Ions Binding to S100 Proteins: Structural Changes Induced by Calcium and Zinc on S100a and S100b Proteins†

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ABSTRACT: The S100a ($\alpha\beta$) and S100b ($\beta\beta$) proteins, from bovine brain, have in their dimer form two typical calcium binding sites as predicted from their amino acid sequence determination [Isobe, T., Ishioka, N., & Okuyama, T. (1981) *Eur. J. Biochem. 115*, 469–474]. The conformations of these proteins in the metal-free forms as well as in the presence of divalents cations have been studied by UV absorption spectroscopy, intrinsic fluorescence, sulfhydryl reactivity, and interaction with a hydrophobic fluorescent probe [6-(*p*-toluidino)naphthalene-2-sulfonic acid, TNS]. It appears that the S100a and S100b proteins interact with Ca^{2+} and Zn^{2+} ions while Mg^{2+} is ineffective. Ca^{2+} and Zn^{2+} induced different conformational changes. Calcium binding to S100a and S100b proteins affects essentially the C-terminal part of the α and β polypeptide chains as revealed by the exposure of one SH group by each subunit (probably cysteine-85α and -84β). In the case of S100a protein, Ca^{2+} ions induce also the exposure of most of the aromatic residues to solvent, an increase of the intrinsic protein fluorescence due to the Trp residues, and the exposure of the C-terminal hydrophobic sequence (74–90) to solvent. In the case of S100b protein, Ca^{2+} ions have only a slight effect on the spectroscopic characteristics of the protein and do not modify the hydrophobicity of the protein. Zinc binding to S100a and S100b proteins induces for both species a conformational change which causes an increase of the intrinsic fluorescence and in the case of S100b only provides a capacity of interacting with hydrophobic fluorescent probe (TNS). The properties of the Ca^{2+} and Zn^{2+} binding sites in both S100a and S100b protein have been studied by using the intrinsic protein fluorescence variations upon ion binding. We present evidence that Ca^{2+} and Zn^{2+} binding sites are different and that K^+ at physiological concentration has an antagonistic effect on the high-affinity Ca^{2+} binding sites. The properties of the Ca^{2+} binding sites are discussed by reference to the model of the crystal structure of the intestinal calcium binding protein. Furthermore, the effect of pH on the Ca^{2+}-free and Ca^{2+}-bound S100a protein was studied and explains the fluorescence decrease vs. pH decrease by static quenching process involving histidine residues instead of a Ca^{2+} release. Finally, the thermal stability of S100a in the presence and absence of Ca^{2+} was investigated by following the intrinsic fluorescence and absorption variations. It appears that in both cases S100a protein is affected upon heating.

The highly acidic, water soluble S100 protein, which was first reported by Moore (1965), is generally considered specific to the nervous system, where it is located mainly in the cytoplasm of glial cell (Hyden & McEwen, 1966; Ghandour et al., 1981; Legrand et al., 1981). However, many authors have recently demonstrated that several nonneuroectodermal cells contained S100 protein-like antigen which could not be distinguished immunologically from S100 protein of the nervous system (Cocchia et al., 1981; Steffanson et al., 1980; Kanamori et al., 1982; Suzuki et al., 1982).

The role of this protein is still unknown, but the fact that it can be detected in the brain of both invertebrates and vertebrates with a remarkably constant immunological response (Moore, 1973) indicates that this protein has been conserved throughout evolution and may therefore be fundamental to the functioning of the nervous system. Interest in this protein has been stimulated by the finding of Calissano et al. (1969, 1974) that it interacts specifically with calcium ions.

More recently Isobe et al. (1977) showed that the bovine brain S100 protein is a mixture of proteins, of which two, named S100a and S100b, predominate. Both these proteins are dimers with subunit composition of $\alpha\beta$ (S100a) and $\beta\beta$ (S100b). Sequence determinations have revealed that the α subunit differs from the β subunit mainly by the presence of a single Trp residue (Trp-90α) while among the 93 amino acids 54 are common to both subunits and 23 differ as a result of a single base mutation (Isobe & Okuyama, 1978, 1981). Moreover, the α and β subunits are characterized by the presence of a common cysteine residue in positions 85α and 84β, and the sequences reveal homology with the test sequence for the "calcium binding sites" proposed by Tufty & Kretsinger (1975), suggesting an evolutionary relationship with the family of the calcium binding proteins.

