REVIEW ARTICLE

Calcium-dependent and -independent interactions of the S100 protein family

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The S100 proteins comprise at least 25 members, forming the largest group of EF-hand signalling proteins in humans. Although the proteins are expressed in many tissues, each S100 protein has generally been shown to have a preference for expression in one particular tissue or cell type. Three-dimensional structures of several S100 family members have shown that the proteins assume a dimeric structure consisting of two EF-hand motifs per monomer. Calcium binding to these S100 proteins, with the exception of S100A10, results in an approx. 40° alteration in the position of helix III, exposing a broad hydrophobic surface that enables the S100 proteins to interact with a variety of target proteins. More than 90 potential target proteins have been documented for the S100 proteins, including the cytoskeletal proteins tubulin, glial fibrillary acidic protein and F-actin, which have been identified mostly from *in vitro* experiments. In the last 5 years, efforts have concentrated on quantifying the protein interactions of the S100 proteins, identifying *in vivo* protein partners and understanding the molecular specificity for target protein interactions. Furthermore, the S100 proteins are the only EF-hand proteins that are known to form both homo- and hetero-dimers, and efforts are underway to determine the stabilities of these complexes and structural rationales for their formation and potential differences in their biological roles. This review highlights both the calcium-dependent and -independent interactions of the S100 proteins, with a focus on the structures of the complexes, differences and similarities in the strengths of the interactions, and preferences for homo- compared with hetero-dimeric S100 protein assembly.

Key words: calcium-binding protein interaction, cytoskeletal protein, dimerization, EF-hand, S100 protein.

INTRODUCTION

Calcium is a ubiquitous second messenger that regulates a diverse array of cellular events, including muscle contraction, neurotransmitter release, fertilization and cell growth. As a result of its pivotal role, the cellular machinery maintains tight control over the concentration of calcium, which ranges from resting levels near 100 nM to signalling levels near 1 μM. The influx of calcium from the extracellular matrix is controlled by voltage-gated or receptor-operated channels that respond to changes in membrane potential or activation via ligand binding. Calcium can also be sourced from the endoplasmic reticulum where the ion is passed to the cytoplasm by the ryanodine or inositol 1,4,5-trisphosphate receptors. Resting calcium levels are re-established by reciprocal mechanisms such as plasma membrane pumps or exchangers or through re-entry to the endoplasmic reticulum via Ca^{2+}-ATPases [1,2]. The intermediary calcium pulse that results from competing influx and efflux of calcium stimulates a variety of cellular activities. The increased calcium level can act as a feedback inhibitor to switch the calcium import machinery off. Furthermore, the bulk of the intracellular calcium is absorbed by calcium-buffering proteins that have high capacities for calcium, tight binding affinities or unique kinetic properties that act to fine-tune the levels and availability of free cytosolic calcium. However, the most important events arising from the calcium signal are the triggering of downstream biological events modulated through binding of calcium to a large number of calcium-sensor proteins (Figure 1). By far the largest group of sensors is the EF-hand calcium-binding proteins, of which more than 600 have been identified from the human genome.

EF-HAND CALCIUM-BINDING PROTEINS

Kretsinger and Nockolds [3] first identified the EF-hand motif, two α-helices with an intervening 12-residue calcium-binding loop, more than 30 years ago. Structural analysis indicates the chelating residues in the calcium-binding loop form a conserved pentagonal bipyramidal arrangement around the Ca^{2+} ion, utilizing the side chains at positions 1, 3, 5, 9 (via water) and 12 and backbone carbonyl group of position 7 [4]. Strong preferences exist for aspartate and glutamate in the 1 and 12 co-ordinating positions respectively, and glycine at the non-co-ordinating position 6 [5]. Functional EF-hands are found in pairs [6] and are required for the correct folding of the proteins and unique variations of calcium binding co-operativity.

The calcium signalling role for EF-hand proteins was first established for calmodulin through its activation of 3′,5′-cyclic nucleotide phosphodiesterase [7] and its ability to bind calcium [8]. Subsequently, the structural basis for calmodulin activation [9] showed a calcium-dependent rearrangement of its helices, resulting in the exposure of a hydrophobic surface used to recruit

Abbreviations used: CacyBP/SIP1, calcyclin-binding protein/Siah-1-interacting protein; CapZ, actin capping protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; 5-HT_{1B} receptor, 5-hydroxytryptamine receptor; MetAP2, methionine aminopeptidase 2; NDR kinase, nuclear Dbf2-related protein kinase; RAGE, receptor for advanced glycation end-products; SERCA2a, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a.

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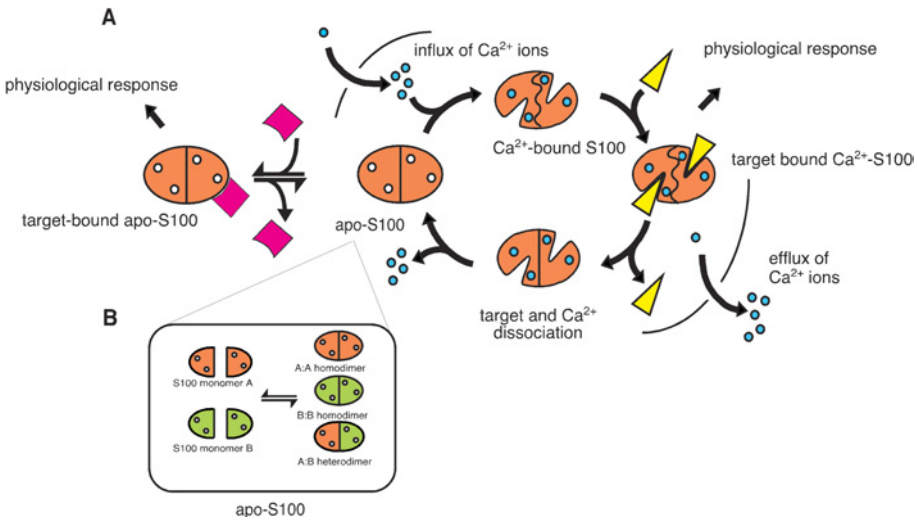


