Supporting Information for

Contributions of the S100A9 C-Terminal Tail to High-Affinity Mn(II) Chelation by the Host-Defense Protein Human Calprotectin

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Experimental Section

Materials and General Methods. All solvents, reagents, and chemicals were obtained from commercial suppliers and used as received unless noted otherwise. Experimental details for the preparation of buffers for metal-binding studies are reported elsewhere.[S1] All aqueous solutions were prepared with Milli-Q water (18.2 MΩ, 0.22 μm filter). For metal-binding experiments, HEPES buffer was prepared with metal-free Ultrol grade HEPES (free acid, Calbiochem) and TraceSELECT NaCl (Sigma), and metal-free aqueous NaOH (Sigma) was used to adjust the pH. To reduce metal-ion contamination, Teflon-coated spatulas were used to transfer buffer reagents, and buffers were treated with Chelex 100 resin (Biorad, 10 g/L) by stirring in a polypropylene beaker for at least 1 h before use. The Chelex resin was removed by centrifugation or by passing the buffer through a 0.22-μm filter, and all buffers were stored in polypropylene containers. All metal-binding studies were conducted at pH 7.5 in 75 mM HEPES, 100 mM NaCl unless specified otherwise. A Tris buffer (1 mM Tris, pH 7.5) prepared from Tris base (J. T. Baker) was used for circular dichroism (CD) spectroscopy experiments requiring Mn(II) addition. This buffer was treated with Chelex resin for 1 h (10 g/L), filtered through a 0.22-μm filter, and the pH was readjusted to 7.5 with hydrochloric acid. Calcium chloride and 99.999% manganese chloride was purchased from Alfa Aesar. Stock solutions of Ca(II) (1 M) and Mn(II) (1 M) were prepared by using Milli-Q water and acid-washed volumetric glassware, and the solutions were immediately transferred to and stored in polypropylene containers. Working M(II) stocks were prepared daily by diluting the appropriate stock to the desired working concentration with buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). Zinpyr-1 (ZP1) was synthesized from 2',7'-dichlorofluorescein and di(2-picolyl)amine as described elsewhere.[S2] Stock solutions of ZP1 (1.8 mM) were prepared in anhydrous DMSO, partitioned into 50-μL aliquots, and stored at -20 ^{o}C. Each aliquot of ZP1 was freeze-thawed only once.

Electrospray Ionization Mass Spectrometry (ESI-MS). An Agilent Poroshell 300SB-C18 column housed in an Agilent 1260 series LC system with an Agilent Jetstream ESI source was employed for high-resolution mass spectrometry. Protein samples for ESI-MS were

prepared with 50 μM protein at pH 8.0 (20 mM HEPES, 100 mM NaCl). For each analysis, 5 μL of the protein sample was injected onto the Poroshell column, and the protein was eluted by using a gradient of 0–65% B over 30 min with a flow rate of 0.2 mL/min (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetronitrile). The resulting mass spectra were deconvoluted using the maximum entropy algorithm in MassHunter BioConfirm (Agilent).

Analytical Size Exclusion Chromatography. An ÄKTA purifier (GE Lifesciences) outfitted with a 500-μL sample loop was used to perform all analytical size exclusion chromatography (SEC) experiments. A Superdex 75 10/300 GL column (GE Lifesciences) was calibrated with a low-molecular-weight calibration mixture containing aprotinin (6.5 kDa, 3 mg/L), ribonuclease A (13.7 kDa, 3 mg/L), carbonic anhydrase (29 kDa, 3 mg/L), ovalbumin (44 kDa, 4 mg/L) and conalbumin (75 kDa, 3 mg/L) obtained from GE Lifesciences and buffered at pH 8.0 (20 mM HEPES, 150 mM NaCl). The calibration mixture was centrifuged briefly prior to use, and the resulting supernatant was injected onto the column. The proteins were eluted over one column volume (20 mM HEPES, 150 mM NaCl, pH 8.0). The dead volume was determined with Blue Dextran 2000 (1 mg/L) (GE Lifesciences). The partition coefficient, K_{av}, for each species was calculated by the relationship:

$$K_{av} = (V_e - V_o)/(V_c - V_o)$$

where V_e is the elution volume of the species, V_o is the dead volume, and V_c is the column volume. K_{av} was plotted against the log of the molecular weight to obtain a linear calibration curve.

For a typical SEC experiment, the protein was buffer-exchanged from the storage buffer into the running buffer if necessary. The protein concentration was adjusted to the indicated concentration, and Ca(II) or Mn(II) was added for the metal-containing samples. For the Ca(II) titrations, the running buffer was 20 mM HEPES, 100 mM NaCl at pH 8.0. For all other experiments, the running buffer was 75 mM HEPES, 100 mM NaCl at pH 7.5. Following addition of Mn(II), the samples were incubated for 0, 2 or 18 h at 4 ^{o}C. Each sample (100 or 200 μL) was loaded onto a 500-μL loop. The column was equilibrated with 1.5 column volumes of running

buffer prior to each run. The loop was emptied with 0.5 mL of running buffer, and the protein was eluted over one column volume at a flow rate of 0.5 mL/min at room temperature. The molecular weights were determined from the elution volume by using the linear relationship provided by the calibration curve.

Circular Dichroism Spectroscopy. An Aviv Model 202 circular dichroism (CD) spectrometer maintained at 25 ^{o}C was used to collect CD spectra. A 1-mm path-length quartz CD cell (Hellma) was employed for all CD measurements. Protein solutions (10 μM, 300 μL) were prepared immediately prior to data acquisition. CD spectra of the CP mutants in the presence and absence of Ca(II) were obtained for samples prepared at pH 8.5 (1 mM Tris, 0.5 mM EDTA) for comparison with literature spectra.[S1,S3] A 6-μL Ca(II) aliquot was added from a freshly-prepared 100 mM solution in buffer to provide a final concentration of 2 mM, and spectra were recorded ca. 15 min after addition. The CD spectra were recorded from 195 nm to 260 nm at 1-nm intervals. Each data point was averaged for 3 seconds before being recorded. Three independent scans were performed for each sample, and the resulting data were averaged to obtain the reported CD spectra. Thermal denaturation experiments were conducted with CP mutant samples prepared in the absence and presence of excess Ca(II) as described above. Manganese-containing samples were prepared at pH 7.5 (1 mM Tris, Chelex-treated). Thermal denaturation experiments were conducted for Mn(II)-CP samples prepared in the absence and presence of Ca(II). A 6-μL aliquot of a 100 mM Ca(II) stock solution (1 mM Tris, pH 7.5, Chelex-treated) was added to the protein to provide a final Ca(II) concentration of 2 mM. Next, a 3-μL Mn(II) aliquot from a 10 mM stock solution in buffer was added immediately before initiating the data collection. The final Mn(II) concentration was 100 μM (10 equiv/CP). For the thermal denaturation experiments, the CD signal at 222 nm was recorded (3 seconds averaging time). The temperature was raised from 25 to 95 ^{o}C in increments of 2 ^{o}C, and the sample was allowed to equilibrate for one minute prior to data collection.

Optical Absorption and Fluorescence Spectroscopy. For spectroscopic metal-binding studies, precautions were taken to minimize metal-ion contamination of buffer solutions

as described in the Materials and General Methods section and as reported previously.[S1] Optical absorption spectroscopy was performed on a Beckman Coulter DU 800 spectrophotometer thermostated at 25 ^{o}C with a Peltier temperature controller or an Agilent 8453 diode array spectrophotometer thermostated at 25 ^{o}C by a circulating water bath. Fluorescence spectra were obtained on a Photon Technologies International QuantaMaster 40 fluorimeter outfitted with a continuous xenon source for excitation, autocalibrated QuadraScopic monochromators, a multimode PMT detector, and a circulating water bath maintained at 25 ^{o}C. This instrument was controlled by the FelixGX software package, and FelixGX was routinely employed to integrate the emission spectra. Quartz cuvettes with 1-cm path lengths (Starna) were used for all optical absorption and fluorescence measurements.

Mn(II) Competition Experiments with ZP1. For competition experiments between ZP1 and the protein of interest (CP, S100A7, S100A9(C3S), S100A12, or S100B), a 2-mL solution of ZP1 (1 μM) was prepared in a quartz cuvette (75 mM HEPES, 100 mM NaCl, pH 7.5) and was allowed to equilibrate in the dark for one hour. The initial emission spectrum was recorded, and the protein was added to the solution from a freshly-thawed aliquot for a final concentration of 4 μM. The solution was allowed to equilibrate in the dark for another 20 min, and the emission spectrum was recorded again. The emission spectrum of apo ZP1 is not affected by the presence of CP/mutant $\alpha\beta$ or $\alpha_2\beta_2$ (Figures S58-S59). Hence, if the intensity of the emission spectrum varied from the initial spectrum indicating a potential metal-ion contamination, the solution was discarded. In experiments requiring calcium, Ca(II) was then added from a 100 mM working solution (75 mM HEPES, 100 mM NaCl, pH 7.5) to afford a final concentration of 200 μM (50 equiv/CP). After Ca(II) addition, the solution was incubated in the dark for 20 min, and the emission spectrum was recorded. A working solution of Mn(II) (500 μM) was prepared daily at pH 7.5 (75 mM HEPES, 100 mM NaCl). The competition experiments were conducted by titrating Mn(II) into the mixture and allowing the solution to equilibrate for 10 min in the dark after each addition prior to recording the emission spectrum. The emission spectra were recorded from 500 to 650 nm (λ_{ex} = 490 nm, 0.4 mm excitation and emission slit widths, 10 nm/sec scan

rate) and integrated over this range. The integrated emission was normalized to that of the Mn(II)-free sample. Each titration was repeated in triplicate and the resulting averages are reported.

Electron Paramagnetic Resonance Spectroscopy. Electron paramagnetic resonance (EPR) spectra (X-band, 9 GHz) were recorded on a Bruker EMX spectrometer outfitted with an ER 4199HS cavity. A flat quartz cell (Bruker Biospin), positioned in an E-field null plane within the cavity, was used for all room-temperature measurements. An ESR900 cryostat was employed for all low-temperature measurements, and the temperature was measured using a Cernox sensor. A copper-EDTA spin standard was used to account for all relevant intensity factors at low temperature. Low-temperature EPR samples were housed in 4-mm (OD) quartz EPR tubes and frozen in liquid nitrogen prior to spectral acquisition. All spectra were analyzed with the SpinCount software package developed by Michael P. Hendrich at Carnegie Mellon University.

Room-Temperature EPR Spectroscopy. Each titration was performed on a 300-μL scale with 100 μM CP. Working Mn(II) stocks were quantified by comparing the intensities of the inner four transitions of the free Mn(II) signal to that of an atomic absorption standard (J. T. Baker). Mn(II) was added to the protein solution from a working stock solution, and the sample was incubated for 5 min following each Mn(II) addition. All spectra were recorded with a power of 2 mW. The concentration of free Mn(II) was determined by comparing the intensities of the inner four transitions to those of an atomic absorption standard. For titrations performed with CP-Ser-AAA and CP-Ser-Δ101, only the peak intensities varied with each Mn(II) addition. Addition of substoichiometric Mn(II) to a sample of S100A7 resulted in distortion of the free Mn(II) signal, and further analysis was not performed.

Antimicrobial Activity Assays. The growth inhibitory activity of CP-Ser, metal-binding site mutants, and C-terminal tail mutants was monitored by using a modified literature protocol.[S1] *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were grown from single colonies with shaking (30 or 37 ^{o}C, 16-20 h) in 5 mL of TSB with or without 0.25%

(w/v) dextrose, respectively. The overnight bacterial culture was diluted 1:100 into 5 mL of fresh TSB and grown with shaking (37 ^{o}C, ~2 h) until the OD_{600} reached 0.6. The culture was diluted by 1:56 into fresh antimicrobial assay (AMA) media prepared from a 62:38 ratio of AMA buffer (20 mM Tris-HCl, 100 mM NaCl, 5 mM BME, 3 mM $CaCl_2$, pH 7.5) and TSB. Protein aliquots were thawed at room temperature and buffer exchanged into AMA buffer three times using 10-kDa molecular weight cutoff 0.5-mL Amicon microcentrifuge tubes (Millipore) that were pre-sterilized by UV irradiation. The protein concentrations were determined by absorbance at 280 nm, and 1.1x protein stocks were prepared in AMA media (1.1 mg/mL to 68.75 μg/mL). To test the effect of Mn(II) supplementation on the antimicrobial activity of CP-Ser and ΔHis_3Asp, 1.0 equiv of Mn(II) was added to each 1.1x protein stock immediately prior to use. The antimicrobial activity assays were performed in sterile polystyrene 96-well plates (Corning). Each well contained 10 μL of the 1:56 diluted bacterial culture and 90 μL of the 1.1x protein stock solution, buffer-only control, or buffer containing 42 μM Mn(II) control. Each condition was set up in duplicate. Each plate was sealed with parafilm and incubated at 30 ^{o}C with shaking at 150 rpm in a tabletop incubator-shaker housing a beaker of water. Bacterial growth was monitored by OD_{600} values, which were measured at varying time points (0 to 20 h) by using a plate reader (BioTek). Three independent replicates were conducted for each species on different days. For these replicates, three different single colonies and at least two different media preparations were employed. The resulting averages and standard errors of the mean are reported (n = 6).

Metal-Ion Analysis. Analyses were performed by staff at the microanalysis laboratory at the University of Illinois at Urbana-Champaign**.** Prior to analysis, 1.5 mL of ca. 500 μM CP-Ser, S100A12, S100B, S100A9(C3S), and S100A7 were buffer exchanged into 20 mM HEPES adjusted to pH 8.0 prepared from metal-free Ultrol-grade HEPES. The samples were stored at -80 ^{o}C prior to shipment on dry ice. For determination of metal-ion content in the antimicrobial assay buffer (*vide supra*), two independent samples were prepared and analyzed. Metal concentrations were quantified by inductively-coupled plasma (ICP) mass spectrometry (Mn, Co, Ni, Cu, and Zn) or ICP optical emission spectroscopy (Ca, Mg, and Fe).

Site-Directed Mutagenesis. A modified Quick-Change site-directed mutagenesis protocol was employed to generate the mutants (Table S3-S4). PCR amplification was conducted using PfuTurbo DNA polymerase. For the AHA(K106H), AAE, and AAA(L109H)(E111H) mutant plasmids, the PCR mix contained 5% DMSO. For the AAA(K106H) mutant plasmid, the PCR mix contained 10% DMSO. The annealing temperature of the PCR protocol for each mutant was modified based on the melting temperature of the primers determined by OligoAnalyzer 3.1 (Integrated DNA Technologies). For the AHA(K106H) mutant, the PCR protocol was: 95 ^{o}C for 30 sec; 95 ^{o}C for 30 sec, 64 ^{o}C for 1 min, 68 ^{o}C for 17 min (25x); and 4 ^{o}C hold. For the AAA(K106H) and AAE mutants, the PCR protocol was: 95 ^{o}C for 30 sec; 95 ^{o}C for 30 sec, 63 ^{o}C for 1 min, 68 ^{o}C for 17 min (25x); and 4 ^{o}C hold. For the AAA(L109H)(E111H) mutant, the PCR protocol was: 95 ^{o}C for 30 sec; 95 ^{o}C for 30 sec, 64 ^{o}C for 1 min, 68 ^{o}C for 17 min (25x); and 4 ^{o}C hold. Following PCR amplification, the template plasmid was digested with *Dpn*I (New England Biolabs) by adding two 1-μL aliquots of the restriction enzyme to a 25-μL PCR reaction at t = 0 and 1.5 h and incubating for 3 h at 37 ^{o}C. The digestion products were transformed into chemically-competent *E. coli* TOP10 cells. Overnight cultures (5 mL, 50 μg/mL kanamycin) were grown from single colonies, and the purified plasmids were isolated by using a miniprep kit (Qiagen). The DNA sequences and the presence of the desired mutations were verified by DNA sequencing.

Preparation of Mutant CP. Mutants of human calprotectin were purified as described previously.[S1] Protein yields ranged from ca. 4 mg (Δ101) to ca. 40 mg (others) per L of culture. The Δ101 mutant was purified multiple times over the course of these investigations, and other CP-Ser mutants were overexpressed and purified once. The chimeric protein CP-Ser-mT, which consists of human A9 residues 1-95 followed by mouse A9 residues 97-113, was lysed in BME-containing buffer and dialyzed into 20 mM HEPES, pH 8.0 without a reducing agent. The purified protein was found to contain a BME-adduct of S100A9 resulting from disulfide bond formation between BME and a cysteine at position 110 (mT chimera numbering). Prior to use in select metal-binding studies, CP-Ser-mT was reduced with TCEP and buffer-exchanged into a

buffer that had been purged with nitrogen or argon. Briefly, a 50-μL aliquot of CP-Ser-mT (ca. 600 μM) was thawed on ice and diluted to 500 μL with metal-binding buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). TCEP was added to a final concentration of 1 mM, and the protein was incubated on ice for 30 min. The protein was buffer exchanged into nitrogen- or argon-purged buffer, and the identity of reduced CP-Ser-mT was verified by ESI-MS (Table S5).

Cloning, Overexpression, and Purification of Human S100A7 Homodimer. The codon-optimized gene for *Nde*I-S100A7-stop-*Xho*I was obtained in the pJ201 vector from DNA 2.0. The gene was amplified by PCR, digested with *Nde*I/*Xho*I, ligated into the pET-41a vector (Invitrogen) to afford pET41a-*S100A7*, and transformed into chemically-competent *E. coli* TOP10 cells. The identity of the miniprepped plasmid was verified by DNA sequencing and transformed into chemically-competent *E. coli* BL21(DE3) cells for subsequent overexpression and purification.

For overexpression of S100A7 in *E. coli*, overnight cultures (50 mL of LB with 50 μg/mL kanamycin) were grown to saturation at 37 ^{o}C (175 rpm, t ~ 16 h). The following day, 1 L of fresh LB (with kanamycin) was inoculated with 10 mL of the overnight culture. The culture was incubated with shaking (37 ^{o}C, 175 rpm) until the OD_{600} reached ca. 0.6. Overexpression was induced with 500 μM of IPTG, and the temperature of the incubator was lowered to 25 ^{o}C. The cells were harvested the following day (t ~ 20 h post-induction) by centrifugation (3 500 rpm, 20 min, 4 ^{o}C), and cell pellets were transferred to sterile 50-mL centrifuge tubes, flash frozen in liquid nitrogen, and stored at -80 ^{o}C for up to 4 months. The typical cell pellet wet weight was 6.0 g/L.

All purification steps were conducted on ice or in a cold room at 4 ^{o}C. A cell pellet from 1 L of culture was thawed on ice, resuspended in 100 mL of Lysis Buffer A (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 0.5% Triton X-100), and sonicated on ice (40% amplitude, 30 sec on, 10 sec off for 2.5 min). The cell lysate was clarified by centrifugation (14 000 rpm, 20 min, 4 ^{o}C). The soluble fraction of the cell lysate contained aggregates of S100A7; thus, a denaturing protocol was utilized to obtain the properly folded homodimer. The

supernatant was collected, and ammonium sulfate was slowly added at 4 ^{o}C to 60% saturation. The solution was stirred at 4 ^{o}C for 1 h, and a precipitate formed. The mixture was clarified by centrifugation (14 000 rpm, 20 min, 4 ^{o}C). The supernatant was transferred to a glass beaker, and ammonium sulfate was slowly added with gentle stirring to 90% saturation. S100A7 precipitated at this concentration of ammonium sulfate. After stirring the mixture at 4 ^{o}C for 1 h, the solution was clarified by centrifugation (14 000 rpm, 20 min, 4 ^{o}C). The supernatant was discarded, and the soft, white pellet was resuspended with Lysis Buffer B (50 mM Tris pH 8.0, 100 mM NaCl, 4 M GuHCl, 5 mM DTT). The protein-containing solution was transferred to a dialysis bag (SpectraPor, 3 500 molecular-weight cut-off) and dialyzed against 20 mM HEPES, 10 mM BME, pH 8.0 (3 x 4 L, > 12 h per dialysis) to refold S100A7 to the homodimer.

S100A7 was purified by successive anion exchange and size exclusion chromatographies. The dialysate was centrifuged to remove precipitated proteins, and the supernatant was passed through a 0.45-μm filter. The protein was loaded onto a pre-equilibrated MonoQ 10/100 GL column via a 150-mL Superloop (GE Lifesciences). S100A7 was eluted with a gradient of 0-10% (Buffer A: 20 mM HEPES, 5 mM DTT, pH 8.0; Buffer B: 20 mM HEPES, 5 mM DTT, 1 M NaCl, pH 8.0) over 15 column volumes. Fractions were analyzed for S100A7 by SDS-PAGE (15% glycine gel), and the S100A7 containing fractions were pooled, concentrated to ~ 10 mL, and loaded onto a HiLoad 26/600 Superdex S75 column which had been pre-equilibrated with 20 mM HEPES, 100 mM NaCl, pH 8.0. The protein was eluted over 1 column volume. The fractions containing S100A7 were pooled, and the protein was dialyzed against 20 mM HEPES, 100 mM NaCl, pH 8.0 with 10 g/L Chelex resin, concentrated to ca. 500 μM, partitioned into 50-μL aliquots, and flash-frozen in liquid nitrogen. The yield for S100A7 ranged from 20 mg/1L to 40 mg/1L of *E. coli* culture. This procedure yields S100A7 with a Cys47—Cys96 disulfide bond in each subunit.

Cloning, Overexpression, and Purification of Human S100A9(C3S) Homodimer. The pET-41a plasmid containing the S100A9(C3S) gene was generated as described previously.[S1] Overnight cultures were prepared by inoculating 50 mL of LB containing 50 μg/mL

kanamycin from -80 °C freezer stocks of *E. coli* BL21(DE3) cells containing the pET41a-*S100A9(C3S)* plasmid. The cultures were grown to saturation by incubating at 37 °C with shaking (175 rpm, ~ 16 h). The following day, 1 L of fresh LB (with 50 μg/mL kanamycin) was inoculated with 10 mL of the overnight culture. The culture was incubated at 37 °C with shaking (175 rpm). Once the cells had grown to OD_{600} ~ 0.6, overexpression was induced with 500 μM IPTG, and the cells were incubated with shaking for an additional 3 h at 37 °C. The cells were harvested by centrifugation (3 500 rpm, 20 min, 4 °C), transferred to sterile 50-mL polypropylene tubes, and flash frozen in liquid nitrogen. Cell pellets were stored at -80 °C until purification of S100A9(C3S). The typical cell pellet wet weight was 3.0 g/L.

The S100A9(C3S) homodimer was purified using a denaturing protocol. All purification steps were performed on ice or in a cold room at 4 °C. Cell pellets containing S100A9(C3S) were thawed on ice, and the pellet was suspended with 30 mL of Lysis Buffer C (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, pH 8.0). PMSF was added to a final concentration of 1 mM. The suspension was transferred to a steel beaker on ice, and the cells were sonicated for 2.5 min (30 sec on, 10 sec off, 40% amplitude). The mixture was then clarified by centrifugation (14 000 rpm, 20 min, 4 °C). The supernatant was discarded, and the pellet was resuspended in Lysis Buffer C, sonicated, and centrifuged. This sequence was repeated once more. Following the third centrifugation step, the pellet was resuspended with 60 mL of Lysis Buffer D (50 mM Tris, 100 mM NaCl, 4 M GuHCl, pH 8.0), sonicated on ice for 5 min (40% amplitude, 30 sec on, 10 sec off). The mixture was centrifuged for 20 min (14 000 rpm, 4 °C), the supernatant was transferred to a dialysis bag (SpectraPor, 3 500 MWCO), and dialyzed against 20 mM HEPES, pH 8.0 (3 x 4 L, > 12 h) to obtain the S100A9(C3S) homodimer.

S100A9(C3S) was purified by anion exchange and size exclusion chromatographies. The dialysate was transferred to 50-mL polypropylene tubes, clarified by centrifugation (3 500 rpm, 30 min, 4 °C), and the supernatant was passed through a 0.45-μm filter. The protein-containing solution was loaded onto a pre-equilibrated MonoQ 10/100 GL column (GE

Lifesciences) via a 150-mL Superloop (GE Lifesciences) and S100A9(C3S) was eluted with a gradient of 0-30% B over 15 column volumes (Buffer A: 20 mM HEPES, pH 8.0; Buffer B: 20 mM HEPES, 1 M NaCl, pH 8.0). Fractions containing S100A9(C3S) were pooled, concentrated to ca. 10 mL, and loaded onto a HiLoad 26/600 Superdex S75 column (GE Lifesciences) pre-equilibrated at pH 8.0 (20 mM HEPES, 100 mM NaCl). The protein was eluted over 1 column volume. S100A9(C3S)-containing fractions were pooled, dialyzed against 20 mM HEPES, 100 mM NaCl, pH 8.0 with 10 g/L Chelex resin, concentrated to ca. 500 μM, partitioned into 50-μL aliquots, flash frozen in liquid nitrogen, and stored at -80 ^{o}C. The yield for S100A9(C3S) ranged from 40 mg/L to 105 mg/L of *E. coli* culture.

Overexpression and Purification of Human S100A12 Homodimer. The pET41a-*S100A12* plasmid was obtained from DNA 2.0 and transformed into chemically-competent *E. coli* TOP10 cells. Several single colonies were selected and used to inoculate 5 mL of LB media containing 50 μg/mL kanamycin. The cultures were grown at 37 ^{o}C to saturation, the plasmids were purified by using a miniprep kit (Qiagen), and the identity of the S100A12 gene was confirmed by DNA sequencing. The plasmid was then transformed into chemically-competent *E. coli* BL21(DE3) cells. Cultures from single colonies were grown to saturation in LB media containing 50 μg/mL kanamycin (37 ^{o}C, 175 rpm, t ~ 16 h), and freezer stocks were prepared by diluting aliquots of the overnight cultures with an equal volume of 1:1 water/glycerol (sterile), which were subsequently flash frozen and stored at -80 ^{o}C.

Overnight cultures of *E. coli* BL21(DE3) containing pET41a-*S100A12* were grown to saturation in 40 mL of LB media with 50 μg/mL kanamycin (37 °C, 175 rpm, t ~ 16 h). Overnight cultures were diluted 1:100 into 200 mL of fresh LB media with 50 μg/mL kanamycin, incubated at 37 ^{o}C with shaking at 175 rpm, and induced with 250 μM IPTG at OD_{600} ~ 0.6. The cultures were incubated at 37 ^{o}C for an additional 3-4 h (OD_{600} ~ 1.7), and pelleted by centrifugation (3 500 rpm x 20 min, 4 ^{o}C). The cell pellet were resuspended in 40 mL of 50 mM Tris pH 8.0, transferred to pre-weighed 50-mL polypropylene centrifuge tubes, pelleted once more by

centrifugation (3 500 rpm x 20 min, 4 ^{o}C), flash frozen in liquid N_2, and stored at -80 °C for up to two months. The typical cell pellet wet weight was 0.8 g per 200 mL of *E. coli* culture.

For the purification of S100A12, all buffers were stored and maintained at 4 ^{o}C, and all purification steps were conducted on ice or in a 4 ^{o}C cold room. A single cell pellet obtained from a 200-mL culture of S100A12 was resuspended in ca. 20 mL of Lysis Buffer C (50 mM Tris, 100 mM NaCl, 1.0 mM EDTA, 0.5% Triton X-100, pH 8.0) and a 200-μL aliquot of 100 mM PMSF was added to provide a final concentration of 1 mM. The presence of Triton X-100 in the lysis buffer was necessary to improve the solubility of S100A12. The resuspended cell pellet was transferred to an ice-cold stainless steel beaker, and the mixture was sonicated on ice (30 sec on, 10 sec off for 2.5 min; 50% amplitude). The crude lysate was clarified by centrifugation (14 000 rpm x 20 min, 4 °C), and the supernatant was transferred to a beaker on ice. In order to isolate S100A12 from the insoluble fraction, the resulting cell pellet was resuspended in ca. 20 mL of Lysis Buffer C supplemented with 1 mM PMSF. The sonication and centrifugation steps were repeated. The resulting supernatant was combined with the supernatant from the previous centrifugation, and the pellet was discarded. The combined supernatant was transferred to a SpectraPor 3 500 MWCO dialysis bag and dialyzed against 20 mM HEPES pH 8.0 (2 x 4 L, 12-24 h each, 4 °C) prior to further purification.

The content of the dialysis bag was clear of any visible precipitate. The dialysate was passed through a 0.45-μm syringe filter and loaded onto a 150-mL Superloop (GE Lifesciences). Chromatographic purification of S100A12 was conducted by using an ÄKTA Purifier FPLC system (GE Lifesciences) housed in a 4 ^{o}C room. Crude S100A12 was first purified by anion exchange chromatography by using a MonoQ 10/100 GL column. Dimeric S100A12 was isolated by utilizing a two-step protocol in which the protein was first eluted with 10% B over 8 column volumes, followed by 25% B over 7 column volumes (Buffer A, 20 mM HEPES, pH 8.0; Buffer B, 20 mM HEPES, 1 M NaCl, pH 8.0). The S100A12 dimer eluted with 10% B, whereas aggregated S100A12 eluted during the 25% B step.

