDI after the addition of matrix (B, C, D, and E, respectively). Masses of the most intense ions and identities of expected digest peptides are indicated. Peptides with spectra characteristic of Cys<sup>41</sup>−Lys<sup>1</sup> with HOCl (see text for details).

Fig. 3. Potential S100A8-HOCl oxidation products. A, A8 amino acid sequence indicating locations of Lys, Met, and Cys residues modified by HOCl oxidation (labeled 8 or *). B, proposed structures of Cys<sup>41</sup>-Lys<sup>6</sup> sulfinamide-bonded products identified after oxidation of A8 with HOCl (see text for details).
to GSH-sulfonyl chloride and then either cyclization with the free N-terminal amine (to yield a cyclic sulfonamide) or the addition of GSH to form a partially oxidized dimer. No sulfonamide bonds between Cys41 and Lys residues were identified in oxidized A8. Differences in the oxidation products of proteins and GSH may be due to stabilization of initially formed Cys(O) by hydrogen bonding to Lys residues, the reactivity of respective Cys residues, the amount of oxidant used, and/or more favorable structural/spatial arrangements in proteins compared with the relative distances of amino acid side chains within GSH. Interestingly, HOCl oxidation of the peptide A832-57 (containing Lys34-35 and Cys41) with a similar molar ratio of reagents yielded a single product that corresponded to fully oxidized peptide containing Met(O) and cysteic acid (not shown) rather than the sulfonamide cross-linked products, suggesting that the full-length protein is in the most favorable structural orientation for sulfonamide formation. The three-dimensional structures of related S100 proteins (e.g. human S100A8, S100B) indicate that residues 32–40 are located with an α-helix (helix II), and the side chains of Lys34-35 and Cys41 are relatively closely spaced (51, 52). A helical wheel representation of A8 also supports an α-helical structure with Lys34-35 and Cys41 residues on adjacent helices and favorably positioned to allow cyclization and intermolecular sulfonamide cross-linking. Although the predominant sulfonamide bond was within monomers and even though A8 forms non-covalent dimers in solution (53), the products containing intramolecular sulfonamides between Lys37/Cys41 and Lys9/Cys41 (Fig. 3) indicate some selective cross-linking between Cys41(O) on one chain with available Lys residues on the second chain.

Confirmation of the participation of lysine in Cys cross-linking was obtained after HOCl oxidation of a mutant in which Lys34-35 were substituted with Ala (Ala34-35A8). Modified proteins with mass additions of m/z +46 and +80 were isolated after C4 RP-HPLC. MALDI reflector TOF peptide mass fingerprinting of the earliest eluting product (mass m/z 10240) after Asp-N digestion showed intense ions with masses corresponding to protonated peptides A832-12, A83-31, and Met(O)–A832-33. The additional ion at m/z 3687.73 was identical to the predicted mass (m/z 3687.71) of Ala34-35A832-57 in which Cys41 is linked via an intermolecular sulfonamide bond to Lys37 in A834-88 (formulas C102H172N62O55S72 (not shown). This product was identical in structure to that of peak 1 (Fig. 1A), but because of the Ala/Lys substitutions, was m/z 114 less than the product generated in A8. A second oxidation product of Ala34-35A8 (m/z 10274) had a mass equivalent to fully oxidized A8 i.e. Met to Met(O) and Cys to cysteic acid). Asp-N digestion–reflector TOF spectra of other minor products were not readily identified, but none contained peptides (predicted m/z 3073.46), which would correspond to the sulfonamide-containing peptides evident in peaks 3 or 4 eluted from C4-HPLC (see Fig. 1A).

