Amino Acids (2011) 41:761–772
DOI 10.1007/s00726-010-0540-4

REVIEW ARTICLE

The role of zinc in the S100 proteins: insights from the X-ray structures

Olga V. Moroz · Keith S. Wilson · Igor B. Bronstein

Received: 12 November 2009 / Accepted: 22 February 2010 / Published online: 20 March 2010

Abstract We here aim to summarise the present knowledge on zinc binding by S100 proteins. While the importance of modulation of the function of the S100 family of EF-hand proteins by calcium is well established, a substantial proportion is also regulated by zinc or copper. Indeed regulation by zinc in addition to calcium was suggested almost as soon as the first S100 protein was discovered and has been confirmed for many family members by numerous experiments. For the first, "His-Zn", group, zinc-binding sites composed of three histidines and an aspartic acid were first proposed based on sequence comparisons and later confirmed by structural studies. A second, "Cys-Zn", group lacks such well-defined zinc-binding motifs and for these cysteines were suggested as the main zinc ligands. There is no three-dimensional structure for a Cys-Zn S100 in the presence of zinc. However, analysis of their sequences together with their X-ray structures in the absence of zinc suggests the possibility of two zinc-binding sites: a conserved site with a degree of similarity to those of the His-Zn group and a less-defined site with a Cys interdimer-binding motif. Some S100 protein-mediated events, such as signalling in the extracellular space, where the levels of calcium are already high, are most unlikely to be calcium regulated. Therefore, a broader knowledge of the role of zinc in the functioning of the S100 proteins will add significantly to the understanding how they propagate their signals.

Keywords S100 proteins · Zinc · Zinc-binding site · X-ray structure · Extracellular signalling

Abbreviations
PDB Protein data bank
Pnt Pentamidine

Introduction

The S100 family, with about 20 members in total, is composed of EF-hand calcium-regulated proteins and is linked to a range of severe diseases including cancer, autoimmune and neurological disorders (recently reviewed in Marenholz et al. 2004; 2006; Santamaria-Kisiel et al. 2006; Schaub and Heizmann 2008). Most S100 proteins are non-covalent homo- or in some cases hetero-dimers. The subunits have very similar architecture, containing two EF-hand motifs linked by a so-called hinge. The hinge is the region with the greatest variation in sequence in the family reflected in differences in the 3D structures. The N-terminal EF-hand (EF-1) is formed by helices αI and αII, and the second C-terminal one (EF-2) by αIII and αIV (Figs. 1, 2). EF-2 is canonical, with high conservation among the whole EF-hand superfamily, with the calcium mostly coordinated by side chain oxygens. EF-1 is only encountered in the S100 family, with the calcium coordinated principally by the main chain oxygens, and there is a much lower conservation for calcium-binding residues than for the canonical EF-2. EF-1 has a lower affinity for calcium, and its conformation

O. V. Moroz (✉) · K. S. Wilson
Structural Biology Laboratory, Department of Chemistry, University of York, Heslington, York YO10 5YW, UK
e-mail: olga@ysbl.york.ac.uk

I. B. Bronstein (✉)
School of Biomedical and Health Sciences, Hodgkin Building, Guy's Campus, King's College, London SE1 1UL, UK
e-mail: igor.bronstein@kcl.ac.uk

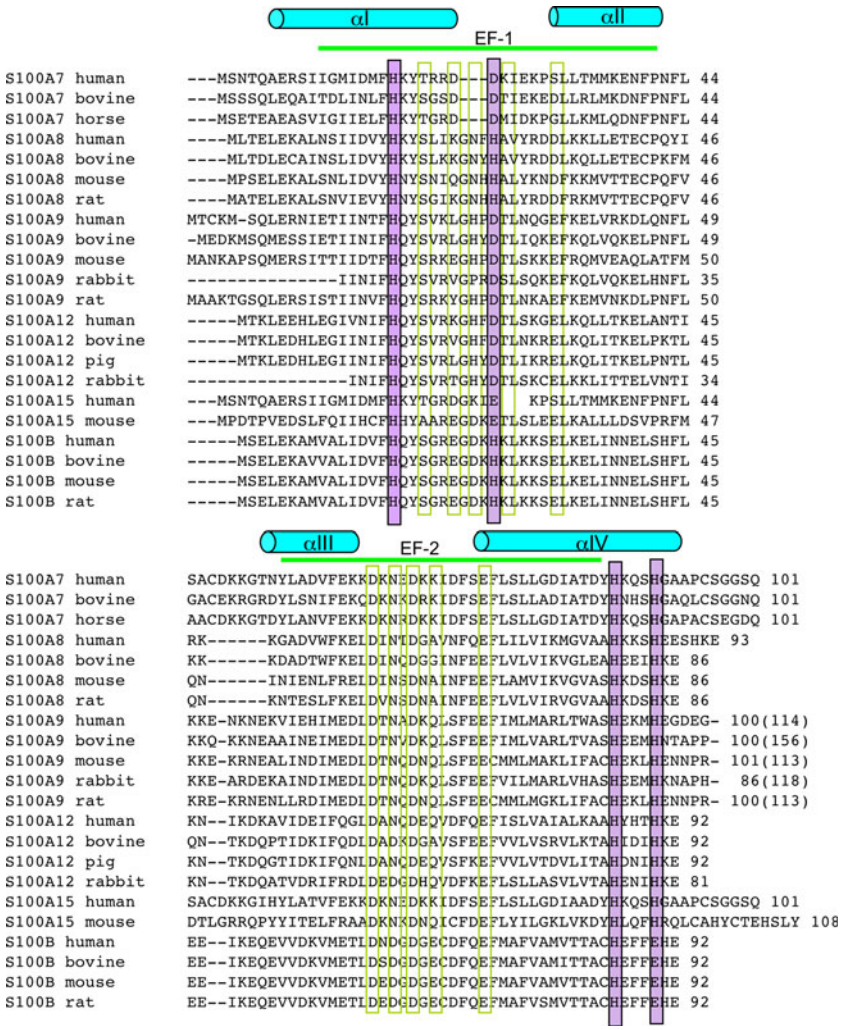

Fig. 1 Sequence alignment of S100 proteins with a His-Zn binding site. Zinc-binding residues are *outlined in magenta*. Calcium-binding residues from EF-1 and EF-2 are outlined with *green boxes*. Secondary structure elements shown above the alignment correspond to the structure of S100A12 in complex with calcium and copper. Sequence alignments here and for Fig. 6 were performed using ClustalW. (Colour available in the online version. In printed version where possible different shades of grey were used). *Green*, *yellow* and *cyan* correspond to *light grey*, *magenta* to *medium grey* and *blue* to *dark grey*

is hardly altered upon calcium binding, while EF-2 has a higher affinity and undergoes substantial changes on calcium binding. It is likely that the differences in structure and calcium affinity of the two EF-hands account for fine-tuning of calcium regulation (Maler et al. 2002; Otterbein et al. 2002). Analysis of several S100-target peptide complexes showed that hydrophobic and charged residues from the hinge region, αIII and the C-terminus contributed to target binding and were responsible for specificity (Bhattacharya et al. 2004). Calcium binding leads to a significant movement of helix αIII, and to exposure of the target-binding site.

Several S100 proteins bind zinc, the zinc binding being linked to an increasing number of target interactions. Zinc binding was first reported for S100B, with the suggestion that zinc as well as calcium was responsible for regulation of its activity. S100B could be purified on a Phenyl-Sepharose column in the presence of zinc (similar to the use of calcium for several other S100 proteins), implying that hydrophobic surfaces were exposed upon zinc binding (Baudier et al. 1982). Three of the four histidines proposed as potential zinc ligands (Baudier et al. 1986) were later confirmed by crystal structure analyses (described below) to be involved in zinc binding. It was subsequently shown that zinc binding increased the calcium affinity of S100B about tenfold (Baudier et al. 1984), and in addition increased the affinity for interaction partners, first described for tau protein (Baudier and Cole 1988).