Fractions containing S100A12 (as determined by SDS-PAGE, 13% Tris-HCl tricine gel) were combined, concentrated to ~10 mL, and purified by gel filtration chromatography (20 mM HEPES, 100 mM NaCl, pH 8.0) using a HiLoad 26/600 Superdex S75 column (GE Lifesciences). Fractions containing pure S100A12 were combined, transferred to a SpectraPor 3 500 MWCO dialysis bag, and dialyzed against 1 L of 20 mM HEPES, 100 mM NaCl, pH 8.0 containing ~10 g Chelex resin (Biorad) at 4 ^{o}C for ~12 h. The dialyzed protein was passed through a 0.45-μm syringe filter to avoid any contaminating Chelex, concentrated to ca. 500 μM in an Amicon 10-kDa molecular weight cut-off spin filter (3 700 rpm, 4 ^{o}C), aliquoted into sterile microcentrifuge tubes, flash frozen in liquid N_2, and stored at -80 ^{o}C. This procedure was repeated in triplicate, and the yield for S100A12 ranged from 13.6 mg/200 mL to 25 mg/200 mL of *E. coli* culture.

Cloning, Overexpression, and Purification of Human S100B Homodimer. The synthetic gene containing the *E. coli* optimized nucleotide sequence for human S100B was obtained in the pJ201-*S100B* vector from DNA 2.0. The plasmid was dissolved in 20 μL of Milli-Q water and transformed into chemically competent *E. coli* TOP10 cells. Overnight cultures were grown from single colonies, and the purified plasmids were isolated by using a miniprep kit (Qiagen) and verified by DNA sequencing. The S100B gene was subcloned into the *Nde*I and *Xho*I sites of pET-41a to afford pET41a-*S100B*. The S100B gene was digested with *Nde*I and *Xho*I (New England Biolabs), ligated into *Nde*I/*Xho*I-digested pET41a by using T4 DNA ligase (New England Biolabs), and transformed into chemically-competent *E. coli* TOP10 cells. Overnight cultures were grown from single colonies, and the purified pET41a-*S100B* plasmid was isolated using a miniprep kit and verified by DNA sequencing.

The pET41a-*S100B* expression plasmids were transformed into chemically-competent *E. coli* BL21(DE3) cells. Overnight cultures were grown from single colonies in LB media containing 50 μg/mL kanamycin to saturation (37 ^{o}C, 175 rpm, t ~ 16 h). Freezer stocks were prepared by diluting the cultures with an equal volume of filter-sterilized 50% (v/v) glycerol, flash-frozen in liquid nitrogen, and stored at -80 ^{o}C.

For protein overexpression, overnight cultures were grown from the freezer stocks in LB media containing 50 μg/mL kanamycin to saturation (37 °C, 175 rpm, t ~ 16 h). The culture was diluted 1:100 into fresh LB media containing 50 μg/mL kanamycin, incubated at 25 °C shaking at 175 rpm, and induced with 500 μM IPTG at OD_{600} ~ 0.6. The cultures were incubated at 37 °C for an additional 3-4 h to an OD_{600} ~ 1.5, and pelleted by centrifugation (3000 rpm x 15 min, 4ºC). These cultures were grown in 1-L portions in 2-L baffled flasks and stored as 1-L cell pellets. Approximately 2-3 g of *E. coli* cells were obtained from each overexpression, and the cell pellets were stored at -80 °C.

The purification of homodimeric S100B was performed on ice or in a 4 °C room, and all buffers were stored and maintained at 4 °C. In a typical purification, a single 1-L cell pellet of S100B was resuspended in ca. 70 mL of Lysis Buffer A (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 0.5% Triton-X, pH 8.0). Resuspended S100B was lysed by sonication (30 sec on, 10 sec off for 2.5 min; 40% amplitude) in an ice-cold stainless steel beaker, and the crude lysate was clarified by centrifugation (14 000 rpm x 20 min, 4 °C). The supernatant containing soluble S100B was decanted into a clean beaker on ice. The resuspension, sonication, and centrifugation steps were repeated once using the resulting cell pellet. An ammonium sulfate precipitation protocol was employed to separate the majority of contaminating *E. coli* proteins from S100B. Solid ammonium sulfate was slowly added to the combined supernatant (ca. 140 mL) with gentle stirring at 4 °C to a final concentration of 100%, causing formation of a precipitate. S100B is soluble at 100% ammonium sulfate, and the insoluble protein was pelleted by centrifugation (14 000 rpm x 20 min, 4 °C). To remove low-density insoluble protein suspended in the supernatant, the supernatant was passed through filter paper. The flow-through (ca. 170 mL) containing S100B was dialyzed against 20 mM HEPES, 1 mM DTT, pH 8.0 (3 x 4 L, ~12 h per dialysis, 4 °C) in a Spectropor3 3500 MWCO dialysis bag. The dialysate was passed through a 0.45-μm syringe filter. The resulting solution containing S100B was loaded onto a 150-mL Superloop (GE Lifesciences), and the purification was performed by using an ÄKTA Purifier FPLC system (GE Lifesciences) housed in a 4 °C

room. Crude S100B was first purified by anion exchange chromatography using a MonoQ 10/100 GL column over three runs. The FPLC protocol was: 0% B for 2 column volumes (CV), 15% B for 5 CV, a gradient of 15-40% B over 2 CV, and 40% B for 2 CV (Buffer A, 20 mM HEPES, 5 mM DTT, pH 8.0; Buffer B, 20 mM HEPES, 1 M NaCl, 5 mM DTT, pH 8.0). This step-wise protocol allowed for the separation of dimeric S100B from soluble *E. coli* proteins and higher-order oligomeric S100B. Fractions containing dimeric S100B (analyzed by SDS-PAGE) were collected, concentrated to ~10 mL, and purified by size exclusion chromatography (20 mM HEPES, 100 mM NaCl, 5 mM DTT, pH 8.0) using a HiLoad 26/600 Superdex S75 column (GE Lifesciences). Fractions containing purified dimeric S100B were collected and dialyzed against 1 L of 20 mM HEPES, 5 mM DTT, 100 mM NaCl, pH 8.0 containing ~10 g Chelex resin (Biorad) at 4 ^{o}C for ~12 h in a Spectropor3 3500 MWCO dialysis bag. The dialysate was passed through a 0.45-μm syringe filter to remove any contaminating Chelex, and the purified protein was concentrated using an Amicon 10-kDa MWCO spin filter (3 750 rpm, 4 ^{o}C) to >500 μM, partitioned into sterile microcentrifuge tubes in 50-μL aliquots, flash frozen in liquid nitrogen, and stored at -80 ^{o}C. The yield for wild-type S100B was ~30 mg/L of culture. The protein was freeze-thawed only once, and prior to an experiment, the protein was thawed on ice and, if necessary, buffer exchanged using an Amicon 10-kDa MWCO spin filter (3 750 rpm, 4 ^{o}C).

Design of the Synthetic Gene for S100A9(C3S)(H103A)(H104A)(H105A)

A synthetic gene for human S100A9(C3S)(H103A)(H104A)(H105A) was ordered from DNA 2.0 and optimized for *E. coli* codon usage. The synthetic gene was designed to include a N-terminal *Nde*I restriction site (N-terminal Met residue encoded by the *Nde*I site) and a C-terminal stop codon followed by a *Xho*I restriction site. This synthetic gene was ligated into the *Nde*I and *Xho*I restriction sites of pET-41a by DNA 2.0.

NdeI-S100A9(C3S)(H103A)(H104A)(H105A)-Stop-**XhoI**

***E. coli* optimized nucleotide sequence:**
CATATGACGAGCAAAATGAGCCAACTGGAACGCAACATCGAGACTATTATCAACACTTTTC
ACCAGTACTCTGTCAAACTGGGCCATCCGGACACCCTGAATCAGGGTGAGTTCAAAGAACT
GGTGCGTAAAGACCTGCAGAATTTTCTGAAAAAGGAGAACAAAAACGAGAAGGTTATCGAG
CACATTATGGAAGATCTGGATACCAATGCCGATAAGCAACTGAGCTTCGAAGAGTTCATTA
TGCTGATGGCGCGTTTGACGTGGGCATCCCACGAAAAGATGCATGAGGGTGACGAAGGTC
CGGGTGCGGCTGCGAAGCCAGGCTTGGGTGAGGGCACCCCGTAA**CTCGAG**

Translated sequence for NdeI-S100A9(C3S)(H103A)(H104A)(H105A)-Stop-XhoI:
H M T S K M S Q L E R N I E T I I N T F H Q Y S V K L G H P D T L N Q G E F K E L V R K D
L Q N F L K K E N K N E K V I E H I M E D L D T N A D K Q L S F E E F I M L M A R L T W A
S H E K M H E G D E G P G A A A K P G L G E G T P Stop **L E**

Design of the Synthetic Gene for S100A9(C3S)(K106A)

A synthetic gene for human S100A9(C3S)(K106A) was ordered from DNA 2.0 and optimized for *E. coli* codon usage. The synthetic gene was designed to include a N-terminal *Nde*I restriction site (N-terminal Met residue encoded by the *Nde*I site) and a C-terminal stop codon followed by a *Xho*I restriction site. This synthetic gene was ligated into the *Nde*I and *Xho*I restriction sites of pET-41a by DNA 2.0.

NdeI-S100A9(C3S)(K106A)-Stop-**XhoI**

***E. coli* optimized nucleotide sequence:**
CATATGACGAGCAAAATGAGCCAACTGGAACGCAACATCGAGACTATTATCAACACTTTTC
ACCAGTACTCTGTCAAACTGGGCCATCCGGACACCCTGAATCAGGGTGAGTTCAAAGAACT
GGTGCGTAAAGACCTGCAGAATTTTCTGAAAAAGGAGAACAAAAACGAGAAGGTTATCGAG
CACATTATGGAAGATCTGGATACCAATGCCGATAAGCAACTGAGCTTCGAAGAGTTCATTA
TGCTGATGGCGCGTTTGACGTGGGCATCCCACGAAAAGATGCATGAGGGTGACGAAGGTC
CGGGTCACCATCACGCGCCAGGCTTGGGTGAGGGCACCCCGTAA**CTCGAG**

Translated sequence for NdeI-S100A9(C3S)(K106A)-Stop-XhoI:
H M T S K M S Q L E R N I E T I I N T F H Q Y S V K L G H P D T L N Q G E F K E L V R K D
L Q N F L K K E N K N E K V I E H I M E D L D T N A D K Q L S F E E F I M L M A R L T W A
S H E K M H E G D E G P G H H H A P G L G E G T P Stop **L E**

Design of the Synthetic Gene for S100A9(C3S)(E111A)

A synthetic gene for human S100A9(C3S)(E111A) was ordered from DNA 2.0 and optimized for *E. coli* codon usage. The synthetic gene was designed to include a N-terminal *Nde*I restriction site (N-terminal Met residue encoded by the *Nde*I site) and a C-terminal stop codon followed by a *Xho*I restriction site. This synthetic gene was ligated into the *Nde*I and *Xho*I restriction sites of pET-41a by DNA 2.0.

NdeI-S100A9(C3S)(E111A)-Stop-**XhoI**

***E. coli* optimized nucleotide sequence:**
CATATGACGAGCAAAATGAGCCAACTGGAACGCAACATCGAGACTATTATCAACACTTTTC
ACCAGTACTCTGTCAAACTGGGCCATCCGGACACCCTGAATCAGGGTGAGTTCAAAGAACT
GGTGCGTAAAGACCTGCAGAATTTTCTGAAAAAGGAGAACAAAAACGAGAAGGTTATCGAG
CACATTATGGAAGATCTGGATACCAATGCCGATAAGCAACTGAGCTTCGAAGAGTTCATTA
TGCTGATGGCGCGTTTGACGTGGGCATCCCACGAAAAGATGCATGAGGGTGACGAAGGTC
CGGGTCACCATCACAAGCCAGGCTTGGGTGCGGGCACCCCGTAA**CTCGAG**

Translated sequence for NdeI-S100A9(C3S)(E111A)-Stop-XhoI:
H M T S K M S Q L E R N I E T I I N T F H Q Y S V K L G H P D T L N Q G E F K E L V R K D
L Q N F L K K E N K N E K V I E H I M E D L D T N A D K Q L S F E E F I M L M A R L T W A
S H E K M H E G D E G P G H H H K P G L G A G T P Stop **L E**

Design of the Synthetic gene for S100A9(C3S)-mT

S100A9(C3S)-mT is an 112-aa chimera of the human and mouse S100A9 amino acid sequences. The N-terminal 95 residues correspond to residues 1-95 of human S100A9(C3S). The C-terminal 17 residues correspond to residues 97-113 of mouse S100A9. The mouse portion is indicated by the italics font below.

A synthetic gene for human S100A9(C3S)-mT was ordered from DNA 2.0 and optimized for *E. coli* codon usage. The synthetic gene was designed to include a N-terminal *Nde*I restriction site (N-terminal Met residue encoded by the *Nde*I site) and a C-terminal stop codon followed by a *Xho*I restriction site. This synthetic gene was ligated into the *Nde*I and *Xho*I restriction sites of pET-41a by DNA 2.0.

NdeI-S100A9(C3S)-*mT*-Stop-**XhoI**

***E. coli* optimized nucleotide sequence:**
CATATGACGAGCAAAATGAGCCAACTGGAACGTAACATCGAGACTATTATCAACACTTTTC
ACCAGTACAGCGTCAAGCTGGGCCATCCGGACACCTTGAACCAGGGTGAGTTCAAAGAGC
TGGTGCGCAAGGATCTGCAGAATTTCCTGAAGAAAGAAAACAAGAATGAGAAAGTTATTGA
ACACATTATGGAAGATCTGGACACCAATGCAGACAAACAACTGTCTTTTGAAGAGTTCATCA
TGCTGATGGCCCGTTTGACCTGGGCGAGCCACGAGAAGATGCAT*GAGAACAATCCGCGTG*
*GCCATGGTCACTCCCACGGTAAGGGTTGCGGCAAA*TAA**CTCGAG**

Translated sequence for NdeI-S100A9(C3S)-mT-Stop-XhoI:
H M T S K M S Q L E R N I E T I I N T F H Q Y S V K L G H P D T L N Q G E F K E L V R K D
L Q N F L K K E N K N E K V I E H I M E D L D T N A D K Q L S F E E F I M L M A R L T W A
S H E K M H *E N N P R G H G H S H G K G C G K* Stop **L E**

Design of the Synthetic Gene for S100A7

A synthetic gene for human S100A7 was ordered from DNA 2.0 and optimized for *E. coli* codon usage. The synthetic gene was designed to include a N-terminal *Nde*I restriction site (N-terminal Met residue encoded by the *Nde*I site) and a C-terminal stop codon followed by a *Xho*I restriction site.

NdeI-S100A7-Stop-**XhoI**

***E. coli* optimized nucleotide sequence:**

CATATGAGCAACACCCAGGCAGAACGTAGCATTATTGGTATGATTGACATGTTTCA
CAAATACACGCGCCGTGATGATAAGATCGACAAACCGTCGCTGCTGACGATGATG
AAAGAGAACTTCCCGAATTTTCTGTCTGCCTGCGATAAGAAAGGCACCAATTATCT
GGCGGACGTGTTCGAAAAGAAAGACAAAAACGAGGACAAGAAGATCGACTTTAGC
GAGTTCTTGTCCCTGCTGGGTGATATCGCGACCGATTACCATAAGCAAAGCCACG
GCGCTGCGCCGTGTAGCGGTGGTAGCCAGTAA**CTCGAG**

Translated sequence for NdeI-S10012-Stop-XhoI:

H M S N T Q A E R S I I G M I D M F H K Y T R R D D K I D K P S L L T M M K E N F
P N F L S A C D K K G T N Y L A D V F E K K D K N E D K K I D F S E F L S L L G D I
A T D Y H K Q S H G A A P C S G G S Q Stop **L E**

Design of the Synthetic Gene for S100A12

A synthetic gene for human S100A12 was ordered from DNA 2.0 and optimized for *E. coli* codon usage. The synthetic gene was designed to include a N-terminal *Nde*I restriction site (N-terminal Met residue encoded by the *Nde*I site) and a C-terminal stop codon followed by a *Xho*I restriction site. This synthetic gene was ligated into the *Nde*I and *Xho*I restriction sites of pET-41a by DNA 2.0.

NdeI-S100A12-Stop-**XhoI**

***E. coli* optimized nucleotide sequence:**
CATATGACGAAACTGGAAGAACACTTGGAAGGCATTGTTAACATTTTTCATCAATACAGCGT
GCGTAAGGGCCACTTCGACACCCTGAGCAAAGGTGAGTTGAAACAGCTGCTGACCAAAGA
GCTGGCAAATACGATCAAGAATATCAAGGATAAGGCTGTCATTGACGAGATTTTCCAGGGT
CTGGATGCCAACCAAGACGAGCAAGTTGATTTCCAGGAGTTTATCTCCCTGGTGGCGATC
GCGCTGAAGGCAGCGCACTATCATACCCACAAAGAATAA**CTCGAG**

Translated sequence for NdeI-S10012-Stop-XhoI:
H M T K L E E H L E G I V N I F H Q Y S V R K G H F D T L S K G E L K Q L L T K E L A N T I
K N I K D K A V I D E I F Q G L D A N Q D E Q V D F Q E F I S L V A I A L K A A H Y H T H K
E Stop **L E**

Design of the Synthetic Gene for S100B

A synthetic gene for human S100B was ordered from DNA 2.0 and optimized for *E. coli* codon usage. The synthetic gene was designed to include a N-terminal *Nde*I restriction site (N-terminal Met residue encoded by the *Nde*I site) and a C-terminal stop codon followed by a *Xho*I restriction site.

NdeI-S100B-STOP-**XhoI**

***E. coli* optimized nucleotide sequence:**
CATATGAGCGAACTGGAGAAAGCAATGGTAGCCCTGATCGACGTTTTTCACCAATACAGCG
GTCGTGAGGGCGATAAGCACAAGCTGAAGAAAAGCGAATTGAAAGAGCTGATCAACAACG
AGCTGTCCCATTTTCTGGAAGAGATTAAAGAGCAGGAAGTCGTGGACAAGGTCATGGAAAC
CCTGGATAATGACGGCGATGGTGAATGTGACTTCCAGGAGTTCATGGCGTTCGTGGCTAT
GGTTACGACCGCGTGCCACGAGTTTTTCGAGCATGAATAA**CTCGAG**

Translated sequence for NdeI-S100B-STOP-XhoI:
H M S E L E K A M V A L I D V F H Q Y S G R E G D K H K L K K S E L K E L I N N E L S H F
L E E I K E Q E V V D K V M E T L D N D G D G E C D F Q E F M A F V A M V T T A C H E F
F E H E Stop **L E**

Table S1. Human Calprotectin Protein Nomenclature.

Protein	**Mutations**	
	S100A8	S100A9
CP	Wild-type	Wild-type
CP-Ser	(C42S)	(C3S)
CP-Ser ΔHis_3Asp	(C42S)(H83A)(H87A)	(C3S)(H20A)(D30A)
CP-Ser ΔHis_4	(C42S)(H17A)(H27A)	(C3S)(H91A)(H95A)
CP-Ser $\Delta\Delta$	(C42S)(H17A)(H27A)(H83A)(H87A)	(C3S)(H20A)(D30A)(H91A)(H95A)
CP-Ser(E96A)	(C42S)	(C3S)(E96A)
CP-Ser(D98A)	(C42S)	(C3S)(D98A)
CP-Ser(E99A)	(C42S)	(C3S)(E99A)
CP-Ser(H103A)	(C42S)	(C3S)(H103A)
CP-Ser(H104A)	(C42S)	(C3S)(H104A)
CP-Ser(H105A)	(C42S)	(C3S)(H105A)
CP-Ser(K106A)	(C42S)	(C3S)(K106A)
CP-Ser(E111A)	(C42S)	(C3S)(E111A)
CP-Ser-AHA	(C42S)	(C3S)(H103A)(H105A)
CP-Ser-AAA	(C42S)	(C3S)(H103A)(H104A)(H105A)
CP-Ser-AAE	(C42S)	(C3S)(H103A)(H104A)(H105E)
CP-Ser-AAA(K106H)	(C42S)	(C3S)(H103A)(H104A)(H105A)(K106H)
CP-Ser-AHA(K106H)	(C42S)	(C3S)(H103A)(H105A)(K106H)
CP-Ser-AAA (L109H)(E111H)	(C42S)	(C3S)(H103A)(H104A)(H105A)(L109H)(E111H)
CP-Ser-Δ101	(C42S)	(C3S)(G102Stop)
CP-Ser-mT	(C42S)	(C3S) with murine C-terminal tail (see Table 1)

Table S2. Molecular Weights and Extinction Coefficients for CP Subunits.

Subunit	Molecular Weight (Da)[a]	ε_{280} ($M^{-1}cm^{-1}$)[b]
S100A8	10 834.5	11 460
S100A8(C42S)	10 818.4	11 460
S100A9	13 241.9	6990
S100A9(C3S)	13 225.9	6990
S100A9(C3S)(E96A)	13 167.7	6990
S100A9(C3S)(D98A)	13 181.8	6990
S100A9(C3S)(E99A)	13 167.7	6990
S100A9(C3S)(H103A)	13 159.8	6990
S100A9(C3S)(H104A)	13 159.8	6990
S100A9(C3S)(H105A)	13 159.8	6990
S100A9(C3S)(K106A)	13 168.8	6990
S100A9(C3S)(E111A)	13 167.7	6990
S100A9(C3S)(H103A)(H105A)	13 093.8	6990
S100A9(C3S)(H103A)(H104A)(H105A)	13 027.7	6990
S100A9(C3S)(H103A)(H104A)(H105E)	13 085.7	6990
S100A9(C3S)(H103A)(H104A)(H105A)(K106H)	13 036.7	6990
S100A9(C3S)(H103A)(H105A)(K106H)	13 102.7	6990
S100A9(C3S)(H103A)(H104A)(H105A)(L109H)(E111H)	13 059.7	6990
S100A9(C3S)(G101Stop)	11766.3	6990
S100A9(C3S)(mT)	13 089.8	6990

[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 S3. Primers Employed for Site-Directed Mutagenesis of the S100A9 C-Terminal Tail.[a]

Primer	Sequence
G102Stop-1	5'-GGTGATGAAGGTCCG**TAA**CACCATCACAAACCGGG-3'
G102Stop-2	5'-CCCGGTTTGTGATGGTG**TTA**CGGACCTTCATCACC-3'
E96A-1	5'-CACGAGAAAATGCATGCGGGTGATGAAGGTCC-3'
E96A-2	5'-GGACCTTCATCACCCGCATGCATTTTCTCGTG-3'
D98A-1	5'-GAGAAAATGCATGAAGGTGCGGAAGGTCCGGGCCACAT-3'
D98A-2	5'-ATGTGGCCCGGACCTTCCGCACCTTCATGCATTTTCTC-3'
E99A-1	5'-GAAAATGCATGAAGGTGATGCGGGTCCGGGCCACCATCA-3'
E99A-2	5'-TGATGGTGGCCCGGACCCGCATCACCTTCATGCATTTTC-3'
AHA(K106H)-1	5'-GGTGCTCATGCGCATCCGGGTTTGGGCGAG-3'
AHA(K106H)-2	5'-CTCGCCCAAACCCGGATGCGCATGAGCACC-3'
AAA(K106H)-1	5'-GGTGCGGCTGCGCATCCAGGCTTGGGTGAG-3'
AAA(K106H)-2	5'-CTCACCCAAGCCTGGATGCGCAGCCGCACC-3'
AAE-1	5'-GTCCGGGTGCGGCTGAAAAGCCAGGCTTGG-3'
AAE-2	5'-CCAAGCCTGGCTTTTCAGCCGCACCCGGAC-3'
(L109H)(E111H)-1	5'-CTGCGAAGCCAGGCCATGGTCATGGCACCCCG**TAA**C-3'
(L109H)(E111H)-2	5'-G**TTA**CGGGGTGCCATGACCATGGCCTGGCTTCGCAG-5'

[a] Bold codons indicate stop codons. The codons containing mutations are underlined and colored red.

Table S4. Template Plasmids and Primer Pairings Employed for Site-Directed Mutagenesis.

Template	Product	Primer Pairing
pET41a-S100A9(C3S)	pET41a-S100A9(C3S)(G102Stop)	G102Stop-1, G102Stop-2
pET41a-S100A9(C3S)	pET41a-S100A9(C3S)(E96A)	E96A-1, E96A-2
pET41a-S100A9(C3S)	pET41a-S100A9(C3S)(D98A)	D98A-1, D98A-2
pET41a-S100A9(C3S)	pET41a-S100A9(C3S)(E99A)	E99A-1, E99A-2
pET41a-S100A9(C3S) (H103A)(H105A)	pET41a-S100A9(C3S) (H103A)(H105A)(K106H)	AHA(K106H)-1, AHA(K106H)-2
pET41a-S100A9(C3S) (H103A)(H104A)(H105A)	pET41a-S100A9(C3S) (H103A)(H104A)(H105A)(K106H)	AAA(K106H)-1, AAA(K106H)-2
pET41a-S100A9(C3S) (H103A)(H104A)(H105A)	pET41a-S100A9(C3S) (H103A)(H104A)(H105E)	AAE-1, AAE-2
pET41a-S100A9(C3S) (H103A)(H104A)(H105A)	pET41a-S100A9(C3S) (H103A)(H104A)(H105A) (L109H)(E111H)	(L109H)(E111H)-1, (L109H)(E111H)-2

Table S5. Mass Spectrometric Analysis of Human CP and Mutants.[a]

Protein	S100A8 Calculated Mass (g/mol)[b]	S100A8 Observed Mass (g/mol)[c]	S100A9 Calculated Mass +/- ^{N}Met (g/mol)[d]	S100A9 Observed Mass (g/mol)
CP	10 834.5	10 834.8	13 241.9	n.f.[e]
			13 110.8 (-Met1)	13 111.0
CP-Ser	10 818.4	10 818.8	13 225.9	13 226.0
			13 094.8 (-Met1)	13 095.0
CP-Ser(E96A)	10 818.4	10 819.0	13 167.7	13 168.6
			13 036.6 (-Met1)	13 037.3
CP-Ser(D98A)	10 818.4	10 819.1	13 181.8	13 182.6
			13050.7 (-Met1)	13 051.5
CP-Ser(E99A)	10 818.4	10 819.1	13 167.7	13 168.4
			13 036.6 (-Met1)	13 037.3
CP-Ser(H103A)	10 818.4	10 819.0	13 159.8	13 160.6
			13 028.6 (-Met1)	13 029.4
CP-Ser(H104A)	10 818.4	10 819.0	13 159.8	13 160.8
			13 028.6 (-Met1)	13 029.4
CP-Ser(H105A)	10 818.4	10 819.1	13 159.8	13 160.7
			13 028.6 (-Met1)	13 029.4
CP-Ser(K106A)	10 818.4	10 818.8	13 168.8	13 169.1
			13 037.6 (-Met1)	13 038.0
CP-Ser(E111A)	10 818.4	10 818.4	13 167.8	13 168.3
			13 036.7 (-Met1)	13 037.1
CP-Ser-AHA	10 818.4	10 819.0	13 093.8	13 094.3
			12 929.6 (-Met1)	12 963.3
CP-Ser-AAA	10 818.4	10 818.6	13 027.7	13 027.1
			12 896.5 (-Met1)	12 896.8
CP-Ser-AAE	10 818.4	10 818.9	13 085.7	13 086.3
			12 954.5 (-Met1)	12 955.0
CP-Ser-AAA(K106H)	10 818.4	10 818.8	13 036.7	13 037.0
			12 905.5 (-Met1)	12 905.8
CP-Ser-AHA(K106H)	10 818.4	10 818.5	13 102.7	13 103.0
			12 971.5 (-Met1)	12 971.6
CP-Ser-AAA(L109H)(E111H)	10 818.4	10 818.9	13 059.7	13 060.0
			12 928.5 (-Met1)	12 928.9
CP-Ser-Δ101	10 818.4	10 818.8	11 920.5	n.f.[e]
			11 789.3 (-Met1)	11 789.42
CP-Ser-mT	10 818.4	10 818.8	13 089.8	13 090.2[f]
			12 958.6 (-Met1)	12 959.2
CP-Ser-mT-BME	10 818.4	10 818.8	13 165.9	13 166.2
			13 034.7 (-Met1)	13 035.1

[a] Masses were determined using a denaturing protocol on an Agilent Poroshell 300SB-C18 column over a 0–65% gradient of acetonitrile in 0.1 % formic acid. [b] Molecular weights were calculated by using the ProtParam tool available at the ExPASy server (http://web.expasy.org/protparam). [c] Masses were calculated with the Agilent MassHunter BioConfirm software package. [d] The N-terminal methionine of S100A9 is sometimes cleaved during overexpression in *E. coli*. Masses are shown for the full-length and shortened forms of S100A9. [e] Mass not found during deconvolution of the raw data. [f] Mass of CP-Ser-mT following treatment with TCEP.

Table S6. Analytical SEC Retention Volumes and Calculated Molecular Weights of S100 Proteins in the Absence and Presence of Ca(II).

