Supporting Information for

Oxidative Post-Translational Modifications Accelerate Proteolytic Degradation of Calprotectin

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This Supporting Information includes:

Experimental Methods

General Materials and Methods. All solvents, reagents, and chemicals were obtained from commercial suppliers and used as received. All buffers and metal solutions were prepared using Mili-Q water (18.2 MΩ•cm, 0.22-μm filter, Millipore). All mobile phases for chromatography experiments were prepared with Ultrol grade HEPES (free acid, Calbiochem) and TraceSELECT NaCl (Fluka), and aqueous TraceSELECT NaOH (Sigma) was used for pH adjustments. Buffers for all other experiments, except where specified otherwise, were prepared using microbiology grade HEPES, NaCl, and NaOH (Sigma) that was treated with Chelex 100 resin (Biorad 10 g/L) for 1 h in a polypropylene beaker. The Chelex resin was removed by filtration with a 0.22-μm bottle-top filter. All buffers were stored in polypropylene bottles. The highest available purity of calcium chloride (99.99%) and manganese chloride (99.99%) were purchased from Alfa Aesar, and anhydrous zinc chloride (99.999%) and $(NH_4)_2Fe(SO_4)_2•6H_2O$ (99.997%) were purchased from Sigma. Stock solutions of Ca(II) (1 M, 100 mL), Mn(II) (1 M, 50 mL), Fe(II) (100 mM, 10 mL), and Zn(II) (100 mM, 100 mL) were prepared by using Mili-Q water and acid-washed volumetric glassware, and the solutions were stored in polypropylene tubes. The Fe(II) solution was prepared under a nitrogen atmosphere in an anaerobic glove box (Vacuum Atmospheres Co.). All iron-containing samples were prepared and manipulated in the glove box. Protein concentrations were determined by optical absorbance at 280 nm using a BioTek Synergy HT plate reader outfitted with a Take3 Micro-Volume plate. The extinction coefficients for the employed proteins are listed in Tables S5 and S6.

General Instrumentation. For analytical high-performance liquid chromatography (HPLC), an Agilent 1200 series instrument equipped with a thermostatted column compartment set to 20 °C, and a multi-wavelength detector set at 220 and 280 nm (500 nm reference wavelength with 100 nm bandwidth), was used. A Proto C4 column (5-μm pore, 4.6 x 250 mm, Higgins Analytical Inc.) set at a flow rate of 1 mL/min was employed for all analytical HPLC experiments. HPLC-grade acetonitrile (MeCN) and trifluoroacetic

acid (TFA) were routinely purchased from EMD and Alfa Aesar, respectively. For all HPLC runs, mobile phase A was 0.1% TFA/H_2O and mobile phase B was 0.1% TFA/MeCN.

For routine high-resolution mass spectrometric characterization of purified protein and the products of oxidation by H_2O_2, an Agilent 1260 series LC system equipped with an Agilent 6230 TOF system housing an Agilent Jetstream ESI source was employed. A Poroshell 300SB-C18 column (5-μm pore, 2.1 x 75 mm, Agilent) and denaturing protocol were utilized for all LC-MS analyses. Mobile phase A was 0.1% formic acid/H_2O. Mobile phase B was 0.1% formic acid/MeCN. The S100A8 and S100A9 subunits were eluted by using a gradient 5–85% over 10 min. The resulting mass spectra were deconvoluted using the maximum entropy algorithm in MassHunter BioConfirm (Agilent).

For mass spectrometry analyses of human mucus and pus samples, an Agilent 1290 Infinity HPLC system equipped with an Agilent 6520 Accurate-Mass ESI-Q-TOF system was employed. A Zorbax 300SB-C3 column (5 μm-pore, 2.1 x 150 mm, Agilent) was utilized for the separation. Mobile phase A was 0.1% formic acid/H_2O. Mobile phase B was 0.1% formic acid/MeCN. Details for the gradients employed are provided in the Preparation of Human Mucus and Pus for LC-MS subsection.

For fluorescence spectroscopy, emission spectra were collected on a Photon Technologies International QuantaMaster 40 fluorimeter outfitted with a continuous xenon source for excitation autocalibrated QuadraScopic monochrometers, a multimode PMT detector, and a circulating water bath maintained at 25 ^oC. This instrument was controlled by the FelixGX software package. FelixGX was used to integrate the emission spectra. Excitation was provided at 490 nm, and the emission spectra were collected and integrated from 500 to 650 nm.

For circular dichroism (CD) spectroscopy, A JASCO Model J-1500 CD spectrometer thermostatted at 25 ^oC was used. A 1-mm path-length CD cell (Hellma) was employed for all CD measurements. All protein samples (10 μM protein, 300 μL, 1 mM Tris, ±2 mM $CaCl_2$, pH 8.5) were made at the same time prior to data acquisition.

Wavelength scans were recorded from 190 nm to 260 nm in 0.5 nm steps at a rate of 50 nm/min. The averages from three scans for each condition are presented.

Site-Directed Mutagenesis. A modified Quick-Change site-directed mutagenesis protocol was employed to generate plasmids encoding S100A9(C3S)(M63A), S100A9(C3S)(M81A), and S100A9(C3S)(M83A). The template plasmid was pET41a-*S100A9(C3S),* which has the S100A9(C3S) gene ligated into the *Nde*I and *Xho*I restriction sites.[S1] The mutagenesis primers are listed in Table S8. PCR amplification was carried out using PfuTurbo DNA polymerase (Agilent, previously Stratagene). The annealing temperature for M63A was 57 °C, and the annealing temperature for M81A and M83A was 62 °C. The PCR protocol was 95 °C for 30 sec, 57 °C (M63A mutation) or 62 °C (M81A or M83A mutations), 68 °C for 17 min, (25 cycles), and 4 °C hold. After PCR amplification, the template DNA was digested at 37 °C for 3 h by *DpnI* (New England Biolabs) by adding 0.75 μL of the enzyme to 25 μL of a PCR reaction, and adding an additional 0.75-μL aliquot of the enzyme after 1.5 h. The digestion products were transformed into chemically competent *E. coli* TOP10, which were plated on LB plates with 50 μg/mL kanamycin and incubated at 37 °C overnight. The following day, overnight cultures (5 mL LB+50 μg/mL kanamycin) were grown from single colonies, and the plasmids were isolated using a miniprep kit (Qiagen). The presence of each mutation and fidelity of the coding sequences were verified by DNA sequencing (Quintara Biosciences).

Preparation and Purification of CP and Variants. CP and CP-Ser were prepared and handled as previously described.[S1] This purification protocol affords the apo CP/CP-Ser heterodimer. The same purification protocol was employed to prepare the new CP-Ser variants reported in this study, CP-Ser(A9-M63A), CP-Ser(A9-M81A), and CP-Ser(A9-M83A). These variants were obtained in yields of 40-50 mg/ 2 L culture (1 L overexpression of each subunit), and each protein was evaluated by ESI-MS, SDS-PAGE, analytical SEC, and CD spectroscopy (Table S3, Figures S3, S4, S5, and S8). ^{15}N-CP-Ser was prepared according to a published protocol.[S2]

Preparation and Purification of Oxidized CP-Ser Variants. The reactions to generate CP with oxidized Met residues produced mixtures of species. The mixture where the dominant species were S100A8 with 1 additional oxygen atom and S100A9 with 3 additional oxygen atoms is referred to as CP-Ser O_4. The more heavily oxidized mixture where the dominant species were S100A8 with 1 additional oxygen atom and S100A9 with 4 additional oxygen atoms is referred to as CP-Ser O_5. The oxidized CP-Ser species CP-Ser O_4 and CP-Ser O_5 were prepared by modifying the standard CP-Ser purification protocol (1). The S100A8(C42S) and S100A(C3S) subunits were overexpressed and a denaturating protocol was employed to obtain a mixture (~120 mL) of the soluble and unfolded subunits as reported previously. This mixture was dialyzed once against 75 mM HEPES, 100 mM NaCl, pH 8.0 (4 L, 4 ^oC, 12 h). Then, the protein was transferred to 50-mL Falcon tubes covered with aluminum foil. To prepare CP-Ser O_4, H_2O_2 (Sigma, 50% w/w in H_2O, 17.6 M) was added to the protein solution to give a final concentration of 50 mM, and the reaction was allowed to proceed for 2.5 h or 5 h at 37 ^oC on a nutating platform. To prepare CP-Ser O_5, the protein was incubated with either 50 mM H_2O_2 for 6 h or 75 mM H_2O_2 for 6.5 h at 37 ^oC on a nutating platform. At the reaction end point, the solution was centrifuged at 3 600 rpm, 4 ^oC for 30 min. The supernatant was transferred to dialysis tubing, and three additional 12-h rounds of dialysis against 4 L of refolding buffer (20 mM HEPES, pH 8.0) were performed at 4 ^oC. The resulting protein sample was purified by anion exchange chromatography and size exclusion chromatography as described for CP-Ser.[S1] The yields for the CP-Ser O_4 and CP-Ser O_5 ranged from 30-40 mg / 2 L of culture (1 L overexpression of each subunit). Protein purity was assessed by SDS-PAGE (Figure S3). The extent of oxidation of CP-Ser was ascertained using mass spectrometry (Figure S7).

Oxidation of CP. CP, which contains two Cys residues, was buffer exchanged from the storage buffer (that contained 10 mM BME) into the assay buffer (75 mM HEPES, 100 mM NaCl, pH 7.5) with three rounds of centrifugal concentration using a 0.5-mL 10-

kDa MWCO Amicon filter. The protein was then diluted to 30 μM in the assay buffer, and Ca(II) was added to give a final concentration of 1.5 mM. After 15 min of incubation at room temperature, H_2O_2 was added to give a final concentration of 100 μM, and the reaction was then incubated at 37 °C. Because we sought to study the protein after forming disulfide bonds, it was essential to remove the H_2O_2 without breaking the disulfide bonds. We employed catalase (Sigma) to rapidly destroy the H_2O_2 by adding the enzyme (10 nM) to the reaction from a freshly prepared 100-nM stock solution in Milli-Q water.

We also sought to study the properties of disulfide-linked CP that contained methionine sulfoxide modifications. First the disulfide-bonds were formed by treating 30 μM CP with 100 μM H_2O_2 for 23 h at 37 °C in 75 mM HEPES, 100 mM NaCl, pH 7.5, as described in the preceding paragraph. To form methionine sulfoxide modifications, the protein was then treated with 100 mM H_2O_2 for 7 h, at which time the reaction was quenched by diluting catalase 10-fold into the reaction to a final concentration of 10 nM.

Collection of Human Mucus. Mucus sampling was approved by the Massachusetts Eye and Ear Infirmary Institutional Review Board as previously described.[S3] All samples were taken from patients undergoing sinonasal surgery. These patients had not been exposed to antibiotics or steroids for at least 4 weeks prior to surgery and sample collection. Mucus samples were taken from the internal valve or middle meatus by placing a compressed polyvinyl alcohol sponge (PVA, Medtronic, Minneapolis, MN) within the nasal cavity, taking care not to abrade the mucosa or contaminate the sponge with blood. Three sponges were placed in each patients' nostril. When ^{15}N-CP-Ser was added, we added 125 μL of 1 μM protein to each sponge immediately after the sponge was removed from the patient's nose. As quickly as possible, the sponges were transferred to 1.7-mL polypropylene tubes, and placed in ice for use later. The mucus was removed from the sponges by placing them into 0.65-mL polypropylene tubes that had the bottom removed with a razor. This tube was placed in a 1.7-mL polypropylene tube, and was centrifuged at 13 000 rpm for 30 min, 4 °C. The

mucus from each patient was pooled, and then flash frozen with liquid nitrogen before storage at -80 °C.

Collection of Human Pus. Pus sampling was approved by the Massachusetts Institute of Technology Institutional Review Board. Both samples were taken from pimples of one graduate student. One pimple was located on the shoulders and the other on the face. After washing his hands, the student used sterile isopropyl alcohol wipes to clean the area before breaking the pimple with his hands. The contents were transferred to a 1.7-mL polypropylene tube. A 2-μL aliquot of 10.4 μM ^{15}N-CP-Ser was immediately added to the pus sample, followed by 50 μL of Milli-Q water, and the resulting mixture was mixed by pipetting. The samples were immediately processed for mass spectrometry.

Preparation of Human Mucus and Pus for LC-MS. Mucus samples 1 and 2 were were thawed on ice, and 5 μL of thawed mucus was combined with 10 μL of an aqueous solution containing 100 mM $(NH_4)HCO_3$ and 20 mM TCEP. The resulting samples were incubated for 45 min at room temperature. Each sample was then centrifuged for 10 min at 13 000 rpm, 4 °C. The supernatant was removed and transferred to a vial for LC-MS analysis. Total protein in the sample was determined by a standard Bradford (Bio-Rad) assay using bovine serum albumin as a standard. An Agilent 6520 mass spectrometer connected to an Agilent 1200 LC system was employed as described above. The injection volume was chosen such that approximately 9 μg of total protein was injected. The sample components were eluted using a gradient that linearly increased from 1% B to 61% B over 9 min at a flow rate of 0.8 mL/min.

All other mucus samples and the two pus samples were prepared and analyzed differently. The mucus samples were thawed on ice and the two pus samples were prepared immediately after collection. We found that an acetone precipitation protocol improved our ability to observe protein signals in proteomics experiments presumably because small molecules that suppressed ionization were removed. The mucus and pus samples were precipitated by combining 10 μL of mucus or pus with 40 μL of acetone

that had been chilled to -20 °C. After mixing, the precipitation samples were stored at -20 °C overnight. The next day, the samples were centrifuged for 10 min, 13 000 rpm, at 4 °C. The supernatant was aspirated, and the pellet was resuspended in a 4:1 mixture acetone:water at -20 °C. The centrifugation repeated, and the supernatant was aspirated. The pelleted protein was dried with a lyophilizer for 1 h, and then redissolved in 75 μL of 4:1 6 M guanidine HCl and 20 mM TCEP: 75 mM HEPES, 100 mM NaCl with the pH adjusted to 7.2. After 2 h, equal volumes of the dissolved protein and 1:1 H_2O:MeCN+0.1% TFA were combined. The protein sample was centrifuged for 10 min, 13 000 rpm, at 4 °C. For LC-MS analysis, 10 μL of the sample was injected, and separated with the same instrumentation described above. A different gradient was used for these samples. The gradient began at 20% B and linearly increased to 45% B over 25 min at a flow rate of 0.8 mL/min.

Analytical Size Exclusion Chromatography. An Äkta purifier (GE Lifesciences) housed in a 4 °C cold room outfitted with a 100-μL or 500-μL sample loop was used for analytical size exclusion chromatography. A Superdex 75 10/300 GL column (GE Lifesciences) equilibrated in mobile phase was calibrated with a low-molecular-weight calibration mixture (GE Lifesciences) as described previously.[S1] The largest protein in the calibration solution had an elution volume of 10.0 mL (77.5 kDa, 500-μL loop), and an analyte eluting in the void volume (8.3 mL, 500-μL loop) would have an apparent molecular weight of 150 kDa. A typical sample volume was 300 μL. All experiments were performed at least twice using two different preparations of the proteins, and representative chromatograms are shown.

