Purification and Cation Binding Properties of the Recombinant Human S100 Calcium-binding Protein A3, an EF-hand Motif Protein with High Affinity for Zinc

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The S100 protein family constitutes a subgroup of Ca2+-binding proteins of the EF-hand type displaying 30% or more sequence identity (Kligman and Hilt, 1988; Hilt and Kligman, 1991). Under physiological conditions their affinity for Ca2+ is rather low but can be increased once S100 proteins are associated with their targets. Different S100 proteins were also found to bind Zn2+ with a fairly high affinity (Baudier et al., 1986; Leung et al., 1987; Filipек et al., 1990; Dell’Angelica et al., 1994). Both intracellular roles, such as activation of enzymes, regulation of motility, and smooth muscle contraction, and extracellular roles, such as neuronal differentiation, glial proliferation, and prolactin secretion (for review, see Donato (1991), Zimmer and Dubuisson (1993), and Heizmann and Braun (1995)), have been proposed. Intriguingly, in different cases where calmodulin was thought to be the regulatory CaBP,1 S100 proteins were finally the real activators (Bianchi et al., 1993). Most S100 proteins interact in vitro with hydrophobic matrices, with membranes, enzymes, cytoskeletal and contractile proteins, and even cell surface receptors (for review, see Donato (1991)). All of these data point to a multifunctional role of the S100 family with a particular function for each of its members. This functional specificity is supported by the fact that their expression is differentially deregulated in different types of cancer cells (Hilt and Kligman, 1991; Weterman et al., 1992; Davis et al., 1993; Pedrocchi et al., 1994a, 1994b), suggesting participation in tumor progression. However, for none of these putative functions have the molecular details been elucidated.

The protein S100A3,2 formerly called S100E, was recognized for the first time as the product of one of the tightest gene clusters discovered in the human genome located on chromosome 1q21 (Engelkamp et al., 1993). The S100A3 gene shows a low but general transcription level in diaphragm, heart, skeletal muscle, stomach, lung, liver, fat tissue, and placenta. A YAC clone from human chromosome 1q21 has been recently isolated on which nine different genes coding for S100 proteins were localized. The clustered organization of S100 genes in the 1q21 region allowed to introduce a new logical nomenclature for these genes (Schäfer et al., 1995). The S100A3 gene product is 101 residues long and possesses one S100-type noncanonical Ca2+-binding loop of 14 residues expanding from Ala-20 to Glu-33, and one canonical EF-hand loop of 12 residues from...
Asp-63 to Glu-74, both flanked by two \( \omega \)-helices. In calbindin D-9k, the prototype of this S100 protein family with a resolved three-dimensional structure (Szejtli and Moffat, 1982; Carlström and Chazin, 1993), the \( \omega \)-helices are oriented in an antiparallel fashion, thus forming a 4-helix barrel. Within the S100 subfamily S100A3 is unique for the exceptionally high number of Cys residues. Despite the Cys frequency, S100A3 does not display the classical zinc-binding motifs seen in metallothioneins (Vallee and Auld, 1990), DNA-binding proteins (Pérez-Alvarado et al., 1994), or protein kinase C (Hommel et al., 1994).

In order to begin to understand the role of S100A3 and the molecular mechanisms by which it exerts its function, we characterized in this study the Ca\(^{2+} \) - and Zn\(^{2+} \)-binding properties of recombinant human S100A3 under physiological conditions. We monitored the cation-dependent changes in the environment of the Trp and Tyr residues, probed the thiol/disulfide state and the cation-dependent reactivity of the thiol, and finally monitored the solvent-exposed hydrophobic surface. The results suggest that under physiological conditions S100A3 is a Zn\(^{2+} \)-binding rather than a Ca\(^{2+} \)-binding protein.

