Title:

Capping Protein Binding to S100B: Implications for the “Tentacle” Model for Capping the Actin Filament Barbed End

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Capping protein S100B Interaction
Summary.

S100B binds tightly to a 12 amino acid peptide derived from heterodimeric capping protein. In native intact capping protein, this sequence is in the C-terminus of the \( \alpha \)-subunit, which is important for capping the actin filament. This C-terminal region is proposed to act as a flexible “tentacle,” extending away from the body of capping protein in order to bind actin. To this hypothesis, we analyzed the interaction between S100B and capping protein in solution. The C-terminal 28 amino acids of the \( \alpha \)-subunit, the proposed tentacle, bound to S100B as a free synthetic peptide or a GST-fusion \((K_d \approx 0.4 - 1 \text{ M})\). In contrast, S100B did not bind to whole native capping protein or functionally affect its capping activity. S100B does not bind, with any significant affinity, to the proposed \( \alpha \) tentacle sequence of whole native capping protein in solution. In the NMR structure of S100B complexed with the \( \alpha \)-subunit derived 12 amino acid peptide, the hydrophobic side of a short \( \alpha \) helix in the peptide, containing an important tryptophan residue, contacts S100B. In the X-ray structure of native capping protein, the corresponding sequence of the \( \alpha \)-subunit C-terminus, including W271, interacts closely with the body of the protein. Therefore, our results suggest the \( \alpha \)-subunit C-terminus is not mobile as predicted by the tentacle model. Addition of non-ionic detergent allowed whole capping protein to bind weakly to S100B, indicating that the \( \alpha \)-subunit C-terminus can be mobilized from the surface of the capping protein molecule, presumably by weakening the hydrophobic binding at the contact site.
Introduction

Capping protein (CP) is an α/β heterodimer that tightly caps ($K_d \sim 0.1 - 1 \text{nM}$) the barbed end of the actin filament, preventing actin subunit addition and loss (reviewed in (1)). CP is important for actin assembly and actin-based motility in vivo in Dictyostelium (2), cultured mammalian cells (3), and striated muscle (4-7). In Drosophila, CP is essential for early development, morphogenesis, and actin organization (8,9). CP is also an essential component of the dendritic nucleation model to account for actin polymerization and protrusion at the leading edge of cells (reviewed in (10)).

The X-ray crystal structure of CP inspired a model where the C-termini (~ 30 amino acids) of the α and β subunits of CP are mobile extensions – “tentacles” – and these regions are responsible for high-affinity binding to, and functional capping of, the barbed end (11). We tested one feature of the tentacle model with recombinant mutant chicken (12) and budding yeast CPs (13). Loss of both tentacles causes a complete loss of capping activity, with the β tentacle contributing much more to capping affinity and kinetics. Loss of the α tentacle reduces the capping affinity by 5,000-fold and the capping on-rate by 20-fold in chicken CP (12). In contrast, removal of the β tentacle reduced the affinity by only 300-fold with no effect on the capping on-rate (12). Qualitatively similar results were observed with budding yeast CP (13). Furthermore, the chicken β1 tentacle alone was sufficient to cap (12). Thus, CP appears to use its two C-terminal regions as independent actin-binding sites to cap the barbed end, and the individual C-termini may interact with more than one actin subunit at a subunit-interface at the barbed end (12).
Another feature of the tentacle model is that in solution the C-termini of both subunits are mobile and flexible extensions. In the X-ray structure the C-terminus of CP\[a\] protrudes away from the body of the protein, making no close contacts. The C-terminus of CP\[a\], however, is folded down, in close contact with the top surface of the body of the protein. W271 of the C-terminal region is oriented downwards, making hydrophobic contacts with the body of the heterodimer (11). An important question is whether the C-terminal region of CP\[a\] is mobile and flexible, functioning as a tentacle, or whether the X-ray structure is representative of the structure in solution. We were able to test this hypothesis because S100B was found to bind to W271 in a peptide derived from the CP\[a\] C-terminus.

S100B is a 21.5 kDa symmetric homodimer and a Ca\(^{2+}\)-dependent conformational change is required to enable it to bind its target proteins, often substrates of kinase-dependent phosphorylation reactions (14-19). A consensus binding sequence for S100B was identified as (K/R)(L/I)XWXXIL by phage display screening (20). The C-terminal region of human CP\[a\] (both \[a\]1 and \[a\]2 isoforms have identical sequences over the last 52 C-terminal residues) contains this consensus sequence. A 12-residue peptide termed TRTK-12 (TRTKIDWNKILS, corresponding to residues T265 – S276 of CP\[a\]), was found to bind tightly (K\(_d\) \sim 0.2 – 1 \(\mu\)M) to S100B (20,21). In an NMR solution structure of TRTK-12 bound to S100B, hydrophobic residues of an amphipathic \(\alpha\)-helix in TRTK-12 make contact with a hydrophobic binding pocket in S100B (22). W7 of TRTK-12, which corresponds to W271 of CP\[a\], is a central component of the hydrophobic interaction. In the X-ray crystal structure of chicken CP(\[a\]1\[b\]1) the majority of the corresponding residues in the \[a\] subunit, W271 - L275, are also found in an amphipathic \(\alpha\)-helix (11). The hydrophobic surface of that \(\alpha\)-helix contacts the body of CP, and W271 is a critical residue of the hydrophobic contact. We were able to test the tentacle model by asking whether
the C-terminal region of CP was sufficiently extended and mobile for S100B to bind to it. If this region is not mobile and remains down on the surface of CP, with the W271 occluded from the solution, then S100B should not be able to bind to whole native CP. A further motivation for testing the interaction of S100B with native CP is that the C-terminus is very important for the capping interaction and a protein, in this case S100B, that interacts with this region is likely to inhibit CP’s activity. This idea has been proposed as a mechanism for regulation of the actin cytoskeleton in vivo by S100 proteins (20,23).