For experiments that monitored the dissociation of the CP-Ser heterotetramer following H_2O_2 treatment, CP-Ser was thawed at room temperature, and diluted to 30 μM into 75 mM HEPES, 100 mM NaCl, 1.5 mM $CaCl_2$, pH 7.5. For experiments that monitored the dissociation of the Mn(II)-bound CP-Ser heterotetramer following H_2O_2 treatment, Mn(II), the samples also contained 30 μM Mn(II). A typical reaction volume was ≈2 mL.

After 15 min of incubation at room temperature, 300 μL of the sample was combined with 33 μL of 5 M aqueous BME to afford the 0 h sample. Another 300-μL aliquot of the protein solution was transferred to a microfuge tube to yield the untreated sample. To initiate the reaction, a final concentration of 100 mM H_2O_2 was added to ≈2-mL of CP-Ser, at which point the reaction and untreated sample were moved to 37 °C. At varying time points, a 300-μL aliquot was removed from the reaction and quenched by addition of 33 μL of freshly prepared 5 M BME. After 23 h, a 33-μL aliquot of BME was added to the untreated sample. The quenched samples were centrifuged at 13 000 rpm, 4 °C for 10 min. The supernatant was loaded onto the 100-μL loop. The loop was emptied with 500 μL of mobile phase (75 mM HEPES, 100 mM NaCl, 1.5 mM $CaCl_2$, pH 7.5), and the protein was eluted over one column volume at a flow rate of 0.5 mL/min. For SEC experiments with the Met→Ala CP-Ser variants, the above procedure was employed except that the final H_2O_2 concentration was 500 mM and the reactions were quenched by combining a 300-μL aliquot of the reaction with 45 μL of 5 M BME.

For experiments that monitored the oligomeric state of disulfide-linked CP, the SEC analysis was performed using similar methods to those above. To reduce the disulfides before elution, a 77-μL aliquot of 75 mM HEPES, 100 mM NaCl, 20 mM TCEP, pH 7.5 was added to a 700-μL aliquot of protein solution and the mixture was incubated at ambient temperature for 1 h. To remove Ca(II) from the protein, a 77-μL aliquot of 75 mM HEPES, 100 mM NaCl, 20 mM EDTA was added to a 700-μL aliquot of protein solution, and the mixture was incubated for 1 h at 4 °C. Before injection onto the SEC column, the solutions were centrifuged at 13 000 rpm, 4 °C, 10 min. The 500-μL loop was loaded with 600 μL of sample, and emptied with 500 μL of elution buffer. For the untreated and TCEP-treated protein, the mobile phase was 75 mM HEPES, 100 mM NaCl, 1.5 mM Ca(II), pH 7.5. For the EDTA-treated protein, the same mobile phase was used except the Ca(II) was omitted. The eluent was collected into 1-mL fractions using an automated fraction collector.

Anion Exchange Chromatography. An Äkta purifier (GE Lifesciences) housed in a 4 °C cold room outfitted with a 500-μL sample loop was used for anion exchange chromatography. A MonoQ 10/100 column was employed. Mobile phase A was 20 mM HEPES, pH 8.0. Mobile phase B was 20 mM HEPES, 1 M NaCl, pH 8.0. The gradient was 0–60% B over 15 column volumes (120 mL) at a flow rate of 1 mL/min. Samples of CP-Ser and the S100A9(C3S) homodimer were thawed at room temperature, diluted to 100 μM into mobile phase A, and centrifuged for 10 min at 13 000 rpm at 4 °C. The final volume of each sample was 300 μL. The samples were injected onto the loop using a 1-mL syringe, and the loop was emptied with 0.5 mL of mobile phase A. All experiments were performed twice, and representative chromatograms are shown.

Analytical Ultracentrifugation (Sedimentation Velocity). A Beckman XL-I Analytical Ultracentrifuge outfitted with an An-60 Ti rotor was employed for all sedimentation velocity experiments. The rotor housed conventional double-sector charcoal filled Epon centerpieces within the sample cells and contained quartz windows. The absorbance wavelength for optical detection was 280 nm. The samples were centrifuged at 42 000 rpm and 20 °C until sedimentation was complete. SEDNTERP (ref S4) was employed to calculate the buffer viscosity (η), buffer density (ρ), and protein specific volume ($\bar{\nu}$). Additional details are provided with Tables S4 and S5.

For sample preparation, 1 L of 75 mM HEPES, 100 mM NaCl, pH 7.5 dialysis buffer containing 10 g of Chelex resin (Biorad) was prepared and chilled at 4 °C. Each protein was thawed at room temperature and then diluted to 30 μM using 75 mM HEPES, 100 mM NaCl, pH 7.5. Each protein sample was transferred to 3.5 k MWCO dialysis tubing (Spectrum Labs) and dialyzed overnight at 4 °C in the dialysis buffer with gentle stirring. The following day, the protein and dialysis buffer aliquots were transferred to 1.7-mL polypropylene tubes and centrifuged at 13 000 rpm, 10 min, 4 °C to sediment any contaminating Chelex resin. Solutions of 1 mM EDTA (diluted from 100 mM in H_2O) and 100 mM Ca(II) were prepared using the centrifuged dialysis buffer. Matched protein and

reference samples were prepared by adding 30 μM EDTA or 600 μM Ca(II) to the protein and buffer-only samples. The protein samples and reference samples were maintained at ambient temperature while the window assemblies were constructed (≈1.5 h). The window assemblies were each loaded with 410 μL of the reference buffer or 400 μL of a protein sample. The samples were equilibrated to 20 °C after the rotor was placed in the AUC. The oxidized proteins were analyzed under both conditions using samples of protein from independent preparations in separate experiments.

Antimicrobial Activity Assays. The growth inhibitory activities of CP-Ser, CP-Ser O_4, and CP-Ser O_5 against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were assayed at 30 °C as described previously.[S1] The antimicrobial activity assay medium, hereafter AMA medium, was a 62:38 ratio of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM β-mercatoethanol (BME), 3 mM $CaCl_2$ and tryptic soy broth (TSB) with 0.25% (w/v) dextrose. To prevent evaporation of the medium, the plates were sealed with parafilm and a beaker of water was housed in the incubator shaker. All proteins were tested in triplicate using two separate preparations of each protein and growth medium. Averages from the three trials are shown with standard error of the mean (SEM) plotted as error bars.

Metal Competition Experiments with ZP1. The ability of CP-Ser O_4 and CP-Ser O_5 to outcompete ZP1 for Mn(II), Fe(II), Ni(II), and Zn(II) was assayed by using a modified competition protocol.[S5] Quartz cuvettes (Starna) were charged with 2 mL of 75 mM HEPES, 100 mM NaCl, pH 7.5. Zinpyr-1 (ZP1)[S6] was stored at -20 °C in DMSO, and a fresh aliquot was thawed daily. ZP1 was added to 1 μM in each cuvette, mixed by pipetting, and stored in the dark for 1 h. The proteins were thawed at room temperature, and diluted to 4 μM, and then Ca(II) was added to 200 μM. After 15 min, emission spectra were collected. Using a 500 μM solution of metal, the metal of interest was added to 3 μM, and after 1 h emission spectra were collected. Next, using a 120 μM solution of metal, the metal concentration was increased to 3.6 μM, and after 10 min emission spectra were

collected. For Zn(II) additional data points were collected. Using a 500 μM solution, the Zn(II) concentration was adjusted to 6 μM, and after 1 h emission spectra were collected. Using a 500 μM solution, the Zn(II) concentration was adjusted to 7.2 μM, and emission spectra were recorded after 10 min. For the experiments with Fe(II), the cuvettes were sealed with screw caps when outside of the glove box, and all additions of Fe(II) were performed in the glove box. Each experiment was performed in triplicate using two independent preparations of protein. Averages from the three trials are shown with standard deviation of the mean (SDM) plotted as error bars.

Protease Digestion of Oxidized CP-Ser. Trypsin (Affymetrix), proteinase K (Affymetrix), and chymotrypsin (Amresco) were obtained as lyophilized powders, stored at 4 °C, and dissolved in water to afford solutions of ≈50-100 μM immediately before use. Extinction coefficients for trypsin, proteinase K, and chymotrypsin are reported in Table S6. Human neutrophil elastase (Enzo Life Sciences) was obtained as a lyophilized powder, and the entire portion of protease was dissolved in 75 mM HEPES, 100 mM NaCl, pH 7.5 to afford a solution with a concentration of ≈1 mg/mL. HNE was divided into single-use aliquots, frozen with liquid nitrogen, and stored at −80 °C. Protease digestion assays with CP-Ser O_4 and CP-Ser O_5 were performed on a 500-μL scale at pH 7.5 (75 mM HEPES, 100 mM NaCl). Aliquots of CP-Ser, CP-Ser O_4, and CP-Ser O_5 were thawed at room temperature and diluted (30 μM, 750 μL) using the assay buffer. Ca(II) (1.5 mM) was added to all samples and Mn(II) or Fe(II) (30 μM) was added to select samples. For the experiments with Fe(II), all materials except for the proteins were brought into the anaerobic glovebox one day prior to the experiment. The digestions were incubated in a heating block set to 37 °C. Samples were removed from the glovebox after they had been quenched. To initiate each digestion assay, an aliquot (between 5- and 10-μL as appropriate) of protease was added to the 500-μL protein solution to afford protease concentrations of 0.45 μM (trypsin), 0.5 μM (HNE), or 1 μM (chymotrypsin, proteinase K). The resulting solution was immediately mixed with a pipette and incubated at 37 °C.

Aliquots (45-μL) of the reaction were quenched with 5 μL of aqueous 6% (v/v) TFA and 150 μL of 6 M guanidinum chloride at t = 0, 0.5, 1, 2, and 3 h (trypsin and proteinase K), t = 0, 4, 8, and 24 h (HNE), or t = 0, 1, 3, 5, and 8 h (chymotrypsin). The quenched solutions were centrifuged (13 000 rpm, 10 min, 4 °C), and the resulting samples were analyzed by analytical HPLC using a gradient of 10–60% B over 50 min. All digestions were performed in triplicate, and two separate preparations of CP-Ser, CP-Ser O_4, and CP-Ser O_4 were used. Representative chromatograms are shown.

Protease Digestion of Oxidized CP. Disulfide-linked CP and disulfide-linked MetO CP were prepared as described in the Oxidation of CP subsection, and stored overnight at 4 °C. The next day, these samples were used in trypsin digestion assays. To monitor the degradation of disulfide-linked and disulfide-linked MetO CP, we quenched the reactions at varying time points and measured the reaction progress using reversed-phase HPLC. At each time point, one 45-μL aliquot of the digest was quenched in the absence of a reducing agent (150 μL of 6 M guanidine hydrochloride and 5 μL of 6% TFA), and a second 45-μL aliquot was quenched in the presence of a reducing agent (40 mM HEPES, 6 M guanidine hydrochloride, 20 mM TCEP, pH 7.5). After rapid mixing with the micropipette, each sample quenched under reducing conditions was heated at 95 °C for 10 min to allow reduction of the disulfide bonds without trypsin activity. After heating, 5 μL of 6% TFA was added, the quenched samples were centrifuged at 13 000 rpm, 4 °C for 10 min, and 100 μL aliquots were analyzed by analytical HPLC, and using a gradient of 10–60% B over 50 min. All digestions were performed in triplicate using two separate preparations of disulfide-linked CP and MetO-disulfide-linked CP. Representative chromatograms are shown.

SDS-PAGE and Western Blots. Samples were prepared for SDS-PAGE by combining 5x Laemmli buffer (312 mM Tris-HCl, 10% SDS, 0.05% bromophenol blue (w/v), BME (v/v), pH 6.8) and protein samples in a 1:4 (v/v) ratio. For analysis of CP disulfide speciation after oxidation, the BME was omitted from the Laemmli buffer. The

samples were heated at 95 °C for 5 min, and then 10 μL was loaded onto the gel. For all experiments, gels of 1-mm width were used. The stacking gel was made from 125 mM Tris-HCl, 0.1% SDS, 5% acrylamide (19:1 acrylamide:bis acrylamide), 0.05% ammonium persulfate, 0.1% tetrmethylethylenediamine, pH 6.8. The resolving gel was made from 250 mM Tris-HCl, 1% SDS, 15% acrylamide (19:1 acrylamide:bis acrylamide), 0.05% ammonium persulfate, 0.05% tetramethylethylenediamine, pH 8.8. The products of the disulfide-bond-formation reactions of CP and the fractions from SEC of disulfide-linked CP were run at 200 V for 90 min. The gels were stained with using Coomassie Brilliant Blue G-250.

For western blots, the protein was transferred to a nitrocellulose membrane using a Trans-Blot Turbo (Bio-Rad). The transfers were performed at 25 V for 10 min. The membrane was blocked for 30 min using 5% milk in Tris-buffered saline (TBS, 2 mM Tris, 138 mM NaCl, pH 7.5). The blot was probed at 4 °C overnight with goat-anti S100A9 (Santa Cruz Biotech, sc-8114, 250-fold diluted) in TBS+5% bovine serum albumin. The next day, the blot was washed three times for 5 min using TBS+0.1% Tween 20, and then rinsed with water. The blot was then stained for 1 h at ambient temperature with 1:10 000-fold diluted donkey anti-goat 800CW (Li-COR) in TBS+5% milk. After rinsing the blot with water, the blot was imaged using an Odyssey scanner (Li-COR). The same probing, washing, and imaging protocol was repeated to detect S100A8. A murine monoclonal antibody (Santa Cruz Biotech, sc-48352) was used to probe for S100A8 and a goat anti-mouse 680CW (Li-COR) antibody was used to visualize S100A8.

Supporting Discussion

Preparation of Oxidized CP-Ser. We followed our reported procedures[S1] for overexpressing the S100A8(C42S) and S100A9(C3) polypeptides and reconstituting the CP-Ser heterodimer except that, following the first round of dialysis in refolding buffer (75 mM HEPES, 100 mM NaCl, pH 7.5), we treated the protein with H_2O_2 at 37 °C. We found that treating the dialyzed protein with 50 mM H_2O_2 for 2.5 or 5 h afforded a mixture of species where the dominant species was oxidized CP-Ser comprised of S100A8 with one additional oxygen atom and S100A9 with three additional oxygen atoms. We named this protein mixture CP-Ser O_4 to denote the most prevalent number of additional oxygen atoms per heterodimer (Table S3 and Figure S7). When we treated the protein with 50 mM H_2O_2 for 6 h or with 75 mM H_2O_2 for 6.5 h, we obtained mixtures where the dominant species was oxidized CP-Ser comprised of S100A8 with one additional oxygen atom and S100A9 with four additional oxygen atoms (Table S3 and Figure S7). We named this protein mixture CP-Ser O_5. Following treatment with H_2O_2, the protein was centrifuged to remove a precipitate that formed during oxidation, dialyzed against refolding buffer, and then the heterodimers were purified by anion exchange and gel filtration chromatography as previously reported for CP-Ser (Figure S3).[S1] The yields for CP-Ser O_4 and CP-Ser O_5 ranged from 30-40 mg / 2 L of overexpression culture.

