Binding of Ca\(^{2+}\) and Zn\(^{2+}\) to Human Nuclear S100A2 and Mutant Proteins

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The Ca\(^{2+}\)-binding protein S100A2 is an unusual member of the S100 family, characterized by its nuclear localization and down-regulated expression in tumorigenic cells. In this study, we investigated the properties of human recombinant S100A2 (wtS100A2) and of two mutants in which the amino-terminal Ca\(^{2+}\)-binding site I (N mutant) and in addition the carboxyl-terminal site II (NC mutant) were replaced by the canonical loop (EF-site) of \&parvalbumin. Size exclusion chromatography and circular dichroism showed that, irrespective of the state of cation binding, wtS100A2 and mutants are dimers and rich in \&helical structure. Flow dialysis revealed that wtS100A2 binds four Ca\(^{2+}\) atoms per dimer with pronounced positive cooperativity. Both mutants also bind four Ca\(^{2+}\) atoms but with a higher affinity than wtS100A2 and with negative cooperativity. The binding of the first two Ca\(^{2+}\) ions to the N mutant occurred with 100-fold higher affinity than in wtS100A2 and a 2-fold increase for the last two Ca\(^{2+}\) ions. A further 2-3-fold increase of affinity was observed for respective binding steps of the NC mutant. The Hummel-Dryer method demonstrated that the wild type and mutants bind four Zn\(^{2+}\) atoms per dimer with similar affinity. Fluorescence and difference spectrophotometry showed that the binding of Ca\(^{2+}\) and Zn\(^{2+}\) induces considerable conformational changes, most attributable to changes in the microenvironment of Tyr\(^{76}\) located in site II. Fluorescence enhancement of 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid clearly indicated that Ca\(^{2+}\) and Zn\(^{2+}\) binding induce a hydrophobic patch at the surface of wtS100A2, which, as in calmodulin, may be instrumental for the regulatory role of S100A2 in the nucleus.

The S100 protein family constitutes a large subgroup of Ca\(^{2+}\)-binding proteins of the EF-hand type and is composed of at least 16 different members displaying 25–65% identity at the amino acid level (1). These proteins contain two distinct EF-hand motifs, separated by a central hinge region. The carboxyl-terminal Ca\(^{2+}\)-binding site (II), called the canonical EF-hand, is composed of 12 amino acids and is common to all EF-hand proteins. The amino-terminal site (site I) contains 14 amino acids and is S100-specific. The three-dimensional structure of S100B (2, 3, 5) and S100A6 (4, 52) revealed a symmetric homodimeric fold that is unique among calcium-binding proteins. It has been suggested that the specificity of interaction with target proteins may be located in the central "hinge" region, which shows the lowest sequence homology among all S100 proteins (5, 6). Members of the S100 family interact with annexins (S100A6, -A10, and -A11); non-muscle tropomyosin and myosin (S100A4); intermediate filaments (S100A8 and -A9); and different enzymes, for example fructose-1,6-bisphosphate aldolase (S100A1 and S100B), guanylate cyclase (S100B), glycogen phosphorylase, adenylate cyclase (S100A1) (reviewed in Refs. 1, 7, and 8), giant myosin-associated twitchin kinase (9), and the SR Ca\(^{2+}\) release channel (both S100A10) (10).

Thirteen S100 genes were found to be localized in a cluster on human chromosome 1q21 (11–13). Contrary to calmodulin, the expression of S100 proteins is cell- and tissue-specific. A wide range of different human diseases has been associated with deregulated expression of S100 genes (1, 7, 14). S100 proteins have been implicated in pleiotropic cellular events, with specific functions for each of the family members, such as cell cycle regulation, cell growth, cell differentiation, and motility.

S100A2 (formerly called S100L) was first detected at high levels in a subset of cells in lung and kidney and moderately in liver, cardiac muscle, and skeletal muscle (15) and was later recognized as the product of a gene located on chromosome 1q21 (16). This protein is an unusual member of the S100 protein family because of its predominantly nuclear localization (17), compared with the cytosolic localization of the other S100 proteins. The cDNA coding for S100A2 was found through subtractive hybridization between normal and tumor-derived human mammary epithelial cells in a search for novel tumor suppressor genes (18). S100A2 is preferentially expressed in normal mammary epithelial cells but is down-regulated in breast tumor cells (19, 20) and in several other tumor types as well (17). Expression of S100A2 can be altered by the calcium ionophore A23187 (down-regulation) and by the demethylation agent 5-azacytosine (up-regulation) in cell culture (19). Investigations of the methylation status of the S100A2 promoter region in normal (HBL100) and tumorogenic (T47D) cell lines showed that S100A2 expression is suppressed by DNA methylation (21). In contrast, S100A4 is reported to be overexpressed in metastatic breast cancer cell lines and human tumor tissues (17). Similarly, the expression of S100A4 in mouse melanoma cells led to a metastatic phenotype (22). These results indicate that S100A2 and S100A4 are oppositely regulated and might have inverse functions in tumorigenesis. The protein S100A2 may play a role in inhibiting tumor initiation or suppressing tumor cell growth.

The relationship between primary structure and cation binding is poorly understood not only in the S100 family but in general (23, 24). Generally, the dimeric S100 proteins display a low affinity for Ca\(^{2+}\) with an intrinsic association constant (K\(_{Ca}\))
of about 3 \times 10^{13} \text{ m}^{-1} (for a review, see Ref. 8). These results were confirmed in more recent studies on S100A4, S100A6, S100B, and S100A11 (25–27). Zn^{2+} can affect the binding of Ca^{2+} to particular S100 proteins (28–30). Indeed, using direct binding methods for S100B (27, 28), S100A4 and S100A6 (25), S100A3 (31), S100A12 (32), and S100A9 (33), it was demonstrated that they are able to bind Zn^{2+} with fairly high affinity. Thus, S100 proteins have variable cation binding properties in agreement with their diversified functions.

