Mapping the Zinc Ligands of S100A2 by Site-directed Mutagenesis*

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S100 family proteins are characterized by short individual N and C termini and a conserved central part, harboring two Ca²⁺-binding EF-hands, one of them highly conserved among EF-hand family proteins and the other characteristic for S100 proteins. In addition to Ca²⁺, several members of the S100 protein family, including S100A2, bind Zn²⁺. Two regions in the amino acid sequences of S100 proteins, namely the helices of the N-terminal EF-hand motif and the very C-terminal loop are believed to be involved in Zn²⁺-binding due to the presence of histidine and/or cysteine residues. Human S100A2 contains four cysteine residues, each of them located at positions that may be important for Zn²⁺ binding. We have now constructed and purified 10 cysteine-deficient mutants of human S100A2 by site-directed mutagenesis and investigated the contribution of the individual cysteine residues to Zn²⁺ binding. Here we show that Cys¹¹³ (the number in parentheses indicating the position in the sequence of S100A2) is the crucial determinant for Zn²⁺ binding in association with conformational changes as determined by internal tyrosine fluorescence. Solid phase Zn²⁺-binding assays also revealed that the C-terminal residues Cys⁴⁸⁷ and Cys⁴⁹⁴ mediated a second type of Zn²⁺ binding, not associated with detectable conformational changes in the molecule. Cys²²², by contrast, which is located within the first EF hand motif affected neither Ca²⁺ nor Zn²⁺ binding, and a Cys “null” mutant was entirely incapable of ligating Zn²⁺. These results provide new information about the mechanism and the site(s) of zinc binding in S100A2.

The S100 family of small cation-binding proteins currently comprises 16 closely related members and two proteins containing the S100 sequence as part of a fusion protein. These and 13 of the S100 genes are clustered in the epidermal differentiation complex located on human chromosome 1 (1). The Ca²⁺-binding sites are well defined as two EF-hands. The C-terminal EF-hand follows the canonical consensus of the common EF hand motif, while the N-terminal one is S100-specific (2, 3). Although Zn²⁺-binding has been demonstrated unequivocally for several S100 proteins, including S100A2 (4), the region(s) involved in this interaction have not yet been identified (5–9). In particular, molecular characterization has been hampered by the absence of a conserved cysteine- or histidine-containing Zinc-finger-like motif (10). Recently, Brodersen and colleagues reported the crystal structures of the human S100A7 (psoriasin) dimer, both in the Zn²⁺-loaded and the free form and determined two zinc binding sites per dimer (11). From their data, these authors further delineated two regions, one located in the first, variable EF-hand of one monomer and the second residing in the C terminus of the other monomer, which could function as potential ligands for zinc ions. Ca²⁺ and Zn²⁺ binding for wild type S100A2 and for mutants in which the EF-hands were substituted for parvalbumin EF-hands have been studied previously (4). However, the detailed mechanism of Zn²⁺ binding remained elusive.

Human S100A2 protein has attracted particular interest based on its potential function as a tumor suppressor-related gene (12). This hypothesis is based on the observations that S100A2 expression is down-regulated in breast cancer (13, 14) and that the S100A2 promoter can be activated by p53 (15). In addition, localization studies have shown that a substantial fraction of the S100A2 protein is present in the nucleus in interphase cells (16–18).

Human S100A2 harbors four cysteines, located at strategically interesting sites, that may be involved in Zn²⁺-binding. One cysteine residue, Cys¹¹³ (the number in parentheses indicating the position in the sequence of S100A2) is located at the very N terminus of the molecule. A cysteine at the same position has been proposed as a potential Zn²⁺ ligand in calcycin (S100A6) by Kordowska et al. (9). The second cysteine, Cys²²² resides within the first EF-hand. The first EF-hand of S100 proteins has only low affinity for Cu²⁺, is 2 amino acid residues longer than canonical EF-hands and, in the present case, contains a cysteine flanked by a highly conserved histidine residue. Moreover, the corresponding site in S100A7 seems to be involved in Zn²⁺-binding (11). The third and fourth cysteine in human S100A2, Cys³⁸⁷ and Cys⁴⁹⁴, are situated in the C terminus adjacent to the penultimate helix-loop-helix motif. In this region, histidine and cysteine residues are found in most human S100 proteins. Based on theoretical models (19), NMR structures of the apo-form of related S100 family proteins, and sequence alignments (10), these residues have been hypothesized to be ligands for Zn²⁺.

In this study, we map the amino acids that can act as ligands for the zinc ion in S100A2 by site-directed mutagenesis and correlate the effects of the absence or presence of a single zinc-ligating amino acid with the binding characteristics of the wild type molecule. Serial substitutions of each of the four cysteines to serine residues did not alter the Cu²⁺-binding properties of human S100A2 but induced significant changes in the Zn²⁺ binding characteristics of the protein. In the wild type protein, Zn²⁺ binding induced conformational changes in the protein. In addition, the affinity for Zn²⁺ were raised 5-fold in the presence of Cu²⁺. In the absence of a conserved sequence motif that would provide all of the four necessary ligand amino acids for the Zn²⁺ ion, we hypothesize an involvement of the
Entire intact dimer to contribute to the Zn$^{2+}$ binding activity of S100A2.

