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The effects of the CapZ peptide (TRTK-12) binding to S100B-Ca²⁺ as examined by NMR and X-ray crystallography

Thomas H. Charpentier¹, Laura E. Thompson¹, Melissa A. Liriano¹, Kristen M. Varney¹, Paul T. Wilder¹, Edwin Pozharski², Eric A. Toth¹, and David J. Weber^{1,✉}

¹Department of Biochemistry and Molecular Biology, The University of Maryland School of Medicine, 108 N. Greene St. Baltimore, MD 21201

²Department of Pharmaceutical Sciences, The University of Maryland School of Pharmacy, 20 Penn St. Baltimore, MD 21201

Abstract

Structure-based drug design is underway to inhibit the S100B-p53 interaction as a strategy for treating malignant melanoma. X-ray crystallography was used here to characterize an interaction between Ca²⁺-S100B and a target, TRTK-12, which binds to the p53 binding site on S100B. The structures of Ca²⁺-S100B (1.5 Å resolution) and S100B-Ca²⁺-TRTK12 (2.0 Å resolution) determined here indicate that the S100B-Ca²⁺-TRTK12 complex is dominated by an interaction between Trp-7 of TRTK-12 and a hydrophobic binding pocket exposed on Ca²⁺-S100B involving residues in helices 2 & 3 and loop 2. As with a S100B-Ca²⁺-p53 peptide complex, TRTK-12 binding to Ca²⁺-S100B was found to increase the proteins Ca²⁺ ion binding affinity. One explanation for this effect was that peptide binding introduced a structural change that increased the number of Ca²⁺ ligands and/or improved Ca²⁺ ion coordination geometry of S100B. This possibility was ruled out when the structures of S100B-Ca²⁺-TRTK12 and S100B-Ca²⁺ were compared and calcium ion coordination by the protein was found to be nearly identical in both EF-hand calcium-binding domains (RMSD=0.19). On the other hand, B-factors for residues in EF2 of Ca²⁺-S100B were found to be significantly lowered with TRTK-12 bound. This result is consistent with NMR ¹⁵N relaxation studies that showed that TRTK-12 binding eliminated dynamic properties observed in Ca²⁺-S100B. Such a loss of protein motion may also provide an explanation for how calcium ion binding affinity is increased upon binding a target. Lastly, it follows that any small molecule inhibitor bound to Ca²⁺-S100B would also have to cause an increase in calcium ion binding affinity to be effective therapeutically inside a cell, so these data need to be considered in future drug-design studies involving S100B.

Keywords

S100B; TRTK-12; calcium; X-ray crystallography; NMR

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*Address all correspondences to: David J. Weber, PhD Professor Biochemistry and Molecular Biology University of Maryland School of Medicine 108 N. Greene St. Baltimore, MD 21201 Phone: (410) 706-4354 Fax: (410) 706-0458 dweber@umaryland.edu.

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Accession Codes: The coordinates for the S100B-Ca²⁺ and S100B-Ca²⁺-TRTK12 X-ray structures were deposited in the Protein Databank⁶¹; ⁶² and assigned the accession numbers 3IQO and 3IQQ, respectively.

Introduction

The small (10.7 kDa) acidic Ca^{2+} -binding protein S100B is a member of the S100 family of proteins that were originally named because of their solubility in 100% ammonium sulfate¹. S100 proteins have no intrinsic enzymatic activity, but like calmodulin (CaM) and most other EF-hand containing proteins, S100 proteins typically function as calcium-activated switches that bind to and regulate the biological function of numerous protein targets^{2; 3}. For S100B, several of these protein targets include cytoskeletal and filament associated proteins (e.g. tubulin, GFAP, tau, desmin, vimentin, CapZ, calponin, calpactin I, and caldesmon), other Ca^{2+} -binding proteins (e.g. annexins II, V, VI, S100A1, S100A6, S100A11, and neurocalcin- δ), membrane associated proteins (e.g. neuromodulin, neurogranin, MARCKS, giant protein AHNAK, and IQGAP1), transcription factors and their regulators (e.g. p53, hdm4, and hdm2) and several enzymes (e.g. aldolase, phosphoglucomutase, photoreceptor guanyl cyclase, Ndr kinase, and protein kinase C)^{4; 5}.

There are several high resolution 3D structures of S100B available that are useful for understanding the calcium-dependent conformational change in S100B, which is necessary for its interaction with protein targets, including p53 and CapZ^{6; 7; 8; 9; 10; 11; 12; 13}. In all of these structures, S100B is a symmetric dimer with a large number of hydrophobic interactions at its dimer interface consistent with its very high stability ($K_D < 1 \text{ nM}$)^{14; 15}. In addition, each S100B subunit has two EF-hand calcium-binding domains that are brought into close proximity by a small two-stranded anti-parallel β -sheet. The second EF-hand (EF2; residues 61–72) has the consensus 12 amino acid sequence that represents a “typical” EF-hand^{16; 17}, and binds calcium more tightly than the N-terminal EF-hand (EF1; residues 18–31). The pseudo-hand or S100-hand, EF1, has 14 rather than 12 residues and several of the ligands to calcium are from backbone carbonyl oxygen atoms rather than from sidechain carboxylate oxygen^{16; 17; 18; 19}. A central loop (loop 2; residues 40–50) connects the two calcium-binding domains and is usually referred to as the “hinge region” of S100 proteins. It is this hinge and the C-terminal loops that are not conserved among the S100 protein family members, and it is these regions of the protein that contribute to specificity for interactions with various target proteins²⁰.

A comparison of 3D structures of apo- and Ca^{2+} -bound S100B illustrates a large rotation ($\sim 90^\circ$) in the position of helix 3 upon Ca^{2+} -binding, exposing a hydrophobic cleft that is responsible for binding protein targets such as p53 and CapZ^{5; 6; 7; 11; 12; 13}. When a peptide derived from the negative regulatory domain of p53 (^{367–388}p53 peptide) binds to S100B, the affinity for Ca^{2+} in the typical EF-hand (EF2) of S100B was found to increase by 3-fold²¹. Consistent with this result, rates of Ca^{2+} -dissociation measured in stopped-flow competition studies with Tb^{3+} indicate that the off-rate of Ca^{2+} is decreased by more than 8-fold in the presence versus the absence of bound p53^{367–388}²². Furthermore, mutation of S100B in the EF-hand calcium-binding domains (E72A or E31A) cause the proteins to exhibit significant broadening of NMR resonances in the presence of calcium, indicative of exchange on the chemical shift timescale, and such broadening is eliminated upon binding the p53 peptide. A similar result was also observed in this study when the TRTK-12 peptide binds to the E72A mutant of S100B. With these data in mind, it was proposed that target peptide binding to S100B- Ca^{2+} was sufficient to stabilize dynamic properties observed in S100B resulting in the overall tightening of calcium-ion binding^{10; 21; 23}. However, an alternative explanation is that peptide binding to S100B- Ca^{2+} caused a conformational change in the EF-hand calcium-binding domain that introduced a new liganding residue and/or a more optimal Ca^{2+} coordination geometry. To test this alternate possibility, we have determined and directly compared crystal structures of S100B- Ca^{2+} and S100B- Ca^{2+} -TRTK12. The structure of S100B- Ca^{2+} -TRTK12 represents the first X-ray structure of S100B- Ca^{2+} bound to a target. These two structures also provide direct evidence that the coordination geometry for both EF-hand calcium-binding domains in S100B- Ca^{2+} are nearly identical in the absence and presence of bound TRTK-12 peptide and rule out

the hypothesis that peptide binding induces a conformational change that increases the affinity of EF2 for calcium. Furthermore, the B-factors for several residues in the canonical EF-hand calcium binding site were significantly lower when TRTK-12 was bound to S100B-Ca²⁺ in the typical EF-hand (residues 61–72) and is consistent with previously reported NMR ¹⁵N relaxation studies of S100B-Ca²⁺²⁴. Thus, the possibility that protein dynamics contribute to weaker calcium binding for S100B-Ca²⁺ in the absence of bound peptide target is discussed, as was suggested previously^{23; 24}. These data are also relevant to studies aimed at developing inhibitors of S100B since any small molecule inhibitor (SBiX; S100B inhibitor) would not only have to bind S100B with high affinity, but it would also need to tighten calcium-binding upon complex formation, as was shown here for TRTK12, to have an effect *in vivo*.

Results

Metal ion binding to S100B in the absence and presence of the target peptide TRTK-12

As described previously for the S100B-Mn²⁺-p53^{367–388} and S100B-Ca²⁺-p53^{367–388} complexes^{21; 23}, metal ion binding to S100B was performed in the absence and presence of the peptide target, TRTK-12, to determine whether a peptide target other than p53^{367–388} can increase the affinity of S100B for metal ions (Mn²⁺ and Ca²⁺). Binding studies with Mn²⁺ were completed since it is a good probe of the high affinity Ca²⁺ binding site on S100B (EF2), as previously shown^{21; 25}. The dissociation of TAMRA-TRTK12 from the S100B-Mn²⁺ and S100B-Ca²⁺ complexes were measured first to verify that TAMRA-TRTK12 could bind to S100B-Ca²⁺ and S100B-Mn²⁺. For these studies, a solution containing S100B-Ca²⁺ or S100B-Mn²⁺ was titrated into a solution of TAMRA-TRTK12 with Ca²⁺ or Mn²⁺ present, and changes in TAMRA fluorescence polarization from the TAMRA-TRTK12 peptide were monitored (Figure 1A and 1B; insets). Data from these titrations were fit to a single hyperbolic curve consistent with a single TAMRA-TRTK12 binding site per S100B subunit and dissociation constants (K_D) of 6 ± 2 and 1.2 ± 0.2 μM for S100B-Mn²⁺ and S100B-Ca²⁺, respectively (Table 1). Unlabeled TRTK-12 was added next in competition binding studies to determine the dissociation constants of unlabeled TRTK-12 from each of the S100B-metal-TRTK12 complexes. These data were fit to a single hyperbolic curve and dissociation constants were calculated using the modified Cheng-Prusoff equation²⁶. It was found that TRTK-12 bound to S100B-Mn²⁺ with a similar affinity (K_D = 3.0 ± 1.0 μM; Table 1) as for S100B-Ca²⁺ (K_D = 2.9 ± 0.5 μM; Table 1) and provided additional evidence that Mn²⁺ is a good probe for the study of EF-hand calcium signaling proteins, such as S100B. The formation of a S100B-Mn²⁺-TRTK12 complex was confirmed in a separate titration and was in agreement with titrations monitoring fluorescent polarization (Table 1); in which a solution of TRTK-12 plus Mn²⁺ was added to S100B-Mn²⁺ and a decrease in enhancement (ε*) of 1/T_{1ρ} of water protons was observed using NMR^{21; 27}. Such a deenhancement of the paramagnetic effect upon TRTK-12 binding is in agreement with structural studies that showed no direct coordination of TRTK-12 to Mn²⁺ in the ternary complex²⁸.