In this paper using spectroscopic methods (absorption, fluorescence) we show, as described by Calissano et al. (1969) for the crude S100 protein, that Ca^{2+} ions induce structural changes in the purified proteins, but furthermore we provide the first evidence that Zn^{2+} ions also specifically affect the conformation of the S100 proteins, Ca^{2+} and Zn^{2+} binding sites being different. Comparative analysis of the effect of Ca^{2+} and Zn^{2+} ions on the structure of the S100a and S100b proteins enabled some difference in the behavior of these proteins to be demonstrated. This allowed us to propose some new path of investigation to elucidate the biological function and the reason for the heterogeneity of this brain-specific protein fraction.

Experimental Procedures

Materials

All chemicals were high-grade commercial products. Ultrapure water (Milli Q Instrument from Millipore Corp.) was used. S100a and S100b proteins were obtained from bovine brain as described (Baudier et al., 1983). All absorption and fluorescence measurements were performed on air-equilibrated solutions contained in quartz cuvettes placed in a thermostatically controlled metallic container.

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Methods

Calcium and Zinc Removal from S100 Protein. S100 proteins were freed of divalent ions either by extensive dialysis successively against buffer plus 2 mM EDTA[1] and appropriate buffer in absence of EDTA or by trichloroacetic acid precipitation according to Haiech et al. (1981). In the last case, the residual calcium was <0.1 mol/mol of protein. Calcium concentration was measured by atomic absorption spectrophotometry by using a Varian Model 1150 apparatus. Note that the absorption, CD, and fluorescence spectra and the emission quantum yield of the S100 proteins deionized by dialysis or by trichloroacetic treatment were indistinguishable.

Absorption spectra and difference spectra were obtained on a Cary 219 spectrophotometer. Measurements were made with a light path of 1.0 cm. Correction for the scattered light was made as indicated by Gérard et al. (1975). The extinction coefficients were determined by assuming a molecular weight of 20900 for S100a and 21000 for S100b and using the protein concentration determined by the Coomassie blue technique (Bradford, 1976). Subsequently the protein concentration was routinely measured by UV spectroscopy assuming an extinction coefficient ϵ_{280} of 11500 and 3400 M^{-1} cm^{-1} for the S100a and S100b, respectively.

Fluorescence Measurements. Fluorescence spectra were obtained on an absolute spectrofluorometer (Fica 55), and the intrinsic fluorescence quantum yield of S100 proteins was determined as described by Gérard et al. (1975), taking free L-tryptophan (ϕ = 0.14) as a reference for S100a fluorescence and free L-tyrosine (ϕ = 0.14) as a reference for S100b. All measurements were made at 20 °C. The solutions (absorbance <0.1) were excited at 295 nm where Trp emission was selectively excited in the case of S100a and at 275 nm for S100b. Lifetimes τ were measured by the single photoelectron technique (Gérard et al., 1972). In the case of S100a, excitation was performed at 295 ± 5 nm, and fluorescence was observed at 342 ± 5 nm. In the case of the S100b protein excitation was performed at 275 nm and fluorescence observed at 310 ± 5 nm. The fluorescence decay profiles were analyzed statistically by using the residuals distribution form (Grinwald & Steinberg, 1974; Grinwald, 1976).

Fluorometric Titration. Titration of metal-free proteins by Ca^{2+} or Zn^{2+} was performed by adding aliquots of $CaCl_2$ or $ZnSO_4$ stock solution and correcting for dilution. pH titration was performed by adding aliquots of concentrated HCl or KOH solution and measuring the pH after each addition with a pH meter E 532 (Metrhom-Herisau).