To test the tentacle model, we analyzed the interaction between recombinant S100B and CP in solution. No binding was observed. S100B also had no effect on CP’s ability to cap the actin filament barbed end in functional assays. In contrast, the isolated tentacle sequence (the C-terminal 28 amino acids) bound S100B with high affinity ($K_d$’s ~ 0.4 – 1 nM). The proposed tentacle sequence can be induced to move off the surface of the protein, by non-ionic detergent, and then S100B can bind to it and inhibit the actin-binding activity of CP. The data suggests that S100B does not bind, at least with any significant affinity, to the tentacle of whole native CP in solution. The tentacle sequence appears to be relatively immobile in solution, confined to the surface of the protein.
Experimental Procedures.

**Molecular Modeling.** To generate a model for a putative CP-S100B structure, we made use of a separate molecular dynamics trajectory previously performed for the C-terminal sequence (R259 - Y277) of CP (Dr. David Sept, Washington University, unpublished results). We selected an extended conformation from this trajectory and fit that conformation to the rest of the CP structure using the backbone atoms of R259 and R260 in the subunit, giving us a structure where the tentacle was lifted off and extended away from the surface of the protein body. The S100B-TRTK-12 NMR structure (22) was then superimposed onto the extended tentacle using the 12 amino acid stretch corresponding to the TRTK-12 peptide (CP residues T265 – S276). This complex was then energy minimized to remove any atomic clashes.

**Protein Purification.** The bacterial expression vector for rat S100B (pET-11b/S100B) was a kind gift from Dr. David Weber (24). Rat S100B was expressed and purified to homogeneity from BL21 Star (DE3) E. coli (Invitrogen) as described (25), with minor modifications. A tandem capping protein (CP) bacterial expression vector (pET-3d/CP), allowing for co-translation of chicken a1 and b1 subunits from a single plasmid was a kind gift from Dr. Takashi Obinata (26). CP was expressed and purified to homogeneity from BL21 Star (DE3) E. coli (Invitrogen), as described (27). The plasmid pET-3d/CP(C28), encoding an C-terminal 28 amino acid deletion mutant (codon R259 to a stop), was constructed, and the protein expressed and purified, as described (12). Plasmids for expression of the C-terminal 28 amino acids of CP (R259 – A286; pGEX-KG/C28), and the C-terminal 34 amino acids of CP(b1 (R244 – N277; pGEX-KG/C34), as GST-fusion proteins were constructed as described (12). GST-fusions were
purified with glutathione-agarose by standard protocols. The synthetic peptides C28 and C34 corresponding to the C-terminal 28 amino acids from chicken CP, (RRQLPVRTKIDWNKILSYKIGKEMQNA), and the C-terminal 34 amino acids from chicken C1 (RSIDAIPDNQKYKQLQRELSQLTQRQIYIQPDN), respectively, were obtained from commercial sources (Biomolecules Midwest Inc., Waterloo, IL) as described (12). Protein concentrations were determined by A280, using the following extinction coefficients: CP(C28) = 78,450 M⁻¹ cm⁻¹; CP(C34) = 71,480 M⁻¹ cm⁻¹; GST-C28 = 47,590 M⁻¹ cm⁻¹; GST-C34 = 43,480 M⁻¹ cm⁻¹; C28 peptide = 6,970 M⁻¹ cm⁻¹; C34 peptide = 2,560 M⁻¹ cm⁻¹; rat S100B = 1,400 M⁻¹ cm⁻¹.

C28 Peptide Fluorescence Spectroscopy. Intrinsic tryptophan fluorescence emission spectra for the C28 peptide, which contains a single tryptophan residue corresponding to W271 in CP, were obtained on a PTI Quantmaster spectrofluorometer (Photon Technology International, Santa Clara, CA), in a 2.5 ml cuvette at 25°C. Tryptophan fluorescence was excited at 259 nm (1 nm band pass), and emission was scanned from 305 to 450 nm (5 nm band pass). Titration of C28 peptide with rat S100B, which contains no tryptophan residues, was followed by monitoring the fluorescence enhancement (DF) at 334 nm. 0.12 mM C28 peptide was incubated in the absence or presence of rat S100B in 10 mM Tris, pH 7.5; 100 mM NaCl; 2 mM CaCl₂; 1 mM MgCl₂; 0.5 mM DTT, or the same buffer minus calcium, for 1 hr at 25°C, and the emission spectra measured. Each spectrum was obtained from a separate incubation mixture, not sequential additions of S100B to the same sample of C28 peptide. The DF at 334 nm was calculated using equation 1.
$\square F = (F_{aC28+S100B} - F_{aC28}) - (F_{S100B} + F_{buffer}) \quad Eq. 1.$

where $\square F$ is the difference between the intrinsic fluorescence of the $a$C28 peptide alone ($F_{aC28}$) and the $a$C28 peptide in the presence of rat S100B ($F_{aC28+S100B}$), background buffer and S100B subtracted ($F_{S100B} + F_{buffer}$). We assumed the $\square F$ at 334 nm is proportional to the concentration of S100B-$a$C28 peptide complex. $\square F$ was plotted versus the total concentration of S100B. The data was least squares fit to equation 2 using Kaleidagraph v3.6 software (Synergy Software, reading, PA);

$$\square F = c \frac{(K_d + [\square C28] + [S100B]) - ((K_d + [\square C28] + [S100B])^2 - (4 \square \square C28 \square [S100B]))}{2} \quad Eq. 2.$$ 

where $\square F$ is the fluorescence enhancement at 334 nm (in arbitrary units, a.u.), $[\square C28]$ is the $a$C28 peptide concentration, [S100B] is the rat S100B concentration, $K_d$ is the equilibrium dissociation constant and $c$ is a proportionality constant.