**EXPERIMENTAL PROCEDURES**

Materials—A protein fusion and expression system was obtained from New England Biolabs. Isopropyl-thio-\( \beta \)-galactopyranoside and restriction endonucleases were from Boehringer Mannheim. Concentrated T4 DNA ligase was obtained from New England Biolabs. EDTA, ampicillin, and lysozyme were purchased from Fluka. Hydroxylapatite (Bio-Gel HTP), polyacrylamide, and electrophoresis equipment were from Bio-Rad.

Oligonucleotides were synthesized on a Gene Assembler DNA synthesizer (Pharmacia Biotech Inc.). The primers used to amplify S100A3 cDNA for cloning into pMal-c2 were as follows: S100A3-M, 5'-ATGGCCAGGCTCAGGAGG-3'; S100A3-B, 5'-GGCAGTCAGGTTGAAGG-3'.

Cloning of Human S100A3 into a Prokaryotic Expression System—Human cDNA of S100A3 was amplified by the polymerase chain reaction (PCR) as described earlier (Engelkamp et al., 1993), using the primers S100A3-M and S100A3-B. The resulting PCR product comprised the complete coding region of S100A3 beginning with the starting codon ATG. The PCR product was blunt ended ligated into the XmnI-digested vector pMal-c2 downstream the initiation codon.

Expression and Purification of Recombinant S100A3—Human and E. coli transformed with the expression vector pMal-c2 downstream the initiation codon were grown in LB medium, 2.5 cm; the fusion protein was eluted as described by the producer's instructions.

The native apparent molecular weight of the metal-free, Ca\(^{2+} \) - and Zn\(^{2+} \)_forms of S100A3 was determined by gel filtration on a 1 \( \times \) 70 cm column of Sephadex G-75 in 50 mM Tris buffer, pH 7.5, 150 mM KCl, 1 mM diithiothreitol (buffer A) containing either no divalent cations, 100 mM CaCl\(_2\), or 100 mM ZnCl\(_2\). The column was standardized with the callithrix mixture of Bio-Rad.

Mass Spectrometry—Electrospray ionization mass spectra were obtained with a Sciex API III and a Finnigan TSQ 700 instrument equipped with an ion-spray source. The protein molecular mass was determined from the acquired spectra with ESI deconvolution software from Finnigan.

Amino Acid Analysis—Amino acid analysis was performed by gas-phase HCl hydrolysis, conversion with dansyl chloride and subsequent evaluation of the derivatized amino acid products with a Beckman System Gold HPLC instrument.

**FIG. 1.** Expression and purification of recombinant human S100A3. Coomassie Blue-stained 15% SDS-Tricine-PAGE under reducing conditions, showing induction and purification steps of MBP-S100A3 fusion protein and of S100A3. Lane 1, 40 \( \mu \)g of crude extract of E. coli; lane 2, 40 \( \mu \)g of flow-through following loading onto amylose-resin column; lane 3, 5 \( \mu \)g of eluate from amylose-resin column; MBP-S100A3 fusion protein; lane 4, 25 \( \mu \)g of fusion protein after factor Xa cleavage; lane 5, 3 \( \mu \)g of MBP; lane 6, 2.5 \( \mu \)g of finally purified S100A3 (monomer).

The native apparent molecular weight of the metal-free, Ca\(^{2+} \) - and Zn\(^{2+} \)-forms of S100A3 was determined by gel filtration on a 1 \( \times \) 70 cm column of Sephadex G-75 in 50 mM Tris buffer, pH 7.5, 150 mM KCl, 1 mM dithiothreitol (buffer A) containing either no divalent cations, 100 mM CaCl\(_2\), or 100 mM ZnCl\(_2\). The column was standardized with the callithrix mixture of Bio-Rad.

Optical Methods to Probe the Environment of Aromatic Residues—Emission fluorescence spectra were taken with a Perkin-Elmer L5-5B spectrofluorimeter. The measurements were carried out on \( 1 \mu \)M tri-chloroacetic acid-treated metal-free S100A3 at room temperature with excitation and emission slits of 5 nm. The excitation wavelength was 280 nm. 100 mM CaCl\(_2\), 30 mM MgCl\(_2\), or 200 mM ZnCl\(_2\) was added to the solution.