The dissociation of Mn²⁺ from the S100B-Mn²⁺ and S100B-Mn²⁺-TRTK12 complexes was compared next using electron paramagnetic resonance (EPR; Supplementary Figure 1S). Under otherwise identical conditions (i.e. [S100B], [Mn²⁺], buffer, etc), the addition of stoichiometric amounts of TRTK-12 to a solution of S100B-Mn²⁺ significantly reduced the amount of free Mn²⁺ as measured by EPR (Fig. 1S). Thus, it was confirmed in a titration that the dissociation constant of Mn²⁺ from the S100B-Mn²⁺ complex (K_D = 71 ± 12 μM), as reported previously²⁹, was 12-fold weaker than that of the dissociation of Mn²⁺ from the S100B-Mn²⁺-TRTK12 complex (K_D = 6 ± 2 μM; Table 1). Such a result was not unexpected since the binding of a peptide derived from p53 (residues 367–388; p53^{367–388}) also increased Mn²⁺ binding affinity; however, in the case of the S100B-Mn²⁺-p53^{367–388} complex, peptide binding only increased Mn²⁺ binding by 3-fold (Table 1)²¹.

Displacement of Mn^{2+} by Ca^{2+} in the S100B- Mn^{2+} -TRTK12 complex was then achieved in competition studies to obtain the dissociation constant of Ca^{2+} from the S100B- Ca^{2+} -TRTK12 complex (Figure 1C; Table 1). Specifically, the appearance of free Mn^{2+} was monitored by EPR upon the addition of Ca^{2+} (Figure 1C), and the data were confirmed by measurements of changes in enhancement (ϵ^*) using NMR with the identical samples (Figure 1C, inset). In the first set of titrations, it was confirmed that Ca^{2+} was readily able to displace Mn^{2+} from the tight site (EF2) of S100B in the absence of TRTK-12, as found previously²¹. A competition experiment between Ca^{2+} and Mn^{2+} for S100B was performed next in the presence of TRTK-12 peptide ($^{\text{C}}\text{K}_2 = 12 \pm 10 \mu\text{M}$; Table 1), and bound TRTK-12 was found to enhance the affinity of Ca^{2+} by 5-fold in the S100B- Ca^{2+} -TRTK12 complex versus in the absence of peptide (Table 1). Thus, as was found previously for S100B-metal ion complexes with the p53³⁶⁷⁻³⁸⁸ peptide²¹, the dissociation constants of Mn^{2+} and Ca^{2+} from the S100B- Mn^{2+} -TRTK12 and S100B- Ca^{2+} -TRTK12 complexes, respectively, were of higher affinity when compared to the analogous complexes in the absence the TRTK-12 target (Table 1). We next examined both the S100B- Ca^{2+} and the S100B- Ca^{2+} -TRTK12 structures by X-ray crystallography to examine whether structural changes at or nearby the calcium-binding site(s) were responsible for the increased Ca^{2+} -binding affinity observed when TRTK-12 is bound.

The X-ray structure of Ca^{2+} -loaded S100B

For direct comparison, it was necessary to first solve the structure of S100B- Ca^{2+} in a manner similar to that used for the structure determination of S100B- Ca^{2+} -TRTK12 (i.e. using the same cryoprotection, collection, and refinement techniques), so the structural differences and the B-factors of the two structures could be most accurately compared. With this in mind, the 1.50 Å resolution structure for S100B- Ca^{2+} was solved and compared to the S100B- Ca^{2+} -TRTK12 (see below). The final asymmetric unit of Ca^{2+} -bound S100B consisted of two S100B subunits (model A and model B) as a symmetric dimer with each subunit consisting of 88-residues for S100B (Ser1 to Phe88), two calcium ions per S100B subunit and a total of 146 water molecules. Nearly all of the residues of the S100B- Ca^{2+} were in the most favorable region of the Ramachandran plot (94.5%) with the remaining residues in the additionally allowed region (5.5%) (Table 2; Figure 2A). The global fold of S100B- Ca^{2+} was nearly identical to that of the X-ray structure reported previously (RMSD^{all atoms} of ~0.26 for both models A and B) with all of the Ca^{2+} -ligands, ligand distances, helical angles, and EF-hand angles found to be very similar (Tables 3–5). Specifically, each subunit of S100B- Ca^{2+} contained four helices (helix 1, S1-G19; helix 2, K28-L40; helix 3, E49-D61; helix 4, D69-F88) with the dimer interface aligned as a symmetric X-type four helix bundle and two helix-loop-helix EF-hand calcium-binding domains including an “S100-type” or “pseudo” EF-hand comprising helices 1, 2 and loop 1, and a “typical” EF-hand with twelve residues contributed by helices 3, 4 and loop 3 (Figure 2). Furthermore, the refined NMR structure of S100B- Ca^{2+} was also very similar to the X-ray structures of calcium-bound S100B²⁴. The only difference observed was with the length of helix 4, which was well defined up to residue Ala-83 in the NMR structure (versus Phe-88 in the X-ray structure). The remaining residues in the C-terminus (C84-E91) of calcium-bound S100B were found to exhibit a significant amount of conformational exchange and fast-time scale motion as a result of its dynamic nature in solution^{10; 12; 24; 30; 31; 32}. In the X-ray crystal structures, it is likely that this C-terminal loop of S100B is stabilized by crystal lattice contacts involving residues at the C-terminus including Phe-87 and Phe-88 of calcium-bound S100B resulting in the stabilization of helix 4 out to Phe-88.

The X-ray structure of S100B- Ca^{2+} -TRTK12 complex

The structure of S100B- Ca^{2+} -TRTK12 represents the first X-ray crystal structure of an S100B- Ca^{2+} -target complex, so it can be used to answer an important unresolved question about whether or not structural changes resulting from target protein binding directly affect Ca^{2+} ion

coordination in either of the two EF-hand calcium binding domains of S100B. If such a change in calcium ion coordination occurred, then this could provide an explanation for how divalent metal ions bind to S100B with a higher affinity when a target peptide is bound (Figure 1; Table 1). This question could not be answered conclusively via the existing NMR structures of S100B-Ca²⁺-target peptide complexes since the position of the bound Ca²⁺ ions could not be measured directly by NMR^{10; 31; 33; 34}. Furthermore, the X-ray structure of S100B-Ca²⁺-TRTK12 determined here will also be useful for resolving a discrepancy between two existing NMR structures^{31; 33; 34}, with regard to the helical conformation of the bound TRTK-12 peptide. Lastly, this structure will also be particularly useful for structure-based drug design studies aimed at inhibiting the S100B-p53 interaction in malignant melanoma since the S100B-TRTK-12 complex directly inhibits full-length p53 from binding Ca²⁺-S100B³⁵.

With these issues in mind, the 2.01 Å resolution X-ray structure for S100B-Ca²⁺-TRTK12 was solved. The final asymmetric unit consisted of 89-residues for S100B (Met0 to Phe88), 9-residues for TRTK-12 (Thr3 to Leu 11), two calcium ions, and 33 water molecules. The biologically significant model is a dimer comprised of the asymmetric unit and a crystallographic symmetry mate. Nearly all of the residues of the S100B-Ca²⁺-TRTK12 were in the most favorable region of the Ramachandran plot (93.3%) with the remaining residues in the additionally (5.6%) and generously (1.1%) allowed region (Table 2; Figure 3B). Unlike what was found for S100B-Ca²⁺ in solution, the NMR and X-ray structures of S100B-Ca²⁺-TRTK12 were in agreement with regard to the length of helix 4, which extended out to residue Phe-87 both in solution and in the crystalline form. This extended helical region was stabilized by direct interactions with the TRTK-12 peptide including hydrophobic interactions between Phe-87 in S100B and hydrophobic residues in the peptide as well as by a hydrogen bond between the backbone carbonyl oxygen of Glu-86 of S100B and the sidechain of Lys-9 in TRTK-12 (Figure 4).

Also consistent with both NMR structures^{31; 33}, the X-ray structure of S100B-Ca²⁺-TRTK12 illustrates that one TRTK-12 peptide binds per S100B subunit. The protein-peptide interaction of TRTK-12 with S100B is mostly hydrophobic with several hydrogen bonds, giving rise to a relatively high affinity interaction when compared to other peptide-protein interactions with S100B^{4; 36}. Of the total TRTK-12 solvent-accessible area 48.1% (586.7/1219.3 Å²) is involved in the S100B-Ca²⁺/peptide interface, and the TRTK-12 peptide occupies only 8.1% of the total solvent-accessible area of each S100B subunit (497.6/6160.7 Å²). The hydrophobic residues Ile-5, Trp-7, Ile-10 and Leu-11 of TRTK-12 contribute most of the buried surface area in the interaction interface. Residues of S100B contributing to hydrophobic peptide interactions are found on helix 2 (I36), loop 2 termed the 'hinge' (H42, F43, L44, E45, I47), helix 3 (V52, K55, V56, T59) and helix 4 (F76, M79, I80, A83, C84, E86, F87). The hinge of S100B connects helices 2 and 3 with residues from this loop including His-42, Phe-43, and Glu-45 found to contribute H-bonds to Thr-3, Lys-4, Ile-5, Asp-6, Trp-7 and Lys-9 of TRTK-12 (Figure 4). This specific interaction between the peptide and the protein created two alpha-helical turns forming hydrogen bonds on the TRTK-12 between Asp-6(O)-Lys-9(N) (3.54 Å) and Trp-7(O)-Leu-11(N) (2.52 Å). The helical nature of the peptide is representative of the helical nature that is found in the crystal structure of the capZ protein (PDB: 1IZN), indicating that TRTK-12 is in its most stable conformation. Furthermore, this helical structure very closely matches that found in solution as reported for one of the previously published NMR structures³³ (Figure 4D).