In order to elucidate the role of S100A2, we investigated the Ca^{2+}- and Zn^{2+}-binding properties of this nuclear protein under physiological conditions. We addressed the question of how the binding of cations to S100A2 is dependent on the intrinsic nature of the loops and on the cross-talk between the paired sites I and II. What is the contribution of each of the sites to ion binding and conformational properties? The approach was to characterize ion binding and conformational properties of wtS100A2 and two mutant proteins, in which the loops of site I and site II were replaced by the canonical loop (EF-site) of parvalbumin in site I; NC mutant, S100A2 with the EF-loop of parvalbumin in site I; PstI/BamHI digested expression vector pSKC-DNA (using primers A2P3/A2P6) was then cloned into the Bluescript SK+ vector (Stratagene). PCR products of wtS100A2 (using primers A2P1/A2P6) and of the N mutant (using primers A2P1/A2P2 and A2P3/A2P6) were cleaved with the corresponding restriction enzymes. These DNA fragments were cloned into PstI/BamHI-digested Bluescript K5+ vector as a three-fragment ligation (pSKC, A2 mutant). The PCR product of the cDNA was then cloned into the Bluescript K5+ vector (Stratagene). PCR products of wtS100A2 (using primers A2P1/A2P6) and of the N mutant (using primers A2P1/A2P2 and A2P3/A2P6) were cleaved with the corresponding restriction enzymes. These DNA fragments were cloned into PstI/BamHI-digested Bluescript K5+ vector as a three-fragment ligation (pSKC, A2 mutant). The PCR product of the cDNA was then cloned into the Bluescript K5+ vector. The PCR product of the cDNA was then cloned into the Bluescript K5+ vector.

**Experimental Procedures**

*Construction of Wild Type and Mutated Expression Vectors—To replace one or both of the functional Ca^{2+} N- or C-terminal domain of wtS100A2 (GenBankTM/EMBL accession number M78076) by a canonical loop (EF-domain) of human a-PV, the human cDNA (GenBankTM/EMBL accession number X63070) was amplified by PCR on Perkin-Elmer and Hybaid Omni-Gene thermal cyclers, using specific oligonucleotides spanning the sequence of the EF-site of PV and specific restriction sites for subsequent cloning into the Bluescript K5+ vector (Stratagene).

PCR products of wtS100A2 (using primers A2P1/A2P6) and of the N mutant (using primers A2P1/A2P2 and A2P3/A2P6) were cleaved with the corresponding restriction enzymes. These DNA fragments were cloned into PstI/BamHI-digested Bluescript K5+ vector as a three-fragment ligation (pSKC, wtS100A2; pSKN, N mutant), following by DNA sequencing using the AutoRead sequencing kit on an A.L.F. Sequencer (Amersham Pharmacia Biotech). The NC mutant was created in two steps; the first PCR products (using primers A2P1/A2P4 and A2P5/A2P6) were cloned into the PstI/BamHI-digested Bluescript K5+ vector as a three-fragment ligation (pSKC, C mutant). The PCR product of the cDNA was then cloned into the Bluescript K5+ vector.

**Oligonucleotide Sequences—**Oligonucleotides were synthesized on an Applied Biosystems 394 DNA synthesizer (Pharmacia LKB). The primers used to amplify the cDNA were as follows: A2P1, 5'-GCGCTGCGACGATCCATGACTGC-3'; A2P2, 5'-CATTTGCGACAGCCATTTCCGTCCTCAGACATTTTGGGCTTTCGTCACCATATT-3'; A2P3, 5'-ATTGGTGTCAGCAAATGGACATTTTGCACACAAG-3'; A2P4, 5'-GGGATCCACACCCAAATTTTGCCGCTCCTTCTTGGAGTTTCTGCTTTCCGGGCTACCAT-3'; A2P5, 5'-GACGAATCTGCTTTTCTGGGGCAGATTTTTG-3'; and A2P6, 5'-CAGGGATCCAGAGGTTCTGGCTTTCCAGACATTTTGGGCTTTCGTCACCATATT-3'.

**Gel Electrophoresis and Mass Spectrometry—**Tricine SDS-PAGE (15%) was performed as described previously (34). For reducing conditions, 150 mM DTT was added to the sample buffer. Proteins were visualized by Coomasie Blue or silver staining. The molecular mass of the purified recombinant S100A2 proteins was analyzed by electrospray ionization mass spectrometry as described (25).

**Western Blot Analysis—**Western blot analysis was performed as described previously (17). The rabbit polyclonal antibodies against human recombinant S100A2 and human recombinant S100A4 were diluted 1:5000 and 1:20,000, respectively. The protein bands were visualized using alkaline phosphatase-conjugated secondary antibodies (Promega) diluted 1:3000.

**Metal Ion Determination and Metal Removal—**Removal of contaminating metals, equilibration of the protein in the assay buffer, and determination of total Ca^{2+} and Mg^{2+} concentrations were performed as described previously (35). The protein concentration was determined from the ultraviolet absorption spectrum using a molar extinction coefficient of 278 nm of 3030, 2920, and 1640 M^{-#1 cm^{-#1} for wild type, N mutant, and NC mutant monomer, respectively.

**Secondary Structure Monitored by Circular Dichroism—**CD spectra were acquired with a Chou J-T15 spectropolarimeter on solutions of 0.25 mg/ml protein in 5 mM Tris-HCl buffer, pH 7.5, in a cell of 1 mm optical path. Ellipticities were normalized to residue concentration using the relationship \( \theta_{new} = \theta_0 M/M_c \), where \( \theta_0 \) represents the observed ellipticity in degrees, \( M_c \) is the average molecular weight of an amino acid residue in the polypeptide, \( l \) is the path length in mm, and \( c \) the protein in g/liter.