**Binding Assays by Supernatant Depletion.** A fixed concentration of rat S100B (2.5 or 0.7 mM) was incubated alone or with increasing concentrations of either GST-$a$C28 or GST-$b$C34 coupled to glutathione sepharose 4B FF beads, $a$C28 peptide or $b$C34 peptide coupled to Affi-gel® 10 (BioRad), or CP($\square 1\square 1$) coupled to Affi-gel® 15 (BioRad), for 10 min at 25°C. Samples were incubated in; 10 mM Tris, pH 7.5; 100 mM NaCl; 2 mM CaCl$_2$; 1 mM MgCl$_2$; 0.5 mM DTT; 1 mM NaN$_3$, or the same buffer minus Ca$^{2+}$. The samples were then spun at 13,000 $\times$ g for 5 min to pellet the beads and any bound proteins. 30 – 50 $\mu$l samples of the supernatant were
loaded onto 15 – 20 % Tricine-SDS-PAGE gels. The amount of S100B present in the supernatant was calculated by densitometry of wet gels from analyzed using NIH Image software. Bound S100B was plotted versus the total bead-coupled ligand concentration and least squares fit to equation 3 using Kaleidagraph v3.6 software (Synergy Software, reading, PA);

\[
[S100B]_{\text{bound}} = \{(K_d + [S100B] + [\text{resin-coupled ligand}]) - (K_d + [S100B] + [\text{resin-coupled ligand}])^2 - (4 \times [S100B] \times [\text{resin-coupled ligand}])\} / 2
\]

Eq 3.

Where \([S100B]_{\text{bound}}\) is the amount of rat S100B bound in complex with the bead-coupled ligand, \([\text{resin-coupled ligand}]\) is the concentration of either GST-[C28] or GST-[C34] coupled to glutathione sepharose, [C28] peptide or [C34] peptide coupled to Affi-gel® 10, or CP[1][1] coupled to Affi-gel® 15, \([S100B]\) is the rat S100B concentration and \(K_d\) is the equilibrium dissociation constant. The amount of S100B bound to the beads was calculated using the equation, \([S100B]_{\text{bound}} = [S100B]_{\text{total}} - [S100B]_{\text{S/N}} - \text{[non-specific binding]}\). The amount of S100B trapped non-specifically by the resin was ~ 4 – 9% for all experiments.

**Competition Binding Assays.** Fixed concentrations of rat S100B (0.5 mM) were incubated with 1 mM GST-[C28] coupled to glutathione sepharose 4B FF beads, in the absence or presence of increasing concentrations free [C28] peptide or whole heterodimeric CP, for 10 min at 25°C. Samples were incubated in 10 mM Tris, pH 7.5; 100 mM NaCl; 2 mM CaCl\(_2\); 1 mM MgCl\(_2\); 0.5 mM DTT; 1 mM NaN\(_3\). The samples were then spun at 13,000 \(\times\) g for 5 min to pellet the resin and any bound proteins. 50 \(\mu\)l samples of the pellet fraction were loaded onto 15 – 20 % Tricine-SDS-PAGE gels. The amount of S100B bound to GST-[C28] was calculated by densitometry of
wet gels from analyzed using NIH Image software. GST-C28 bound S100B was plotted versus the total C28 peptide or CP(1-1) concentration. The amount of S100B bound to the beads was corrected for non-specific binding, ~4 – 9% for all experiments.

**Spectrin-F-Actin Seeded (SAS) Actin Polymerization Assay.** Proteins were prepared and assays performed essentially as described (12), with minor modifications. Mg²⁺-ATP-actin was used at a final concentration of 2 µM (3 - 10 % pyrene labeled) with final buffer conditions of either 10 mM Imidazole, pH 7.0; 100 mM NaCl; 3 mM CaCl₂; 1 mM MgCl₂; 1 mM EGTA; 0.2 mM ATP; 0.5 mM DTT; 1 mM NaN₃, for calcium containing conditions (2 mM free Ca²⁺), or 10 mM Imidazole, pH 7.0; 100 mM NaCl; 0.2 mM CaCl₂; 1 mM MgCl₂; 1 mM EGTA; 0.2 mM ATP; 0.5 mM DTT; 1 mM NaN₃, for non-calcium containing conditions (7 nM free Ca²⁺). CP and S100B were added to the actin followed by addition of a 1/20th volume of 200 mM Imidazole, pH 7.0; 2 M NaCl; 20 mM MgCl₂; 20 mM EGTA; plus or minus 60 mM CaCl₂, and finally addition of SAS. Free Ca²⁺ concentrations were calculated using ‘EGTA’ software (PM Smith, The University of Liverpool, UK; [http://www.liv.ac.uk/luds/people/cds/bds/pms/cal.htm/](http://www.liv.ac.uk/luds/people/cds/bds/pms/cal.htm)).

**Kinetic Modeling.** The kinetic mechanism used for the modeling of rate constants for the interaction between S100B and CP is indicated below. Rate constants were determined by least squares minimization fitting of the data using the Berkeley Madonna package, version 8.01 (URL [http://www.BerkeleyMadonna.com](http://www.BerkeleyMadonna.com)). A is the actin monomer concentration, Nₛ is the concentration of barbed ends (equal at the start to the concentration of SAS added and equivalent to the filament number), CP is the capping protein concentration, and S100 is the concentration of rat S100B.
1. \( A + N_b \leftrightarrow N_b \quad k_{+b} : k_{-b} \)

2. \( CP + N_b \leftrightarrow CPN_b \quad k_{+cap} : k_{cap} \)

3. \( S100 + CP \leftrightarrow S100CP \quad k_{+S100} : k_{-S100} \)

The concentration of SAS was determined by fitting the experimental data for seeded polymerization of actin alone (reaction 1), with \( k_{+b} \) and \( k_{-b} \) of 11.6 \( \text{M}^{-1} \text{s}^{-1} \) and 1.4 \( \text{s}^{-1} \), respectively (28). Actin polymerization of was unaffected by addition of 1.4% TX-100. Addition of 1% TX-100 had no effect on CP’s activity. 1.4% TX-100 had a small reproducible effect on CP activity; the on- and off-rate constants of reaction 2 were 2.1 \( \text{M}^{-1} \text{s}^{-1} \) and 3.8 \( 10^{-4} \text{s}^{-1} \), compared to 5.8 \( \text{M}^{-1} \text{s}^{-1} \) and 5.6 \( 10^{-4} \text{s}^{-1} \) in the absence of TX-100. These rate constants were determined using a range of CP concentrations as previously described (12). The on- and off-rate constants for the interaction between S100 and CP (reaction 3), \( k_{+S100} \) and \( k_{-S100} \), respectively, were determined from SAS seeded actin polymerization assays in the presence of CP and 1.4 % TX-100. The mechanism assumes no pointed-end growth from the SAS, which we confirmed by determining that no actin polymerization occurred over 1000 s with SAS and 24 nM cytochalasin D. The contribution of spontaneous nucleation by actin monomers to these assays was so small as to be negligible.