Zinc binding was next demonstrated for pig S100A12, with zinc increasing the calcium affinity by ~1,500-fold, and a zinc-binding site was subsequently predicted in several other S100 proteins based on conserved sequence motifs (Dell'Angelica et al. 1994). This zinc site, henceforth referred to as His-Zn, was distinct from the calcium-binding sites in the EF-hands and consisted of either three histidine and one aspartate, or four histidine residues. S100B, S100A7, S100A12 have to date been proven by structural studies to have such zinc sites, and S100A8, S100A9 and S100A15 are also believed to possess one on the basis of sequence alignments and 3D comparisons of their zinc-free structures with those of homologues with

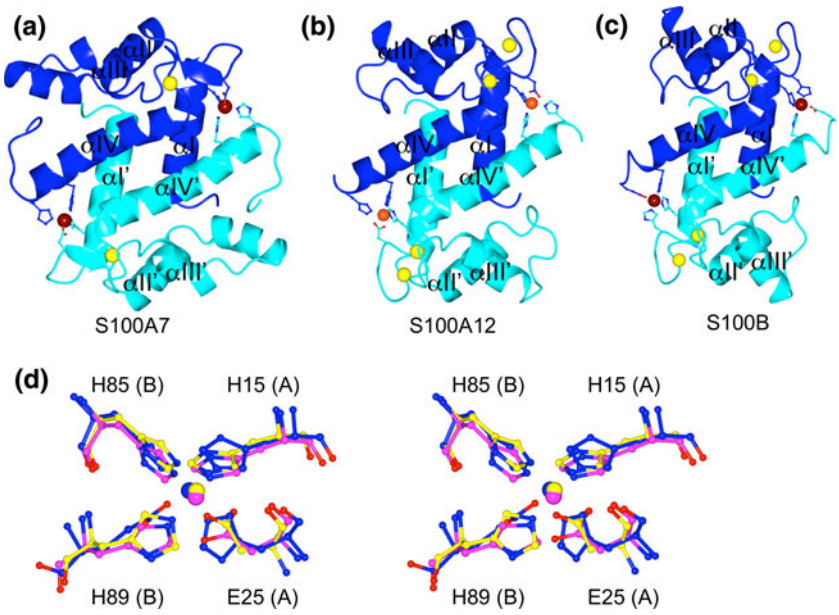

Fig. 2 Zinc and copper binding sites of the His-Zn group of S100 proteins. **a** The S100A7 dimer with bound calcium and zinc. One subunit is shown in *blue* and the other in *cyan*, calcium ions are in *yellow* and zinc in *brown*. Zinc is coordinated by His and Asp from one subunit, and two His from another subunit. **b** The S100A12 calcium–copper complex, coloured as for S100A7, with *copper in orange*. **c** The S100B calcium–zinc complex. **d** Stereoview of superposition of the zinc-binding sites of Zn-S100A12 (PDB code 2wc8, *yellow*), S100A7 (3psr, *magenta*) and S100B (3cr2, *blue*). Zinc ions are shown in the corresponding colours. This figure and Figs. 3, 4, 5 were generated using CCP4mg (Potterton et al. 2004)

zinc bound. Another likely candidate is S100A6, although its His-Zn site is not complete, discussed later.

In addition to this His-Zn group, zinc binding has been reported for several other S100 proteins, including S100A1, S100A2, S100A3, S100A5, S100A6 and probably S100A4. These proteins were suggested to have cysteine-containing zinc-binding sites (henceforth termed Cys-Zn), but no three-dimensional structures in a zinc-bound state have been determined. The zinc affinity constants reported for several S100 proteins are summarised in Table 1.

As for S100B, zinc binding has been directly linked to the increase in the affinity of several other S100 proteins for their targets (Table 2). In addition for some S100 members while the partner protein has not been identified as yet, there is nevertheless indirect evidence for a functional role of zinc. For instance, zinc-regulated binding has been demonstrated for S100A8/A9 to MM46 tumour cells (Nakatani et al. 2005) and S100A12 to gastric carcinoma MKN74 cells (Moroz et al. 2009b). Among recent direct disease association reports are those of zinc-dependent up-regulation of S100A8 leading to early esophageal carcinogenesis (Taccioli et al. 2009), and S100A8/A9 amyloid formation in the aging prostate promoted by calcium or zinc (Yanamandra et al. 2009). Zinc also promotes homo- or hetero-oligomerisation of several S100 proteins, e.g. formation of S100A8/A9 heterotetramers (Vogl et al. 2006; Yousefi et al. 2007), S100B heterocomplexes with S100A6 and S100A11 (Deloulme et al. 2000), as well as S100A2 (Koch et al. 2007) homotetramers and S100A12 homotetra- and hexamers (Moroz et al. 2009a, b). Evidence

Table 1 Zinc binding constants reported for S100 proteins

Protein	K_d (μM)	Reference	Method
S100B	0.094	Wilder et al. (2003)	Isothermal titration calorimetry
S100A2	0.025	Koch et al. (2007)	Competition with zinc chelator (PAR_2)
S100A3	0.004	Fritz et al. (2002)	Atomic absorption spectroscopy
S100A5	1-3	Schafer et al. (2000)	Equilibrium gel filtration
S100A6	0.1	Kordowska et al. (1998)	Fluorescence spectroscopy
S100A7	100	Vorum et al. (1996)	Equilibrium dialysis
S100A12	0.01–4.5	Dell'Angelica et al. (1994), Moroz et al. (2009b)	Fluorescence spectroscopy

PAR_2 4-(2-Pyridylazo)-resorcinol

Table 2 Zinc-enhanced S100-target interactions

Protein	Target	Reference
S100B	TRTK-12 peptide	Barber et al. (1999)
	Tau protein	Baudier and Cole (1988)
	AHNAK	Gentil et al. (2001)
	IQGAP1 protein	Mbele et al. (2002)
S100A1	Twitchin kinase	Heierhorst et al. (1996)
	Synapsin	Heierhorst et al. (1999)
S100A7	RAGE	Wolf et al. (2008)
S100A9	RAGE	Bjork et al. (2009)
S100A12	RAGE	Xie et al. (2007), Moroz et al. (2009b)
	Paramyosin	Moroz et al. (2009a)

for an increasing number of S100 proteins being regulated by zinc is accumulating fast, but the picture is far from complete, with much more functional and structural information needed to understand fully the role of zinc in these systems.

The aim of the present review is to summarise the available structural information and explain some of the mechanisms and likely consequences of zinc binding. Investigation of zinc regulation is of interest because for a number of S100 proteins, regulation by calcium seems unlikely, and indeed some have modifications in the EF-hands which render them incapable of binding calcium. In particular, none of the extracellular S100 protein-mediated events are likely to be calcium regulated, because the levels of calcium in the extracellular space are already high and the EF-hands would be saturated by calcium all the time. In addition, binding of intracellular targets by S100 proteins between calcium waves may also be regulated by zinc. We conclude that further investigation of zinc binding by S100 proteins not only as individuals, but considered as a whole group, will shed fresh light on S100 protein-mediated signalling.

The His-Zn S100 group

Zinc binding in S100A7

S100A7 was the first family member for which the structure of a zinc-bound form was determined (Brodersen et al. 1999). Analysing the effect of zinc binding was of particular importance, because S100A7 has unusual calcium-binding properties. First, its N-terminal EF-1 hand is distorted, and probably incapable of binding calcium. Second (unlike other family members), loss of calcium from its C-terminal EF-2 hand led to denaturation and is therefore unlikely to provide a reversible regulation mechanism (Brodersen et al. 1999). Since this strongly suggests that S100A7 is unlikely to be regulated by calcium, it is reasonable to suggest that, as it is known to bind zinc, it may instead be regulated by this metal.

