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New perspectives on S100 proteins: a multi-functional Ca^{2+}-, Zn^{2+}- and Cu^{2+}-binding protein family

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S100 proteins (16 members) show a very divergent pattern of cell- and tissue-specific expression, of subcellular localizations and relocations, of post-translational modifications, and of affinities for Ca^{2+}, Zn^{2+}, and Cu^{2+}, consistent with their pleiotropic intra- and extracellular functions. Up to 40 target proteins are reported to interact with S100 proteins and for S100A1 alone 15 target proteins are presently known. Therefore it is not surprising that many functional roles have been proposed and that several human disorders such as cancer, neurodegenerative diseases, cardiomyopathies, inflammations, diabetes, and allergies are associated with an altered expression of S100 proteins. It is not unlikely that their biological activity in some cases is regulated by Zn^{2+} and Cu^{2+}, rather than by Ca^{2+}. Despite the numerous putative functions of S100 proteins, their three-dimensional structures of, e.g., S100B, S100A6, and S100A7 are surprisingly similar. They contain a compact dimerization domain whose conformation is rather insensitive to Ca^{2+} binding and two lateral α-helices III and III, which project outward of each subunit when Ca^{2+} is bound. Target docking depends on the two hydrophobic patches in front of the paired EF-hand generated by the binding of Ca^{2+}. The selectivity in target binding is assured by the central linker between the two EF-hands and the C-terminal tail. It appears that the S100-binding domain in some target proteins contains a basic amphiphilic α-helix and that the mode of interaction and activation bears structural similarity to that of calmodulin.

Keywords: Ca^{2+}-binding S100 proteins, EF-hand, protein structures, Ca^{2+}, Zn^{2+}, Cu^{2+}

Introduction

S100 proteins (*S*oluble in *100* % ammonium sulfate) were first isolated from bovine brain more than three decades ago (Moore 1965). Subsequent studies revealed that this fraction contained the two-dimeric proteins, S100A1 and S100B (Hilt & Kligman 1991; Donato 1991). To date, some 16 different proteins, which display various degrees of amino acid sequence homology from 25 to 65 %, have been assigned to the S100 protein family (Schäfer & Heizmann 1996). S100 proteins are characterized by two distinct EF-hand motifs displaying different affinities for Ca^{2+}. Both EF-hands are flanked by hydrophobic regions at either terminal and are separated by a central hinge region with significant amino acid sequence divergence. The C-terminal EF-hand (rich in acidic amino acids) contains the canonical Ca^{2+}-binding loop encompassing 12 amino acids, common to all EF-hand Ca^{2+}-binding proteins (more than 200 members are now known). The N-terminal EF-hand (rich in basic amino acids), consisting of 14 amino acids, is specific for S100 proteins. The large differences in the Ca^{2+} affinities of the N- and C-terminal EF-hands indicate that S100 proteins are heterobifunctional (Hilt & Kligman 1991; Krebs *et al*. 1995). A large number of target proteins was detected binding in the presence (or absence) of Ca^{2+} to each individual S100 family member. Modulation of Ca^{2+} affinities to S100 proteins at different ionic

strength and after posttranslational modification of S100 proteins adds further complexity to an understanding of the pleiotropic cellular events regulated by S100 proteins. Furthermore, Zn^{2+} can affect the binding of Ca^{2+} to particular S100 proteins, further contributing to their variable cation-binding properties and their diversified functions.

S100 proteins show tissue- and cell-specific expression (Schäfer & Heizmann 1996), a characteristic they share with parvalbumin, calbindin D-28K, and most other EF-hand Ca^{2+}-binding proteins (Heizmann & Hunziker 1991) but not with the multifunctional calmodulin (Cohen & Klee 1988; Crivici & Ikura 1995), which is ubiquitously distributed.

Expression patterns of individual S100 proteins, however, vary greatly from very restricted expression, such as that shown by S100A3 in human hair cuticles (Kizawa *et al.* 1996; Böni *et al.* 1997a) to a much wider distribution in a large number of tissues and cells as shown by S100A2 (Glenney *et al.* 1989; Wicki *et al.* 1997) and S100A13 (Wicki *et al.* 1996a). There are also a few cells that co-express several S100 proteins, e.g., smooth muscle cells, cardiomyocytes, and tumor cells, with a very distinct subcellular localization.

For example, human smooth muscle cells co-express at least four S100 proteins (Mandinova *et al.* 1998). S100A1 and S100A4 are associated with active stress fibers (probably regulating contraction) and the sarcoplasmic reticulum (regulating Ca^{2+} homeostasis), S100A6 is mainly associated with a network-like structure around the cell nucleus, and S100A2 was found exclusively in the cell nucleus (possibly regulating fundamental nuclear functions).

Specific relocations of some S100 proteins were observed in human smooth muscle cells (Mandinova *et al.* 1998) in response to a rise of the cytosolic Ca^{2+} levels within the physiological range. These relocations, which are also found for S100A8 and S100A9 in monocytes (Rammes *et al.* 1997), are very subtle and different for each family member. In the case of S100A1 in smooth muscle cells, only the SR-associated S100A1 protein relocates upon Ca^{2+} increase to vesicle-like structures in the nuclear envelope, whereas the S100A1 bound to F-actin remains firmly attached. S100A2 located in the cell nucleus of smooth muscle cells remained unaffected by a rise of cytoplasmatic Ca^{2+}, indicating that it might regulate specific nuclear Ca^{2+}-dependent processes. During these relocation processes, S100 proteins must attach/detach to/from various target proteins that have yet to be identified.

A large number of diverse functions are attributed to S100 proteins, ranging from calcium-buffering (calbindin 9k) through intracellular functions (modulation of enzyme activities, energy metabolism, motility, secretion) and nuclear functions (transcription and apoptosis) to extracellular activities (secretion, neurite extension, and chemotaxis). Apart from the monomeric calbindin, most intracellular S100 proteins probably function as non-covalent dimers. In the extracellular space, however, and as shown for S100B, neurotrophic activity depends on the disulfide-linked dimerization (Barger *et al.* 1992). This indicates a tremendous variability of different structures (monomeric, homo-/heterodimeric), postranslationally modified, with different affinities for metal ions interacting with a large variety of target proteins/extracellular receptors. Overall, this is an even more complex situation than is found for calmodulin.