Electrophoresis and Western Blotting—Analytical SDS gel electrophoresis on 10–26% gradient polyacrylamide mini slab gels and Western blotting onto nitrocellulose (Hybond; Amersham Pharmacia Biotech) were performed essentially as described elsewhere (20). Transferred proteins were detected by incubation with a monoclonal anti-S100A2 antibody (SH-L1; Sigma) and visualized using horseradish peroxidase-coupled secondary antibodies and the ECL chemiluminescence system (Amersham Pharmacia Biotech).

Cloning of Wild Type and Mutant S100A2—S100A2 and cysteine-deficient mutants were cloned from a human A431 DNA clone (ATCC, cat. no. Z37914) into the NdeI and EcoRI sites of the bacterial expression vector pMW 172 (21). A set of eight primers was used, allowing every possible combination of substituting one or more cysteines for serine by site-directed mutagenesis. The primers were as follows: 1, 5′-GGAGAACAGACATATGTTGAGTGCTTCTTG-3′; 2, 5′-GGAGAGAGACATGTTGAGTGCTTCTTG-3′; 3, 5′-CTACATCCACGAAATCTCTCCATCCGCAGG-3′; 4, 5′-CTGGTACTGCTTCAACCTCTTGGGAAGAAGAAGAGACAT-3′; 5′-GGAGGATCCATTGGGTGTTGGAGGAGGAGACAT-3′; 6, 5′-GGAGGATCCATTGGGTGCTTGCGGAGGAGGAGACAT-3′; 7, 5′-GGAGGATCCATTGGGTGTTGGAGGAGGAGACAT-3′; 8, 5′-GGAGAAGTTCCAGGGTGCTTGCGGAGGAGGAGACAT-3′. All mutants in which the second cysteine (Cys 154) was substituted were cloned as follows. The N-terminal part and an overlapping C-terminal part were amplified and then combined in a third step using the corresponding end primers. The clones used for expression of the recombinant proteins were confirmed by nucleotide sequencing using a Li-Cor 400 automated sequencer (MWG Biotech) and selected for a maximum of expression by SDS-gel electrophoresis.

Purification of Recombinant S100A2 Mutants—Recombinant proteins were expressed in the Escherichia coli host strain BL21. Purification was carried out according to a modified protocol by Becker et al. (22). Bacteria were grown in LB medium containing 100 μM ampicillin and induced at logarithmic growth by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. After growth for an additional 3 h, bacteria were harvested by centrifugation in a Sorvall GS-3 rotor at 20,000 × g for 30 min at 4 °C. The supernatant was dialyzed against buffer C1. Proteins were applied onto a Phenyl-Sepharose column (120 ml) in buffer C1.

Gel filtration column (120 ml) in buffer C1. The molecular mass of the complexes in solution was determined in the presence or absence of Ca$^{2+}$, Zn$^{2+}$, or EDTA concentrations. The maximum change in fluorescence at 315 nm was plotted as a function of the cation concentration against the fluorescence emission at 315 nm. Before the titrations, recombinant S100A2 and mutant proteins were reduced by an overnight incubation with 100 mM dithioerythritol in buffer C1 at room temperature followed by gel filtration on a PD10 column (Amersham Pharmacia Biotech) with dithioerythritol.

The volume of free sulphhydryls was determined by measurement of the absorbance at 412 nm after a 15-min incubation with 1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). During the reaction with free sulphhydryl residues, an equivalent amount of 5-nitro-2-benzoxo anions is released, which can be monitored by titrating the absorption at 412 nm. The extinction coefficient of the reaction product is 13,600 OD/M g/mol/1 mm × 1 mg/ml. Data were processed as described elsewhere (4).

Solid Phase 65Zn Binding Assays—2–10 μg of the recombinant human S100A2 wild type or mutants diluted in 20 μl of buffer C1 was spotted onto nitrocellulose membrane (PALL, Linz, Austria), employing a vacuum dot blot apparatus (Bio-Rad). After 20 min of incubation, the blot was washed twice in buffer C1 while still in the apparatus and subsequently transferred to a Petri dish and equilibrated in metal binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl). The membrane was then cut into strips (11 × 0.7 cm) containing 12 spots corresponding to the different mutant proteins (or protein concentrations) and incubated for 20 min in 2 ml of metal binding buffer containing varying ZnCl$^2$ concentrations traced with $^{65}$ZnCl$^2$. After incubation, the blot was washed twice in metal binding buffer containing no metal ions and exposed to an x-ray film (Eastman Kodak Co.) overnight.

Cell Fractionation Experiments—Cells (porcine LLC-PK1 or human A431) were grown to 90% confluence in 10-cm tissue culture plate Petri dishes (Falcon). After 25 h of culture, the monolayers were washed with a linear gradient from 20 mM to 1 mM NaCl. The fractions containing recombinant S100A2 were pooled, dialyzed against buffer C1 containing 2 mM Ca$^{2+}$, and applied onto a 10-ml phenyl-Sepharose column (Amersham Pharmacia Biotech). After elution of the recombinant protein with 4 mM EGTA, the protein was >90% pure. For further purification, the protein was applied to an Amersham Pharmacia Biotech S100-HR gel filtration column (120 ml) in buffer C1.