Two structures of S100A7-zinc complexes revealed for the first time the architecture of the His-Zn binding site which lies at the subunit–subunit interface. In the first structure only one zinc site is occupied, in the second both have zinc bound. In both structures the zinc is coordinated by His17 and Asp24 from one subunit, and His86 and His90 from the other (Fig. 2a). The C-terminal ligands were precisely those predicted by several groups based on sequence alignments (Dell'Angelica et al. 1994; Marti et al. 1996). Although zinc binding did not bring about dramatic conformational changes, there were some significant differences from the zinc-free structure. The first was the conformation of the EF-1 calcium-binding loop in the "one-zinc" structure (PDB: 3psr). EF-1 became wider, and could in theory accommodate a calcium ion, implying the possibility of calcium binding by S100A7 being fine-tuned by changes in local zinc concentrations. In addition, the authors proposed that the contraction of the molecule in the region of the putative target-binding site may account for changes in affinity for its interaction partners.

S100A12: zinc-induced increase in affinity for calcium

The X-ray structure of zinc-bound S100A7 was followed by that of a copper-bound S100A12 (Moroz et al. 2003) (Fig. 2b). Copper has also been proposed as a regulator of S100 function (Nishikawa et al. 1997; Landriscina et al. 2001). In S100A12, the copper was coordinated by the same ligands as those predicted for zinc, and the binding site superimposed very well on that of Zn-S100A7. In both Zn-S100A7 and Cu-S100A12, as well as in Zn-S100B (Fig. 2c), determined later, calcium was bound to the EF-hands (excluding the atypical EF-1 hand of S100A7). Although biochemical data indicated that zinc binding leads to an increase in the affinity for calcium in S100B and S100A12, no significant differences in the conformations of the calcium-binding EF-hands in zinc-free and zinc (or copper)-bound states were seen in the 3D structures, and the question as to how zinc influenced the calcium affinity remained open.

An answer was suggested by the structure of zinc-bound S100A12 in the absence of calcium (Moroz et al. 2009a). As expected, this structure revealed the same zinc-binding site as in zinc-bound S100A7 and S100B (Fig. 2d); however, the overall fold of the protein was strikingly different from both the calcium-bound and apo-structures (Fig. 3a). The dimerisation core of the protein adopted the calcium-bound conformation, while αIII, which undergoes the most significant changes upon calcium binding, was found to be

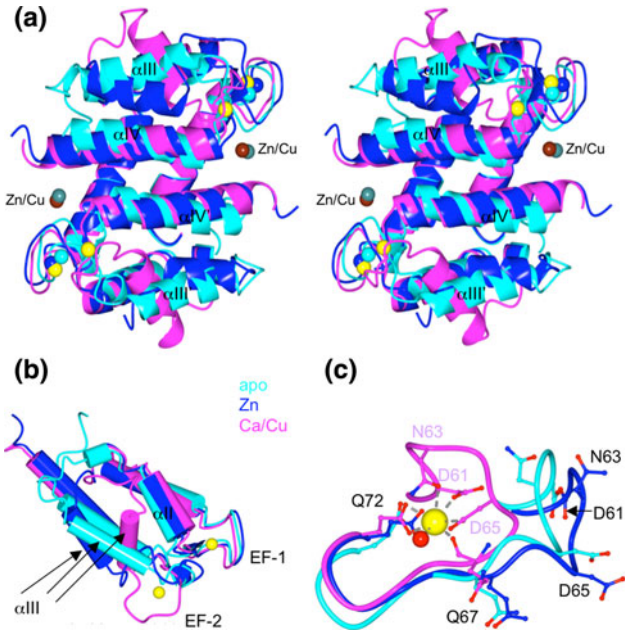

Fig. 3 The conformational changes in S100A12 seen upon metal-binding. **a** Stereo view of superimposed dimers of the apo (PDB code 2wce, *cyan*), zinc (2wc8, *blue*), and calcium–copper (1odb, *magenta*) S100A12. The helices αIII and αIV from two subunits of the dimer are labelled and distinguished by apostrophes. Calciums are shown in *yellow*, sodiums from apo and zinc-only structures are in *cyan and blue*, correspondingly. **b** Movement of αIII upon zinc, and then calcium binding. Same superposition as for **a**, shown for one subunit of S100A12. Colours are the same as for **a**, helices are represented as cylinders. Calciums from calcium–copper structure are shown in *yellow*, to indicate better the location of the EF-hands. **c** Close-up of EF-2, *colours* are the same as for subfigures **a** and **b**

in an intermediate position between the apo and calcium-bound states (Fig. 3b). Moreover, structures of Zn-S100A12 were determined in two crystal forms, with six independent protomers in total, in one of which the conformation of αIII was very close to that in the calcium-bound state. Taken together, the different positions of the αIII helices were proposed to represent different stages of the transition from the apo to metal-bound state. Another important feature of Zn-S100A12 was the restructuring of EF-2, whose structure was significantly different from that in the apo form, and was very flexible with high temperature factors and poorly defined electron density (totally disordered for two of the six subunits). Conformational changes in αIV also contribute to the transition of EF-2 to the calcium-ready state, with the calcium-binding Q72 superimposing on Q72 from the calcium-bound form (Fig. 3c). Together with the movement of αIII this suggests that zinc binding induces EF-2 to take up the calcium-binding conformation.

Zinc-induced increase in calcium affinity was also reported for S100B [although at a lower level, tenfold (Baudier et al. 1984)]. This is probably the result of conformational changes in S100B similar to those in S100A12. A structure of S100B with zinc and no calcium is needed to confirm this hypothesis. An equivalent mechanism is likely to apply to the other proteins from the His-Zn subgroup.

S100B: insights as to how zinc may modulate target affinity

Several structures of zinc/calcium-bound S100B have recently been deposited in the PDB. One is a complex with the drug pentamidine (Pnt; Charpentier et al. 2008) that had been shown to prevent S100B-p53 complex formation and to inhibit growth of malignant melanoma cells (Markowitz et al. 2004). ITC and fluorimetry did not reveal any detectable differences in the affinity of calcium-bound S100B for Pnt in the presence or absence of zinc (Charpentier et al. 2008), but some interesting differences can be seen in the structures of the zinc-free and zinc-bound complexes (PDB codes 3cr4 and 3cr5), Fig. 4. In both structures two Pnt molecules are located between adjacent S100B dimers leading to a continuous superstructure of linked dimers through the crystal. However, the conformations and positions of Pnt are quite different in the two structures, with the C-terminus moving to ligate the zinc, and creating a new space into which the Pnt can move. In addition, the Pnt molecules have two alternate conformations with ~half occupancies in both structures, but for the zinc-bound form the alternate conformations are much closer to one another than in the calcium-only. This reflects substantial changes in the protein conformation around the Pnt sites between the two forms, with the Pnt being confined between a set of large hydrophobic residues in the zinc form. A close-up of the target-binding sites in the two forms illustrates this (Fig. 4c, d). Although the Pnt-binding affinity to this more rigid zinc-induced site is the same as for the more flexible, calcium-only state, since the ligand itself is very flexible, the better ordered pocket may be important for binding the real biological target of S100B. This is consistent with zinc increasing the specificity in target recognition.

The proximity of symmetry-related molecules raises the question as to whether the Pnt-linked dimers form a biologically significant tetramer. The former receives some support from estimates of buried surface area [1,680A^2, calculated using Areaimol, (Collaborative Computational Project 4: 1994)]. The hypothetical tetramer interface is quite different from those reported earlier for the X-ray structure of the octameric assembly of S100B (Ostendorp et al. 2007). Formation of the Pnt-induced tetramer could provide an additional explanation for its inhibitory effect; the Pnt-bridged tetramers preventing the formation of biologically active octamers. However, it cannot be excluded that this is an artefact of crystallisation.

