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S100 Proteins in the Innate Immune Response to Pathogens

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Abstract

S100 proteins are distinct dimeric EF-hand Ca^{2+}-binding proteins that can bind Zn^{2+}, Mn^{2+}, and other transition metals with high affinity at two sites in the dimer interface. Certain S100 proteins, including S100A7, S100A12, S100A8, and S100A9, play key roles in the innate immune response to pathogens. These proteins function via a “nutritional immunity” mechanism by depleting essential transition metals in the infection that are required for the invading organism to grow and thrive. They also act as damage-associated molecular pattern ligands, which activate pattern recognition receptors (e.g., Toll-like receptor 4 RAGE) that mediate inflammation. Here we present protocols for these S100 proteins for high-level production of recombinant protein, measurement of binding affinities using isothermal titration calorimetry, and an assay of antimicrobial activity.

Keywords

S100 proteins; S100A7; S100A12; S100A8; S100A9; Calprotectin; Nutritional immunity; Metal binding; Host-pathogen interaction; Inflammatory response; Protein expression; Protein purification; Isothermal titration calorimetry; Antimicrobial growth assay

1 Introduction

1.1 S100 Proteins

S100 proteins are a unique subfamily of EF-hand calcium (Ca^{2+})-binding proteins that have an integrated dimeric structure and can form higher-order oligomers [1]. Although EF-hand proteins are widely known as second messengers that transduce Ca^{2+} signals, S100 proteins also function in the extracellular milieu [2, 3]. Remarkably, several S100 proteins have been shown to play central roles in the innate immune response to infection by pathogenic organisms, with dual functions via “nutritional immunity” and activation of inflammation [4]. Of the 25 different S100 genes, only S100A7, S100A8, S100A9, and S100A12 have been identified as innate immune modulators.

1.2 Structure and Function of S100 Proteins

S100 proteins contain N-terminal S100-specific and C-terminal canonical, “EF-hand” helix-loop-helix calcium-binding motifs that come together to form the stable four-helix bundle

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characteristic of all EF-hand proteins. However, S100 proteins are distinct among the EF-hand proteins in that the basic structural unit is a completely integrated, obligate dimer [1]. All S100 proteins except the ancestral and C-terminally truncated S100G form homodimers, and a select few form heterodimers. However, S100A8 and S100A9 are unique in that they preferentially form heterodimers [5].

Ca^{2+} binding results in a significant shift in the orientation of helix 3 (Fig. 1), exposing a hydrophobic cleft that mediates binding to target proteins [6]. Because the levels of Ca^{2+} present in the extracellular milieu are so high (in the mM range), when outside cells S100 proteins are constitutively bound to Ca^{2+} and activated for target binding. Besides binding Ca^{2+}, many S100 proteins also bind transition metal ions (e.g., Mn^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+}) [7]. Each S100 dimer has two transition metal-binding sites at symmetrically disposed locations across the dimer interface (Fig. 2) [7, 8]. Binding of transition metals has been characterized for S100A12 [9], S100A7 [10], and S100A8/S100A9 heterodimer (calprotectin, CP) [8]. S100A7 and S100A12 have canonical sites comprised of three histidine and one aspartic acid residue that coordinate Zn^{2+} or Cu^{2+} in a tetrahedral fashion [7]. CP has two distinct transition metal-binding sites. One is specific to CP and is comprised of six histidine residues arranged in an octahedral coordination sphere, capable of binding Mn^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+} and Fe^{2+} (S1). The other (S2) is a canonical site that can bind Zn^{2+} and Cu^{2+}. Although binding of transition metal ions minimally perturbs S100 protein structures, high concentrations of these ions (and also Ca^{2+}) induce the formation of tetramers and higher-order oligomers. In some cases, it appears that these oligomeric states have a key role for function [3, 11].