Tandem MS Identification of Covalently Linked Amino Acids in the Major A8 Oxidation Product—Low energy CID MS/MS spectra of the Asp-N digest peptide A832-57 of oxidized and native A8 (Fig. 4A) isolated by RP-HPLC showed characteristic fragmentation patterns (y4 to y16) confirming that residues 43–57 were not modified (data not shown). Peptides containing fewer amino acids were required for definitive MS/MS characterization. Fig. 4 summarizes the masses of peptides isolated by C18 RP-HPLC after Lys-C, Glu-C, and pepsin digestion of native (Fig. 4B) and oxidized A832-57 (Fig. 4C). The peptides generally contained the usual sequence-specific b- or y-type MS/MS fragmentations, but several also had characteristic fragmentations that could be attributed to intramolecular sulfonamide bonds (Fig. 4). Peptides with masses of m/z 409 and 2640 and corresponding to the predicted masses of A832-34 and A836-57 were isolated after complete digestion of A832-57 generated from digestion at Lys34-35 and Lys9-C (Fig. 4B). In contrast, three peptides of masses m/z 409, 2798, and 3206 and corresponding to oxidized A832-34, A835-57, and A832-57 + H2O, were generated from oxidized A832-57 (Fig. 4C and 5A). Those with masses of m/z 409 and 2798 were expected products of oxidized A832-57 generated from digestion at Lys34-35, confirming that amino acids 32–34 were not modified. The peptide with mass m/z 3206 was m/z 18 more than oxidized A832-57, and the mass increase corresponded to the addition of H2O. These results suggest that an unusual intramolecular covalent link (sulfonamide) within this peptide prevented dissolution of peptide DFKK after digestion at Lys35 and indicates that Lys-C cleaved between residues Lys34–Lys35 and Lys57–Met(O)56 to generate two peptides (Fig. 5A). Taken together, covalent sulfonamide bonds between Cys41 and Lys34 or Lys57 were indicated, and oxidized A832-57 contained a mixture of isomers, A832-57 Cys41/Lys34-sulfonamide and A832-57 Cys34/Lys57, sulfonamide, which were not initially separated by C18 RP-HPLC of the Asp-N digest.

MALDI PSD Mass Spectrometry—Although PSD tandem MS suffers from low resolution precursor ion selection (M/ΔM ~80) when using the timed ion selector of the Voyager mass spectrometer, useful structural information can be obtained from the fragmentations obtained from HPLC-fractionated peptides,
where the homogeneity of the precursor ion can be assured (54). The PSD spectra of protonated oxidized A832-57 + H2O and A835-57 showed characteristic fragmentations that confirmed the most likely covalently linked residues as Cys41(O) and Lys34 or Lys35 forming sulfinamide bonds (Fig. 5B and data not shown). Four major fragments were evident in the PSD spectra of the [M + H]+ ion of oxidized A832-57 + H2O (Fig. 5B). The ion at m/z 2622.8 corresponds to loss of m/z 584, which could be attributed to the loss of DFKK + NISOH (calculated mass, m/z 585) and formation of dehydro-alanine at the Cys residue. A proposed fragmentation mechanism is outlined (Fig. 5C). Processes whereby protonated, oxidized, and substituted Cys residues preferentially undergo elimination reactions to form protonated peptides containing dehydro-alanine and substituted sulfenic acids have recently been described (55). Furthermore, the ion at m/z 2555.9 corresponds to the loss of methanesulfenic acid (m/z 64) from the ion at m/z 2622.8, a characteristic fragmentation of Met(O) with numerous examples reported (56). Loss of methanesulfenic acid from the precursor ion is also evident, forming the ion at 3141 (Fig. 5C). Another fragmentation producing an ion at 1974.6, and corresponding to y′-16 (i.e. loss of M2VTTE and DFKKC0), also indicated that Lys34 is covalently linked to oxidized Cys41. Further support for our assigned structure was obtained by low energy CID MS/MS of the [M + 4H]+ ion of A832-57 + H2O (data not shown). The two major fragment ions at m/z 537.3 and 890.7 corresponded to protonated A812-35 (DFKK, calculated mass, 537.6) and [M + 3H]+ A816-57 containing Cys(O) (calculated mass, m/z 890.7). Simple fragmentation of the sulfinamide bond forming two protonated products accounts for the observed spectrum (data not shown).

The PSD spectra of the [M + H]+ ion of oxidized A835-57 contained one major ion at m/z 1974.6, which corresponded to y′-16 and indicated favorable loss of the complete N-terminal sulfinamide-containing fragment KM2VTTEO (data not shown), supporting the proposed sulfinamide bond between Lys35 and Cys41(O).