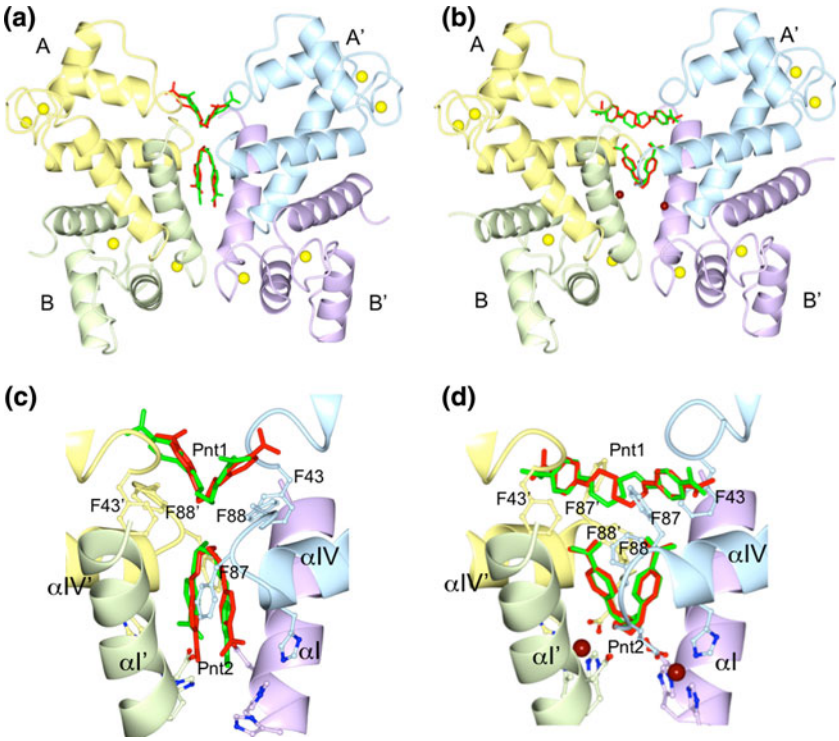

Fig. 4 The influence of zinc on target-binding affinity of S100B. Pnt molecules are bound to the S100B tetramer, with two conformations shown in *green* and *red*. Subunits of one S100B dimer are shown in *pale yellow* and *green*, subunits of the second dimer are in *light blue* and *violet*. Calcium ions are in *yellow* and zinc ions in *brown*. **a** Two adjacent symmetry-related S100B dimers in the absence of zinc (PDB code 3cr4). **b** Same dimers in the presence of zinc (3cr5). **c** Close-up, in the absence of zinc. Residues involved in the formation of Pnt-binding pocket, as well as zinc-binding residues are shown in *ball-and-stick* here and in **d**. **d** Close-up in the presence of zinc. Alternate conformations for both Pnt molecules shown in *red* and *green* are closer to each other, than in **c**. Phenylalanines Phe43, Phe87 and Phe88 from adjacent dimers form a well-defined binding pocket for Pnt-1

In addition, three recently released PDB structures of S100B (PDB codes 3czt, 3doy, 3d10) demonstrate the possibility of rearrangement of the zinc site depending on pH. In one structure the C-terminal Glu90 is substituted by His91 which reflects a degree of flexibility of the zinc-binding site in the absence of bound targets (Gunter Fritz, personal communication).

S100A8/9, S100A15 and S100A6

Other potential His-Zn S100 proteins include S100A8, S100A9, the recently discovered S100A15, and possibly S100A6. Although no structures of these proteins in zinc-bound states have been reported to date, the predicted zinc sites of the S100A8/A9 heterocomplex in the absence of zinc (Fig. 5a, d) superimpose well on those of the zinc-bound S100A7, S100A12 and S100B. Interestingly, in the structure of homo-S100A9 the C-termini are disordered, but in the heterocomplex with S100A8, they become well-defined and two types of potential zinc sites per heterodimer were identified. The first had three histidines and an aspartate as in the other group members (Fig. 5a, d). The second site consists of only histidines and was reported to have a lower affinity for zinc (Korndorfer et al. 2007). The S100A8 homodimer has two His-only sites (Fig. 5b, e), which would lead to a lower affinity for zinc than does the heterodimer. These authors suggested that a homodimer of S100A9 would be unlikely to bind zinc, because of the flexibility of its C-terminus.

S100A6 has only three potential zinc-binding residues, two histidines and an asparagine, which does however superimpose reasonably well on those of the other His-Zn proteins (Fig. 5c, f), and it can be proposed that this would be one of the two zinc sites per subunit set proposed earlier (Kordowska et al. 1998). Another site includes Cys2 and could be involved in interdimer zinc binding, linking dimers into tetramers. Such a mode of zinc binding and zinc-induced oligomerisation is a feature of the Cys-Zn site group, discussed below.

No structures of S100A15 are available, however, this protein has an extremely high sequence similarity to S100A7 (Fig. 1), and can be expected to have a similar mode of zinc binding.

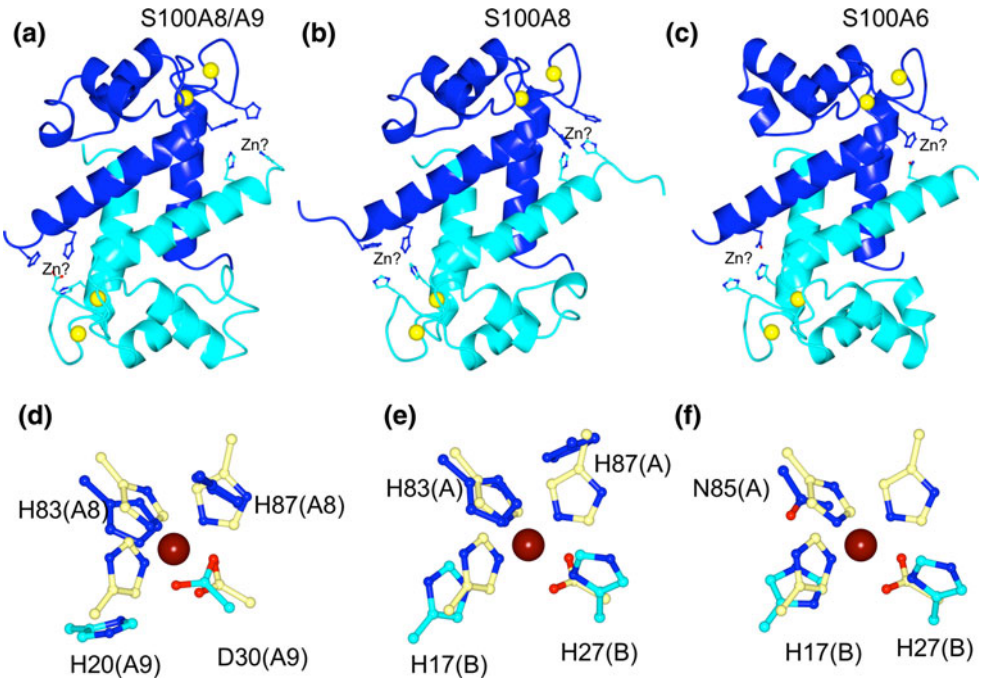

Fig. 5 Putative zinc-binding sites of **a** the S100A8/A9 heterodimer (PDB code 1xk4), **b** S100A8 (1mr8) and **c** S100A6 (1k96) homodimers. For all three subfigures one subunit of the dimer is shown in *blue* and the other in *cyan*, calcium ions are in *yellow*. **d–f** Close-ups of the putative zinc-binding sites of S100A8/A9, S100A8 and S100A6 superimposed on the zinc-binding site of S100A7. S100A7 residues are shown in *yellow*, zinc ion is shown in *brown*. Putative zinc-binding residues are indicated for S100A8/A9, S100A8 and S100A6 in **d**, **e**, **f** correspondingly

The Cys-Zn S100 subgroup

While no structures of zinc-bound Cys-Zn site S100 proteins have been deposited in the PDB to date, zinc binding has been reported for S100A1, S100A2, S100A3, S100A4, S100A5 and S100A6.