1.3 S100 Proteins in Nutritional Immunity

During infections, pathogens acquire nutrient metals from the host, while the host in turn limits the availability of nutrient metals [12, 13]. S100 proteins play a central role in restricting the bio-availability of nutrient transition metals, a mechanism termed “nutritional immunity” [14]. In keeping with this, increasing dietary transition metals can overcome this defense and in turn cause detrimental effects for the host during infection. For example, dietary iron supplementation enhances *Mycobacterium avium* [15] and *Mycobacterium tuberculosis* [16] infection, excess dietary zinc heightens *Clostridium difficile* colonization of the gut [17], and elevated dietary manganese increases susceptibility of the heart to *Staphylococcus aureus* infection [18]. Consistent with CP playing a critical role during infection, a deficiency in CP enhances susceptibility of the murine host to numerous bacterial and fungal pathogens [19–24]. S100A12 inhibits *Campylobacter jejuni* [25] and *Helicobacter pylori* [26] through Zn^{2+}-dependent sequestration. Additionally, S100A12 exhibits antimicrobial activity against multiple parasites, possibly through the binding of copper, which enhances the production of superoxide [27, 28]. In 2007, it was reported that S100A7 and S100A15 have antimicrobial activity toward *Escherichia coli* during infections of the skin [29, 30]. In addition to limiting metal nutrients, S100A7 can directly adhere to and reduce the survival of *E. coli* in the skin [31]. These observations suggest that the ability of S100 proteins to bind a variety of nutrient metal ions is critical in establishing nutritional immunity and protection during infections.

1.4 S100 Proteins in the Inflammatory Response

In addition to nutritional immunity, S100 proteins play diverse roles as immunomodulators. S100A7 and S100A15 proteins are expressed in keratinocytes [32, 33], whereas CP and S100A12 are primarily expressed in monocytes, macrophages, and neutrophils [28, 34, 35]. However, during the inflammatory response, CP and S100A12 are also expressed in keratinocyte, endothelial, and epithelial cells [36–39]. Secreted CP can act as a potent chemoattractant for monocytes, macrophages, and neutrophils during inflammation [40, 41]. As a result, CP inhibits immune cell growth or induces apoptosis [17]. In addition, S100 proteins can act as damage-associated molecular patterns by binding pattern recognition receptors including TLR4 [42], RAGE [43, 44], and CD33 [45]. Misregulation of S100 protein expression is implicated in chronic inflammation [46], as overactivation of the pattern recognition receptors TLR4 and RAGE drives NF-κB production and recruitment of neutrophils, monocytes, and macrophages. For example, high levels of calprotectin and S100A12 have been associated with inflammatory bowel disease and rheumatoid arthritis and high levels of S100A7 with inflammatory skin diseases (e.g., psoriasis, atopic dermatitis) [17, 30, 44]. Altered expression of S100 proteins also correlates with multiple autoimmune disorders including systemic lupus erythematosus, Still's, and Sjogren's [47–50]. Given the numerous associations to inflammation and infections, S100 proteins have been targeted for potential therapeutic effects [51].

2 Materials

2.1 Expression and Purification of S100 Proteins

2.1.1 Reagents and Solutions

1. pGEMEX plasmid containing S100A12.
2. BL21 (DE3) competent cells.
3. LB/agar plates supplemented with 100 μg/mL ampicillin.
4. Autoclaved Luria broth (LB) growth media.
5. 100 mg/mL ampicillin solution sterile-filtered prior to use
6. Ammonium sulfate.
7. Lysis buffer: 20 mM Tris-HCl (pH 8.0).
8. Q buffer A: 20 mM Tris-HCl (pH 8.0).
9. Q buffer B: 20 mM Tris-HCl (pH 8.0), 1 M NaCl.
10. SEC buffer: 20 mM Tris-HCl (pH 8.0), 100 mM NaCl.
11. SDS-PAGE buffers and Coomassie stain.

2.1.2 Equipment

1. Autoclave.
2. 0.45 and 0.2 μm syringe filters.

3. Plastic syringes.
4. Appropriate flasks and petri dishes.
5. Shaking incubator.
6. Sonication system.
7. Centrifuges.
8. Dialysis tubing with a 1 kDa MWCO.
9. 10 kDa MWCO concentrators.
10. FPLC system.
11. Q-sepharose column.
12. S75 column.
13. SDS-PAGE system and gels.