Sulfhydryl Measurements. The sulfhydryl groups in the S100 protein were titrated by the method of Ellman (1959). The rate of reaction of sulfhydryl groups with DTNB was determined by following the increase in absorbance at 412 nm recorded in terms of the amount of free 5-nitro-2-thiobenzoate (RSH) produced. The extinction coefficient of RSH was the same in buffer and in 6 M guanidinium chloride and was assumed to be 13600 M^{-1} cm^{-1}.

Results

(1) Spectral Characteristics of the S100a Protein. The UV absorption spectrum of the S100a protein (Figure 1a) is typical of a Phe-rich Trp-containing protein. The shoulder at 293 nm is due to the single Trp residue, the maximum at 277 nm and the peak at 283 nm are due to tyrosyl residues in an apolar environment (Gérard et al., 1972), and the secondary peaks at 253, 259, and 268.5 nm are due to the vibronic absorption bands of Phe residues. The extinction coefficient is ϵ_{280} = 11500 M^{-1} cm^{-1}. When the protein is denatured in 6 M guanidinium chloride (Figure 1a), the absorption spectrum is highly modified; there is a blue shift of the maximum absorption, the peak at 283 nm disappears, and the extinction coefficient decreases, ϵ_{280} = 10000 M^{-1} cm^{-1}. The negative difference spectrum between the native and denatured proteins (Figure 1b) shows that the blue shift involves the absorption bands of all the aromatic residues, resulting from the unfolding of the polypeptide chains and the exposure of these residues to the aqueous solvent (Donovan, 1973). More precisely, the negative peak at 293 nm is characteristic of a blue shift of the Trp absorption while the double negative troughs at 285 and 278 nm are characteristic of a blue shift of the Tyr absorption