Several S100 proteins are released from cells despite the absence of a conventional signal peptide sequence of the kind essential for conventional ER-Golgi mediated secretion. Recently, a novel tubulin-dependent pathway for S100A8 and S100A9 release from activated monocytes was proposed (Rammes *et al.* 1997), suggesting a common mechanism for other S100 family members and possibly also for other proteins, including growth factors, which are also secreted lacking a signal peptide sequence.

Extracellular activities have been reported for S100A2, S100A4, A100A7, S100 A8, S100A9, and S100B, but the search for high-affinity cell surface receptors is still at its infancy. First experiments (Donato *et al.* 1975; Donato 1982) demonstrated a specific binding of S100A1 and S100B to synaptosomal membranes in the cerebral cortex. Later, Komada *et al.* 1996, reported that S100A2 interacts with eosinophil receptors, presumably coupled to a G protein, leading to the intracellular Ca^{2+} response. The biochemical nature of these S100 receptor proteins, however, has not been elucidated.

A wide range of different human diseases has been associated with a deregulated expression of S100 genes (Schäfer & Heizmann 1996; Heizmann 1996). It has been shown (Schäfer *et al.* 1995; Wicki *et al.* 1996a; Wicki *et al.* 1996b) that S100 genes (A1-A13) are clustered on human chromosome 1q21, a sensitive region where a number of rearrangements (deletions, translocations, duplications) occur in a number of cancer cells (Weterman *et al.* 1996), possibly affecting the differential expression of some S100 proteins (especially S100A2, S100A4, S100A6).

Recently we found that the S100 gene cluster is structurally conserved between human (chromosome 1q21), and mouse (chromosome 3), with some rearrangements (Ridinger *et al.* 1998).

There is also considerable interest in exploring the possibility of using S100 proteins/specific antibodies in clinical diagnostics. For example, specific antibodies have been generated for the immunohistochemical classification and diagnosis of tumors (Ilg *et al*. 1996a; Huang *et al*. 1996; Böni *et al*. 1997b; Maelandsmo *et al*. 1997; Camby *et al.* 1999) as well as for the diagnosis of cardiomyopathies (Usui *et al*. 1989; Remppis *et al*. 1996), chronic inflammations (Goebeler *et al*. 1994; Kocher *et al*. 1996), neurodegenerative disorders (Heizmann & Braun 1995 and 1997), and diabetes (Zimmer *et al*. 1997). S100 proteins are presently also being explored as targets for therapeutic interventions , e.g., for treatment of cardiovascular disorders (Nakatani *et al*. 1996) and allergies (Oyama *et al*. 1997).

This review will focus on recent developments in our understanding of the protein structure of S100 proteins, and discuss their metal-binding properties, posttranslational modifications, and interaction with target proteins, to provide a basis for future functional and clinical investigations.

The three-dimensional structures of S100B and S100A6

In the last years the three-dimensional structures of the metal-free forms of rabbit S100A6 (calcyclin) (Potts *et al*. 1995), and of bovine (Kilby *et al*. 1996) and rat (Drohat *et al*. 1996) S100B (ββ) and S100A12 (Nonato *et al*. 1997) were reported. 1998 was a vintage year with the structure elucidations of Ca^{2+}-bound rabbit S100A6 (Sastry *et al*. 1998), of Ca^{2+}-bound rat (Drohat *et al*. 1998), human (Smith & Shaw, 1998), and bovine (Matsumura *et al*. 1998) S100B(ββ), and of metal-bound S100A7 (psoriasin) (Brodersen *et al*. 1998). Whereas the three above described structures are characterized by perfect symmetry, the case of S100A8 and S100A9 is different: these proteins have a strong preference to form a heterodimer with a better complementarity of the interface then in homodimers (Hunter & Chazin 1998). All the S100 proteins are dimeric and, given the extended hydrophobic interface observed in all the three-dimensional structures, it is unlikely that in solution the monomer exists in the apoform or any of the metal-bound states ($^{dimer}K_D$ = 0.5 nM, Drohat *et al*. 1997). The perfectly symmetrical dimer looks like a 25 × 25 × 49 Å box (Ca^{2+} form) with a central groove at the top (Fig. 1), thus very different from the bi-lobed calmodulin. Each monomer displays two helix-loop-helix motifs, typical for EF-hands. The α-helices I and IV (subunit 1) and I′ and IV′ (subunit 2) form an X-type four-helix bundle (center of Fig. 1) which constitutes the dimer interface. The central groove is delineated by the antiparallel helices IV and IV′. The protein segments involved in the dimerization form a stable basement, i.e., their conformation does not change upon binding or dissociation of Ca^{2+}. Binding of Ca^{2+} induces a reorientation in the loop of the second EF-hand and helices III and III′, refolded towards the protein center in the apo-state, swing out over their C-terminal anchoring point with an angle change of 130°, entraining also an outward movement of the central linkers (Fig. 1). This ample swing-out movement on both sides of the protein dimer increases the solvent-accessible surface by