Mass Spectrometry—Mass spectra of wild type S100A2 or mutant proteins were generated on a Perkin-Elmer SCIEX API 365 LC/MS/MS electrospray ionization mass spectrometer (Perkin-Elmer). The protein samples were generated on a PE SCIEX API 365 LC/MS/MS electrospray ionization mass spectrometer (Perkin-Elmer). The protein samples were generated on a PE SCIEX API 365 LC/MS/MS electrospray ionization mass spectrometer (Perkin-Elmer). The protein samples were generated on a PE SCIEX API 365 LC/MS/MS electrospray ionization mass spectrometer (Perkin-Elmer).

RESULTS

Biophysical Characterization of Wild Type S100A2

Recombinant S100A2 was purified to homogeneity (Fig. 1A) as described under "Materials and Methods." Similar to other members of this family S100A2 formed dimers both in the presence and absence of metal ions, as shown by analytical ultracentrifugation (Fig. 1B). The conformational changes taking place during Ca$^{2+}$ binding resulted in a significant change of the tyrosine fluorescence, presumably due to the exposition of a hydrophobic protein interface (Fig. 1C). The half-maximal saturation for Ca$^{2+}$ in the presence and absence of Zn$^{2+}$ was in the range of 350 μM. The addition of Zn$^{2+}$ to the wild type S100A2 molecule in the presence or absence of Ca$^{2+}$ led to an

$^1$ The abbreviations used are: ESI-MS, electrospray ionization mass spectrometry; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.
S100A2. Gel filtration purification step revealed one major peak for wild type S100A2.

Fig. 1. Biophysical characterization of recombinant human wild type S100A2. A, the elution profile from the final Sephacryl S100-HR gel filtration purification step revealed one major peak for wild type S100A2. Insets, C, Coomassie-stained gel; W, Western blot probed with monoclonal anti-S100A2 recognizing both the monomer (M) and the dimer (D). B, pure S100A2 in solution is a dimer as determined by analytical ultracentrifugation. The dimeric state is stable in buffer C1 containing 1 mM Ca\(^{2+}\) analyzing the addition of Ca\(^{2+}\) alone. (see below). Upon exposure to low concentrations of Zn\(^{2+}\), the recombinant protein oligomerizes (Fig. 1B) and starts to precipitate in the upper micromolar range. CD spectra also detected conformational changes in the \(\alpha\)-helical content during Ca\(^{2+}\) binding in the absence of Zn\(^{2+}\), whereas no change was observed during Zn\(^{2+}\) binding in the absence of Ca\(^{2+}\). Remarkably, the change induced in CD spectra by Ca\(^{2+}\) is reversed upon further addition of Zn\(^{2+}\) (Fig. 1D).

Recombinant S100A2 can bind Zn\(^{2+}\) above its physiological range causing the protein to precipitate. This is assumed to be due to the presence of four cysteines in the protein, which are all capable of chelating Zn\(^{2+}\) as shown in the DTNB test. Upon the addition of 40 \(\mu\)M Zn\(^{2+}\), the reactivity of all four sulphhydryl residues of S100A2 to DTNB was dramatically decreased (see also Fig. 6). To overcome this problem and to identify the cysteine(s) involved in Zn\(^{2+}\) binding, we constructed a battery of cysteine-deficient mutants. The cysteines are tentatively named Cys\(^{1(3)}\), Cys\(^{2(22)}\), Cys\(^{3(86)}\), and Cys\(^{4(94)}\), the numbers 1–4 referring to the order in the amino acid sequence and the numbers in parentheses indicating the positions in the amino acid sequence. The mutants were numbered as \(\Delta\)n, where the number n refers to the cysteine(s) substituted for serine. The mutations and tentative names are summarized in Table I. The actual molecular mass as detected by ESI-MS and the theoretical mass calculated with the program GPMAW is shown in Table II. The deviation of 1–2 daltons between calculated and actual molecular mass as detected by ESI-MS and the theoretical mass is determined under metal-free conditions (solid line) compared with control conditions in the absence of metal ions (solid line). Upon the addition of 50 \(\mu\)M Zn\(^{2+}\), in the presence of 1 mM Ca\(^{2+}\), the spectrum is reversed to the level determined under metal-free conditions (dashed line).

### Table I

| Clone | Cysteines present | \(c(Ca^{10})\) | \(c(Zn^{10})\) | \(c(Zn^{10}, Ca)\) | Tyrosine fluorescence |
|-------|-------------------|----------------|----------------|---------------------|----------------------|
| WT    | 1 2 3 4           | 347           | 150            | 30                  | Increase             |
| \(\Delta 1\) | 2 3 4           | 348           | 250            | NR                  | No change            |
| \(\Delta 2\) | 1 3 4           | 276           | 85             | 75                  | Increase             |
| \(\Delta 3\) | 1 2 4           | 336           | 100            | 75–100              | Increase             |
| \(\Delta 4\) | 1 2 3 4         | 308           | 75             | 75                  | Increase             |
| \(\Delta 12\) | 1               | 301           | 300            | 50–75               | Increase             |
| \(\Delta 134\) | 2               | 330           | 350–400        | NR                  | Decrease             |
| \(\Delta 124\) | 3 4             | 301           | >500           | NR                  | Decrease             |
| \(\Delta 123\) | 3               | 368           | >900           | NR                  | No change            |
| \(\Delta 1234\) | 4               | 279           | >900           | NR                  | Decrease             |
| \(\Delta 12\) | 3 4             | 334           | >800           | NR                  | No change            |