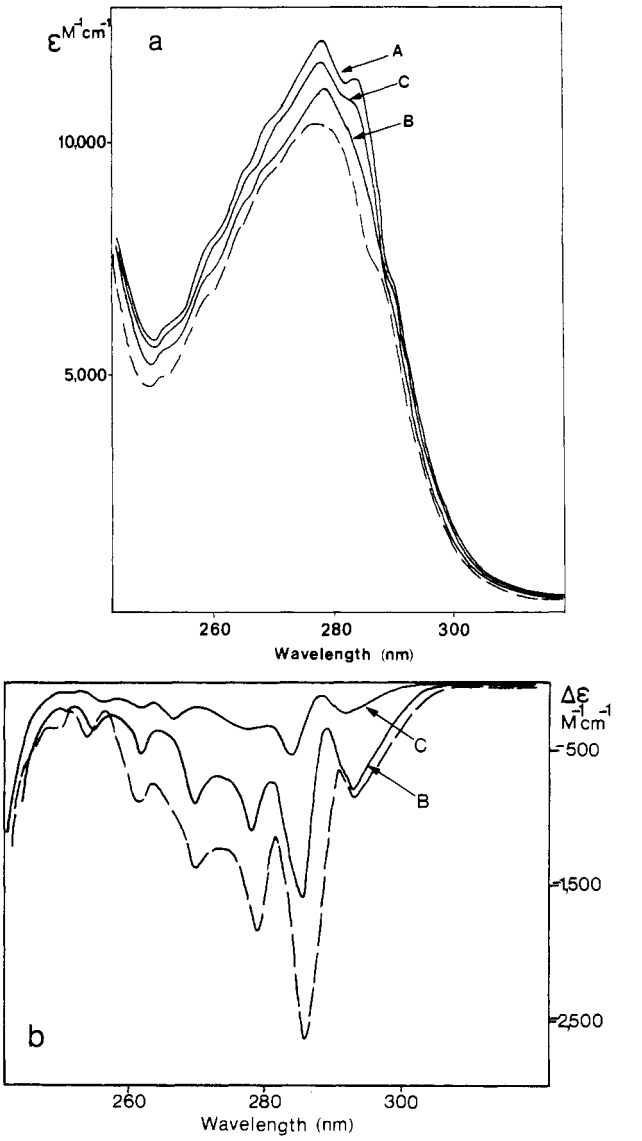

FIGURE 1: (a) Absorption spectra of (A) the S100a protein free of divalent cations in 100 mM Tris, pH 8.3; (B, C) the same protein in the presence of 4 mM Ca^{2+} and in the presence of 0.1 mM Zn^{2+}, respectively. The spectrum shown as a broken line was obtained in the presence of 6 M guanidinium chloride. (b) Difference spectra of the S100a protein in 100 mM Tris, pH 8.3. The reference cell contained S100a protein in buffer, and the sample cell contained S100a protein in buffer plus 4 mM $CaCl_2$ (B), 0.1 mM $ZnSO_4$ (C), and 6 M GdnCl (---).

[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ICaBP, intestinal calcium binding protein; pCa, −log [Ca^{2+}]; Phe, phenylalanine; pM, −log [metal]; pZn, −log [Zn^{2+}]; TNS, 6-(*p*-toluidino)naphthalene-2-sulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Trp, tryptophan; Tyr, tyrosine; RSH, 5-nitro-2-thiobenzoate; EDTA, ethylenediaminetetraacetic acid; GdnCl, guanidinium chloride.

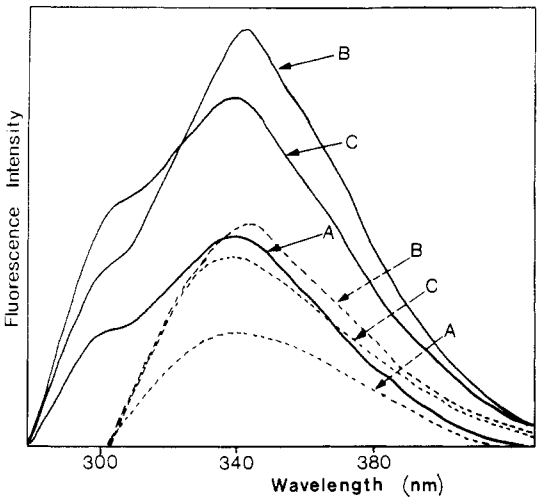

FIGURE 2: Fluorescence spectra for excitation at 275 (—) and 295 nm (---) of (A) the S100a protein free of divalent cations (8×10^{-6} M in 100 mM Tris, pH 8.3) and (B, C) the same protein in the presence of 4 mM $CaCl_2$ and in the presence of 0.1 mM $ZnSO_4$, respectively.

band. Moreover, the difference in the extinction coefficient at 285 nm, $\Delta\epsilon = -2500$ M^{-1} cm^{-1}, corresponds to the movement of three model residues (*N*-acetyl-D-tyrosinamide) from a hydrophobic to an aqueous environment (Gérard et al., 1972). This indicates that in the native S100a protein the three tyrosine residues are enclosed within the hydrophobic interior of the protein. (At the same wavelength, $\Delta\epsilon$ of the Trp residue is independent of the polarity of the environment and therefore does not interfere with $\Delta\epsilon$ of the Tyr residue.)

In the presence of 4 mM Ca^{2+}, the absorption spectrum of S100a is greatly modified (Figure 1a): the absorption maximum shifts to 279 nm, and the peak at 283 nm disappears. The differential spectrum (Figure 1b) recorded between the Ca^{2+}-free and the Ca^{2+}-bound proteins indicates that the environment of the aromatic chromophores is modified; the negative differential peaks indicate the exposure of these residues to the solvent. Quantitative analysis shows that Ca^{2+} binding induces the same modification of the Trp absorbance as denaturation, whereas it causes slightly more than half this variation in the Phe and Tyr residues.

In the presence of 0.1 mM zinc, the UV spectrum (Figure 1a) is also modified, but the differential spectrum (Figure 1b) shows that the environmental changes affecting the aromatic residues are less important than when calcium is present.

It should be noted that there is no modification of the absorption spectrum upon addition of 5 mM Mg^{2+}.