Figure 1 Calcium-dependent and -independent interactions of the S100 family

(**A**) S100 proteins generate diverse physiological responses by interacting with target molecules (pink and yellow). At low calcium concentrations, S100 proteins (orange) reside in their calcium-free (apo) state. Upon influx of calcium via voltage-gated or receptor-mediated channels, the S100 protein binds calcium and undergoes a conformational change that modifies its hydrophobic surface properties. This allows the protein to interact with a wide spectrum of target proteins (yellow) to stimulate a physiological response. Release of calcium through Ca^{2+}-ATPase activity results in the dissociation of calcium and target protein from the S100 protein, returning it to its apo state. Although the majority of S100-target interactions are calcium-dependent, some S100 members have been shown to interact with target proteins (pink) in a calcium-independent fashion. (**B**) Dimeric S100 proteins can exchange subunits with other members of the S100 proteins to form homo- and hetero-dimers in the same cell type as shown by *in vitro* and *in vivo* experiments. The populations of each species are dependent on the concentration of the S100 protein in the cell and the relative affinities for the S100 homo- and hetero-dimers.

target proteins. A related calcium-sensitive mechanism exists for the muscle contractile EF-hand protein troponin-C [10,11], where calcium-binding modulates its interactions with troponin-I within the muscle complex. More recently, it has been established that the S100 proteins comprise a complex grouping of EF-hand calcium-sensors that have diverse tissue distributions and many protein interactions that result in multiple physiological responses [12] (Figure 1). Interest in the S100 proteins has been sparked by their involvement in several human diseases, such as Alzheimer's disease, cancer and rheumatoid arthritis, usually due to modified levels of expression of the S100 members [13,14].

S100 PROTEINS

The S100 proteins are small acidic proteins (10–12 kDa) that are found exclusively in vertebrates [15]. With at least 25 members found to date in humans, the S100 proteins constitute the largest subfamily of the EF-hand proteins. Of these, 21 family members (S100A1–S100A18, trichohylin, filaggrin and repetin) have genes clustered at chromosome locus 1q21, while other S100 proteins are found at chromosome loci 4p16 (S100P), 5q14 (S100Z), 21q22 (S100B) and Xp22 (S100G). First identified by Moore in 1965 [16], the S100 proteins have 25–65 % identity at the amino acid level and contain two EF-hand motifs flanked by conserved hydrophobic residues and separated by a linker region [15]. The sequences of the linker region and the C-terminal extension are the most variable among the S100 proteins.

Three features are unique to the S100 proteins when compared with other EF-hand proteins. First, the two EF-hands in each monomer differ in sequence and mechanisms of calcium co-ordination. The 12-residue C-terminal EF-hand ligates calcium in a similar manner to calmodulin and troponin-C, resulting in a higher calcium affinity site with $K_d \approx 10$–50 μM [17]. The N-terminal or 'pseudo-canonical' EF-hand is formed by 14 residues and binds calcium mostly through main-chain carbonyl groups, except for the bidentate side chain of glutamate at the last position of the loop. This results in a weaker calcium affinity with $K_d \approx 200$–500 μM [17]. This presents an intriguing scenario for the S100 proteins in the cell whereby the C-terminal EF-hand has an affinity that would allow it to be populated during calcium influx, while the N-terminal site affinity is likely to be too weak to bind calcium at any appreciable level. The second unique characteristic of the S100 proteins is their dimeric nature. *In vivo* and *in vitro* experiments have shown that the S100 proteins can form non-covalent homo- and hetero-dimers. This indicates that dynamic exchange of the S100 subunits may occur, depending on the populations of the individual S100 protein members in a cellular compartment (Figure 1). Finally, S100 proteins are expressed in a tissue- and cell-specific fashion [12]. For example, S100A1 and S100A2 are found in the cytoplasm and nucleus respectively of smooth-muscle cells [18], whereas S100P is located in the cytoplasm of placental tissue [19,20]. Together, this results in a complex picture of calcium signalling by the S100 proteins governed by the interchange of homo- and hetero-dimeric protein species, calcium binding to the proteins, interaction with target proteins, cell specificity and regulation of biological function (Figure 1).

STRUCTURES OF S100 PROTEINS

In the last 10 years, three-dimensional structures of S100 proteins have been determined in the calcium-free (apo) [21–32], calcium-bound [24,28,33–41] and target-bound [25,42–47] states. These structures have revealed that the S100 proteins undergo a significant calcium-induced conformational change that, as with calmodulin and troponin-C, results in the exposure of a hydrophobic surface allowing interaction with a target protein (Figure 2). In all S100 structures determined to date, with the exception of S100G (calbindin D_{9k}), the S100 protein exists as a symmetric dimer, with each monomer containing two EF-hand motifs (Figure 2). The N-terminal EF-hand comprises helix I,

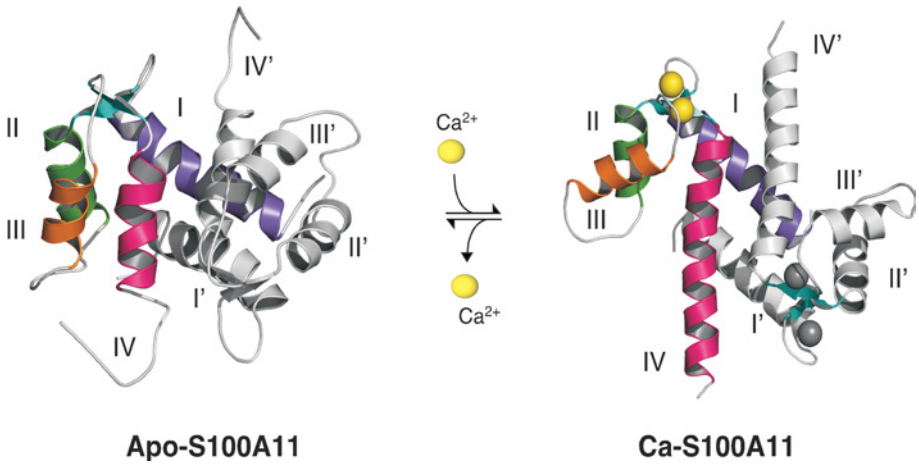


Figure 2 Calcium-dependent conformational change in S100 proteins

The three-dimensional structures of calcium-free S100A11 (apo-S100A11) and calcium-bound S100A11 (Ca-S100A11) are shown to demonstrate the calcium-induced conformational change. In the symmetrical dimer, helices of one monomer (I–IV) are highlighted in different colours, while the other monomer (helices I'–IV') is coloured grey. As sensors, the S100 proteins experience a conformational change upon calcium binding (four atoms/dimer). The rearrangement of helix III (orange) exposes previously buried residues that are essential for target recognition (not shown, see Figure 3) and further biological response. This Figure was drawn using MacPyMOL (http://delsci.com/macpymol/).