Comparisons of the structures of S100B-Ca²⁺-TRTK12 and S100B-Ca²⁺

Upon the addition of calcium to dimeric apo-S100B, helix 3 of both subunits reorients by ~90° relative to helix 4 as is found for most S100 proteins^{3; 5; 6; 8; 11; 12; 21; 24; 30; 31; 33}. This conformational change exposes residues on loop 2 termed the 'hinge' (residues H42, F43, L44,

E45, E46, I47), helix-3 (V51, K55, V56, T59), and helix-4 (F76, M79, I80, A83, C84, F87) and provides the “open” Ca^{2+} -bound form of S100B, which binds target peptides such as TRTK-12 (Figures 2–4). To determine the structural consequences of binding the target peptide, TRTK-12, we determined and compared the crystal structures of S100B- Ca^{2+} and S100B- Ca^{2+} -TRTK12 (Figures 5 and 6). As a control, we first compared the two asymmetric units of Model A (subunit 1) and Model B (subunit 2) of the symmetric S100B- Ca^{2+} dimer for which very minimal changes were observed (overall $\text{mainchain RMSD} = 0.2 \text{ \AA}$; $\text{sidechain RMSD} = 0.5 \text{ \AA}$). These included differences in positioning of the mainchain for residues in the pseudo-EF-hand (E21, G22) and helix 4 (E86, F87, F88) and differences in sidechain positioning for residues in helix 1 (E2, K5, V13), the pseudo-EF-hand (E21, K24, H25, K28), helix 3 (E58) and helix 4 (Q71, T81, E86, F87, F88) (Figures 5A & 5B). Such differences between Model A and B are not surprising in a small protein such as S100B, and are likely the result of varying crystal packing environments, although it cannot be ruled out that these differences represent two conformations of nearly the same free energy. As expected, additional differences were observed when the structures of Ca^{2+} -S100B (both models) and S100B- Ca^{2+} -TRTK12 were examined side-by-side that were not observed when models A and B of Ca^{2+} -S100B were compared (colored red in Figures 5C, 5D, 5E, & 5F only when the difference was observed for both models). Specifically, main chain residues that changed position when TRTK-12 bound to Ca^{2+} -S100B ($\text{RMSD} > 0.4 \text{ \AA}$; highlighted in red on Figs. 5C, 5E) included residues in helix 1 (S1, E2, L3, E4, K5, V8, A9), loop 2 (Q50, V53), and helix 4 (H85). Likewise, several sidechains changed positions ($\text{RMSD} > 1.0 \text{ \AA}$; highlighted in red on Figs. 5D, 5F) in similar regions as the mainchain including residues in helix 1 (Q16), loop 2 (E45, Q50), and helix 4 (M79, H85). In conclusion, several regions of the protein were conclusively found to change position upon the addition of TRTK-12 to S100B- Ca^{2+} while many residues that have direct interaction with TRTK-12 did not change position. These side chain residues form hydrophobic contribution of TRTK-12 binding. TRTK-12 directly interacting with these hydrophobic residues may help stabilize the S100B in the “open” Ca^{2+} bound position.

Calcium-coordinating residues in S100B are the same and positioned nearly identically when the structures of S100B- Ca^{2+} and S100B- Ca^{2+} -TRTK12 are compared

Although there were some changes in the conformation and orientation of specific residues of S100B- Ca^{2+} upon binding TRTK-12 (Figure 5), the residues that coordinate calcium in S100B- Ca^{2+} -TRTK12 were found to be the same as those for S100B- Ca^{2+} with minimal differences in coordinate distances and positions ($\text{RMSD}: 0.19 \text{ \AA}$; Table 3; Figure 2B). Specifically, calcium coordination in the S100-hand of S100B- Ca^{2+} and S100B- Ca^{2+} -TRTK12 was from four backbone carbonyl oxygen atoms including Ser-18 (X position), Glu-21 (Y position), Asp-23 (Z position), and Lys-26 (–Y position), a water molecule (–X position), and the two sidechain carboxylate oxygen atoms of Glu-31 (–Z position; bidentate ligand). In the typical EF-hand, calcium was coordinated by sidechain carboxylate oxygen atoms of Asp-61 (X position), Asp-63 (Y position), Asp-65 (Z position), the backbone carbonyl of Glu-67 (–Y position), a water molecule (–X position), and two sidechain carboxylate oxygen atoms of Glu-72 (–Z position; bidentate ligand) (Figure 2B). As discussed previously³⁷, a side-chain carboxylate oxygen atom of Glu-67 formed a hydrogen bond with a water molecule coordinated to calcium at the –X position of the pseudo-EF hand in S100B- Ca^{2+} -TRTK12 (Figure 2B, Table 3). Thus, it can be concluded that TRTK-12 binding did not affect calcium-coordination distances or the positioning of the residues involved in binding calcium in either EF1 or EF2 (Figure 2B).

Ca^{2+} -binding is restored to the E72A mutant of S100B in the presence of TRTK-12 as determined by NMR spectroscopy

Wild-type S100B binds calcium in the typical EF-hand (residues 61–72; EF2 $K_D = 56 \pm 9 \text{ \mu M}$; Table 1) more tightly than the pseudo EF-hand (residues 18–31; EF1 $K_D > 350 \text{ \mu M}$), and

a conformational change occurs upon binding EF2, which is necessary to bind target proteins such as TRTK-12^{8; 10; 30}. One notable NMR chemical shift perturbation that occurs upon the addition of calcium to wild-type S100B is a 2.12 ppm downfield shift in the amide ¹H for Gly-66. This residue is in position 6 of the 12 consensus residues in EF2, and the chemical shift perturbation for this glycine residue upon binding calcium is very typical for all EF-hand calcium-binding domains³⁰. This and several other perturbations (>400 Hz) are slow on the chemical shift timescale and provide a limiting value for the rate of calcium ion dissociation (<400/sec). Furthermore, TRTK-12 peptide binding to Ca²⁺-S100B is found to enhance calcium binding in the typical EF-hand by 5-fold (Table 1), despite the fact that no TRTK-12 peptide binding occurs in the absence of calcium even at high protein and peptide concentrations (>3 mM). Therefore, not surprisingly, the chemical shift of Gly-66 is also found to be significantly downfield shifted in the S100B-Ca²⁺-TRTK12 complex, and this chemical shift value (¹H, 10.11 ppm; ¹⁵N, 114.21 ppm) is only very slightly shifted from that found in Ca²⁺-S100B (¹H, 10.13 ppm; ¹⁵N, 114.28 ppm).

To examine the calcium-dependent S100B-target protein interaction more closely, the EF-hand mutant, E72A, was examined next in the absence and presence of bound TRTK-12 via NMR spectroscopy. As previously observed, the chemical shift values for the apo-E72A mutant were minimally different from those reported previously for wild-type S100B^{16; 23}, and the perturbations that were observed are for the most part localized to the general area of the mutation, which is indicative that no large structural rearrangement or problem with folding occurred as a result of the mutation. However, unlike what was observed previously in calcium ion titrations with wild-type S100B³⁰, the addition of calcium severely broadens several of the ¹H-¹⁵N correlations for the E72A mutant of S100B, including the HSQC correlation for Gly-66, which completely disappears. Furthermore, the spectra did not improve even at very high levels of calcium (20 mM). For Gly-66, this broadening effect is most readily explained by the weakened calcium-binding affinity of the E72A mutant protein giving rise to intermediate exchange on the chemical shift timescale or severe conformational exchange, as reported previously²³. Whereas, for wild-type S100B, several residues, including Gly-66, disappear and then reappear later in the titration consistent with slow exchange kinetics on the chemical shift timescale³⁰. For the E72A mutant, calcium binding could be measured, but it was consistent with only a single calcium ion binding to the pseudo EF hand of each S100B subunit (EF1 $K_D = 480 \pm 130 \mu\text{M}$)²³. The fact that this residual calcium binding (EF1 $K_D = 480 \pm 130 \mu\text{M}$) was indeed due to the pseudo EF-hand was confirmed by observations of tyrosine sensitized Tb³⁺ luminescence from Tyr-17 to Tb³⁺, which occurs only when Tb³⁺ is bound to EF1 as is described in detail previously^{23; 38}. Consistent with what was observed by NMR titrations, it was also found that binding of the TRTK-12 peptide to the E72A mutant is nearly identical to that found for the wild-type protein ($K_D = 3.2 \pm 1.5 \mu\text{M}$; Supplemental Fig. 2S). This binding occurred despite their large decreases in calcium binding in the absence of peptide. Furthermore, TRTK-12 binding to the E72A mutant of Ca²⁺-S100B eliminated much of the conformational exchange observed for the E72A-Ca²⁺ complex in the absence of peptide, and the appearance of the downfield shifted correlation for Gly-66, which typifies calcium binding in the slow-exchange regime to EF2, was restored (Fig. 2S; ¹H=10.07 ppm; ¹⁵N=113.83 ppm). These results indicate that the presence of a target peptide, such as TRTK-12, restored the ability of the E72A mutant of S100B to undergo the calcium-dependent conformational change involving EF2 as is necessary to bind target peptide. The restoration of calcium binding to the E72A mutant of S100B was also observed previously in titrations with a peptide derived from the C-terminus of p53²³, so this effect on calcium-binding is not specific for a single target peptide.

Discussion

In the absence of calcium, the TRTK-12 peptide does not bind to S100B, even at mM concentrations³³. Thus, as with several target protein interactions involving S100B, the interaction between S100B and TRTK-12 is dependent upon the addition of calcium^{4; 5; 20; 21; 31; 33; 39; 40; 41; 42}. As observed previously, most of the residues that interact with TRTK-12 (11 of 15; Fig. 4) are buried in the apo-S100B structure, but are exposed due to a large change in the position of helix 3 upon the addition of calcium explaining the calcium-dependent S100B-TRTK12 interaction^{6; 8; 11; 12; 13; 30; 31; 33; 43}. Furthermore, the relative orientation of all four helices in both subunits of dimeric S100B in the S100B-Ca²⁺-TRTK12 structures are nearly identical when the X-ray structure determined here and the two NMR structures determined previously are compared (Table 4 and 5)^{31; 33}. We also have found that a major component of the S100B-TRTK12 interface involves a hydrophobic interaction between the tryptophan residue of TRTK-12 (W7) and several hydrophobic residues from helices 3, 4, and loop 2 termed the 'hinge' region of Ca²⁺-S100B (Figure 4). There is also agreement in the identity and relative position of several sidechains at the S100B-TRTK12 interface when the X-ray structure of bovine S100B determined here is compared to the NMR structure of rat S100B bound to TRTK-12. This is likely because both of these structures illustrate that TRTK-12 bound to S100B forms a very small helix (Figure 4D). Thus, in addition to interactions with Trp-7, several other hydrophobic residues of TRTK-12 at the peptide-protein binding interface are the same including Ile-5, Ile-10, and Leu-11. In contrast, in another NMR structure of the human S100B-TRTK12 complex, it was reported that the peptide is in an extended conformation with several of the sidechain moieties of TRTK-12 positioned differently. Nonetheless, in all three structures, the tryptophan residue of the TRTK-12 peptide, Trp-7, is at the center of the S100B-TRTK12 interaction and is proximal to nine hydrophobic residues of S100B (Figure 4), indicating that this residue is critically important for binding under all conditions. In fact, when a peptide derived from p53 is mutated to a tryptophan at the analogous position (F387W), the affinity for this peptide, p53^{367-388; F387W} goes up by 3- to 4-fold²¹. Thus, the hydrophobic residues that define the S100B-TRTK12 interface include Ile-5, Trp-7, Ile-10, and Leu-11 of TRTK-12 and are extensively involved in a mini-hydrophobic core that likely determines the orientation of TRTK-12 peptide in the Ca²⁺-S100B binding pocket (Figure 4). While some ionic interactions and hydrogen bonds are also observed here (Figure 4C), they are fewer in number and probably do not contribute as much to the binding of TRTK-12, as discussed previously³⁶.