Protein Reduction and Thiol Reactivity—The protein solution was reduced by overnight incubation with 100 mM DTT at pH 8.5 and chromatographed on a Sephadex G-25 column (0.7 \( \times \) 40 cm). The protein was concentrated to 1 mg/ml and applied to an amylose resin column (0.7 \( \times \) 40 cm). The fusion protein was eluted as described by the producer's instructions.

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Mass Spectrometry—Electrospray ionization mass spectra were obtained with a Sciex API III and a Finnigan TSQ 700 instrument equipped with an ion-spray source. The protein molecular mass was determined from the acquired spectra with ESI deconvolution software from Finnigan.

Amino Acid Analysis—Amino acid analysis was performed by gas-phase HCl hydrolysis, conversion with dansyl chloride and subsequent evaluation of the derivatized amino acid products with a Beckman System Gold HPLC instrument.

Direct Zn\(^{2+} \)-binding Studies—For removal of contaminating metals, S100A3 was precipitated with 3% trichloroacetic acid and then passed through a 1 \( \times \) 40 cm Sephadex G-25 column equilibrated in the assay buffer. The protein concentration was determined from the UV absorption spectrum using a molar extinction coefficient at 280 nm of 14,500 M\(^{-1}\) cm\(^{-1}\) for metal-free S100A3. These values were measured on protein stock solutions in bidistilled water whose concentrations were determined by quantitative amino acid analyses.

Zn\(^{2+} \)-binding was measured at room temperature by the equilibrium gel filtration method of Hummel and Dryer (1962). A Sephadex G-25 column (0.7 \( \times \) 50 cm) was equilibrated in buffer A containing variable concentrations of Zn\(^{2+} \). 0.5–1 ml of 50–200 \( \mu \)M metal-free protein was applied to the column. In the eluant Zn\(^{2+} \) concentrations were determined by atomic absorption with a Perkin-Elmer 2380 atomic absorption spectrophotometer. For the atomic absorption measurements EDTA up to 1 mM was added to all solutions, including the standards (Tritisol, Merck). Protein concentrations were measured by ultraviolet absorption.

Optical Methods to Probe the Environment of Aromatic Residues—Emission fluorescence spectra were taken with a Perkin-Elmer L5-5B spectrofluorimeter. The measurements were carried out on \( 1 \mu \)M tri-chloroacetic acid-treated metal-free S100A3 at room temperature with excitation and emission slits of 5 nm. The excitation wavelength was 280 nm. 100 mM CaCl\(_2\), 30 mM MgCl\(_2\), or 200 mM ZnCl\(_2\) was added to the solution.

Protein Reduction and Thiol Reactivity—The protein solution was reduced by overnight incubation with 100 mM DTT at pH 8.5 and chromatographed on a Sephadex G-25 column (0.7 \( \times \) 35 cm) equilibrated in nitrogen-saturated buffer A. The thiol reactivity was assayed by monitoring spectrophotometrically at 412 nm the kinetics of the reduction of Ellman’s reagent according to Riddles et al. (1983). The reaction was initiated upon mixing the protein solution with 10 \( \mu \)M DTNB to a final concentration of 0.3 mM. Titrations of Exposed Hydrophobic Sites—The Ca\(^{2+} \) - and Mg\(^{2+} \)-dependent changes in hydrophobic matrices of S100A3 was followed by monitoring the fluorescence properties of 2-p-toluidinynaphthalene-6-sulfonate (TNS) as described by...
Expression and Purification of Recombinant S100A3—To express and isolate S100A3 in large amounts, human S100A3 cDNA was cloned into the prokaryotic expression vector pMal-c2. A PCR product of human S100A3 cDNA was introduced at the protease factor Xa cleavage site behind the maltose binding protein (MBP) gene of pMal-c2 to generate a MBP-S100A3 fusion construct.