**Critical Concentration Assays.** The critical concentration for polymerization of actin and the equilibrium dissociation constant for capping of the barbed end (\( K_{cap} \)), were measured essentially as described (29,30). Mg\(^{2+}\)-ATP Actin at 30 \% pyrene labeled) was polymerized in 10 mM Tris, pH 7.5; 100 mM NaCl; 1 mM MgCl\(_2\); 0.2 mM ATP; 0.5 mM DTT; 1 mM NaN\(_3\), for 2
hrs at 25°C. Actin was then diluted to a final concentration of 2 μM with final buffer conditions of either 10 mM Tris, pH 7.5; 100 mM NaCl; 3 mM CaCl₂; 1 mM MgCl₂; 1 mM EGTA; 0.2 mM ATP; 0.5 mM DTT; 1 mM NaN₃, for calcium containing conditions (2 mM free Ca²⁺), or 10 mM Tris, pH 7.5; 100 mM NaCl; 0.2 mM CaCl₂; 1 mM MgCl₂; 1 mM EGTA; 0.2 mM ATP; 0.5 mM DTT; 1 mM NaN₃, for non-calcium containing conditions (7 nM free Ca²⁺). CP alone or CP pre-incubated with S100B (at the indicated final concentrations), for 1 hr at 25°C in the respective buffer, were added, the mixture incubated overnight at 25°C and the fluorescence intensity recorded.

**Actin Filament Sedimentation Assays.** Actin at 5 μM was polymerized in the absence or presence of 0.9 μM CP, 48 μM S100B, or both in 10 mM Tris, pH 7.5; 100 mM NaCl; 3 mM CaCl₂; 1 mM MgCl₂; 1 mM EGTA; 0.2 mM ATP; 0.5 mM DTT; 1 mM NaN₃ (2 mM free Ca²⁺), in a total volume of 100 μl. Samples were incubated at 25°C for 5 hrs. The polymerized mixture was then carefully placed onto a 10% sucrose cushion and centrifuged at 90,000 rpm in a Beckman TLA 100.1 rotor for 30 min at 25°C. 25 μl of the supernatant was removed from the meniscus and added to 25 μl 2× SDS sample buffer. The remaining liquid was removed, avoiding the pellet, and the pellet dissolved in 50 μl 1× SDS sample buffer. Equal loading volumes of the supernatant and pellets fractions were analyzed on 10 – 20% acrylamide Tricine-SDS-PAGE gels. Protein amounts were calculated by densitometry of wet gels stained with Coomassie Blue using NIH Image software.

**Non-Denaturing Polyacrylamide Gel Electrophoresis.** Native-PAGE was preformed essentially as described (31,32), with minor modifications. CP alone, S100B alone or mixtures of both (at
the indicated concentrations) were incubated for 1 hr at 25°C in 10 mM Tris, pH 7.5; 100 mM NaCl; 1 mM MgCl₂; 0.5 mM DTT; 1 mM NaN₃, plus or minus 2 mM CaCl₂. Gels (7 % acrylamide) were run for 35 min at 200 volts in 25 mM Tris; 194 mM glycine, pH 9.0; 0.5 mM DTT; minus or plus 2 mM CaCl₂. In the absence of Ca²⁺, S100B migrates primarily as a smear, while in the presence of Ca²⁺ it migrates as a distinct focused band. This difference can be reconciled with the conformational changes that occur in S100 proteins upon Ca²⁺ binding (14-16,22). This structural rearrangement changes the charge distribution of the S100B homodimer and, in addition to the change in native charge that likely occur upon binding the Ca²⁺ ions themselves, is undoubtedly the cause of the differing electrophoretic characteristics observed.

**Analytical Gel-filtration Chromatography.** 200 µl samples of whole CP alone, S100B alone or a mixture of both (7 µM CP and 16.3 µM S100B), pre-incubated in 10 mM Tris, pH 7.5; 100 mM NaCl; 2 mM CaCl₂; 1 mM MgCl₂; 0.5 mM DTT; 1 mM NaN₃, for 1 hr at 25°C, were applied to a Superdex-200 column (Pharmacia) (Vᵣ ≈ 22 ml; 1.0 cm ¥ 28 cm) pre-equilibrated in the same buffer. The column was run at 0.6 ml min⁻¹. The first fraction collected was 5 ml and all subsequent fractions were 0.31 ml. The apparent molecular weights of CP (80.2 kDa.) and S100B (dimer, 25 kDa.; monomer, 10.7 kDa.) alone, were calculated from a curve of protein standards.

**Miscellaneous.** Covalent coupling of C28 peptide, C34 peptide and CP(1-1) to Affi-gel® resin (BioRad) was performed according to the manufacturers recommendations. SDS-PAGE (33) and Tricine-SDS-PAGE (34) were performed as described, with minor modifications. Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).
Results

To investigate whether the C-terminus of CP is a “tentacle” in the sense of being flexible and extending out away from the rest of the protein in solution, we asked whether S100B is able to bind to whole CP in solution, since S100B is known to bind tightly to a peptide corresponding to the region in question. In particular, a tryptophan residue (corresponding to W271 of CP) is involved in binding to S100B and in the contact of the C-terminus with the body of CP.