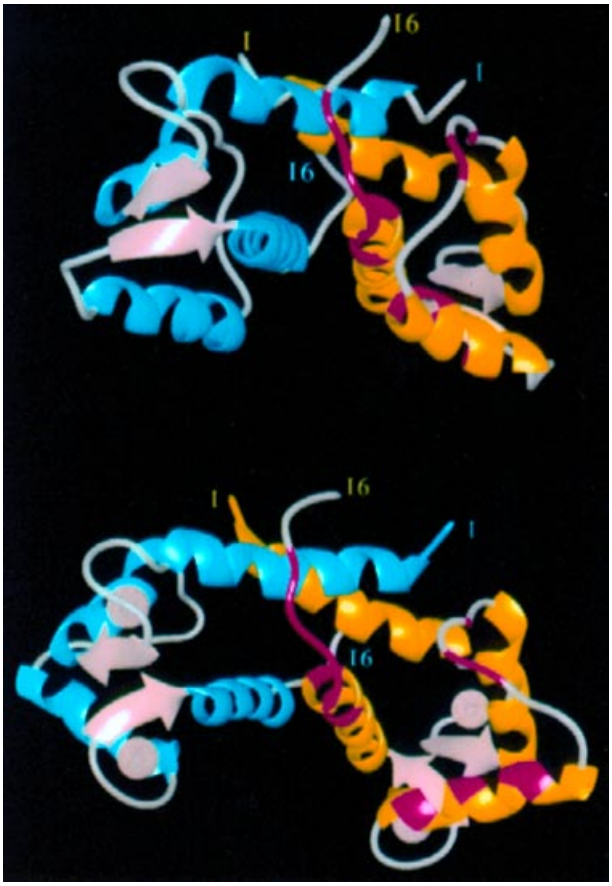

Figure 1. Comparison of the 3D structures of Ca^{2+}-bound (top) and apo-S100B(ββ) (bottom). Upon binding of Ca^{2+} only helices III and III' (horizontal at top) and the central linkers undergo a swing-out movement, thus uncovering two hydrophobic patches (in front, in the yellow subunit; in the back, behind the Ca^{2+}-binding β-sheet in the blue subunit). Residues whose orientation change upon binding of target p53 peptide, are shown in purple. The figure was kindly provided by David J. Weber.

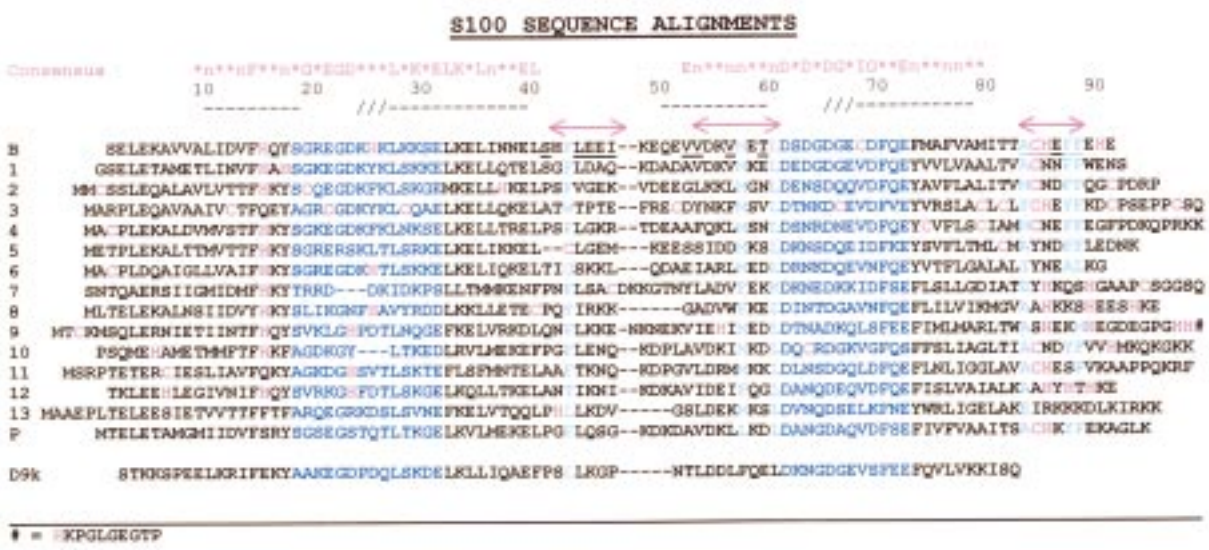

Figure 2. S100 sequence alignments. Numbering is based on S100B(ββ), the best-known structure. The consensus sequence is taken from Kretsinger (1987 Cold Spring Harbor Symp Quant Biol 52, 499–510). The code for the secondary structure is: —-, α-helix; //, β-sheet. The Ca^{2+}-binding loops are in blue; Ca^{2+}-exposed hydrophobic residues are in green; potential Zn^{2+} ligands His and Cys are shown in red. The segments forming the target-binding patch are indicated by the double arrow and residues directly involved in it are underlined. The sequence of S100A2 deposited in the GenBank (M87068) contains an error, resulting in an amino acid exchange from N62 to S62 (as reported by Wicki *et al.* 1997). This exchange has, therefore, been corrected in the above sequence.

420 Å and leads to unmasking of hydrophobic residues in two segments of the protein (purple arrows in Fig. 2). These unmasked residues form two exposed hydrophobic patches, adjacent to the two ends of the central groove. These patches have been shown to be instrumental for the binding of at least one target protein of S100B, namely tumor suppressor protein p53 (Rustandi *et al.* 1998). Since these residues are well conserved in the sequence (Fig. 2) and all the Ca^{2+}-loaded forms of S100 proteins bind in a Ca^{2+}-dependent way to phenyl-sepharose columns, it is likely that the Ca^{2+}-generated hydrophobic patches are formed in most of the S100 proteins. The interaction of the S100 proteins with the fluorescent hydrophobic probes, TNS, ANS and bis-ANS, also points to the functional importance and the ubiquitous character of this switch.

Interestingly, the model of Ca^{2+}-dependent conformational changes of S100B seems not to be valid for S100A6. The three-dimensional structures of apo- and Ca^{2+}-bound S100A6 are very similar and the monomer structure resembles that of apo- or Ca^{2+}-bound calbindin D9k (Potts *et al.* 1996). Since the helices I and I′ cross each other rather than being antiparallel, S100A6 is heart- rather than box-shaped and the central groove is deeper. The helices III and III′ and central linkers are oriented outward as in the Ca^{2+}-bound form of rabbit S100B, although not as far as in the rat protein. From these studies the question arises whether S100A6 is always interactive, independently of Ca^{2+}. However, there is a considerable body of *in vitro* evidence to support the Ca^{2+}-dependent binding of S100A6 to annexin II and XI, tropomyosin, glyceraldehyde-3-phosphate dehydrogenase, calponin and caldesmon, but a Ca^{2+}-independent binding to different target proteins might still occur.