Fluorescence spectra were obtained by exciting the protein solution at 275 nm where both Tyr and Trp residues absorb and at 295 nm where Trp emission was selectively excited (Figure 2). The fluorescence spectrum of the native S100a protein excited at 275 nm exhibits a shoulder at 303 nm due to the tyrosyl contribution and a maximum at 340 nm due to Trp emission. When the protein is denatured in 6 M guanidinium chloride, the peak of tryptophan fluorescence shifts to 346 nm owing to the exposure of this chromophore to the aqueous solvent (result not shown).

In the presence of 4 mM Ca^{2+}, the fluorescence spectra are characterized mainly by a red shift of the maximum emission of Trp to 343 nm, indicating a partial exposure of this residue to the aqueous solvent.

The effect of 0.1 mM Zn^{2+} on the fluorescence spectrum of the S100a protein excited at 275 nm induces an increase of the tyrosine contribution at 303 nm, whereas the maximum of the Trp emission does not change.

As observed on the absorption spectrum, Mg^{2+} has no effect on the fluorescence spectrum of S100a protein.

(*2*) *Spectral Characteristics of the S100b Protein. The UV absorption spectrum* of the S100b protein is typical of a Tyr-containing protein characterized by a high Phe/Tyr ratio which lacks tryptophanyl residue (Figure 3a). The peaks at 277 and 283 nm correspond to the absorption bands of tyrosine in a nonpolar environment (Gérard et al., 1972); the peaks at 253, 258.5, 261.5, and 268 nm correspond to the fine structure of the phenylalanine absorption bands. The extinction coef-