First, we asked whether it was theoretically possible to sterically accommodate the binding of S100B to CP. We generated a model for the CP-S100B structure by moving the proposed tentacle sequence in the X-ray structure (residues R259 – A286) off the top surface of the molecule, exposing W271 (compare Figure 1A and 1B). The S100-TRTK-12 NMR structure (22) was then superimposed onto the 12 amino acids corresponding to the TRTK-12 peptide (CP residues T265 – S276) (Figure 1C). This CP-S100B complex was then energy minimized to remove steric clashes (Figure 1D). The result shows that S100B can theoretically bind CP, without steric clashes, if the CP C-terminus is extended with W271 removed from the surface of CP.

Second, we asked whether a 28 amino acid residue peptide (termed “C28” peptide here) corresponding to the proposed tentacle sequence (CP residues R259 – A286) was able to bind S100B. Previous studies had shown that a shorter peptide, TRTK-12 (CP residues T265 – S276), binds tightly (20,21). We measured binding of a synthetic C28 peptide to recombinant S100B (Figure 2A) by intrinsic tryptophan fluorescence. The C28 peptide has a single tryptophan residue, corresponding to W271, while S100B has none. Titration caused a fluorescence enhancement and a blue shift in the emission wavelength maximum from 355 nm to
334 nm (Figure 2B). Similar results were observed with the TRTK-12 peptide in previous studies (20,21). The blue shift reflects a change in the environment of the tryptophan residue to a less polar one, in agreement with the hydrophobic nature of the binding interaction between TRTK-12 and S100B in the NMR solution structure (22). The equilibrium dissociation constant ($K_d$) for the \textsuperscript{\textit{a}C28} peptide binding to S100B was $0.5 \pm 0.07 \ \mu\text{M}$ (mean $\pm$ SEM, $n = 3$) (Figure 2C). TRTK-12 showed a similar affinity for S100B with $K_d$ values of $0.2$ – $1 \ \mu\text{M}$ in previous studies (20,21). Experiments performed in the absence of Ca\textsuperscript{2+} showed no significant binding of \textsuperscript{\textit{a}C28} peptide to S100B, at concentrations of S100B up to $42 \ \mu\text{M}$ (data not shown), consistent with previous results for the TRTK-12 peptide (20,21).

Next, we asked whether S100B could bind to whole heterodimeric CP. We performed pull-down experiments, with CP covalently coupled to resin (termed Affi-CP[\textit{a}1\textit{b}1]). The S100B concentration was constant at $2.5 \ \mu\text{M}$, and increasing amounts of beads, providing a total concentration of CP up to $27.2 \ \mu\text{M}$, were added. No depletion of S100B from the supernatant was observed (Figure 3A) An upper limit for the $K_d$ based on this result is $\sim 100 \ \mu\text{M}$. As positive controls, we used the \textit{a} tentacle sequence immobilized on resin, both as a GST-fusion, (termed GST-\textit{a}C28), or as the synthetic \textit{a}C28 peptide covalently coupled to resin (termed Affi-\textit{a}C28). Increasing amounts of beads with either one of the \textit{a} tentacle species (used over similar concentration ranges as covalently coupled whole CP) resulted in depletion of S100B from the supernatant (Figure 3B – D). The mean $K_d$ for S100B binding to GST-\textit{a}C28 was $0.36 \pm 0.08 \ \mu\text{M}$ ($\pm$ SEM, $n = 4$) and to Affi-\textit{a}C28 was $1.2 \pm 0.15 \ \mu\text{M}$ ($\pm$ SEM, $n = 3$) (Figure 3B and C, respectively). These $K_d$ values are similar to the $K_d$ obtained from the tryptophan fluorescence titration experiments. In the absence of Ca\textsuperscript{2+}, binding to S100B was not observed for either GST-\textit{a}C28 or Affi-\textit{a}C28 (data not shown). The specificity of this interaction for the C-terminal 28
amino acids of CP was confirmed by the lack of binding of S100B to the proposed tentacle sequence of CP (the C-terminal 34 amino acids) covalently coupled to resin (termed Affi-C34) (Figure 3E).

We considered that the covalent coupling of CP to the resin might have rendered CP unable to bind S100. To address this possibility, we asked whether CP free in solution was able to bind to S100B free in solution and thus inhibit S100B binding to GST-C28 beads. The addition of CP did not decrease the binding of S100B to the GST-C28 beads, at concentrations of CP up to 35 □M (Figure 4). As a positive control, addition of the C28 peptide did decrease the amount of S100B associated with the GST-C28 beads with a $K_d$ of 1.3 ± 0.4 □M (mean ± SEM, n = 3) (Figure 4).

We also looked for evidence of whole CP binding to S100B with native gel electrophoresis and analytical gel-filtration. No binding was observed with either approach, either in presence or absence of Ca$^{2+}$ (Figure 5). In native-PAGE experiments, the mobilities of CP and S100B in the mixture were the same as those of the individual proteins alone (Figure 5A). In the gel-filtration analysis, the elution profiles for the proteins in the mixture were the same as those of the individual proteins alone (Figure 5B).

We next used a functional approach to determine if S100B binds and inhibits CP. This experiment has implications beyond testing the tentacle model, in that previous studies reporting the S100B/CP-derived peptide interaction have led to speculation that S100B might regulate the activity of CP in vivo (20). The C-terminal region of CP is important for binding actin, which is one facet of the tentacle model (12,13). Truncation mutants lacking 28 amino acids at the C-terminus of CP have a 5,000-fold loss of binding affinity, with a ~20-fold decrease in
both the capping on- and off-rate constants (12). Binding of S100B to whole CP in solution should inhibit CP’s ability to cap the filament.

We measured the actin binding activity of CP using capping assays, with and without S100B. In a barbed-end nucleated actin polymerization assay, S100B, up to 7.8 µM, had no effect on the inhibition by CP present in low nM concentrations (Figure 6A and B). Similar results were found in the absence or presence of Ca\textsuperscript{2+} (Figure 6A and B, respectively). In a steady-state actin critical concentration assay, addition of S100B in high molar excess (9.2 µM) did not affect the ability of nM concentrations of CP to shift the critical concentration for actin polymerization to that of the pointed end (Figure 6C and D). Experiments performed in the absence or presence of Ca\textsuperscript{2+}, gave essentially identical results (Figure 6C and D, respectively). The equilibrium dissociation constant for CP binding the barbed end ($K_{\text{cap}}$) was 0.2 – 0.4 nM for all conditions tested (measured and calculated as described (29,30)).