**Low Energy CID MS/MS Analysis of Native and Oxidized S100A8** —Fragmentations consistent with a Lys-Cys sulfinamide were identified using ESI low energy CID MS/MS analysis of peptides isolated after protease digestion. Two peptides generated from native and oxidized A832-57 by pepsin and isolated by C18 RP-HPLC had m/z values of 1574.1 and 1604.3 and corresponded to A832-44 and oxidized A832-44, respectively. Low energy CID MS/MS analysis of [M + 2H]+ ions supported the proposed location of the oxidized amino acids and an intramolecular sulfinamide bond between Lys34 or Lys35 and oxidized Cys41 (Fig. 6A and B). Pepsin digestion of A8 caused loss of the very basic Arg residue near the C terminus, shifting the fragmentation pattern from predominantly y- to b-type. The al stacked complete series of b ions (b16-b20) in the MS/MS spectrum of protonated A832-44 indicated loss of individual amino acids from the N terminus and retention of charge at the C terminus (Fig. 6A). Predominant fragmentations around Cys41 (b19 and b20) were obvious. Except for the addition of 16 Da to fragments b > 4 (corresponds to conversion of Met36 to Met36(O)) and an additional 14 Da for fragments b > 9 (e.g. b16 m/z 1214.3) (Fig. 6B), spectra of protonated A832-44 and oxidized A832-44 were similar. Additional fragments attributed to
Novel Sulfinamide Bonds in HOCl-oxidized S100A8

Fig. 7. Comparison of C4 RP-HPLC chromatograms after incubation of S100A8 with various oxidants. A, unmodified A8. Similar chromatograms were obtained after the oxidation with reagent HOCl (molar ratio protein:oxidant ~1:5) (B) or myeloperoxidase/H2O2/Cl− (molar ratio protein:H2O2 ~1:5) (C) for 10 min. The reaction of H2O2 without MPO (D) (molar ratio protein:H2O2 ~1:5) converted a small amount of monomer to disulfide-linked dimer in 10 min. PMA-activated human PMN also converted exogenous A8 into oxidized forms, although the chromatogram was complicated by secreted PMN proteins.

The relative amounts of modified A8 monomers and dimers were dependent on the concentration of HOCl used and reaction time (not shown), and aggregation predominated with molar ratios >~10 fold (not shown), suggesting a two-step process. Oxidation-dependent modifications in A8 may represent a physiologically significant regulatory mechanism con-
trolling A8-provoked leukocyte recruitment (10). In conditions of low HOCl production, sulfamine bond formation may stabilize the monomeric chemotactic form and favor continued leukocyte influx, whereas higher amounts of HOCl could generate intra-chain cross-linked inactive products, resulting in decreased inflammation. Importantly, the sensitivity of A8 to oxidation may represent a novel means of protecting host tissue from excessive oxidative damage when high levels of HOCl are generated. A8 constitutes ~20% of the cytoplasmic protein of neutrophils, and large amounts of extracellular murine A8 are found in acute inflammatory sites, including bacterial infection (19) and lung alveolitis (18). High levels of the human protein are found in serum from patients with numerous acute and chronic inflammatory conditions (5). Furthermore, A8 gene transcription and protein secretion by macrophages activated by endotoxin or interferon γ is markedly amplified by interleukin 10 (IL10), and IL10 from LPS-activated cells is partially responsible for the LPS-provoked response. Interleukin 10 acts via a prostaglandin E2-CAMP pathway, supporting the notion that A8 may be involved in protection/resolution of cell-mediated immune responses (60).

Inside cells, S100 proteins occur mainly as non-covalent homo/heterodimers with reduced sulphhydryl groups (32), although we recently identified A8 S-S homodimers exclusively in inflammatory sites (61), suggesting that this protein is responsible for the LPS-provoked response. Interleukin 10 acts by inflammatory sites (60), suggesting that this protein is responsible for the LPS-provoked response. Interleukin 10 acts via a prostaglandin E2-CAMP pathway, supporting the notion that A8 may be involved in protection/resolution of cell-mediated immune responses (60).

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Novel Intra- and Inter-molecular Sulfinamide Bonds in S100A8 Produced by Hypochlorite Oxidation

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