Protein	Elution Volume –Ca(II) (mL)	Calculated Molecular Weight –Ca(II) (kDa)	Elution Volume +Ca(II) (mL)	Calculated Molecular Weight +Ca(II) (kDa)
CP-Ser	11.4	35.5	10.6	49.5
CP-Ser(E96A)	11.6	32.4	10.8	45.3
CP-Ser(D98A)	11.6	32.0	10.8	44.9
CP-Ser(E99A)	11.6	32.1	10.8	45.2
CP-Ser(H103A)	11.5	33.8	10.7	46.5
CP-Ser(H104A)	11.5	33.5	10.7	46.3
CP-Ser(H105A)	11.5	33.7	10.7	46.5
CP-Ser(K106A)	11.4	34.2	10.6	48.3
CP-Ser(E111A)	11.5	33.3	10.7	46.7
CP-Ser-AHA	11.5	33.3	10.7	46.7
CP-Ser-AAA	11.5	34.0	10.6	48.3
CP-Ser-AAE	11.4	34.8	10.5	49.9
CP-Ser-AAA(K106H)	11.4	34.2	10.7	47.0
CP-Ser-AHA(K106H)	11.3	35.6	10.7	47.0
CP-Ser-AAA (L109H)(E111H)	11.6	31.9	10.9	44.0
CP-Ser-Δ101	11.8	29.9	11.0	41.6
CP-Ser-mT	11.7	30.4	10.9	42.9
S100A7	12.0	27.0	12.1	26.5
S100A9(C3S)	11.2	37.4	11.3	35.6
S100A12	12.3	24.1	12.2	25.1
S100B	11.7	31.3	11.8	29.4

Table S7. Melting Temperatures (T_m) of CP-Ser and Mutants.[a]

Protein	Melting Temperature –Ca(II) (°C)	Melting Temperature –Ca(II) +Mn(II) (°C)	Melting Temperature +Ca(II) (°C)	Melting Temperature +Ca(II) +Mn(II) (°C)
CP-Ser	61	87	79	>95
CP-Ser-Δ101	59	65	79	88
CP-Ser-AAA	61	65	79	88
CPSer-AHA	n.d.	67	n.d.	88

[a] The manganese samples contained 10 equiv of Mn(II).

Table S8. Slope Analysis of Room-Temperature EPR Titrations.[a]

Sample	Slope	R^2
Buffer[b]	1.00	0.9967
CP-Ser ΔHis_4[b]	1.0452	0.9957
	0.9412	0.9627
CP-Ser-AAA	0.7738	0.9902
	0.7957	0.9894
CP-Ser-Δ101	0.7114	0.9889
	0.7432	0.9960

[a] The slopes were obtained from linear fits of the plots of $[Mn(II)_{free}]$ versus $[Mn(II)_{total}]$. The plots are provided in Figure S4. Each entry represents an independent titration. [b] Data taken from ref. S4.

Table S9. Molecular Weights and Extinction Coefficients for Human S100 Proteins. [a]

Protein	Molecular Weight (Da)[b]	ε_{280} ($M^{-1}cm^{-1}$)[c]
S100A7[d]	22 909.8	9190
S100A9(C3S)	26 451.8	13 980
S100A12	21 150.0	5960
S100B	21 163.6	2980

[a] Molecular weights and extinction coefficients are given for the dimeric forms of each protein. [b] Molecular weights were calculated by using the ProtParam tool available on the ExPASy server (http://web.expasy.org/protparam). [c] Extinction coefficients (280 nm) were calculated by using the ProtParam tool. [d] Each subunit of S100A7 has a Cys47—Cys96 disulfide bond.

Table S10. Mass Spectrometric Analysis of Human S100 Proteins.[a]

	Monomer		Dimer	
Protein	Calculated Mass (g/mol)[b]	Observed Mass (g/mol)[c]	Calculated Mass (g/mol)[b]	Observed Mass (g/mol)[c]
S100A7[d]	11 454.9	11 455.2	22 909.8	22 910.0
	11 323.7[e]	11 324.1	22 647.4	22 647.5
S100A9(C3S)	13 225.9	13 226.2	26 451.8	26 452.1
	13 094.7[e]	13 095.1	26 189.4	26 189.8
S100A12	10 575.0	10 575.5	21 150.0	21 150.4
	10 443.8[e]	10 444.1	20 887.6	20 887.7
S100B	10 713.0	10 713.1	21 426.0	21 426.3
	10 581.8[e]	10 582.0	21 163.6	21 163.4

[a] Masses were determined using a denaturing protocol on an Agilent Poroshell 300SB-C18 column over a 0–65% gradient of acetonitrile in 0.1% formic acid. [b] Molecular weights were calculated by using the ProtParam tool available at the ExPASy server (http://web.expasy.org/protparam). [c] Masses were calculated with the Agilent MassHunter BioConfirm software package. [d] Each S100A7 subunit contains a Cys47—Cys96 disulfide bond. [e] The N-terminal methionine of various S100 proteins is sometimes cleaved during overexpression in *E. coli*. The data for the –Met truncated forms are listed in the second row of each entry. The masses of the full-length and truncated proteins are given.

Table S11. Metal content of purified S100 proteins. [a,b]

Protein [c]		Ca	Mg	Mn	Fe	Co	Ni	Cu	Zn
Buffer	ppm	0.039	0.017	0.0 [d]	0.0	0.0	0.0	0.0051	0.0045
	μM	0.098	0.070	0.0	0.0	0.0	0.0	0.0080	0.0069
CP	ppm	0.71	0.06	0.004	0.0	0.003	0.021	0.013	0.044
	μM	18.0	2.5	0.06	0.0	0.06	0.36	0.20	0.67
	equiv./CP	0.041	0.006	1e-4	0.0	1e-4	0.001	5e-4	0.002
A7	ppm	9.8	0.020	0.019	0.0	0.005	0.048	0.034	0.67
	μM	240.0	0.84	0.35	0.0	0.08	0.82	0.54	10.0
	equiv./A7	0.66	0.002	0.001	0.0	2e-4	0.002	0.001	0.028
A12	ppm	0.018	0.06	0.038	0.061	0.053	0.32	0.30	0.21
	μM	0.45	2.47	0.69	1.09	0.90	5.45	4.69	3.15
	equiv./A12	8e-4	0.005	0.001	0.002	0.002	0.010	0.009	0.006
S100B	ppm	0.42	0.05	0.012	0.0	7e-4	0.035	0.014	0.49
	μM	10.0	2.1	0.22	0.0	0.01	0.60	0.22	7.5
	equiv./S100B	0.018	0.004	4e-4	0.0	2e-5	0.001	4e-4	0.013
A9(C3S)	ppm	0.33	0.042	0.053	0.029	0.021	0.48	0.44	0.25
	μM	8.3	1.7	0.97	0.52	0.36	8.1	6.9	3.8
	equiv./A9(C3S)	0.012	0.003	0.001	8e-4	5e-4	0.012	0.010	0.006

[a] Metals were quantified by inductively-coupled plasma (ICP) mass spectrometry (Mn, Co, Ni, Cu, and Zn) or ICP optical emission spectroscopy (Ca, Mg, and Fe). [b] Each sample was analyzed once. [c] Metal content is given in parts per million (ppm), concentration in μM, and the ratio of metal/protein. [d] An entry of 0.0 indicates that the metal-ion concentration was below the detection limit.

Table S12. Metal content of the antimicrobial activity assay media. [a,b]

Sample [c]		Ca	Mg	Mn	Fe	Co	Ni	Cu	Zn
1	ppm	82.3	3.76	0.007	0.25	0.002	0.015	0.048	0.23
	μM	2,400	160	0.13	4.4	0.041	0.26	0.76	3.4
2	ppm	73.7	3.57	0.007	0.23	0.002	0.014	0.048	0.20
	μM	1,800	150	0.12	4.2	0.036	0.24	0.76	3.1

[a] Metals were quantified by inductively-coupled plasma (ICP) mass spectrometry (Mn, Co, Ni, Cu, and Zn) or ICP optical emission spectroscopy (Ca, Mg, and Fe). [b] Two independent samples were analyzed. [c] Metal content is given in parts per million (ppm) and concentration in μM.

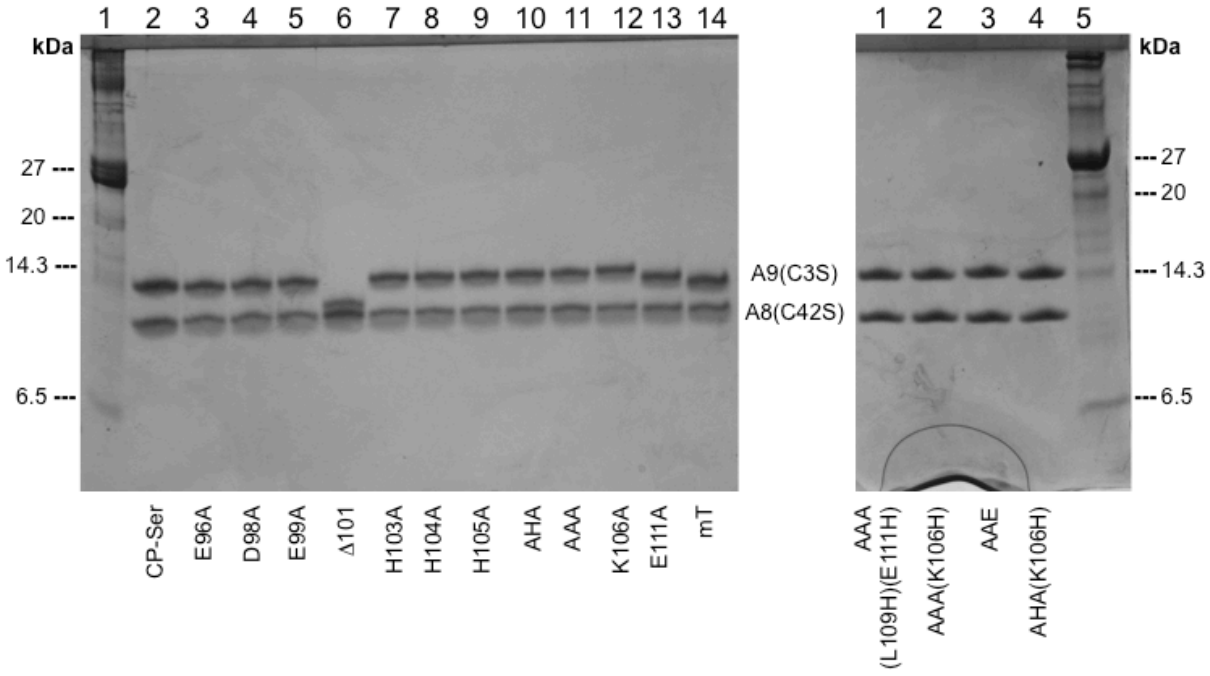


Figure S1. SDS-PAGE (13% Tris-HCl, tricine gel) of the CP mutant family utilized in this work. Left panel: P7702 broad-range protein marker (New England Biolabs, lane 1), CP-Ser (lane 2), E96A (lane 3), D98A (lane 4), E99A (lane 5), Δ101 (lane 6), H103A (lane 7), H104A (lane 8), H105A (lane 9), AHA (lane 10), AAA (lane 11), K106A (lane 12), E111A (lane 13), and mT (lane 14). Right panel: AAA(L109H)(E111H) (lane 1), AAA(K106H) (lane 2), AAE (lane 3), AHA(K106H) (lane 4), and P7702 broad-range protein marker (lane 5).

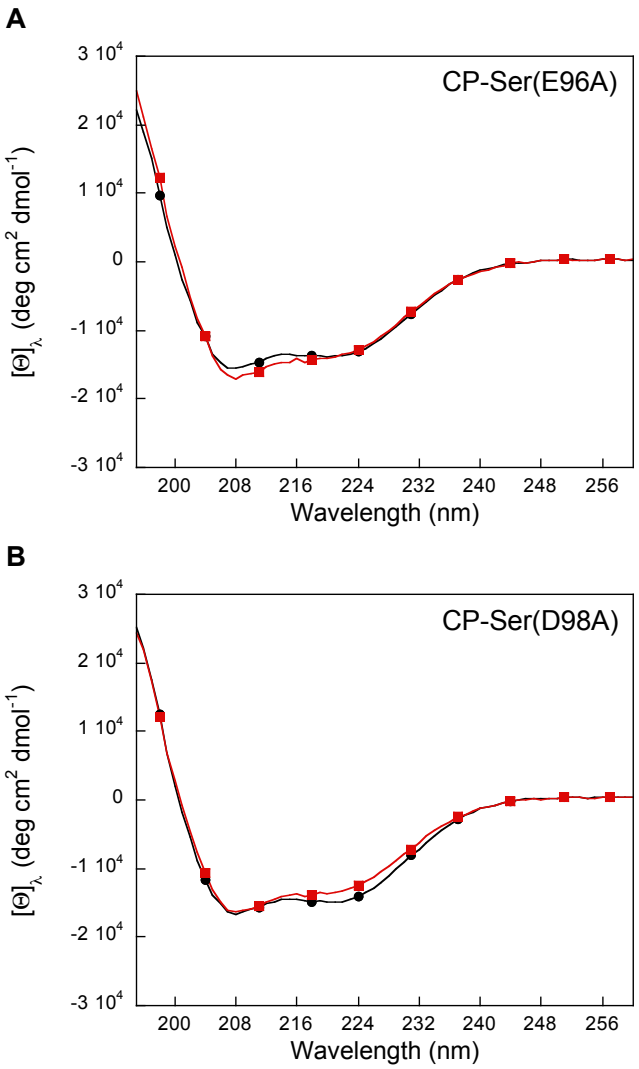


Figure S2. CD spectra of 10 μM CP-Ser(E96A) (**A**) and CP-Ser(D98A) (**B**) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 ^{o}C).

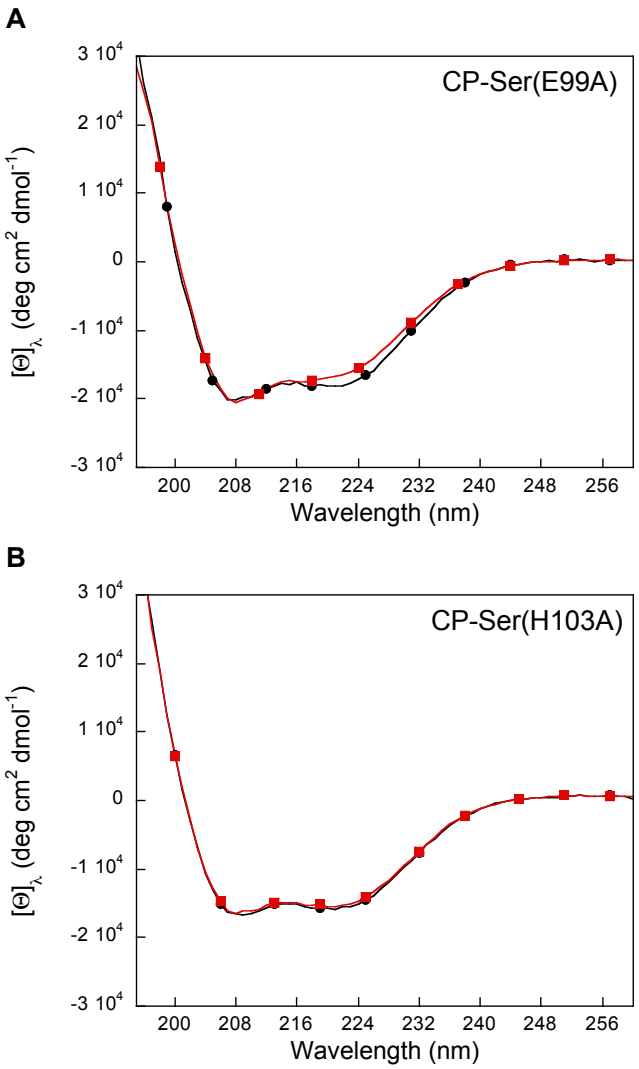


Figure S3. CD spectra of 10 μM CP-Ser(E99A) (**A**) and CP-Ser(H103A) (**B**) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 ^{o}C).

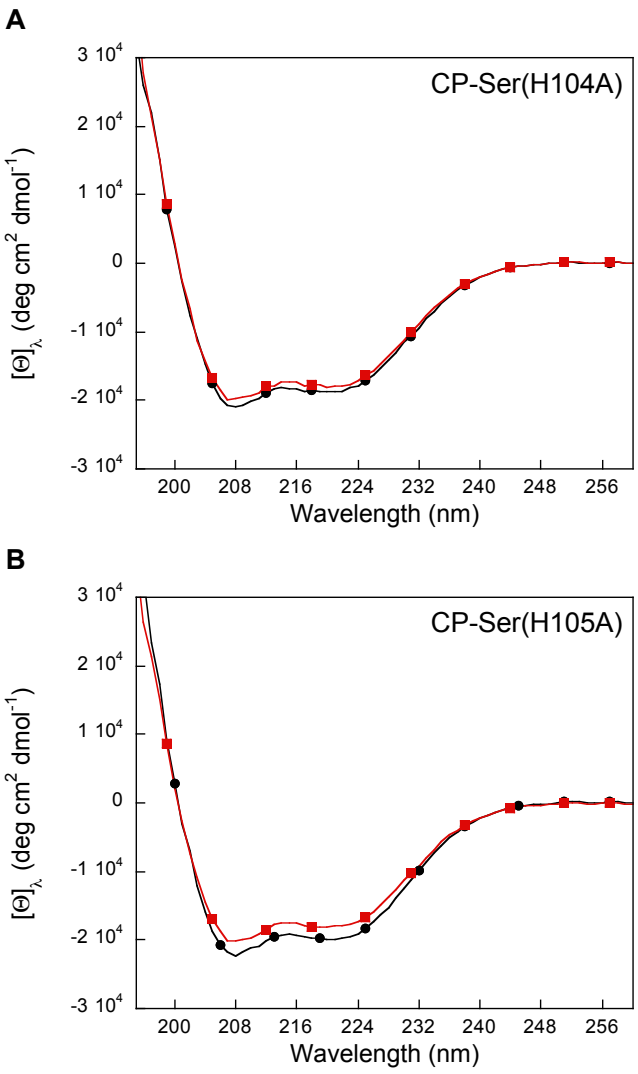


Figure S4. CD spectra of 10 μM CP-Ser(H104A) (**A**) and CP-Ser(H105A) (**B**) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 ^{o}C).

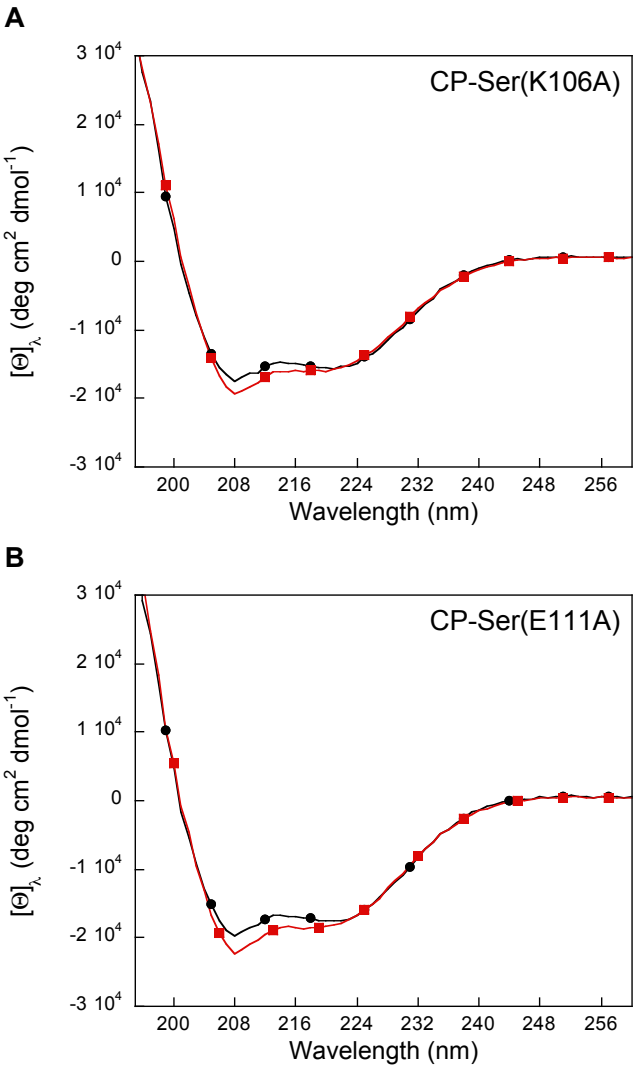


Figure S5. CD spectra of 10 μM CP-Ser(K106A) (**A**) and CP-Ser(E111A) (**B**) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 ^{o}C).

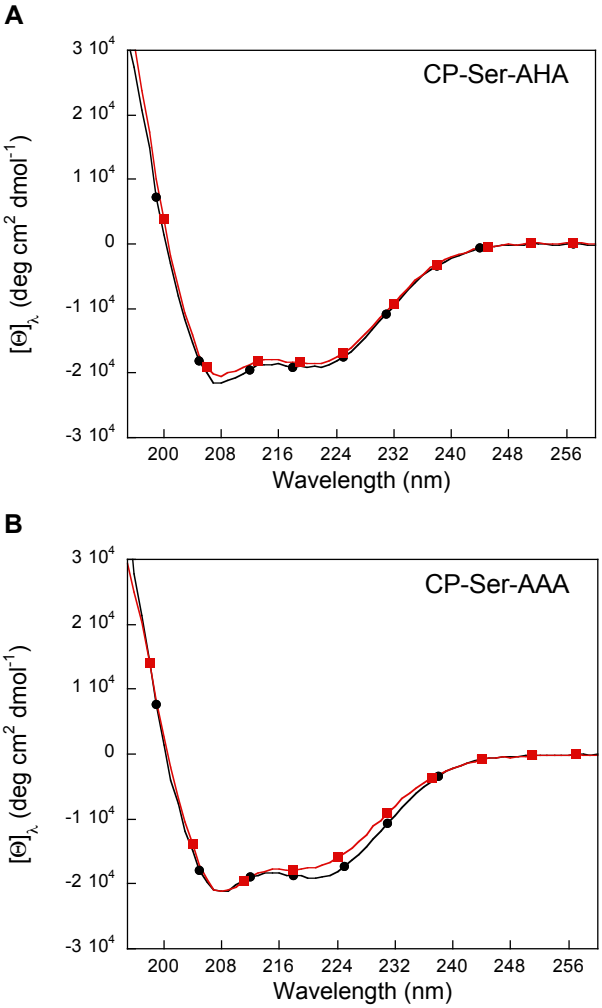


Figure S6. CD spectra of 10 μM CP-Ser-AHA (**A**) and CP-Ser-AAA (**B**) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 ^{o}C).

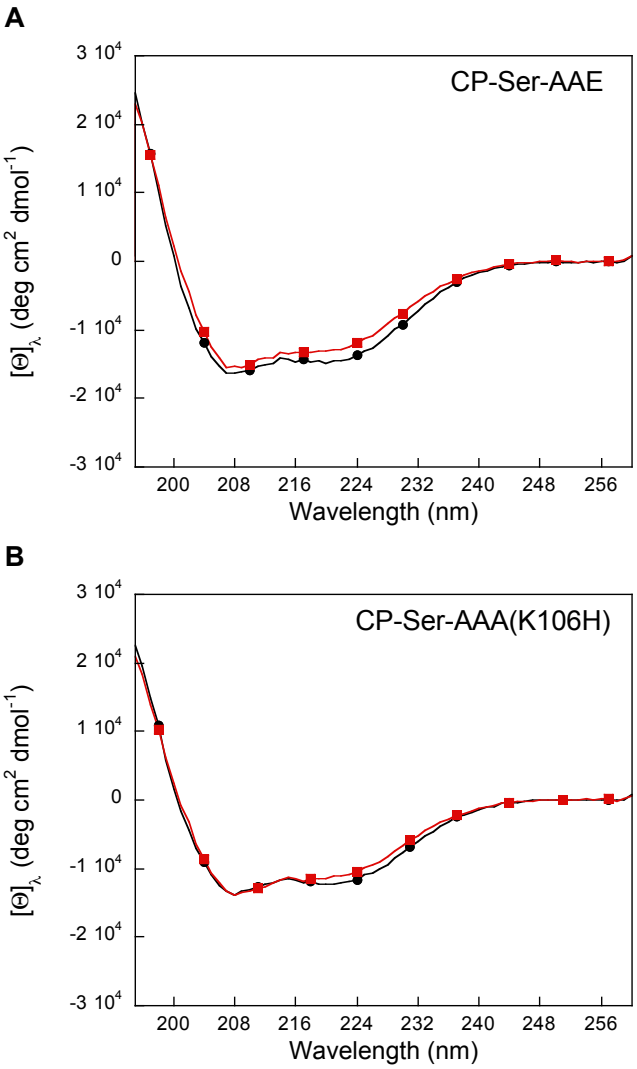


Figure S7. CD spectra of 10 μM CP-Ser-AAE (**A**) and CP-Ser-AAA(K106H) (**B**) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 ^{o}C).

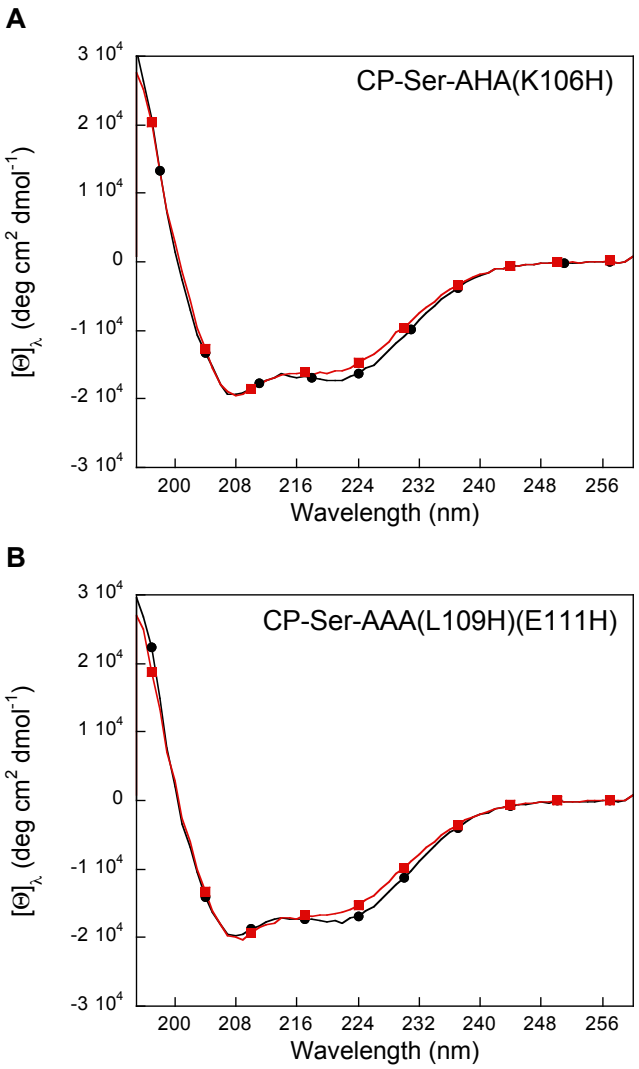


Figure S8. CD spectra of 10 μM CP-Ser-AHA(K106H) (**A**) and CP-Ser-AAA(L109H)(E111H) (**B**) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 ^{o}C).

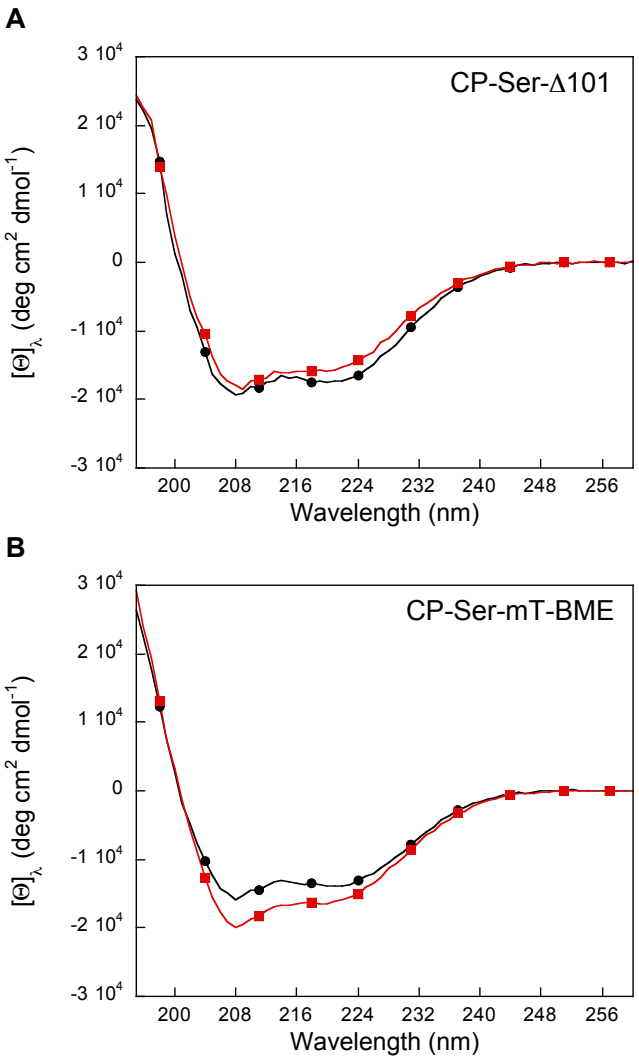


Figure S9. CD spectra of 10 μM CP-Ser-Δ101 (**A**) and CP-Ser-mT (**B**) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 ^{o}C).