The table lists all S100A2 constructs, their tentative names, and half-maximal saturation concentrations for Ca\(^{2+}\), Zn\(^{2+}\), and Zn\(^{2+}\) in the presence of Ca\(^{2+}\).
Biophysical Characterization of Cysteine-deficient S100A2 Mutants

Ca$^{2+}$ binding and associated conformational changes are unaffected by the substitution of cysteines. Almost identical elution profiles were obtained for all S100A2 mutants on an S100HR gel filtration column. Purified recombinant S100A2 and mutant proteins and their reactivity with the monoclonal anti-S100A2 antibody is shown in Fig. 2A. Additional evidence for the structural integrity of all mutant proteins is the characteristic UV absorption spectrum that was identical for all mutants (Fig. 2B). Moreover, the virtually identical Ca$^{2+}$ binding characteristics indicated that none of the mutations influenced the dimeric state and/or EF-hand flexibility of the mutants compared with wild type S100A2. The half-maximal calcium saturation concentrations for all mutants were in the range of $c(Ca)_{50} = 310 \pm 30 \mu M$ (Fig. 2C). The value of the mutant lacking all four cysteine residues ($\Delta 1234$) showed the highest affinity for Ca$^{2+}$ ($c_{50} = 280 \mu M$). This value may be taken as the estimated Ca$^{2+}$ affinity of the native S100A2 wild type protein in the cell, as it may be completely reduced in the cytoplasm of living cells. For freshly reduced wild type protein, similar values could be determined, but heavy oxidation was measured when monitoring the DTNB reactivity during Ca$^{2+}$ titration. After several hours of incubation in the Ca$^{2+}$-free state (and shorter in the Ca$^{2+}$-bound state) wild type S100A2 harbors less than one free cysteine per monomer, and the Ca$^{2+}$ affinity is in the range of 1 mM (data not shown). Therefore, all measurements were carried out immediately after complete reduction of the proteins in dithioerythritol (see "Materials and Methods"). The state of reduction was measured as a function of the DTNB reactivity. The different cysteines contribute to this oxidation in different amounts as documented by the differential sensitivity to the reaction with DTNB. For example, the enhanced reactivity of Cys$^{2(22)}$ upon Ca$^{2+}$ binding is most likely elicited through exposure of this residue to the solvent (see also Fig. 6). We employed ESI-MS to test whether S100A2 or mutants oligomerize during Zn$^{2+}$-binding and to calculate the population of S100A2 dimers (or oligomers) that are bound to one or more zinc ions at a certain concentration. ESI-MS has been used before to analyze the Ca$^{2+}$-binding and associated conformational changes of several highly conserved residues located within helix 4 and helix 1, respectively (28). Thus, the organic solvent used in the ESI-MS will disfavor the formation and stabilization of the dimer. However, we could show that the monomers of recombinant human S100A2, forming the major particle population under these conditions, are sufficient to bind to two Ca$^{2+}$ ions per monomer although with lower affinity, due to the low pH and the absence of cooperativity, mediated only by the intact dimer. No significant affinity for Zn$^{2+}$ could be detected for monomers.

Zn$^{2+}$ Binding and Associated Conformational Changes Are Impaired by Serial Substitution of Cysteines

Wild Type S100A2—The addition of Zn$^{2+}$ to the wild type S100A2 molecule led to a strong increase in tyrosine fluorescence. Precise determination of the binding characteristics was restricted due to gradual precipitation of wild type S100A2 before a clear end point of the titration was reached. The estimated half-maximal Zn$^{2+}$ saturation concentration in the absence of Ca$^{2+}$ was $c(Zn)_{50} = 150 \mu M$. When Zn$^{2+}$ was added in the presence of 500 $\mu M$ Ca$^{2+}$ (70–75% saturation according to the Ca$^{2+}$ titration), this value increased about 5-fold to $c(Zn)_{50} = 30 \mu M$ (compare Table I).
Cys(1(3)) Is Sufficient for the Aspect of Zn$^{2+}$ Binding That Can Be Detected by Tyrosine Fluorescence Changes

Cys(1(3))—Cysteine 1 is situated at position 3 in the amino acid sequence of human S100A2. All mutants containing Cys(1(3)) (Δ2, Δ3, Δ4, Δ234), exhibited high affinity Zn$^{2+}$ binding, which was associated with an increase in tyrosine fluorescence as a result of conformational changes in the molecule upon metal ion binding. When Cys(1(3)) was replaced by serine, no Zn$^{2+}$ binding was detectable in all corresponding mutants (Δ1, Δ12, Δ13, Δ14, Δ123, Δ124, Δ134, Δ1234). Fig. 3 shows two representative curves for this series of experiments. A typical curve indicating Zn$^{2+}$ binding associated with increased tyrosine fluorescence is demonstrated by the measurements for the Δ2 mutant. The "Cys-null" mutant Δ134, by contrast, displays no Zn$^{2+}$ binding before the protein starts to precipitate. All mutants missing Cys(1(3)) lacked specific Zn$^{2+}$ binding accompanied by conformational changes. In the absence of cysteines 2, 3, and 4, however, Zn$^{2+}$ binding was restored in the presence of Ca$^{2+}$ by the sole presence of the first cysteine only (mutant Δ234) (Fig. 4). From these data, we conclude that Cys(1(3)) is essential for the aspect of Zn$^{2+}$ binding that induces a conformational change leading to an increased tyrosine fluorescence and that this is strongly enhanced (or even induced) only after Ca$^{2+}$ binding of S100A2. A summary of all half-maximal concentrations for Ca$^{2+}$ and for Zn$^{2+}$ in the presence and absence of Ca$^{2+}$ for all mutants is given in Table I.