2.2 Determination of Transition Metal Affinities of S100 Proteins

2.2.1 Reagents and Solutions

1. CP protein (~2 mL at 10–50 μM) in ITC buffer.
2. ITC buffer: 20 mM HEPES (pH 7.5), 75 mM NaCl.
3. $Zn(OAc)_2$ in ITC buffer.
4. 1 M $CaCl_2$.

Equipment

1. VP-ITC titration calorimeter.
2. Degassing station.

2.3 Antimicrobial Growth Assay

Reagents and Solutions

1. Tryptic Soy Agar (TSA) plate.
2. Tryptic Soy Broth (TSB).
3. CP protein in CP buffer.
4. CP buffer: 20 mM Tris (pH 7.5), 100 mM NaCl, 3 mM $CaCl_2$, 10 mM BME, filtered.

Equipment

1. 96-well microtiter plate.
2. Plastic wrap.
3. UV/Vis spectrophotometer.

3 Methods

3.1 Expression and Purification of S100 Proteins

A lac operon-based system is used for bacterial expression of S100 proteins. Following standard protocols, *Escherichia coli (E. coli)* cells are transformed with a plasmid containing the gene of interest, grown to a high optical density, and stimulated to express the protein of interest by the addition of isopropyl β-D-1-thiogalacto-pyranoside (IPTG). After cell lysis, the protein is purified from the soluble lysate. Purification takes advantage of the extraordinary structural stability of S100 proteins. The name S100 is derived from the observation that these proteins remain soluble in 100% ammonium sulfate [52]. Hence, the first step of the purification is an ammonium sulfate precipitation that increases the ionic strength of the solution and results in the precipitation of most of the endogenous *E. coli* proteins, while S100 remains in solution. The next step is anion-exchange chromatography for further separation of impurities by differences in isoelectric point. The last step of the purification involves size-exclusion chromatography to separate the S100 protein from its impurities by size. Finally, mass spectrometry of the purified product confirms the identity of the protein of interest. Protocols for S100A7, S100A8, S100A9, S100A12, and CP have been published [5, 14, 53–55]. Here we provide a specific detailed example, an optimized 1 L expression and purification protocol for human S100A12.

3.1.1 Procedure

Expression

1. Transform pGEMEX-S100A12 into BL21 (DE3) chemically competent cells using a standard transformation protocol [56] (*see* Note 1).
2. Plate 100 μL of transformation solution on LB/agar plate supplemented with ampicillin, and grow 12–16 h at 37 °C.
3. Pick a single colony from the freshly transformed plate, and inoculate 25 mL of LB media supplemented with antibiotic.
4. Grow this culture in a shaking incubator at 250 rpm for 12–16 h at 37 °C.
5. In the morning, inoculate 10 mL of the culture to inoculate 1 L of LB supplemented with antibiotic (*see* Note 2).
6. Grow this culture at 37 °C in a shaking incubator at 250 rpm.
7. While shaking the culture, monitor cell growth. Once the optical density at 600 nm ($O.D._{600}$) is between 0.6 and 0.8 absorbance units, add IPTG to a concentration of 0.5 mM.
8. Allow the culture to grow at 37 °C in a shaking incubator at 250 rpm for 12–16 h.
9. Harvest the cells by centrifugation at 5000 × *g* for 20 min, and freeze the pellets at –80 °C.