More recently, S100B has gained attention because it binds directly to the p53 tumor suppressor protein in melanoma, reduces p53 protein levels, and inhibits wild-type p53 functions in malignant melanoma^{44; 45}. Therefore, several groups are working to develop inhibitors of S100B with the goal of restoring functional p53 into cancers such as malignant melanoma. Such structure-based drug designed approaches will benefit greatly by this new X-ray structure of S100B bound to TRTK-12 particularly because TRTK-12 directly inhibits full length p53 from binding to Ca²⁺-S100B³⁵. Specifically, methods in designing/identifying small molecule inhibitors of S100B should make use of the fact the Trp-7 of TRTK-12 binds a well-defined hydrophobic pocket of S100B and that the majority of the S100B-TRTK12 interactions are hydrophobic with only a few hydrogen bonds. Thus, it is now clear that any small molecule inhibitor of S100B would benefit by binding this same hydrophobic pocket on S100B as defined by this structure of S100B bound to TRTK-12.

In addition to defining the S100B-TRTK12 interface, another major purpose for solving the structure of S100B-Ca²⁺-TRTK12 was to evaluate whether any changes in calcium-ion coordination resulted in Ca²⁺-bound S100B upon binding the TRTK-12 peptide. This question is of importance because of the observation here and elsewhere that target peptide binding increases the affinity of S100B for calcium²³. While some changes were observed in the

positioning of specific residues in Ca^{2+} -S100B upon binding TRTK-12 (Figure 5), the residues that coordinate calcium in S100B- Ca^{2+} -TRTK12 do not move when compared to those for S100B- Ca^{2+} (RMSD: 0.19 Å; Table 3; Figure 2B). Thus, TRTK-12 binding did not induce a conformational change in Ca^{2+} -bound S100B that could explain the increase in calcium affinity observed (Table 1). A closer look at the structure reveals that there are some residues on S100B that form hydrophobic interactions with TRTK-12, which are nearby the typical EF hand. This includes residues on helix 3 (V56 and T59) and helix 4 (F76). For example, Val-56 and Phe-76 each form i,i+4 hydrogen bonds to residues Leu-60 and Glu-72, respectively. Leu-60 is shown to be directly below the EF-hand while Glu-72 is the bidentate coordinating residue of EF2. Thus, TRTK-12 binding, may stabilize the EF-hand and increase Ca^{2+} affinity for the typical EF-hand in S100B through secondary interactions and H-bonds.

We next examined whether temperature factors (or B-factors) differed between the crystal structures of S100B- Ca^{2+} and S100B- Ca^{2+} -TRTK12. In such a comparison, the B-factors account for random displacements of atoms from their mean position as a result of either lattice disorder and/or from thermal motion; however, the timescale of such motions cannot be ascertained from a standard diffraction experiment. An interesting observation was made when the B-factors from the S100B- Ca^{2+} and S100B- Ca^{2+} -TRTK12 structures were compared (Figure 6). The B-factors were nearly the same for most residues in the protein with the exception of residues in EF2. Specifically, several residues in EF2 (i.e. S62, D63, G64, D65 and G66) had significantly higher temperature factors when the target peptide was not present (Figure 6). These changes are observed in both models A and B of S100B- Ca^{2+} , thus excluding the possibility of model-specific variations (Fig. 6). One explanation for the change in the B-factor values is that there was one variation in the lattice involving Lys-48 and two residues in EF2 (D65, D67). Specifically, Lys-48 is nearby the backbone carbonyl oxygen of Asp-65 in S100B- Ca^{2+} (3.26 Å) and may form a very weak hydrogen bond; whereas, in the S100B- Ca^{2+} -TRTK12 structure, the sidechain of Lys-48 is no longer within a hydrogen bond distance of Asp-65, but rather it approaches a carboxylate oxygen atom of Glu-67 (2.79 Å) and may form an ionic interaction instead. Thus, it is possible that this change in a lattice contact between crystallographic symmetry mates may contribute to the variation in B-factor values observed for residues in EF2 when S100B- Ca^{2+} and S100B- Ca^{2+} -TRTK12 are compared. However, this explanation is unlikely since the binding of a small molecule inhibitor of S100B, pentamidine, also causes the B-factors to lower for residues in EF2 (Fig. 4A and 4B; insets) even though a different lattice interaction is observed. In this structure, one residue at the beginning of the EF2 loop (S62) has two hydrogen bonds across the lattice with a backbone nitrogen atom (G22; 2.65 Å) and carboxylate oxygen atom (E21; 2.66 Å). Another explanation is that a single-body motion involving the loop comprising EF2 (residues S62 to G66) in the Ca^{2+} -bound form of S100B contributes to the lower B-factors and that TRTK-12 binding eliminates such motion. This explanation is supported by the fact that we observe weaker Ca^{2+} ion binding for S100B in the absence of bound TRTK-12 in solution. Further support for this interpretation is provided by ^{15}N -relaxation rate data published previously in which Glu-62 illustrates fast time-scale motion for S100B- Ca^{2+} (i.e. via ^{15}N - ^1H NOE)²⁴. That TRTK-12 binding to Ca^{2+} -S100B can eliminate dynamic motion in EF2 is also provided with NMR data from a mutant of S100B (E72A), which exhibits extensive broadening in EF2 for the Ca^{2+} -bound form of S100B that is eliminated upon binding to TRTK-12. Furthermore, the calcium-affinity in EF2 was significantly reduced for this mutant as was previously established²³; however, the highly characteristic downfield glycine proton- ^{15}N correlation (G66) in EF2, which is absent in Ca^{2+} -S100B for the E72A mutant of S100B, is rescued and returns clearly when TRTK-12 is bound (Fig. 2S). Such a result is consistent with TRTK-12 binding rescuing the ability of EF2 of the E72A mutant protein to bind Ca^{2+} tightly such that it is once again interacting with the protein in the slow-exchange regime.

Summary

The S100B-Ca²⁺-TRTK12 complex was found to have a 5-fold higher affinity for Ca²⁺ than S100B alone. One possible explanation for increased Ca²⁺-ion binding affinity explored here is that TRTK-12 binding to S100B-Ca²⁺ introduced a structural change that increased the number of Ca²⁺ ligands and/or improved the protein's Ca²⁺ ion coordination geometry. However, this possibility was ruled out when the structures of S100B-Ca²⁺-TRTK12 and Ca²⁺-S100B were compared using X-ray crystallography. Specifically, the calcium liganding residues were found to be the same in both EF-hand domains (S100-EF1, EF2) in the absence or presence of bound TRTK12 (S100-EF1, residues 18–31; EF2, residues 61–72), and the orientation of the Ca²⁺-coordinating sidechains were nearly identical in both EF-hand domains. However, the B-factors for several residues in the canonical EF-hand calcium binding site (EF2) were significantly lower when TRTK12 was bound to S100B-Ca²⁺. Furthermore, a similar observation was made when a small molecule inhibitor binds to calcium-S100B. These observations were also consistent with NMR data for which dynamic properties of Ca²⁺-S100B observed on both fast and slow timescales were eliminated when TRTK-12 or other target peptides bind to S100B-Ca²⁺^{23; 24; 32}. Thus, a model in which TRTK-12 binding to S100B-Ca²⁺ eliminates dynamic properties in EF2 and helix 4 is a mechanism that should be considered further as an explanation for the 5-fold increase in calcium-binding affinity when the TRTK-12 peptide target is bound. Lastly, it follows that like TRTK12, any small molecule inhibitor bound to Ca²⁺-S100B would also have to cause an increase in calcium ion binding affinity to be effective therapeutically inside a cell making these data relevant to future drug-design studies involving S100B.

Material and Methods

Materials

All chemicals and reagents were ACS-grade or higher and typically purchased from Sigma — Aldrich unless otherwise indicated. ¹⁵NH₄Cl was purchased from Cambridge Isotope Laboratories (Andover, MA). All buffers were passed through Chelex-100 resin to remove trace metals prior to use.

Bacterial expression and purification of the wild-type S100B

Recombinant S100B protein and the E72A mutant were expressed in *E. coli* (HMS174(DE3) strain) and purified as previously described³⁰. Yields of wild-type and mutant S100B protein were typically 20–30 mg of purified protein per liter of bacterial culture. For NMR experiments, S100B protein was prepared using defined media that included ¹⁵N-labeled NH₄Cl as the only nitrogen source³⁰.

Peptides

All peptides were synthesized using solid-state peptide synthesis and their purity was determined to be >95% by HPLC and mass spectrometry (Biosynthesis Inc., Lewisville, Texas). The TAMRA-TRTK12-am is an N-terminal 5-carboxytetramethylrhodamine (TAMRA) labeled derivative of the TRTK-12 peptide derived from the actin capping protein, CapZ, residues 265–276 (TAMRA-TRTKIDWNKILS-am) with an amidated C-terminus (-am). The TAMRA-TRTK12-am peptide was suspended in H₂O at 450 μM, and the pH adjusted to 7.2 with stocks stored frozen in 50 μL aliquots. The concentration of TAMRA-TRTK12-am was determined at pH 7.2 in H₂O using the extinction coefficient for TAMRA, ε₅₄₇ = 65,000 cm⁻¹M⁻¹. The TRTK-12 peptide was suspended in H₂O and then eluted through a G15 column and dialyzed twice in 0.25 mM Tris pH 7.5 and then lyophilized. The powder was suspended in 98% D₂O to a final concentration of 6.15 mM TRTK-12 and 4.9 mM Tris-D₁₁ and stored in 50 μL aliquots. The concentration of TRTK-12 was determined at pH 7.5 in 0.5

mM Tris-D₁₁, 98% D₂O using the extinction coefficient for TRTK12-am, $\epsilon_{280} = 8,850 \text{ cm}^{-1}\text{M}^{-1}$.