Cys(2(22))—Cys(2(22)) is situated within the helix-loop-helix motif of the first S100-specific EF-hand of S100A2 between the first helix and the calcium-binding loop. Substitution of Cys(2(22)) with serine (Δ2) did not cause any detectable changes in the Zn$^{2+}$ binding characteristics of this mutant compared with the wild type protein as determined in conformational titrations in the presence of Ca$^{2+}$. But in this mutant, the affinity for Zn$^{2+}$ in the absence of Ca$^{2+}$ was remarkably increased (see also Table I). Notably, mutant Δ2 was also more resistant to oxidation and precipitation during the time course of the experiments than the wild type molecule or any of the mutants carrying Cys(2(22)) (Δ1, Δ3, Δ4). When only Cys(2(22)) was present (Δ134), no specific Zn$^{2+}$-binding could be detected monitoring the tyrosine fluorescence both in the presence and absence of Ca$^{2+}$ (Fig. 4). However, in the radioactive $^{65}$Zn$^{2+}$ binding studies we observed a signal at higher Zn$^{2+}$ concentrations (Fig. 5). With the S100A2 mutant Δ134, we could also determine an enhanced reactivity to DTNB upon the addition of Ca$^{2+}$, which was significantly reduced upon the addition of Zn$^{2+}$ (Fig. 6).

Thus, this residue appears to become exposed to the solvent phase upon Ca$^{2+}$ binding, thereby increasing its sensitivity to oxidation.
Cys387 and Cys494 Are Involved in the Formation of a Zn$^{2+}$-binding Site That Does Not Alter the Overall Conformation of S100A2 upon Metal Binding

Cys387 located at position 87 and Cys494 at position 94 are placed C-terminal of the canonical second EF-hand motif of S100A2. Substitution of Cys387 alone (Δ3) did not alter the Zn$^{2+}$-binding characteristics of S100A2, and the sole presence of Cys387 (Δ124) led to low Zn$^{2+}$ binding (Fig. 5). Since the complementing mutant Δ3 displayed wild-type-like Zn$^{2+}$-binding characteristics in all tests, we conclude that the Zn$^{2+}$-binding capacity attributable to Cys387 alone (displayed by mutant Δ124 in the radioactively solid-phase assay) is not relevant for specific Zn$^{2+}$ binding of the intact S100A2 molecule. By contrast, substitution of Cys494 (Δ4) led to a clear decrease in the Zn$^{2+}$-binding capacity as can be seen in the $^{65}$Zn$^{2+}$-binding assay (Fig. 5), whereas the conformational change induced by Zn$^{2+}$ in the presence or absence of Ca$^{2+}$ was not affected.

In mutants where both Cys494 and Cys493 are missing (Δ124, Δ134, and to a lesser degree Δ134), a decrease of the tyrosine fluorescence could be observed when Zn$^{2+}$ was added in the presence of Ca$^{2+}$ (Fig. 4). Cys494 counteracted this conformational effect when Zn$^{2+}$ was added to mutant proteins missing Cys493. This effect represents a conformational change that cannot be observed in the wild type S100A2 protein. In radioactive binding studies using the mutant containing Cys494 only (Δ123), $^{65}$Zn$^{2+}$ binding could be detected, but in fluorescent measurements this mutant showed no increased tyrosine fluorescence upon Zn$^{2+}$ binding, similar to all mutants lacking Cys493 (see also above).

Based on sequence alignments, it has been proposed that a Zn$^{2+}$-finger-like structure with at least two possible ligands for Zn$^{2+}$ (CX,C) is present in the C terminus of several members of the S100 protein family and that this may represent a conserved motif for Zn$^{2+}$ binding in this family (10). To test the hypothesis that Cys387 and Cys494, which are 7 residues apart act cooperatively in the ligation of a zinc ion (or form a zinc finger-like structure), an additional mutant, exclusively harboring these two cysteine residues (mutant Δ12) was constructed. As determined in the radioactive $^{65}$Zn$^{2+}$-binding studies, this mutant exhibited Zn$^{2+}$ binding greater than that seen for the mutant containing only Cys494 (Fig. 5), but no conformational change was associated with this activity.

Cys Null Mutant—Finally, we designed a mutant in which all four cysteines were substituted for serine (Δ1234). No Zn$^{2+}$ binding could be detected in any of the two assays employed in this study. This result further supports the assumption that the highly conserved histidine in position 18, His118, or His840 alone is not sufficient for Zn$^{2+}$ binding.