Purification

1. Thaw the frozen cell pellet at room temperature, and resuspend in 30 mL of lysis buffer.
2. Sonicate on ice at 40% amplitude (5 s on, 5 s off) for 5 min (*see* Note 3).
3. Clarify the lysate by centrifugation at 20,000 × *g* for 25 min at 4 °C.
4. The S100A12 protein will be in the supernatant (*see* Note 4). Decant supernatant into 100 mL glass beaker and place the beaker on ice. Add a stir bar and slowly add ammonium sulfate to 60% saturation (*see* Note 5). Allow the solution to stir in the cold room for 1 h.
5. Clarify the solution by centrifugation at 20,000 × *g* for 25 min at 4 °C.
6. Transfer the supernatant into dialysis tubing. Dialyze against 2 L of Q buffer A at 4 °C. Change the dialysis buffer twice, allowing at least 4 h in between changes (*see* Note 6).
7. Using the FPLC system, equilibrate a Q-Sepharose column with 4 column volumes (CV) of Q Buffer A.
8. Load the protein sample and collect flow through.
9. Wash the column with four CV of Q buffer A.
10. Elute proteins with a 0–30% NaCl gradient over 15–20 CV using Q buffer B, collecting 5 mL fractions.
11. Take an aliquot of each fraction and run SDS-PAGE with Coomassie staining.
12. Pool the fractions containing S100A12 protein. The protein has a molecular weight of ~10.5 kDa.
13. Concentrate the fractions to <5 mL using a 10 kDa MWCO concentrator.
14. Equilibrate an S75 column with 1 CV of SEC buffer (*see* Note 7).
15. Load the concentrated S100A12 protein onto the SEC column.
16. Elute the protein over 1 CV, collecting 5 mL fractions.
17. Take an aliquot of each fraction, and run SDS-PAGE with Coomassie staining.
18. Pool together the S100A12 protein fractions, and validate the protein identity using mass spectrometry.
19. Measure the absorbance of the protein using a spectrophotom¬eter in order to obtain the concentration. The extinction coef¬ficient of S100A12 is 5960 M–1 cm–1 (*see* Note 8).
20. Concentrate protein to ~5–10 mg/mL. Concentrated protein will reduce the risk of degradation. Aliquot into 1.5 mL Eppendorf tubes, and flash freeze using liquid nitrogen. Store the protein at –80 °C (*see* Note 9).

3.2 Determination of Transition Metal Affinities of S100 Proteins by Isothermal Titration Calorimetry

Determining transition metal ion-binding affinities of S100 proteins is one key to understanding their role at the host-pathogen interface as this informs on their ability to function via the nutritional immunity mechanism, i.e., outcompete bacterial metal ion transporters for the nutrient metals [53]. Many techniques have been implemented to determine the dissociation constants (K_d) for S100 proteins, which have been reported in the μM-pM range [7]. Isothermal titration calorimetry (ITC), which measures the amount of heat released or absorbed upon binding, has been used to measure μM-nM transition metal ion-binding affinities of S100 proteins [8, 57, 58]. ITC estimates of the binding affinities of CP for Zn^{2+} (S1–3 nM, S2–8 nM) and Mn^{2+} (S1–6 nM) have been reported (Fig. 3). Additional methods for determining metal ion affinities include optical absorption spectroscopy [53], electron paramagnetic resonance spectroscopy [53], competition chelator fluorescence experiments [53, 59], and equilibrium gel filtration [60, 61], each method having its own set of advantages and limitations. Competition chelator fluorescence measurements are the most sensitive and are able to measure K_d values in the pM range, although this method requires transition metal-specific fluorescence dyes and can be complicated by interference from Ca^{2+} [62]. Here, we provide a protocol for measuring transition metal binding to CP protein using ITC. This is the only experimental method that allows for the complete thermodynamic characterization of a reaction (ΔH, ΔG, and ΔS) and also provides the stoichiometry of the reaction.