Fluorescent polarization competition assay (FPCA)

Fluorescence polarization competition assays (FPCA) were performed in Corning 96-well, flat bottom plates (Corning, NY) in a final volume of 200 μl . The S100B titration into TAMRA-TRTK12-am contained 0 – 40 μM S100B, 50 nM TAMRA-TRTK12-am, 50 mM Tris pH 7.5, 10 mM MnCl₂ or CaCl₂, and 0.10% Triton X-100. The polarization was read from the top of the well with a PolarStar fluorescent plate reader (BMG Labtech, Durham, NC) using a 544 $\pm$ 10 nm excitation and a 590 $\pm$ 10 nm emission filter with the temperature maintained at 30°C. Titrations of TRTK-12 into a solution of TAMRA-TRTK12-am bound to Ca²⁺- or Mn²⁺-S100B (wild-type or E72A mutant) were performed by monitoring the change in polarization of TAMRA-TRTK12-am with a Varian Cary Eclipse fluorescence spectrophotometer in quartz cuvettes. In these competition binding experiments, the sample contained 5 μM S100B, 50 nM TAMRA-TRTK12, 0–100 μM TRTK-12, 50 mM Tris pH 7.2, 15 mM NaCl, 100 mM KCl, 10 mM CaCl₂ or MnCl₂, 0.10% Triton X-100, and the temperature was maintained at 30°C using a circulating constant-temperature bath. The binding data were fit using a single site binding model with Origin software (OriginLab Corp., Northampton, MA), with one peptide bound per symmetrical S100B subunit. For the fluorescence polarization competition titrations, an equation derived by Nikolovska-Coleska et al. was used to calculate the dissociation constant (K_D) using the IC₅₀ values as follows: $K_D = [I]_{50}/([L]_{50}/K_D + [P]_0/K_D + 1)$ where $[I]_{50}$ is the concentration of the unlabelled TRTK-12 at 50% inhibition, $[L]_{50}$ is the concentration of the free TAMRA-TRTK12 at 50% inhibition, $[P]_0$ is the concentration of the free protein at 0% inhibition, and K_D is the dissociation constant for the dissociation of TAMRA-TRTK12-am from the S100B-Ca²⁺-TAMRA-TRTK12-am complex²⁶.

Metal and TRTK-12 peptide binding to S100B

The concentration of free Mn²⁺ in a mixture of free and bound Mn²⁺ was determined by electron paramagnetic resonance²⁷ using a Varian E-4 EPR spectrometer with the temperature maintained at 22°C in a buffer of 50mM Tris at pH 7.2. These data were supplemented by studies of bound Mn²⁺ under the same conditions by measuring the longitudinal relaxation rates of water protons at 24.3 MHz with a 180°- τ -90° pulse sequence using a Seimco pulsed NMR spectrometer as described previously^{27; 46}. The observed enhancement of the water proton relaxation rate is defined as $\epsilon^* = (1/T_{1P}^*)/(1/T_{1P})$ where $1/T_{1P}$ is the paramagnetic contribution to the longitudinal relaxation rate in the presence (*) and absence (no symbol) of S100B²⁷. The NMR and EPR data were analyzed to determine the stoichiometry (n) of Mn²⁺ bound, the dissociation constant (K_D), and the enhancement factor (ϵ_B) of the S100B-Mn²⁺ complex as previously described^{21; 27; 47; 48; 49; 50; 51; 52}. In addition the binding of Mn²⁺ (41, 60 and 82 μM) to S100B (65 μM) in the presence of the TRTK-12 (150 μM) peptide ($^{Mn}K_2$) was monitored by EPR and by changes in $1/T_{1P}^*$ of water protons and the observed enhancement factor (ϵ^*). A dissociation constant ($^{Mn}K_3$) of the TRTK-12 peptide from the S100B-Mn²⁺-TRTK12 (S100B: 65 μM ; TRTK-12: 0–150 μM) complex was analyzed using a noncooperative binding model to find the dissociation constant ($^{Mn}K_3$) and enhancement factors (ϵ_T) as previously described at two concentrations of Mn²⁺ (82 and 60 μM)^{21; 27; 50; 53}. The apparent dissociation constants of Ca²⁺ (0–5mM) from the tight site of S100B (CaK_{app}) in the absence and presence of TRTK-12 peptide were obtained in competition experiments in which the S100B-Mn²⁺($\pm$ TRTK12) were titrated with Ca²⁺ and monitoring the displacement of Mn²⁺ by changes in the enhancement (ϵ^*) of $1/T_{1P}^*$ and independently by the appearance of free Mn²⁺ in the EPR spectrum as previously described²¹. There was no detectable binding for EF1 (pseudo EF hand) when TRTK-12 was present as seen previously for the p53 peptide²¹; therefore, the subsequent competition experiments with Ca²⁺ only report

on the binding to the tight site (EF2). In addition, any binding of calcium to EF1 that may occur at the highest calcium concentrations, in such competition experiments, do not significantly affect the total calcium available to bind EF2 (< 1%). Thus, any effect of EF1 sequestering small amounts of calcium, under the conditions used in these experiments, have no effect on the calculations of K_2 values for EF2, within the error limits reported (Table 1). From these data, the actual dissociation constants of Ca^{2+} from the S100B- Mn^{2+} ($\pm$ TRTK12) were calculated using the competition equation $^{\text{Ca}}K_D = ^{\text{Ca}}K_{\text{app}} / (1 + ([\text{Mn}^{2+}] / ^{\text{Mn}}K_D))$. All calcium stocks were prepared from a calibrated 1.00 M CaCl_2 standard stock that was purchased from Fluka Analytical and the MnCl_2 concentration was determined by comparing the $1/T_{\text{IP}}$ of stock solutions to that of a Mn^{2+} sample of known concentration as previously described²⁷.

Protein crystallization

Bovine S100B protein was dialyzed into buffer (0.1 mM TES, pH 7.2, and 0.05 mM DTT), lyophilized, and dissolved in ddH_2O to 80–100 mg/mL (~ 8–10 mM subunit concentration) and stored frozen. Diffraction quality crystals for the S100B- Ca^{2+} -TRTK12 complex were obtained by sitting drop vapor diffusion at 22 °C by mixing 2 μL of S100B protein (40 mg/mL S100B, 7.5 mM CaCl_2 , 3.8 mM TRTK-12, 20 mM cacodylate buffer pH 7.2) with 2 μL of reservoir solution (7.5 mM CaCl_2 , 0.1 M cacodylate buffer pH 6.8, and 26% PEG3350) and equilibrating for 2–3 days. After crystals formed, they were cryoprotected in a harvest solution (7.5 mM CaCl_2 , 3.8 mM TRTK-12, 0.07 M cacodylate buffer pH 6.8, 28% PEG3350, and 5% glycerol) for 30–60 seconds and then flash-cooled in liquid nitrogen. The space group and unit cell parameters are given in Table 2. The above crystal forms had one S100B- Ca^{2+} -TRTK12 complex subunit in the asymmetric unit.

Diffraction quality crystals for the S100B- Ca^{2+} were obtained in a similar manner as S100B- Ca^{2+} -TRTK12 with 2 μL of S100B protein sample buffer (40 mg/mL S100B, 15 mM CaCl_2 , 20 mM cacodylate buffer pH 6.5) and 2 μL of reservoir solution (7.5 mM CaCl_2 , 0.1 M cacodylate buffer pH 6.3, and 25% PEG3350) and were cryoprotected (15 mM CaCl_2 , 0.1 M cacodylate buffer pH 6.3, 28% PEG3350, and 5% glycerol). The space group was C2 (Table 2) which was different and a lower symmetry than the previously published S100B- Ca^{2+} (1MHO)¹² and S100B- Ca^{2+} -TRTK12 that had a spacegroup of C222₁. The above crystal form of S100B- Ca^{2+} had two monomer subunits, which are labeled model A and model B in the asymmetric unit.

X-ray data collection, model building, and refinement

X-ray data for S100B- Ca^{2+} -TRTK12 were collected at 100 K using an in-house X-ray generator (MSC micromax 7; Rigaku Texas, USA) and a Raxis4⁺⁺ image plate detector (Rigaku Texas, USA). X-ray data for S100B- Ca^{2+} were collected remotely at the SSRL 12–2 beamline (Stanford Synchrotron Radiation Laboratory, Menlo Park, CA). The reflection intensities were integrated and scaled with the HKL2000 suite of computer programs⁵⁴. The crystal of S100B- Ca^{2+} and S100B- Ca^{2+} -TRTK12 diffracted to 1.50 Å and 2.01 Å resolutions, respectively. Both structures were solved by molecular replacement using the structure of Ca^{2+} -bound S100B with calcium ions removed (PDB file: 1MHO;¹²) as a search model and the computer program PHASER from the CCP4 program suite^{55; 56}. Model building and refinement of the S100B structure were completed using COOT and REFMAC^{57; 58}. The locations of the TRTK-12 molecule and several water molecules were determined by visual inspection electron density maps calculated with the $2mF_o - DF_c$ and $mF_o - DF_c$ coefficients with COOT⁵⁸. It was evident from visual inspection of the $2mF_o - DF_c$ and $mF_o - DF_c$ electron density maps that the only way to accurately model the bound TRTK-12 was to remove residues Thr-1 and Arg-2 from the N-terminal side and Ser-12 from the C-terminal side from the model. The C-terminal region of TRTK-12 is found on the crystallographic two-fold axis affecting Thr-1 and Arg-2, and there was no convincing density for these residues. The residue Thr-3 was found at the

crystallographic two-fold axis with convincing density for this residue, where the atoms C α and C β of Thr-3 were set to an occupancy value of 0.5 by REFMAC5. The stereochemistry was checked with the programs WHATCHECK and PROCHECK^{59; 60}. The quaternary structure and accessible surface areas were analyzed using the PISA server (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver). Figures were generated with the program PyMol (<http://www.pymol.org>).

NMR Spectroscopy

Purified ¹⁵N-labeled S100B was dialyzed against 0.1 mM TES pH 7.2, lyophilized, and hydrated in a small aliquot of ddH₂O and stored at −80 or −20 °C. The apo and Ca²⁺-loaded E72A S100B mutant NMR sample was prepared in a manner similar to that previously described²³ and contained 0.25 mM E72A mutant S100B subunit concentration, 0.625 mM TRTK-12, 0.34 mM NaN₃, 15 mM NaCl, 15 mM CaCl₂, 10% D₂O, 10 mM TES buffer, and adjusted to pH 7.2 with HCl. The Apo-S100B sample contained 1 mM EDTA, and the resonance assignments determined previously for the E72A mutant of S100B in the apo and Ca²⁺-bound states were confirmed here with standard 3D ¹⁵N-edited NOESY and ¹⁵N-edited HMQC-NOESY-HSQC²³. Likewise, the observable backbone ¹H and ¹⁵N assignments for the E72A mutant of S100B-Ca²⁺ bound to TRTK-12 were assigned in a straightforward manner in 2D HSQC titrations and confirmed using 3D ¹⁵N-edited NOESY and ¹⁵N-edited HMQC-NOESY-HSQC. Heteronuclear single quantum coherence (HSQC) NMR data, 3D ¹⁵N-edited NOESY, and ¹⁵N-edited HMQC-NOESY-HSQC data were collected at 37 °C with an Avance 800 US2 (800.27 MHz for protons) NMR spectrometer both equipped with pulsed-field gradients, four frequency channels, and triple resonance, z-axis gradient cryogenic probes⁶³. Data were processed with NMRPipe and analyzed with nmrDraw and NMRViewJ^{64; 65}. Proton chemical shifts were reported with respect to the H₂O or HDO signal taken as 4.658 ppm relative to external TSP (0.0 ppm). The ¹⁵N chemical shifts were indirectly referenced as previously described using the following ratio of the zero-point frequency: 0.10132905 for ¹⁵N to ¹H^{66; 67; 68}.