**Cation Binding—**Ca^{2+} binding was measured at 25 °C by the flow dialysis method (36) in 50 mM Tris-HCl pH 7.5, 150 mM KCl (buffer A). Protein concentrations were 40–70 \( \mu \)g. Treatment of the raw data and evaluation of the metal binding constants were as described earlier (35). Since the \( \text{Ca}^{2+} \)-binding isotherms showed either positive or negative cooperativity, the data were analyzed with the equation of Adair,

\[
\gamma = (K_1[Ca^{2+}] + 2K_2[Ca^{2+}]^2 + \ldots + nK_n[Ca^{2+}]^n)/(1 + K_1[Ca^{2+}]) + K_2[Ca^{2+}]^2 + \ldots + K_n[Ca^{2+}]^n)
\]

where \( K_1, K_2, \ldots, K_n \) are the stoichiometric association constants for the binding of the first, second, and \( n \)th \( \text{Ca}^{2+} \) to the protein. The data were fitted according to two binding models. In the first, it was assumed that, given the structural symmetry in the dimer, S100A2 and the mutants display two pairs of identical sites; in the second, it was assumed that the proteins display four different \( \text{Ca}^{2+} \)-binding sites, implying allosteric interactions between the monomers. 

**Optical Methods to Probe the Environment of Tyr—**Emission fluorescence spectra were taken with a Perkin-Elmer LS-5B spectrophluorimeter on metal-free proteins at room temperature, with excitation and emission slits of 2.5 and 10 nm, respectively. The excitation wavelength was 280 nm. 5 mM EGTA or 5 mM CaCl_2 or 0.5 mM ZnCl_2 was added to obtain the metal-free, \( \text{Ca}^{2+} \) or \( \text{Zn}^{2+} \) forms, respectively. Denaturation curves were obtained by monitoring the Tyr fluorescence change upon titration of the metal-free proteins with guanidine-HCl and analyzed according to the method of Pace et al. (37).

\[
\Delta G_0 = -RT\ln K = \Delta G(H_2O) - m[GdnHCl]
\]

where \( \Delta G(H_2O) \) represents the intrinsic stability of the protein, and \( m \) is the factor that describes the sensitivity on denaturant concentration [GdnHCl].

In titration experiments, the data were normalized between 0 (ap-
oprotein) and 1 (fully ligand-saturated protein). In the fluorimetric titration of wtS100A2 with Ca\(^{2+}\) described in Fig. 4, the concentration of protein–bound Ca\(^{2+}\) can be neglected compared with that of added Ca\(^{2+}\), so that the latter is equal to the free Ca\(^{2+}\) concentration.

**Titrations of Exposed Hydrophobicity**—The Ca\(^{2+}\) - and Mg\(^{2+}\) -dependent changes in hydrophobic matrices of S100A2 or derivatives were followed by monitoring the fluorescence properties of 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS) as described by Pan and Johnson (38). Solutions of 40 \(\mu\)M bis-ANS and 2 \(\mu\)M metal-free proteins (after the addition of 25 mM EGTA) were excited at 390 nm, and the emission spectrum was recorded with slits of 5 nm. 2 mM CaCl\(_2\) or 1 mM ZnCl\(_2\) was added to obtain the Ca\(^{2+}\) or Zn\(^{2+}\) form, respectively.

**Protein Reduction and Thiol Reactivity**—The thiol reactivity was assayed after overnight incubation with 100 mM DTT at pH 8.5 followed by desalting on a Sephadex G-25 column equilibrated in nitrogen-saturated buffer A. The thiol reactivity was monitored by measuring the kinetics of the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) or Ellman's reagent by spectrophotometry at 412 nm according to the kinetics of the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) or Ellman's reagent by spectrophotometry at 412 nm according to the kinetics of the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) or Ellman's reagent by spectrophotometry at 412 nm according to Riddles et al. (39).

**RESULTS**

**Expression, Purification, and General Properties of Recombinant Human S100A2 Protein and Two Mutants**—S100A2 mutants were designed to produce proteins with alterations in Ca\(^{2+}\)-binding sites I and/or II; the introduced amino acid sequence changes are shown in Fig. 1. In the N mutant, the 14-residue-long loop of site I was replaced by a canonical 12-residue-long loop, corresponding to the sequence of the EF-site of human α-FV. To create the NC mutant, site II of S100A2 was replaced by the same EF-site of PV (see “Experimental Procedures”). The NC mutant has an additional amino acid exchange, Y76F, due to the EcoRI restriction site, which was introduced for the cloning procedure. Both mutants should possess two functional HLH-type Ca\(^{2+}\)-binding sites. The cDNA coding for all S100A2 proteins was then cloned into the prokaryotic expression vector pGEMEX for bacterial expression, giving rise to the construct pX2 (wtS100A2), pXN (N mutant), or pXNC (NC mutant). Extracts of bacterial cultures, before and after induction with isopropyl-β-D-thiogalactopyranoside, were subjected to SDS-PAGE (Fig. 2). Due to very low expression, low solubility, and unusual biochemical properties, it was not possible to purify sufficient amounts of the analogous C-mutant for this study. The reason for this is still not known.

High expression levels of wtS100A2 and mutants were observed only after induction. Purification of wtS100A2 and two mutant proteins was achieved by ammonium sulfate precipitation followed by hydrophobic interaction chromatography on a phenyl-Sepharose column. The average yield of purified proteins was 5–12 mg/250 ml of bacterial culture. Recombinant proteins were homogeneous as judged by SDS-PAGE (Fig. 2A). The wtS100A2, N mutant, and NC mutant proteins (lanes 1–3) migrated as monomers and dimers. The occurrence of dimers, despite the treatment with DTT, has already been observed previously (14) and suggests that some thiols are very rapidly reoxidized during SDS-PAGE (also see below). The ratio between monomers and dimers varied from the wtS100A2 to the mutants, since the NC mutant (lane 3) showed significantly fewer dimers than wtS100A2 and N mutant (lanes 1 and 2).