3.2.1 Procedure

1. Dialyze CP protein (10–50 μM) in ITC buffer for Replace with fresh buffer twice for a total of three dialysis exchanges (*see* Note 10).
2. Prepare a 100–750 μM $Zn(OAc)_2$ solution using the dialysis ITC buffer. Save the remaining dialysis buffer for rinsing the syringe and sample cell of the ITC instrument (*see* Note 11).
3. Add stoichiometric amount of $CaCl_2$ to both the protein and the metal solutions.
4. Degas the protein and metal ion solutions for 10 min.
5. Rinse the ITC sample cell three times with ITC buffer.
6. Add the CP protein to the sample cell of the ITC instrument. Ensure that no air bubbles are present in the syringe.
7. Load the $Zn(OAc)_2$ solution into the ITC titration syringe.
8. Titrate the metal ion solution into the protein. We recommend starting with 50 injections, each of 6 μL and 210 s between each injection. The number of injections, injection volumes, and the time between injections must be optimized depending on the metal-binding affinities and the heat absorbed or released upon binding of the system. Before performing another injection, make sure that the heat from each injection is fully dissipated (*see* Note 12).

9. To correct for the heat of dilution, perform a metal solution to buffer titration after thoroughly rinsing out the sample cell with the ITC buffer. Repeat **steps 2–8**, except add only ITC buffer to the sample cell (*see* Note 13).

10. To process the data, subtract the buffer isotherm from the protein isotherm. Fit a single-site binding model to the data using the VP-ITC Origin software (Northampton, MA), and obtain the thermodynamic parameters (*see* Note 14). An example of data depicting the titration of CP mutants with $ZnSO_4$ is shown in Fig. 3.

3.3 Antimicrobial Growth Assay

The ability of S100 proteins to sequester nutrient transition metals inhibits growth of pathogens [20, 22, 26]. Here we describe an antimicrobial growth assay that measures the activity of WT and mutant CP, monitoring the ability of CP to inhibit the growth of *Staphylococcus aureus.* This assay verifies the ability of CP to sequester transition metal ions. The results of the assay are reported as $O.D._{600}$ plotted over time, quantifying bacterial growth in the presence of decreasing concentrations of CP (Fig. 4)[20]. Typically, CP at a concentration ≥ 75 μg/mL is sufficient to inhibit the growth of *S. aureus* in minimal media; however, the inhibition of bacterial growth is also species dependent with different concentrations of CP required for inhibition. *Salmonella* is greater than 125 μg/mL. *Acinetobacter baumannii* is greater than 37.5 μg/mL. *Helicobacter pylori* is greater than 300 μg/mL. *Pseudomonas aeruginosa* and *Clostridium difficile* require 1 mg/mL. Inhibition of bacterial growth by CP could vary based on transition metal enrichment of the media chosen to facilitate bacterial growth. This same assay can be implemented to probe the antimicrobial activity of other S100 proteins such as S100A12 [26].

1. Streak a TSA plate with *S. aureus*, and incubate at 37 °C for 16–18 h (*see* Note 15).

2. Select a single colony of *S. aureus*, start a 5 mL overnight culture in TSB, and incubate at 37 °C on a shaker for 16–18 h at 180 rpm.

3. Back dilute the overnight culture 1:50 in fresh TSB, and incu¬bate at 37 °C on a roller drum for 1 h at 40 rpm.

4. During the incubation, prepare a 96-well microtiter plate as follows:

   (a) Rows B-H will each contain 62 μL CP buffer and 38 μL TSB (*see* Note 16).

   (b) Row A will contain the highest concentration of CP with CP buffer to a total volume of 124 μL. In addition, each well in this row will have 76 μL TSB. For example, if you want 200 μg of protein in row A and have a protein stock that is 10 μg/μL, you would add 20 μL protein stock to 104 μL CP buffer.

   (c) The CP stock should be thawed and supplemented with 3 mM $CaCl_2$ prior to use (*see* Note 17).

(d) To create a 1:2 serial dilution of CP for this assay, transfer 100 μL from row A into row B. Pipet to mix, and transfer 100 μL from row B to row C. Repeat until you transfer 100 μL from row F into row G. After mixing row G, remove 100 μL from row G so that there is a final volume of 100 μL. Do not transfer row G to row H. Row H should only contain CP buffer and TSB without protein (*see* Note 18).

(e) After adding all the media components to the wells, briefly centrifuge (800 r.c.f., 2 min) the plate to remove bubbles.