These results indicate that the hydrophobic residues in CP\[ involved in binding S100B are unavailable in free CP. We hypothesized that the CP\[ C-terminus might still flip up off the body of the molecule but only upon binding the barbed end of the actin filament, which might expose W271 and other residues important for binding S100B. To test this hypothesis, we analyzed the physical interaction of CP and S100B with the actin filament in sedimentation assays (Figure 6E). CP sedimented with the filamentous actin by virtue of binding to the barbed end. S100B, up to 48 µM, had no effect on the amount of sedimenting CP. The amount of S100B that pelleted with the filamentous actin was small (~ 3 – 4 %) due to non-specific inclusion in the pellet. The addition of CP did not increase the amount of S100B found in the pellet. These results indicate that S100B does not bind to CP already bound to the filament end, and that S100B does not compete with F-actin for binding to CP. In other words, the conformations of
whole CP, free in solution and when bound to F-actin, do not include ones in which the S100 binding site is available. Thus, the C-terminus of CP in solution would appear to have a relatively constrained and immobile conformation similar to that in the X-ray structure (11) in which the residues on the hydrophobic side of the amphipathic α-helix involved in binding S100B (W271, I274 and L275 (22)) are occluded and unavailable.

We asked whether we could induce the CP C-terminus to become able to bind S100B under conditions short of denaturation, which is known to permit binding of S100B to CP (20). Since the CP C-terminus is bound to its body via hydrophobic interactions, we tested a non-ionic detergent, Triton-X100 (TX-100). In the presence of 1.4% TX-100, increasing concentrations of S100B were able to completely inhibit CPs barbed end capping activity (Figure 7A). Addition of 1.4% TX-100 alone, without S100B, resulted in only a small effect on the capping activity of CP (Figure 7A, compare trace a and c). Actin assembly was unaffected by 1.4% TX-100.

We used kinetic modeling to quantitate these effects and test the mechanism. In the presence of 1.4% TX-100 the capping on- and off-rate constants were 2.1 M⁻¹s⁻¹ and 3.8 × 10⁻⁴ s⁻¹, respectively. Kinetic modeling with a model where S100B binds CP and completely inhibits its interaction with the barbed end gave good fits (red lines, Figure 7A). For the S100B / CP interaction, the on-rate (k⁺S100) was 0.7 ± 0.3 M⁻¹s⁻¹ and the off-rate (k⁻S100) was 11 ± 1 s⁻¹, giving a $K_d$ of 16 ± 3.5 M (all values are mean ± SEM, n = 3). To understand the significance of this binding affinity, we asked how well the C28 peptide bound to S100B in the presence of 1.4% TX-100. In a pull-down assay, the $K_d$ for Affi-C28 binding S100B was 34 ± 10 M (mean ± SEM, n = 3) (black line, Figure 7B). As a negative control, the tentacle sequence showed no binding to S100B in a similar experiment (Figure 7B). To confirm that the binding of S100B to
whole CP in 1.4% TX-100 is via the tentacle sequence we used an tentacle deletion mutant, CP[DC28] (Figure 7C). We previously showed that this mutant retains a decreased level of barbed end capping activity (12). S100B had no effect on the capping activity of CP[DC28] in 1.4% TX-100 (Figure 7C), showing that the tentacle sequence is required for the S100B interaction (Figure 7A).
Discussion.

One of the elements of the proposed tentacle model for how CP binds to the barbed end of the actin filament is that the C-terminal regions of each subunit are hypothesized to be mobile in solution and extend away from the body of CP in order to bind actin (11). Our results here argue strongly against this element of the model for the CP subunit. The C-terminus of CP appears to be folded down onto the surface of the protein and relatively immobile. We were able to test this aspect of the model for the CP subunit because of previous results showing that S100B can bind to a peptide derived from the C-terminus of CP (20,21). We found that S100B was able to bind the isolated C-terminal 28 amino acids of CP, with Kd in the 0.4 – 1 μM range. However, we found no evidence for S100B binding to whole native CP in solution, either free or bound to the barbed end of the actin filament.

In the X-ray structure of chicken CP(1b1) the subunit C-terminus is folded down and lies on the top surface of the body of the protein (11). A tryptophan residue at position 271 is part of the hydrophobic face of a small amphipathic α-helix, and W271 is oriented downwards and makes hydrophobic contacts with the body of the heterodimer (11). In the X-ray crystallography study, the C-terminal residues of CP had higher temperature factors than other regions of the molecule, which helped support the hypothesis that it might be mobile and flexible in solution (11). Under this hypothesis, the orientation observed in the X-ray structure may have been acquired merely as a result of molecular packing during crystal formation (11).

A separate aspect of the tentacle model is that the C-terminal regions of both subunits bind actin (11). A number of results support this aspect of the model (12,13,35). Loss of both tentacles caused a complete loss of actin binding activity. Removal of the C-terminal 28 amino
acids of CP decreased capping affinity by 5,000-fold and the on- and off-rates of capping by 20-fold (12). Removal of the tentacle (C-terminal 34 amino acids) decreased the capping affinity by only 300-fold and had very little effect on the capping on-rate (12). Point mutations in conserved residues in the proposed tentacle of CP also resulted in significant reductions in the capping affinity (12). We concluded that these residues might be part of the actin contact site or that they were important for the structure of the actin contact site elsewhere in the C-terminal region. Changing W271 (part of the hydrophobic contact between the C-terminal region of CP and the body of CP) to R produced a ~30-fold decrease in capping affinity reflected in ~3-fold and ~10-fold reductions in the capping on- and off-rates, respectively (12). Residue R259 occupies a position at the base of the proposed tentacle, and its side chain protrudes inward to make apparent ionic and hydrogen bond contacts with residues in the body of the protein (Y107, E221 and N222 of the subunit (11)). An R259A mutant also had a ~30-fold decrease in capping affinity mostly due to ~20-fold decreased on-rate (12). Thus, R259 may influence the structure and/or the orientation of the actin binding C-terminal region of CP.