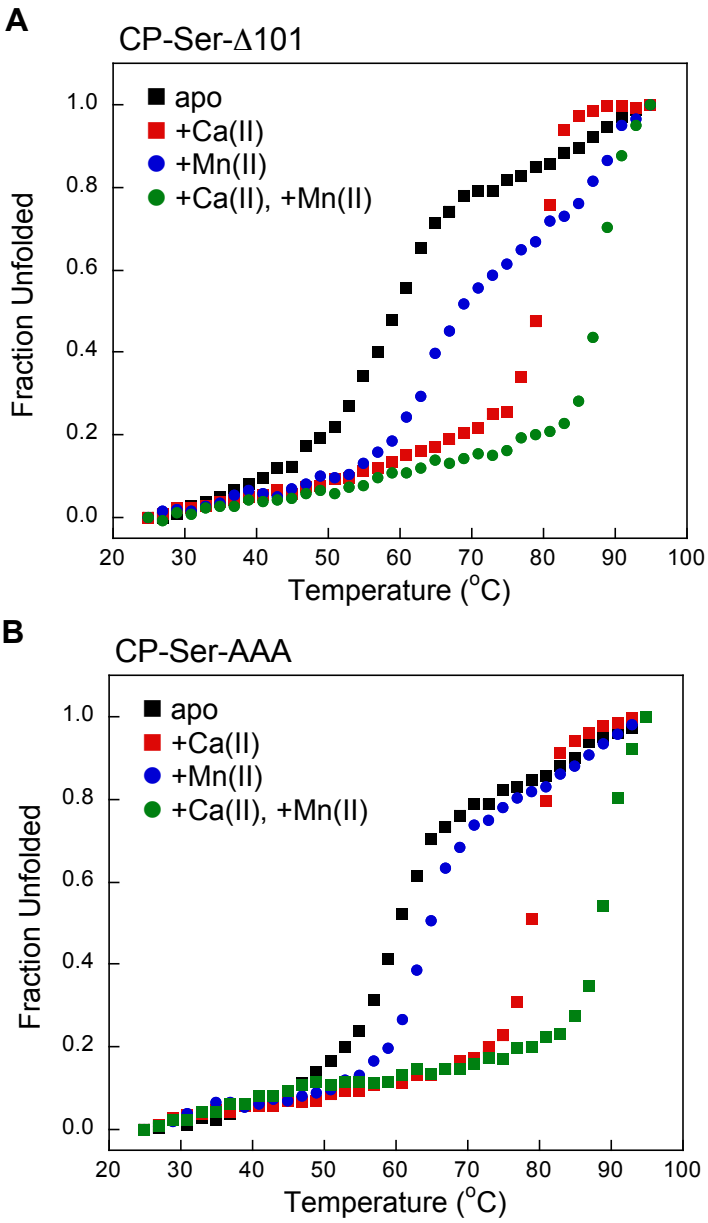


Figure S10. Thermal denaturation of CP-Ser-Δ101 (**A**) and CP-Ser-AAA (**B**) in the absence and presence of 10 equivalents Mn(II) with or without 2 mM Ca(II) (1mM Tris-HCl, pH 7.5).

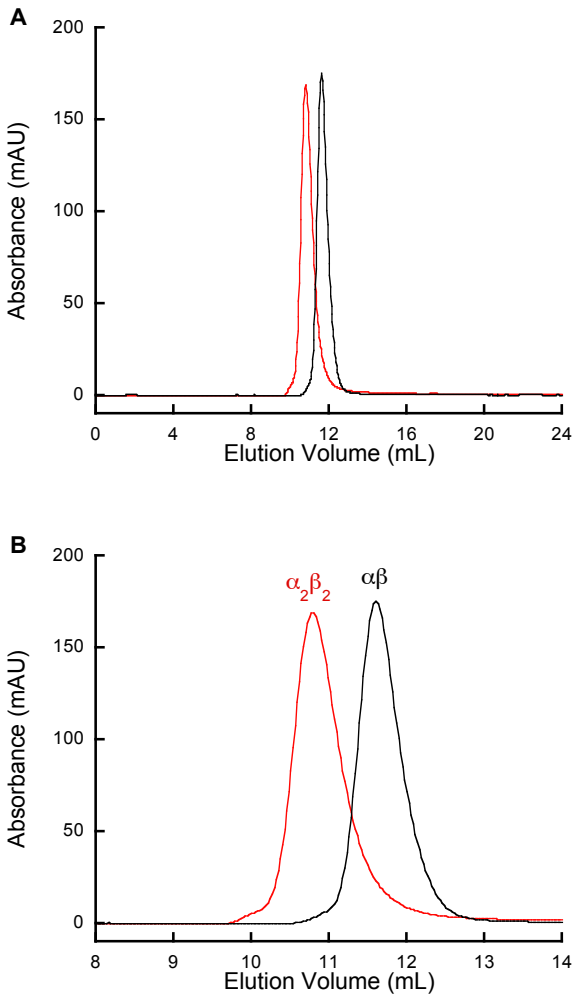


Figure S11. Analytical size exclusion chromatography of 100 μM CP-Ser(E96A) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

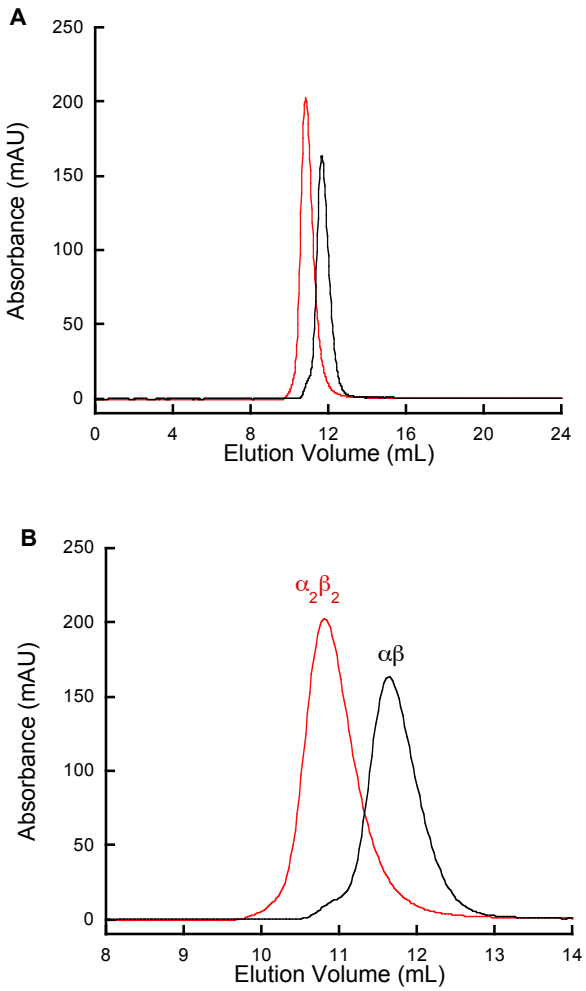


Figure S12. Analytical size exclusion chromatography of 100 μM CP-Ser(D98A) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

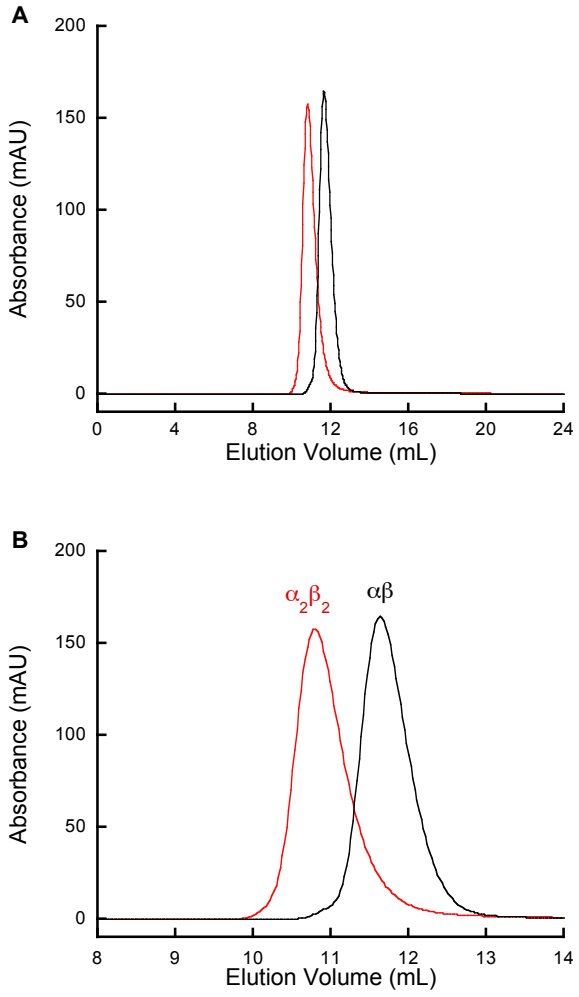


Figure S13. Analytical size exclusion chromatography of 100 μM CP-Ser(E99A) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

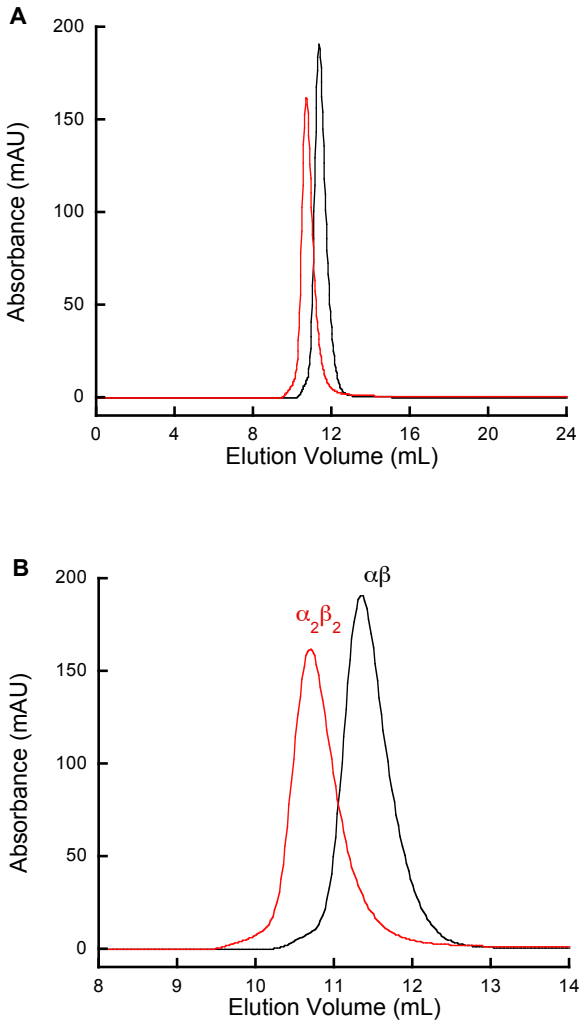


Figure S14. Analytical size exclusion chromatography of 100 μM CP-Ser(H103A) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

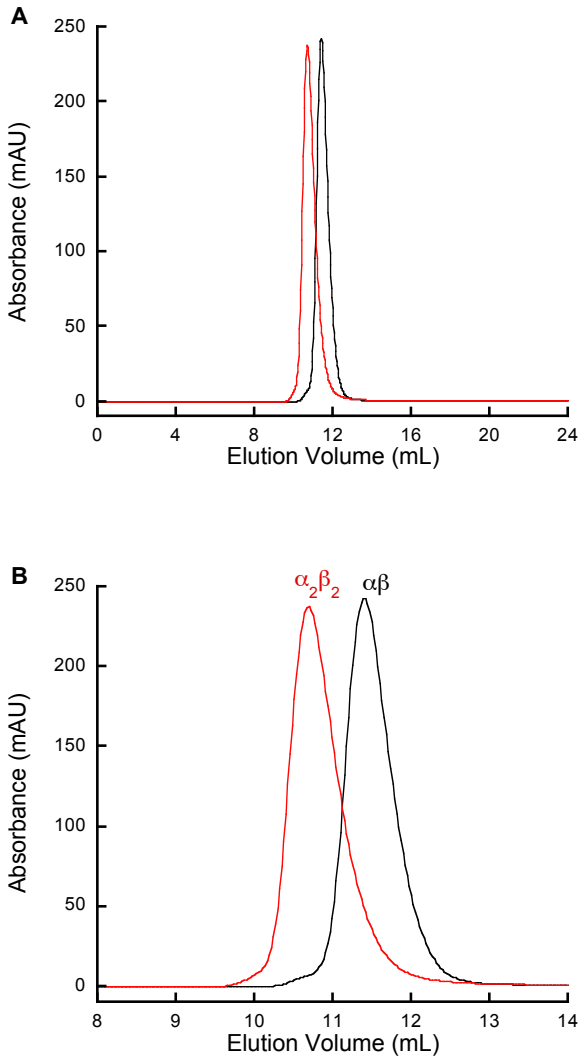


Figure S15. Analytical size exclusion chromatography of 100 μM CP-Ser(H104A) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

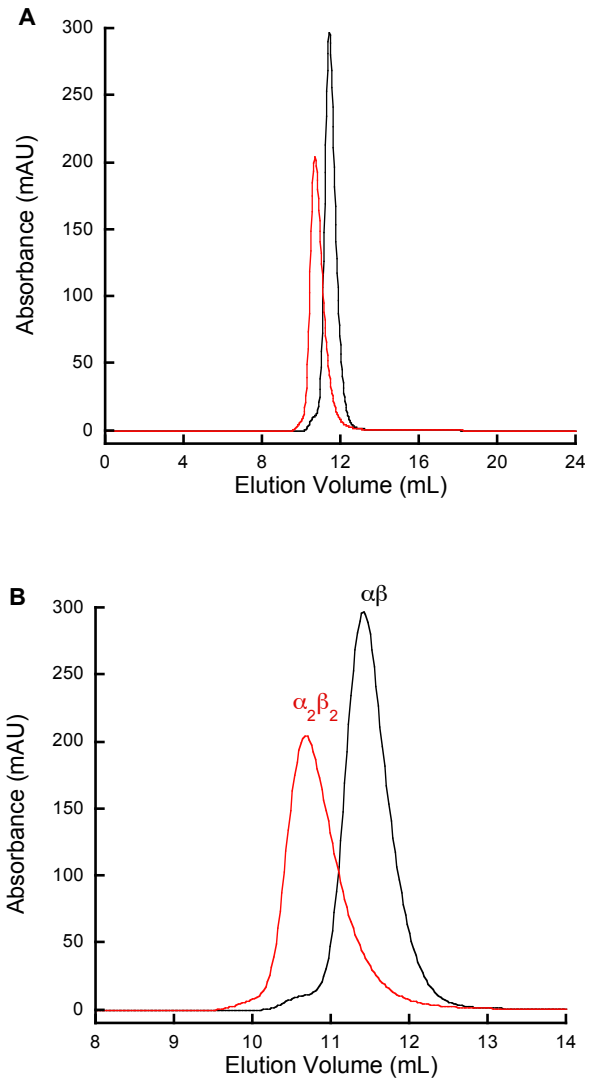


Figure S16. Analytical size exclusion chromatography of 100 μM CP-Ser(H105A) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

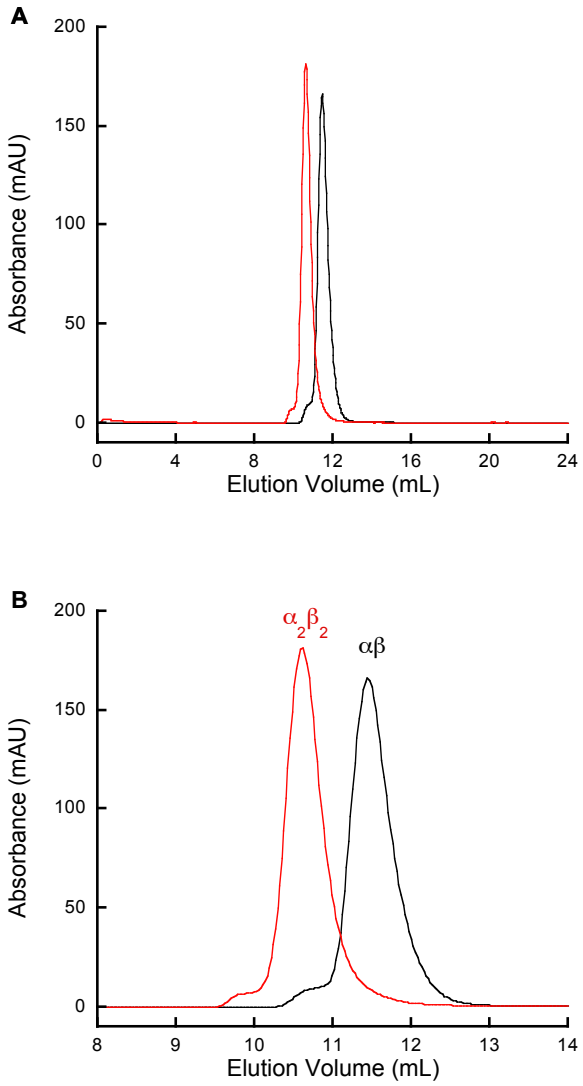


Figure S17. Analytical size exclusion chromatography of 100 μM CP-Ser(K106A) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

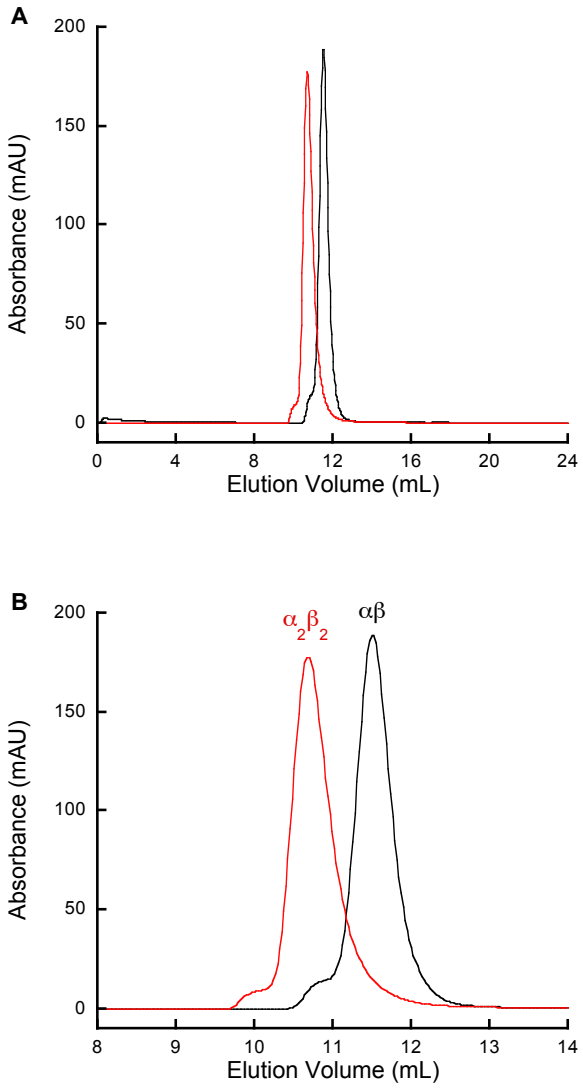


Figure S18. Analytical size exclusion chromatography of 100 μM CP-Ser(E111A) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

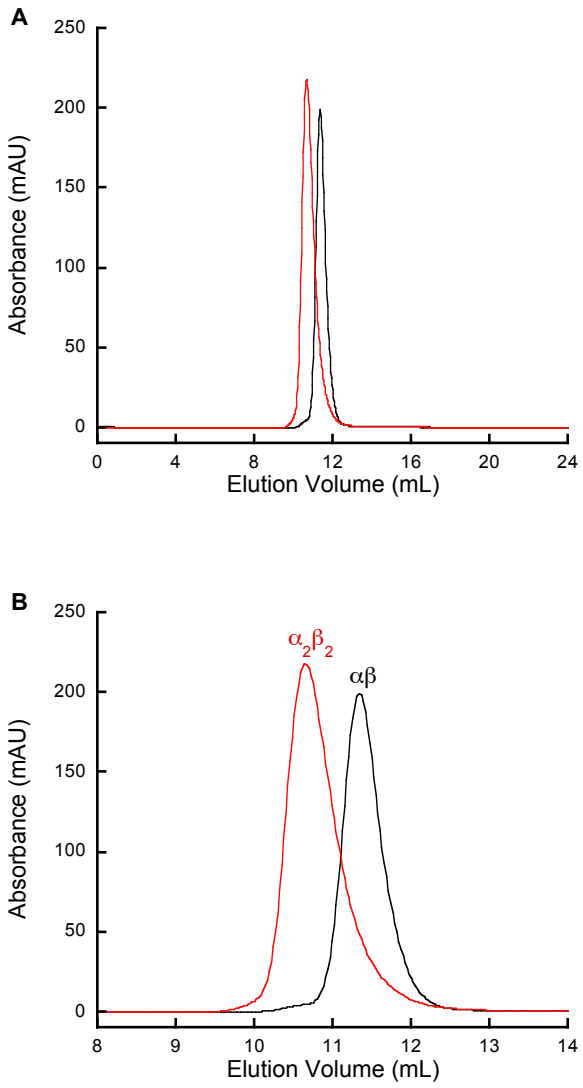


Figure S19. Analytical size exclusion chromatography of 100 μM CP-Ser-AHA (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

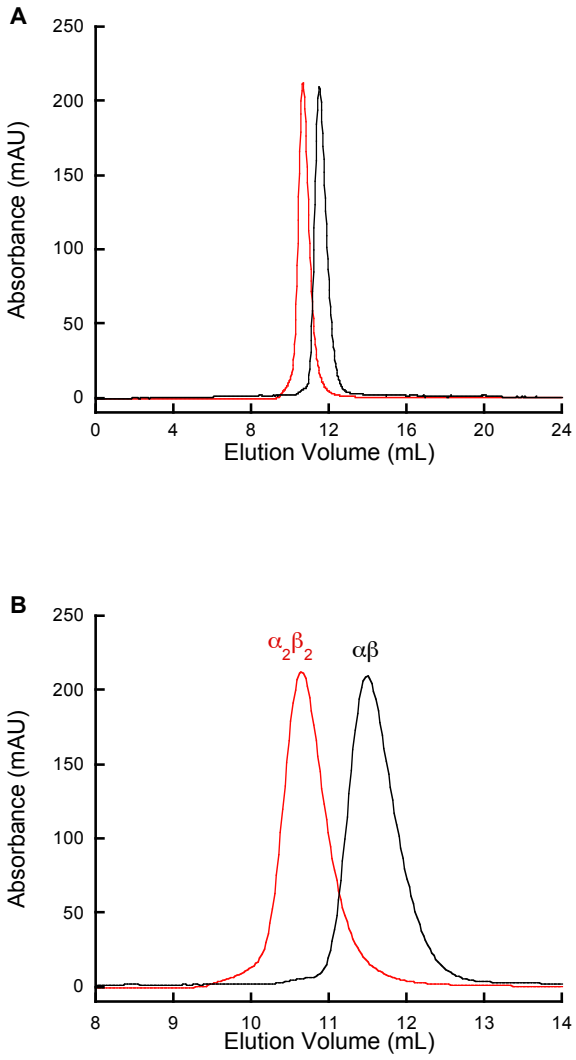


Figure S20. Analytical size exclusion chromatography of 100 μM CP-Ser-AAA (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

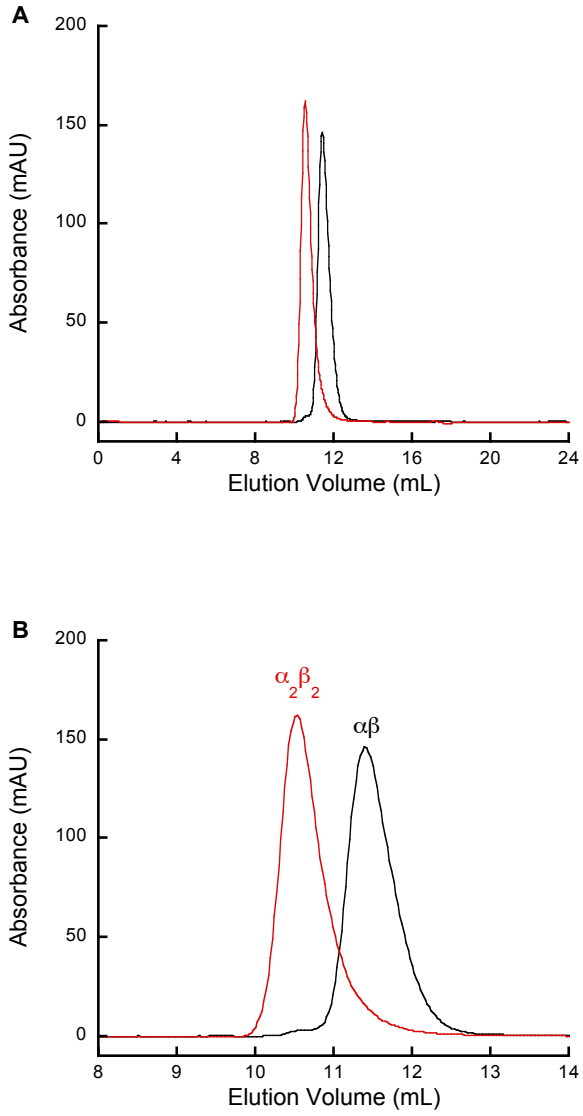


Figure S21. Analytical size exclusion chromatography of 100 μM CP-Ser-AAE (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

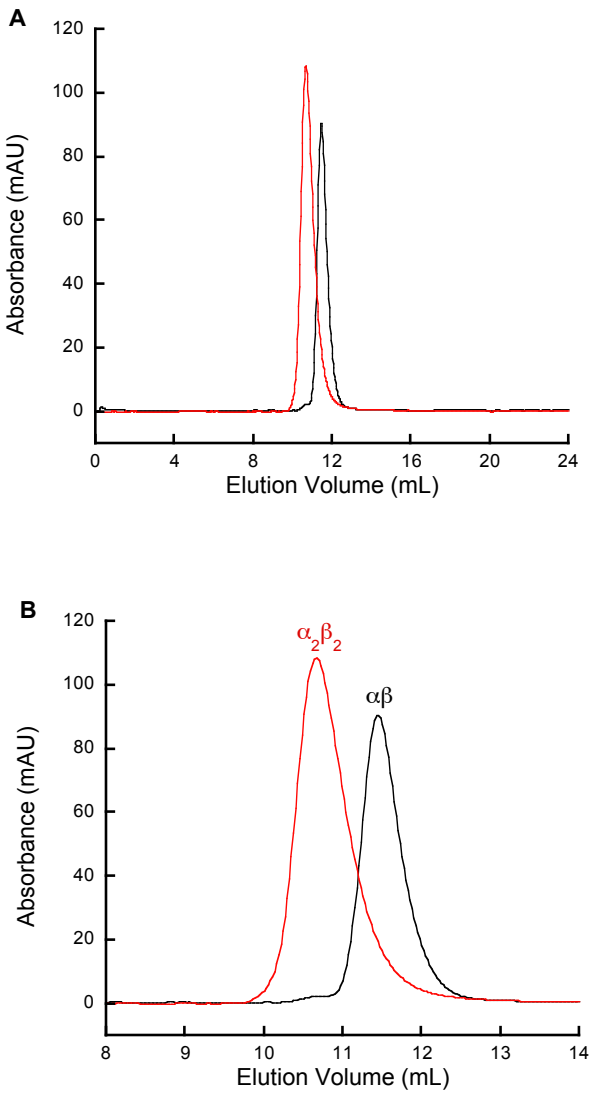


Figure S22. Analytical size exclusion chromatography of 100 μM CP-Ser-AAA(K106H) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