5. After 1 h, add 1 μL of back-diluted culture from **step 3** to each well.

6. Measure initial $O.D._{600}$ to establish background and 0 h time point (*see* Note 19).

7. Incubate the microtiter plate at 37 °C with shaking. Wrap the microtiter plate in plastic wrap to prevent evaporation *(see* Note 20).

8. Take time points every 1–2 h. Vortex the microtiter plate prior to reading the $O.D._{600}$ to resuspend any bacteria that have settled to the bottom. Representative data for antimicrobial activity of WT CP against *S. aureus* are shown in Fig. 4.

4 Notes

1. S100 proteins may also be purified from *E. coli* using a lac operon-based system with a plasmid-containing N-terminal 6 × His tag. The protein can then be purified by immobilized metal affinity chromatography (IMAC). This is the case for S100A7 and S100A15 [55]. A plasmid-containing N-terminal 6× His tag has been reported for S100A4 with purification via IMAC followed by anion-exchange chromatography [63].

2. The use of auto-inducing media in place of LB can significantly increase yields for S100A12 [54, 64]. Whether this is true for other S100 proteins must be experimentally verified.

3. S100 proteins are stable enough to perform the purification at room temperature with the exception of the sonication, centrifugation, and ammonium sulfate precipitation steps.

4. Purification of CP requires a protein-refolding step before the anion-exchange column [5, 14, 53], as S100A8–S100A9 are expressed into inclusion bodies rather than in the supernatant.

5. The optimum ammonium sulfate percentage must be optimized for each protein.

6. For S100 proteins containing cysteine residues, it may be necessary to include BME (or other reducing agents) to the buffers right before use to prevent disulfide bond formation.

7. Adding 0.5–5 mM of $CaCl_2$ to the chromatography buffers may increase protein stability and yields. However, this must be removed by the addition of EDTA and dialysis prior to storing the protein, as Ca^{2+} induces S100 protein oligomerization.

8. Typical yields for S100 proteins using this protocol are 35–45 mg per 1 L of culture. However, the optimal induction time must be empirically determined for each protein.

9. To maintain S100 protein activity, avoid repeated freeze-thaw cycles.

10. The protein and metal ion concentrations have to be accurately determined as this will affect the results. Protein concentrations can be experimentally determined with the use of the extinction coefficients and absorbance at 280 nm.

11. It is critical that the protein in the sample cell and the metal ion solutions in the syringe have the *exact* same buffer. The best way to ensure that the buffer is exactly the same is to prepare the syringe solutions in the dialysis buffer that the protein was dialyzed in. If the buffers are not exactly the same, fluctuations in the data will give false binding results.

12. This ITC procedure can be used for all S100 proteins; however, the number of injections and concentrations might need to be adjusted. It should also be noted that the typical sensitivity of ITC places a lower limit on K_d in the nM range. Methods described above might need to be used if the binding affinities fall outside of this range.

13. Reducing agents can adversely affect the linearity of the base¬line. Whether your system can tolerate the reducing agent in the buffer must be determined empirically. In general, TCEP is preferable to BME, which is preferable to DTT. Another alternative is to make Cys to Ser mutations in your protein.

14. Repeat the experiments in triplicate.

15. This assay can use a variety of different bacterial strains, other than *S. aureus*. If another strain is used, the appropriate media for the chosen bacteria needs to be implemented.

16. A lower amount of CP buffer relative to growth media can be used in order to facilitate bacterial growth; however, this will usually increase the concentration of CP necessary to inhibit growth.

17. Add BME to the CP buffer right before use.

18. For most assays, a twofold dilution of CP starting at a concentration of 100 μg/100 μLin rows A–G will suffice for *S. aureus*.

19. With *S. aureus*, there will be some initial growth at the highest dose of CP, most likely due to residual metal ions stored within the bacteria.

20. It is possible to use an automated plate reader to quantify the $O.D._{600}$. In this situation, it is recommended for the lid to remain closed to avoid evaporation.