S100A1

Bovine S100A1 was the first member of the Cys-Zn site group to be identified and the first to be investigated with respect to zinc binding. It was shown by fluorescence studies that zinc caused conformational changes, different from those caused by calcium (Leung et al. 1987). Later the importance of zinc for S100A1 function was confirmed by studies on the interactions with twitchin kinase (Heierhorst et al. 1996) and synapsins (Heierhorst et al. 1999), which were enhanced by zinc.

S100A2

However, zinc binding has been best characterised for S100A2, for which two zinc-binding sites were proposed (Koch et al. 2007). One site, with a higher zinc affinity, includes Cys21 and possibly His17 and Gln22, and a second site of two (or four) Cys2 from adjacent dimers. Binding of zinc to this second site was proposed to lead to association of the dimers into tetramers. In contrast to the His-Zn proteins, zinc binding to S100A2 at the lower affinity Cys2 site causes a decrease in calcium affinity (Koch et al. 2007). Recent results (Botelho et al. 2009) on the influence of metal ions on folding and stability of S100A2 suggest an explanation with zinc binding leading to protein destabilisation which in its turn causes reduced calcium affinity. The authors proposed a similar mode of regulation for S100A3 and S100A4.

S100A3

S100A3 had been shown to lose 40% of its α-helical content in the presence of zinc, which also resulted in partial dissociation of the dimers (Fritz et al. 2002). An unusual feature of S100A3 is that it has a relatively low affinity for calcium (Fohr et al. 1995; Fritz et al. 1998) (4–30 mM) and a very high affinity for zinc (Fritz et al. 2002) (4 nM), and was therefore proposed to be mainly zinc-regulated. Analysis of the structure of apo S100A3 combined with biophysical/biochemical experiments allowed modelling of one of its zinc-binding sites, which was proposed to consist of His87, Cys83 and Cys86 from the C-terminus (Fritz et al. 2002).

S100A4

There is no direct evidence for zinc binding to S100A4; however, it was mentioned as unpublished results by Fritz and Koch (Botelho et al. 2009). Indeed, for human S100A4 it would be logical to assume that Cys2 could play a role similar to that reported for S100A2 and S100A6, forming

an interdimer zinc-binding site that links the dimers into tetramers.

S100A5

S100A5 was reported to bind two zincs per dimer, with no effect on calcium binding (Schäfer et al. 2000). In addition, it bound copper (like S100B, S100A12 and S100A13) with negative effect on calcium binding, and it was suggested that copper bound to some of the calcium-binding ligands. However, in the absence of independent structural information it appears more likely that copper binds to the same sites as zinc, and variations in the effect on calcium binding result from the differences in the bond lengths for copper and zinc coordination, as we observed in S100A12, where zinc induced formation of tetramers with a tighter interface than did copper (Moroz et al. 2009a).

S100A6

S100A6 is more similar to the His-Zn group than are other Cys-Zn group members. As mentioned above, it has two potential zinc-binding N-terminal histidines (although one of these is substituted by asparagine in three species: pig, horse and chick; Fig. 6). Five species, including human, have an N-terminal cysteine that could play a similar oligomerisation promotion role to the Cys2 from human S100A2.

In general, the Cys-Zn group of S100 proteins does not have a well-defined consensus zinc-binding motif such as that seen in the His-Zn group. The sequence alignment (Fig. 6), reveals the cysteines are not highly conserved, and sometimes even vary for the same protein between different organisms (for e.g., there is only a Cys2 close to the N-terminus in human S100A4, while there are cysteines at the C-termini only for mouse and rat. Human S100A2 has four cysteines but bovine protein only one (Fig. 6). However, one common feature can be observed; there is a histidine (usually His17), corresponding to the first histidine of the His-Zn group, only in S1003 is it replaced by asparagine. In addition, there is a high conservation of asparagine at the C-terminus, again at the position corresponding to first of the C-terminal histidines of the His-Zn group. In S100A3 this asparagine is substituted for histidine (while it has asparagine at the N-terminus instead of histidine). Interestingly, for those Cys-Zn group proteins where zinc-binding residues were proposed from biophysical/biochemical characterisation and/or modelling, the N-terminal His (for S100A2), or C-terminal His (for S100A3) were proposed as possible zinc ligands (Fritz et al. 2002; Koch et al. 2007). It is tempting to suggest that these His/Asn at the N- and C-termini contribute to zinc binding for the whole Cys-Zn group; making it somewhat similar to the His-Zn one. If this is correct, zinc binding can be proposed for S100A10, S100Z and possibly S100A11, based on sequence alignment (Fig. 6, shown only for human S100A10, S100A11 and S100Z, but true for all other species available from SWISSPROT, apart from zebra fish S100A11).

While asparagine is not a common zinc-binding residue, it has been observed in zinc binding, as can be seen from Metalloprotein Database analysis (PDB codes 1mdh, 1ush, 1kbp) (MDB, http://metallo.scripps.edu/) (Castagnetto et al. 2002). Asn22 was suggested as one of the zinc-binding residues for site 1 in S100A2 (Koch et al. 2007). In the same study, a contribution to zinc binding from three nitrogens was detected by spectroscopic studies of Co^{2+}-substituted S100A2 and its mutants, so the third N was thought to come from Tris; this could in fact come from Asn87. While in the structure of apo-S100A2 this asparagine is too far from the putative zinc-binding site to be a zinc ligand, structure superposition of S100A6 (Fig. 5, discussed earlier, or S100A4, not shown) on those of zinc-bound S100 proteins suggests that it could move to a similar position in calcium-bound S100A2.

We therefore propose that one of the Cys-Zn group zinc sites would have these histidine and asparagine as zinc ligands, complemented with cysteines specific for the individual proteins. Additional zinc sites, where present (like site 2 suggested for S100A2) might include N-terminal cysteines that would link dimers into tetramers. Three-dimensional structures are required to confirm or disprove this hypothesis.

Zinc-induced oligomerisation of S100 proteins

As described in the "Introduction", zinc has been shown to promote homo- or hetero-oligomerisation of several S100 proteins. No common oligomerisation mechanisms have been detected for the His-Zn group to date, and it is known that the mode of tetramer formation differs for S100B, S100A12 and calprotectin (S100A8/S100A9 heterodimer). In contrast, the predicted zinc-binding site between two N-terminal cysteines is common to several members of the Cys-Zn group. Oligomers have been shown to be functionally relevant for S100B (Ostendorp et al. 2007), S100A4 (Novitskaya et al. 2000; Kiryushko et al. 2006; Klingelhofer et al. 2007; Oslejskova et al. 2008), calprotectin (Vogl et al. 2006) and S100A12, although not all are dependent on zinc. For S100A12 binding of zinc induced tetramerisation in the absence and hexamerisation in the presence of calcium, while S100A12 hexamers were shown to be the active species for interaction with RAGE and possibly other cell surface receptors (Xie et al. 2007; Moroz et al. 2009b). Zinc-induced oligomerisation could provide an additional