The amount of expressed MBP-S100A3 fusion protein corresponded to about 12% of total cell protein of a bacterial culture. After isolating the fusion protein by amylose resin affinity chromatography S100A3 was cleaved off from MBP by the protease factor Xa. S100A3 was finally purified by ion exchange chromatography and a second amylose resin affinity chromatography. The correct cleavage of the fusion protein and the purity and concentration of S100A3 was controlled by amino acid analysis and SDS gel electrophoresis. Fig. 1 shows the expression and purification of the fusion protein and of S100A3.

Biochemical Properties of Human Recombinant S100A3—After classical treatments of S100A3, such as dialysis, ultrafiltration, and freezing, the S100A3 protein is partly insoluble, but dissolves quickly and completely when solid DTT up to 50 mM is added. SDS-PAGE clearly shows that different disulfide-bonded S100A3 forms are linked oligomers are formed, which are reduced to the monomer after DTT treatment (data not shown).

To verify recombinant S100A3 for correct synthesis in bacteria we determined its exact mass by electrospray ionization mass spectrometry. Before desalting with butyl-300 microbore reversed-phase HPLC it was again necessary to mix the protein probe with 50 mM DTT to prevent precipitation on the column and to obtain any mass signal. In acidic solvent a molecular mass of 11,713.3 Da was obtained, which is in good agreement with the calculated molecular weight, including the unprocessed amino-terminal methionine.

SDS-PAGE after reduction of S100A3 with 10 mM DTT for 30 min at 37 °C also yielded a band with a molecular mass of 11 kDa. Determination of the apparent molecular mass by gel filtration on Sephadex G-75 after thorough reduction yielded different values depending on the presence of divalent cations: 22.4 kDa in the absence of divalent cations, 24.9 kDa in the presence of 100 μM Zn²⁺, and 39.0 kDa in the presence of 100 mM Ca²⁺. Thus, as in other members of the S100 family, S100A3 forms a noncovalent homodimer. Moreover, 100 mM Ca²⁺ promotes formation of a higher order oligomer (likely tetramers). It is not clear if the latter phenomenon is to be attributed to the specific binding of Ca²⁺ or to an ionic strength effect, which is known to stabilize hydrophobic interactions.

We assessed the isoelectric point of S100A3 by two-dimensional gel electrophoresis under reducing conditions using an immobilized pH gradient ranging from pH 3.5 to 10. In contrast to the calculated pI of 4.53, the determined pI of the denatured protein was found to be 5.5 (data not shown). This divergence may be caused by the experimental conditions and not by any modifications of the protein as the measured mass of recombinant S100A3 was found to be identical to the calculated value.

Direct Cation Binding Studies—Trichloroacetic acid can be used to remove ions, concentrate the protein, and prepare the sample correctly for Hummel-Dryer experiments. Gel filtration on Sephadex G-25 in 350 mM free Ca²⁺ shows very little binding (<0.2 mol of Ca²⁺/mol of S100A3), indicating that a binding study of this cation cannot be carried out by direct means. Zn²⁺ binding by the Hummel-Dryer method (Fig. 3) yields an isotherm of which the maximum value is somewhat difficult to evaluate since the protein shows a tendency to aggregate above 100 μM free Zn²⁺. The Scatchard plot, although curved upward indicating positive cooperativity, allowed a rather precise extrapolation to four binding sites per monomer (not shown). Assuming a maximal binding of 4 Zn²⁺ per monomer, the Hill plot was calculated and yielded a Hill coefficient (nH) of 1.4 and a [Zn²⁺]₀.₅ of 11 μM (inset). A Hummel-Dryer experiment in the presence of both Ca²⁺ and Zn²⁺ indicated that there is neither competition between the cations nor reinforcement of affinity, as was described for S100A1 (Leung et al., 1987) or calgranulin C (Dell’Angelica et al., 1994).