Supplementary Material

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Acknowledgments

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Abbreviations

EPR	electron paramagnetic resonance
NMR	Nuclear Magnetic Resonance
DTT	dithiothreitol
TPPI	time-proportional phase incrementation
TSP	3-(trimethylsilyl)-propionic acid-D4 sodium salt
PEG	polyethylene glycol

TAMRA	Carboxytetramethylrhodamine
HSQC	heteronuclear single-quantum coherence
EDTA	ethylenediaminetetraacetic acid
NOE	Nuclear Overhauser Effect
TES	(2-[2-hydroxyl-1,1-bis[hydroxymethyl]ethyl)-amino]ethanesulfonic acid)
Tris-HCl	tris(hydroxymethyl)-aminomethane hydrochloride
ddH ₂ O	deionized and doubly distilled H ₂ O

References

1. Moore B. A soluble protein characteristic of the nervous system. *Biochem. Biophys. Res. Comm* 1965;19:739–744. [PubMed: 4953930]
2. Donato R. Intracellular and extracellular roles of S100 proteins. *Microsc Res Tech* 2003;60:540–51. [PubMed: 12645002]
3. Heizmann CW. The multifunctional S100 protein family. *Methods Mol Biol* 2002;172:69–80. [PubMed: 11833360]
4. Wilder PT, Lin J, Bair CL, Charpentier TH, Yang D, Liriano M, Varney KM, Lee A, Oppenheim AB, Adhya S, Carrier F, Weber DJ. Recognition of the tumor suppressor protein p53 and other protein targets by the calcium-binding protein S100B. *Biochim Biophys Acta*. 2006
5. Santamaria-Kisiel L, Rintala-Dempsey AC, Shaw GS. Calcium-dependent and -independent interactions of the S100 protein family. *Biochem J* 2006;396:201–14. [PubMed: 16683912]
6. Drohat AC, Amburgey JC, Abildgaard F, Starich MR, Baldisseri D, Weber DJ. Solution structure of rat apo-S100B as determined by NMR spectroscopy. *Biochemistry* 1996;35:11577–11588. [PubMed: 8794737]
7. Drohat AC, Baldisseri DM, Rustandi RR, Weber DJ. Solution structure of calcium-bound rat S100B (betabeta) as determined by nuclear magnetic resonance spectroscopy. *Biochemistry* 1998;37:2729–40. [PubMed: 9485423]
8. Drohat AC, Tjandra N, Baldisseri DM, Weber DJ. The use of dipolar couplings for determining the solution structure of rat apo-S100B. *Protein Science* 1999;8:800–9. [PubMed: 10211826]
9. Inman KG, Yang R, Rustandi RR, Miller KE, Baldisseri DM, Weber DJ. Solution NMR structure of S100B bound to the high-affinity target peptide TRTK-12. *Journal of Molecular Biology* 2002;324:1003–14. [PubMed: 12470955]
10. Rustandi RR, Baldisseri DM, Weber DJ. Structure of the negative regulatory domain of p53 bound to S100B. *Nat Struct Biol* 2000;7:570–4. [PubMed: 10876243]
11. Kilby PM, Van Eldik LJ, Roberts GC. The solution structure of the bovine S100B protein dimer in the calcium-free state. *Structure* 1996;4:1041–52. [PubMed: 8805590]
12. Matsumura H, Shiba T, Inoue T, Harada S, Kai Y. A novel mode of target recognition suggested by the 2.0 Å structure of holo S100B from bovine brain. *Structure* 1998;6:233–41. [PubMed: 9519413]
13. Smith SP, Shaw GS. A novel calcium-sensitive switch revealed by the structure of human S100B in the calcium-bound form. *Structure* 1998;6:211–222. [PubMed: 9519411]
14. Drohat AC, Nenortas E, Beckett D, Weber DJ. Oligomerization state of S100B at nanomolar concentration determined by large-zone analytical gel filtration chromatography. *Protein Sci* 1997;6:1577–82. [PubMed: 9232658]
15. Ferguson PL, Shaw GS. Role of the N-terminal helix I for dimerization and stability of the calcium-binding protein S100B. *Biochemistry* 2002;41:3637–46. [PubMed: 11888280]
16. Amburgey JC, Abildgaard F, Starich MR, Shah S, Hilt DC, Weber DJ. 1H, 13C and 15N NMR assignments and solution secondary structure of rat Apo-S100 beta. *J Biomol NMR* 1995;6:171–9. [PubMed: 8589606]

17. Strynadka NCJ, James MNG. Crystal structures of the helix-loop-helix calcium-binding proteins. *Ann. Rev. Biochem* 1989;58:951–998. [PubMed: 2673026]
18. Kretsinger RH, Rudnick SE, Sneden DA, Schatz VB. Calmodulin, S-100, and crayfish sarcoplasmic calcium-binding protein crystals suitable for X-ray diffraction studies. *Journal of Biological Chemistry* 1980;255:8154–6. [PubMed: 7410354]
19. Baudier J, Gerard D. Ions binding to S100 proteins. II. Conformational studies and calcium-induced conformational changes in S100 alpha alpha protein: the effect of acidic pH and calcium incubation on subunit exchange in S100a (alpha beta) protein. *J Biol Chem* 1986;261:8204–12. [PubMed: 3722150]
20. Weber, DJ.; Rustandi, RR.; Carrier, F.; Zimmer, DB. Interaction of dimeric S100B($\beta\beta$) with the tumor suppressor protein: A model for Ca-dependent S100-target protein interactions. In: Pochet, R., editor. *The molecular basis of calcium action in biology and medicine*. Kluwer Academic Publishers; Dordrecht, The Netherlands: 2000.
21. Rustandi RR, Drohat AC, Baldisseri DM, Wilder PT, Weber DJ. The Ca^{2+} -dependent interaction of S100B with a peptide derived from p53. *Biochemistry* 1998;37:1951–60. [PubMed: 9485322]
22. Markowitz J, Rustandi RR, Varney KM, Wilder PT, Udan R, Wu SL, Horrocks WD, Weber DJ. Calcium-Binding Properties of Wild-Type and EF-Hand Mutants of S100B in the Presence and Absence of a Peptide Derived from the C-Terminal Negative Regulatory Domain of p53. *Biochemistry* 2005;44:7305–7314. [PubMed: 15882069]
23. Markowitz J, Rustandi RR, Varney KM, Wilder PT, Udan R, Wu SL, Horrocks WD, Weber DJ. Calcium-binding properties of wild-type and EF-hand mutants of S100B in the presence and absence of a peptide derived from the C-terminal negative regulatory domain of p53. *Biochemistry* 2005;44:7305–14. [PubMed: 15882069]
24. Wright NT, Inman KG, Levine JA, Cannon BR, Varney KM, Weber DJ. Refinement of the solution structure and dynamic properties of $\text{Ca}(2+)$ -bound rat S100B. *J Biomol NMR* 2008;42:279–86. [PubMed: 18949447]
25. Mildvan AS, Granot J, Smith GM, Liebman M. *Adv. Inorg. Biochem* 1979;2:211–236.
26. Nikolovska-Coleska Z, Wang R, Fang X, Pan H, Tomita Y, Li P, Roller PP, Krajewski K, Saito NG, Stuckey JA, Wang S. Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. *Anal Biochem* 2004;332:261–73. [PubMed: 15325294]
27. Mildvan AS, Engle JL. Nuclear relaxation measurements of water protons and other ligands. *Methods Enzymol* 1972;26(PtC):654–82. [PubMed: 4376581]
28. Mildvan AS, Engle JL. Nuclear relaxation measurements of water protons and other ligands. *Methods Enzymol* 1972;49D:322–359.
29. Rustandi RR, Drohat AC, Baldisseri DM, Wilder PT, Weber DJ. The $\text{Ca}(2+)$ -dependent interaction of S100B(beta beta) with a peptide derived from p53. *Biochemistry* 1998;37:1951–60. [PubMed: 9485322]
30. Drohat AC, Baldisseri DM, Rustandi RR, Weber DJ. Solution structure of calcium-bound rat S100B as determined by nuclear magnetic resonance spectroscopy. *Biochemistry* 1998;37:2729–2740. [PubMed: 9485423]
31. McClintock KA, Shaw GS. A novel S100 target conformation is revealed by the solution structure of the Ca^{2+} -S100B-TRTK-12 complex. *J Biol Chem* 2003;278:6251–7. [PubMed: 12480931]
32. Rustandi RR, Baldisseri DM, Drohat AC, Weber DJ. Structural changes in the C-terminus of Ca^{2+} -bound rat S100B upon binding to a peptide derived from the C-terminal regulatory domain of p53. *Protein Science* 1999;8:1743–1751. [PubMed: 10493575]
33. Inman KG, Yang R, Rustandi RR, Miller KE, Baldisseri DM, Weber DJ. Solution NMR structure of S100B bound to the high-affinity target peptide TRTK-12. *J Mol Biol* 2002;324:1003–14. [PubMed: 12470955]
34. Bhattacharya S, Large E, Heizmann CW, Hemmings B, Chazin WJ. Structure of the Ca^{2+} /S100B/NDR kinase peptide complex: insights into S100 target specificity and activation of the kinase. *Biochemistry* 2003;42:14416–26. [PubMed: 14661952]
35. Wilder PT, Lin J, Bair CL, Charpentier TH, Yang D, Liriano M, Varney KM, Lee A, Oppenheim AB, Adhya S, Carrier F, Weber DJ. Recognition of the tumor suppressor protein p53 and other protein

targets by the calcium-binding protein S100B. *Biochim Biophys Acta* 2006;1763:1284–97. [PubMed: 17010455]