CP-Ser O_4 and CP-Ser O_5 Interconvert Between Heterodimers and Heterotetramers. Velocity analytical ultracentrifugation (AUC) is a powerful technique for studying protein oligomerization that is capable of differentiating between interacting and non-interacting systems.[S7,S8] Along these lines, two scenarios that would give rise to the observed SEC peak shapes for CP-Ser O_4 and CP-Ser O_5 can be considered: (i) the oxidized proteins containing a population of tetramers and a population of dimers that do not interconvert or (ii) the oxidized proteins are in a dynamic equilibrium between heterodimer and heterotetramer. For a system of two species that interconvert on a slow timescale or do not interconvert, analysis of AUC data by SEDFIT will afford a

sedimentation profile where the two species have S values identical to those of the species in isolation and the S values will be invariant with protein concentration, but the relative amount of each species will change with protein concentration.[S7,S8] In contrast, systems that interconvert on the timescale of sedimentation will have peak profiles that change as a function of protein concentration, and the S value(s) observed will be in between those of the two species in isolation when analyzed by SEDFIT.[S7,S8]

Oxidized CP-Ser is Protease Sensitive – Degradation Assays with Proteinase K. In prior work, we did not compare the protease stability of the CP heterodimer and heterotetramer against proteinase K; however, we demonstrated that proteinase K cleaves after (A8)E89 and (A9)H104 in the presence of excess Ca(II).[S9] In this work, S100A8+O displayed a peak shift consistent with cleavage after E89 within 30 min of incubation with proteinase K (Figure S17). At later times in the reaction, the CP-Ser O_4 and CP-Ser O_5 subunits were proteolyzed to peptides, whereas the truncated CP-Ser subunits persisted (Figure S17).

Oxidation of CP Yields Disulfide-Linked Oligomers. For the SEC analyses, we treated aliquots of the sample from the 23-h time point in three different ways such that the effects of Ca(II) ions and disulfide bond formation on protein oligomerization could be assessed (Figure S21). The first protein aliquot was untreated and eluted with a mobile phase that contained 1.5 mM Ca(II). The second protein aliquot was treated with 2 mM EDTA to remove bound Ca(II) ions from the protein and eluted with a mobile phase that did not contain added Ca(II). The third protein aliquot was treated with 2 mM TCEP to reduce the disulfide bonds, and this sample eluted with a mobile phase that contained excess Ca(II) ions. When disulfide-linked CP was eluted in the presence of excess Ca(II) ions, two peaks with elution volumes of 10.2 mL (77.5 kDa) and 11.1 mL (42.6 kDa) were observed. In the presence of EDTA and no Ca(II) ions added to the mobile phase, disulfide-linked CP eluted as two major species with elution volumes of 9.8 mL (64.8 kDa) and 11.7 mL (33.0 kDa). Reduction of disulfide-linked CP with TCEP in the presence of

Ca(II) caused the protein to elute as a single peak indicative of a heterotetramer (11.1 mL, 43.8 kDa), demonstrating that the effects of disulfide bond formation could be reversed via reduction. We note that the larger oligomers observed in this experiment are within the linear range of the column; therefore, the calculated molecular weights should be reasonable estimates, indicating that the disulfide-forming reaction conditions did not produce aggregates.

SDS-PAGE analysis of the fractions obtained from SEC revealed that, regardless of the sample treatment, all fractions contained S100A8 and S100A9 subunits (Figure S21), suggesting that CP retained native-like noncovalent heterooligomers after the formation of disulfide bonds. Moreover, all of the fractions contained the disulfide-linked S100A8–S100A9 species, and the fractions corresponding to the relatively low-molecular-weight peaks (11.1 mL, 42.6 kDa, +Ca(II); 11.7 mL, 33.0 kDa, +EDTA) exclusively contained the disulfide-linked S100A8–S100A9 species. The 11.7 mL (33.0 kDa) peak in the +EDTA sample was close to the apparent molecular weight of the CP heterodimer (34.9 kDa), and in the presence of Ca(II) in the mobile phase this species appeared to shift to an apparent molecular weight comparable to the CP heterotetramer (45.9 kDa). We reason that, in the absence of Ca(II), this protein was dimeric CP and contained an intramolecular disulfide bond, which would explain why the S100A8–S100A9 disulfide linkage is more prevalent in the lower molecular weight fractions. Examination of CP-Ser crystal structures revealed that the N-terminus of S100A9 is in close proximity to (A8)C42 (Figure S22), indicating that formation of an (A9)C3–(A8)C42 “intradimer” disulfide bond is reasonable. The molecular identity of the 64.8-kDa peak in the sample that was treated with EDTA is unclear; however, a trimer of $\alpha\beta$ heterodimers or a larger oligomer are possible species. The 64.8 kDa species shifted to 77.5 kDa when Ca(II) ions were present in the mobile phase, indicating that this species binds Ca(II) ions, causing formation of a higher-order oligomer. In conclusion, we established that (i) all species in the disulfide-linked CP displayed Ca(II)-induced oligomerization (ii) the major

species in the disulfide-linked CP mixture was the CP heterotetramer that contained at least one (A9)C3–(A8)C42 intradimer disulfide bond, and (iii) the minor product was a small disulfide-linked CP polymer.

Preparation of MetO-disulfide-linked CP. Because we sought to prepare and study disulfide-linked CP species that contain oxidized methionine residues, we developed a two-step procedure to obtain these proteins. First, disulfide-linked CP was prepared in the presence of excess Ca(II) ions using the disulfide-bond formation reaction described for generating disulfide-linked CP. In the second step, disulfide-linked CP was treated with 100 mM H_2O_2 at 37 ^oC for up to 23 h. Mass spectrometry of the reaction mixture at the 7-h time point demonstrated that S100A8+O, S100A9+3O and S100A9+4O were the predominant species (Figure S20). SDS-PAGE of reaction aliquots taken at varying time points during the 23-h reaction revealed a gradual loss of the disulfide-linked S100A9–S100A9 species and increase in the disulfide-linked S100A8–S100A8 species (Figure S23). We also observed a small increase in the apparent molecular weight of all the bands after Met oxidation, which is a known phenomenon.[S13] This analysis indicated the disulfide bonds were not cleaved to cysteic acid under the reaction conditions. We concluded that treating disulfide-linked CP for 7 h with 100 mM H_2O_2 afforded the sufficient Met oxidation to resemble CP-Ser O_4 and CP-Ser O_5 while minimally perturbing the disulfide bonds, and we named this reaction product MetO-disulfide-linked CP.

Table S1. Table of observed and theoretical masses for species assigned in the mass spectrometry of human specimens.

Peak Number	Assignment	Observed Mass (Da)	Theoretical Mass (Da)
1	S100A8	10834.9	10834.5
2	S100A8+O	10850.8	10850.5
3	S100A9(ΔM1,acetyl)	13153.5	13152.8
4	S100A9(ΔM1,acetyl)+O	13169.5	13168.8
5	S100A9(ΔM1,acetyl)+2O	13185.4	13185.8
6	S100A9(ΔM1,acetyl)+3O	13201.6	13200.8
7	S100A9(ΔM1,acetyl)+4O	13218.0	13216.8
8	S100A9(ΔM1,acetyl)+5O	13233.0	13232.8
9	S100A9(ΔM1,acetyl)+6O	13249.3	13248.8
10	S100A9(ΔM1,acetyl)+7O	13266.1	13264.8
11	S100A9(ΔM1,acetyl)+8O	13281.3	13280.8
12	S100A9(ΔM1-M5, acetyl)	12689.6	12689.1
13	S100A9(ΔM1-M5, acetyl)+O	12706.2	12705.1
14	S100A9(ΔM1-M5, acetyl)+2O	12722.8	12721.1
15	S100A9(ΔM1-M5, acetyl)+3O	13737.1	12737.1
16	S100A9(ΔM1-M5, acetyl)+5O	12770.4	12769.1
17	S100A9(ΔM1-M5, acetyl)+6O	12786.2	12785.1
18	S100A8	10834.9	10834.5
19	S100A8+O	10851.1	10850.5
20	^{15}N-S100A8(C42S)	10945.0	10944.1
21	S100A9(ΔM1,acetyl)	13152.9	13152.8
22	S100A9(ΔM1,acetyl)+O	13169.4	13168.8
23	S100A9(ΔM1,acetyl)+5O	13233.9	13232.8
24	^{15}N-S100A9(ΔM1, C3S)	13253.8	13253.8
25	^{15}N-S100A9(ΔM1, C3S)+O	13266.8	13269.8
26	S100A8	10835.0	10834.5
27	S100A8+O	10851.6	10851.6
28	^{15}N-S100A8(C42S)	10945.0	10944.1
29	S100A9(ΔM1,acetyl)	13153.2	13152.8
30	S100A9(ΔM1,acetyl)+O	13169.9	13168.8
31	S100A9(ΔM1,acetyl)+2O	13184.1	13184.8
32	S100A9(ΔM1,acetyl)+5O	13233.3	13232.8
33	^{15}N-S100A9(ΔM1, C3S)	13253.3	13253.8
34	S100A8	10834.6	10834.5
35	S100A9(ΔM1,acetyl)	13153.5	13152.8
36	S100A9(ΔM1,acetyl)+O	13168.0	13168.8
37	S100A9(ΔM1,acetyl)+2O	13184.5	13184.8
38	S100A8	10834.9	10834.5
39	S100A9(ΔM1,acetyl)	13152.9	13152.8
40	S100A9(ΔM1,acetyl)+O	13170.6	13168.8

Peak Number	Assignment	Observed Mass (Da)	Theoretical Mass (Da)
41	S100A9(ΔM1,acetyl)+5O	13233.3	13232.8
42	S100A8	10834.7	10834.5
43	S100A8+O	10851.5	10850.5
44	^{15}N-S100A8(C42S)	10945.1	10944.1
45	S100A9(ΔM1,acetyl)	13153.3	13152.8
46	S100A9(ΔM1,acetyl)+O	13170.1	13168.8
47	S100A9(ΔM1,acetyl)+5O	13232.8	13232.8
48	^{15}N-S100A9(ΔM1, C3S)	13253.1	13253.8
49	^{15}N-S100A9(ΔM1, C3S)+O	13268.9	13269.8

Table S2. Summary of mucus samples analyzed.[a]

Sample	A8	A8+O	A9	A9+O	A9+2O	A9+3O	A9+4O	A9+5O	A9+6O	A9+7O	A9+8O
Mucus 1	X	-	X	X	X	-	-	-	-	-	-
Mucus 2	X	X	X	X	X	X	X	X	X	X	X
Mucus 3	-	-	-	-	-	-	-	-	-	-	-
Mucus 4	-	-	-	-	-	-	-	-	-	-	-
Mucus 5	-	-	-	-	-	-	-	-	-	-	-
Mucus 6	X	-	-	X	-	-	-	-	-	-	-
Mucus 7	X	-	-	X	-	-	-	-	-	-	-
Mucus 8	-	-	-	-	-	-	-	-	-	-	-
Mucus 9	-	-	-	-	-	-	-	-	-	-	-
Mucus 10	X	-	X	X	-	-	-	X	-	-	-
Mucus 11	X	-	X	-	-	-	-	-	-	-	-
Mucus 12	-	-	-	-	-	-	-	-	-	-	-
Mucus 13	X	-	X	-	-	-	-	-	-	-	-
Mucus 14	X	-	X	-	-	-	-	-	-	-	-
Mucus 15	-	-	X	-	-	-	-	-	-	-	-
Mucus 16	-	-	-	-	-	-	-	-	-	-	-
Mucus 17	X	-	X	-	-	-	-	-	-	-	-
Mucus 18	-	-	-	-	-	-	-	-	-	-	-
Mucus 19	-	-	-	-	-	-	-	-	-	-	-
Mucus 20	-	-	-	-	-	-	-	-	-	-	-
Mucus 21	X	-	X	X	-	-	-	-	-	-	-
Mucus 22	X	X	X	X	-	-	-	X	-	-	-
Mucus 23	X	X	X	-	-	-	-	-	-	-	-
Mucus 24	X	X	X	X	-	-	-	-	-	-	-
Mucus 25	X	-	X	-	-	-	-	-	-	-	-
Mucus 26	X	-	X	-	-	-	-	-	-	-	-

[a] Species that were observed are marked with an "X."

Table S3. Mass spectrometric analysis of CP and variants.

Protein	S100A8 Observed Mass (g/mol)	S100A9 Observed Mass[a] (g/mol)
CP-Ser	10 818.6	13 094.7
CP-Ser O_4	10 834.4	13 142.7
CP-Ser O_5	10 834.5	13 159.0
CP-Ser(A9-M63A)	10 818.9	13 034.6
CP-Ser(A9-M81A)	10 818.6	13 034.6
CP-Ser(A9-M83A)	10 818.8	13 034.6
CP	10 834.1	13 110.4
MetO-disulfide-linked CP	10 850.5, 10 866.6	13 159.0, 13 174.0

[a] In all preparations, the dominant purified species of the S100A9 subunit lacked the N-terminal methionine. The masses reported here are the observed values for S100A9(C3S) lacking the N-terminal Met residue.

Table S4. Calculated sedimentation coefficients and molecular weights using SEDFIT.[a]

Protein	Concentration (μM)	$s_{20,w}$ (S)	MW (kDa)	Partial Specific Volume (mL/g)
CP-Ser	30	2.2, 3.9	22.8, 52.0	0.7388
CP-Ser+Ca(II)	30	4.0	41.4	0.7388
CP-Ser O_4 [b]	30	2.2	23.8	0.7388
CP-Ser O_4 [c]	30	2.4	22.6	0.7388
CP-Ser O_4+Ca(II) [b]	30	2.5, 3.5	22.4, 37.5	0.7388
CP-Ser O_4+Ca(II) [c]	30	2.7, 4.5	23.2, 49.8	0.7388
CP-Ser O_5 [d]	30	2.2	23.3	0.7388
CP-Ser O_5 [e]	30	2.4	23.1	0.7388
CP-Ser O_5+Ca(II) [d]	30	3.3	40.7	0.7388
CP-Ser O_5+Ca(II) [e]	30	2.4, 3.5	22.9, 35.9	0.7388

[a] All experiments were conducted at 20 °C. The units of viscosity are in centipoise (cP) (1 Poise = g cm^{-1} s^{-1}). For experiments without Ca(II), the buffer was 75 mM HEPES, 100 mM NaCl, 30 μM EDTA, pH 7.5. For experiments in the presence of Ca(II), the buffer was 75 mM HEPES, 100 mM NaCl, 600 μM $CaCl_2$, pH 7.5. For both conditions $s_{20,w}$ values were adjusted with solvent density (ρ) of 1.0082 g/mL, solvent viscosity (η) of 1.0565 cP and pH 7.5 at 20 °C. The *c(s)* method was used for fitting the data. All scans that began at the baseline were used in fitting. The units of viscosity are in centipoise (cP) (1 Poise g cm^{-1} s^{-1}). Sedimentation coefficients are in Svedbergs (1 Svedberg = 100 fs = 1 x 10^{-13} s). [c] Data obtained using the second preparation of protein. [b] Data obtained using protein oxidized with 50 mM H_2O_2 for 2.5 h. [c] Data obtained using protein oxidized with 50 mM H_2O_2 for 5 h. [d] Data obtained using protein oxidized with 50 mM H_2O_2 for 6 h. [e] Data obtained with protein oxidized for 75 mM H_2O_2 for 6.5 h.