Fluorescence Characteristics—Fluorescence spectra (Fig. 4) indicate that denatured S100A3 has a fluorescence maximum 10-fold lower than that of the metal-free form. In the latter
form and in the presence of Mg$^{2+}$ the Trp is very well shielded with $\lambda_{\text{max}}$ at 340 nm. Mg$^{2+}$ does not affect the spectrum, suggesting that no binding occurs. Ca$^{2+}$, Zn$^{2+}$, or Co$^{2+}$ binding leads to a 3-fold fluorescence decrease, in the case of Ca$^{2+}$ with a 7-nm blue shift, in the case of Zn$^{2+}$ or Co$^{2+}$ without any shift. Given the very good signal change, Ca$^{2+}$, Zn$^{2+}$, and Co$^{2+}$ titrations could be carried out by fluorimetry on a 2 $\mu$M solution of S100A3. Since S100A3 displays a very low affinity for Ca$^{2+}$, the ratio of bound to added Ca$^{2+}$ is negligible. For Ca$^{2+}$ a smoothly increasing sigmoid was observed (Fig. 5A), indicating more than one site with different affinities or displaying negative cooperativity. This isotherm can be analyzed with $\left[\text{Ca}^{2+}\right]_{0.5} = 35$ mM and $n_h = 0.76$ (Fig. 5B). The same isotherm was obtained in 30 mM Mg$^{2+}$, indicating that the sites are specific. For Zn$^{2+}$ there are two levels of signal change (Fig. 5A), one with a midpoint of 8 $\mu$M and one at 600 $\mu$M. The high affinity compartment, with four Zn$^{2+}$ binding sites (see above), shows pronounced positive cooperativity with $n_h$ equal 1.3 as analyzed in Fig. 5C. Zn$^{2+}$ binding to the lower affinity compartment (at 200–1000 $\mu$M free Zn$^{2+}$) induces protein precipitation, as evidenced from strongly increased signals at emission wavelengths (285–290) close to the excitation wavelength (280 nm). Addition of both Ca$^{2+}$ and Zn$^{2+}$ leads to much stronger protein precipitation. Co$^{2+}$ binding seems monophasic with a $K_D$ of 1 mM (data not shown). Apparently this Co$^{2+}$-binding compartment corresponds to the high affinity compartment of Zn$^{2+}$ binding. Difference spectrophotometry on the S100A3 Co$^{2+}$ complex in the 240–800 nm zone shows that the complex does not display the peaks in the 650–750 nm, which are so characteristic for classical zinc fingers. Thus, the absence of these bands and the comparable low affinities for Zn$^{2+}$ and Co$^{2+}$ (nM for Zn$^{2+}$ and \muM for Co$^{2+}$ in zinc fingers) suggest that in S100A3 there is no such motif.
**Fig. 6.** Difference spectra of S100A3 (66 µM) in buffer A at room temperature after addition of 100 mM Ca²⁺ (-----) or 390 µM Zn²⁺ (-----) to the metal-free protein. The difference in optical density was expressed for a protein solution with an optical density of 1.0 at 280 nm.

**Fig. 7.** Thiol reactivity in S100A3 as monitored by the absorbance at 412 nm after addition of DTNB. Metal-free S100A3 (-----); S100A3 in the presence of 200 mM Ca²⁺ (-----), 100 µM Zn²⁺ (-----), and 200 mM Ca²⁺ + 100 µM Zn²⁺ (-----). Protein concentration was 6.7 µM. After 15 min of reaction time 31 µM thiols were titrated. The reactions do not follow pseudo first-order kinetics.

**Fig. 8.** Hydrophobic exposure in S100A3 as monitored by the fluorescence of TNS after excitation at 326 nm. Protein and TNS concentrations were 4 and 0.5 µM, respectively. TNS alone (thin solid line); metal-free S100A3 (-----); S100A3 + 180 mM Ca²⁺ (-----); S100A3 + 180 mM Ca²⁺ + 86.5 µM Zn²⁺ (-----); S100A3 + 190 µM Zn²⁺ (-----); S100A3 + 180 mM Ca²⁺ + 190 µM Zn²⁺ (-----); S100A3 + 190 µM Zn²⁺ + 90 mM Ca²⁺ (thin dotted line).