pseudo calcium-binding site I and helix II, separated by a flexible linker from the C-terminal EF-hand that includes helix III, calcium-binding site II and helix IV. The dimer interface consists of helices I (I′) and IV (IV′) of each monomer arranged in a bicornate or X-type four-helix bundle. The interhelical relationship between these helices is strictly maintained in both the apo- and calcium-bound states. Calcium binding to site I results in only minor alterations of its backbone conformation, consistent with the site adopting a 'calcium-ready' state identified many years earlier for the EF-hand protein S100G (calbindin D_{9k}) [48]. The apo-S100 proteins have a more 'closed' arrangement of helices III and IV. Calcium binding causes helix III to reorient and repack itself forming a more 'open' structure. For example, in apo-S100A11 helix III is nearly antiparallel to helix IV (154°), but opens by approx. 40° upon calcium binding with respect to both helices II and IV [21,46]. The degree of opening is similar to that observed for the C/D helices in the N-terminal domains of both troponin-C (60–70°) [49] and calmodulin (35–40°) [9], although these latter proteins have additional helix movements (i.e. helix B in troponin-C) that do not occur in the S100 proteins. Nevertheless, the calcium-induced structural change in the S100 proteins results in an exposure of residues from helices III and IV in the C-terminal EF-hand, and linker region that facilitates target protein interaction.

BIOLOGICAL ROLES OF S100 PROTEINS

S100 proteins are proposed to have intracellular and extracellular roles in the regulation of many diverse processes such as protein phosphorylation, cell growth and motility, cell-cycle regulation, transcription, differentiation and cell survival [12] (Table 1). In the last 5 years, a wealth of information has become available, supporting these diverse biological roles and concentrating on quantifying direct interactions between an S100 protein and its biological target. Furthermore, *in vivo* methods such as the yeast two-hybrid assay and co-immunoprecipitation have uncovered a wide spectrum of new biological targets and roles for the S100 proteins, raising many interesting questions about the calcium-dependent and -independent functions of some S100 family members. Despite the extensive summary of S100 protein interactions, biological targets have not yet been identified for S100A3, S100A5, S100A14-S100A18, S100G (calbindin D_{9k}), S100Z and the epidermal proteins filaggrin, trichohyalin and repetin.

Calcium-dependent protein interactions

The calcium-dependent signalling roles of the S100 proteins arise because their affinities for calcium are comparable with the free calcium concentration in the cytoplasm during a calcium wave ($\sim 1\ \mu$M). Thus the binding of S100 proteins to their targets in the presence of calcium and their release, or lack of binding with EDTA, have been used to provide evidence of a calcium-dependent interaction using *in vitro* techniques such as affinity chromatography, optical biosensing and gel overlay, among others. Despite more than 90 potential protein complexes for the S100 proteins (Table 1), only two examples are available that use a yeast two-hybrid screen to identify calcium-dependent interactions (S100A4 with the cell growth regulator protein CCN3 [50], and methionine aminopeptidase 2, MetAP2 [51]). The lack of calcium-dependent interactions observed in yeast is likely to be a function of their tightly controlled intracellular calcium levels (~ 200 nM) [52] that are well below the calcium K_d values of most S100 proteins (~ 10–$50\ \mu$M) [17] and resulting in very low populations of the calcium-bound S100 protein during these experiments. S100 proteins have been found to interact with biological targets using co-immunoprecipitation or co-localization experiments. For instance, S100A2 and its interacting partner p53 co-localize in the nucleus and the cytoplasm in oral carcinoma cell lines, suggesting a relevant interaction of these two proteins [53]. Furthermore, binding of p53 to S100A2 was confirmed using pull-down assays and electrophoretic mobility-shift assays [53]. In contrast, S100B interactions with p53 [47], MARCKS (myristoylated alanine-rich C-kinase substrate) [54] and caldesmon [55] have been observed *in vitro*, but no evidence of co-localization has been demonstrated [12]. This lack of co-localization evidence not only for S100B, but also for other S100 proteins and their targets, has generated some confusion regarding the biological relevance of some *in vitro* findings.

In general, the calcium-dependent roles of the S100 proteins can be divided into five major functional groupings: (i) regulation of

Table 1 Calcium-dependent interactions of the S100 proteins

Support refers to the techniques used to study interactions. Abbreviations used: AB, **Ab** epitope mapping; AC, **a**ffinity **c**hromatography; C, **c**ompetition assays; CC, **c**hemical **c**ross-linking; CD, **c**ircular **d**ichroism; CE, **c**o-**e**xpression; CI, **c**o-**i**mmunoprecipitation; CL, **c**o-localization; CP, **c**o-**p**urification; CS, **c**o-**s**edimentation; E, native gel **e**lectrophoresis; F, **f**luorescence; FT, **f**luorescence resonance energy **t**ransfer; GO, **g**el **o**verlay; IF, **i**soelectric **f**ocusing; M, **m**utagenesis; MS, **m**ass **s**pectrometry; NMR, **n**uclear **m**agnetic **r**esonance; O, **o**thers; OB, **o**ptical **b**iosensor; Ph, **ph**age display; X-Ray, **X-ray** crystallography; Y2H, **y**east two-**h**ybrid. The regions involved in protein interaction are shaded in black or have a heavier line in the schematic of EF-hand motifs in S100 members (N-terminus–HelixI–Site1–HelixII–Linker–HelixIII–Site2–HelixIV–C-terminus). K_d is the dissociation constant for S100–target interaction. Ranges of K_d reflect multiple measurement reports. AHNAK, giant phosphoprotein, identical with desmoyokin; CCN3, cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed; EC, excitation–contraction; IQGAP1, IQ motif GTPase-activating protein; MAG, myelin-associated glycoprotein; MRP, myeloid-related protein; MyOD, myogenic determination gene; PLB, phospholamban; S100PBPR, S100P-binding protein.