The finding that S100B bound to a peptide derived from CP led to speculation that S100B might target or regulate CP in cells (20). This hypothesis now seems unlikely, given the lack of binding between S100B and native whole CP. However, high concentrations of non-ionic detergent (1.4% Triton X-100) were sufficient to allow S100B to bind to whole CP and prevent it from capping actin. The non-ionic detergent did not denature CP, in that the two subunits remained associated with each other and capped the barbed end of the filament. Therefore, it remains possible that some environment in the cell has a similar effect, enabling CP to bind S100B and be inhibited from binding actin. In skeletal muscle, CP(1/1) (CapZ) is concentrated at the Z-disc, but S100B is not found at the Z-disc (36,37). High Ca\(^{2+}\) concentrations (~2 mM)
are required for binding of S100B to the C28 and TRTK-12 peptides, which also suggests that between S100B and CP is unlikely to occur in cells.

One can still envision a scenario in which the orientation of the C-terminus in free CP does change when CP binds to the actin filament. If so, such a conformational change must occur only upon interaction of CP with the actin filament, in such a manner that the C-terminal region is hidden from S100B. We tested this hypothesis by looking for S100B binding to CP during the time when CP was binding to actin and also when CP was already bound to actin - none was observed.

In previous studies reporting evidence for binding of S100B to the whole of the CP subunit, the CP was denatured (20). The single exception to this statement is that chemical cross-linking of native CP in solution with S100B did show a Ca\textsuperscript{2+} dependent interaction (20). In light of our current results, we suggest that the chemical cross-linking either trapped denatured CP or induced a local change in the C-terminal region comparable to what occurred here with non-ionic detergent.

In summary, our data suggest that the C-terminus of whole CP in solution is constrained and immobile. The conformation of this sequence in the X-ray structure – where it is folded down on top of the molecule – appears to be representative of the orientation of this region in solution. Further structural studies directly assessing the conformation of the C-terminal regions of CP in solution will be required to address the question of whether these regions do indeed behave as tentacles when they interact with the barbed end of the actin filament.
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References.


**Figure legends.**

**Figure 1. Structural model for the putative CP-S100B complex.** (A) Ribbon diagram of the X-ray crystal structure of chicken CP(\[1\]1) (11). The colour scheme is the same for all panels. The CP \[ subunit is yellow, and its proposed tentacle (R259 - A286) is cyan. Trp 271 is labelled and shown in space-filling representation. The CP \[ subunit is red, and its proposed tentacle (R244 - N277) is green. (B) Model of CP with an extended \[ tentacle. (C) Model of rat S100B (gold) bound to the whole extended \[ tentacle sequence. (D) Model of S100B bound to whole CP. In (C) and (D), for clarity, only a single subunit of the S100B dimer is shown. Fitting and energy minimization of this complex structure were performed with the dimer.

**Figure 2. S100B binds to the isolated \[ tentacle sequence of CP.** (A) SDS-polyacrylamide gels of the purified preparations of the recombinant proteins; rat S100B, wt chicken CP(\[1\]1\]) and an \[ tentacle deletion mutant, CP(\[\[\C28]\]). Appropriate molecular weight standards (kDa.) are indicated by the side of each gel. (B) Intrinsic fluorescence emission spectra of 0.12 \[M \[C28] peptide alone (spectrum a), 2.7 \[M rat S100B alone (spectrum b) and 0.12 \[M \[C28] peptide in the presence of 2.7 \[M rat S100B (spectrum c). Spectrum d (red) shows the spectrum that would be obtained if the contribution of the components in the reaction mixture of spectrum c were simply additive. All spectra were performed in 2 mM Ca\(^{2+}\) and background buffer has been subtracted. (C) The fluorescence enhancement (\[F\]) at 334 nm (a.u.) of the \[C28] peptide is plotted versus the rat S100B concentration in \[M. The data from two repeat experiments are shown (circles), along with a least squares fit of the data (black line) to equation 2 (see experimental procedures).
Figure 3. **Whole CP coupled to resin does not bind S100B.** (A) Supernatant depletion assay performed with resin-coupled whole CP. A representative Tricine-SDS-polyacrylamide gel (15 – 20% acrylamide) is shown. *Lane 1*, 2.5 μM S100B alone; *lane 2*, Concentration of S100B remaining in the supernatant (S/N) after incubation with 27.2 μM of immobilized whole CP; *lane 3*, Concentration of S100B present in the pellet (Plt.) fraction. The results shown are those performed in 2 mM free Ca\(^{2+}\). Essentially identical results were obtained in the absence of Ca\(^{2+}\) (data not shown). Panels (B) and (C) show the binding of S100B, in 2 mM free Ca\(^{2+}\), to the isolated ⧺ tentacle sequence of CP as a GST-fusion (GST-⧺C28), or the ⧺C28 peptide covalently coupled to resin (Affi-⧺C28), (B) and (C), respectively. The upper portion of each panel shows representative Tricine-SDS-polyacrylamide gels (15 – 20% acrylamide) illustrating the depletion of soluble S100B from the supernatant by increasing concentrations of immobilized ⧺ tentacle. The lower portion of each panel is a plot of the concentration of bound S100B *versus* the concentration of immobilized ⧺ tentacle in μM. 2 repeat experiments are shown in (B) and 3 are shown in (C). The data were least squares fit (black lines) to equation 3 (see experimental procedures). (D) Section from a Tricine-SDS-polyacrylamide gel (15 – 20%) showing controls for panels (B) and (C). *Lane 1*, 2.5 μM S100B alone; *lane 2*, concentration of S100B bound in the 6 μM GST-⧺C28 pellet fraction; *lane 3*, non-specific binding of S100B to glutathione-sepharose resin alone; *lane 4*, concentration of S100B bound in the 22 μM Affi-⧺C28 pellet fraction; *lane 5*, non-specific binding of S100B to Affi-gel\(^{®}\) resin alone. (E) Covalently coupled ⧺C34 peptide does not bind S100B. A portion of a representative Tricine-SDS-polyacrylamide gel (15 – 20% acrylamide) is shown. *Lane 1*, 2.5 μM S100B alone; *lane 2*, S100B remaining in the supernatant (S/N) after incubation with 33 μM immobilized ⧺C34 peptide; *lane 3*, S100B present in the pellet (Plt.) fraction.
Figure 4. **Free whole CP does not compete with GST-[^C]28 for the binding of S100B.** (A) The concentration of S100B bound to GST-[^C]28 is plotted *versus* the concentration of either free whole CP or free[^C]28 peptide, in M. The[^C]28 peptide (open and red-filled circles show two repeat experiments) shows a concentration dependent competition for GST-[^C]28-bound S100B, whereas free whole CP (black-filled circles) does not. The solid black line is a least squares fit of the data where the free[^C]28 peptide competes with GST-[^C]28 for S100B binding with a $K_d$ of $1.3 \pm 0.4$ M (mean ± SEM, n = 3). (B) Composite image from representative Tricine-SDS-polyacrylamide gels (15 – 20% acrylamide) illustrating the amount of GST-[^C]28-bound and supernatant-free S100B for the points * and ** in (A). Lane 1, amount of S100B bound to GST-[^C]28 in the absence of[^C]28 peptide or whole CP; lane 2, amount of S100B present in the supernatant in the presence of 35 M free whole CP; lane 3, amount of S100B bound to GST-[^C]28 in the presence of 35 M free whole CP, point ** in (A); lane 4, amount of S100B bound to GST-[^C]28 in the presence of 35 M[^C]28 peptide, point * in (A); lane 5, amount of S100B present in the supernatant in the presence of 35 M[^C]28 peptide (lower band corresponds to the[^C]28 peptide); lane 6, non-specific binding of S100B to glutathione beads alone.