The crystal structure of S100A10 (p11; which does not bind Ca^{2+}) resembles that of a S100 protein locked in the Ca^{2+}-bound conformation (Réty *et al.* 1999). Despite a stronger sequence homology to S100B, the S100A10 structure resembles more the monomeric cytosolic calbindin D9k. Réty *et al.* (1999) found a disulfide-linked tetramer in the crystal as well as in solution, demonstrating that this form may also be considered to be functional. The authors also reported on the crystal structure of a S100A10 complexed with a tetra-decapeptide corresponding to the N-terminal of annexin II, the first report on the crystal structure of a S100 protein in target peptide complex. The peptide was attached to the hydrophobic groove built up by the dimerization of helices I and IV of S100A10.

The three-dimensional structure of Ho^{3+}-substituted S100A7 has very recently been determined by MAD-phasing and refined to 1.05 Å resolution

(Brodersen *et al*. 1998). It is the most accurate structure of a Ca^{2+}-binding protein. Due to a 3-residue deletion in the non-canonical EF-hand, only 2 Ho^{3+} can bind to the dimer. This protein displays the heart-shape of S100A6, as well as the swing-out orientation of helices III and III′. The novelty in the S100A7 structure is the presence of an additional 8-residue long α-helix in the central linker of each subunit. Since only the structure of the metal-bound protein is known, it offers no clues as to the ubiquitousness of the S100 activation model.

Ca^{2+}, Zn^{2+} and Cu^{2+} binding

The Ca^{2+}- and Zn^{2+}-binding parameters of those S100 proteins that have been studied in detail are shown in Table 1. Generally, the dimeric S100 proteins bind four Ca^{2+} per dimer and display a low affinity for Ca^{2+} with K'_{Ca} values around 3×10^3 M^{-1} (for review, see Donato 1991). These results were confirmed in more recent studies on S100A2, S100A4, S100A6, S100B and S100A11 (Pedrocchi *et al*. 1994; Allen *et al*. 1996; Durussel *et al*. 1997; Franz *et al*. 1998). It should be noted that at low ionic strength the affinity is about ten times higher (Baudier *et al*. 1986). Two examples illustrate this ionic strength dependence: in 20 mM Tris-HCl, pH 7.5, the $[Ca^{2+}]_{0.5}$ for S100A1 is 36 μM, but 290 μM in the same buffer plus 120 mM KCl (Baudier *et al*. 1986). For S100A6 $[Ca^{2+}]_{0.5}$ is 3 μM at low ionic strength (Mani & Kay, 1990), but 320 μM in 50 mM Tris-HCl, pH 7.5, 150 mM KCl (Pedrocchi *et al*. 1994).

Zn^{2+} binds to most of the S100 proteins; the Zn^{2+} and Ca^{2+} sites are distinct and in several cases Zn^{2+} can modify the affinity for Ca^{2+}. Since for lack of space not all the cases can be treated here in more detail, we present a short anthology on the most striking cases. In physiological salt concentrations S100B binds 4 Zn^{2+} with high affinity (1 μM) and binding of Zn^{2+} decreases the $[Ca^{2+}]_{0.5}$ from 500 to 40 μM (Baudier *et al*. 1986). S100A12 dimer (calgranulin C) binds 2 Ca^{2+} per dimer with low affinity and 2 Zn^{2+} with high affinity, and binding of Zn^{2+} induces 2 very high-affinity Ca^{2+}-binding sites with $[Ca^{2+}]_{0.5} < 0.1$ μM (Dell'Angelica *et al*. 1994). S100A3 binds Ca^{2+} with such low affinity that it can be inferred alone from fluorescence studies, but binds 8 Zn^{2+} with a $[Zn^{2+}]_{0.5}$ of 11 μM (Föhr *et al*. 1995). Contrary to S100A2, Zn^{2+} binding to S100A3 does not lead to an affinity increase for Ca^{2+}. In S100A2 the 4 Ca^{2+}-binding sites in the dimer form an allosteric unit. The dimer binds 4 Zn^{2+} with a $[Zn^{2+}]_{0.5}$ of 5 μM, but Zn^{2+} is rather antagonistic with Ca^{2+} (Franz *et al*. 1998). S100A6 also binds 4 Zn^{2+} with a $[Zn^{2+}]_{0.5}$ of 0.1 μM, but it is not known what effect this cation has on the affinity of Ca^{2+} for this

Table 1. Ca^{2+}- and Zn^{2+}-binding parameters of the S100 proteins.

	Ca^{2+} sites*	$[Ca^{2+}]_{0.5}$ (mM)	n_H	Zn^{2+} sites*	$[Zn^{2+}]_{0.5}$ (μM)	n_H	Ca^{2+}/Zn^{2+} effect	Ref.
S100B	4	0.50	1.0	6–8	500	nd	+	a
S100A1	4	0.29	1.1				–	a
S100A2	4	0.47	2.04	4	4.5	1	–	b
S100A3	nd	35	0.76	8	11	1.4	no	c
S100A4	4	0.15	1.15					d
S100A5	4	0.013	1.76	2	nd		+	e
S100A6	4	0.32	1.33	4	2000		(–)	d,f
S100A10	no				nd			
S100A11	4	0.52	1.4					g
S100A12	2	0.05	1	2	< 0.1	nd	+	h
S100P	4	0.002/0.8	na					i

* per dimer; na, not applicable; nd, not determined; no, no binding.
The Ca^{2+}-binding parameters of S100A7, S100A8, S100A9 and S100A13 have not been reported.

a, Baudier J, Glasser N, Gerard DJ. 1986 *J Biol Chem* **261**, 8192–8203; and Leung IKM, Mani RS, Kay CM. 1987 *FEBS Lett* **214**, 35–40.
b, Franz C, Durussel I, Cox J, Schäfer BW, Heizmann CW. 1998 *J Biol Chem* **111**, 273, 18826–18834.
c, Föhr UG, Heizmann CW, Engelkamp D, Schäfer BW, Cox JA. 1995 *J Biol Chem* **270**, 21056–21061.
d, Pedrocchi M, Schäfer BW, Durussel I, Cox JA, Heizmann CW. 1994 *Biochemistry* **33**, 6732–6738.
e, study in progress.
f, Kordowska J, Stafford WF, Wang CLA. 1998 *Eur J Biochem* **253**, 57–66.
g, Allen BG, Durussel I, Walsh MP, Cox JA. 1996 *Biochem Cell Biol* **74**, 687–694.
h, Dell'Angelica EC, Schleicher CH, Santome JA. 1994 *J Biol Chem* **269**, 28929–28936.
i, Becker T, Gerke V, Kube E, Weber K. 1992 *Eur J Biochem* **207**, 541–547.