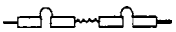
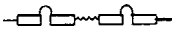
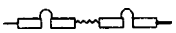
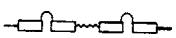
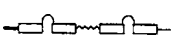
Protein	Target	Support	Region	K_d (nM)	Function	Reference(s)
S100A1	Aldolase C	GO			Stimulation of aldolase C activity	[60]
	Annexin A5	F, CC				[73]
	Annexin A6	CL, CI, F, CC		<1000	Regulation of calcium flux and IF assembly	[72,73,116]
	CacyBP/SIP1	GO, AC, F, CI			Regulation of CacyBP/SIP	[79]
	Caldesmon	E, F, CC		170	Decrease in inhibition of actomyosin by caldesmon	[55]
	CapZ (TRTK-12)	GO, M, CC, F, CS, O		5550	Modulation of actin organization	[83,117–119]
	Desmin	F, O			Inhibition of desmin intermediate filament assembly	[120]
	F-actin	CL, CS			Regulation of actin filament polymerization	[18]
	GFAP	F, CC, CS		500	Inhibition of GFAP assembly	[88,121]
	Microtubules/tubulin	F, CC, O		6000	Regulation of microtubule dynamics	[122,123]
	MyOD	AC, CC, E, F, CI		2	Inhibition of MyoD phosphorylation and DNA binding	[124,125]
	NDR	O			Activation of NDR kinase activity	[57]
	p53	F, CS			Disruption of tubulin–S100A1 complex formation	[117]
	Phosphoglucomutase	GO, AC, O			Inhibition of phosphoglucomutase activity	[61]
	RAGE	O			Promotion of cell survival	[68]
	Ryanodine receptor	CI, OB, AC, O		214	Regulation of ryanodine and cardiac contractility	[70,71]
	SERCA2a and PLB	AC, CI, CL, CE			Regulation of EC-coupling in the heart	[126]
	Synapsin I	CL, C, AC, OB		245	Regulation of catalytic activity of synapsins	[127,128]
	Titin (PEVK domain)	GO, OB, O			Inhibition of the actin– or nebulin–PEVK interaction	[129,130]
	Twitchin kinase	OB, O			Activation of twitchin kinase	[131,132]
S100A2	ΔNp63	O			Downstream mediation of ΔNp63	[133]
	p53	CL, CI, AC, O			Activation of p53 transcriptional activity	[53]
	Tropomyosin	CC, CI, CL, AC, O			Modulation of the actin cytoskeleton organization	[134]
S100A4	CCN3	Y2H, AC			Modulation of S100A4 affinity to calcium and function	[50,135]
	F-actin	CS		34000		[85,136]
	MetAP2	AC, CL, CI, Y2H			Regulation of MetAP2	[51]
	Myosin heavy chain II-A	AC, M, CI, FT, GO, CS, CL, C, OB, O		220–600	Regulation of cytoskeletal dynamics	[77,77a,80,85,92–96]
	Myosin heavy chain II-B	CS, O		23100		[85,94,138]
	Tropomyosin isoform2	AC, CL			Regulation of tropomyosin–actin association	[86]
	p37	F, AC			Increase affinity of S100A4 to calcium	[139]
	p53	OB, CI, CE, AC, F, O		9–14	Enhancement of p53-dependent apoptosis in tumours	[75–77]
	Sept2, Sept6 and Sept7	AC				[140]
S100A6	Annexin A11	AC, GO, CC, CI, CL, M, O		1610	Regulation of annexin A11 function	[141–146]
	Annexin A2	AC, GO		Very weak		[74,89,145]
	Annexin A5	GO		Very weak		[145]
	Annexin A6	AC, O				[74]
	CacyBP	F, M, NMR, AC, GO, O		960		[141,147]
	CacyBP/SIP	AC, CI, O			Regulation of CacyBP/SIP ubiquitinylation complex	[79]
	Caldesmon	F				[148]
	Fetuin (biotinylated)	O				[149]
	GADPH	AC, GO, F, CS, O		100		[74,89,150]
	Lysozyme	GO				[151]
	Tropomyosin	F, O		0.3–1	Regulation of smooth muscle contraction	[152]
	Sgt1	AC, CC, CI			Regulation of protein ubiquitination via Stg1	[59]
S100A8/S100A9	Cytochrome b_{558}	O			Activation of cytochrome b_{558}	[153]
	Glycosaminoglycans	O		6	Localization of MRPs to endothelium	[154]
	S100A8/S100A9 (tetramer)	MS, O			Increasing affinity for calcium	[155,156]
S100A11	Actin	CS		1000	Regulation of actin activated myosin ATPase	[157]
	Annexin A1	X-Ray, F, M, CL, AC, CI, CS, O		15000	Targeting and membrane cross linking	[46,66,158,159]
S100A12	Annexin A5	AC, OB		621		[160]
	CacyBP/SIP	AC, O			Degradation of α/β-catenin	[79]
	Paramyosin	E, O			Development of keratitis	[161]
	RAGE	CD, E, O			Inflammatory processes	[67,162]
	S100A12 (hexamer)	X-Ray			Receptor signaling	[163]
S100B	AHNAK	AC, CI, OB		50	Regulation of calcium homoeostasis	[164]
	Aldolase C	GO			Stimulation of aldolase C activity	[60]

Table 1 Contd.

Protein	Target	Support	Region	K_d (nM)	Function	Reference(s)
	Annexin A6	CL, CI, F, CC		< 1000	Regulation of calcium flux and IF assembly	[72,73,116]
	CacyBP/SIP	GO, AC, CI, F			Regulation of ubiquitination	[55]
	Caldesmon	E, F, CC		500	Decrease in inhibition of actomyosin by caldesmon	[79]
	CapZ (TRTK-12)	NMR, F, AC, CC, O		150–1000	Regulation of actin filament extension	[43,45,65,84,165]
	FtsZ	CL, AC				[64]
	GFAP	O			Assembly of intermediate filaments	[63]
	Guanylate cyclase	O		2000	Activation of guanylate cyclase	[166]
	Intermediate filaments	CC, CL, O			Regulation of IF assembly and disassembly	[167]
	IQGAP1	CL, CI, AC			Membrane rearrangement	[168]
	MAG	AC, CC, O		7000	Regulation of glial cell cytoskeleton	[169]
	Microtubules	CI, O			Regulation of microtubule dynamics	[62]
	NDR	NMR, AC, O		500	Modulation of NDR kinase activity	[42,57]
	Neuromodulin	CC			Inhibition of neuromodulin phosphorylation by PKC	[170]
	p53	NMR, AC, F, OB, O		24–23500	Inhibition of p53 function	[47,78,110,171–173]
	Phosphoglucomutase	GO, AC, M, O			Stimulation of phosphoglucomutase activity	[61,174]
	RAGE	O			Promotion of cell survival	[68]
	Tau	AC, CC		100–1000	Inhibition of tau phosphorylation by protein kinase II	[56,175]
S100P	CacyBP	O				[79]
	Dormant ezrin	AC, CL			Regulation of ezrin ability to bind actin	[176]
	Melittin	F, CD, O		5000		[97]
	RAGE	CI			Stimulation of cell proliferation and survival via RAGE	[69,177]
	S100PBPR	AC, CI, O			Involvement in early pancreatic cancer	[178]
	Sgt1	AC				[59]

phosphorylation mediated by protein kinases, (ii) modulation of enzymatic activity, (iii) maintenance of cell shape and motility, (iv) influence of some signal-transduction pathways, and (v) promotion of calcium homoeostasis. For example, S100B has been implicated in the phosphorylation of tau protein [56], and the modulation of kinase activity by NDR kinase (nuclear Dbf2-related protein kinase) and protein kinase II [42,56–58]. The mechanism of this inhibition is through direct interaction with the kinase rather than recruitment of substrate. The regulation of the yeast ubiquitination pathway protein Sgt1 by S100A6 appears to operate by a similar mechanism [59]. The enzymatic activity of aldolase (isoforms A and C) and phosphoglucomutase, occurs via interactions with S100A1 [60,61] or S100B [60,61], whereas MetAP2 interacts with S100A4 [51]. It is surprising that reversed biological function can be promoted by different S100 proteins. For example, the interaction of S100A1 with phosphoglucomutase inhibits enzyme activity, whereas S100B interaction seems to stimulate this enzyme's function [61]. Furthermore, S100A1 binding to aldolase C requires calcium, whereas the interaction with aldolase A is calcium-insensitive [60].