The apparent molecular weights of S100 protein monomers estimated from SDS-PAGE are always too low when compared with the molecular mass calculated from the sequence data. To verify the correct synthesis and molecular weight of the S100A2 proteins, we determined their exact masses by electrospray ionization mass spectrometry. The molecular masses of all proteins were found to be in good agreement with the calculated values (Table I). From these data, we conclude that our recombinant S100A2 proteins represent the full-length proteins.

**Immunological Properties of wtS100A2 and Mutants**—The immunological reactivity of the wild-type and mutant proteins with polyclonal S100A2 and α-FV antibodies was tested in Western blot analyses in the presence of Ca\(^{2+}\) (Fig. 2, B and C). With the polyclonal antibody against S100A2 (Fig. 2B), we observed a strong immunoreaction for the wtS100A2 monomer as well as for the dimer (lane 1). Additionally, a weak signal of higher molecular weight was detected for wtS100A2 (lane 1), probably representing a trimeric form of the protein. In contrast, only faint signals were detected with the mutants, especially with their dimeric forms (lanes 2 and 3). This immunoreaction indicates that the amino acid exchanges modify the protein structure of the mutants and therefore decrease the reactivity against the S100A2 antibody. The polyclonal antibody against human α-FV detected the monomer and dimer bands of the N mutant and an additional band of ~16 kDa of unknown origin (Fig. 2C, lane 2). This band was barely visible by protein staining and may represent the Ca\(^{2+}\) form of this mutant. In the case of the NC mutant, only the monomers showed a signal with the antibody against human PV (lane 3). The stronger immunoreaction with the N mutant indicates...
that the additional EF-loop in the NC decreases the immuno-reactivity of this mutant. No reactivity was observed against wtS100A2 (Fig. 2C, lane 1), indicating that the PV antibody recognizes the EF-site of PV in the mutant proteins.

**Biophysical Characterization of wtS100A2 and Mutants—**
Size exclusion chromatography on a TSK125 gel filtration column (300 × 7.8 mm) equilibrated in buffer A under reducing conditions was carried out to determine if S100A2 and its mutant proteins existed as monomers or dimers in solution (data not shown). All protein forms eluted as single sharp peaks except for the Zn2+-loaded N mutant. Using the calibration mixture of protein standards from Bio-Rad, we found apparent molecular masses of 26–28 kDa, suggesting that they behave as dimers. This is in agreement with several other S100 proteins, even those that cannot form an intermolecular disulfide bridge. Zn2+-loaded N mutant showed an apparent molecular mass of 16 kDa, but the elution peak is broad and asymmetric, suggesting that this mutant partially dissociates into monomers upon binding of Zn2+.

**Secondary Structure—** Fig. 3 shows the far UV CD spectra of the apo, Ca2+, and Zn2+ forms of wtS100A2 and the two mutants. The spectra of the Ca2+ and metal-free form of the wild type protein are superimposable with an estimated α-helix content of 50%. Zn2+ binding causes a slight, but significant, decrease in α-helix content. The ellipticities of the N and NC mutants are about 1.3-fold lower than that of wtS100A2, suggesting that the “normalization” of site I is at the expense of some α-helical conformation or implies a simple change in the orientation of the α-helices (40). The ellipticity of these mutants is minimally influenced by the binding of Ca2+ or Zn2+.

In summary, the secondary structure does not change significantly upon binding of Ca2+ or Zn2+ to wtS100A2 or its mutants. This feature is reminiscent of calbindin D9k, for which the exclusive role as buffer was invoked as rationale (41).

**Direct Cation Binding Studies—** Ca2+ binding studies by flow dialysis revealed that the wtS100A2 dimer binds four Ca2+ with pronounced positive cooperativity (H = 2.04) and a [Ca2+]0.5 of 470 μM (Fig. 4). The N and NC mutants also bind four Ca2+ per dimer but at independent sites of different affinities or sites with negative cooperativity. The binding iso-

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**TABLE I**
Molecular masses of wtS100A2 and mutant proteins

|                | Theoretical | Measured  |
|----------------|-------------|-----------|
| wtS100A2       | 11,112.7    | 11,119.4  |
| N mutant       | 10,805.3    | 10,807.8  |
| NC mutant      | 10,583.2    | 10,586.8  |
therms were analyzed with the Adair equation assuming four sites with either pairwise identical affinities or with different affinities. The stoichiometric constants are summarized in Table II. Both models offer a reasonably good fit (thick and thin lines in Fig. 4), but for wtS100A2 the first model yields a calculated Hill coefficient of 1.87 with very discrepant values for the stoichiometric binding constants. This and the fact that the experimental value of the Hill coefficient is above 2 strongly suggest that the second model is more appropriate and that allosteric interactions occur between subunits of wtS100A2. Therefore, only the model of four different binding constants is further investigated in this study. In the N and NC mutants the low affinity sites have changed to high affinity sites, since binding of the first Ca\(^{2+}\) to the mutants occurs with a 100–400-fold higher affinity than in wtS100A2. In contrast, the second Ca\(^{2+}\) binds with 2–7-fold higher affinities in the N and NC mutant, respectively.

Punctuate Zn\(^{2+}\) binding studies by the Hummel-Dryer method revealed that S100A2, N mutant, and NC mutant bind two Zn\(^{2+}\) atoms per monomer without apparent cooperativity (Fig. 5). Scatchard analysis yielded intrinsic affinity constants \((K)\) of \(2.2 \times 10^5, 5.3 \times 10^4,\) and \(1.1 \times 10^5\) M\(^{-1}\) for wtS100A2, N mutant, and NC mutant, respectively. Hummel-Dryer experiments on S100A2 in the presence of both Ca\(^{2+}\) and Zn\(^{2+}\) (data not shown) suggest that the two cations do not bind to the same sites but nevertheless weaken each other’s affinity. The NC mutant fractions containing more than 300 µM protein are turbid.