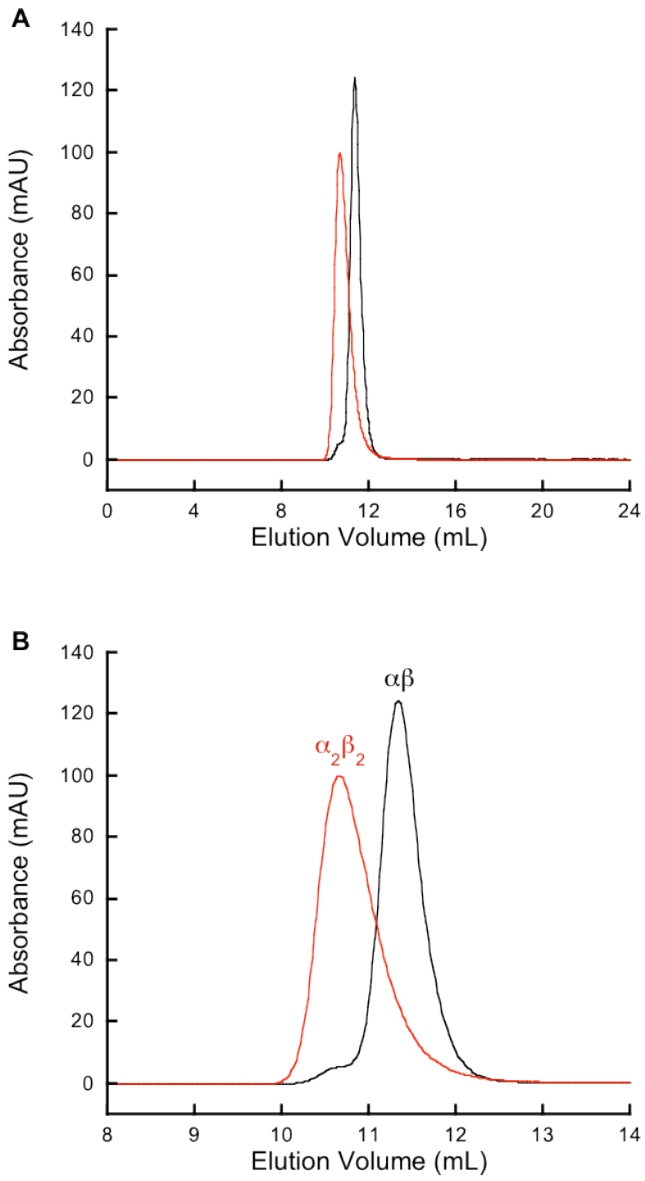


Figure S23. Analytical size exclusion chromatography of 100 μM CP-Ser-AHA(K106H) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

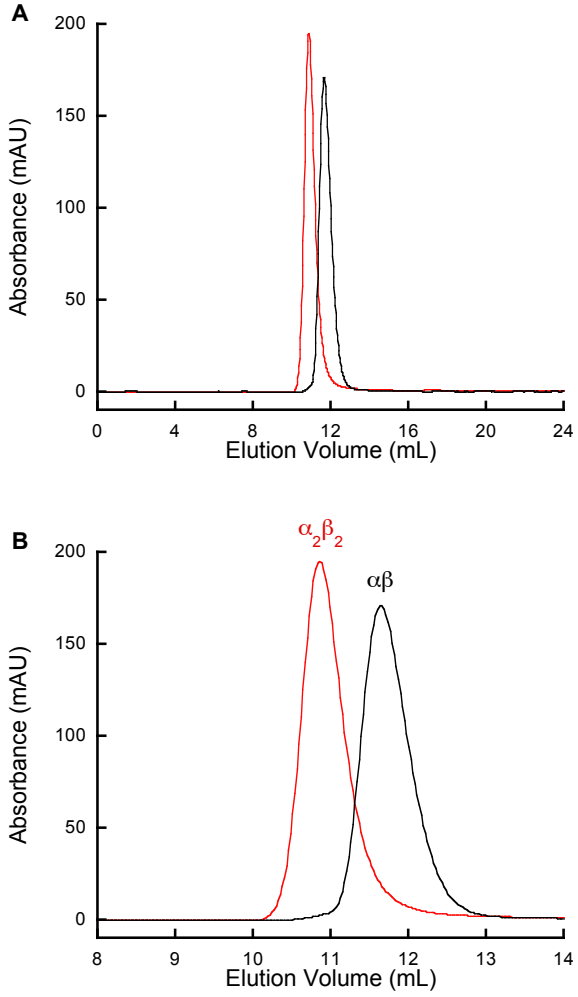


Figure S24. Analytical size exclusion chromatography of 100 μM CP-Ser-AAA(L109H)(E111H) (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

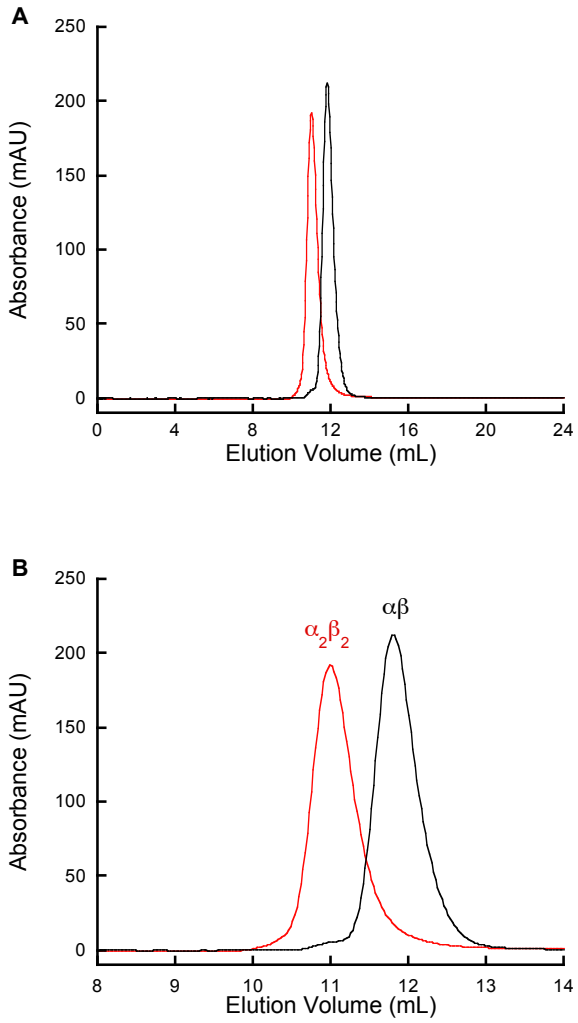


Figure S25. Analytical size exclusion chromatography of 100 μM CP-Ser-Δ101 (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

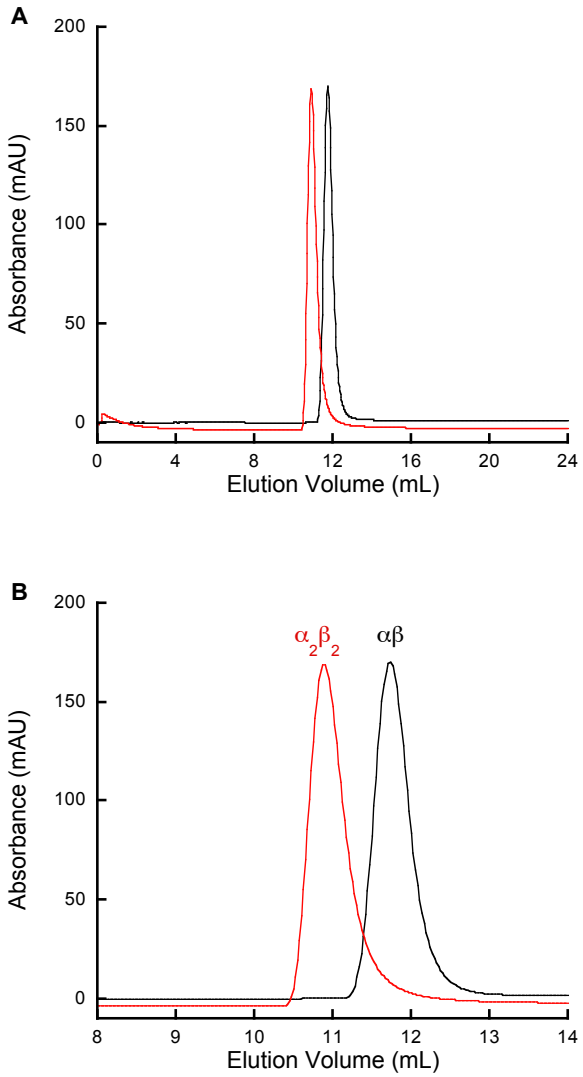


Figure S26. Analytical size exclusion chromatography of 100 μM CP-Ser-mT-BME (100 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**A**) Full chromatograms. (**B**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8. The TCEP-treated species was not analyzed by analytical SEC.

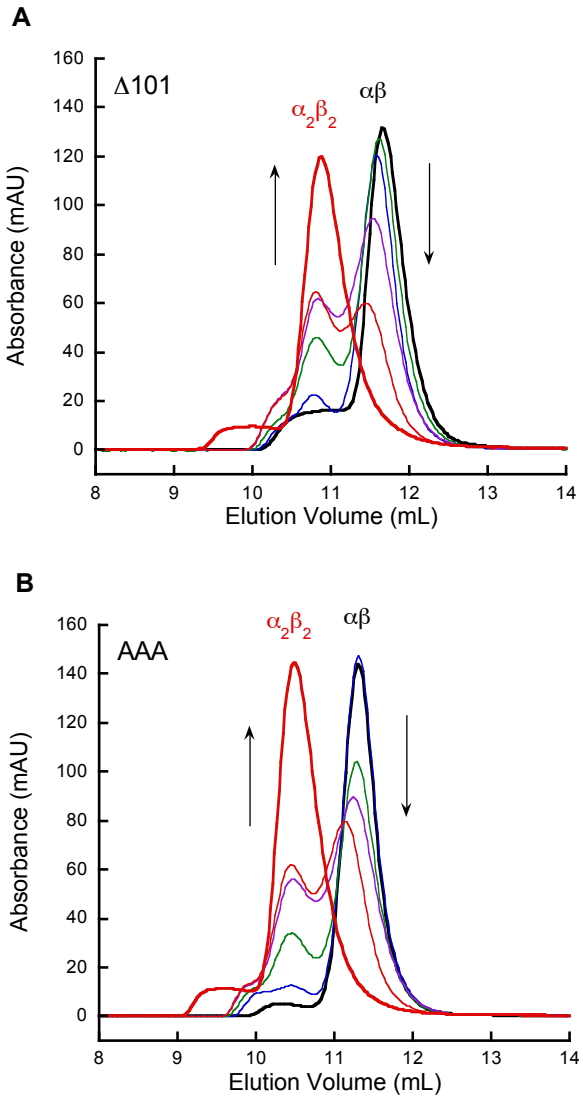


Figure S27. Calcium-binding titrations of 100 μL of 100 μM CP-Ser-Δ101 (**A**) and 100 μM CP-Ser-AAA (**B**) monitored by SEC at pH 8.0 (20 mM HEPES, 100 mM NaCl). The bold black line is the CP mutant in the absence of Ca(II). The bold red line is a standard for the Ca(II)-bound heterotetramer and corresponds to each mutant eluted with 2 mM Ca(II) in the running buffer. The thin blue, green, purple, and red traces correspond to addition of 1.0, 2.0, 4.0, and 8.0 equiv of Ca(II), respectively, to CP prior to SEC. Absorbance was monitored at 280 nm at room temperature.

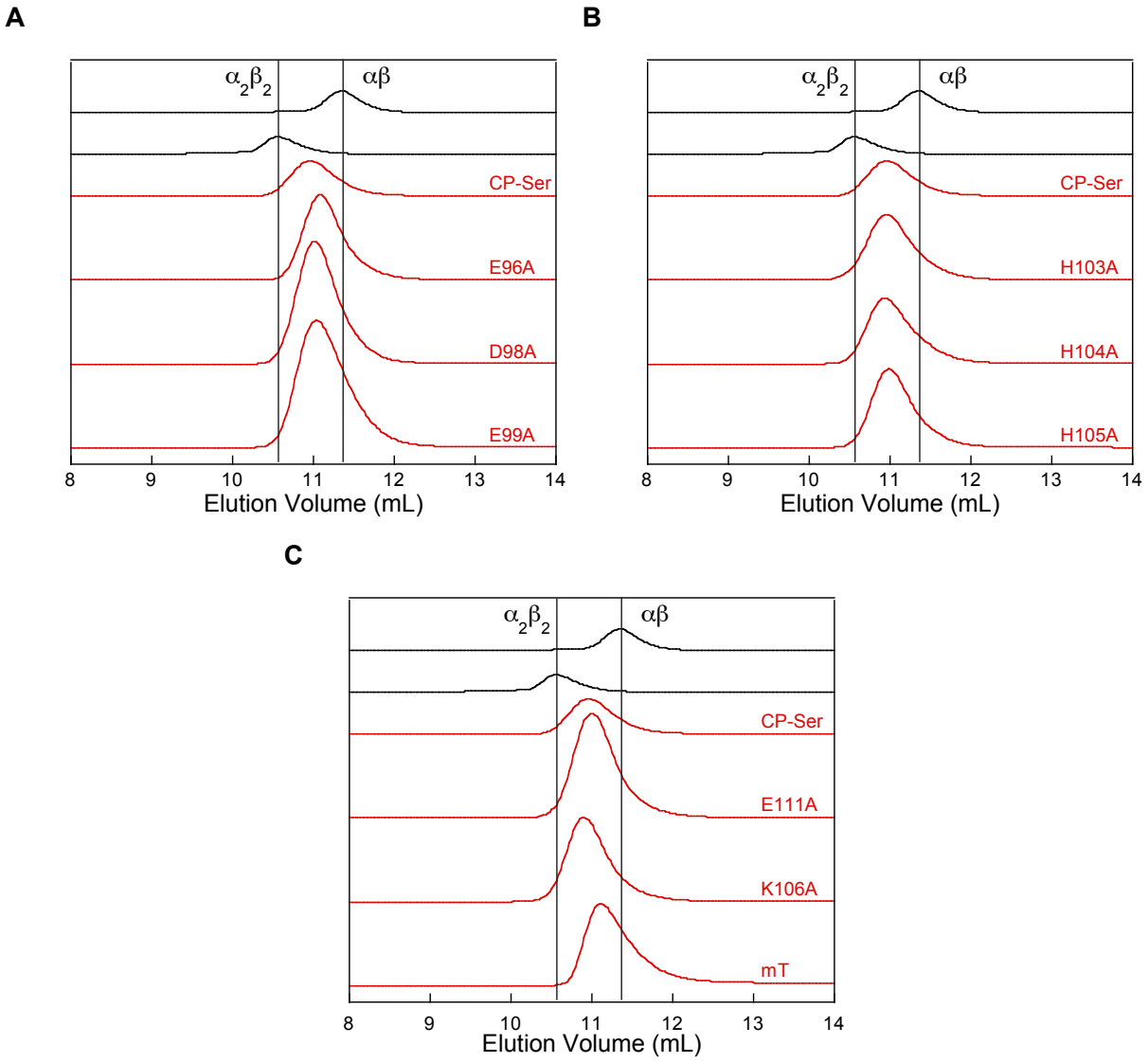


Figure S28. Analytical SEC of CP-Ser and mutant proteins in the presence of Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). The red traces are CP-Ser and mutants in the presence of 10 equiv of Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). The black traces are standards of CP-Ser in the absence and presence of 2 mM Ca(II). The protein concentrations were 100 μM (100 μL, black traces) and 200 μM (200 μL, red traces). In each panel, the vertical lines indicate the elution volumes for the CP-Ser $\alpha\beta$ and $\alpha_2\beta_2$ forms.

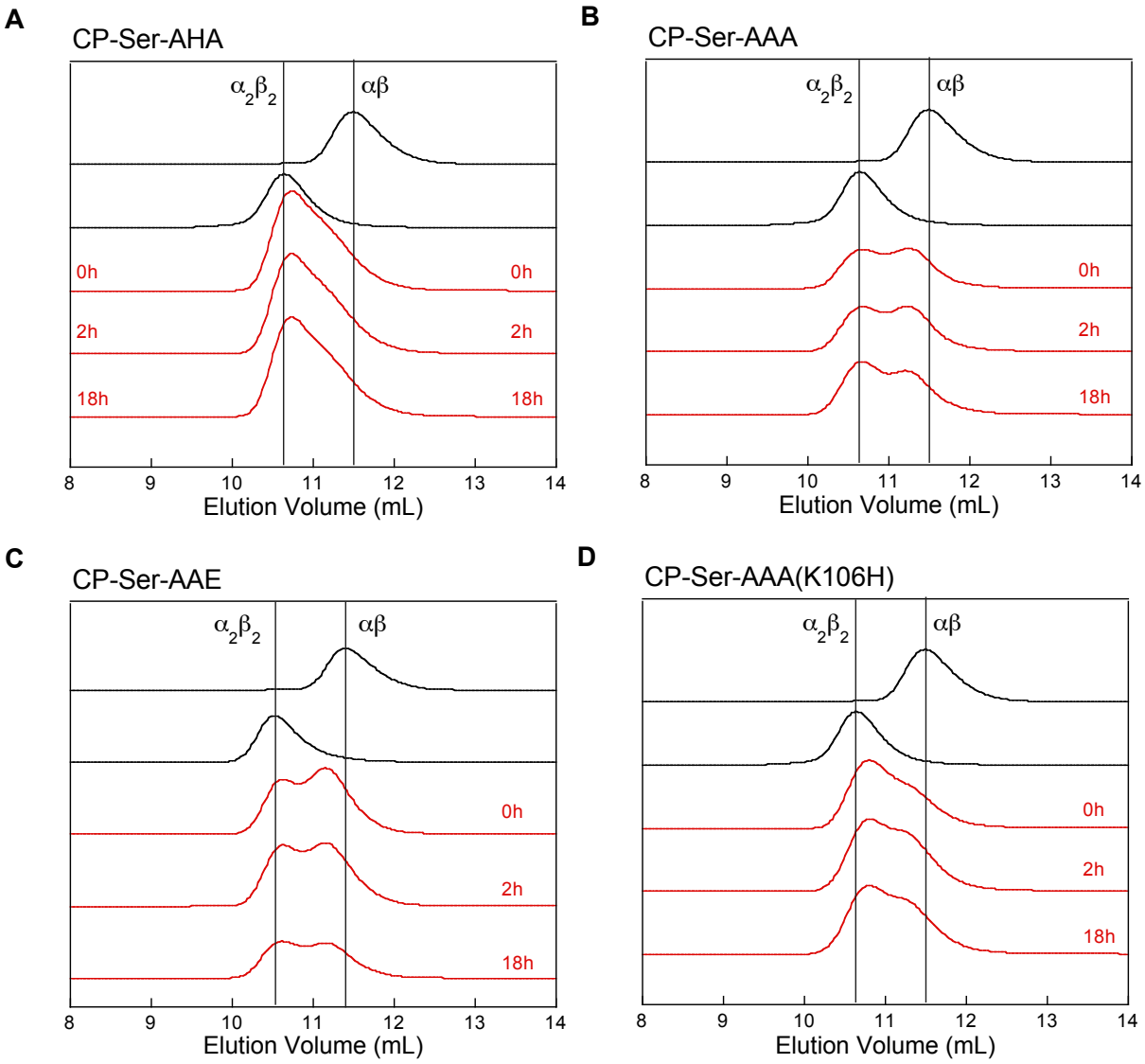


Figure S29. Analytical SEC of CP-Ser-AHA (**A**), CP-Ser-AAA (**B**), CP-Ser-AAE (**C**) and CP-Ser-AAA(K106H) (**D**) in the presence of Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). The black traces are standards of the indicated mutant in the absence and presence of 2 mM Ca(II). The red traces are the CP-Ser mutants in the presence of 10 equiv of Mn(II) after a 0, 2 and 18 h incubation time. The protein concentrations were 100 μM (100 μL, black traces) and 200 μM (200 μL, red traces). The vertical lines indicate the elution volumes for the αβ and $\alpha_2\beta_2$ forms of the indicated mutant.

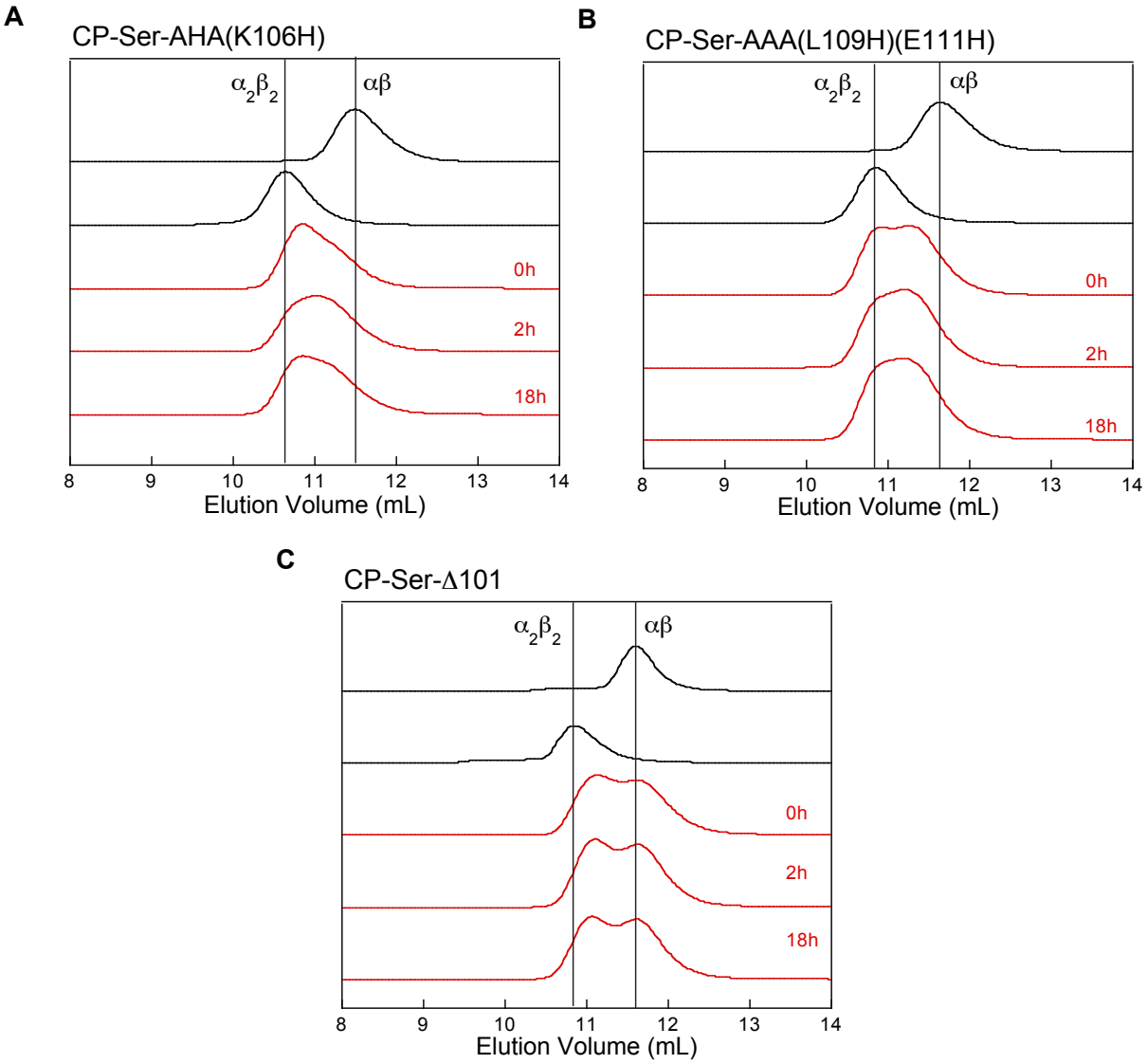


Figure S30. Analytical SEC of CP-Ser-AHA(K106H) (**A**), CP-Ser-AAA(L109H)(E111H) (**B**) and CP-Ser-Δ101 (**C**) in the presence of Mn(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). The black traces are standards of the indicated mutant in the absence and presence of 2 mM Ca(II). The red traces are the CP-Ser mutants in the presence of 10 equiv of Mn(II) after a 0,, 2 and 18h incubation time. The protein concentrations were 100 μM (100 μL, black traces) and 200 μM (200 μL, red traces). The vertical lines indicate the elution volumes for the αβ and $\alpha_2\beta_2$ forms of the indicated mutant.

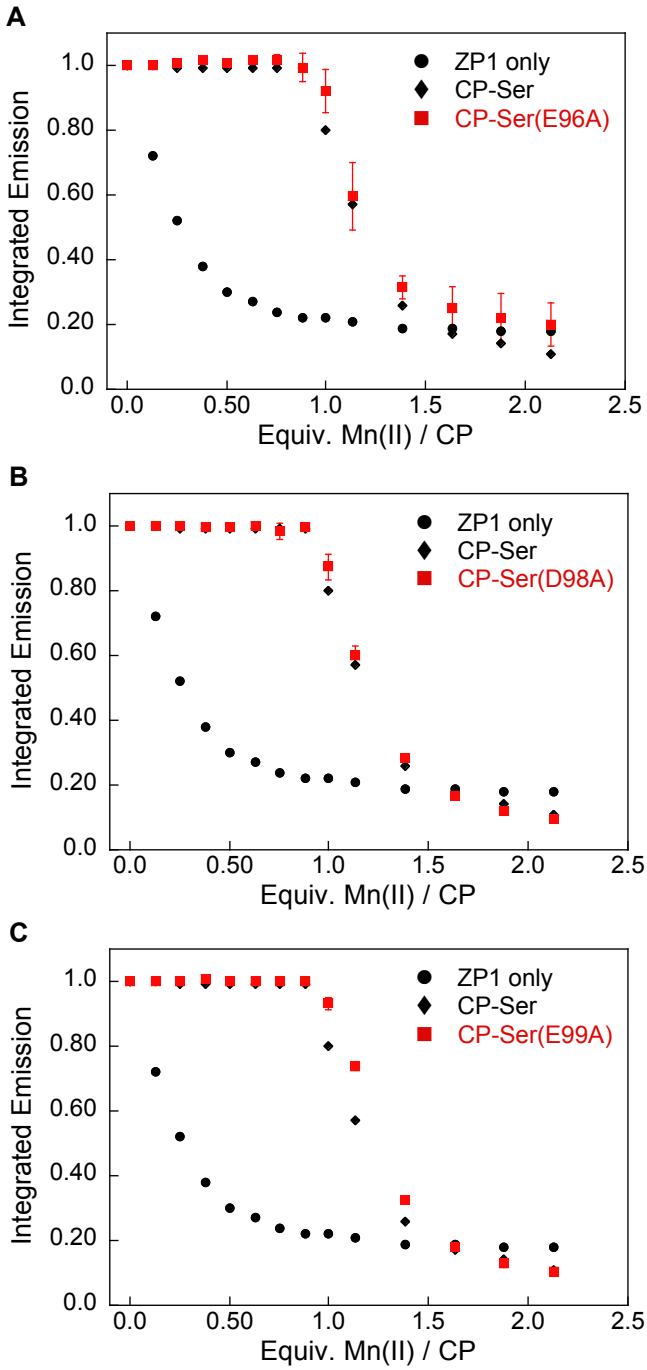


Figure S31. Competition between ZP1 and CP-Ser(E96A) (**A**), CP-Ser(D98A) (**B**), and CP-Ser(E99A) (**C**) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The integrated emission of apo ZP1 was normalized to a value of one.

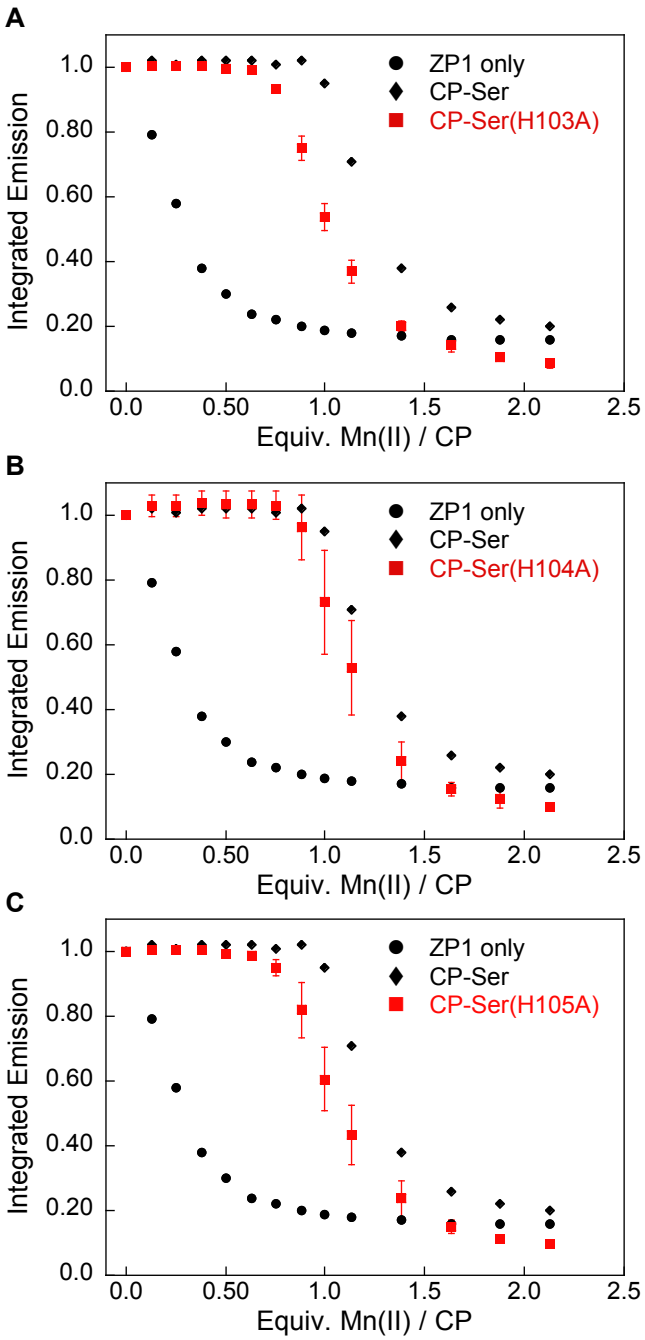


Figure S32. Competition between ZP1 and CP-Ser(H103A) (**A**), CP-Ser(H104A) (**B**), and CP-Ser(H105A) (**C**) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The integrated emission of apo ZP1 was normalized to a value of one.