protein (Mani & Kay 1990; Filipek *et al.* 1990; Kordowska *et al.* 1998). Most of the other members have not been studied in detail, but there are qualitative hints about Ca^{2+} and Zn^{2+} binding. In the presence of 100 μM Zn^{2+} S100A1 shows a 30-fold increase of its affinity for twitchin kinase (Heierhorst *et al.* 1997). In a blotting and $^{65}Zn^{2+}$ overlay experiment Raftery *et al.* (1996) showed that murine S100A9 binds indeed Zn^{2+} with a higher affinity than S100B. The much larger S100-related protein profilaggrin also displays 2 low affinity Ca^{2+}-binding sites with dissociation constants of 0.14 and 1.2 mM, respectively (Presland *et al.* 1995). Thus S100 proteins have variable cation-binding properties in agreement with their diversified functions.

S100B was identified in brain as an inhibitor of the Cu^{2+}-catalyzed oxidation of L-ascorbate (Nishikawa *et al.* 1997). The dimer binds 4 Cu^{2+} ions with a K_D of 0.46 μM, most of which can be displaced with 1 mM Zn^{2+}, suggesting that Zn^{2+} and Cu^{2+} share the same binding sites. Given the high concentration of S100B in brain, it is possible that it protects against oxidative damage and plays a role in copper homeostasis. In this regard it must be noted that the βA4 amyloid precursor protein reduces Cu^{2+} to Cu^{1+}, leading to neurodegeneration in Alzheimer's disease (Multhaup *et al.* 1996). The physiological importance of Cu^{2+} sequestration by S100 proteins must be considered in the light of the recent finding that cellular prion protein binds Cu^{2+} *in vivo* (Brown *et al.* 1997). It is likely that the different S100 proteins also show different binding properties with respect to Cu^{2+}.

Little is known about the physiological function of intracellular Zn^{2+}, except for its structural role in transcription factors and its catalytic role in enzymes. Zn^{2+} homeostasis is mainly regulated by transporters (Reyes 1996), belonging to the family of cation diffusion facilitators (Eide 1997) and, whereas vesicular and synaptic cleft Zn^{2+} can amount to 200–400 μM (Frederickson 1989), the cytosolic Zn^{2+} concentration is exceedingly low, i.e. < 0.1 nM. Thanks to an ingenious mechanism based on redox-modulated changes in their Zn^{2+} affinity, metallothioneins can provide or withdraw Zn^{2+} from transcription factors, metalloenzymes and proteins (Maret & Vallee 1998; Jiang *et al.* 1998; Jacob *et al.* 1998). Metallothioneins keep the cellular Zn^{2+} concentrations remarkably low, since they bind Zn^{2+} with a K_d of $1.4\ 10^{-13}$ M; but with their special Zn^{2+}-thiol clusters, the ion is kinetically very labile and easily exchanged with Zn^{2+}-binding proteins. Thus, the cellular redox state, metallothionein and natural Zn^{2+} chelators such as citrate and glutathion may regulate also the state of occupancy of the Zn^{2+} sites in the S100 proteins, however, this view is still debated. Since metallothioneins also bind Cu^{2+}, a similar mechanism may prevail for this latter cation. As Zn^{2+}- and Cu^{2+}-binding proteins, the S100 proteins may assume conformations different from the Ca^{2+} or apo conformation, with possibly new functions in the cytoplasm or nucleus. Alternatively, as Zn^{2+} chelators S100 proteins may be instrumental in the redox/metallothionein-orchestrated redistribution of Zn^{2+} to or from various metalloproteins (Jacob *et al.* 1998).

A novel Zn^{2+}- (Cu^{2+}?)-binding motif

Although the sequences of all S100 proteins contain a number of Cys and His residues capable of chelating Zn^{2+} (Fig. 2), it is not possible to propose a Zn^{2+}-binding site and a consensus sequence. Now that the three-dimensional structures of S100B, S100A6, S100A7 (Brodersen *et al.* 1998), S100A10 (Réty *et al.* 1998), and S100A12 (Nonato *et al.* 1997) are available, it is possible to predict locations of putative Zn^{2+} sites. Smith & Shaw (1998) pointed out that in S100B His15 and His25 are closely spaced (5 Å) and not far from His85 or Cys84 in the complementary subunit (8 Å). A study with *Lanthanides* suggests that binding of Zn^{2+} to His15 and His25 would explain the observed reduction of the intersite distance (Chaudhuri *et al.* 1997). S100A6, S100A8 and S100A9 also possess a similar chelating geometry. In S100A6, which binds two Zn^{2+} per monomer at sites different from the Ca^{2+}-binding sites, Cys3 has been shown to be one ligand (Kordowska *et al.* 1998). Intriguingly, intra- or interchain Cys-Cys bridges are easily formed in several S100 proteins and the respective free thiols may be potential Zn^{2+}-binding sites, where the cation just bridges and mimicks the disulfide bridge.

Another motif was pointed out by Clohessy & Golden (1996): the Zn^{2+}-chelating S100A8, S100A9 and S100A12 contain at the C-terminal end a His-x-x-x-His motif followed by a proximal upstream Glu. When present in an α-helix this motif can bind Zn^{2+} with high affinity, as was found in a number of naturally occurring proteins (Higaki *et al.* 1992). This motif was also found in S100A7. S100B, which binds 8 Zn^{2+} per dimer with sites of high and low affinity (Baudier *et al.* 1986), contains a His-x-x-x-x-His motif instead at the C-terminal end.