Table S5. Calculated sedimentation coefficients and molecular weights using DCDT+.[a]

Protein	Concentration (μM)	$s_{20,w}$ (S)	D (F)	MW (kDa)	Partial Specific Volume (mL/g)
CP-Ser	30	2.6	12.5	18.9	0.7388
CP-Ser+Ca(II)	30	4.0	9.1	41.2	0.7388
CP-Ser O_4 [b]	30	2.3	10.6	20.4	0.7388
CP-Ser O_4 [c]	30	2.5	10.9	21.0	0.7388
CP-Ser O_4+Ca(II) [b]	30	3.3	11.0	28	0.7388
CP-Ser O_4+Ca(II) [c]	30	2.9	13.8	19.6	0.7388
CP-Ser O_5 [d]	30	2.3	10.0	21.3	0.7388
CP-Ser O_5 [e]	30	2.4	10.3	22.1	0.7388
CP-Ser O_5+Ca(II) [d]	30	3.4	9.0	35.0	0.7388
CP-Ser O_5+Ca(II) [e]	30	3.1	12.1	23.9	0.7388

[a] All experiments were conducted at 20 °C. The units of viscosity are in centipoise (cP) (1 Poise = g cm^{-1} s^{-1}). Sedimentation coefficients are in Svedbergs (1 Svedberg = 100 fs = 1 x 10^{-13} s). Diffusion coefficients correspond to the best-fit molecular mass in Fick units (1 Fick = 1 x 10^{-7} cm^2/s. The dc/dt method was used to fit all data. Between 10 and 12 scans were used for fitting. The first and last scans used ranged from the 19th to the 47th. The peak broadening limit was always greater than 80 kDa. For experiments without Ca(II), the buffer was 75 mM HEPES, 100 mM NaCl, 30 μM EDTA, pH 7.5. For experiments in the presence of Ca(II), the buffer was 75 mM HEPES, 100 mM NaCl, 600 μM $CaCl_2$, pH 7.5. For both conditions $s_{20,w}$ values were adjusted with solvent density (ρ) of 1.0082 g/mL, solvent viscosity (η) of 1.0565 cP and pH 7.5 at 20 °C. [b] Data obtained using protein oxidized with 50 mM H_2O_2 for 2.5 h. [c] Data obtained using protein oxidized with 50 mM H_2O_2 for 5 h. [d] Data obtained using protein oxidized with 50 mM H_2O_2 for 6 h. [e] Data obtained with protein oxidized for 75 mM H_2O_2 for 6.5 h.

Table S6. Molecular weights and extinction coefficients for the S100A8 and S100A9 subunits of proteins examined in this study.

Protein	Molecular Weight (Da)	ε_{280} (M^{-1} cm^{-1})[b]
A8(C42S)	10 818.5 [a]	11 460
A8(C42S)+O	10 834.5 [a]	11 460
A8	10 834.5 [a]	11 460
A8+O	10 850.5 [a]	11 460
A8+2O	10 866.5 [a]	11 460
^{15}N-A8(C42S)	10 944.1 [c]	11 460
^{15}N-A9(C3S)	13 253.8 [c, d]	6 990
A9(C3S)	13 094.7 [a, d]	6 990
A9(C3S)+3O	13142.7 [a, d]	6 990
A9(C3S)+4O	13 158.7 [a, d]	6 990
A9(C3S)(M63A)	13 034.6 [a, d]	6 990
A9(C3S)(M81A)	13 034.6 [a, d]	6 990
A9(C3S)(M83A)	13 034.6 [a, d]	6 990
A9	13 110.8 [a, d]	6 990
A9+2O	13 142.8 [a, d]	6 990
A9+3O	13 158.8 [a, d]	6 990
A9+4O	13 174.8 [a, d]	6 990

[a] Extinction coefficients (280 nm) were calculated by using the ProtParam tool. (http://web.expasy.org/protparam). [b] Molecular weights were calculated by using the ProtParam tool available on the ExPASy server. [c] The theoretical mass was calculated using 99% ^{15}N and 1% ^{14}N. [d] In all preparations, LCMS revealed that the dominant purified species lacked the N-terminal methionine. The molecular weight shown is the theoretical value for S100A9(C3S) lacking the N-terminal Met residue.

Table S7. Molecular weights and extinction coefficients for commercial proteins.

Protein	Molecular Weight (Da)[a]	ε_{280} (M^{-1} cm^{-1})	ε_{405} (M^{-1} cm^{-1})
Trypsin	23 300	30 000 (ref S11)	-
Chymotrypsin	25 000	50 000 (ref S12)	-
Proteinase K	28 900	36 580 [b]	-
Human neutrophil elastase	28 500	-	-
Catalase	250 000	-	324 000 (ref S13)

[a] Molecular weights were calculated by using the ProtParam tool available on the ExPASy server (http://web.expasy.org/protparam). [b] Extinction coefficients (280 nm) were calculated by using the ProtParam tool.

Table S8. Primers employed for site-directed mutagenesis.

Primer	Sequence[a]
M63A-1	5'-GTGATTGAACACATT**GCG**GAGGATCTGGACACC-3'
M63A-2	5'-GGTGTCCAGATCCTC**CGC**AATGTGTTCAATCAC-3'
M81A-1	5'-CTTTGAAGAGTTCATC**GCG**CTGATGGCCCGTCTG-3'
M81A-2	5'-CAGACGGGCCATCAG**CGC**GATGAACTCTTCAAAG-3'
M83A-1	5'-GAGTTCATCATGCTG**GCG**GCCCGTCTGACGTGG-3'
M83A-2	5'-CCACGTCAGACGGGC**CGC**CAGCATGATGAACTC-3'

[a] The codons containing mutations are underlined and colored red. The template plasmid was pET41a-*S100A9(C3S),* which has the S100A9(C3S) gene ligated into the *Nde*I and *Xho*I restriction sites.[S1]

Table S9. Elution volume and calculated molecular weights from SEC experiments.

Ca(II)	Mn(II)	Protein	Elution Volume (mL)	Calculated Molecular Weight (kDa)
−	−	CP-Ser	11.5	34.9
+	–	CP-Ser	10.9	45.9
–	+	CP-Ser	11.2	40.0
+	+	CP-Ser	11.0	43.8
−	−	CP-Ser O_4	11.6	33.4
+	–	CP-Ser O_4	11.7	31.9
-	+	CP-Ser O_4	11.1	41.9
+	+	CP-Ser O_4	11.2	43.8
−	−	CP-Ser O_5	11.6	33.4
+	–	CP-Ser O_5	11.6	33.4
–	+	CP-Ser O_5	11.2	43.8
+	+	CP-Ser O_5	11.1	41.9

[a] Each sample contained 30 μM protein (75 mM HEPES, 100 mM NaCl, pH 7.5). The +Ca(II) samples contained 1.5 mM Ca(II) in the sample and mobile phase. The +Mn(II) samples contained 300 μM Mn(II) in the sample only.For the +Ca(II) +Mn(II) the samples and the mobile phase contained 1.5 mM Ca(II) and only the sample contained 30 μM Mn(II). The experiments were performed at 4 °C.

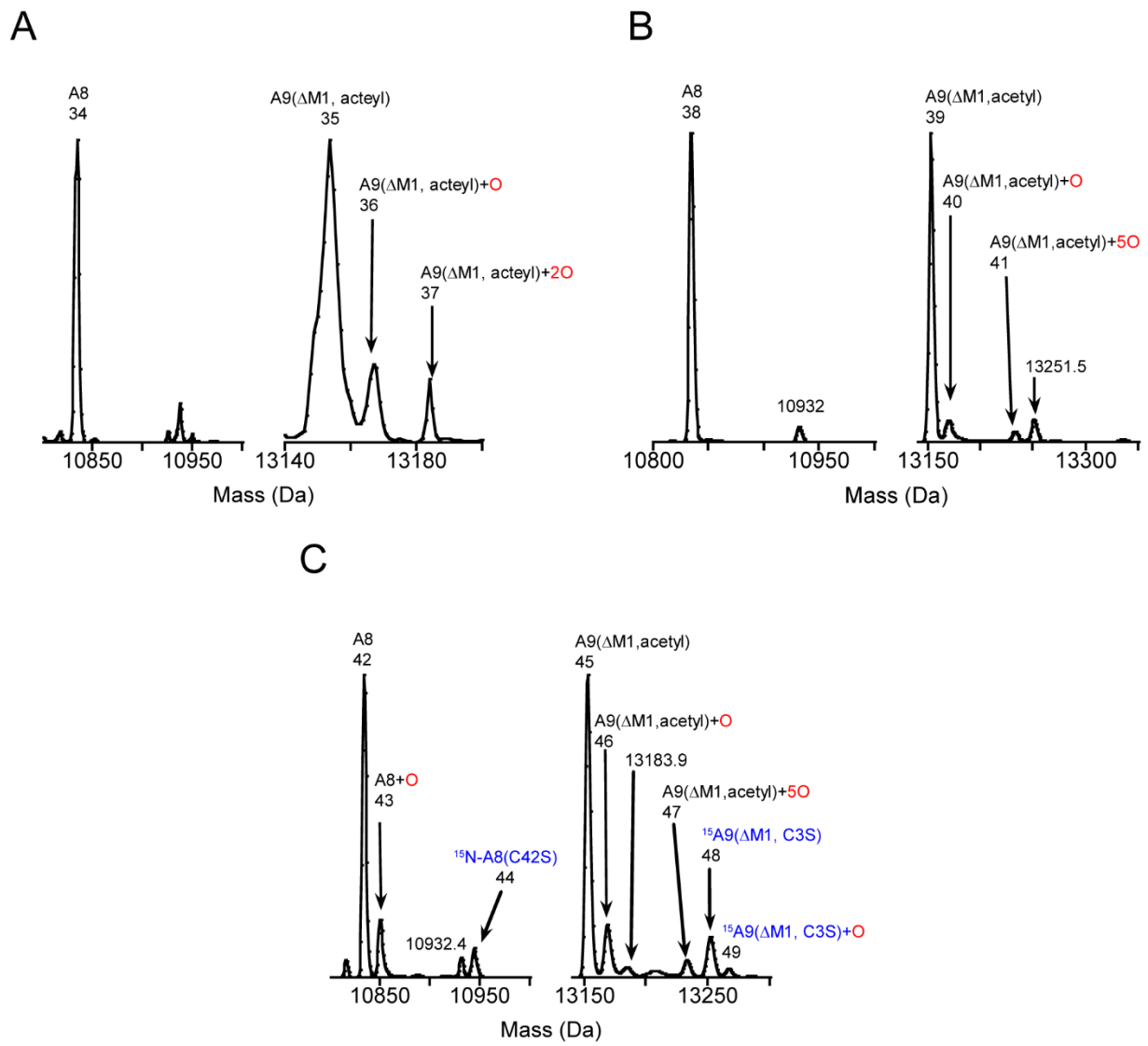


Figure S1. Mass spectrometry of additional human pus and nasal mucus samples. The observed and theoretical masses for each numbered peak are listed in Table S1. Deconvoluted mass spectra expanded around S100A8 and S100A9. (A) Mucus sample 1. (B) Mucus sample 10. (C) Pus sample 1 with ^{15}N-CP-Ser added after collection.

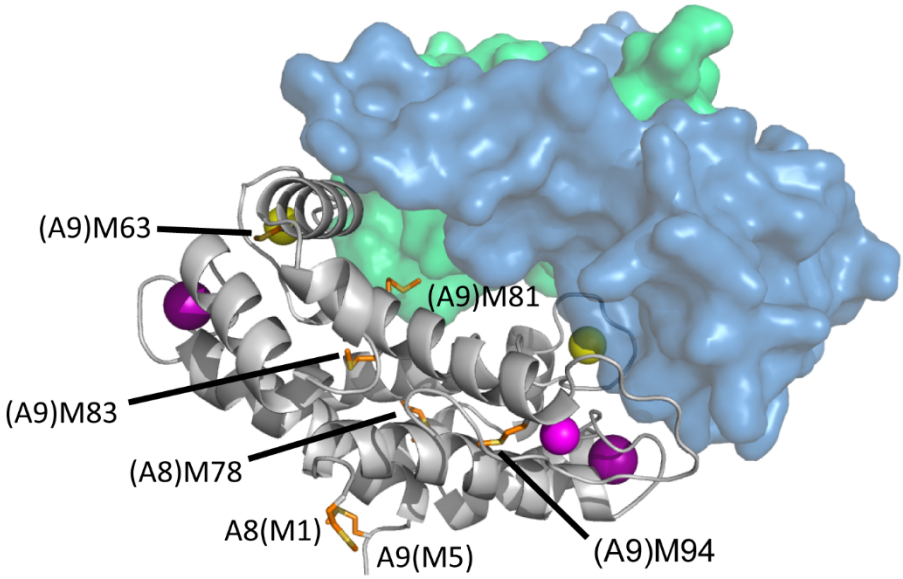


Figure S2. Crystal structure of the Ca(II)-, Na(I)- and Mn(II)-bound CP-Ser heterotetramer with all Met residues shown as sticks (PDB: 4XJK).[S2] The surface of one dimer unit is shown with S100A8 in green and S100A9 in blue. Both subunits of the other dimer are shown in silver with secondary structure depicted. The yellow spheres are Ca(II) ions, the purple spheres are Na(I) ions, and the pink sphere is a Mn(II) ion. The only Met residue at the dimer-dimer interface is M81 of S100A9.The N-terminus of S100A9 is disordered in this structure, and M1 of S100A9 is cleaved during overexpression, and thus not observed in this structure.