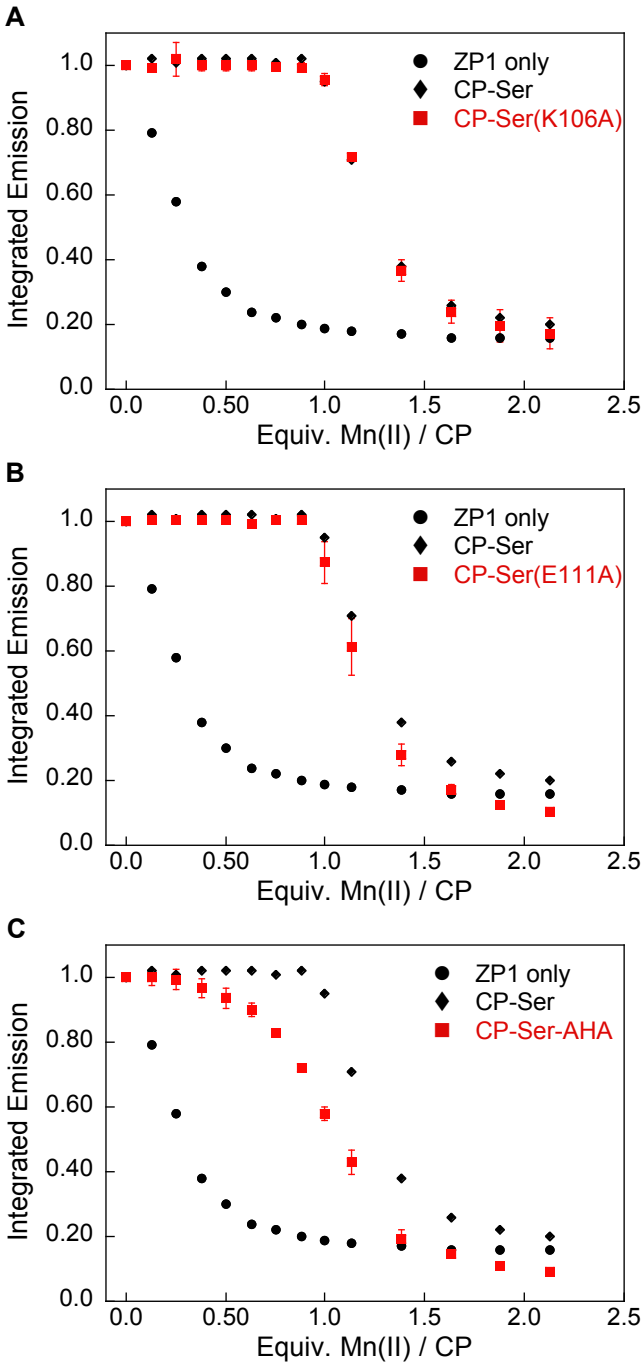


Figure S33. Competition between ZP1 and CP-Ser(K106A) (**A**), CP-Ser(E111A) (**B**), and CP-Ser-AHA (**C**) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The integrated emission of apo ZP1 was normalized to a value of one.

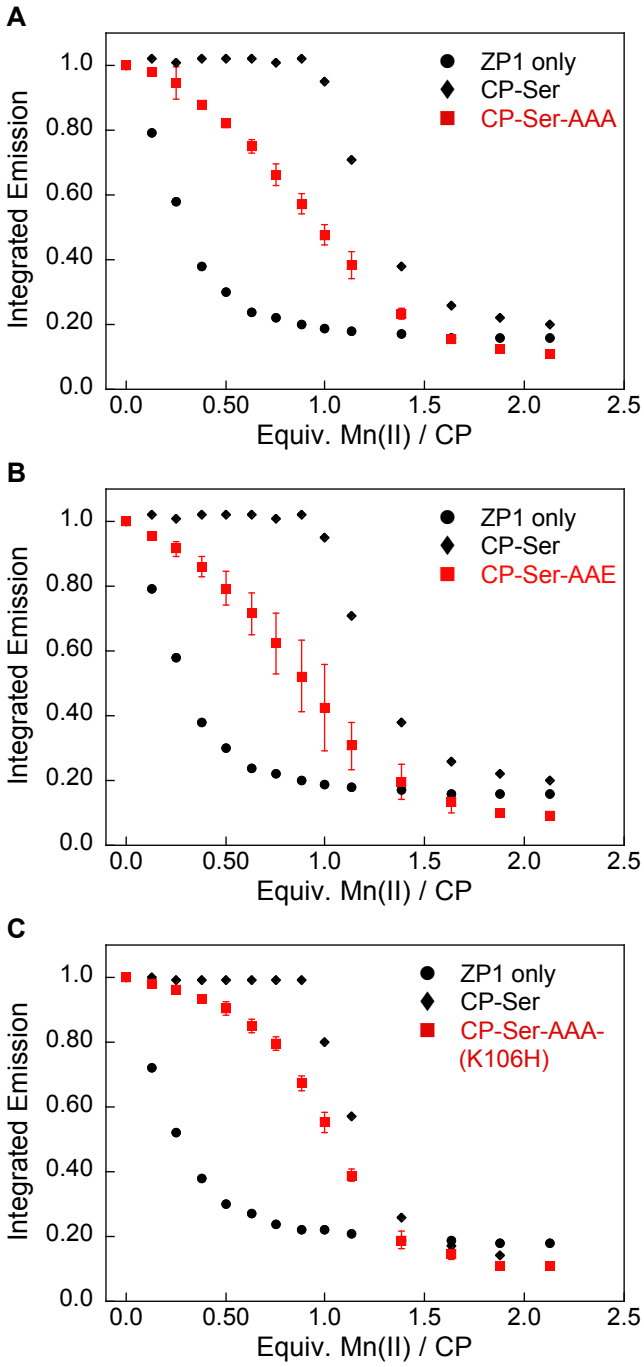


Figure S34. Competition between ZP1 and CP-Ser-AAA (**A**), CP-Ser-AAE (**B**), and CP-Ser-AAA-(K106H) (**C**) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The emission of apo ZP1 was normalized to a value of one.

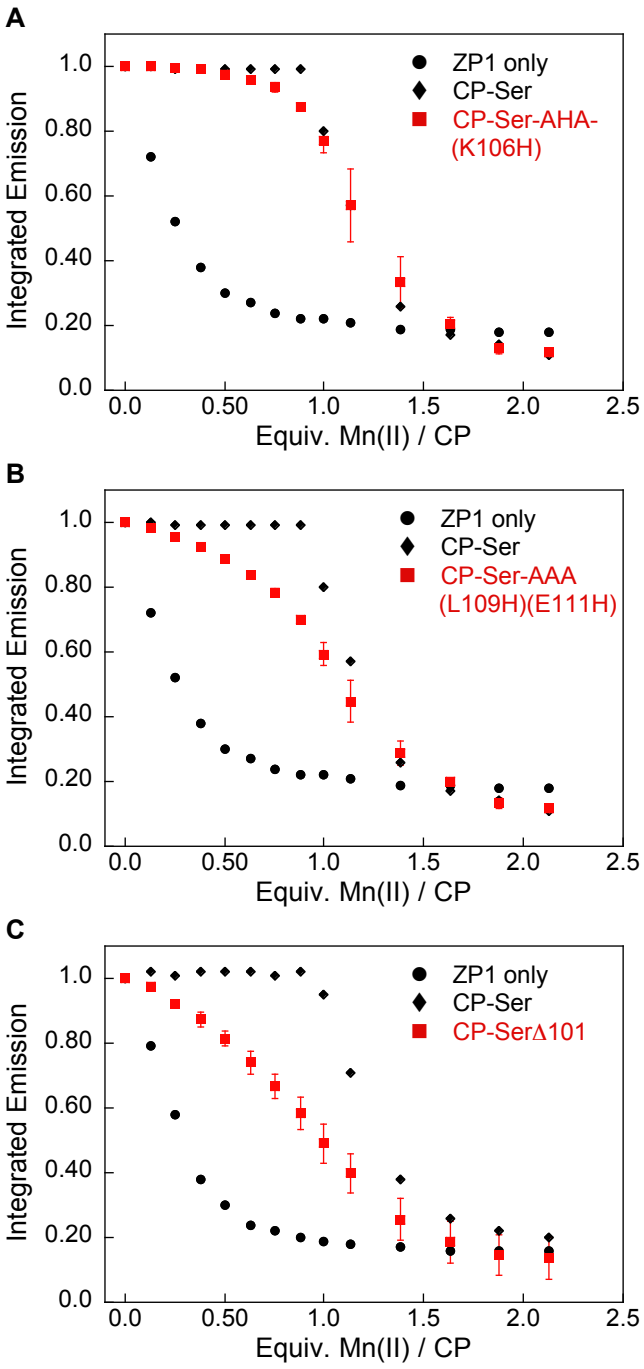


Figure S35. Competition between ZP1 and CP-Ser-AHA(K106H) (**A**), CP-Ser-AAA(L109H)(E111H) (**B**), and CP-Ser-Δ101 (**C**) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The emission of apo ZP1 was normalized to a value of one.

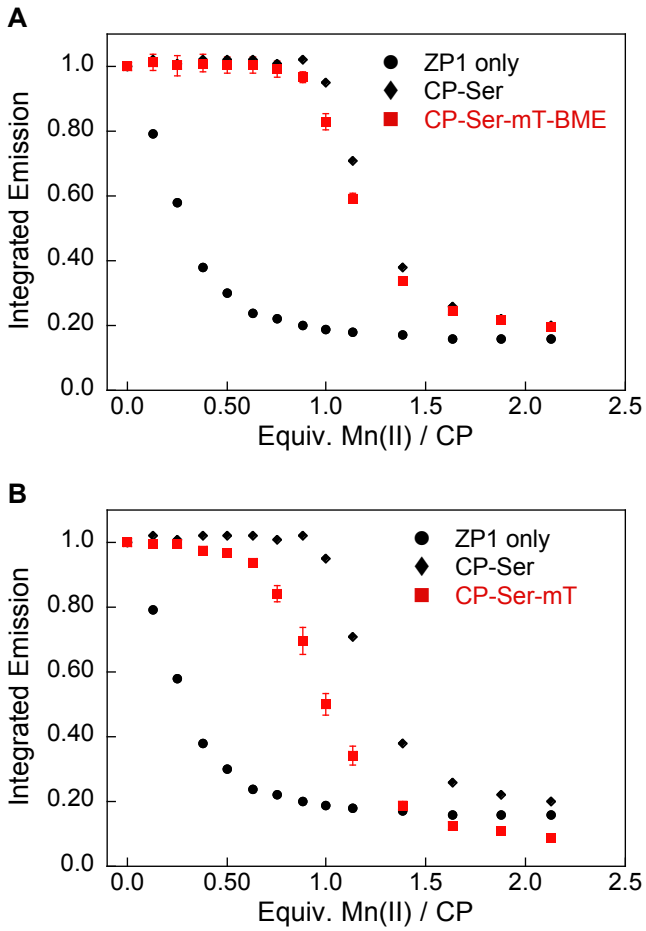


Figure S36. Competition between ZP1 and CP-Ser-mT-BME (**A**) and CP-Ser-mT (**B**) for Mn(II). All titrations were conducted with 1 μM ZP1, 4 μM CP, and 200 μM Ca(II) at pH 7.5 (75 mM HEPES, 100 mM NaCl). Excitation was provided at 490 nm, and the ZP1 emission was integrated from 500–650 nm. The emission of apo ZP1 was normalized to a value of one.

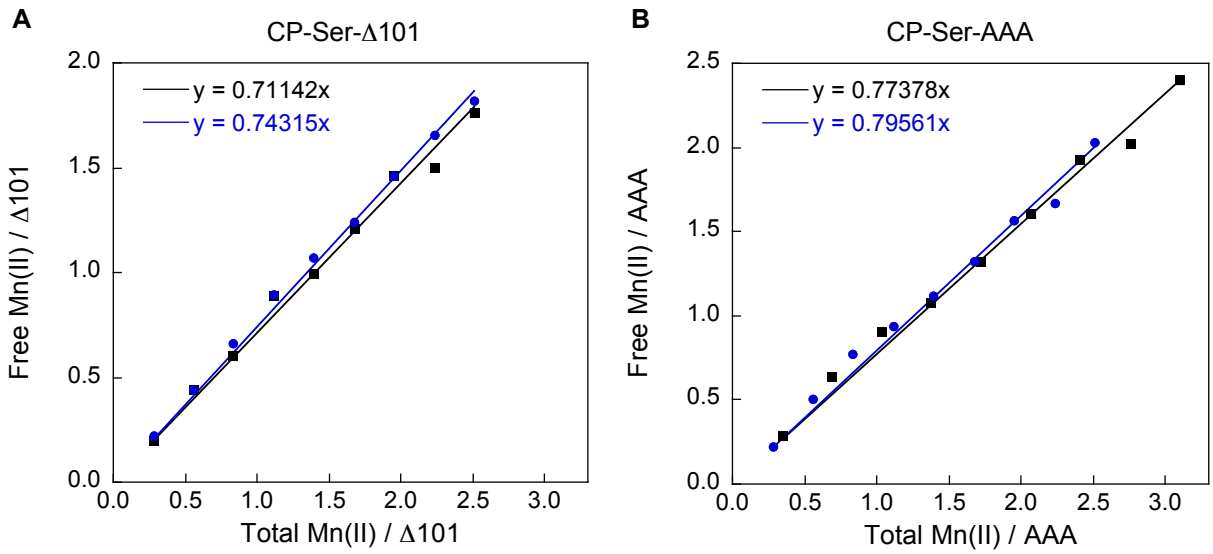


Figure S37. Slope analysis of the room-temperature EPR titrations of CP-Ser-Δ101 (**A**) and CP-Ser-AAA (**B**). Plots of $[Mn(II)_{free}]$ versus $[Mn(II)_{total}]$ are shown. All titrations were performed with 100 μM protein (75 mM HEPES, 100 mM NaCl, pH 7.5).

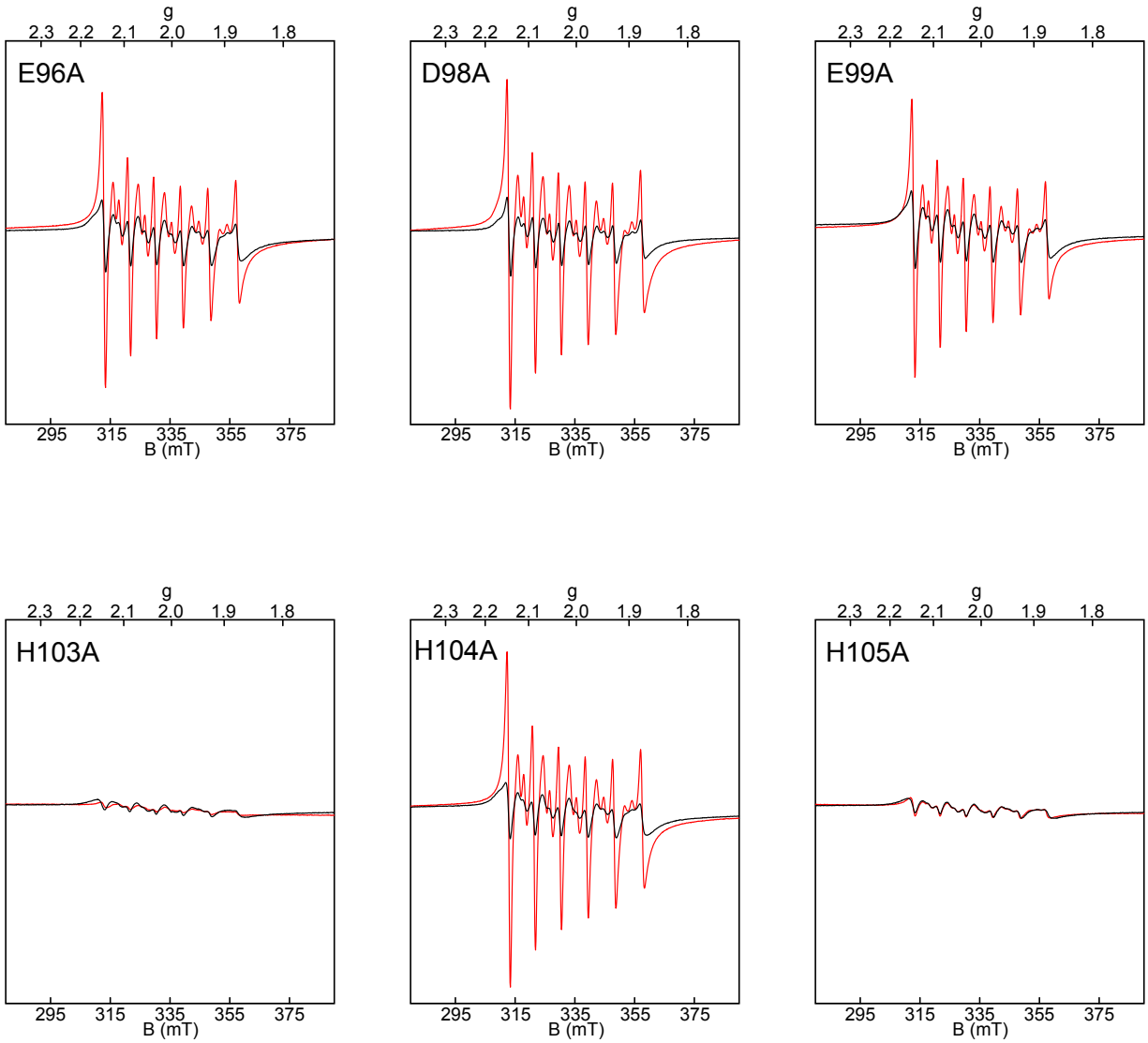


Figure S38. Low-temperature EPR spectra of the CP mutant proteins E96A, D98A, E99A, H103A, H104A, and H105A. All samples contained 100 μM protein and 30 μM Mn(II) in absence (black line) or presence (red line) of 1 mM Ca(II). All panels contain the same y-axis scale. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.

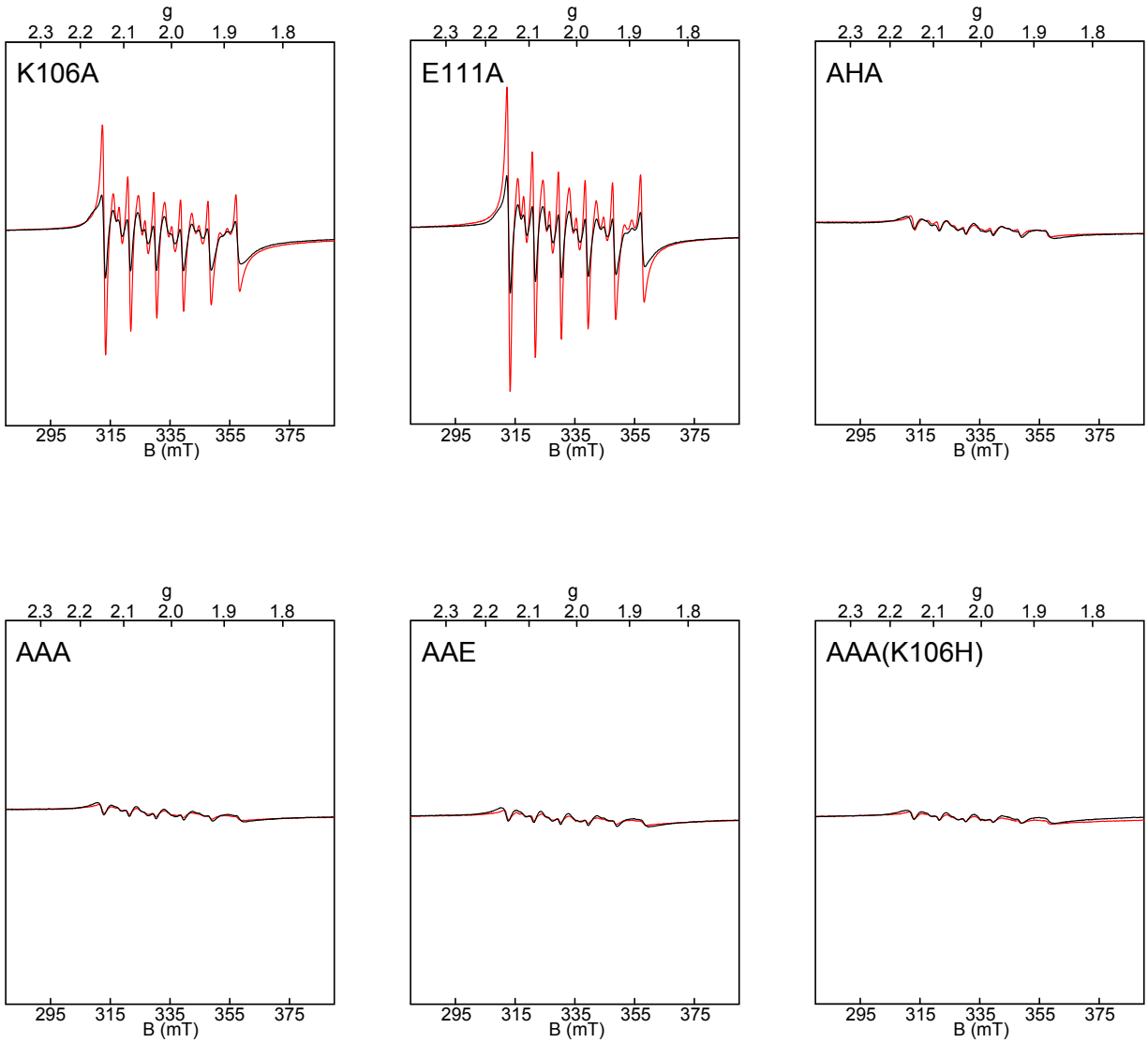


Figure S39. Low-temperature EPR spectra of the CP mutant proteins K106A, E111A, AHA, AAA, AAE, and AAA(K106H). All samples contained 100 μM protein and 30 μM Mn(II) in absence (black line) or presence (red line) of 1 mM Ca(II). All panels contain the same y-axis scale as used in Figure S38. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.

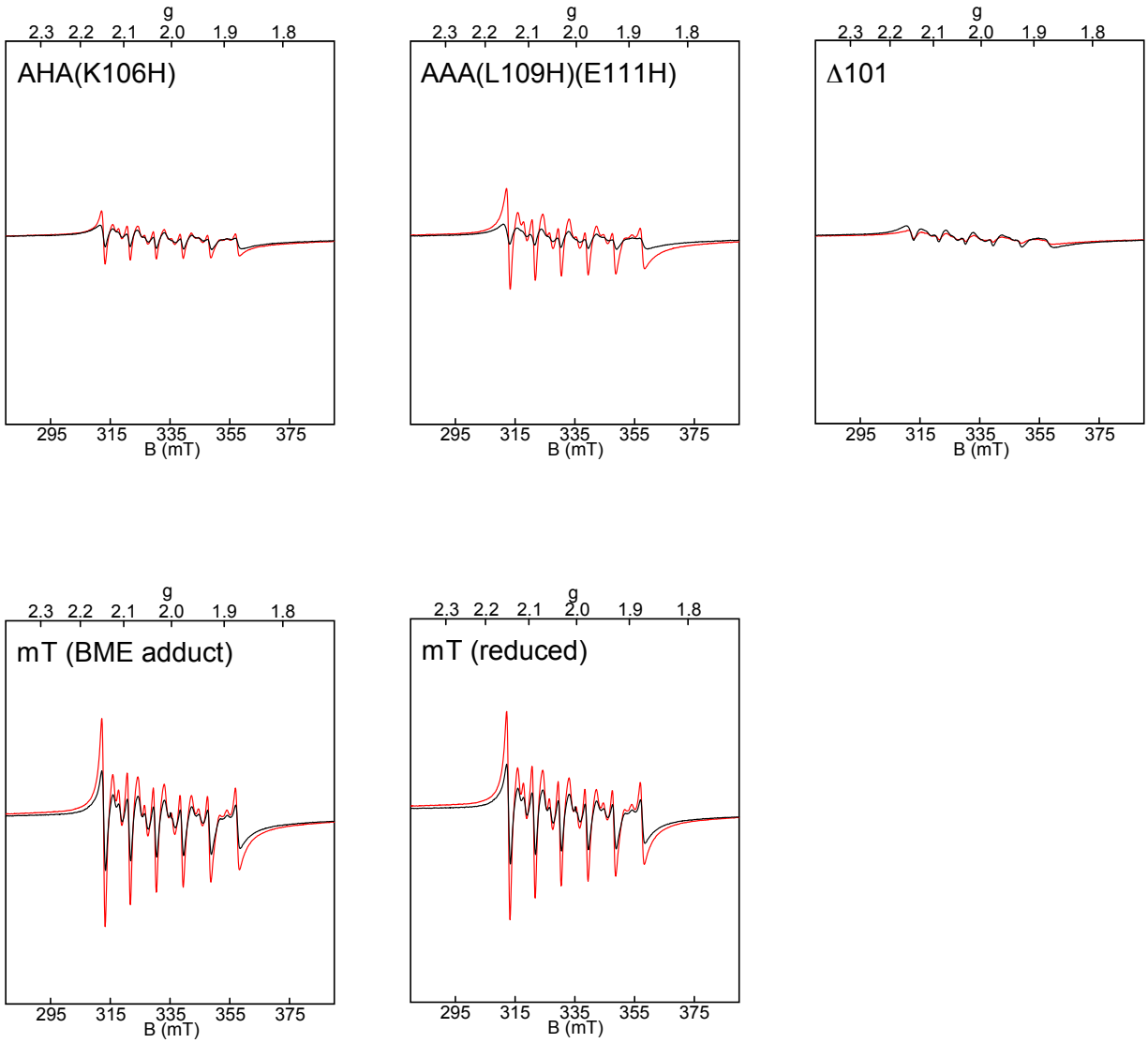


Figure S40. Low-temperature EPR spectra of the CP mutant proteins AHA(K106H), AAA(L109H)(E111H), Δ101, mT-BME, and mT. All samples contained 100 μM protein and 30 μM Mn(II) in absence (black line) or presence (red line) of 1 mM Ca(II). All panels contain the same y-axis scale as used in Figures S38 and S39. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.

Additional Discussion of Low-Temperature EPR Spectra

The low-temperature Mn(II) EPR signatures of CP-Ser-AHA(K106H) and CP-Ser-AAA(L109H)(E111H) each exhibit a sharp feature at g = 1.88 attributed to semi-forbidden transitions; however, the signals are of significantly lower intensity than for CP-Ser ($\alpha_2\,\beta_2$). These data are consistent with the presence of a relatively small amount of six-coordinate Mn(II) in each sample. It is possible that these samples contain mixtures of five- and six-coordinate Mn(II) complexes, which would account for the attenuated six-coordinate EPR signals and ZP1 competition assays, the latter of which support higher-affinity Mn(II) coordination than Δ101 and AAA.

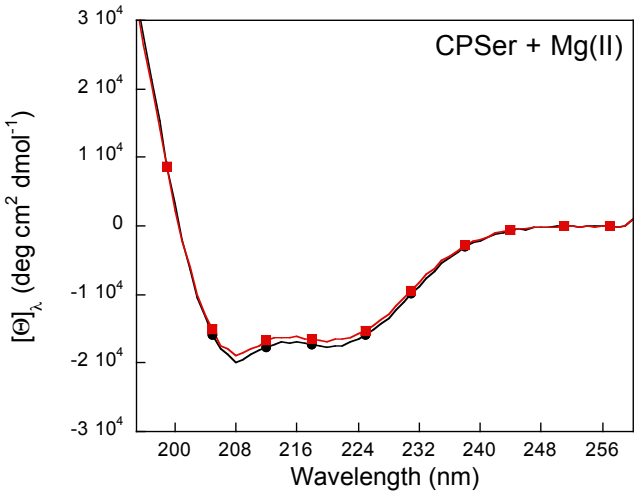


Figure S41. CD spectra of 10 μM CP-Ser in the absence (black circles) and presence (red squares) of 2 mM Mg(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 °C).