Figure 5. **Native-PAGE and gel-filtration analysis show no evidence of an interaction between CP and S100B in solution.** Panels (A) and (B) show native gels of whole CP alone, S100B alone and a mixture of both (at the indicated concentrations) performed in the absence or presence of Ca^{2+}, (A) and (B), respectively. (C) Superdex-200 gel filtration chromatography shows no evidence of an interaction between CP and S100B in solution. A280 (a.u.) monitored elution profiles of whole CP alone (red), S100B alone (green) or of a mixture of both (7 M
whole CP and 16.3 μM S100B (black), pre-incubated for 1 hr at 25°C in the presence of 2 mM Ca²⁺. Below the elution profiles is a Commassie-stained Tricine-SDS-polyacrylamide (10 – 20%) gel showing the protein components of the indicated column fractions from the incubation mixture of CP and S100B. Peak-elution fractions of either whole CP alone or S100B alone are indicated below the gel. The peak elution positions and molecular weights of protein standards are indicated above the elution profiles.

Figure 6. S100B has no effect on CPs barbed end capping activity. Pyrene-actin fluorescence (a.u.) is plotted in panels (A) – (D). Panels (A) and (B) show inhibition of barbed end actin polymerization (2 μM) nucleated by the addition of spectrin-F-actin seeds (SAS), in the absence (A) (7 nM free Ca²⁺), or presence (B) (2 mM free Ca²⁺) of Ca²⁺. In (A) and (B) black and red curves were performed in the absence or presence of S100B, respectively. a, 2 μM actin alone; b, 2 μM actin + 7.8 μM S100B; c, 2 μM actin + SAS; d, 2 μM actin + SAS + 7.8 μM S100B; e, 2 μM actin + SAS + 2 nM CP ; f, 2 μM actin + SAS + 2 nM CP + 7.8 μM S100B. Panels (C) and (D) show the effect of CP on the steady-state actin filament concentration, in the absence (open circles) or presence of 9.2 μM S100B (closed triangles), either in the absence (7 nM free) or presence (2 mM free) of Ca²⁺, (C) and (D) respectively. (E) Filamentous actin pelleting assay. The pellet (Plt) and supernatant (S/N) fractions from a representative 10 – 20 % Tricine-SDS-polyacrylamide gel are shown illustrating the amount of actin, CP and S100B present following incubation and high-speed ultra-centrifugation. 5 μM actin was polymerized in the absence or presence of CP alone, S100B alone, or with both (at the indicated concentrations) for 5 hrs at 25°C. Equal loadings of the Plt and S/N fractions were then run on the gel.
Figure 7. S100B can inhibit CPs capping activity in the presence of 1.4% TX-100. Pyrene actin fluorescence is plotted in panels (A) and (C). Panels A and C show inhibition of actin polymerization (2 μM) from barbed ends (nucleated by spectrin-F-actin seeds, SAS) under the indicated conditions by wt CP (A), and an α tentacle deletion mutant, CPα(C28)(C). (A) In the presence of 1.4% TX-100, high concentrations of S100B inhibit wt CPs capping activity. Red lines are a least squares fit of the data to a model where S100B can bind CP and inhibit its interaction with the filament end. (B) In the presence of 1.4% TX-100, the C28 peptide covalently coupled to Affi-gel® 10 resin (Affi-C28), binds to S100B. The α tentacle peptide (Affi-C34) does not. The upper portion of the panel shows a representative Tricine-SDS-polyacrylamide gel (15 – 20% acrylamide) illustrating the depletion of soluble S100B from the supernatant by increasing concentrations of Affi-C28 or Affi-C34. The lower portion is a plot of the concentration of bound S100B versus the concentration of Affi-C28 in μM. The data (three repeat experiments are shown) were least squares fit (black line) to equation 3 (see experimental procedures). (C) Addition of S100B in 1.4% TX-100 has no effect on the barbed end capping activity of the α tentacle deletion mutant, CPα(C28).
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A

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Capping protein binding to S100B: Implications for the "tentacle" model for capping the actin filament barbed end
Martin A. Wear and John A. Cooper

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