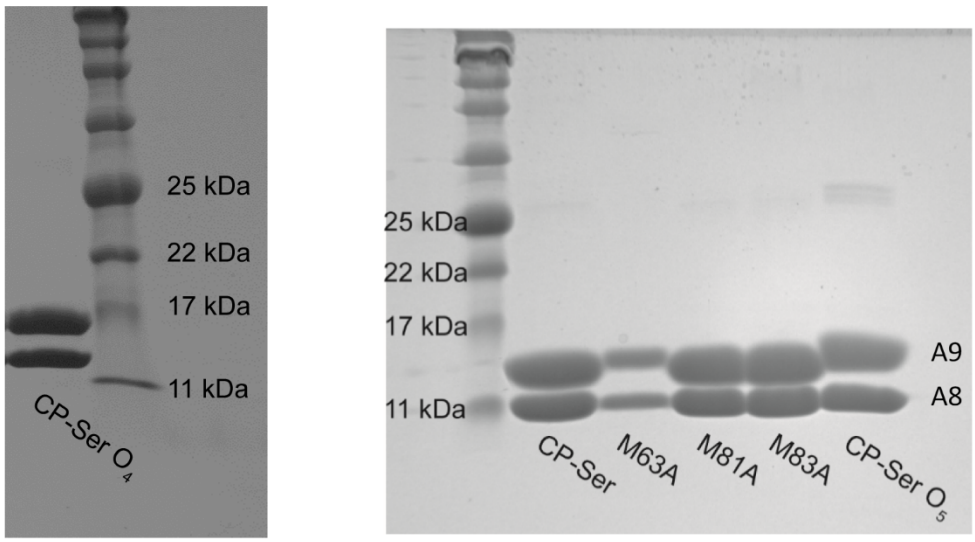


Figure S3. SDS-PAGE (Tris-HCl glycine, 15% acrylamide) visualized with Coomassie Blue of CP variants used in this study. The ladder is P7712S from New England Biolabs.

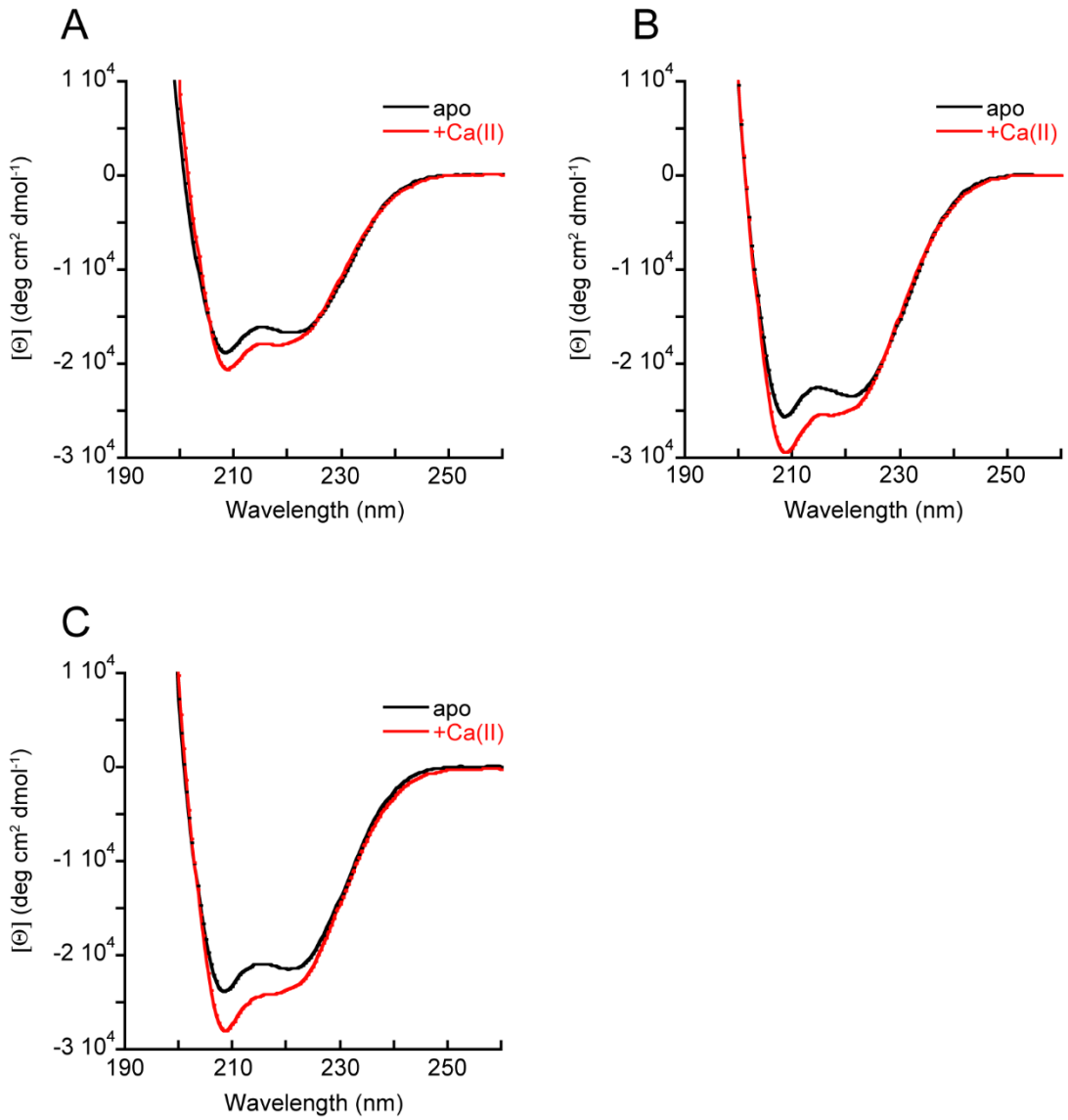


Figure S4. Circular dichroism spectra of Met→Ala variants of CP-Ser. (A) M63A, (B) M81A, (C) M83A. Conditions: 10 μM protein, 300 μL, 1 mM Tris, ±2 mM $CaCl_2$, pH 8.5, 25 °C.

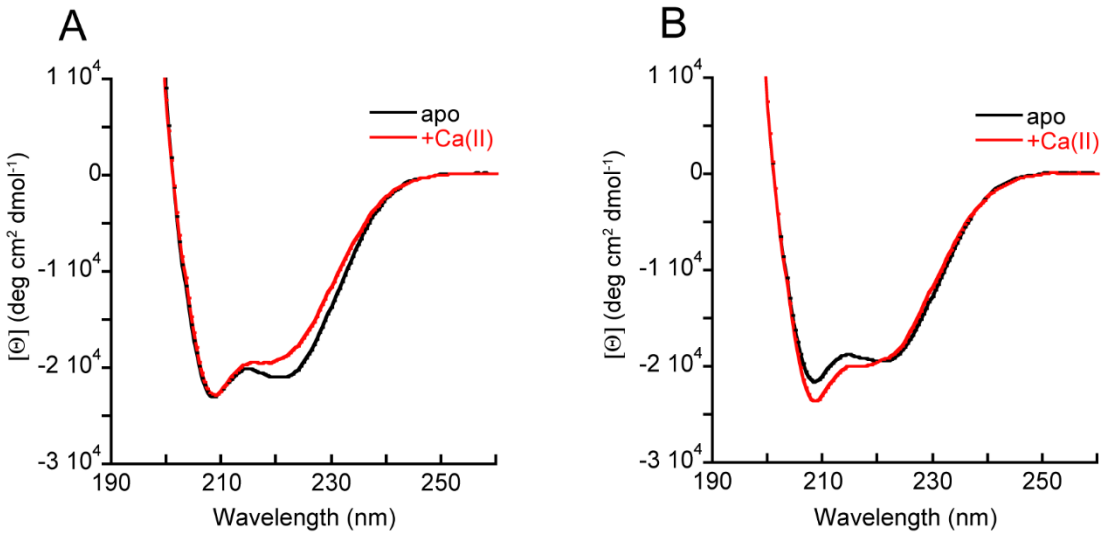
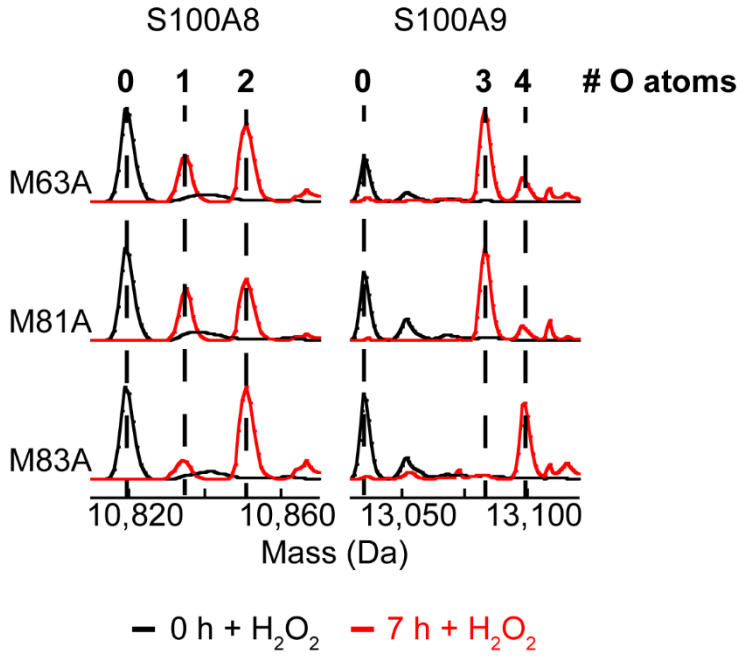


Figure S5. Circular dichroism spectra of oxidized CP-Ser. (A) CP-Ser O_4. (B) CP-Ser O_5. Conditions: 10 μM protein, 300 μL, 1 mM Tris, ±2 mM $CaCl_2$, pH 8.5, 25 °C.



Protein	Masses
S100A8(C42S)	Theoretical: 10 818.4 Da Observed: 10 818.6 Da
S100A8(C42S)+O	Theoretical: 10 834.4 Da Observed: 10 834.2 Da
S100A8(C42S)+2O	Theoretical: 10 850.4 Da Observed: 10 850.4 Da
S100A9(ΔM1,C3S,M→A)	Theoretical: 13 034.6 Da Observed: 13 034.6 Da
S100A9(ΔM1,C3S,M→A)+3O	Theoretical: 13 082.6 Da Observed: 13 082.4 Da
S100A9(ΔM1,C3S,M→A)+4O	Theoretical: 13 098.6 Da Observed: 13 098.5 Da

Figure S6. Mass spectra data of CP-Ser variants with Met→Ala mutations before and after treatment with H_2O_2. (A) Deconvoluted mass spectra of each Met→Ala variant before and after H_2O_2 treatment. (B) Table of theoretical and representative observed masses. Conditions: 75 mM HEPES, 100 mM NaCl, 1.5 mM Ca(II), pH 7.5, 7 h, 37 ºC.

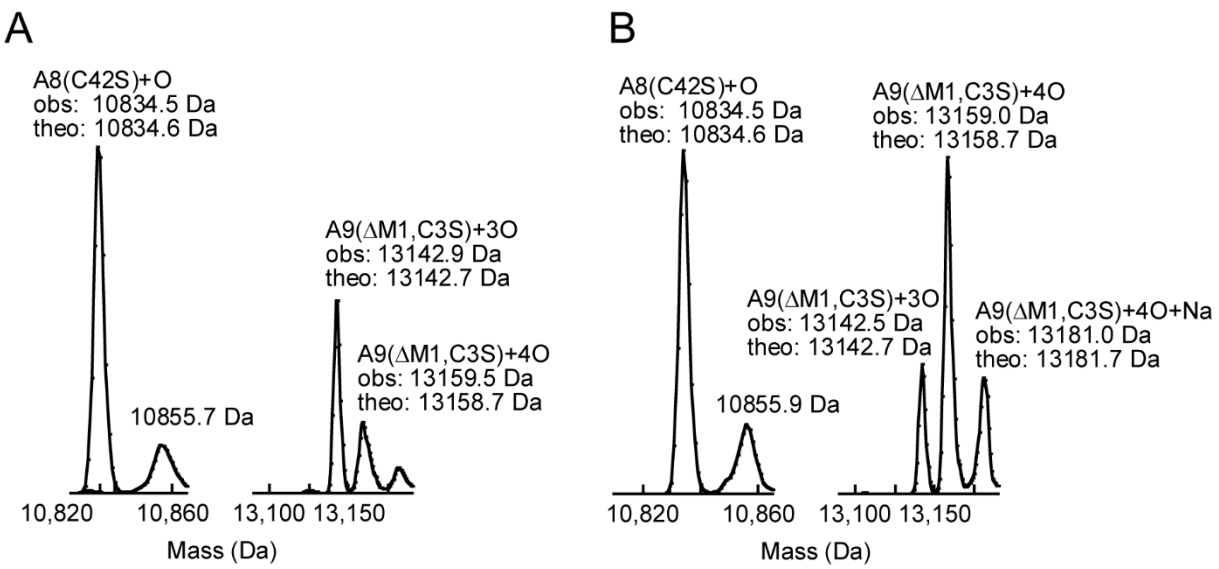


Figure S7. Representative deconvoluted mass spectra of (A) CP-Ser O_4 and (B) CP-Ser O_5 after purification.

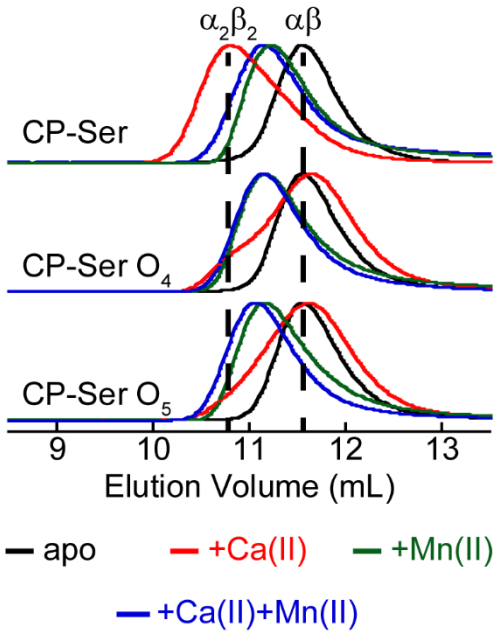


Figure S8. Analytical SEC chromatograms of 30 μM CP-Ser, CP-Ser O_4, and CP-Ser O_5. The sample and mobile phase buffers were 75 mM HEPES, 100 M NaCl, pH 7.5. For all samples with added Ca(II), 1.5 mM Ca(II) was included in the sample and mobile phase buffers. For the Mn(II)-only runs, 300 μM Mn(II) was added only to the sample. For the +Ca(II)+Mn(II) runs, 30 μM Mn(II) was added only to the sample

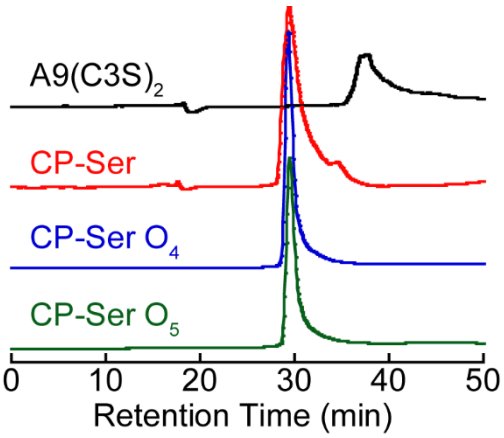


Figure S9. Anion exchange chromatography of the S100A9(C3S) homodimer, CP-Ser, CP-Ser O_4, and CP-Ser O_5. Conditions: 20 mM HEPES, 100 μM protein, pH 8.0. Mobile phase A: 20 mM HEPES, pH 8.0. Mobile phase B: 20 mM HEPES, 1 M NaCl, pH 8.0.