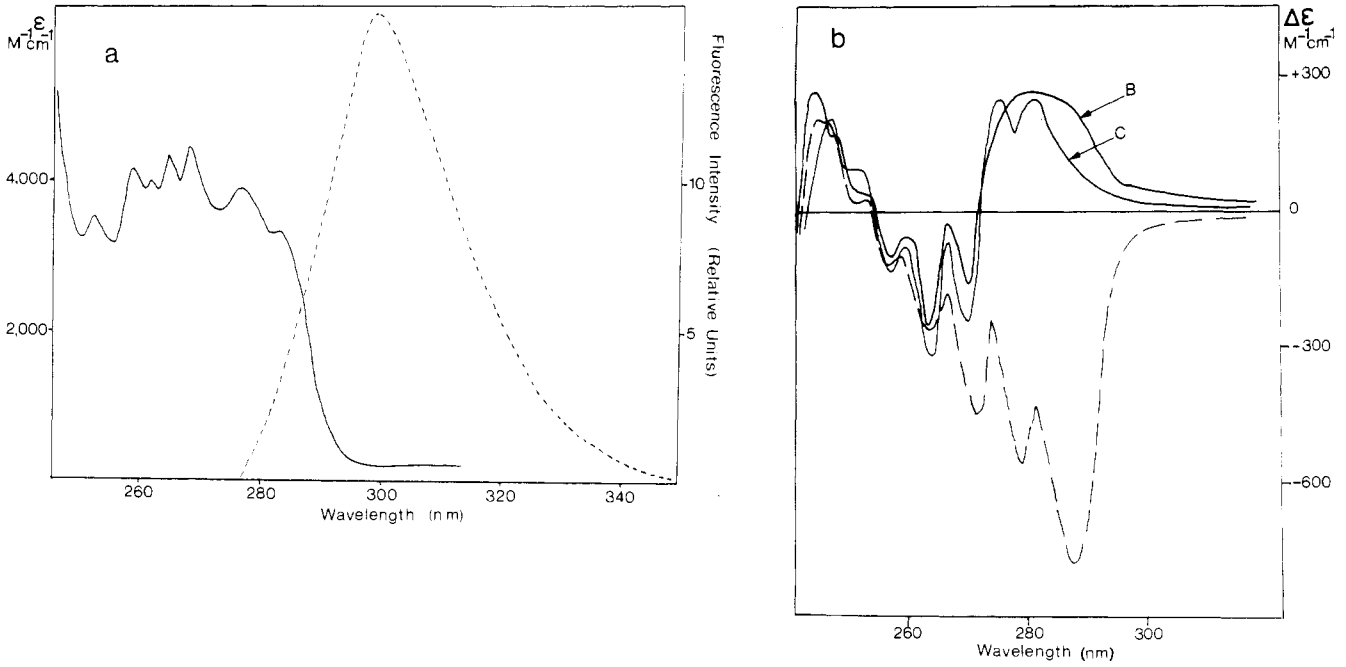

FIGURE 3: (a) Absorption and fluorescence spectra of the S100b protein in 100 mM Tris, pH 7.5. (b) Difference spectra of the S100b protein in 100 mM Tris, pH 7.5. The reference cell contained the S100b protein in buffer, and the sample cell contained S100b protein in buffer plus 2 mM $CaCl_2$ (B), 0.3 mM $ZnSO_4$ (C), and 6 M GdnCl (---).

Table I: Tryptophan Fluorescence Characteristics of the S100a Protein

conditions[b]	$-Ca^{2+}$ λ_{max}[a] (nm)	ϕ^{295}[a]	τ[a] (ns)	$\phi/\tau \times 10^{-7}$ (s^{-1})	ω[a]	$+Ca^{2+}$ λ_{max}[a] (nm)	ϕ^{295}[a]	τ[a] (ns)	$\phi/\tau \times 10^{-7}$ (s^{-1})	ω[a]
free of cations, pH 7.3	340	0.028	1.1	2.54	0.45	343	0.045	2.8	1.6	0.66
free of cations, pH 8.3	340	0.030	1.1	2.72	0.42	343	0.072	2.8	2.57	0.45
0.1 mM Zn^{2+}, pH 7.3	340	0.054	2	2.7	0.42					
0.1 mM Zn^{2+}, pH 8.3	340	0.054	2	2.7	0.42	341	0.087	3.2	2.7	0.42
denatured	346	0.085	2.1	4.1	0.12					
free L-tryptophan in water	350	0.14	3	4.67						

[a] λ_{max}, fluorescence maximum emission (±1 nm); ϕ^{295}, tryptophan fluorescence quantum yield (±0.0025); τ, fluorescence lifetime (±0.2 ns); ω, fraction of static quenching (see text). [b] All protein solutions (S100a at 8×10^{-6} M) were in 100 mM Tris buffer.

Table II: Tyrosine Fluorescence Characteristics of the S100b Protein

conditions[b]	ϕ^{275}[a]	τ[a] (ns)	$\phi/\tau \times 10^{-7}$ (s^{-1})	ω[a]
free of cations	0.01	0.5	2	0.5
4 mM Ca^{2+}	0.007	0.9	0.77	0.8
0.3 mM Zn^{2+}	0.06	2.1	2.8	0.3
denatured	0.038	1.4	2.4	0.4
free L-tyrosine in water	0.14	3.5	4	

[a] ϕ^{275}, tyrosine fluorescence quantum yield (±0.002); τ, fluorescence lifetime (±0.2 ns); ω, fraction of static quenching (see text). [b] All protein solutions (S100b at 8×10^{-6} M) were in 100 mM Tris buffer, pH 7.5.

ficient ϵ_{280} = 3400 M^{-1} cm^{-1}. When denatured in 6 M guanidinium chloride, the differential spectrum (Figure 3b) shows a blue shift of the absorption bands of the aromatic residues owing to their exposure to the aqueous solvent.

As seen on the differential spectrum (Figure 3b), addition of 2 mM Ca^{2+} as well as 0.3 mM Zn^{2+} results in an alteration in the tyrosine and phenylalanine environment. The positive differential peaks between 277 and 290 nm can be interpreted as a decrease in the exposure of tyrosine to the solvent while the negative bands between 275 and 250 nm correspond to a higher exposure of the Phe residues to the solvent.

The fluorescence spectra are typical of a Trp lacking protein (Figure 3a), with a maximum at 303 nm. As expected, the fluorescence spectra were not affected by denaturation in 6 M guanidinium chloride.