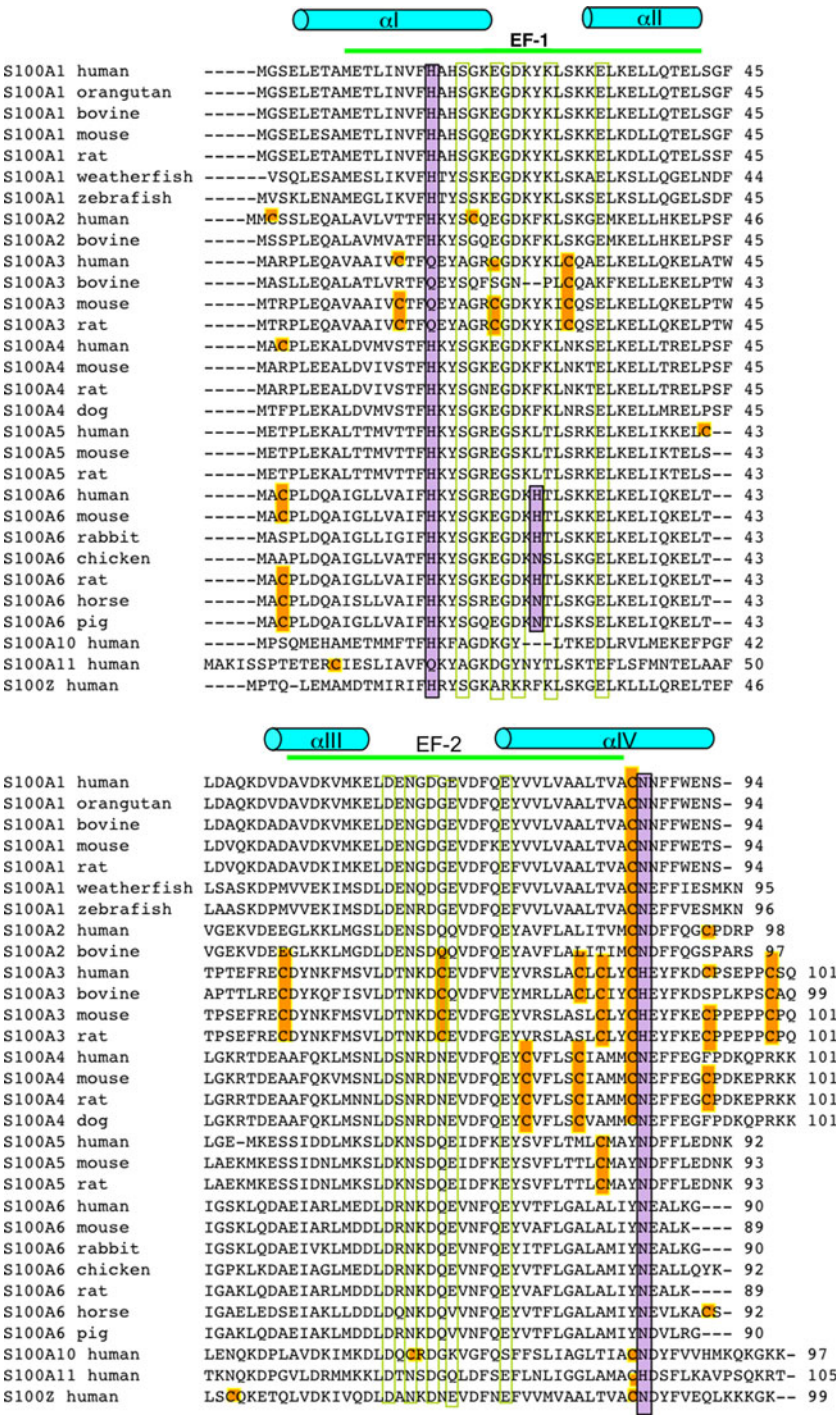

Fig. 6 Sequence alignment of the Cys-Zn group. Predicted non-cysteine zinc-binding residues are *outlined in magenta*, cysteines are *shaded in orange*. Calcium-binding residues from EF-1 and EF-2 are outlined with *green boxes*. Secondary structure elements shown above the alignment correspond to the structure of S100A12 in complex with calcium and copper

means of regulation of S100 proteins by zinc in addition to zinc-induced local conformational changes in their target-binding sites, and influence of zinc on calcium binding.

Conclusions

The evidence for zinc being involved in cell signalling pathways is increasing. Zinc-inhibited calcium influx is the result of binding to store-operated calcium channels (Gore et al. 2004). Increase of free zinc levels originating in the endoplasmic reticulum was detected following FcεRI-mediated mast cell activation and was termed a "zinc wave" (Yamasaki et al. 2007). Following this and other discoveries, reviewed in (Murakami and Hirano 2008), zinc has been suggested to play the role of a novel intracellular second messenger. Total cellular zinc concentration is in the hundred micromolar range; however, most zinc is

tightly bound leaving much lower "free zinc" levels. For eukaryotic cells there are different estimates of free zinc levels depending on the methods of measurement and cell type; from low picomolar (Bozym et al. 2006) to micromolar (Brand and Kleineke 1996), but mostly in the hundred picomolar range, reviewed in (Maret and Li 2009). However, even in cells with picomolar free zinc, buffering metallothioneines can release it in response to oxidative stress making it available to other zinc-binding proteins, even those that have nanomolar (or maybe even lower) zinc affinities (Krezel and Maret 2008; Maret 2009).

The situation is similar with extracellular zinc: although the concentrations of free extracellular zinc were traditionally considered insufficient (again in the picomolar range) to bind to proteins with lower zinc affinities, there is emerging evidence of the importance of exchangeable zinc. A recent study suggested that zinc released from human serum albumin under certain pathological conditions, e.g. when the level of fatty acids in the blood is increased, could bind to histidine-rich glycoprotein resulting in thrombotic disorders. It was shown by structure comparisons that binding of myristic acid led to two putative zinc-binding residues moving away from the suggested zinc site (Blindauer et al. 2009). Studies on hippocampal mossy fibre synapses suggested that zinc could act as a neurotransmitter. Zinc concentrations during synaptic release were estimated as 10–30 μM, which is another illustration of the dramatic fluctuations in local free zinc concentrations in the extracellular space (Vogt et al. 2000; Frederickson et al. 2005; Ketterman and Li 2008).

For S100 proteins the role of zinc in addition to calcium was described in the original studies on S100B and S100A1. Moreover, in some proteins there is regulation by zinc and not calcium, such as S100A3, which has a very high affinity for zinc, and such a low affinity for calcium that its binding seems physiologically irrelevant. Another interesting example is the zinc-promoted S100B interaction with tau protein, which is calcium independent (Yu and Fraser 2001). In addition, S100 proteins are unlikely to be regulated by binding calcium in the extracellular space because the EF-hands would be already saturated by calcium. Hence some could instead be regulated by such mechanisms as zinc-induced oligomerisation, or even zinc-induced calcium release, for those S100 proteins with negative effects of zinc on calcium affinity. The zinc-only structure of S100A12 revealed a conformation of the potential target-binding site different from those in the apo- and calcium-bound states (Moroz et al. 2009a). This could enable S100A12 to bind intracellular targets that would be released by a subsequent conformational change upon calcium binding. A similar mode of intracellular regulation could exist for other S100 proteins.