Ca²⁺ binding induces a 30-fold increase in fluorescence enhancement, whereas no enhancement at all is observed upon binding of Zn²⁺. It should be noted that the development of the Ca²⁺-induced hydrophobic patch(es) occurs in a biphasic manner: one phase is very rapid (occurs within the time of mixing) and may well present the exposure of hydrophobic residues in each monomer; the second phase occurs over a range of 10s of minutes and may correspond to the transition of the dimer to the 38-kDa oligomer as shown by gel filtration. It should be noted that addition of even 220 mM Ca²⁺ does not lead to noticeable aggregation, as monitored by turbidimetry (not shown). But in the presence of both 55 mM Ca²⁺ and 95 µM Zn²⁺ the enhancement is half of that of Ca²⁺ alone, indicating that the binding is noncompetitive. Very similar results have been obtained with the fluorescent probe 1-anilinonaphthalene-8-sulfonate (data not shown).
In this study we report the biochemical characterization and cation-binding properties of S100A3, a new member of the S100 family with an unusually high content of Cys residues. The protein is a dimer and contains two EF-hand motifs per monomer. But, whereas most other S100 proteins display Ca$^{2+}$-dissociation constants of 0.1-1 mM, S100A3 is able to bind Ca$^{2+}$-only in the 10-100 mM free Ca$^{2+}$ range, i.e. very far from the cytosolic Ca$^{2+}$ levels. Nevertheless, this binding seems specific since it is accompanied by Tyr and Trp conformational changes very similar to those caused by Zn$^{2+}$ binding, by a well defined absence of hydrophobicity and an oligomerization. The reason for this low affinity for Ca$^{2+}$ is not clear, since its primary structure is quite classical for a S100 member. However, our data indicate that each dimer contains five disulfide bridges. This may stabilize the protein but can impose strong constraints for the efficient binding of Ca$^{2+}$. Reduction of all the disulfide bridges of S100A3 under denaturing conditions and alkylation of the thiol yields a protein product which binds Ca$^{2+}$ with a dissociation constant of 0.8 mM, i.e. an affinity close to that of most other S100 proteins. It is still possible that S100A3 displays a real Ca$^{2+}$-dependent function when associated with its target or when secreted in the Ca$^{2+}$-rich extracellular fluid. In contrast to calgranulin C (Dell’Angelica et al., 1994) and S100B (Baudier et al., 1986), S100A1 (Leung et al., 1987) binds Zn$^{2+}$ with a rather low affinity. S100A6 (calcyclin) binds Zn$^{2+}$ with a (Zn$^{2+}$)$_{10}$S$_5$ of about 2 mM (Filipek et al., 1990; Pedrocchi et al., 1994b). But S100A4 is the only S100 protein with 1 Cys residues, most of which are clustered at the N-terminal side of the two Ca$^{2+}$-binding loops. This abundance of sulfur atoms and the fact that the fully reduced and alkylated protein does not bind Zn$^{2+}$ at all anymore suggest that the Zn$^{2+}$ ions are bound in thiolate clusters of the Kagi and Kojima type (reviewed in Vallee and Auld (1990)). Direct binding of Zn$^{2+}$ to the Cys residues would explain the strong reduction of the thiol reactivity in the presence of Zn$^{2+}$, but not of Ca$^{2+}$. The metallothioneins bind Zn$^{2+}$ per mol (8 for the S100A3 dimer) to 20 cysteinyl residues in clusters of the type Zn$_n$S$_n$ and Zn$_n$S$_2$. The Zn$^{2+}$-thiolate cluster has recently also been observed in DNA-binding proteins (Pan and Coleman (1990). However, since Co$^{2+}$-binding does not induce the characteristic absorption bands at 700 nm as it does in metallothioneins, and since half of the Cys residues in S100A3 are not in the free thiol form, it is tempting to postulate that a new type of cluster is present in the latter protein. Structural work is in progress to provide a more detailed description of this novel Zn$^{2+}$-binding motif.

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