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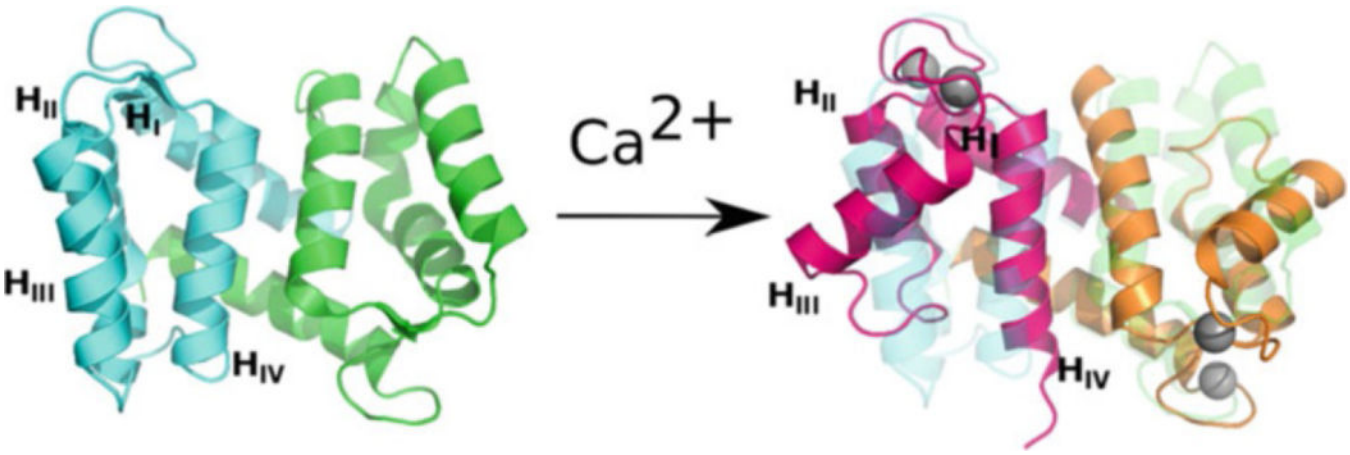

Fig. 1.

The effect of Ca^{2+} binding on the structure of S100A12. The Ca^{2+}-free protein (green and cyan) is shown on the left, and the Ca^{2+}-bound state (magenta and orange) is shown on the right, superimposed on the Ca^{2+}-free state. Note the change in position of helix 3 between the Ca^{2+}-free and Ca^{2+}-bound states. PDB ID: 2WCE and 2M9G

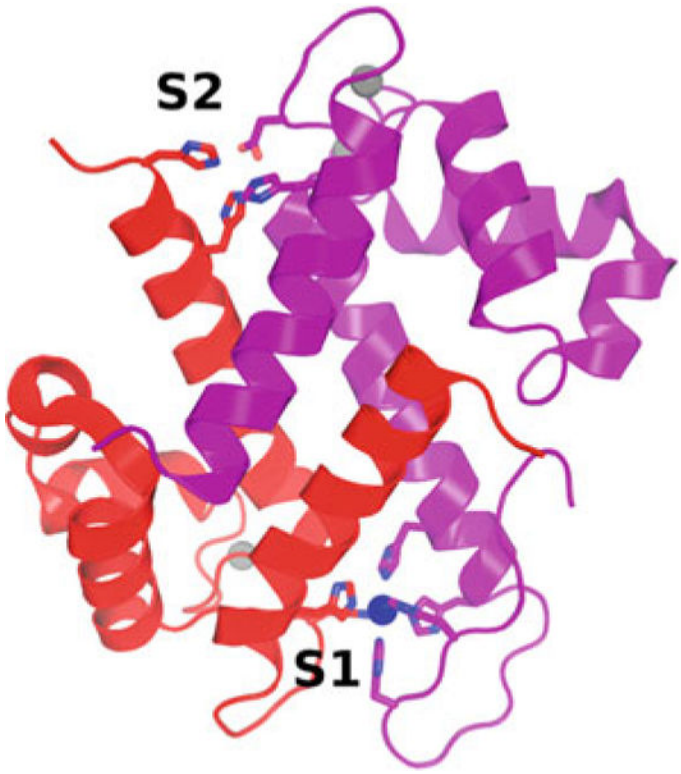

Fig. 2.