S100 proteins have been termed evolutionary newcomers (Schaub and Heizmann 2008). Within a relatively short time span they have diverged to exert very different functions in diverse environments depending on the tissue in which they are expressed. Striking examples are S100A7 and S100A15, which differ only in three residues, but interact with different targets, RAGE for A7 and G protein coupled receptor for A15 (Wolf et al. 2008). Until recently only mammalian S100 proteins were identified and investigated; however, recent studies revealed S100 family members in teleosts (bony fish), with direct orthologues for S100A1, S100A10, S100A11, S100B, S100P and S100Z (Ravasi et al. 2004; Kraemer et al. 2008). Sequence alignment of the S100 proteins from the Cys-Zn group shows that S100A1 from zebra fish and loach already has His17 on αI and Asn90 on αIV close to the C-terminus. If these residues do indeed contribute to zinc binding, this would imply that the ancient architecture, at least for a group of S100 proteins was already adapted for this additional, zinc-related fine-tuning, allowing cross talk not only between two EF-hands with a completely different mode of calcium binding, but with the zinc-binding site in the close vicinity of the EF-hands. Because most S100 proteins have zinc and calcium sites that can affect each other, they are likely candidates as members of a group of "zinc-sensing receptors"; a term first suggested after the discovery that extracellular zinc could mobilise intracellular calcium in colon cancer cells HL-29 (Hershfinkel et al. 2001). Such as yet undiscovered receptors were proposed to translate zinc signals into calcium ones, or vice versa. As described above, for several S100 proteins binding of zinc leads to an increase in the affinity to calcium and/or to their targets. In contrast, it was suggested that binding of zinc to S100A2 under conditions of oxidative stress lead to decreased calcium affinity and protein destabilisation which would prevent its interaction with p53 and thus disrupt cell cycle regulation (Botelho et al. 2009).

To summarise, a more general view on zinc binding to S100 family members would provide a better understanding of their function. Some members appear most unlikely to be regulated by calcium since they lack the appropriate amino acid content and/or architecture to coordinate the calcium ion in one, or both, of the EF-hands. Moreover, in the extracellular space regulation by calcium is improbable for any S100 protein, at least not by binding of calcium to the EF-hands, because the concentration of calcium is already high. Therefore, further investigation of a range of zinc binding S100 family members and the role of zinc–calcium crosstalk in their function will shed more light on common features and differences as to how they propagate their signals. In practical terms, this will provide valuable information relevant to the treatment of numerous S100-related pathologies.

Acknowledgments This work was supported by European Commission funding through the SPINE2–COMPLEXES project LSHG–CT–2006–031220. We thank Alexei Murzin for the useful discussions of oligomerisation interfaces.

References

Barber KR, McClintock KA, Jamieson GA Jr, Dimlich RV, Shaw GS (1999) Specificity and Zn^{2+} enhancement of the S100B binding epitope TRTK-12. J Biol Chem 274:1502–1508

Baudier J, Cole RD (1988) Interactions between the microtubule-associated tau proteins and S100b regulate tau phosphorylation by the Ca^{2+}/calmodulin-dependent protein kinase II. J Biol Chem 263:5876–5883

Baudier J, Holtzscherer C, Gerard D (1982) Zinc-dependent affinity chromatography of the S100b protein on phenyl-Sepharose. A rapid purification method. FEBS Lett 148:231–234

Baudier J, Glasser N, Haglid K, Gerard D (1984) Purification, characterization and ion binding properties of human brain S100b protein. Biochim Biophys Acta 790:164–173

Baudier J, Glasser N, Gerard D (1986) Ions binding to S100 proteins. I. Calcium- and zinc-binding properties of bovine brain S100 alpha alpha, S100a (alpha beta), and S100b (beta beta) protein: Zn^{2+} regulates Ca^{2+} binding on S100b protein. J Biol Chem 261:8192–8203

Bhattacharya S, Bunick CG, Chazin WJ (2004) Target selectivity in EF-hand calcium binding proteins. Biochim Biophys Acta 1742:69–79

Bjork P et al (2009) Identification of human S100A9 as a novel target for treatment of autoimmune disease via binding to quinoline-3-carboxamides. PLoS Biol 7:e97

Blindauer CA et al (2009) Structure, properties, and engineering of the major zinc binding site on human albumin. J Biol Chem 284:23116–23124

Botelho HM, Koch M, Fritz G, Gomes CM (2009) Metal ions modulate the folding and stability of the tumor suppressor protein S100A2. FEBS J 276:1776–1786

Bozym RA, Thompson RB, Stoddard AK, Fierke CA (2006) Measuring picomolar intracellular exchangeable zinc in PC-12 cells using a ratiometric fluorescence biosensor. ACS Chem Biol 1:103–111

Brand IA, Kleineke J (1996) Intracellular zinc movement and its effect on the carbohydrate metabolism of isolated rat hepatocytes. J Biol Chem 271:1941–1949

Brodersen DE, Nyborg J, Kjeldgaard M (1999) Zinc-binding site of an S100 protein revealed. Two crystal structures of Ca^{2+}-bound human psoriasin (S100A7) in the Zn^{2+}-loaded and Zn^{2+}-free states. Biochemistry 38:1695–1704

Castagnetto JM, Hennessy SW, Roberts VA, Getzoff ED, Tainer JA, Pique ME (2002) MDB: the metalloprotein database and browser at The Scripps Research Institute. Nucleic Acids Res 30:379–382

Charpentier TH et al (2008) Divalent metal ion complexes of S100B in the absence and presence of pentamidine. J Mol Biol 382:56–73

Collaborative Computational Project 4 (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr D Biol Crystallogr 50:760–763

Dell'Angelica EC, Schleicher CH, Santome JA (1994) Primary structure and binding properties of calgranulin C, a novel S100-like calcium-binding protein from pig granulocytes. J Biol Chem 269:28929–28936

Deloulme JC, Assard N, Mbele GO, Mangin C, Kuwano R, Baudier J (2000) S100A6 and S100A11 are specific targets of the calcium- and zinc-binding S100B protein in vivo. J Biol Chem 275:35302–35310

Fohr UG, Heizmann CW, Engelkamp D, Schafer BW, Cox JA (1995) Purification and cation binding properties of the recombinant human S100 calcium-binding protein A3, an EF-hand motif protein with high affinity for zinc. J Biol Chem 270:21056–21061

Frederickson CJ, Koh JY, Bush AI (2005) The neurobiology of zinc in health and disease. Nat Rev Neurosci 6:449–462

Fritz G, Heizmann CW, Kroneck PM (1998) Probing the structure of the human Ca^{2+}- and Zn^{2+}-binding protein S100A3: spectroscopic investigations of its transition metal ion complexes, and three-dimensional structural model. Biochim Biophys Acta 1448:264–276

Fritz G, Mittl PR, Vasak M, Grutter MG, Heizmann CW (2002) The crystal structure of metal-free human EF-hand protein S100A3 at 1.7-A resolution. J Biol Chem 277:33092–33098

Gentil BJ et al (2001) The giant protein AHNAK is a specific target for the calcium- and zinc-binding S100B protein: potential implications for Ca^{2+} homeostasis regulation by S100B. J Biol Chem 276:23253–23261

Gore A, Moran A, Hershfinkel M, Sekler I (2004) Inhibitory mechanism of store-operated Ca^{2+} channels by zinc. J Biol Chem 279:11106–11111

Heierhorst J et al (1996) Ca^{2+}/S100 regulation of giant protein kinases. Nature 380:636–639

Heierhorst J et al (1999) Synapsins as major neuronal Ca^{2+}/S100A1-interacting proteins. Biochem J 344(Pt 2):577–583

Hershfinkel M, Moran A, Grossman N, Sekler I (2001) A zinc-sensing receptor triggers the release of intracellular Ca^{2+} and regulates ion transport. Proc Natl Acad Sci USA 98:11749–11754

Ketterman JK, Li YV (2008) Presynaptic evidence for zinc release at the mossy fiber synapse of rat hippocampus. J Neurosci Res 86:422–434

Kiryushko D et al (2006) Molecular mechanisms of $Ca^{(2+)}$ signaling in neurons induced by the S100A4 protein. Mol Cell Biol 26:3625–3638

Klingelhofer J et al (2007) Up-regulation of metastasis-promoting S100A4 (Mts-1) in rheumatoid arthritis: putative involvement in the pathogenesis of rheumatoid arthritis. Arthritis Rheum 56:779–789