Intrinsic Tyr Fluorescence—wtS100A2 and the N and NC mutants each possess a Tyr\(^{20}\), adjacent to the amino terminus of the site I Ca\(^{2+}\)-binding loop (Fig. 1). In addition, wtS100A2 and the N mutant possess a Tyr\(^{76}\) adjacent to the carboxyl terminus of the site II loop. The microenvironments of both Tyr residues may thus be strongly influenced by Ca\(^{2+}\) binding. Indeed, the metal-free forms of the three proteins show unusual fluorescence emission spectra, i.e. maxima at 320 nm that are blue-shifted to 310 in the presence of Ca\(^{2+}\) or Zn\(^{2+}\) (Fig. 6). In wtS100A2, cation binding is accompanied by an important (Zn\(^{2+}\)) to moderate (Ca\(^{2+}\)) enhancement of the fluorescence intensity. In the different native forms of the N mutant, the fluorescence intensities are lower but qualitatively similar to wtS100A2; they resemble those of S100A6, which possesses two Tyr in exactly the same positions. The fluorescence of the NC mutant yields low signals, and the spectra are qualitatively different from those of wtS100A2 and N mutant. This suggests that the environment of Tyr\(^{76}\) is much more perturbed by ion binding than Tyr\(^{20}\), the only probe in NC. They are similar to S100B, which also has one Tyr in the same position as the NC mutant. For all three proteins, the spectra in the presence of Mg\(^{2+}\) are the same as those of the apo forms (not shown), thus confirming the general conclusion that S100 proteins do not interact significantly with Mg\(^{2+}\). Positive cooperativity implies an allosteric conformational change after binding of the first Ca\(^{2+}\). A Ca\(^{2+}\) titration of the fluorescence change yielded a steep curve for wtS100A2 (Fig. 4, asterisks), slightly preceding the mean saturation curve.

Denaturation curves (Fig. 7A) of the metal-free proteins are very steep, and for the three proteins the deduced \(\Delta G\) varies linearly with the guanidine-HCl concentration (Fig. 7B), indicating a two-state folding mechanism (42). The characteristics are summarized in Table III. Interestingly, loop grafting does not modify dramatically the free energy change between the folded and unfolded form with a \(\Delta G(H_2O)\) varying from 4.0 and 5.2 kcal/mol. In contrast, grafting strongly affects the denaturant sensitivity factor \(m\). The 1.7-fold larger \(m\) value for wtS100A2 is an indication of increased cooperativity in folding. Thus, the metal-free mutant proteins may have the same packing as S100A2 but are more accessible to the denaturant.

Near UV Difference Spectra—The UV difference spectra (data not shown) of wtS100A2 show intense positive peaks at 280 and 288 nm (Fig. 7A) and are quite similar to those of wild type N mutant NC mutant

![FIG. 5. Zn\(^{2+}\) binding to wtS100A2 (triangles), N mutant (rectangles), and NC mutant (circles) as determined by the Hummel-Dryer method.](image)

The Scatchard plot yielded \(K\) values of \(2.2 \times 10^5, 5.3 \times 10^4,\) and \(1.1 \times 10^5\) M\(^{-1}\) and maximal binding of 1.95, 1.76, and 2.0 Zn\(^{2+}\) atoms for wtS100A2, N mutant, and NC mutant, respectively.

| Table II |
| Stoichiometric Ca\(^{2+}\) binding constants of wtS100A2, N mutant, and NC mutant |
| Analysis assuming four different Ca\(^{2+}\)-binding sites per dimer |
| Wild type | N mutant | NC mutant |
| \(K_1\) | \(1.7 \times 10^3\) | \(3.7 \times 10^3\) | \(4.2 \times 10^3\) |
| \(K_2\) | \(2.4 \times 10^4\) | \(1.2 \times 10^4\) | \(3.6 \times 10^4\) |
| \(K_3\) | \(2.4 \times 10^3\) | \(6.3 \times 10^3\) | \(1.4 \times 10^4\) |
| \(K_4\) | \(2.0 \times 10^3\) | \(4.9 \times 10^3\) | \(1.8 \times 10^4\) |
| \((K_1 \times K_2)^{-1}\) | \(2.0 \times 10^3\) | \(2.1 \times 10^4\) | \(3.9 \times 10^4\) |
| \((K_3 \times K_4)^{-1}\) | \(2.2 \times 10^3\) | \(5.6 \times 10^3\) | \(1.6 \times 10^4\) |

| Analysis assuming two pairs of identical Ca\(^{2+}\)-binding sites per dimer |
| Wild type | N mutant | NC mutant |
| \(K_1\) | \(3.0 \times 10^2\) | \(2.2 \times 10^3\) | \(3.9 \times 10^2\) |
| \(K_2\) | \(1.5 \times 10^3\) | \(6.0 \times 10^3\) | \(1.8 \times 10^4\) |
S100A6 (25), which has an identical topography of Tyr residues. The difference spectra of the N mutant are similar to those of S100A2 but show 2–3-fold lower intensities, which corroborates the fluorescence data. The difference spectra of the NC mutant are small. Together, our data indicate that Tyr76, adjacent to the Z-position of site II, is highly responsive to cations, whereas the environment of Tyr20, located in the N-terminal helix of site I, is minimally perturbed by Ca$^{2+}$ and Zn$^{2+}$.