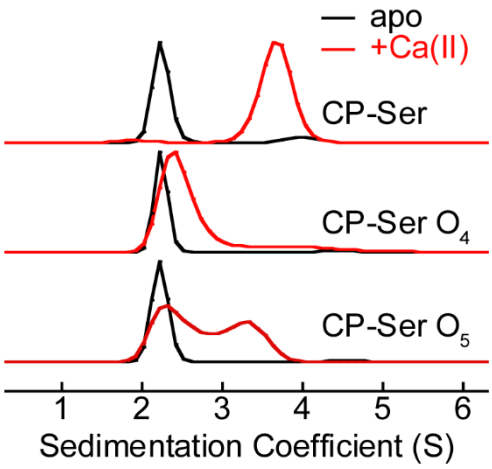


Figure S10. SEDFIT analysis of sedimentation velocity experiments with CP-Ser O_4 that had been oxidized with 50 mM H_2O_2 for 5 h and CP-Ser O_5 that had been oxidized with 75 mM H_2O_2 for 6.5 h. Conditions: 75 mM HEPES, 100 mM NaCl, 30 μM protein, ±30 μM EDTA, ±600 μM Ca(II), pH 7.5, 20 °C. In the presence of Ca(II), the S values for CP-Ser O_4 and CP-Ser O_5 are greater than the CP-Ser dimer and less than the CP-Ser tetramer, indicating that dynamic exchange between the dimer and tetramer oligomeric states is occurring. Sedimentation coefficients and fitting details can be found in Table S4.

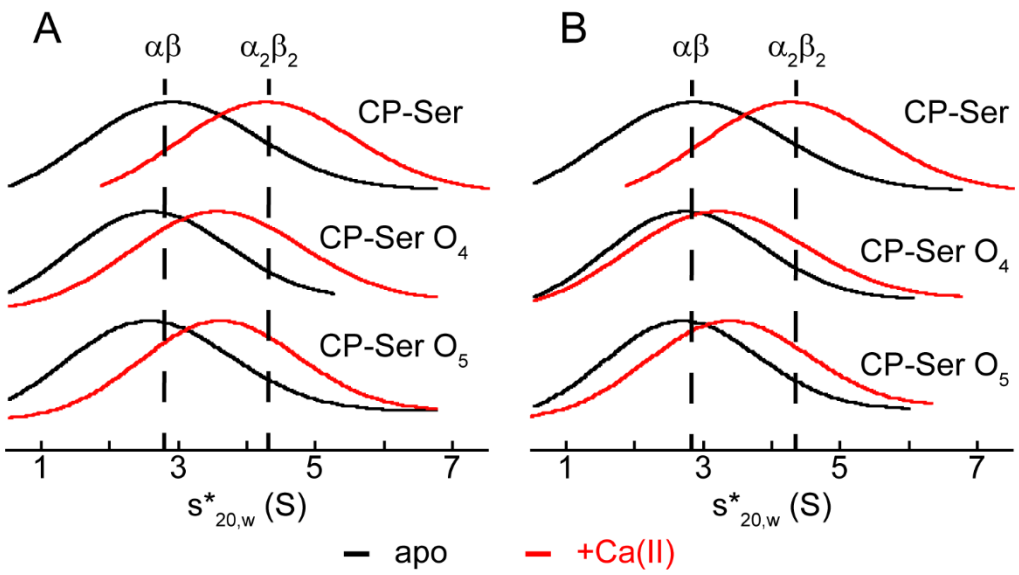


Figure S11. DCDT+ analysis of sedimentation velocity experiments with CP-Ser, CP-Ser O_4, CP-Ser O_5. (A) Sedimentation profiles with data from the first preparations of CP-Ser O_4 and CP-Ser O_5. (B) Sedimentation profiles with data from the second preparations of CP-Ser O_4 and CP-Ser O_5. Conditions: 75 mM HEPES, 100 mM NaCl, 30 μM protein, ±30 μM EDTA, ±1.5 mM Ca(II), 20 °C. Sedimentation coefficients and fitting details can be found in Table S5.

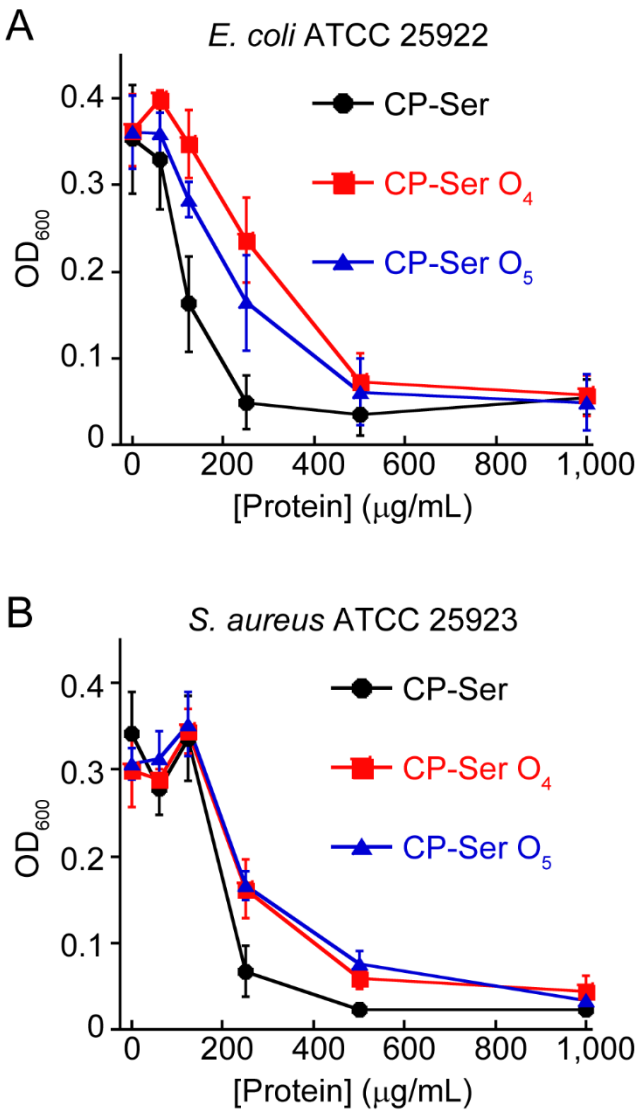


Figure S12. AMA assays with CP-Ser, CP-Ser O_4 and CP-Ser O_5 against (A) *E. coli* ATCC 25922 and (B) *S. aureus* ATCC 29523. OD_{600} was recorded after 20 h (*n*=3, ±SEM).

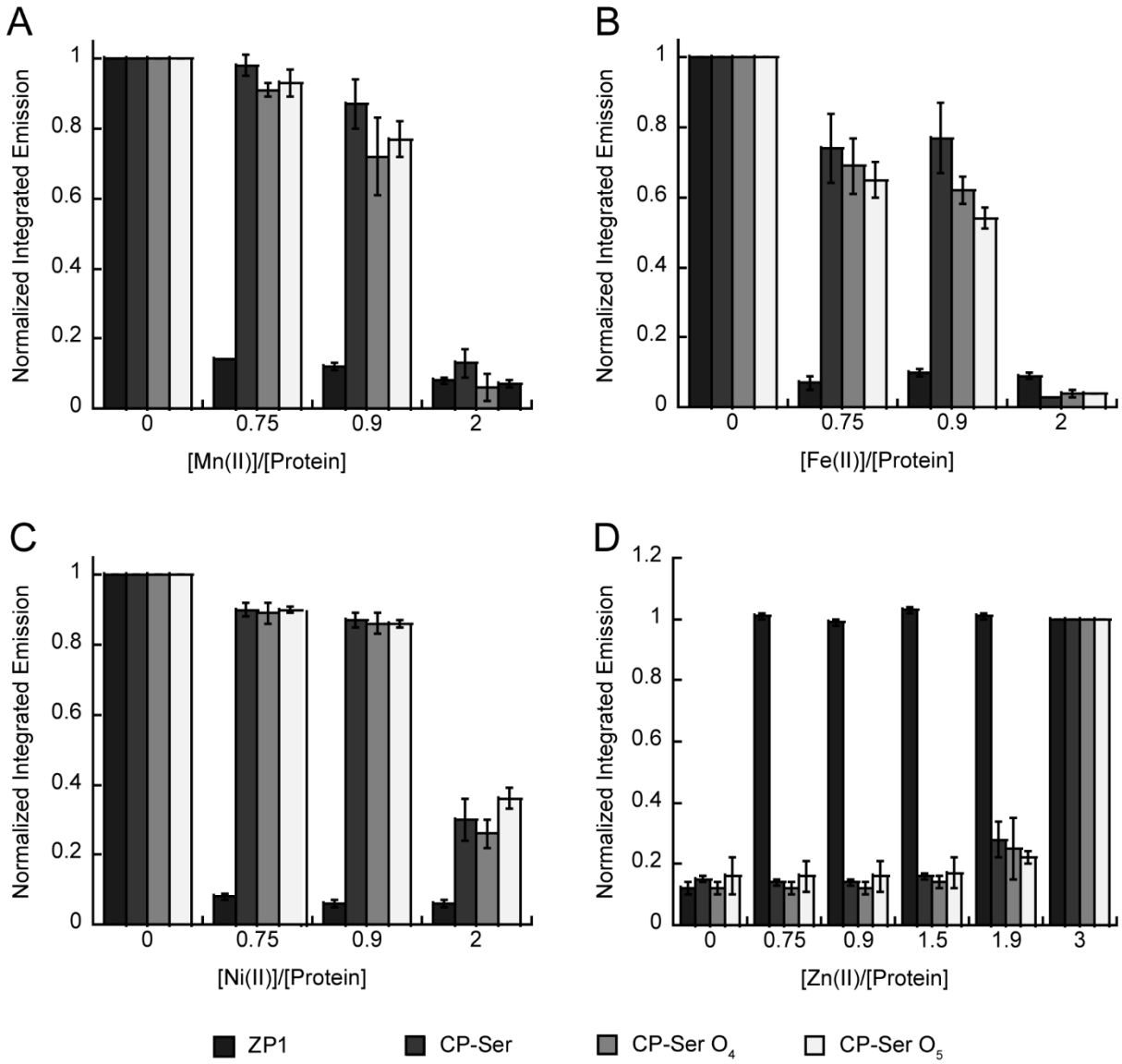


Figure S13. Competition between ZP1 and CP for Mn(II), Fe(II), Ni(II), and Zn(II) in the presence of Ca(II). The emission was normalized with respect to apo ZP1 emission for the Mn(II), Fe(II), and Ni(II) experiments (mean ± SDM, *n*=3). For the Zn(II) experiment, peak area was integrated with respect to emission after adding 12 μM Zn(II) (mean ± SDM, *n*=3). Conditions: 75 mM HEPES, 100 mM NaCl, pH 7.5, 4 μM CP, 200 μM Ca(II), 1 μM ZP1, 25 °C.

ZP1 is a fluorescent small molecule that exhibits fluorescence quenching as a result of Mn(II), Fe(II), and Ni(II) binding, and fluorescence enhancement as a result of Zn(II) complexation.[S6] In this experiment, CP-Ser O_4 and CP-Ser O_5 behave like CP-Ser and out-compete ZP1 for Mn(II), which indicates high-affinity Mn(II) binding in the presence of excess Ca(II). The apparent $K_{d1,Mn(II)}$ for ZP1 is 550 nM,[S14] $K_{d1,Fe(II)}$ is 2.2 pM,[S15] and $K_{d1,Zn(II)}$ is 0.7 nM.[S6] The apparent $K_{d,Ni(II)}$ is undetermined.

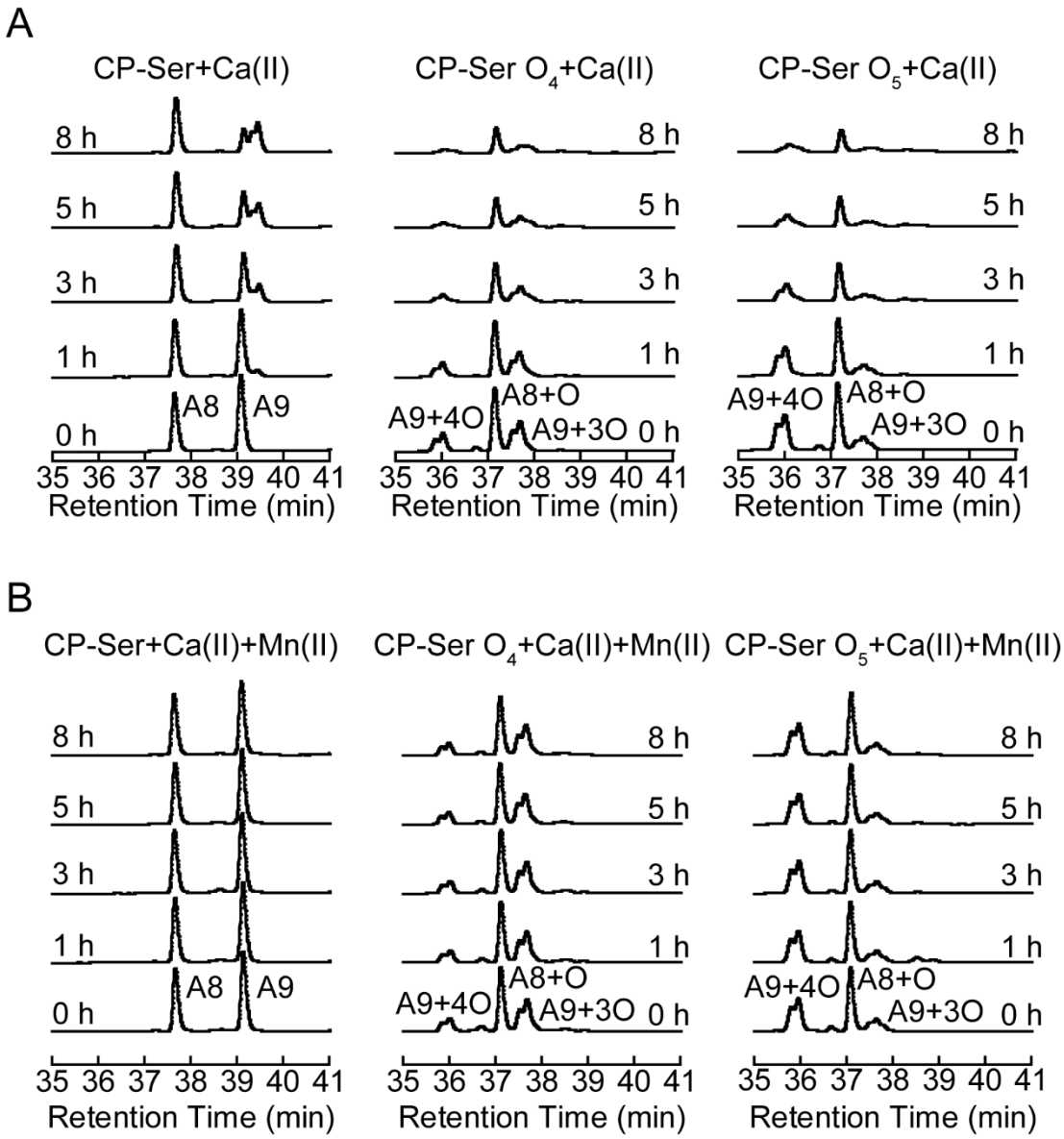


Figure S14. Digestions of CP-Ser, CP-Ser O_4 and CP-Ser O_5 by chymotrypsin. (A) Digestions in the presence of Ca(II). (B) Digestions in the presence of Ca(II) and Mn(II). Conditions: 75 M HEPES, 100 mM NaCl, 30 μM CP, 1.5 mM Ca(II), ±30 μM Mn(II), 1 μM chymotrypsin, pH 7.5, 37 °C.