Association of Endogenous and Recombinant S100A2 with Membranes

To test if the different conformational effects induced in S100A2 by the addition of Ca$^{2+}$ and Zn$^{2+}$ in vitro are also mirrored by altered subcellular compartmentalization, cell fractionation experiments were carried out monitoring the behavior of endogenous porcine S100A2, recombinant wild type, and the cysteine null mutant Δ1234. Endogenous S100A2 from porcine LLC-PK1 cells was found in the microsomal fraction of porcine LLC-PK1 cells in a Ca$^{2+}$-dependent manner. The protein was efficiently removed from this fraction by further addition of EGTA. The sole presence of 50 μM Zn$^{2+}$ was not sufficient for significant targeting of S100A2 to membranes (Fig. 7A). These tests were also carried out with extracts of human A431 cells that contain only low detectable levels of endogenous S100A2. We selected these cells for the assay because a cell line negative for endogenous S100A2 could be devoid of the correct binding partners. Cell extracts were mixed
with recombinant human wild type S100A2 (Fig. 7B) or mutant Δ1234 (Fig. 7C) and treated similarly to LLC-PK1 extracts. This assay revealed targeting of S100A2 to the microsomal fraction in a strictly Ca\textsuperscript{2+}-dependent manner. The behavior of mutant Δ1234 in this assay was indistinguishable from human recombinant wild type and endogenous porcine S100A2, demonstrating that Ca\textsuperscript{2+} is the prime determinant for membrane association of S100A2 and that Zn\textsuperscript{2+} is not involved in this mechanism.

**DISCUSSION**

In this study, we aimed at determining the Zn\textsuperscript{2+} binding characteristics of human S100A2 and its dependence on Ca\textsuperscript{2+} binding to the EF-hands. Detailed knowledge of these features of the protein is important for a thorough understanding of the potential involvement of this protein in signal transduction and transcriptional regulation or in events related to apoptosis. For the latter, several indications point toward a direct causal relation between programmed cell death and the Zn\textsuperscript{2+} binding of S100 proteins. The role of Zn\textsuperscript{2+} binding in the regulation of p53 by S100B is currently under debate (29–32), and for human S100A2 a correlation of its nuclear localization (16), Zn\textsuperscript{2+} binding (4), and transcriptional regulation by p53 (15) has been documented. Since the sites responsible for Zn\textsuperscript{2+}-binding in S100 proteins are still ill defined, we purposely selected S100A2 for this study because it harbors potentially important residues in critical positions, i.e. the very N and C terminus.

Human S100A2 is highly sensitive to oxidation due to the presence of four cysteines in each monomer, affecting the affinities for both Ca\textsuperscript{2+} and Zn\textsuperscript{2+}. The reduction of the Ca\textsuperscript{2+} affinity in human S100A2 after oxidation is most likely a result of the formation of disulfide bonds impeding EF-hand flexibility. The individual cysteines contribute to this oxidation in different amounts as shown in the altered reactivities to DTNB in the presence or absence of metal ions (see also Fig. 6). Especially, residue Cys\textsuperscript{20,22} in the sequence of human S100A2 appears to be involved when oxidation affects the metal binding in vitro (see below).

| Ca\textsuperscript{2+} | Zn\textsuperscript{2+} | EGTA |
|---------------------|---------------------|------|
| P S                 | P S                 | P S  |

Fig. 7. Ca\textsuperscript{2+}- but not Zn\textsuperscript{2+}-dependent association of S100A2 with membranes. The membranous microsomal fraction of cell extracts of porcine LLC-PK1 cells containing endogenous S100A2 (A) and a corresponding fraction of human A431 cells mixed with wild type recombinant human S100A2 (B) or the null mutant Δ1234 (C) was pelleted in the presence of 1.5 mM Ca\textsuperscript{2+}, 50 μM Zn\textsuperscript{2+}, or 2 mM EGTA. Note the presence of S100A2 in the membranous fraction also for the null mutant Δ1234, which is unable to bind Zn\textsuperscript{2+}.

Ca\textsuperscript{2+} binding is mediated through the two EF-hand motifs and displays a high level of cooperativity (4). Ca\textsuperscript{2+}-mediated conformational changes in S100 proteins have been studied by monitoring 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonate-mediated or internal fluorescence (4, 9) and by NMR-based studies (28, 33–35), and these changes revealed highly similar characteristics in several S100 family members. The increase of internal tyrosine fluorescence upon Ca\textsuperscript{2+} binding has been interpreted as a result of the exposition of a hydrophobic target interface in human S100A2 (4). In contrast to these findings, the conformational effects induced by the addition of Zn\textsuperscript{2+} to human S100A2 (but also resulting in increased internal tyrosine fluorescence) are presumed to be distinct from those induced by Ca\textsuperscript{2+}, since they can also be achieved when Zn\textsuperscript{2+} is added in the presence of saturating amounts of Ca\textsuperscript{2+}. In addition, CD spectra showed that changes in the α-helical content induced by Ca\textsuperscript{2+} cannot be induced by the addition of Zn\textsuperscript{2+} and, moreover, that the Ca\textsuperscript{2+}-induced changes are reversed when Zn\textsuperscript{2+} is added in the presence of Ca\textsuperscript{2+}. Because the complete substitution of all cysteine residues for serines does not affect the Ca\textsuperscript{2+} binding characteristics but completely abolishes Zn\textsuperscript{2+} binding, we conclude that Ca\textsuperscript{2+} and Zn\textsuperscript{2+} binding are separate features employing different sites in the protein. These results also support the hypothesis that Ca\textsuperscript{2+}-dependent targets of S100A2 may be different from Zn\textsuperscript{2+}-dependent binding partners. From several studies addressing a similar question for other S100 family members (36–39), it can be hypothesized that the Ca\textsuperscript{2+}-dependent membrane association of S100A2 is mediated by members of the annexin family, whereas Zn\textsuperscript{2+}-dependent targets are almost unknown except for one peptide that binds to S100B in a Zn\textsuperscript{2+}-enhanced manner (30). It has been discussed earlier that Zn\textsuperscript{2+} binding to S100 family members could be antagonistic to Ca\textsuperscript{2+} binding at least in respect to target recognition (5, 7, 8). For human S100A2, it has been suggested that Ca\textsuperscript{2+} and Zn\textsuperscript{2+} may act antagonistically (4). In contrast, we here report a 5-fold increase in the affinity of S100A2 for Zn\textsuperscript{2+} in the presence of Ca\textsuperscript{2+}.