The most significant number of protein interactions for the S100 proteins are with components of the cytoskeleton, including the tubulins, intermediate filaments, actin, myosin and tropomyosin. For example, S100B controls the assembly of microtubules [62] and GFAP (glial fibrillary acidic protein) [63] interacts with the tubulin homologue FtsZ [64] and is proposed to regulate actin filament extension through interaction with CapZ (actin capping protein) [65]. Other S100 proteins, including S100A1 S100A2, S100A4, S100A6 and S100A11, have been shown to affect similar components of the cytoskeleton (Table 1). In particular, elegant mechanisms for calcium-dependent membrane aggregation, important for cell vesiculation, have been proposed for the interaction of S100A11 with annexin A1 [66]. Several S100 proteins have been implicated in a variety of signal-transduction pathways. For example, S100B, S100A1, S100A12 and S100P can bind to the RAGE (receptor for advanced glycation end-products) [67–69], activating an intracellular signal cascade that contributes to cell proliferation and survival. Finally, the involvement of the S100 proteins on calcium homoeostasis has been suggested through interactions of S100A1 with the ryanodine receptor [70,71].

A few general observations that can be made from Table 1. Clearly, some S100 members are proposed to regulate the activity of the same target molecule. For example, S100A1, S100A6 and S100B bind annexin A6 [72–74]; S100A1, S100A2, S100A4 and S100B interact with the tumour suppressor p53 ([47,53,75–77], but see [77a], [78]); and S100B, S100A6, S100A12 and S100A1 form complexes with the ubiquitination protein CacyBP/SIP (calcyclin-binding protein/Siah-1-interacting protein) [55,79]. This is perhaps not surprising, given the significant sequence similarities between many of the S100 proteins. The apparent multi-S100 protein interactions could result from differential expression in tissues such that two different S100 proteins might control similar processes but in different tissues. It will be important to link these interactions with the expression and availability of both S100 protein and target in the same cell type and tissue. This has been elegantly shown for S100A4 and the non-muscle myosin A heavy chain that is thought to have a role in metastasis [80].

For most of the S100-target protein complexes, the dissociation constant of the S100 member for the target is approx. 1 μM (Table 1). This affinity is similar to that obtained for other calcium-dependent EF-hand protein complexes, such as troponin-C with troponin-I (0.1–30 μM) [81,82]. In some cases, affinity measurements may provide evidence for target selectivity amongst the S100 proteins. For example, the CapZ peptide TRTK-12 (TRTKIDWNKILS) binds 10-fold more tightly to S100B than to S100A1, indicative of a strong preference for S100B [83,84]. It will be important to extend these studies to off-rate measurements to determine whether the release rates are consistent with

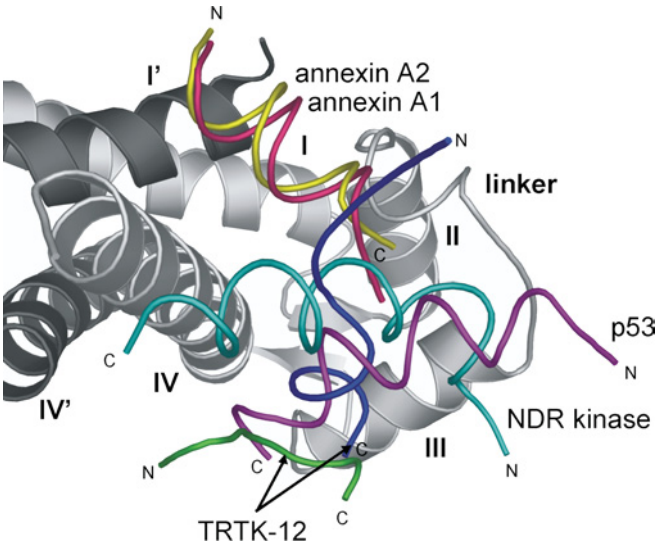


Figure 3 Target protein orientation for S100 proteins

A ribbon representation of calcium-bound S100A11 is presented, showing one of the monomers in light grey (helices labelled as I, II, III and IV) and the other monomer in dark grey (helices labelled as I' and IV'). The S100 portion from the three dimensional complexes of S100A10–annexin A2, S100B–p53, S100B–TRTK and S100B–NDR kinase was superimposed with S100A11 to give the relative orientations of each of the target peptides. Annexin A1 (residues 1–11) is shown in pink, annexin A2 (residues 1–11) in yellow, NDR kinase (residues 71–87) in cyan, p53 (residues 99–112) in purple and TRTK-12 (residues 1–12) in both green and blue. The N- and C-termini of each of the peptides are indicated with either an N or C respectively. The S100A11 and S100B complexes are in the calcium-bound state, although no calcium ions are shown in the Figure. S100A10 is in the calcium-free state. This Figure was drawn using MacPyMOL (http://delsci.com/macpymol/).

the lifetimes of the biological function as shown recently for S100A4 [80]. Table 1 also reveals that there are some disagreements regarding some S100 complexes. For example, co-sedimentation experiments show an interaction of F-actin with S100A4 [85], but this binding could not be observed using optical biosensor assays ([77], but see [77a]). In the same manner, the S100A4–tropomyosin (TM2) complex was observed using affinity chromatography [86], but not using surface plasmon resonance detection ([77], but see [77a]) and the binding of annexin A2 to S100A6 was reported using affinity-chromatographic techniques, but was not detected by fluorescence or chemical cross-linking [74]. These results emphasize the need to use several methods and/or sample conditions to monitor the *in vitro* S100–target interactions.