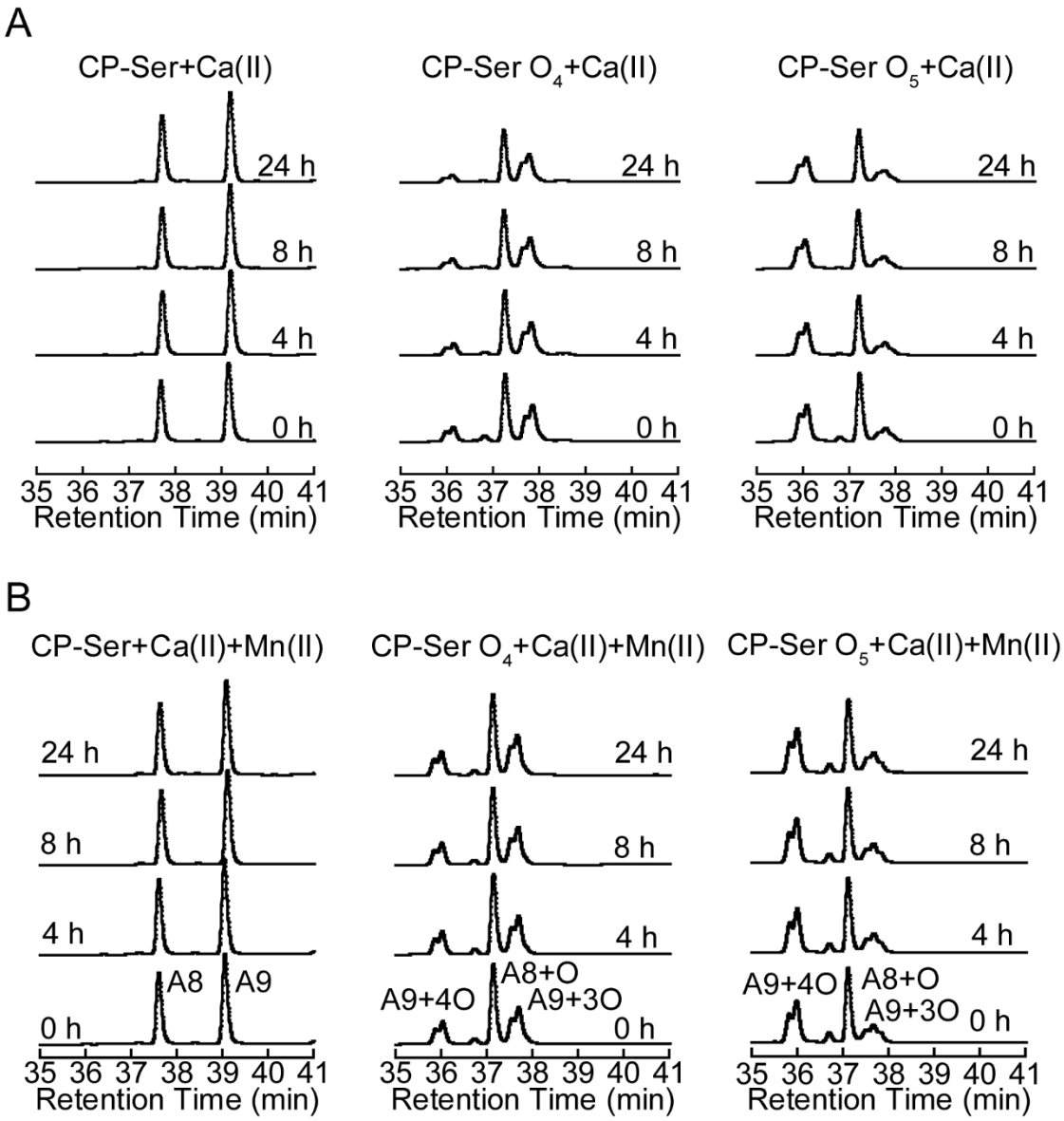


Figure S15. Digestions of CP-Ser, CP-Ser O_4 and CP-Ser O_5 by HNE. (A) Digestions in the presence of Ca(II) (reproduced from the main text). (B) Digestions in the presence of Ca(II) and Mn(II). Conditions: 75 M HEPES, 100 mM NaCl, 30 μM CP, 1.5 mM Ca(II), ±30 μM Mn(II), 1 μM HNE, pH 7.5, 37 °C.

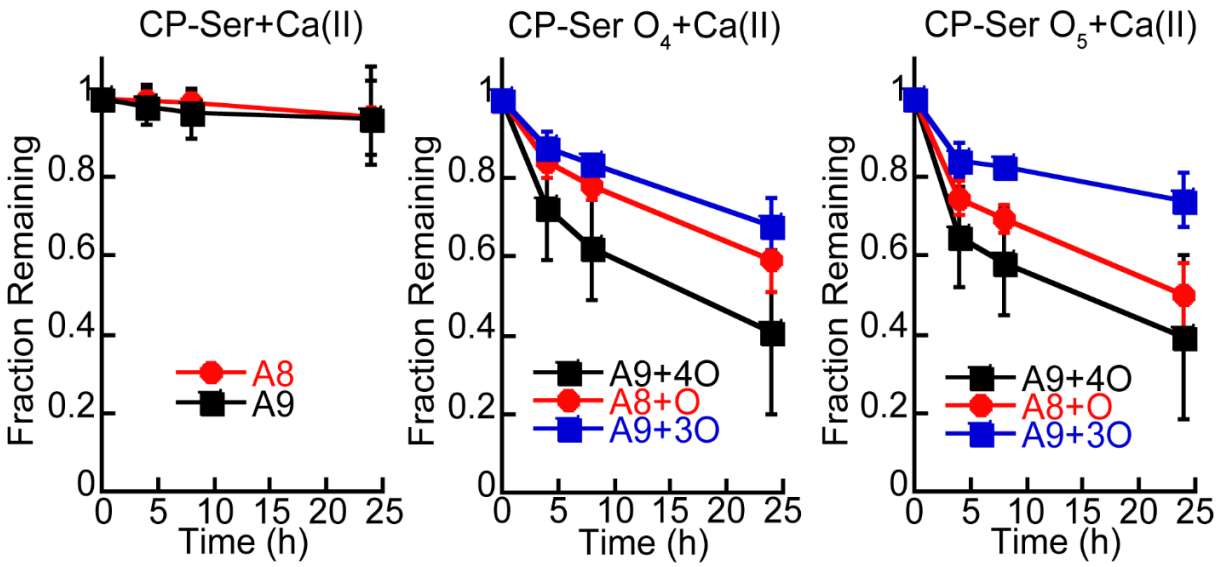


Figure S16. Plots of peak area from HPLC chromatograms of HNE digestions in the presence of Ca(II). The areas were normalized to the values obtained from the 0 h chromatograms (mean ± SDM, *n*=3). Conditions: 75 mM HEPES, 100 mM NaCl, 1.5 mM $CaCl_2$, pH 7.5, 30 μM CP, 1 μM HNE at 37 °C.

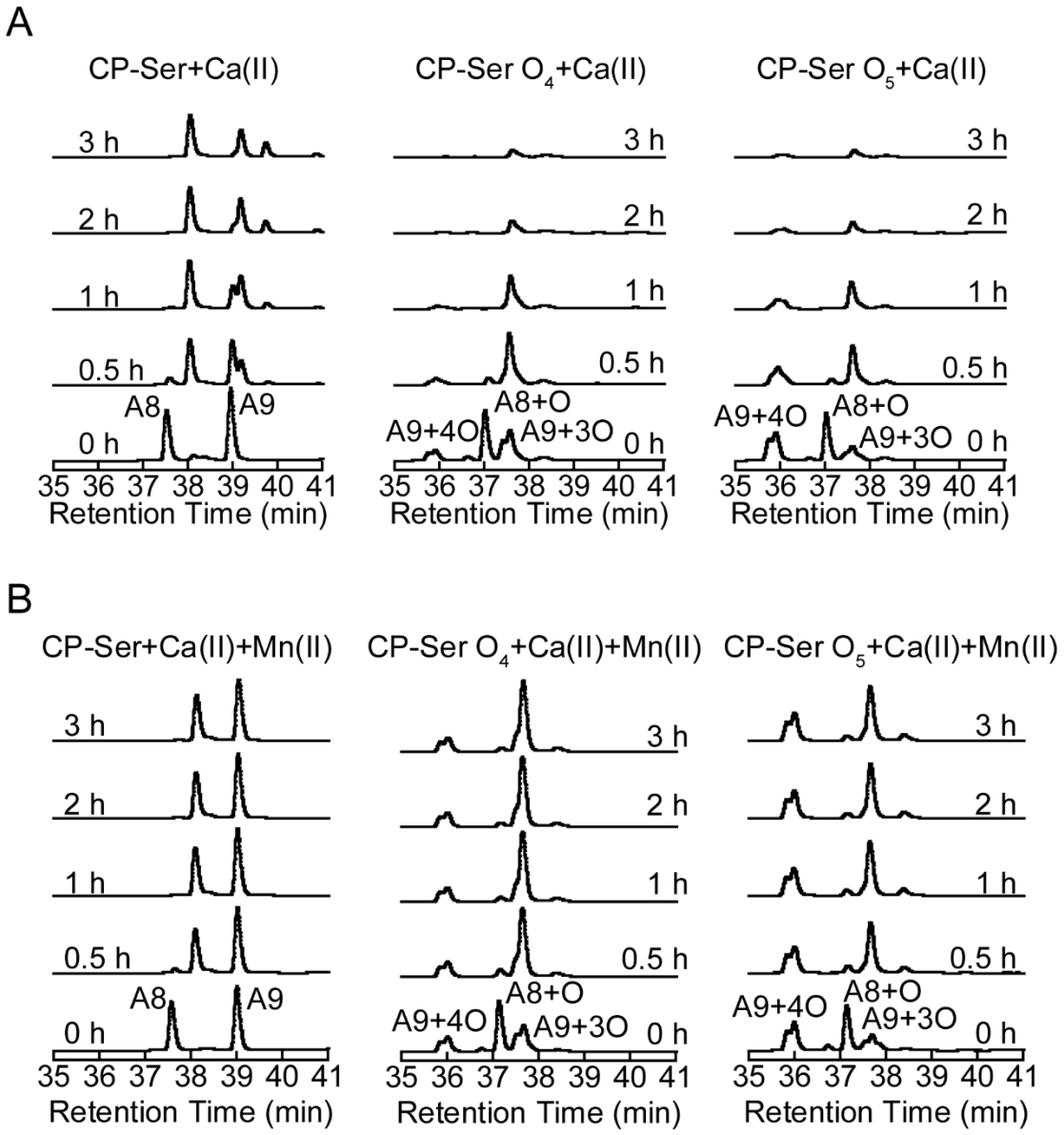


Figure S17. Digestions of CP-Ser, CP-Ser O_4 and CP-Ser O_5 by proteinase K. (A) Digestions in the presence of Ca(II). (B) Digestions in the presence of Ca(II) and Mn(II). Conditions: 75 M HEPES, 100 mM NaCl, 30 μM CP, 1.5 mM Ca(II), ±30 μM Mn(II), 1 μM proteinase K, pH 7.5, 37 °C.

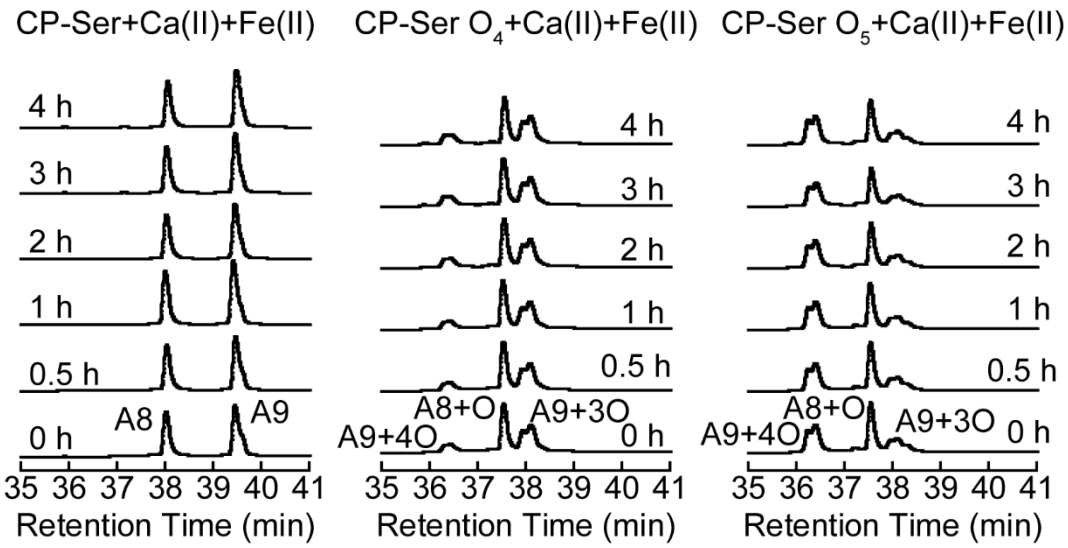


Figure S18. Digestions of CP-Ser, CP-Ser O_4 and CP-Ser O_5 by trypsin in the presence of Ca(II) and Fe(II). Conditions: 75 M HEPES, 100 mM NaCl, 30 μM CP, 1.5 mM Ca(II), 30 μM Fe(II), 0.45 μM trypsin, pH 7.5, 37 °C.