Koch M et al (2007) Implications on zinc binding to S100A2. Biochim Biophys Acta 1773:457–470

Kordowska J, Stafford WF, Wang CL (1998) Ca^{2+} and Zn^{2+} bind to different sites and induce different conformational changes in human calcyclin. Eur J Biochem 253:57–66

Korndorfer IP, Brueckner F, Skerra A (2007) The crystal structure of the human (S100A8/S100A9)2 heterotetramer, calprotectin, illustrates how conformational changes of interacting alpha-helices can determine specific association of two EF-hand proteins. J Mol Biol 370:887–898

Kraemer AM, Saraiva LR, Korsching SI (2008) Structural and functional diversification in the teleost S100 family of calcium-binding proteins. BMC Evol Biol 8:48

Krezel A, Maret W (2008) Thionein/metallothionein control Zn(II) availability and the activity of enzymes. J Biol Inorg Chem 13:401–409

Landriscina M et al (2001) Copper induces the assembly of a multiprotein aggregate implicated in the release of fibroblast growth factor 1 in response to stress. J Biol Chem 276:25549–25557

Leung IK, Mani RS, Kay CM (1987) Fluorescence studies on the Ca^{2+} and Zn^{2+} binding properties of the alpha-subunit of bovine brain S-100a protein. FEBS Lett 214:35–40

Maler L, Sastry M, Chazin WJ (2002) A structural basis for S100 protein specificity derived from comparative analysis of apo and $Ca^{(2+)}$-calcyclin. J Mol Biol 317:279–290

Marenholz I, Heizmann CW, Fritz G (2004) S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophys Res Commun 322:1111–1122

Marenholz I, Lovering RC, Heizmann CW (2006) An update of the S100 nomenclature. Biochim Biophys Acta 1763:1282–1283

Maret W (2009) Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals. Biometals 22:149–157

Maret W, Li Y (2009) Coordination dynamics of zinc in proteins. Chem Rev 109:4684–4707

Markowitz J et al (2004) Identification and characterization of small molecule inhibitors of the calcium-dependent S100B-p53 tumor suppressor interaction. J Med Chem 47:5085–5093

Marti T, Erttmann KD, Gallin MY (1996) Host-parasite interaction in human onchocerciasis: identification and sequence analysis of a novel human calgranulin. Biochem Biophys Res Commun 221:454–458

Mbele GO et al (2002) The zinc- and calcium-binding S100B interacts and co-localizes with IQGAP1 during dynamic rearrangement of cell membranes. J Biol Chem 277:49998–50007

Moroz OV et al (2003) Structure of the human S100A12-copper complex: implications for host-parasite defence. Acta Crystallogr D Biol Crystallogr 59:859–867

Moroz OV, Blagova EV, Wilkinson AJ, Wilson KS, Bronstein IB (2009a) The crystal structures of human S100A12 in apo form and in complex with zinc: new insights into S100A12 oligomerisation. J Mol Biol 391:536–551

Moroz OV et al (2009b) Both Ca^{2+} and Zn^{2+} are essential for S100A12 protein oligomerization and function. BMC Biochem 10:11

Murakami M, Hirano T (2008) Intracellular zinc homeostasis and zinc signaling. Cancer Sci 99:1515–1522

Nakatani Y, Yamazaki M, Chazin WJ, Yui S (2005) Regulation of S100A8/A9 (calprotectin) binding to tumor cells by zinc ion and its implication for apoptosis-inducing activity. Mediators Inflamm 2005:280–292

Nishikawa T, Lee IS, Shiraishi N, Ishikawa T, Ohta Y, Nishikimi M (1997) Identification of S100b protein as copper-binding protein and its suppression of copper-induced cell damage. J Biol Chem 272:23037–23041

Novitskaya V et al (2000) Oligomeric forms of the metastasis-related Mts1 (S100A4) protein stimulate neuronal differentiation in cultures of rat hippocampal neurons. J Biol Chem 275:41278–41286

Oslejskova L, Grigorian M, Gay S, Neidhart M, Senolt L (2008) The metastasis associated protein S100A4: a potential novel link to inflammation and consequent aggressive behaviour of rheumatoid arthritis synovial fibroblasts. Ann Rheum Dis 67:1499–1504

Ostendorp T et al (2007) Structural and functional insights into RAGE activation by multimeric S100B. EMBO J 26:3868–3878

Otterbein LR, Kordowska J, Witte-Hoffmann C, Wang CL, Dominguez R (2002) Crystal structures of S100A6 in the $Ca^{(2+)}$-free and $Ca^{(2+)}$-bound states: the calcium sensor mechanism of S100 proteins revealed at atomic resolution. Structure 10:557–567

Potterton L et al (2004) Developments in the CCP4 molecular-graphics project. Acta Crystallogr D Biol Crystallogr 60:2288–2294

Ravasi T et al (2004) Probing the S100 protein family through genomic and functional analysis. Genomics 84:10–22

Santamaria-Kisiel L, Rintala-Dempsey AC, Shaw GS (2006) Calcium-dependent and -independent interactions of the S100 protein family. Biochem J 396:201–214

Schäfer BW et al (2000) Brain S100A5 is a novel calcium-, zinc-, and copper ion-binding protein of the EF-hand superfamily. J Biol Chem 275:30623–30630

Schaub MC, Heizmann CW (2008) Calcium, troponin, calmodulin, S100 proteins: from myocardial basics to new therapeutic strategies. Biochem Biophys Res Commun 369:247–264

Taccioli C et al (2009) Zinc replenishment reverses overexpression of the proinflammatory mediator S100A8 and esophageal preneoplasia in the rat. Gastroenterology 136:953–966

Vogl T, Leukert N, Barczyk K, Strupat K, Roth J (2006) Biophysical characterization of S100A8 and S100A9 in the absence and presence of bivalent cations. Biochim Biophys Acta 1763:1298–1306

Vogt K, Mellor J, Tong G, Nicoll R (2000) The actions of synaptically released zinc at hippocampal mossy fiber synapses. Neuron 26:187–196

Vorum H et al (1996) Expression and divalent cation binding properties of the novel chemotactic inflammatory protein psoriasin. Electrophoresis 17:1787–1796

Wilder PT, Baldisseri DM, Udan R, Vallely KM, Weber DJ (2003) Location of the $Zn^{(2+)}$-binding site on S100B as determined by NMR spectroscopy and site-directed mutagenesis. Biochemistry 42:13410–13421

Wolf R et al (2008) Chemotactic activity of S100A7 (Psoriasin) is mediated by the receptor for advanced glycation end products and potentiates inflammation with highly homologous but functionally distinct S100A15. J Immunol 181:1499–1506

Xie J, Burz DS, He W, Bronstein IB, Lednev I, Shekhtman A (2007) Hexameric calgranulin C (S100A12) binds to the receptor for advanced glycated end products (RAGE) using symmetric hydrophobic target-binding patches. J Biol Chem 282:4218–4231

Yamasaki S et al (2007) Zinc is a novel intracellular second messenger. J Cell Biol 177:637–645

Yanamandra K et al (2009) Amyloid formation by the pro-inflammatory S100A8/A9 proteins in the ageing prostate. PLoS One 4:e5562

Yousefi R, Imani M, Ardestani SK, Saboury AA, Gheibi N, Ranjbar B (2007) Human calprotectin: effect of calcium and zinc on its secondary and tertiary structures, and role of pH in its thermal stability. Acta Biochim Biophys Sin (Shanghai) 39:795–802

Yu WH, Fraser PE (2001) S100beta interaction with tau is promoted by zinc and inhibited by hyperphosphorylation in Alzheimer's disease. J Neurosci 21:2240–2246