Several three-dimensional structures of calcium-bound S100 proteins, bound to target molecules, have been solved using either NMR or X-ray-crystallographic methods. The structures of S100A11 bound to the N-terminal peptide of annexin A1 [46], and S100B in complex with peptides derived from CapZ (TRTK-12) [43,45], p53 [47] and NDR kinase [42] provide atomic details about the protein–protein interface that can be used to rationalize the specificity of the interactions. The first structure of a calcium-bound S100 protein in complex with a region of its biological target was the crystal structure of S100A11 bound to the N-terminal 14 residues of annexin A1 [46]. This structure reveals that the S100A11–annexin A1 complex remains symmetrical, binding two annexin molecules per S100 dimer on the periphery of the protein. The annexin peptide lies across the linker, helix III and helix IV of one monomer and contacts helix I of the partner monomer, thus bridging the two S100 subunits (Figure 3). Residues near the C-terminus of helix IV in S100A11 are necessary for the annexin interaction. This region is not helical in apo-S100A11 (Figure 2), indicating that induction of an α-helix is important for target protein binding. The annexin peptide forms an amphipathic α-helix with its hydrophobic surface (Ala^{1}, Met^{2}, Val^{3}, Phe^{6}, Leu^{7}, Ala^{10}, Trp^{11}) facing the S100 protein, thereby identifying some of the amino acid determinants for the S100 specificity.

Three-dimensional structures of calcium-bound S100B with peptides derived from p53, CapZ (TRTK-12) and NDR kinase have been determined by NMR spectroscopy (Figure 3). These structures provide initial details for the specificity of different targets for the same S100 protein. All four of these structures utilize a similar binding region of the S100B protein, namely the surface formed by the linker and helices III and IV. The p53 and NDR targets adopt α-helical conformations that are 12–14 residues in length. Both peptides have their N-termini positioned towards the N-terminus of helix III, but the orientation of the two peptides diverges by more than 8 Å (1 Å = 0.1 nm) as they approach helix IV. Structures of calcium-bound S100B with TRTK-12 show these peptides have little regular secondary structure and are arranged roughly at 90° to the orientations of the p53 or NDR peptides. These four structures point towards a malleable flat binding surface in S100B that could accommodate many different target proteins, and is shallower than that found in calmodulin [42]. This is consistent with observations in Table 1 showing that S100B can interact with at least 20 different proteins in a calcium-dependent manner. The possibility does exist that the peptide segments being used to map S100 target interactions are void of secondary, or supplementary, binding sites. Evidence for this arises from the calcium-dependent interaction of S100B with p53, where peptides comprising residues 319–393 possess very tight binding to S100B ($K_d = 24$ nM) [87], but this is weakened more than 100-fold with shorter constructs (367–381 or 367–393) [75]. In order to define further the binding surfaces on the S100 proteins with specific binding partners it will be important to expand the number of structures of calcium-bound S100 family members with targets, including interactions with intact target proteins or much larger peptide sequences. Furthermore, the precision of the structures may need to be improved, especially for side-chain interactions involving the bound peptides, in order to develop better rationales for binding specificity.

All of the calcium-bound S100A11 and S100B complexes utilize helix IV for their target protein interaction. The TRTK-12 and p53 sequences interact with regions near Val^{80}–Cys^{84}, whereas the NDR peptide interacts mostly downstream of this (Cys^{84}–Phe^{88}). In contrast, annexin A1 utilizes the opposite face of helix IV in S100A11 (Figure 3). This shows the central adaptive role that helix IV plays to modify S100 target protein interactions. Examination of Table 1 corroborates this idea, as more than 25 calcium-dependent binding partners with different S100 proteins utilize helix IV or the subsequent C-terminus. For example, deletion of the S100A1 C-terminus abolishes its interaction with TRTK-12, GFAP, p53, SERCA2a (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a) and phospholamban [88]. However, not all S100 target proteins follow this trend. For instance, the N-terminal region of S100A6 is essential for its interaction with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and annexin A2 [89]. These observations and the variations in the three-dimensional structures of calcium-bound S100–target complexes provide some insight into the level of target specificity among the S100 family members. It is clear that, although several target proteins appear to 'cross-react' with more than one S100 protein and most S100 proteins interact with several targets, there remains a fine level of discrimination that does not allow random interaction of targets with all S100 proteins. The broad selection of the S100 targets such as TRTK-12, NDR and p53 is likely

to be a reflection of the moderate hydrophobicity in the short target peptide sequence and the extensive hydrophobic patch that exists on the S100 structures. Nevertheless, this exposed region comprises the most divergent portions of the S100 sequences (the linker and C-terminal regions) and this must be enough to discriminate against different target proteins.

Calcium-independent protein interactions

Although the majority of S100 protein interactions are calcium-dependent, several calcium-independent interactions have been reported (Table 2). The most common binding partners for the apo-S100 proteins are enzymes. For example, S100B and S100A1 bind with glycogen phosphorylase [90], whereas S100A10 and S100A11 show interactions with transglutaminase [91]. Some discrepancies for the calcium-sensitivity of S100-target interactions are encountered. For example, optical biosensor techniques show a calcium-insensitive complex between S100A4 and non-muscle myosin (heavy chain II, isoform A), and between S100A4 and p53 ([77], but see [77a]), whereas strict calcium-dependence was observed using other techniques ([77], but see [77a], [80,85,92–96]). In some cases, such as the calcium-insensitive interaction of S100A4 with F-actin (filamentous actin), the dissociation constant is very weak ($K_d \approx 500\ \mu$M), but strengthens significantly upon calcium binding to the S100 protein ($K_d \approx 30\ \mu$M) [85]. Similar observations have been noted for the interaction of melittin with S100P [97]. These poor binding affinities in the absence of calcium probably indicate that the *in vivo* interaction is insignificant in the absence of calcium.

Although questions remain about the significance of some apo-S100–protein interactions, there is overwhelming support for the notion that S100A10 has multiple binding partners in the calcium-free state. The calcium-insensitivity of S100A10 results from its inability to bind calcium owing to mutations in both calcium-binding sites. Early evidence showed that S100A10 was co-purified as a heterotetramer with annexin A2 [98]. More recently, an important interaction between S100A10 and the 5-hydroxytryptamine (serotonin) receptor 5-HT_{1B} has been identified using a yeast two-hybrid screen. Further experiments have provided strong evidence that this S100A10–5-hydroxytryptamine complex may have a role in the onset of depression [99]. Other complexes with S100A10, including those with the viral proteins NS3 and hepatitis virus B polymerase, have been identified using yeast two-hybrid assays [100,101] (Table 2), as these protein interactions are very strong ($K_d \approx 30$ nM for annexin A2) [98]. The S100A10–annexin A2 complex was the first three-dimensional structure of an S100 complex to be determined [25]. As with S100A11, the S100A10 protein interacts with the N-terminus of the annexin molecule. The fold of the S100A10 protein is nearly identical with that of calcium-bound S100A11 (Figure 2), adopting the more 'open' conformation that provides a hydrophobic surface for protein binding, even in the absence of calcium. The location and interactions of the annexin A2 peptide with apo-S100A10 are remarkably similar to those identified for S100A11 (root mean square deviation 0.87 Å) [46], interacting with residues from helices III and IV, and the linker of one monomer, and helix I of the other monomer (Figure 3). Other protein partners, including tissue-type plasminogen activator, appear to utilize the same helix IV region (Table 2a), while targets such as the sodium channel NaV1.8 interact at different sites [102].