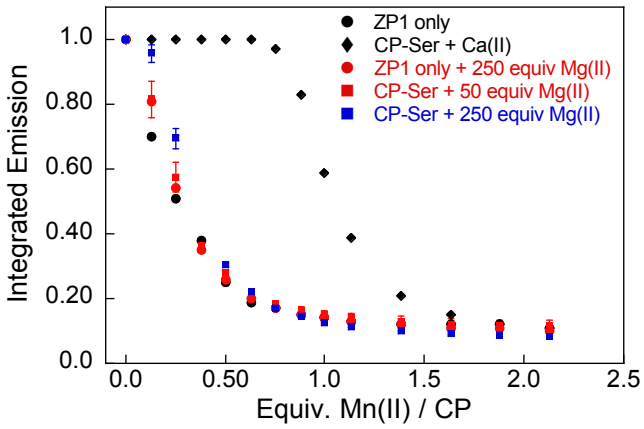


Figure S42. Competition between ZP1 and CP-Ser for Mn(II) in the absence and presence of Mg(II). All titrations were conducted with 1 μM ZP1 and 4 μM CP at pH 7.5 (75 mM HEPES, 100 mM NaCl).

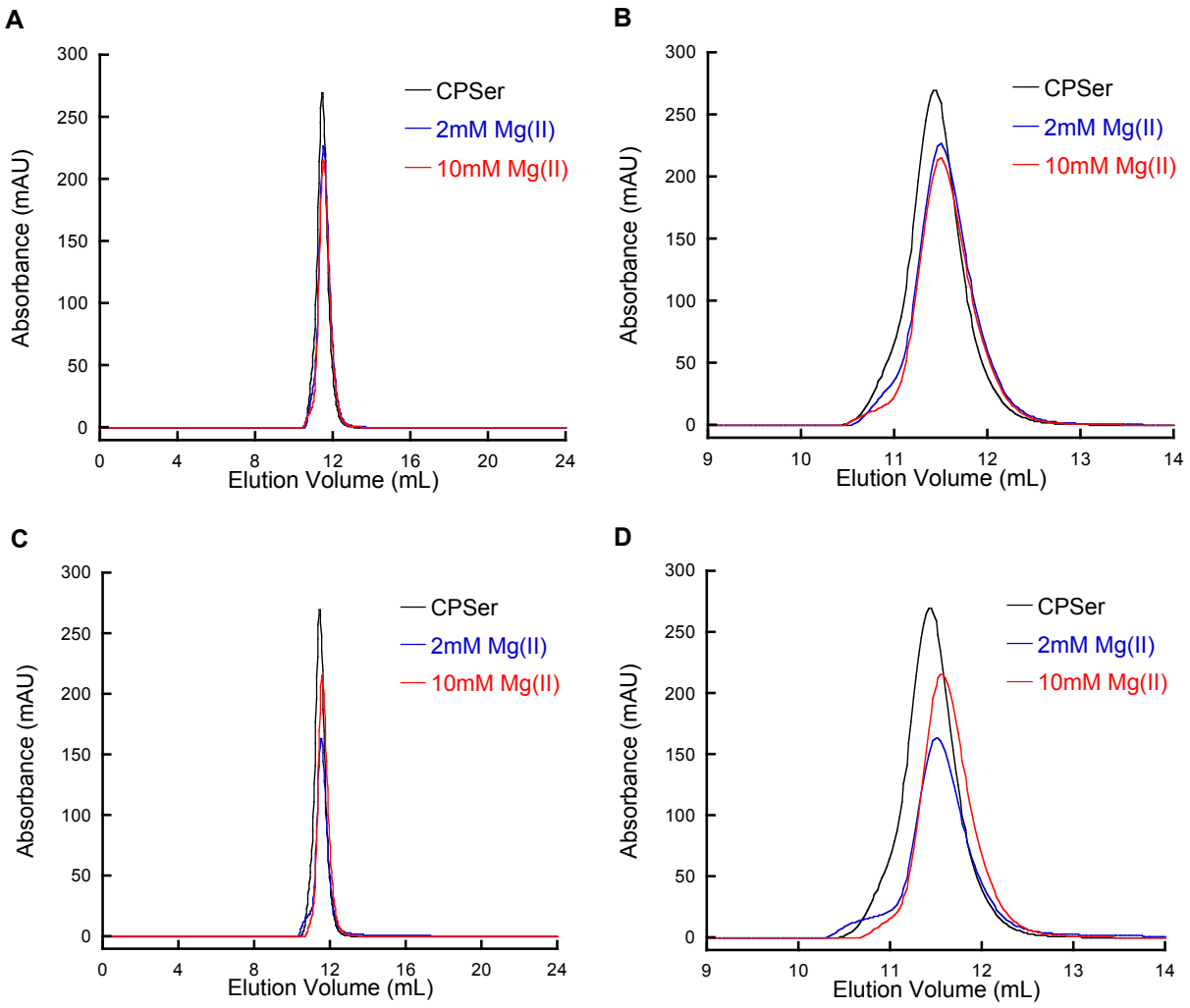
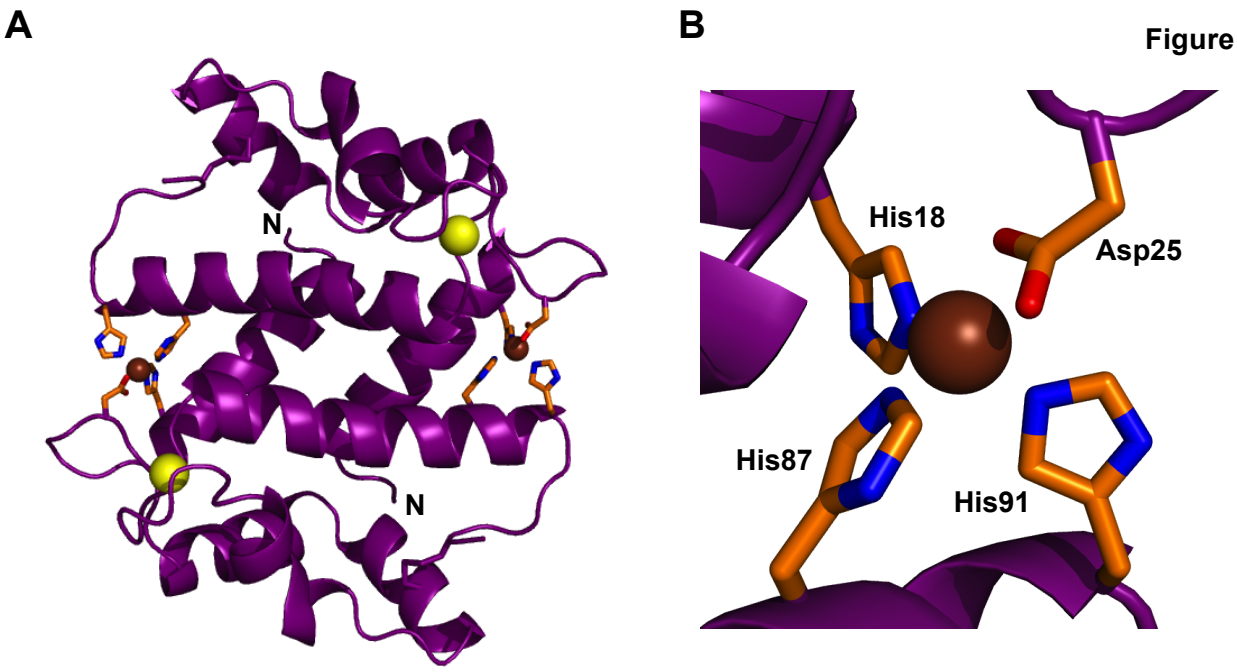


Figure S43. Analytical SEC of CP-Ser in the presence of Mg(II). (**A**) No Mg(II) in the running buffer, overnight equilibration at 4 ^{o}C, full chromatogram. (**B**) No Mg(II) in the running buffer, overnight equilibration at 4 ^{o}C, zoom. (**C**) Mg(II) in the running buffer, full chromatogram. (**D**) Mg(II) in the running buffer, zoom. All samples (200 μL) contained 100 μM CP-Ser (75 mM HEPES, 100 mM NaCl, pH 7.5). Absorbance was monitored at 280 nm at room temperature.

Figure S44. Complete amino acid sequence alignment of mammalian S100A9 polypeptides.



Figure

S45. X-ray crystal structure of Ca(II)- and Zn(II)-bound human S100A7 (PDB: 2PSR).[S5] (**A**) The S100A7 homodimer. Each S100A7 monomer is shown in purple and Zn(II)-binding residues are presented in orange. The Ca(II) ions are shown as yellow spheres, and the Zn(II) ions are shown as chocolate spheres. (**B**) An expansion of the Zn(II)-binding site. Zn(II) is coordinated at the dimer interface by His18 and Asp25 from one subunit, and His87 and His91 from the second subunit. The residues are numbered starting from Met1.

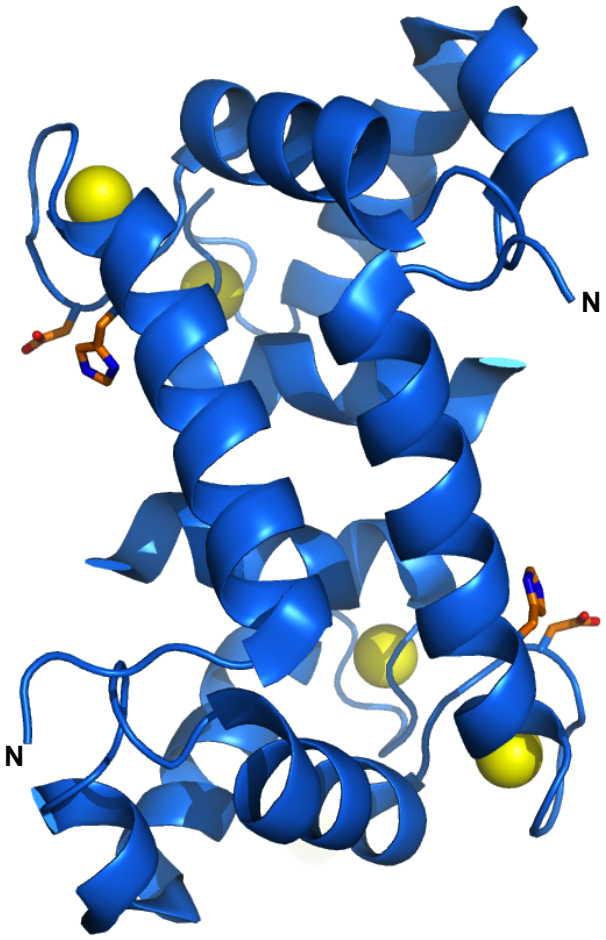


Figure S46. X-ray crystal structure of the Ca(II)-bound human S100A9 homodimer (PDB: 1IRJ).[S6] The Ca(II) ions are shown as yellow spheres, and the side chains of His20 and Asp30 are shown as orange sticks. Residues 87-114 are disordered, and no transition metal-binding motifs are observed in the human A9 homodimer. Nevertheless, the murine form of this protein is reported to coordinate Zn(II).[S7] The residues are numbered starting from Met1.

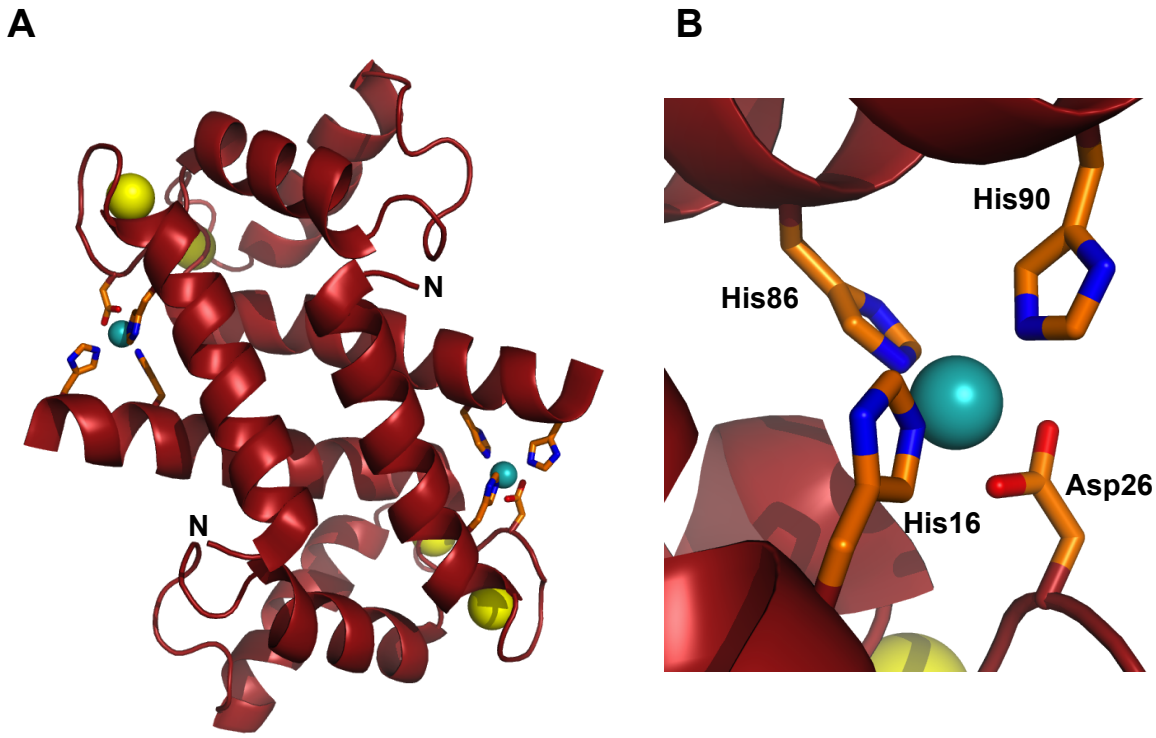


Figure S47. X-ray crystal structure of the Ca(II)- and Cu(II)-bound form of S100A12 (PDB: 1ODB).[S8] (**A**) The S100A12 dimer is shown in red, and the Cu(II)-binding residues are shown as orange sticks. Ca(II) ions are shown as yellow spheres, and Cu(II) ions are shown as teal spheres. (**B**) An expansion of the Cu(II)-binding site. Cu(II) is coordinated at the dimer interface by His16 and Asp26 from one subunit, and His86 and His90 from the second subunit. The residues are numbered starting from Met1.

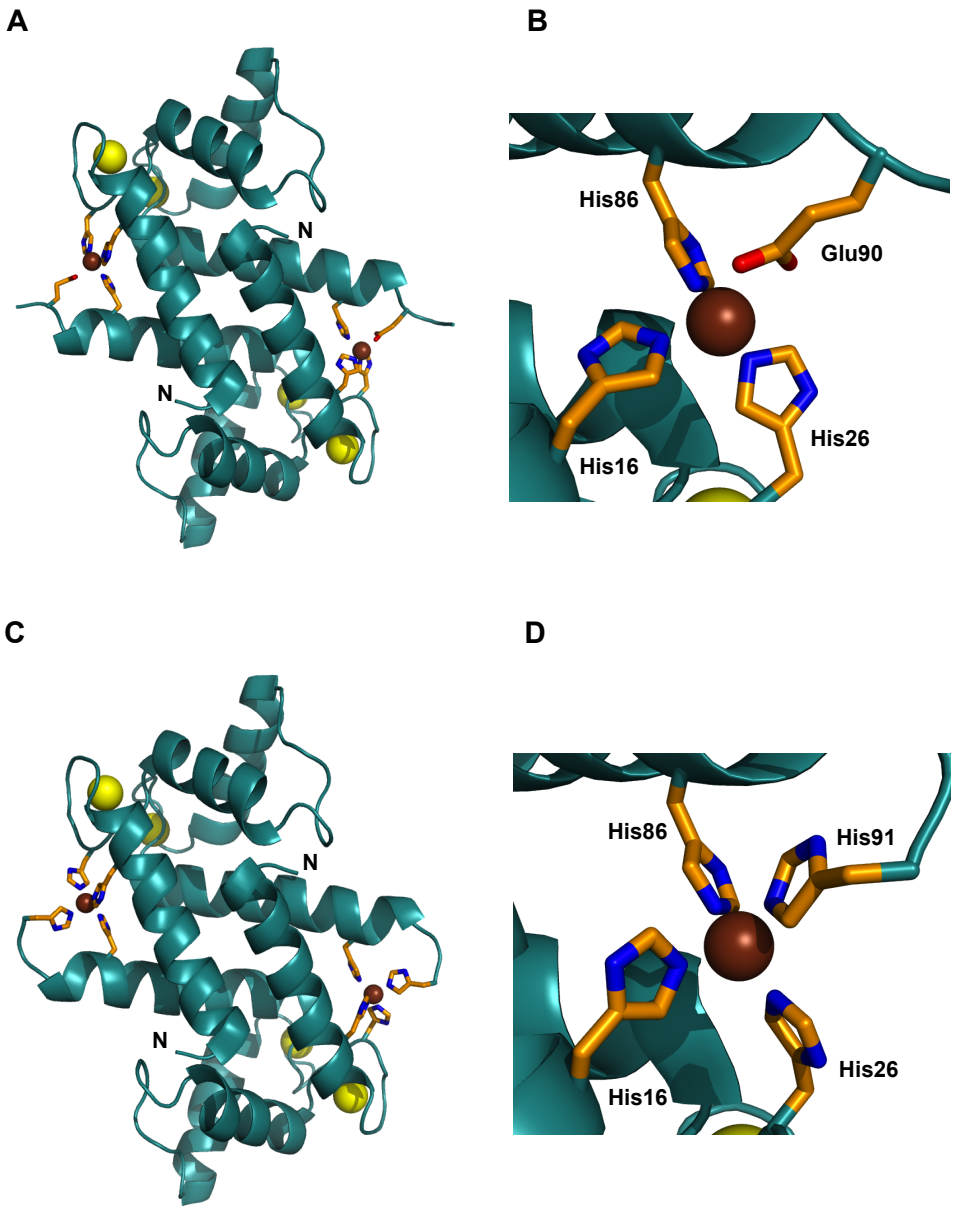


Figure S48. X-ray crystal structures of the Ca(II)- and Zn(II)-bound forms of S100B at pH 10.0 (**A,B**) and pH 9.0 (**C,D**). (**A**) The S100B dimer crystallized at pH 10.0 (PDB 3D10).[S9] S100B is shown in teal, and metal-binding residues are shown as orange sticks. Ca(II) ions are shown as yellow spheres, and Zn(II) ions are shown as chocolate spheres. (**B**) The His_3Glu Zn(II)-binding motif of S100B at pH 10.0 is formed by His16 and His26 from one subunit, and His86 and Glu90 from the second subunit. (**C**) The S100B dimer crystallized at pH 9.0 (PDB 3CZT).[S7] (**D**) The His_4 Zn(II)-binding motif of S100B at pH 9.0 is formed by His16 and His26 from one subunit, and His86 and His91 from the second subunit.

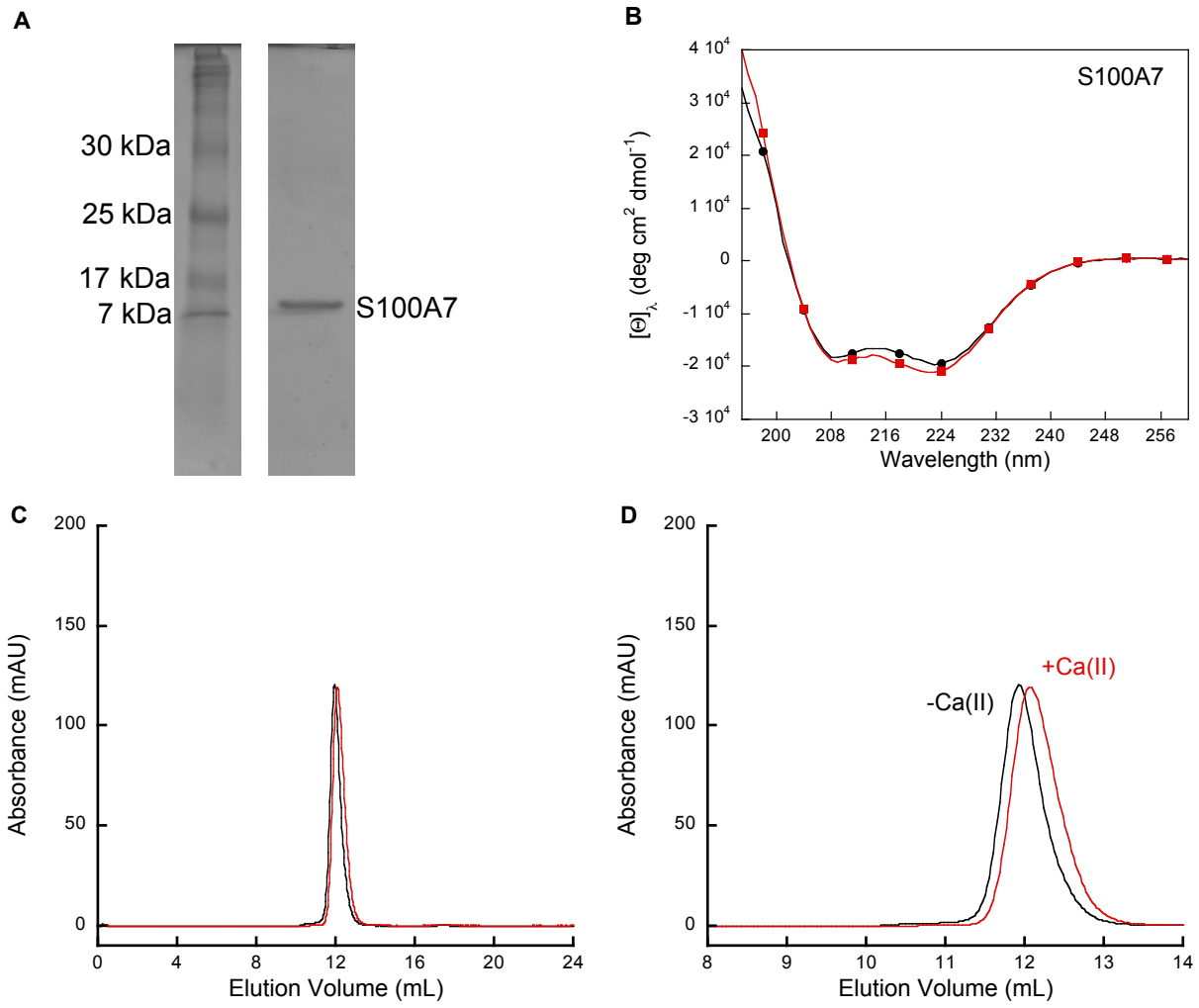


Figure S49. Characterization of homodimeric S100A7. (**A**) SDS-PAGE of purified S100A7 (15% Tris-HCl, glycine gel). Lane 1: P7708S pre-stained gel ladder (New England Biolabs). Lane 2: Pure S100A7 after gel filtration chromatography. The two lanes are from the same gel. (**B**) CD spectra of 10 μM S100A7 in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 °C). (**C**) and (**D**) Analytical SEC of 100 μM S100A7 (200 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**C**) Full chromatograms. (**D**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

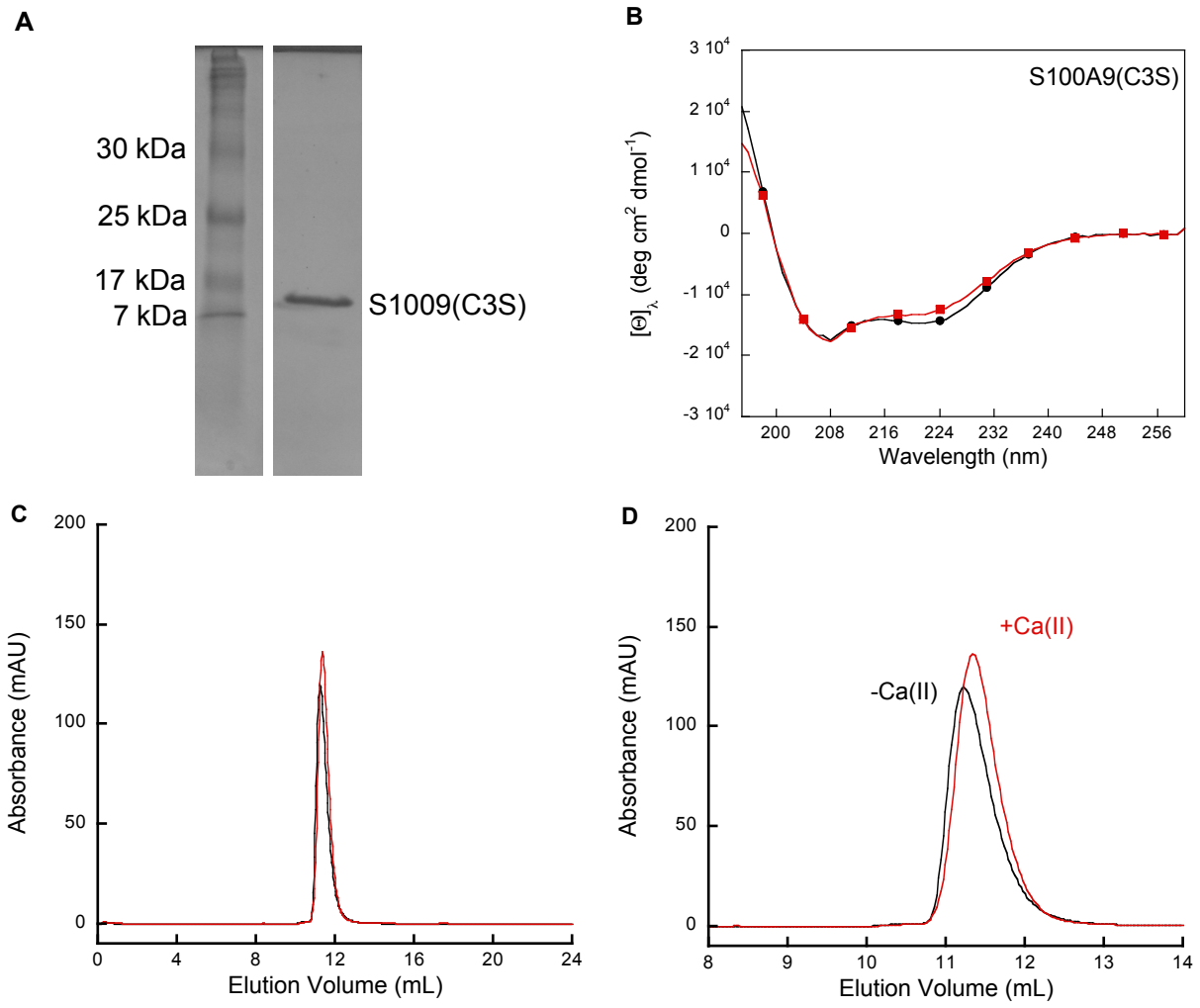


Figure S50. Characterization of homodimeric S100A9(C3S). (**A**) SDS-PAGE of purified S100A9(C3S) (15% Tris-HCl, glycine gel). Lane 1: P7708S pre-stained gel ladder (New England Biolabs). Lane 2: Pure S100A9(C3S) after gel filtration chromatography. The two lanes are from the same gel. (**B**) CD spectra of 10 μM S100A9(C3S) in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 °C). (**C**) and (**D**) Analytical SEC of 100 μM S100A9(C3S) (200 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**C**) Full chromatograms. (**D**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