In this work, we were able to attribute the aspect of Zn\textsuperscript{2+} binding resulting in profound conformational changes in human S100A2 specifically to the very N-terminal cysteine residue, tentatively named Cys\textsuperscript{13}. This cysteine residue is both necessary and sufficient for all known features of this aspect of Zn\textsuperscript{2+} binding including the induction of increased tyrosine fluorescence upon the addition of Zn\textsuperscript{2+} and a 5-fold increase in Zn\textsuperscript{2+} affinity in the presence of Ca\textsuperscript{2+}. Additional support for the hypothesis that this Zn\textsuperscript{2+} binding site employs only the first cysteine (Cys\textsuperscript{13}) residue of the four tested stems from the solid phase \textsuperscript{65}Zn\textsuperscript{2+}-binding assay. The binding capacity for Zn\textsuperscript{2+} in the Δ1 mutant is reduced by 40%, and mutant Δ234 retains about 40% of the Zn\textsuperscript{2+}-binding of the wild type molecule.

These results of an N-terminal binding site are consistent with the findings of Kordowska et al. (9), who demonstrated that the Zn\textsuperscript{2+} binding of S100A6 (calcyclin) induces a conformational change in the molecule, which can be monitored by internal tyrosine fluorescence and that Zn\textsuperscript{2+} binding characteristics are altered upon derivatization of the single aminoterminal cysteine. Their results further implied a Zn\textsuperscript{2+}-binding site that is distinct from the Ca\textsuperscript{2+}-binding EF-hands.

The other ligands necessary for ligating the zinc ion remain elusive. However, assuming that the three-dimensional structure of the S100A2 dimer is closely related to other known S100 structures, one more putative ligand for this "N-terminal" Zn\textsuperscript{2+}-binding site is the histidine residue His\textsuperscript{240} contributed by the other part of the S100A2 dimer. His\textsuperscript{240} is located in the hinge region between the two EF-hand motifs, a region previously inferred to be involved in Zn\textsuperscript{2+} binding (10). Together with our data from ESI-MS studies, this suggests a zinc-binding site employing the entire intact S100 dimer, thus compensating for the lack of a conserved sequence motif for Zn\textsuperscript{2+} binding in this protein family.

We also detected a second aspect of Zn\textsuperscript{2+} binding in the very C terminus of recombinant human S100A2. We show that the
cysteine residues Cys\(^{3(87)}\) and Cys\(^{4(94)}\) of recombinant S100A2 form at least part of a second Zn\(^{2+}\)-binding site in S100A2 and that they contribute to this binding site in different amounts. The sole presence of Cys\(^{4(94)}\) (\(\Delta 123\)) was sufficient for the binding of radioactive \(^{65}\text{Zn}\) ; however, with lower affinity than was seen in the additional presence of Cys\(^{3(86)}\) (\(\Delta 12\)). Also, the sum of the Zn\(^{2+}\) bound by the mutants \(\Delta 124\) and \(\Delta 123\) was always lower than the amount of Zn\(^{2+}\) bound by mutant \(\Delta 12\) harboring both residues, pointing toward a synergism between these two cysteine residues. This assumption is also supported by the fact that the reduction of zinc binding in mutant \(\Delta 4\) is significantly higher than the binding capacity retained by the mutant \(\Delta 123\) (compare with Fig. 5).

Stable ligation of a zinc ion requires a minimum of four ligands (three amino acid residues and one solvent molecule) (40). Thus, these two cysteines are not sufficient for the complete ligation of Zn\(^{2+}\). From the crystal structures that were obtained from Zn\(^{2+}\)-loaded S100A7 (psoriasin) (11), it may be concluded that other N-terminal residues like His\(^{1(18)}\) act in concert on the ligation of the metal ion, since the C terminus of one monomer is in close enough proximity to the first helix of the first EF-hand motif of the second monomer in the dimer to interact with each other. Based on the model of Brodersen et al. (11), residue His\(^{1(18)}\) in S100A2 would correspond to His\(^{17}\) in S100A7 in one half of the dimer, and residues Cys\(^{3(87)}\) and Cys\(^{4(94)}\) of S100A2 would correspond to His\(^{86}\) and His\(^{90}\) of S100A7 in the other half of the dimer, respectively. This hypothesis is further supported by the finding that under assay conditions that affect the integrity of the dimer like in ESI-MS, Zn\(^{2+}\)-binding is abolished. Thus, Zn\(^{2+}\)-binding in S100 family proteins appears to depend on the formation of an intact dimer.