The most important calcium-independent interactions of the S100 proteins are their abilities to form homo- and hetero-dimers, as well as some higher-order complexes (Table 2b). This property results in a dynamic interplay between formation of the dimeric species, calcium binding and interactions with a biological target protein (Figure 1). Traditionally, homodimeric interactions have been the focus of the S100 proteins; however, the use of yeast two-hybrid experiments has uncovered a large number of heterodimeric complexes. Furthermore, optical biosensor experiments have been used to quantify the strength of the homo- and hetero-dimeric interactions *in vitro*, so that the importance of these can be assessed *in vivo*.

The association of several homodimeric S100 proteins has been quantified, providing an indication of the overall stability of S100B, S100A4, S100A12 and S100P. These studies show a wide range of K_d values for the monomer–dimer equilibrium. For example, S100B forms the tightest dimer ($K_d < 500$ pM) in the calcium-free state [103] and, remarkably, is nearly 5000-fold more stable than either the S100A4 or S100P homodimers ($K_d \approx 1$–$2\ \mu$M). In some cases (S100P and S100A12), dimerization is enhanced by more than 100-fold in the presence of calcium. In other cases (S100B, S100A4), calcium binding has a negligible effect. *In vivo*, the extent of homodimerization will be dependent on the concentration of the S100 protein and the K_d for dimer formation. For example, S100B has been found at high concentrations (nearly $10\ \mu$M) in glial cells [104], indicating that this protein would be completely in the dimeric form. On the other hand, proteins such as S100A4 and S100P, if found in similar or lower concentrations than S100B, would have a significant population of monomeric protein in the cell. This would facilitate formation of heterodimers with other S100 proteins (Figure 1).

The observation that dynamic exchange occurs for the S100 subunits indicates that heterodimeric proteins probably exist *in vivo*. Consistent with this, at least ten different heterodimeric S100 species have been identified. The S100A8–S100A9 heterodimer is probably the most well characterized of these. Originally isolated from synovial fluid [105], X-ray structures are available for the S100A8 and S100A9 homodimers [36,44]. However, other experiments have shown there is a strong preference for the S100A8–S100A9 heterodimer, especially in the presence of calcium, and it has been suggested that the S100A8–S100A9 heterodimeric species is the only relevant biological species [106]. A similar calcium stabilization of the heterodimer has been observed for S100B–S100A6 and S100B–S100A11. In the case of the other S100 heterodimers, there are several intriguing possibilities. The first, of course, is that the relevant S100 proteins must be found in the same cell type to substantiate heterodimer formation. This has been confirmed for several species, including S100A1–S100A4 found in several mammary cell lines, and S100B–S100A6, found in some human melanoma cells [107,108]. Furthermore, the formation of the S100 heterodimeric proteins will be governed by the thermodynamics of the equilibria involved, shown previously for homo- and hetero-dimeric calcium-binding peptides [109], and for tropomyosin [110]. Using the example of S100A1 and S100A4, the S100A1–S100A4 heterodimer will be preferentially formed when $\Delta G_{S100A1-S100A4} < {}^1/_2[\Delta G_{S100A1} + \Delta G_{S100A4}] + R \cdot T \cdot \ln 2$. On the basis of Table 2(b), the stability of S100A1–S100A4 ($K_d \approx 300$ nM) is nearly 10-fold that of the S100A4 homodimer ($K_d \approx 1$–$2\ \mu$M), as determined using yeast two-hybrid, optical biosensor and analytical ultracentrifugation experiments [107,111,112]. Furthermore, yeast two-hybrid studies have shown the S100A1 homodimer probably has a similar stability as the S100A1–S100A4 heterodimer. On the basis of these observations and the above inequality, the S100A1–S100A4 heterodimer would be favoured thermodynamically by approx. 3 kJ/mol. More complicated situations may arise in the cell, including interactions with other S100 proteins or binding partners, or large differences

Table 2 Calcium-independent interactions of the S100 proteins

For details see the legend to Table 1. Homodimer proteins are listed only if the K_d has been reported. E-FABP, epidermal-type fatty-acid-binding protein; FGF, fibroblast growth factor; HBV pol, hepatitis B virus polymerase; TASK-1, TWIK (two-pore domain weak inwardly rectifying K^+ channel)-related acid-sensitive K^+ channel; TRPV, transient receptor potential channel (subfamily V).

(a) Interactions with other proteins

Protein	Target	Support	Region	K_d (nM)	Function	Reference(s)
S100A1	Adenylate cyclase	O		200	Stimulation of adenylate cyclase	[179]
	Aldolase A	O				[83]
	Glycogen phosphorylase	GO, AC, CL			Inhibition of glycogen phosphorylase activity	[90]
	Microtubules	O			Microtubule assembly	[180]
S100A4	Annexin A2	CI, NMR, O			Mediation of plasmin production from plasminogen	[181]
	F-actin	OB		543000		[85]
	Liprin β1	CI, CL, MS, O			Inhibition of liprin β1 phosphorylation	[182]
	Myosin heavy chain II-A	OB		220–600	Regulation of cytoskeletal dynamics	[77]
	p53	OB		9–14	Enhancement of p53-dependent apoptosis in tumours	[77]
	S100A4 (tetramer)	MS			Stimulation of angiogenesis and neurite growth	[183]
S100A7	E-FABP	CL, CI, CP, GO			Shuttle E-FABP to membrane or ligands	[184–186]
	Jab-1	Y2H, CI, O			Pro-survival pathway	[187,188]
	RanBPM	Y2H, CI			Cytoskeletal functions, adhesion and migration	[189]
	Transglutaminase	O			Regulation of S100A7 function	[91]
S100A8/S100A9	Carboxylated glycans	AC			Inflammation	[190]
	CD36	AC			Fatty acid uptake	[191]
	Phox proteins	CP, CI			Scaffold for phox and NADPH oxidase activation	[153,192]
S100A10	5-HT_{1B} receptor	Y2H, CI, CL, O			Localization of 5-HT_{1B} receptors to the cell surface	[99]
	Annexin A2	X-Ray, CD, F, M, CC, CL, O		$\sim$30	Signal transduction	[98,193–203]
	NS3	Y2H			Mediation of virus release	[100]
	Connexin 31	Y2H				[204]
	Phospholipase A2	CI, O			Regulation of phospholipase A_2 activation	[205]
	HBV Pol	Y2H, AC, CI, CL, O			Inhibition of the DNA polymerase activity of HBV pol	[101]
	TASK-1 K^+ channel	Y2H, AC, CI, M, O			Trafficking of TASK-1 to the plasma membrane	[206]
	Plasminogen activator	AC, O				[207]
	Sodium channel $Na_v1.8$	Y2H, AC, O			Translocation of $Na_v1.8$ to the plasma membrane	[102,208,209]
	Transglutaminase	O			Regulation of S100A10 function	[91]
	TRPV5/TRPV6	Y2H, AC, CI			Translocation TRPV5/TRPV6 to the plasma membrane	[210]
S100A11	Transglutaminase	O			Regulation of S100A11 function	[91]
	Isocitrate dehydrogenase	AC, OB				[160]
	Aldolase A					[160]
	GAPDH	AC, OB		83		[160]
S100A13	FGF-1, p40 Syt-1	CP, E, CL, O			Regulation of FGF-1 release	[211–213]
	Interleukin-1α	CL, CI			Stress induced release of interleukin-1α	[214]
S100B	Aldolase A	O			Stimulation of aldolase A activity	[60]
	Glycogen phosphorylase	GO, AC				[90]
S100P	Melittin	F, CD, O		200000		[97]