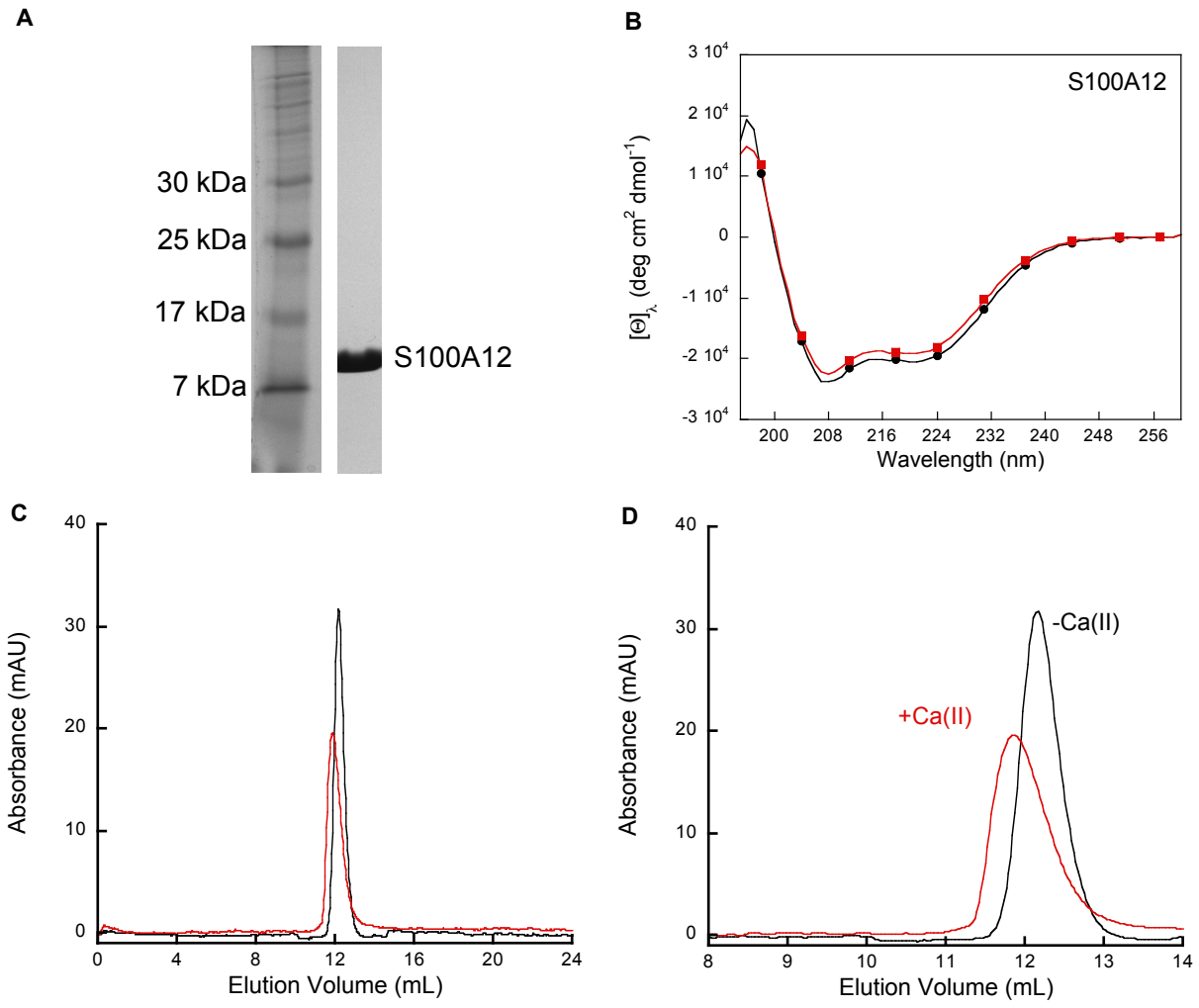


Figure S51. Characterization of homodimeric S100A12. (**A**) SDS-PAGE of purified S100A12 (15% Tris-HCl, glycine gel). Lane 1: P7708S pre-stained gel ladder (New England Biolabs). Lane 2: Pure S100A12 after gel filtration chromatography. The two lanes are from the same gel. (**B**) CD spectra of 10 μM S100A12 in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 °C). (**C**) and (**D**) Analytical SEC of 100 μM S100A12 (200 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**C**) Full chromatograms. (**D**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

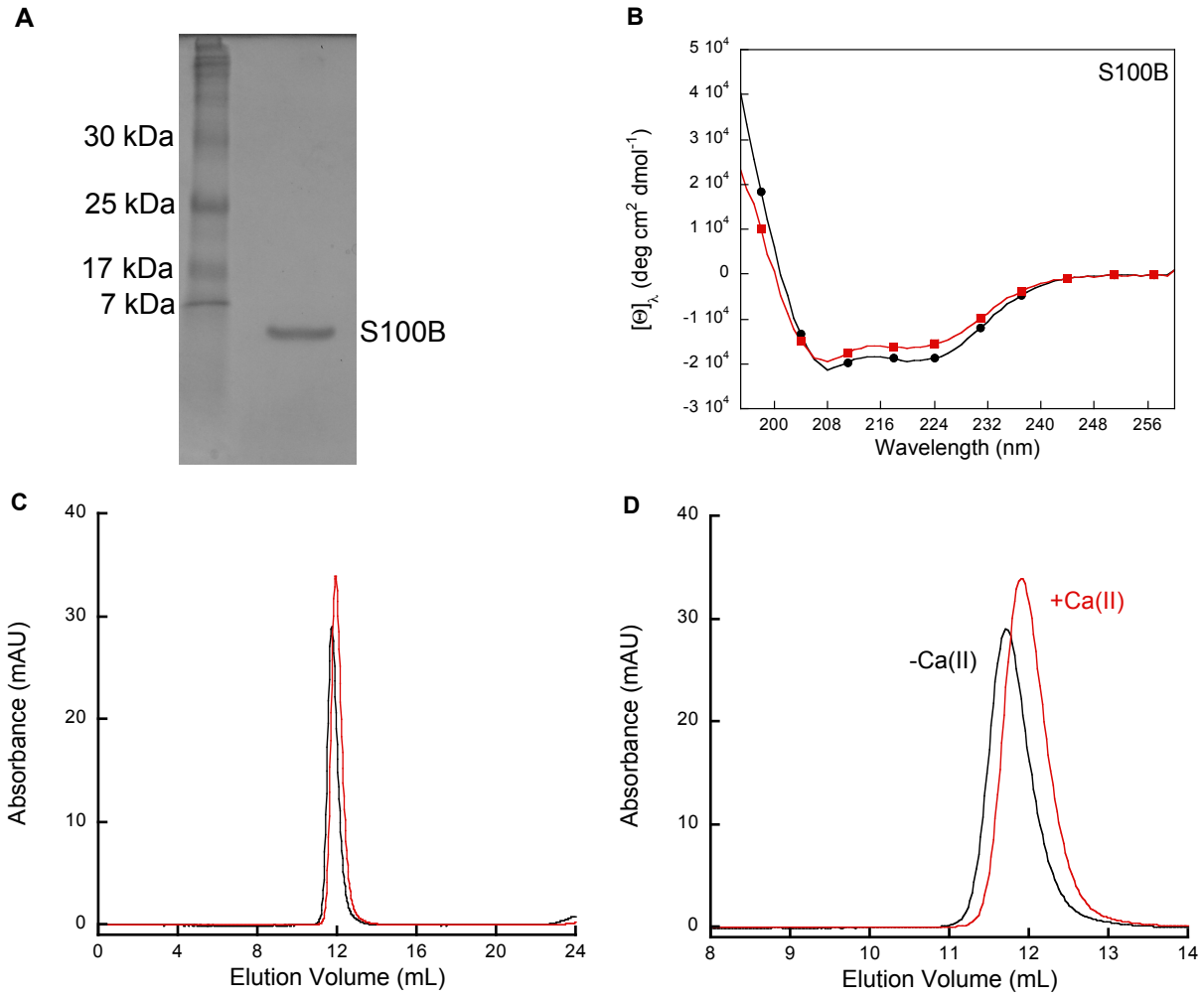


Figure S52. Characterization of homodimeric S100B. (**A**) SDS-PAGE of purified S100B (15% Tris-HCl, glycine gel). Lane 1: P7708S pre-stained gel ladder (New England Biolabs). Lane 2: Pure S100B after gel filtration chromatography. The two lanes are from the same gel. (**B**) CD spectra of 10 μM S100B in the absence (black circles) and presence (red squares) of 2 mM Ca(II) (1 mM Tris-HCl, 0.5 mM EDTA, pH 8.5, T = 25 ^{o}C). (**C**) and (**D**) Analytical SEC of 200 μM S100B (200 μL) in the absence (black line) and presence (red line) of 2 mM Ca(II) in the running buffer (75 mM HEPES, 100 mM NaCl, pH 7.5). (**C**) Full chromatograms. (**D**) Zoom. Absorbance was monitored at 280 nm at room temperature. Elution volumes are provided in Table S8.

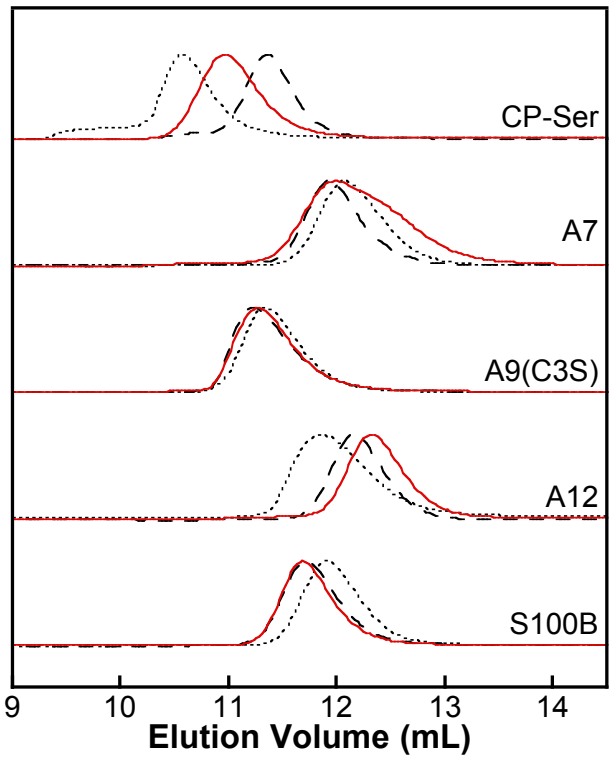


Figure S53. Analytical SEC of S100 proteins in the presence of 10 equiv of Mn(II). The SEC profile in the absence (black dashes) and presence (black dots) of 2 mM Ca(II) is shown for each protein, and the SEC profile in the presence of Mn(II) is shown as a solid red line. The SEC chromatograms for CP-Ser are shown for reference. Protein samples (100 μM or 200 μM) were eluted with 75 mM HEPES, 100 mM NaCl, pH 7.5. The maximum peak height of each trace is normalized to one.

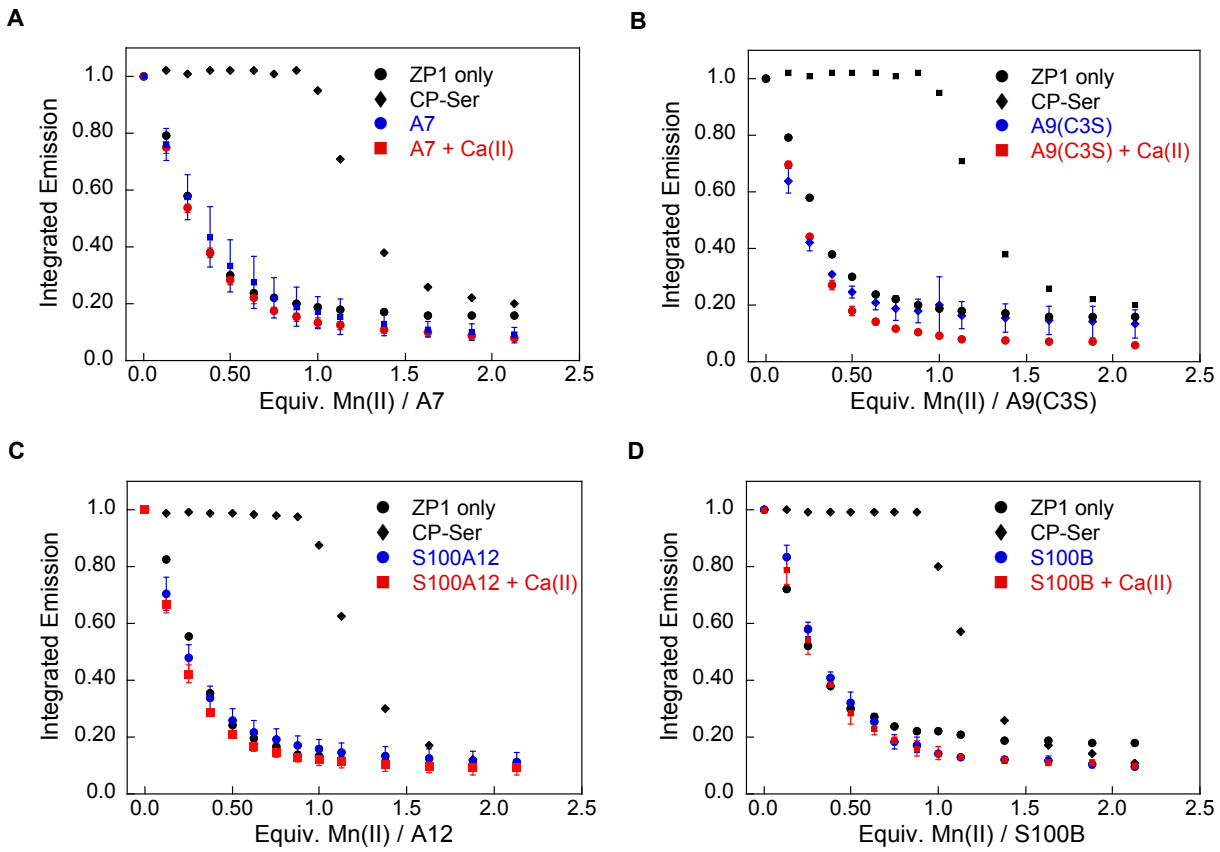


Figure S54. Human S100 homodimers do not compete with ZP1 for Mn(II). (**A**) S100A7, (**B**) S100A9(C3S), (**C**) S100A12, and (**D**) S100B do not compete with ZP1 for Mn(II) in the absence or presence of 200 μM Ca(II) (75 mM HEPES, 100 mM NaCl, pH 7.5). All titrations were performed with 4 μM protein and 1 μM ZP1. All error bars represent the standard deviation from the mean (n = 3).

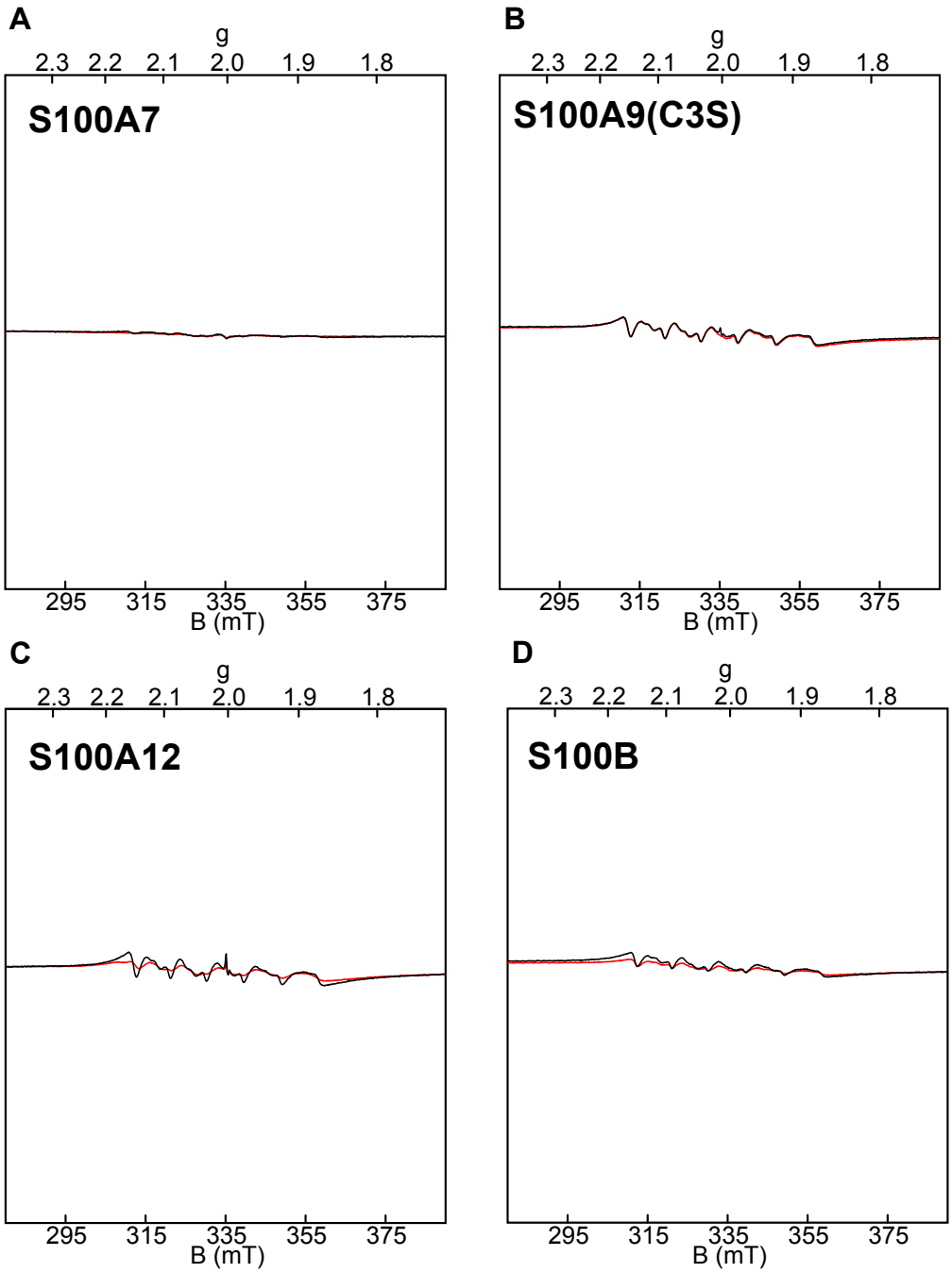


Figure S55. Low temperature EPR spectroscopy of (A) S100A7, (B) S100A9(C3S), (C) S100A12, and (D) S100B. All samples contain 100 μM protein and 30 μM Mn(II) in absence (black line) or presence (red line) of 1 mM Ca(II). All panels contain the same y-axis scale as Figures S38-S40. Instrument conditions: temperature, 20 K; microwaves, 0.2 mW at 9.4 GHz; modulation amplitude, 0.5 mT.

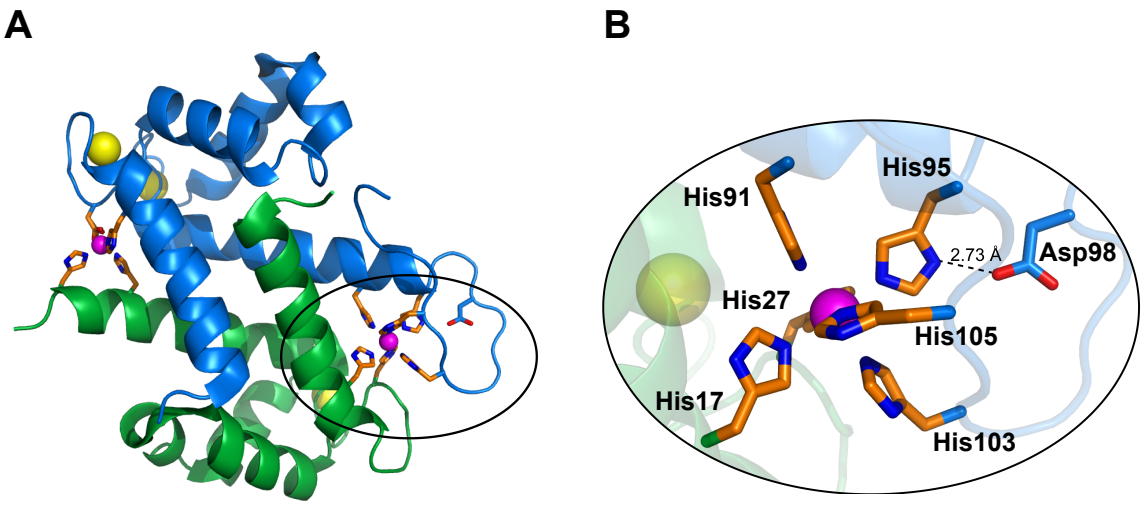


Figure S56. The X-ray crystal structure of Mn(II)- and Ca(II)-bound CP-Ser reveals a hydrogen bonding interaction between (A9)His95 and (A9)Asp98 (PDB: 4GGF).[S10] (**A**) A representation of the Mn(II)- and Ca(II)-bound CP heterodimer taken from the structure of the heterotetramer. The A9 subunit is blue and the A8 subunit is green. The Mn(II)-binding residues are shown as orange sticks, and Asp98 is shown as blue sticks. The Ca(II) ions are depicted as yellow spheres, and the Mn(II) ions are shown as magenta spheres. The region of the protein containing the Mn(II)-His_6 site is circled. (**B**) An expansion of the region circled in panel A. The dashed line represents a hydrogen bond between Nδ of His95 and $O\delta^1$ of Asp98.

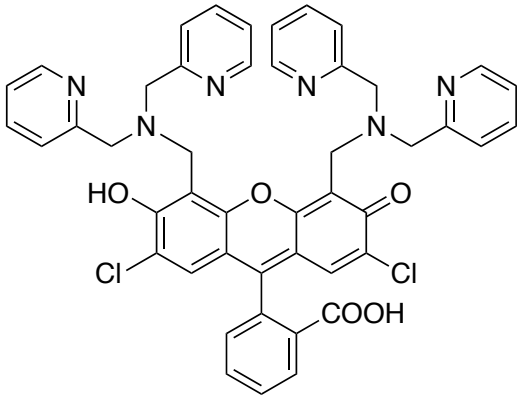


Figure S57. Structure of ZP1.

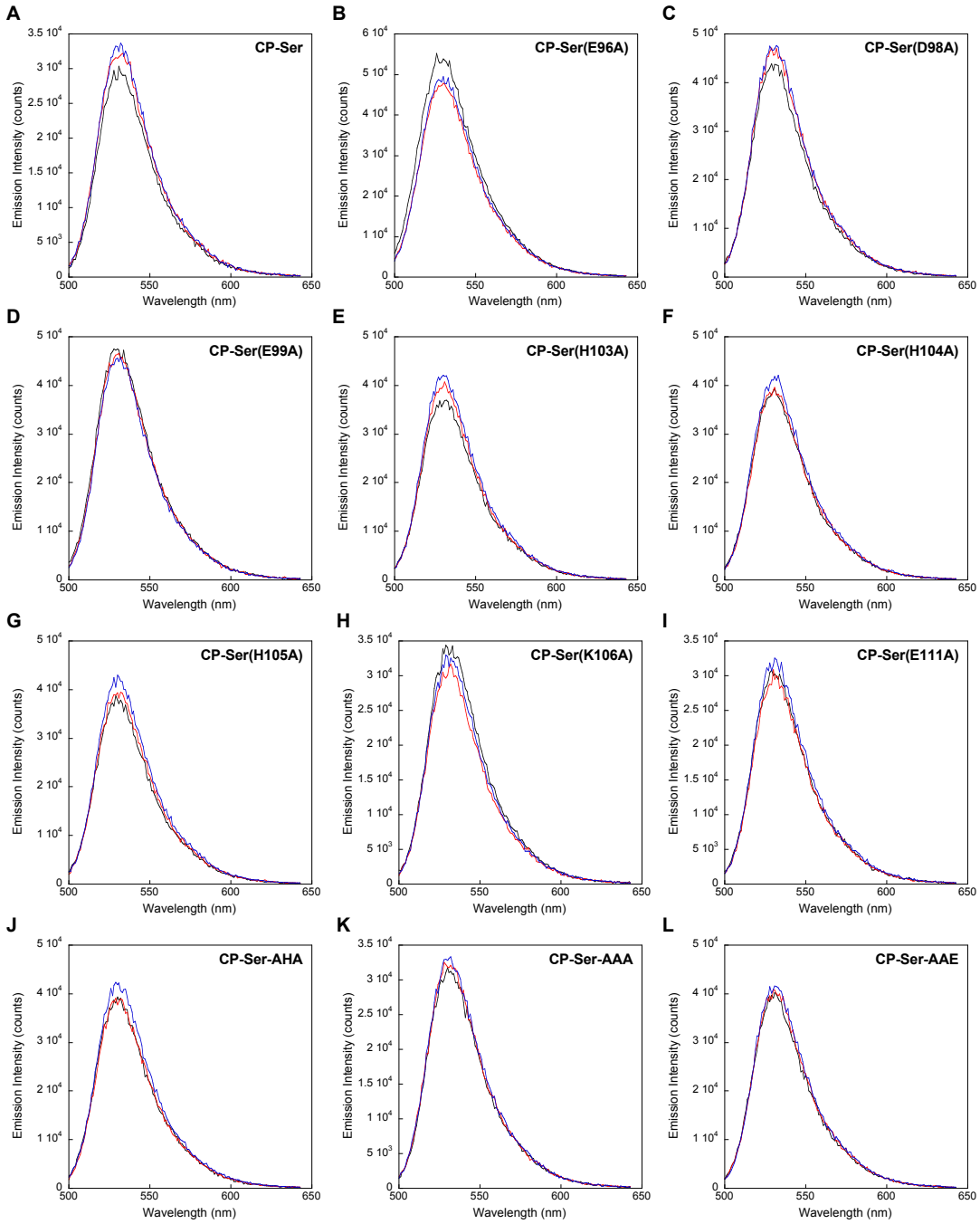


Figure S58. Emission spectra of apo ZP1 in the absence and presence of CP. The emission spectra of 1 μM ZP1 (black), 1 μM ZP1 after the addition of 4 μM protein (red), and after the addition of 50 eq of Ca(II) to the ZP1/protein mixture (blue) at pH 7.5 (75 mM HEPES, 100 mM NaCl). (A) CP-Ser, (B) CP-Ser(E96A), (C) CP-Ser(D98A), (D) CP-Ser(E99A), (E) CP-Ser(H103A), (F) CP-Ser(H104A), (G) CP-Ser(H105A), (H) CP-Ser(K106A), (I) CP-Ser(E111A), (J) CP-Ser-AHA, (K) CP-Ser-AAA, and (L) CP-Ser-AAE. Excitation was provided at 490 nm with slit widths of 0.4 mm and 0.1 sec integration time.

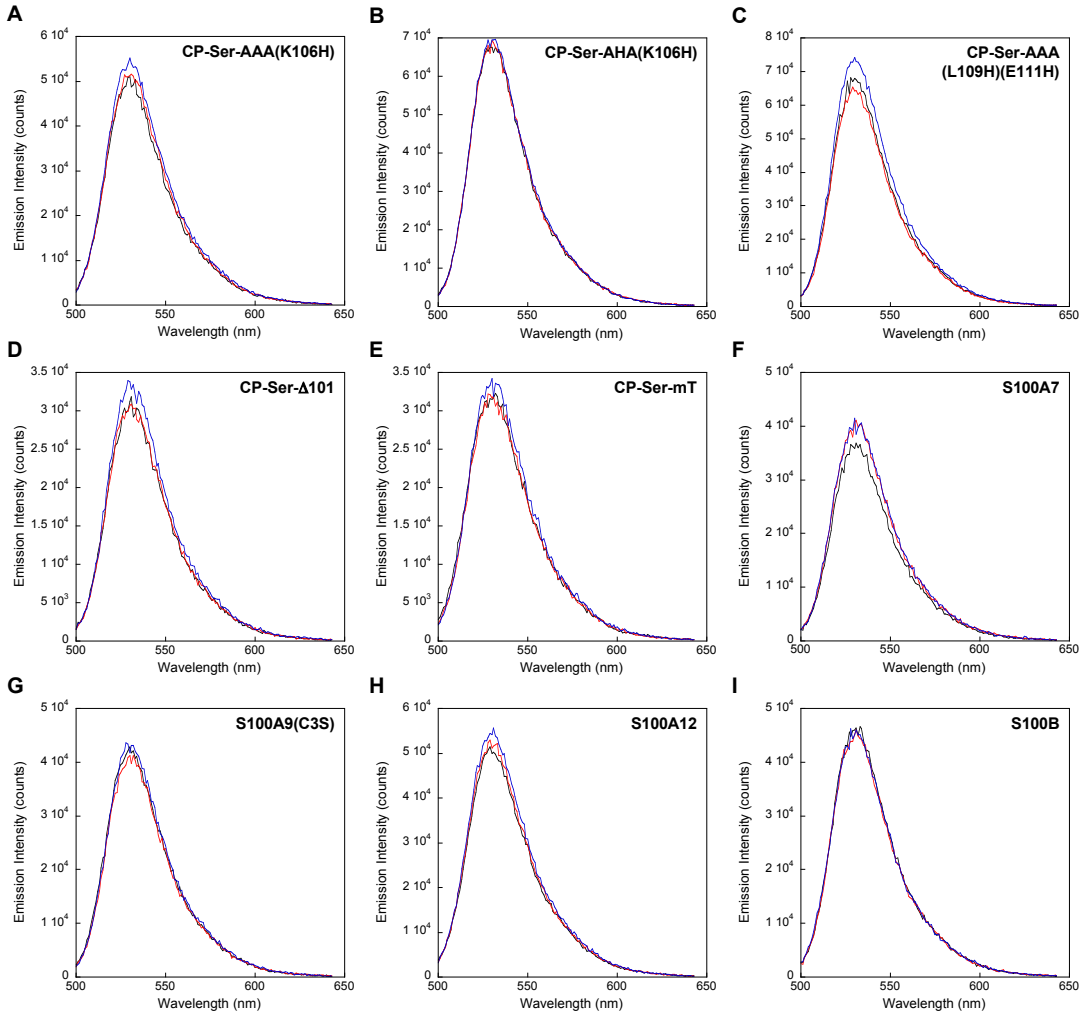


Figure S59. Emission spectra of apo ZP1 in the absence and presence of CP or other S100 proteins. The emission spectra of 1 μM ZP1 (black), 1 μM ZP1 after the addition of 4 μM protein (red), and after the addition of 50 eq of Ca(II) to the ZP1/protein mixture (blue) in at pH 7.5 (75 mM HEPES, 100 mM NaCl). (A) CP-Ser-AAA(K106H), (B) CP-Ser-AHA(K106H), (C) CP-Ser-AAA(L109H)(E111H), (D) CP-Ser-Δ101, (E) CP-Ser-mT (F) S100A7, (G) S100A9(C3S), (H) S100A12, and (I) S100B. Excitation was provided at 490 nm with slit widths of 0.4 mm and 0.1 sec integration time.

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