A 3-D structural model of S100A3 in its Zn^{2+} form was proposed (Fritz *et al.* 1999). Two Zn^{2+} ions bind to one S100A3 monomer in a concerted manner, forming one Zn^{2+} $(S_{Cys})_4$ and one $Zn^{2+}(S_{Cys})_3(N_{His})$ site. This binuclear cluster at the C–terminus resem-

Table 2. Posttranslational modifications

Proteins	Modifications
S100A8	phosphorylation (a)
S100A9	phosphorylation (a,b) methylation (c)
S100A8/S100A9 heterodimer (FA-p34) complex	binding of unsaturated fatty acids with high affinity (d)
S100A11	transglutaminase-reactive residues; forms multimers (e)
S100A12	S-sulfo-cysteine (f)

(a) Guignard F, Mauel J, Markert M. 1996 *Eur J Biochem* **241**, 265–271.
(b) Edgeworth J, Freemont P, Hogg N. 1989 *Nature* **342**, 189–192.
(c) Raftery MJ, Harrison CA, Alewood P, Jones A, Geczy CL. 1996 *Biochem J* **316**, 285–293.
(d) Siegenthaler G, Roulin K, Chatelard-Gruaz D *et al*. 1997 *J Biol Chem* **272**, 9371–9377.
(e) Robinson NA, Eckert RL. 1998 *J Biol Chem* **273**, 2721–2728.
(f) Yang Z, de Veer MJ, Gardiner EE *et al*. 1996 *J Biol Chem* **271**, 19802–19809.

bles the $(Zn^{2+})_2$ cluster found in transcription factors of the GAL4 type.

In conclusion, with the exception of S100A13 and to a lesser degree S100A5, all the S100 proteins have at least 2 residues of His and Cys in the N-terminal part of the pseudo EF-hand and in the C-terminal end loop. Since these regions come together in the dimeric protein, Zn^{2+}-binding sites can easily be formed.

Do post-translational modifications modulate the regulatory role of S100 proteins?

Modifications of a number of S100 proteins (Table 2) have been reported that might influence their metal-binding properties, their interactions with target proteins, activation of enzyme, and their relocations in cells upon stimulation.

S100A8 and S100A9 have been found to be phosphorylated. S100A9 phosphorylation (Edgeworth *et al*. 1989) might influence the above mentioned functions. However, there are species differences, e.g., the murine S100A9 (in contrast to human S100A9) lacks the Thr at the C-terminus and is probably not phosphorylated upon Ca^{2+} increase in the cell. Phosphorylation of S100A8 and S100A9 might also influence their translocation to the cell membrane during human neutrophil activation (Guignard *et al*. 1996).

Inter- and intrasubunit disulfide-bridged S100B is one of the best substrates for casein kinase II and the product becomes highly mitogenic (Scotto *et al*. 1998).

The hetero complex S100A8/S100A9 in human keratinocytes was found to be associated with unsaturated fatty acids (FA) (Siegenthaler *et al*. 1997, Klempt *et al*. 1997). FA are implicated in membrane synthesis and energy delivery and have to be translocated by specific carrier proteins (e.g., albumin and fatty acid-binding protein). It has been suggested that S100A8/A9 might be a novel family of FA-binding proteins mediating the effects of FA in a Ca^{2+}-dependent manner during cell differentiation and inflammation. Binding of FA to S100 proteins might also be a mechanism involved in the relocation/secretion of S100 proteins in other cells.

S100A11, a component of the keratinocyte cornified envelope (Robinson & Eckert 1998) was found to be a transglutaminase substrate and crosslinked to form multimers at specific sites.

S100A12 was detected as a major sulfite acceptor in the sulfate reduction pathway in neutrophils (Yang *et al*. 1996). It was suggested that this modification is involved in the secretion of S100A12, exhibiting extracellular cytostatic activities as found for S100A8 and S100A9. This modification has been detected in rabbit S100A12. Since human and porcine S100A12 do not contain this Cys residue, S-sulfation might be species-specific.

There are a number of modifications not listed in Table 2 that might influence biochemical and functional properties of S100 proteins. For instance, modern mass spectrometry (Smith *et al*. 1997; Raftery *et al*. 1996; Ilg *et al*. 1996b; Smith *et al*. 1997) has revealed heterogenous modifications at the N-terminal of S100 proteins (acetylation, formylation), as has been found for recoverin (Johnson *et al*. 1997). The heterogeneity of these modifications in recoverin seems to be tissue-specific. A myristoyl (or related N-acyl group) covalently attached to the N-terminus of recoverin confers Ca^{2+}-dependent membrane-binding, referred to as the calcium-myristoyl switch (Ames *et al*. 1997), a mechanism that also might occur in some S100 proteins during translocations and binding to the plasma membrane followed by secretion.

Furthermore, some S100 proteins are able to form intra- and/or interdisulfide bonds. In addition, cysteine residues of S100 proteins can be oxidized, influencing target binding (Baudier & Cole 1988; Johnsson & Weber 1990; Yao *et al.* 1996; Fritz *et al.* 1998), indicating a tremendous spectrum of possible modifications of S100 proteins modulating their biological activities.

Site-directed mutagenesis studies on S100 proteins

Site I coordinates Ca^{2+} by the α-carbonyls and, therefore, the site has turned inside-out and displays a sharper bend in the first 6 residues of the loop (illustrated in Smith & Shaw 1998) as compared to the situation in the archetypal EF-hand (closed *vs* open configuration). Elegant site-directed mutagenesis on calbindin D9k, which also possesses such an N-terminal EF-hand, revealed that 2 mutations and 2 deletions in the loop are necessary and sufficient to invert the geometry of the site from closed to open, while the affinity remains as high as in the wild-type protein (Johansson *et al.* 1991, 1993). Is this also true for S100 proteins?

Systematic point mutations to convert the pseudo site were not carried out for S100 proteins, but a complete exchange of the 14-residue-long loop of site I by the 12-residue-long loop of a canonical EF-hand has been reported for S100B (Durussel *et al.* 1997) and S100A2 (Franz *et al.* 1998). Neither the stability, nor the secondary and quaternary structures of the mutant proteins differed from the wild type, although local conformational changes occurred, likely in the region involved in hydrophobic interactions. In both S100B and S100A2 the exchange led to an increase in the affinity for Ca^{2+} without any notable change in the affinity for Zn^{2+}. In S100B the affinity of the two sites increased threefold. In S100A2, where the wild type protein shows strong positive cooperativity (Table 1), the 'normalization' of site I led to a 100-fold affinity increase.