(b) Observed heterodimers of the S100 proteins

Monomer I	Monomer II	Support	Region	K_d (nM)	Function	Reference(s)
S100A1	S100A4	Y2H, F, FT, MS, OB, AC, GO, CP, CE, M		300–500 (+ Ca^{2+})	Modulation of metastasis in cancer cells	[107,112,114]
	S100B	Y2H, O				[108,113,215]
	S100P	Y2H, OB, FT, CE, M		1100–1800 (apo); 10–20 (+ Ca^{2+})	Target binding and function regulation	[115]
S100A4	S100A4	Y2H, OB, NMR, O		4000 (apo); 670–1000 (+ Ca^{2+})		[107,111,112,114]
S100A6	S100B	Y2H, CI, CL			Melanoma cell growth	[108,113]
S100A7	S100A10	MS, CI, O				[216]
S100A8	S100A9	Y2H, MS, AC, M, Ph, AB, CC, CD, F, NMR, X-Ray, E, IF			Inflammatory processes	[106,217–220]
	S100A10	MS, CI, O				[216]
S100A9	S100A12	AC, CI, OB, M, O		1520 (apo)		[160]
S100A11	S100B	Y2H, CL, CI			Modulation of target binding	[113,158]
S100A12	S100A12	X-Ray, OB		4 (+ Ca^{2+})		[160]
S100B	S100B	NMR, O		< 0.5 (apo); < 0.5 (+ Ca^{2+})		[103]
S100P	S100P	Y2H, X-Ray, OB		1400–2500 (apo); 40–120 (+ Ca^{2+})	Involved in various diseases	[41,115,221]
	S100Z	Y2H, IF, E				[222]

in cellular concentrations of the S100 protein. However, the thermodynamic point of view would indicate that the S100A1–S100A4 heterodimer is the dominant *in vivo* species. For other heterodimers, this approach indicates that the homodimer is the major species. For example, the extremely tight dimer association of S100B indicates that heterodimers such as S100B–S100A11 and S100B–S100A6 would be poorly formed *in vivo*. Although K_d values are not available for these two heterodimers, two-hybrid experiments show an approx. 2-fold poorer β-galactosidase activity for the heterodimers [108,113]. Furthermore, experiments will be needed for other S100 homo- and hetero-dimers to establish their strengths of interaction and relative populations in different cell types.

Some important differences regarding the specificity of interaction at the dimer interface have been noted for S100 homo- and hetero-dimers using site-directed mutagenesis and yeast two-hybrid experiments. For example, deletion of the C-terminal eight residues for S100B abolishes heterodimer formation with both S100A6 and S100A11, whereas S100B homodimer formation is unaffected [113]. Within this region, Phe^{87} and Phe^{88} are particularly important. These residues are in an unstructured region following helix IV and have very few intersubunit contacts in the apo-S100B structure. In order to contribute substantially to the heterodimer complex, these interactions must be significantly altered in the heterodimeric structures. Similar observations have been made for S100A1–S100A4 and S100A1–S100P where Cys^{76} and Cys^{81} in S100A4 [114] and Val^{76} in S100P [115] are required for heterodimerization with S100A1, but have little affect on the respective homodimer formation. More recently, the first X-ray structure of a heterodimeric S100 protein, S100A8–S100A9 shows that the heterodimer might be energetically driven by a slightly more extensive burial of solvent-accessible surface area compared with either the S100A8 or S100A9 homodimers (I. Korndoefer and A. Skerra, personal communication). These experiments will form an excellent initial framework for assessing homo- and hetero-dimer formation.

FUTURE INSIGHTS

The S100 proteins are a unique family of EF-hand calcium-binding proteins involved in a large network of calcium-dependent, and independent protein–protein interactions. Although structures exist for the S100 proteins in the apo, calcium-bound and target-bound states, there are still many unresolved questions about the *in vivo* biological functions of the proteins. The dimeric structure of S100 family members suggests that these proteins, in principle, interact with either two identical, or two non-identical, target proteins in the case of homodimers and heterodimers respectively. For some S100–protein complexes, such as those with the oligomeric targets GFAP, tubulin and p53, this presents an attractive mechanism whereby the S100 can modulate the assembly/disassembly of the target protein complex. *In vitro* experiments support this to some extent, whereas *in vivo* evidence is not as conclusive. Other hypotheses exist such as those for S100A10 and S100A11 where the protein acts to bridge adjacent phospholipid membrane surfaces en route to vesiculation, further providing a rationale for the S100 dimeric structure.

There is a great deal of excitement in the calcium signalling field as the S100 protein story unfolds. Clearly, some inroads have been made towards the structural recognition of biological target proteins through recent three-dimensional structures. As with calmodulin and troponin-C, further quantification and structural information will undoubtedly allow researchers to understand the specificity of the S100 proteins for target recognition. Furthermore, the formation of the homo- and hetero-dimers presents a seemingly unending variation in S100 dimer composition. Is it possible that the monomeric S100 form has a biological role in some cells? This, and other questions, will provide researchers with many challenges in the future.

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