36. McClintock KA, Van Eldik LJ, Shaw GS. The C-terminus and linker region of S100B exert dual control on protein-protein interactions with TRTK-12. *Biochemistry* 2002;41:5421–8. [PubMed: 11969402]
37. Otterbein LR, Kordowska J, Witte-Hoffmann C, Wang CL, Dominguez R. Crystal structures of S100A6 in the Ca(2+)-free and Ca(2+)-bound states: the calcium sensor mechanism of S100 proteins revealed at atomic resolution. *Structure* 2002;10:557–67. [PubMed: 11937060]
38. Chaudhuri D, Horrocks WD Jr, Amburgey JC, Weber DJ. Characterization of lanthanide ion binding to the EF-hand protein S100 beta by luminescence spectroscopy. *Biochemistry* 1997;36:9674–80. [PubMed: 9245399]
39. Donato R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol* 2001;33:637–68. [PubMed: 11390274]
40. Heizmann CW, Cox JA. New perspectives on S100 proteins: a multi-functional Ca(2+)-, Zn(2+)- and Cu(2+)-binding protein family. *Biometals* 1998;11:383–97. [PubMed: 10191501]
41. Ivanenkov VV, Jamieson GA Jr, Gruenstein E, Dimlich RV. Characterization of S-100b binding epitopes. Identification of a novel target, the actin capping protein, CapZ. *J Biol Chem* 1995;270:14651–8. [PubMed: 7540176]
42. Delphin C, Ronjat M, Deloulme JC, Garin G, Debussche L, Higashimoto Y, Sakaguchi K, Baudier J. Calcium-dependent interaction of S100B with the C-terminal domain of the tumor suppressor p53. *J Biol Chem* 1999;274:10539–44. [PubMed: 10187847]
43. Wright NT, Cannon BR, Wilder PT, Morgan MT, Varney KM, Zimmer DB, Weber DJ. Solution structure of S100A1 bound to the CapZ peptide (TRTK12). *J Mol Biol* 2009;386:1265–77. [PubMed: 19452629]
44. Lin J, Blake M, Tang C, Zimmer D, Rustandi RR, Weber DJ, Carrier F. Inhibition of p53 transcriptional activity by the S100B calcium-binding protein. *J Biol Chem* 2001;276:35037–41. [PubMed: 11454863]
45. Lin J, Yang Q, Yan Z, Markowitz J, Wilder PT, Carrier F, Weber DJ. Inhibiting S100B Restores p53 Levels in Primary Malignant Melanoma Cancer Cells. *J Biol Chem* 2004;279:34071–7. [PubMed: 15178678]
46. Carr HY, Purcell EM. Effects of Diffusion on Free Precession in Nuclear Magnetic Resonance Experiments. *Phys. Rev* 1954;94:630–638.
47. Mildvan AS, Cohn M. Kinetic and magnetic resonance studies of the pyruvate kinase reaction. II. Complexes of enzyme, metal, and substrates. *J Biol Chem* 1966;241:1178–93. [PubMed: 5933875]
48. Mildvan AS, Cohn M. Magnetic Resonance Studies of the Interaction of the Manganous Ion with Bovine Serum Albumin. *Biochemistry* 1963;2:910–9. [PubMed: 14087380]
49. Serpersu EH, Shortle D, Mildvan AS. Kinetic and magnetic resonance studies of effects of genetic substitution of a Ca²⁺-liganding amino acid in staphylococcal nuclease. *Biochemistry* 1986;25:68–77. [PubMed: 3513826]
50. Serpersu EH, Shortle D, Mildvan AS. Kinetic and magnetic resonance studies of active-site mutants of staphylococcal nuclease: factors contributing to catalysis. *Biochemistry* 1987;26:1289–300. [PubMed: 3567171]
51. Weber DJ, Meeker AK, Mildvan AS. Interactions of the acid and base catalysts on staphylococcal nuclease as studied in a double mutant. *Biochemistry* 1991;30:6103–14. [PubMed: 1676297]
52. Weber DJ, Serpersu EH, Shortle D, Mildvan AS. Diverse interactions between the individual mutations in a double mutant at the active site of staphylococcal nuclease. *Biochemistry* 1990;29:8632–42. [PubMed: 1702994]
53. Reed GH, Cohn M, O'Sullivan WJ. Analysis of equilibrium data from proton magnetic relaxation rates of water for manganese-nucleotide-kinase ternary complexes. *J Biol Chem* 1970;245:6547–52. [PubMed: 5482765]
54. Otwinowski Z, Minor W. Processing of X-Ray diffraction data collected in oscillation mode. *Methods Enzymol* 1997;276:307–326.

55. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. *J Appl Crystallogr* 2007;40:658–674. [PubMed: 19461840]
56. Vagin AA, Isupov MN. Spherically averaged phased translation function and its application to the search for molecules and fragments in electron-density maps. *Acta Crystallogr D Biol Crystallogr* 2001;57:1451–6. [PubMed: 11567159]
57. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 1997;53:240–55. [PubMed: 15299926]
58. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 2004;60:2126–32. [PubMed: 15572765]
59. Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: a program to check the stereochemical quality of protein structures. *Journal of Applied Crystallography* 1993;26:283–91.
60. Laskowski RA, MacArthur MW, Thornton JM. Validation of protein models derived from experiment. *Curr Opin Struct Biol* 1998;8:631–9. [PubMed: 9818269]
61. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. The Protein Data Bank. *Nucleic Acids Res* 2000;28:235–42. [PubMed: 10592235]
62. Yang H, Guranovic V, Dutta S, Feng Z, Berman HM, Westbrook JD. Automated and accurate deposition of structures solved by X-ray diffraction to the Protein Data Bank. *Acta Crystallogr D Biol Crystallogr* 2004;60:1833–9. [PubMed: 15388930]
63. Mori S, Abeygunawardana C, Johnson MO, van Zijl PC. Improved sensitivity of HSQC spectra of exchanging protons at short interscan delays using a new fast HSQC (FHSQC) detection scheme that avoids water saturation. *J Magn Reson B* 1995;108:94–8. [PubMed: 7627436]
64. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995;6:277–93. [PubMed: 8520220]
65. Johnson BA. Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol Biol* 2004;278:313–52. [PubMed: 15318002]
66. Edison AS, Abildgaard F, Westler WM, Mooberry ES, Markley JL. Practical introduction to theory and implementation of multinuclear, multidimensional nuclear magnetic resonance experiments. *Methods Enzymol* 1994;239:3–79. [PubMed: 7830587]
67. Live DH, Davis DG, Agosta WC, Cowburn D. Long range hydrogen bond mediated effects in peptides: ^{15}N NMR study of gramicidin S in water and organic solvents. *J. Am. Chem. Soc* 1984;106:1939–1941.
68. Spera S, Bax A. Empirical correlation between protein backbone conformation and $\text{C}\alpha$ and $\text{C}\beta/^{13}\text{C}$ nuclear magnetic resonance chemical shifts. *Journal of American Chemical Society* 1991;113:5490–5492.

Figure 1.

Thermodynamic binding constants for S100B-metal and S100B-metal-TRTK12 complexes. (a) Displacement of the TAMRA-TRTK12 peptide from the S100B- Ca^{2+} -TAMRA-TRTK12 complex by unlabeled TRTK12 as monitored by fluorescence polarization. The solution contained 1.5 μM S100B, 50 nM TAMRA-TRTK12, and 10 mM $CaCl_2$ in 50mM Tris-HCl pH 7.5. (a, inset) Binding of TAMRA-TRTK12 to S100B- Ca^{2+} as monitored by fluorescence polarization. The solution contained 50 nM TAMRA-TRTK12, and 10 mM $CaCl_2$ in 50 mM Tris-HCl, pH 7.5. (b) Displacement of the TAMRA-TRTK12 peptide from the S100B- Mn^{2+} -TAMRA-TRTK12 complex by unlabeled TRTK12 as monitored by changes in fluorescence polarization (ΔmP). The solution contained 4 μM S100B, 50 nM TAMRA-TRTK12, and 10 mM $MnCl_2$ in 50mM Tris-HCl pH 7.5. (b, inset) Binding of TAMRA-TRTK12 to S100B- Mn^{2+} as monitored by changes in fluorescence polarization. The solution contained 50 nM TAMRA-TRTK12, and 10 mM $MnCl_2$ in 50 mM Tris-HCl pH 7.5. (c) Displacement of bound Mn^{2+} by Ca^{2+} from S100B in the presence of TRTK as detected by EPR and NMR (inset). The solutions for both experiments contain 65 μM S100B, 150 μM TRTK-12, and 82 μM $MnCl_2$ in 50mM Tris-HCl pH 7.5. The data were fit with the Hill equation to determine

K_{app} with the corresponding dissociation constants calculated using competition equations described in Material and Methods.

Figure 2.

Calcium ion coordination in the X-ray structures of S100B- Ca^{2+} in the presence and absence of TRTK-12. (a) X-ray crystal structure of S100B- Ca^{2+} (3IQO) shown in a ribbon diagram with two Ca^{2+} ions per subunit (cyan spheres) labeled I (EF1, Pseudo EF-hand) and II (EF2, Typical EF-hand) with the subunits of the symmetric S100B homodimer colored red and blue with alpha helices labeled (I–IV). (b) The positions for the sidechains of the Ca^{2+} coordinating residues are compared for the pseudo-EF-hand (Ser18, Glu21, Asp23, Lys26 and Glu31) and the canonical EF-hand (Asp61, Asp63, Asp65, Glu67, and Glu72) calcium-binding sites of S100B- Ca^{2+} (green) and S100B- Ca^{2+} -TRTK12 (Magenta) (RMSD = 0.18 Å).

Figure 3.

The X-ray and NMR structures of the S100B-Ca²⁺-TRTK12 complex. (a) Stereo view (walleye mode) of the TRTK-12 peptide when bound to S100B with the electron density maps calculated with the 2mF_o-DF_c coefficients (1.0σ) for TRTK-12. (b) Ribbon and surface diagram of the X-ray structure of S100B-Ca²⁺-TRTK12 (3IQQ) illustrating the location of TRTK-12 (green) together with residues of S100B that interact with the peptide (in yellow). (c) Ribbon and surface diagram of the NMR structure of S100B-Ca²⁺-TRTK12 (1MWN) illustrating the location of TRTK-12 (green) bound to rat S100B with residues colored (in yellow) that have intermolecular NOE correlations to TRTK-12³³.

Figure 4.

Side-chains of S100B and TRTK-12 that are involved in the peptide-protein interface. (a) TRTK-12 peptide residues that are observed in the X-ray crystal structure are illustrated (T3-L11) highlighting residues on S100B that are within 4.0 Å of TRTK peptide (in boxes) and residues that are involved in hydrogen bonding in red. (b) View of TRTK-12 (green) and S100B (blue) illustrating side-chains involved in hydrophobic interactions (yellow) between the TRTK-12 and S100B. (c) View of TRTK-12 (green) and S100B (blue) illustrating side-chains involved in hydrogen bonds (dashed lines) between TRTK-12 and S100B. (d) The X-ray structure of the bovine S100B- Ca^{2+} -TRTK12 complex (shown in blue and green) superposed on the NMR solution structure of the same complex with rat S100B (red PDB: 1MWN).

Figure 6.