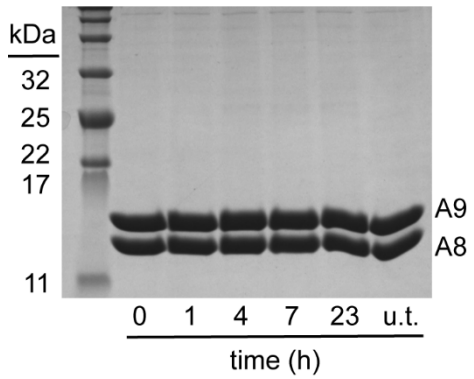
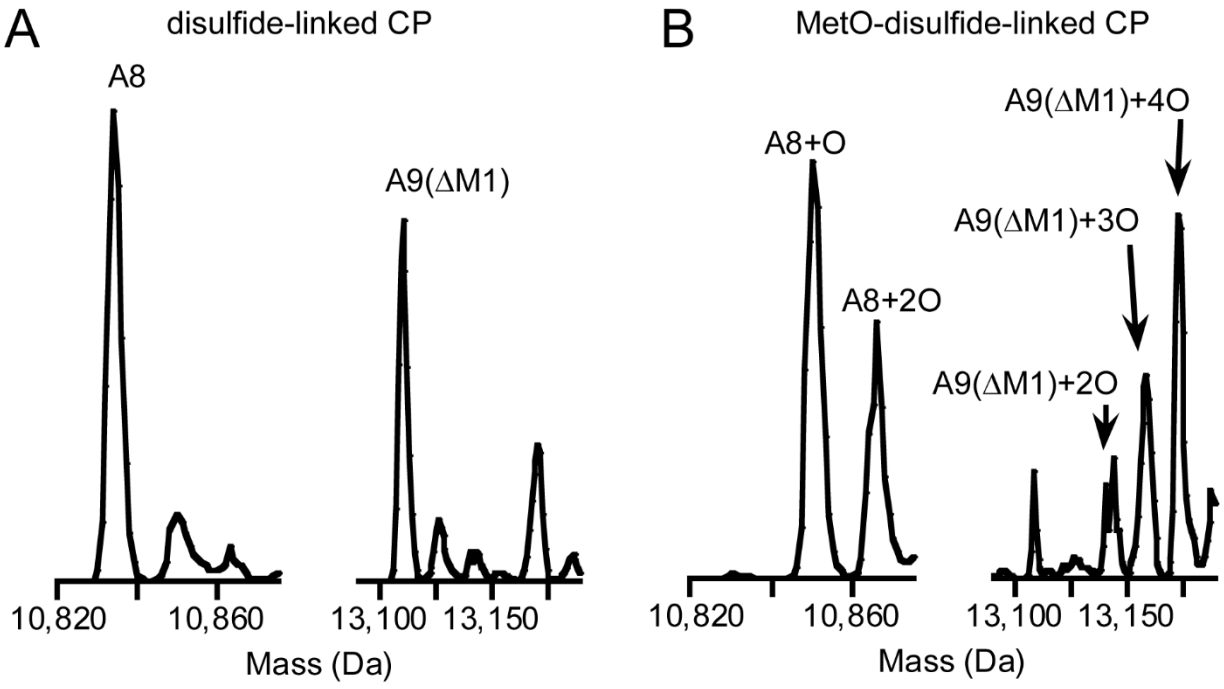


Figure S19. SDS-PAGE gel (Tris-HCl glycine, 15% acrylamide) of CP(C3S) after before and after treatment with 100 μM H_2O_2. The abbreviation u.t. stands for untreated. Reaction conditions: 75 mM HEPES, 100 mM NaCl, 30 μM CP, 1.5 mM Ca(II), 100 μM H_2O_2, pH 7.5, 37 ^{o}C. The ladder was P7712S from New England Biolabs.



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Protein	Mass
S100A8	Theoretical: 10 834.5 Da
	Observed: 10 834.4 Da
S100A8+O	Theoretical: 10 850.5 Da
	Observed: 10 850.5 Da
S100A8+2O	Theoretical: 10 866.5 Da
	Observed: 10 865.6 Da
S100A9	Theoretical: 13 110.8 Da
	Observed: 13 110.4 Da
S100A9+2O	Theoretical: 13 143.8 Da
	Observed: 13 142.1 Da
S100A9+3O	Theoretical: 13 158.8 Da
	Observed: 13 159.0 Da
S100A9+4O	Theoretical: 13 174.8 Da
	Observed: 13 174.0 Da

Figure S20. Mass spectra data from oxidation of wild-type CP. (A) Deconvoluted mass spectrum of disulfide-linked CP. (B) Deconvoluted mass spectrum of disulfide-linked MetO (C) Table of theoretical and representative observed masses of oxidized CP. Disulfide-linked CP conditions: 75 mM HEPES, 100 mM NaCl, 30 μM CP, 1.5 mM Ca(II), 100 μM H_2O_2, pH 7.5, 37 °C, 23 h. Disulfide-linked MetO CP conditions: 75 mM HEPES, 100 mM NaCl, 30 μM CP, 1.5 mM Ca(II), 100 mM H_2O_2, pH 7.5, 37 °C, 7 h. Prior to mass spectrometry analysis, the protein was combined with an equal volume of 75 mM HEPES, 100 mM NaCl, 20 mM TCEP, pH 7.5 and incubated for 10 min at ambient temperature.

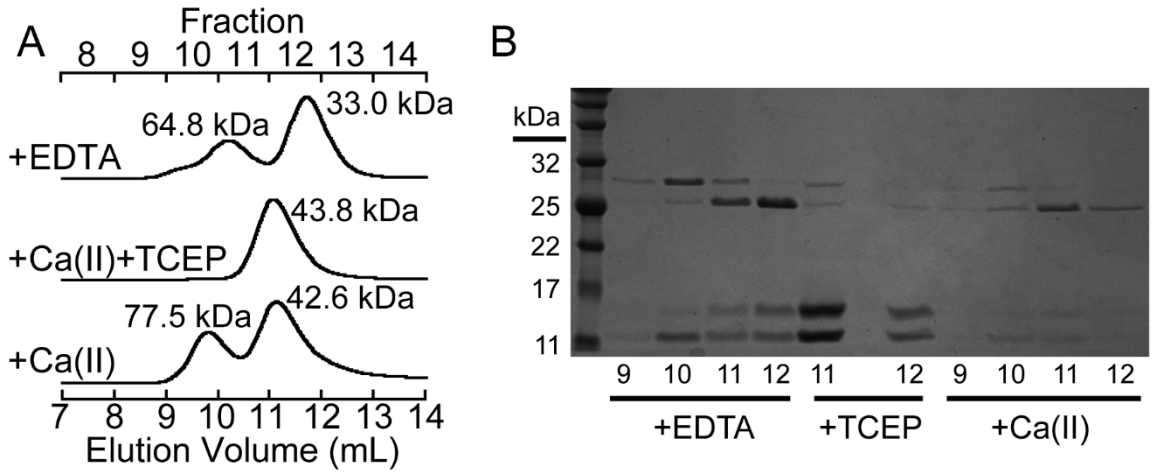

Figure S21. (A) SEC chromatograms of 30 μM CP after a disulfide-bond forming reaction with 100 μM H_2O_2 in the presence of 1.5 mM Ca(II) for 23 h at 37 °C. Prior to elution, the protein was treated with 2 mM EDTA, 2 mM TCEP, or left untreated. A 500 μL injection loop was employed; therefore, the elution volume for a given molecular weight is different than prior SEC experiments. (B) Nonreducing SDS-PAGE of the fractions collected from the SEC runs. We note that the protein reduced with TCEP contained a detectable disulfide-linked protein, indicating that the reduction was not quantitative. The S100A8–S100A9, and S100A9–S100A9 disulfide linked species had apparent molecular weights of 25 kDa and 30 kDa, respectively.

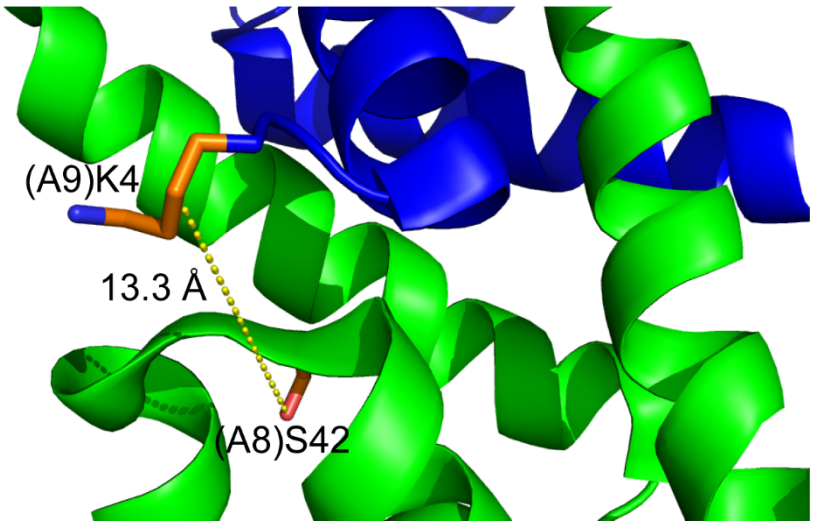

Figure S22. The N-terminus of S100A9 and Cys42 of S100A8 are in close proximity. In this crystal structure of Ca(II)-, Na(I)- and Mn(II)-bound CP-Ser (PDB:4XJK),[S2] the protein variant containing S100A8(C42S) was employed and the residues prior to Lys4 of S100A9(C3S) are disordered. The dotted line shows the 13.3-Å distance between γ-carbon of Lys4 of S100A9(C3S) and the γ-oxygen of Ser42 of S100A8(C42S).

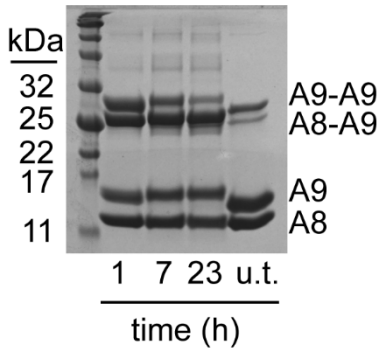

Figure S23. SDS-PAGE gel (Tris-HCl glycine, 15% acrylamide) of 30 μM disulfide-linked CP after treatment with H_2O_2. The abbreviation u.t. stands for untreated. Conditions: 75 mM HEPES, 100 mM NaCl, 30 μM disulfide-linked CP, 1.5 mM Ca(II), pH 7.5, 37 °C, 100 mM H_2O_2. Prior to this experiment, CP was treated with 100 μM H_2O_2 in the same buffer for 23 h at 37 °C. The u.t. lane contained CP that was on the benchtop for 46 h at 37 °C in the same buffer without H_2O_2. The gel was visualized with Coomassie Blue. The ladder is P7712S from New England Biolabs.

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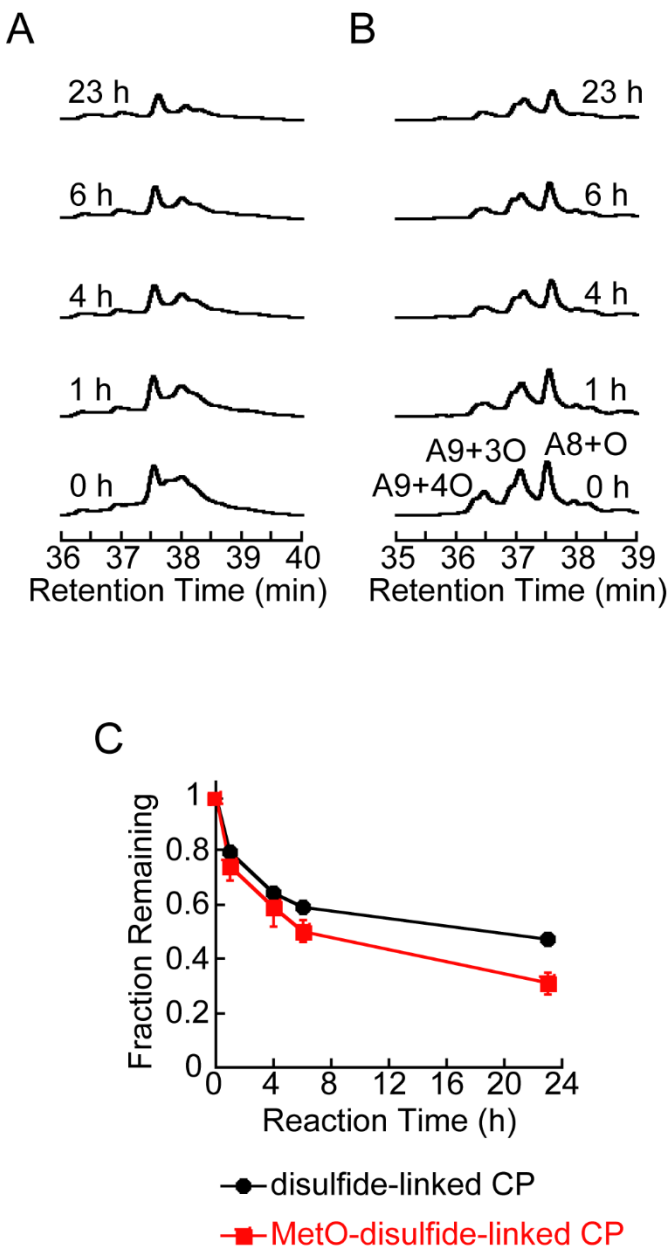

Figure S24. Representative HPLC chromatograms of MetO-disulfide-linked CP digested by trypsin Time points were quenched in the (A) absence and (B) presence of TCEP. (C) A plot of the total area in the –TCEP disulfide-linked CP and MetO-disulfide-linked CP chromatograms for the retention windows shown as a function of digestion time. The areas were normalized to the area of the 0 h chromatograms. Conditions: 75 mM HEPES, 100 mM NaCl, 1.5 mM $CaCl_2$, 30 μM CP, 0.45 μM trypsin, 37 °C.

Figure S25. Alignment of all protein sequences corresponding to genes annotated as S100A8 from the Uniprot database. Only one organism from a given genus is shown. If there were two sequences for a single organism, then the shorter sequence was used. The excluded S100A8 genes had the same amino acid at position 42. This alignment demonstrates that Cys42 (shown in red) is highly conserved. Ord's kangaroo rat, the African elephant, and the Tasmanian devil encoded Val, Gly, and Ile, respectively.

Supporting References

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