**Bis-ANS Fluorescence Enhancement**—In polar solvents, the...
fluorescence of bis-ANS is weak, but it is strongly enhanced if the probe is associated with hydrophobic surface patches of proteins or other biomaterials (35). Fig. 8A shows how the different forms of wtS100A2 enhance the fluorescence of bis-ANS: 2-fold for the metal-free and Mg$^{2+}$ form, 4- and 6-fold for the Ca$^{2+}$ and Zn$^{2+}$ forms, respectively. The N mutant also shows a 2-, 3- and 4-fold enhancement in the metal-free, Ca$^{2+}$, and Zn$^{2+}$ forms, respectively (Fig. 8B). These data clearly indicate that Ca$^{2+}$ and Zn$^{2+}$ induce a hydrophobic patch at the surface, which is qualitatively similar in the wild type and N mutant. The metal-free form of the NC mutant enhances the bis-ANS fluorescence 3-fold, Zn$^{2+}$ and Ca$^{2+}$ forms do not show a significant enhancement, and Ca$^{2+}$ binding even leads to a slight decrease (Fig. 8C).

Thiol Reactivity—wtS100A2 contains four Cys residues per monomer in positions 3, 22, 87, and 94 (i.e. three outside the Ca$^{2+}$-binding sites and one in site I), whereas the N and NC mutants lack Cys$^{22}$ (Fig. 1). After thorough reduction and DTT removal as described by Föhr et al. (31), thiol titration with DTNB revealed that 2.8–3.0 thios reacted rapidly in wtS100A2 ($t_{1/2} < 10$ s), 2.0–2.3 in N mutant, and 1.8–1.9 in NC mutant. The thiol reactivity is not affected by Ca$^{2+}$, and the addition of 4 M guanidine-HCl did not uncover more thiols than could be titrated in the native proteins. It is likely that one Cys residue is very reactive and rapidly reoxidized to cystine after removal of the excess DTT, since during SDS-PAGE such disulfide bridges are not resolved.

Addition of 4 M guanidine-HCl did not uncover more thiols than could be titrated in the native proteins. It is likely that one Cys residue is very reactive and rapidly reoxidized to cystine after removal of the excess DTT, since during SDS-PAGE such disulfide-bridged covalent dimers are also observed (Fig. 2A and Ref. 14).

**DISCUSSION**

S100A2 exerts its function in the cell nucleus by some presently unknown mechanism. Even in the dimeric state, S100A2 is small enough to enter the nucleoplasm via the nuclear pore complex by passive diffusion (for a review, see Ref. 43). Nuclear accumulation may occur if S100A2 is selectively retained through binding to a nondiffusible component in the nucleoplasm, thus offering a plausible explanation why S100A2 is predominantly found in the nucleus. The inner membrane of the nuclear envelope contains inositol 1,4,5-trisphosphate- and cADPR-modulated Ca$^{2+}$-channels, which upon stimulation may raise the local Ca$^{2+}$ to high levels. Nuclear and cytoplasmic Ca$^{2+}$ signals in turn act on gene expression by coincidental activation of different components of the basic transcription machinery (44, 45). Hence, as a candidate tumor suppressor gene, S100A2 may well link the nuclear Ca$^{2+}$ signal to one of the above mentioned transcription components. Indeed, some S100 members activate nuclear protein kinases (e.g. S100B activates the new Ndr kinase and binds to bHLH transcription factors (46, 47). The question arises as to whether S100A2 has the correct Ca$^{2+}$ sensitivity and protein-interactive properties. The first purpose of this study was to evaluate these features. We expressed and purified the protein to electrophoretic homogeneity, verified the correct molecular weight, and observed, as for all other S100 members, that the protein is homodimeric, not merely by noncovalent interaction but also through formation of disulfide bridges. Usually, S100 proteins occur as dimers in vitro and in vivo, but S100A2 is also able to form higher polymers under nonreducing conditions (17).

In common with most S100 members, S100A2 binds four Ca$^{2+}$ ions per protein dimer with moderate affinity but with much greater positive cooperativity than in S100A4, S100A6, S100A11, etc. Our study does not allow us to identify the site to which Ca$^{2+}$ binds first or even to determine whether the binding to this allosteric unit is sequential as in Nereis sarcoplasmic Ca$^{2+}$-binding protein SCP (48). But it is likely that site I has a lower affinity than in other S100 members, since the sequence CQ (underlined in Fig. 1) in the Ca$^{2+}$-binding loop of site I deviates strongly from the G(R/K) sequence encountered in the other members. Our data indicate that the four Ca$^{2+}$-binding sites form an allosteric unit and that binding of the first two Ca$^{2+}$ ions overcomes an energetically expensive conformational change and leads to a facilitated binding of the next two Ca$^{2+}$ ions. wtS100A2 binds also two Zn$^{2+}$ per monomer with an affinity of $2 \times 10^5$ M$^{-1}$, i.e. 2-fold higher than in the case of the unusual S100A3 (31). We have direct evidence that Zn$^{2+}$ does not bind to the same sites as Ca$^{2+}$ and that, contrary to the case of some other S100 members, Ca$^{2+}$ and Zn$^{2+}$ do not act synergistically but rather in an antagonistic manner. Where are the two potential Zn$^{2+}$-binding sites located? The high affinity for

### Table III

Parameters characterizing the guanidine-HCl denaturation of wtS100A2 and the two mutant proteins

| [GdnHCl]$_{0.5}$ | $M$ | $\Delta G_{H_{2}O}$ |
|------------------|-----|-------------------|
|                  | kcal mol$^{-1}$ | kcal mol$^{-2}$ |
| wtS100A2         | 1.9  | 2.50             | 4.46             |
| N mutant         | 3.6  | 1.45             | 5.16             |
| NC mutant        | 2.9  | 1.38             | 3.98             |

**Fig. 8. Hydrophobic exposure as monitored by fluorescence enhancement of bis-ANS.** Spectra are shown of 40 $\mu$M bis-ANS in the presence of 2 $\mu$M wtS100A2 (A), N mutant (B), or NC mutant (C) in the absence of divalent cations (dotted lines) or in the presence of 0.5 $\mu$M Zn$^{2+}$ (dashed lines), 5 $\mu$M Ca$^{2+}$ (solid lines), or 5 $\mu$M Mg$^{2+}$ (thin dashed line). The dashed and dotted lines represent the fluorescence of 40 $\mu$M bis-ANS alone.