In conclusion, in calbindin D9k as in the more complex S100 proteins the pseudo and canonical sites can very easily be interchanged. The changes in conformation are rather small, although inside-out inversion must take place. The increase in affinity is linked either to the intrinsically higher affinity of the canonical site over the pseudo site, or to less hydrophobic exposure in the mutant proteins. Another interesting mutation concerns the inactivation of the canonical EF-hand in S100B (Durussel *et al.* 1997): in this so-called NoEF mutant the remaining pseudo site has a $[Ca^{2+}]_{0.5}$ of 10 mM, suggesting that it has a very low intrinsic affinity for Ca^{2+}, but gains affinity in the context of an active paired site.

The diversity of the target proteins of S100

It is believed that binding of Ca^{2+} to a S100 protein will induce a significant conformational change, exposing hydrophobic domains to facilitate the interaction with a hydrophobic region of the secondary effector protein, leading to the physiological response.

This view, however, does not seem to be true for all S100 proteins. For example, in S100A6 (Sastry *et al.* 1998) only very modest Ca^{2+}-induced changes have been observed, different from S100B (Drohat *et al.* 1996; Kilby *et al.* 1996; Drohat *et al.* 1998; Matsumura *et al.* 1998; Smith & Shaw 1998) and in sharp contrast to calmodulin (Finn *et al.* 1995; Kuboniwa *et al.* 1995; Zhang *et al.* 1995). Therefore, the minute Ca^{2+}-induced changes in S100A6 may not strongly influence protein-target interactions, suggesting a different mode of Ca^{2+}-signal transduction.

Similarly, S100A7 (Brodersen *et al.* 1998) was found to bind only one Ca^{2+}/monomer with a modest conformational change, further challenging the role of S100 proteins in Ca^{2+}-sensing. Binding of S100 proteins to target proteins could, however, increase their affinity for Ca^{2+} into the range of intracellular Ca^{2+} signalling. One example is S100A10, lacking an intact EF-hand and being unable to bind Ca^{2+}. In the hetero complex $(S100A10)_2$/ $(\text{annexin II})_2$, a Ca^{2+}-dependent conformational change occurs with subsequent binding to the cell membrane (Gerke 1991). This model might also be valuable for other S100 members where few changes in their conformation have been observed upon Ca^{2+}-binding.

The large number of target proteins interacting in a Ca^{2+}-dependent manner is listed in Table 3 and is consistent with the increasing number of intra- and extracellular functions of S100 proteins. Recently, we were able to demonstrate (Franz *et al.* 1998) that nuclear S100A2 dimer binds four Zn^{2+} ions with a high affinity, inducing conformational changes, including a hydrophobic patch on the protein surface, possibly essential for binding to nuclear target proteins. Therefore, future research should also try to identify target proteins binding to S100 proteins in a Ca^{2+}-independent but Zn^{2+}- and Cu^{2+}-dependent manner.

Table 3.

Protein	Target Proteins	Postulated Functions
S100A1	fructose-1,6-biphosphate aldolase, glycogen phosphorylase, adenylate cyclase, tubulin, GFAP, myoD, twitchin kinase (a), desmin (b), CapZ (c,d), F-actin (e), caldesmon (f), novel proteins (g), myogenic bHLH transcription factors (h), ryanodine receptor (i)	inhibition of microtubule assembly, inhibition of PKC-mediated phosphorylation, regulation of Ca^{2+}-release channel (ryanodine receptor) (i), desmin assembly (b), regulation of transcription factors (h)
S100A2	nuclear proteins (e,j), tropomyosin (k), chemotactic receptor (l)	tumor suppressor gene function (m), Ca^{2+}-dependent functions in the cell nucleus (e,j), chemotactic for eosinophils (l), organization of the actin cytoskeleton via interaction with tropomyosin (k)
S100A3	–	hair cuticle differentiation (n,o,xx), association with breast cancer (p)
S100A4	non-muscle tropomyosin, p53, non-muscle myosin, MAP, association with stress fibers and ER (e), p37 (q)	motility and invasion of tumor cells, tubulin polymerization, regulation of cytoskeleton (e)
S100A5	–	–
S100A6	annexin II, annexin VI, annexin XI (r), glyceraldehyde-3-phosphate dehydrogenase, caldesmon (s), caltropin (t), other proteins (u)	stimulates Ca^{2+}-dependent insulin release, stimulates prolactin secretion, exocytosis, regulation of Ca^{2+} homeostasis and tumor progression (j,v,w)
S100A7		chemotactic for neutrophils and T lymphocytes, breast cancer progression, psoriasis (x) and acting as allergen (y)
S100A8	intermediate filaments (vimentin), cytoskeletal structure and cell membrane (z)	inhibits casein kinase, myeloid cell differentiation and activation factor, inflammation, chemotactic activities (aa,bb), fatty acid metabolism (cc), apoptosis (dd), adhesion of neutrophils via Mac-1 (yy)
S100A9	intermediate filaments (vimentin), cytoskeletal structure and cell membrane (z)	inhibits casein kinase, myeloid cell differentiation, inflammation, neutrophil immobilizing activity, cytostatic activity (aa,bb), apoptosis (dd), fatty acid metabolism (cc), stimulator of neutrophil adhesion (yy)
S100A10	$(\text{annexin II})_2(\text{S100A10})_2$ complex, 85-kDa phospholipase A_2 (ee)	anti-inflammatory, regulation of neurotransmitter release, inhibition of phospholipase A_2 (ee)
S100A11	annexin I (ff,gg)	suppression of annexin I function, phosphorylation by protein kinase C (hh), structural organization of early endosomes (ii)
S100A12	association with the cytoskeleton (jj)	regulation of neutrophil activation (jj), differentiation of squamous epithelial cells (kk), host-parasite interaction (ll), secretion (mm), pathology of autoimmune disease (nn)
S100A13	FGF-1/p40Syn-1/A13 complex (oo)	functions in muscle (pp), regulation of FGF-1 and p40 Syn-1 release (oo)
S100B	fructose-1,6-biphosphate, aldolase, calponin, tau protein, tubulin, caldesmon, neurocalcin, GAP43, GFAP, CapZ (qq), p53 (rr,ss), nuclear Ser/Thr protein kinase (vv), bHLH transcription factor (h)	neurite extension, proliferation of melanoma cells, inhibition of PKC-mediated phosphorylation, axonal proliferation and astrocytosis, inhibits microtubule assembly, guanylate cyclase activation (tt), inhibition of the hypertrophic phenotype (uu,zz), regulation of transcription (h), regulation of a nuclear kinase (Ndr) (vv), suppression of Cu^{2+}-induced cell damage (ww)