Structure of Ca^{2+}- and Mn^{2+}-bound CP. S100A8 is colored red, and S100A9 is colored purple. Ca^{2+} ions are gray spheres and the Mn^{2+} ion is a blue sphere. The side chains chelating transition metal ions in S1 and S2 are shown. PDB ID: 4GGF

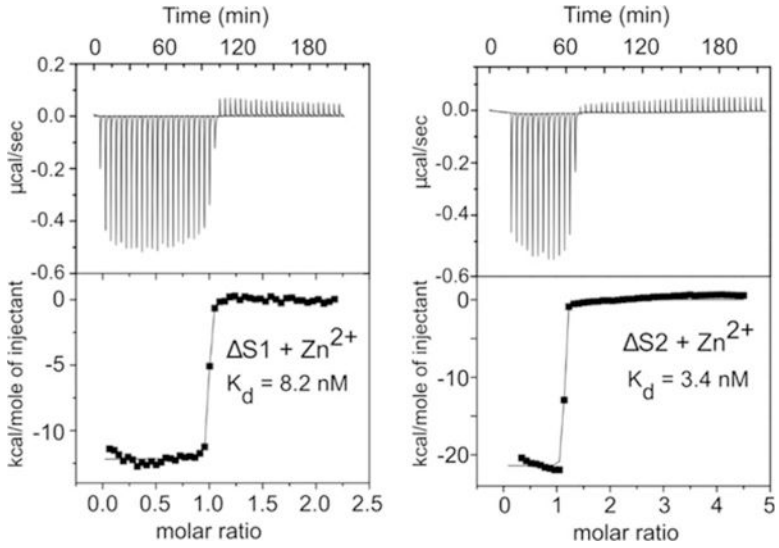

Fig. 3.

The binding of Zn^{2+} to CP monitored by ITC. ITC isotherms of CP as Zn^{2+} are titrated from the syringe into the cell with S1 or S2 knockout mutants (ΔS1, ΔS2). Note the modest differences in K_d values between S1 and S2. These panels were taken from Fig. 1 in the original publication in Damo et al. [8]

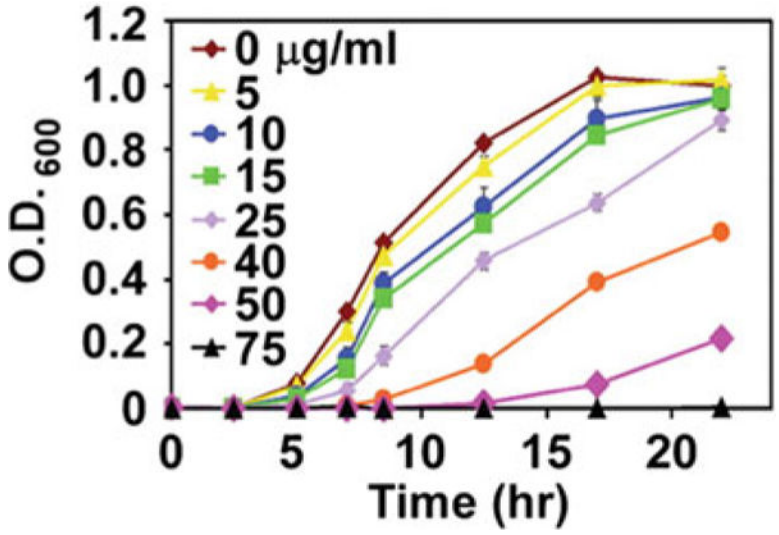

Fig. 4.

Representative growth assay of *S. aureus* in the presence of increasing concentrations of recombinant CP. Growth was quantified by $0.D._{600}$. Error bars represent the standard deviation of at least three replicates. Panel is adapted from the original publication in Corbin et al. [20]