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\[2\] B. W. Schäfer, and C. W. Heizmann, unpublished data.
Ca\(^{2+}\) and Zn\(^{2+}\) Binding to S100A2 and Mutants

Zn\(^{2+}\) suggests that each binding site contains clusters of at least two His and/or Cys residues (49). If human S100A2 has a three-dimensional structure similar to S100A6 or S100B, two clusters are observed per monomer: His\(^{18}\) + Cys\(^{22} +\) His\(^{40}\) and Cys\(^{3} +\) Cys\(^{87} +\) Cys\(^{93}\). Since our data showed that the thiol pools were well exposed to the polar solvent, these may well represent potential Zn\(^{2+}\)-binding sites and would constitute a novel Zn\(^{2+}\)-binding motif.

Ca\(^{2+}\) and Zn\(^{2+}\) binding to S100A2 proceeds with minimal changes in the secondary structure but with marked changes in the environment of the two Tyr residues, as probed by fluorescence and near UV difference spectrophotometry. Bis-ANS fluorescence and near UV difference spectrophotometry. A very similar loop grafting mutation did not dramatically affect the global folding, although local conformational changes occur (see bis-ANS fluorescence). A very similar phenomenon is expected to occur in the case of Tyr fluorescence and near UV difference spectrophotometry. Bis-ANS fluorescence and near UV difference spectrophotometry. A very similar loop grafting mutation did not dramatically affect the global folding, although local conformational changes occur (see bis-ANS fluorescence). A very similar phenomenon is expected to occur in the case of Tyr fluorescence and near UV difference spectrophotometry. Bis-ANS fluorescence and near UV difference spectrophotometry. A very similar loop grafting mutation did not dramatically affect the global folding, although local conformational changes occur (see bis-ANS fluorescence). A very similar phenomenon is expected to occur in the case of Tyr fluorescence and near UV difference spectrophotometry. Bis-ANS fluorescence and near UV difference spectrophotometry. A very similar loop grafting mutation did not dramatically affect the global folding, although local conformational changes occur (see bis-ANS fluorescence). A very similar phenomenon is expected to occur in the case of Tyr fluorescence and near UV difference spectrophotometry. Bis-ANS fluorescence and near UV difference spectrophotometry. A very similar loop grafting mutation did not dramatically affect the global folding, although local conformational changes occur (see bis-ANS fluorescence). A very similar phenomenon is expected to occur in the case of Tyr fluorescence and near UV difference spectrophotometry. Bis-ANS fluorescence and near UV difference spectrophotometry. A very similar loop grafting mutation did not dramatically affect the global folding, although local conformational changes occur (see bis-ANS fluorescence). A very similar phenomenon is expected to occur in the case of Tyr fluorescence and near UV difference spectrophotometry.

In summary, the dimeric S100A2 displays an unusually low affinity for Ca\(^{2+}\) but very strong positive cooperativity. The latter is completely lost, but the Ca\(^{2+}\) affinity is strongly increased, in mutants with a canonical Ca\(^{2+}\)-binding loop in site I. Two Zn\(^{2+}\)-ions bind with an high affinity and induce, as Ca\(^{2+}\), conformational changes including an hydrophobic patch on the protein surface that may be instrumental for the interaction of S100A2 with nuclear targets.