In human S100A2, all cysteines except for one are located outside the EF-hands and have no effects on the Ca\(^{2+}\)-binding characteristics. Only Cys\(^{2(22)}\) is positioned next to the first Ca\(^{2+}\)-binding loop and could be potentially involved in Ca\(^{2+}\)-binding. Cys\(^{2(22)}\) is situated 4 amino acid residues (one complete helical turn) C-terminal of the highly conserved histidine residue (His\(^{1(18)}\)) that has been suggested to be involved in the Zn\(^{2+}\)-binding site of S100A2 (compare with Fig. 5).

As shown in the DTNB assay, this residue becomes exposed to the surface upon Ca\(^{2+}\)-binding (compare Fig. 6), and ligation with Zn\(^{2+}\) does not interfere with Ca\(^{2+}\)-binding. On the other hand, substitution of this residue does not alter the Zn\(^{2+}\)-binding characteristics or affinity of recombinant human S100A2. Moreover, this residue is conserved neither among members of the S100 family nor among species. At the position corresponding to Cys\(^{2(22)}\) in human S100A2, all other known sequences of S100A2 and most other S100 family members contain a highly conserved glycine. From this, we conclude that Cys\(^{2(22)}\) is not involved in Zn\(^{2+}\)-binding or plays a highly subordinate role.

Substitution of Cys\(^{4(94)}\) in mutants incapable of Zn\(^{2+}\)-binding via Cys\(^{3(83)}\) (\(\Delta 124\), \(\Delta 134\), \(\Delta 1234\)) leads to conformational changes different from those seen for the wild type protein (i.e., a decrease of tyrosine fluorescence). We conclude that Cys\(^{4(94)}\) plays an important role in the maintenance of the structural integrity of the protein during Zn\(^{2+}\)-binding. One possible explanation may include the prevention of the Zn\(^{2+}\)-ion from binding to Cys\(^{2(22)}\) by Cys\(^{4(94)}\). In conclusion, Cys\(^{4(94)}\) appears essential for the high affinity Zn\(^{2+}\)-binding in S100A2 not associated with increased internal tyrosine fluorescence and acting synergistically with Cys\(^{3(87)}\). However, the precise role of Cys\(^{4(94)}\) in zinc binding leading to increased tyrosine fluorescence remains unclear.

The S100A2 mutant in which all cysteines have been converted to serine did not show detectable zinc binding in any of the assays employed in this study. However, incubation with high concentrations of ZnCl\(_2\) (>0.5 mM) leads to precipitation of the sample solution. The inability of this null mutant (\(\Delta 1234\)) to bind to Zn\(^{2+}\) may also be interpreted as a control for His\(^{1(18)}\) and His\(^{3(87)}\), which are not sufficient for zinc binding even in concert with additional potential ligands (like asparagine) as has been proposed for S100A7.

Finally, our cell extraction experiments have shown that the different metal binding features in human S100A2 are also mirrored by their metal ion-dependent localization in different cellular compartments. A similar mechanism has been shown for S100A6 (41, 42). Membrane association of S100A2 is strictly Ca\(^{2+}\)-dependent and can be hypothesized to be mediated by members of the annexin family, since several S100 proteins interact with annexins in a Ca\(^{2+}\)-dependent manner. Thus, Zn\(^{2+}\)-dependent targets of S100A2 may be different from Ca\(^{2+}\)-dependent binding partners, since Zn\(^{2+}\) is unable to translocate S100A2 to membranous cellular compartments and as the null mutant \(\Delta 1234\) displays Ca\(^{2+}\)-dependent membrane localization.

Conclusions—Zn\(^{2+}\)-binding to the "N-terminal" site is closely linked to conformational alterations in the dimeric S100A2 molecule, and the affinity for Zn\(^{2+}\) increases in response to Ca\(^{2+}\) although the accessibility of the cysteine residue Cys\(^{1(18)}\) itself may not be altered upon Ca\(^{2+}\)-binding. Since all S100 family members have been shown to form dimers in solution and all three-dimensional structures determined so far reveal a highly similar molecular arrangement, it appears likely that those residues unaccessible in one S100A2 monomer to form a complete Zn\(^{2+}\)-binding site are situated in (and contributed by) the other half of the dimer. The second "C-terminal" Zn\(^{2+}\)-binding site employs Cys\(^{3(87)}\) and Cys\(^{4(94)}\) from one monomer and most likely also His\(^{1(18)}\) of the other half of the dimer. In contrast to the amino-terminal site, Zn\(^{2+}\)-binding at the carboxyl terminus is not associated with conformational alterations and appears to be independent of the Ca\(^{2+}\)-associated properties of human S100A2. Histidine and cysteine residues can be found in most S100 family members in corresponding or neighboring positions, as is the highly conserved His\(^{1(18)}\) or residues in the hinge region and C-terminal histidine and cysteine residues. We thus hypothesize that two separate regions in the S100A2 monomer are responsible for two distinct types of Zn\(^{2+}\)-binding in vitro but that only the intact dimer is capable of exhibiting full Zn\(^{2+}\)-binding activity and displays the Zn\(^{2+}\)-related functional alterations.

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