Graph showing B-factor values for each residue in S100B- Ca^{2+} in the absence and presence of bound TRTK-12. (a) Average of main-chain atoms B-factor values per residue of S100B for S100B- Ca^{2+} ; model A (blue diamonds), S100B- Ca^{2+} ; model B (green squares), S100B- Ca^{2+} -TRTK (red triangle). (inset) Average of main-chain atoms B-factor values per residues of S100B for S100B- Ca^{2+} -pentamidine (orange pluses) and S100B- Ca^{2+} -TRTK (red triangle). (b) Average of all atoms B-factors per residue of S100B for S100B- Ca^{2+} ; model A (blue diamonds), Ca^{2+} -S100B; model B (green squares), S100B- Ca^{2+} -TRTK (red triangle). (inset) Average of all atoms B-factors per residue of S100B for S100B- Ca^{2+} -pentamidine (orange pluses) and S100B- Ca^{2+} -TRTK (red triangle). (c) Ribbon diagram of the X-ray structure of S100B- Ca^{2+} -TRTK (3IQQ) illustrating the residues of S100B (in red) that showed significant changes in B-factor values when the X-ray structural models for S100B- Ca^{2+} -TRTK and S100B- Ca^{2+} (i.e. for both models A and B) were compared.

Table 1
Dissociation of Metal Ions or Peptides from S100B-Metal or S100B-Metal-Peptide Complexes^a

Metal Ions	K _D (μM) ^b	p53K ₂ (μM) ^c	TRTKK ₂ (μM) ^d	p53K ₃ (μM) ^e	TRTKK ₃ (μM) ^f	Tamara-TRTKK ₃ (μM) ^g
Ca ²⁺	56 ± 9 (2) ^f	20 ± 3 (5) ^f	12 ± 10 (5)	24 ± 7 (3) ^f	2.9 ± 0.5 (2)	1.2 ± 0.2 (2)
Mn ²⁺	71 ± 12 (2) ^f	27 ± 4 (2) ^f	6.0 ± 2.0 (3)	94 ± 20 (4) ^f	3.0 ± 1.0 (3)	6.0 ± 2.0 (2)

^aThe number of experiments (n) is shown in parentheses.

^bDissociation constants of metals from the tight site (EF2) of S100B.

^cDissociation constants (p53K₂) of metals from the tight site of S100B (EF2) in the presence of p53 peptide.

^dDissociation constants (TRTKK₂) of metals from the tight site of S100B (EF2) in the presence of TRTK peptide.

^eDissociation constants of p53 (p53K₃) peptide from S100B-metal complexes.

^fValues reported in Rustandi et al, 1998.

^gDissociation constants (TRTKK₃) of TRTK-12 from S100B-metal complexes as determined by performing competition experiments with TAMARA-TRTK12.

^hDissociation constants (Tamara-TRTKK₃) of TAMARA-TRTK from metal-S100B complexes as measured directly by changes in fluorescence polarization.

Table 2**X-ray Diffraction and Model Refinement Statistics**

	Ca²⁺-S100B^a	TRTK-Ca²⁺-S100B
Diffraction Statistics		
Space Group	C2 ₁	C222 ₁
Cell dimensions a, b, c (Å)	89.6, 35.0, 58.1	33.0, 89.8, 58.9
Cell angles α, β, γ (deg)	90, 92.6, 90	90, 90, 90
Resolution (Å)	58.03–1.50 (1.54–1.50) ^b	44.90–2.01 (2.06–2.01) ^a
No. of unique reflections	24746 (1408)	5763 (421)
Completeness (%)	89.1 (68.4)	98.7 (97.8)
R _{sym} ^c	0.061 (0.247)	0.066 (0.200)
Average I/σ	13.9 (2.8)	28.8 (13.4)
Multiplicity	2.6 (1.5)	12.7 (12.9)
Refinement Statistics		
R _{crys} ^d (%)	20.2 (23.8)	22.3 (22.1)
R _{free} ^d (%)	24.2 (35.6)	26.7 (27.2)
Protein Atoms	1452	796
Water Molecules	146	33
Non-Hydrogen Atoms	1599	832
RMSD		
Bond Length (Å)	0.015	0.014
Bond Angles (Å)	1.333	1.736
Mean B values (Å ²)	32.56	34.61
Ramachandran plot (%)^e		
Most Favored	94.5	93.3
Additionally Allowed	5.5	5.6
Generously Allowed	0.0	1.1
PDB identification	3IQO	3IQQ

^aThe previously published Ca²⁺-S100B (1MHO) was compared to Model A and Model B of the Ca²⁺-S100B structure represented here, the RMSD changes were very minimal at 0.251 and 0.266, respectively.

^bNumbers in parentheses represent the last outer shell.

^cR_{sym} = $\sum h \sum i (|l(h)| - \langle |l(h)| \rangle) / \sum h \sum i l(h)$, where $l(h)$ = observed intensity, and $\langle |l(h)| \rangle$ = mean intensity obtained from multiple measurements.

^dR_{crys} and R_{free} = $\sum ||F_o| - |F_c|| / \sum |F_o|$, where $|F_o|$ = observed structure factor amplitude and $|F_c|$ = calculated structure factor amplitude for the working and test sets, respectively.

^eFor Ca²⁺-S100B the calculations had 155 residues in the most favored region and 9 residues in additionally allowed regions and for TRTK-Ca²⁺-S100B the calculations had 84 residues in the most favored region, 5 residues in additionally allowed regions, and 1 residue in the generously allowed regions.

Table 3Divalent metal ion and water coordinate distances^{a,b}

	Ca ²⁺ -S100B (A)	Ca ²⁺ -S100B (B)	TRTK-Ca ²⁺ -S100B
Pseudo EF Hand (EF1)			
Ser-18 (C=O)	2.3	2.4	2.3
Glu-21 (C=O)	2.4	2.4	2.3
Asp-23 (C=O)	2.3	2.3	2.4
Lys-26 (C=O)	2.4	2.4	2.4
Glu-31 (Oε1, Oε2)	2.5, 2.6	2.4, 2.6	2.4, 2.7
H ₂ O ^d	2.6	2.3	2.3
Canonical EF Hand (EF2)			
Asp-61 (Oδ1 or Oδ2)	2.3	2.3	2.2
Asp-63 (Oδ1 or Oδ2)	2.3	2.3	2.4
Asp-65 (Oδ1 or Oδ2)	2.5	2.4	2.4
Glu-67 (C=O)	2.3	2.3	2.3
Glu-72 (Oε1, Oε2)	2.4, 2.6	2.4, 2.6	2.5, 2.7
H ₂ O ^e	2.4	2.4	2.4
Ca²⁺-bound H₂O			
Asp-65 (Oδ1 or Oδ2) ^c	2.6	2.8	2.8
Glu-67 (Oε1 or Oε2) ^d	2.6	2.7	2.5

^a All distances are in Å and are from Nitrogen, Oxygen, or Ca²⁺.^b Distances between the two Ca²⁺ ions in EF1 and EF2 for the Ca²⁺-S100B (A), Ca²⁺-S100B (B), and the TRTK-Ca²⁺-S100B structures are all 11.4 Å.^c Distances for the H₂O ligands to the Ca²⁺ ions in the pseudo EF Hand (EF1).^d Distances for the H₂O ligands to the Ca²⁺ ions in the canonical EF Hand (EF2).

Table 4

Interhelical angles and distances for structures of S100B

	Interhelical Angles (°) ^{a,b}				Interhelical distance (Å) ^{a,b}			
	I to II	I to III	I to IV	II to III	II to IV	III to IV	I to II	I to III
Ca ²⁺ -S100B (X-ray) ^c	140	-121	125	99	-32	110	15	22
Ca ²⁺ -S100B-A(X-ray)	143	-120	126	97	-32	109	14	22
Ca ²⁺ -S100B-B(X-ray)	143	-120	127	98	-31	109	14	22
TRTK-Ca ²⁺ -S100B (X-ray)	143	-117	128	99	-32	110	14	22
Ca ²⁺ -S100B (NMR) ^d	138	-127	124	93	-36	106	17	25
TRTK-Ca ²⁺ -S100B (NMR; Inman et al)	132	-116	122	108	-35	119	15	22
TRTK-Ca ²⁺ -S100B (NMR; Shaw et al)	134	-105	132	115	-48	111	15	21

^aInterhelical angles and distances were calculated using INTERHLX software (K. Yap, University of Toronto).

^bThe residues defining each helix of S100B were as follows: helix I, residues 2–18; helix II, residues 29–40; helix III, residues 50–62; and helix IV, residues 70–82.

^cCalculated using PDB entry 1MHO.

^dCalculated using PDB entry 2K7O.

^eCalculated using PDB entry 1MWI.

^fCalculated using PDB entry 1MQ1.

Table 5

EF hand angles determined using the VGM method^{ab}

	N-terminal coordinate of second helix			
	Θ (°)	Φ (°)	Ω (°)	
<i>Pseudo EF hand</i>				
Ca ²⁺ -S100B (X-ray) ^c	8.2	-0.1	-6.8	50.0 86.8 109
Ca ²⁺ -S100B (A; X-ray)	8.3	0.0	-6.7	48.6 85.4 110
Ca ²⁺ -S100B (B; X-ray)	8.2	-0.1	-6.7	50.0 85.7 110
TRTK-Ca ²⁺ -S100B (X-ray)	8.1	0	-6.6	48.3 85.4 111
Ca ²⁺ -S100B (NMR) ^d	10.4	-3.2	-7.9	48.6 75.8 120
TRTK-Ca ²⁺ -S100B (Imman et al.) ^e	9.4	-1.3	-7.5	54.4 93.3 103
TRTK-Ca ²⁺ -S100B (Shaw et al.) ^f	10.3	0.1	-4.1	52.3 101.4 128
<i>Typical EF hand</i>				
Ca ²⁺ -S100B (X-ray) ^a	8.9	0.3	-6.6	74.3 101.9 75
Ca ²⁺ -S100B (A; X-ray)	8.7	0.3	-6.9	74.4 102.0 73
Ca ²⁺ -S100B (B; X-ray)	8.7	0.3	-6.8	75.0 102.0 73
TRTK-Ca ²⁺ -S100B (X-ray)	8.8	0.5	-6.6	74.3 102.0 75
Ca ²⁺ -S100B (NMR) ^d	8.9	-2.6	-8.3	71.3 82.5 94
TRTK-Ca ²⁺ -S100B (Imman et al.) ^e	8.6	1.8	-5.6	67.0 116.2 67
TRTK-Ca ²⁺ -S100B (Shaw et al.) ^f	8.8	-2.7	-4.6	75.5 91.3 113

^aVector Geometry Mapping (VGM) angles were calculated using VGM software (K. Yap, University of Toronto) with the helices and structures as indicated.^bThe residues used for the VGM calculations were as follows: helix I, residues 10–17; helix II, residues 29–39; helix III, residues 53–60; and helix IV, residues 70–80.^cCalculated using PDB entry 1MHO.^dCalculated using PDB entry 2K7O.^eCalculated using PDB entry 1MWV.^fCalculated using PDB entry 1MQ1.