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REFERENCES

1. Schäfer, B. W., and Heizmann, C. W. (1996) Trends Biochem. Sci. 21, 134–140
2. Kilby, P. M., Van Eldik, L. J., and Roberts, G. C. K. (1996) Structure 4, 1041–1052
3. Drohat, A. C., Amburgey, J. C., Abildgaard, F., Starich, M. R., Baldisseri, D., and Weber, D. J. (1996) Biochemistry 35, 11577–11588
4. Potts, B. C. M., Carlstrom, G., Okazaki, K., Hidaka, H., and Chazin, W. (1996) Protein Sci. 5, 2162–2174
5. Kligman, D., and Hilt, D. C. (1988) Trends Biochem. Sci. 13, 437–443
6. Lackmann, M., Rajasekharai, F., Ismaa, S. E., Jones, G., Cornish, C. J., Hu, S., Simpson, R. J., Moritz, R. L., and Geczy, C. L. (1995) J. Immunol. 156, 2981–2991
7. Hilt, D. C., and Kligman, D. (1991) in Novel Calcium-binding Proteins (Heizmann, C. W., ed) pp 65–103, Springer-Verlag, Berlin
8. Donato, R. (1991) Cell Calcium 12, 713–726
9. Heierhorst, J., Mann, R. J., and Kemp, B. E. (1997) Eur. J. Biochem. 249, 127–133
10. Treves, S., Scutari, E., Robert, M., Groh, S., Ottolina, M., Prestipino, G., Ronjat, M., and Zoratto, F. (1997) Biochemistry 36, 11496–11503
11. Schäfer, B. W., Wieki, R., Engelkamp, D., Matter, M. G., and Heizmann, C. W. (1996) Genomics 35, 635–640
12. Wicki, R., Marenholz, I., Mischke, D., Schäfer, B. W., and Heizmann, C. W. (1996) Cell Calcium 20, 459–464
13. Wicki, B., Schäfer, B. W., Erne, P., and Heizmann, C. W. (1996) Biochem. Biophys. Res. Commun. 227, 592–599
14. Heizmann, C. W. (1996) Mol. Cells 6, 629–636
15. Glenney, J. R., Kindy, M. S., and Zokas, L. (1989) J. Cell Biol. 108, 569–578
16. Engelkamp, D., Schäfer, B. W., Matter, M. G., Erne, P., and Heizmann, C. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6547–6551
17. Ilg, E. C., Schäfer, B. W., and Heizmann, C. W. (1996) Int. J. Cancer 68, 325–332
18. Lee, S. W., Tomasetto, C., and Sager, R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2825–2829
19. Lee, S. W., Tomasetto, C., Swisshelm, K., Keyomarsi, K., and Sager, R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2504–2508
20. Pedrocchi, M., Schäfer, B. W., Mueller, H., Eppenberger, U., and Heizmann, C. W. (1994) Int. J. Cancer 57, 684–690
21. Wicki, R., Fras, C., Schull, F. A., Heizmann C. W., and Schäfer, B. W. (1997) Cell Calcium 22, 243–254
22. Parker, C., Lakshmi, B., Pürer, B., and Sherer, G. V. (1994) DNA Cell Biol. 13, 343–351
23. Falke, J. J., Drake, S. E., Hazard, A. L., and Peersen, O. B. (1994) Q. Rev. Biophys. 27, 219–290
24. Linse, S., and Farsi, S. (1995) Adv. Second Messenger Phosphoprotein Res. 36, 89–151
25. Pedrocchi, M., Schäfer, B. W., Durussel, I., Cox, J. A., and Heizmann, C. W. (1994) Biochemistry 33, 6732–6738
26. Allen, B. G., Durussel, I., Walsh, M. P., and Cox, J. A. (1996) Biochem. Cell Biol. 74, 687–694
27. Durussel, I., Van Eldik, L. J., and Cox, J. A. (1997) Biochem. Biophys. Acta 1343, 139–143
28. Baufeld, J., Glasser, N., and Gerard, D. (1988) J. Biol. Chem. 263, 8192–8203
29. Leung, I. K. M., Mani, R. S., and Kay, C. M. (1987) FEBS Lett. 214, 35–40
30. Filipek, A., Heizmann, C. W., and Uzzick, J. (1990) FEBS Lett. 264, 263–266
31. Fehr, U. G., Heizmann, C. W., Engelkamp, D., Schäfer, B. W., and Cox, J. A. (1995) J. Biol. Chem. 270, 21056–21061
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32. Dell’Angelica, E. C., Schleicher, C. H., and Santome, J. A. (1994) J. Biol. Chem. 269, 28929–28936
33. Baffery, M. J., Harrison, C. A., Alewood, P., Jones, A., and Gezzy, C. L. (1996) J. Biochem. (Tokyo) 316, 285–293
34. Föhr, U. G., Weber, B. R., Müntener, M., Staudenmann, W., Hughes, G. J., Frutiger, S., Banville, D., Schafer, B. W., and Heizmann, C. W. (1993) Eur. J. Biochem. 215, 719–727
35. Cox, J. A. (1996) in Guidebook to the Calcium Binding Proteins (Celio, M., ed) pp. 1–14, Oxford University Press, Oxford
36. Colowick, S. P., and Womack, F. C. (1969) J. Biol. Chem. 244, 774–777
37. Pace, C. N., Shirley, B. R., and Thomson, J. A. (1990) in Protein Structure: A Practical Approach (Creighton, T. E., ed) pp. 311–330, IRL Press at Oxford University, Oxford
38. Pan, B.-S., and Johnson, R. G., Jr. (1996) J. Biol. Chem. 271, 817–823
39. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1983) Methods Enzymol. 91, 49–60
40. Gagne, S. M., Tsuda, S., Li, M. X., Chandra, M., Smillie, L. B., and Sykes, B. D. (1994) Protein Sci. 3, 1961–1974
41. Potts, B. C. M., Smith, J., Akke, M., Macke, T. J., Okazaki, K., Hidaka, H., Case, D. A., and Chazin, W. J. (1995) Nature Struct. Biol. 2, 790–796
42. Pace, C. N. (1996) Trends Biotechnol. 8, 93–98
43. Perez-Terzic, C., Javoni, M., and Clapham, D. E. (1997) BioEssays 19, 787–792
44. Hardingham, G. E., Chawla, S., Johnson, C. M., and Bading, H. (1997) Nature 385, 260–265
45. Finkbeiner, S., and Greenberg, M. E. (1997) BioEssays 19, 657–660
46. Baudier, J., Bergeret, E., Bertacchì, N., Weintraub, H., Gagnon, J., and Garin, J. (1995) Biochemistry 34, 7834–7846
47. Onions, J., Hermann, S., and Grundström, T. (1997) J. Biol. Chem. 272, 23930–23937
48. Précheur, B., Cox, J. A., Petrova, T., Mispelter, J., and Craescu, C. T. (1996) FEBS Lett. 395, 89–94
49. Vallee, B. L., and Auld, D. S. (1990) Biochemistry 29, 5647–5659
50. Johansson, C., Ullner, M., and Drakenberg, T. (1993) Biochemistry 32, 8429–8438
51. Smith, S. P., and Shaw, G. S. (1998) Structure 6, 211–222
52. Matsumura, H., Shiba, T., Inoue, T., Harada, S., and Kai, Y. (1998) Structure 6, 233–241
53. Drohat, A. C., Baldisseri, D. M., Rustandi, R. R., and Weber, D. J. (1998) Biochemistry 37, 2729–2740
54. Sastry, M., Ketchum, R. R., Cresczenzi, O., Weber, C., Luhieski, M. J., Hidaka, H., and Chazin, W. J. (1998) Structure 6, 223–231
55. Pauls, T. L., Durussel, I., Berchtold, M. W., and Cox, J. A. (1994) Biochemistry 33, 10395–10400
56. Rhynier, J. A., Durussel, I., Cox, J. A., Ilg, E., Schafer, B. W., and Heizmann, C. W. (1996) Biochim. Biophys. Acta 1313, 179–186
57. Johansson, C., Brodin, P., Grundström, T., Forsén, S., and Drakenberg, T. (1993) Eur. J. Biochem. 202, 1283–1290