References: This table is updated from Schäfer BW & Heizmann CW. 1996 *Trends Biochem Sci* **21**, 134–140, and therefore only new references are added. For further references see Kligman D, Hilt DC. 1988 *Trends Biochem Sci* **13**, 437–443; Hilt DC, Kligman D. 1991 In: Heizmann CW, ed. Novel Ca^{2+}-binding Proteins. Fundamentals and Clinical Implications. Berlin: Springer Verlag: 65–103; Donato R. 1991 *Cell Calcium* **12**, 713–726; Zimmer DB, Cornwall EH, Landar A, Song W. 1995 *Brain Res Bull* **4**, 417–429; Fano *et al.*1995 *Prog Neurobiol* **46**, 71–82.

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Structural aspects of target interaction with S100 proteins

The elucidation of the Ca^{2+}- and apo-forms of rat S100B(ββ) allowed for the first time to make an educated guess on the molecular aspects of S100-target interactions. In each β subunit the binding of Ca^{2+} unlocks a hydrophobic patch defined by the residues indicated with purple arrows in Fig. 2. These patches, located at each side of the central groove (Fig. 1), are characterized by a hydrophobic center containing Cys84 in the middle and surrounded by acidic groups. They ideally represent the docking site for targets, such as a synthetic peptide of tumor suppressor protein p53 (Baudier *et al.* 1992). S100B binds to the p53 peptide, which, like most calmodulin targets, likely forms an amphiphilic positively charged α-helix, in a Ca^{2+}-dependent manner with a stoichiometry of 2 peptides per dimer and a K_D of 20–50 μM. P53 peptide perturbs essentially only some residues located at the hydrophobic patches (Rustandi *et al.* 1998). The importance of the exposed Cys84 for the S100B-

Table 3 *Continued*

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target interaction may depend on the nature of the target: the Cys84Ala mutant does not affect aldolase but alters very specifically the regulation of two other targets (Landar *et al.* 1997).

But some general mechanism may emerge: in at least four cases S100B directly binds to and inhibits the PKC-phosphorylation of a short basic α-helical segment in the target (Wilder *et al.* 1998). Screening of a peptide library with an S100B-binding assay revealed the consensus (K/R)(I/L)xWxxIL and the synthetic peptide TRTKIDWNKILS present in the target CapZ binds to S100B with high affinity and in a Ca^{2+}-specific manner (Ivanenkov *et al.* 1995).

Is the S100B-p53 model of target interaction also valid for other S100 proteins? Well documented studies on some members, such as S100A4, yield a positive answer. In the presence of Ca^{2+}, the S100A4 dimer interacts with a 29-amino acid region at the C-terminal end of the heavy chain of non-muscle myosin II. This segment is composed of two α-helices and a 4-residue linker. S100A4 inhibits phosphorylation of a Ser residue by PKC in this segment (Kriajevska *et al.* 1998). Interestingly, if this target forms a 1 to 1 complex with the S100 dimer, one can speculate that each of the two α -helices binds to one hydrophobic patch and that the linker passes through the central groove of S100A4. This model would bear similarity to the classical molecular model of calmodulin-target interaction.

One of the most spectacular targets of S100A1 is the giant protein kinase twitchin, which binds only this S100 protein in a Ca^{2+}-dependent, Zn^{2+}-modulated manner with a K_{S100A1} of 3 μM and is activated 1000-fold (Heierhorst *et al.* 1996). The S100A1-binding region is located in the intrasteric autoinhibitory domain of twitchin kinase and has the characteristics of a calmodulin-binding, 18-residue-long amphiphilic basic α-helix. The crystal structure of the large twichin fragment comprising the catalytic domain and autoregulatory sequence revealed that this α-helical peptide region lies in the groove that normally binds to the kinase substrate (Kobe *et al.* 1996). Calmodulin and S100A6 also can bind to this peptide but they do not activate the enzyme (Heierhorst *et al.* 1997), indicating that the interaction occurs in two phases: initial binding to a degenerate basic amphiphilic helix, followed by a very selective removal of the autoinhibitory peptide, which only S100A1 is known to be capable of achieving.

But for the other S100 proteins target interaction may be quite different since the interacting residues of S100B (underlined in Table 1) are not at all conserved in the other S100 proteins. The diversity of molecular models describing the interaction of S100 proteins with their targets may be at the basis of the high functional diversity observed in the S100 family.

Conclusions and perspectives

Now that the three dimensional structures of the metal-free and Ca^{2+} states of several S100 proteins are available and the target interactive regions are being identified, it might soon be possible to design very efficient cell-permeant drugs selective for each S100 subfamily member in order to probe its function *in vivo*. Not only the structure of the Ca^{2+}-form, but also of the Zn^{2+}-, Cu^{2+}-, or Ca^{2+}/Zn^{2+}- and Ca^{2+}/Cu^{2+}-forms need to be elucidated, since these heavy cations show important *in vitro* effects likely also occurring *in vivo*. In this context it is important to resolve the basic question if and how metallothioneins might regulate the metal content of S100 proteins. In the near future we likely will see the three-dimensional structures of different complexes between S100 proteins and peptides of their specific target proteins and these results will provide a basis for future functional studies and therapeutic interventions.

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