(3) Fluorescence Quantum Yields and Decay Times. The values of quantum yields and decay times were determined for both proteins under different experimental conditions: with or without denaturation of the proteins and in the absence or presence of divalent cations. The main results are indicated in Table I for the S100a protein and in Table II for the S100b protein.

For the S100a protein in the absence of divalent cations, at pH 8.3 the Trp quantum yield (ϕ = 0.03) and decay time (τ = 1.1 ns) are low compared with those of most Trp- and Tyr-containing proteins, and they are not considerably affected by pH changes ϕ = 0.025, and τ = 1.1 ns at pH 7.3. Denaturation in 6 M guanidinium chloride induces an increase in quantum yield (ϕ = 0.085) and decay time (τ = 2.1 ns) values.

Calcium titration of the S100a protein was carried out as illustrated in Figure 4. Calcium binding induces a pH-dependent increase in the quantum yield of the Trp fluorescence which is associated with a red shift to 343 nm of the maximum of emission.

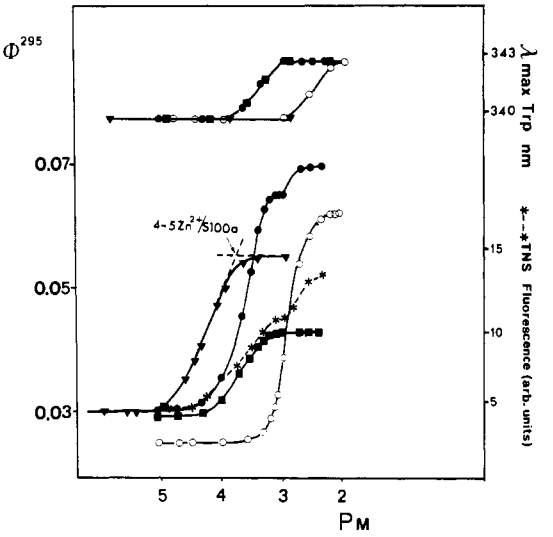

FIGURE 4: Effect of divalent cations on the intrinsic fluorescence quantum yield (ϕ^{295}) and emission maximum (λ_{max}) of the S100a protein. Effect of Ca^{2+} (S100a protein at 6×10^{-6} M in 100 mM Tris) at pH 8.3 (●), at pH 7.5 (■), and in the presence of 120 mM KCl at pH 8.3 (○). Effect of Zn^{2+} and S100a protein at 3×10^{-5} M in 100 mM Tris, pH 8.3 (▼). Similar experiments were performed by adding Ca^{2+} to the S100a protein (10^{-5} in 100 mM Tris, pH 8.3) in the presence of 20 μM TNS (*). The solution was excited at 365 nm, and the TNS fluorescence emission was measured at 440 nm.

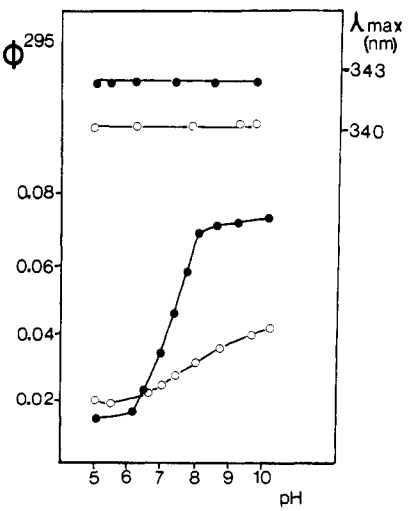

FIGURE 5: Variation of the tryptophan fluorescence quantum yield (ϕ^{295}) and maximum of emission (λ_{max}) in the S100a protein upon pH titration in the absence of (○) and in the presence (●) of 4 mM $CaCl_2$ (S100a at 6×10^{-6} M in 100 mM Tris).

The pH-dependent effect of Ca^{2+} on the fluorescence of S100a was studied following the Trp fluorescence quantum yield and emission maximum of the Ca^{2+}-free and Ca^{2+}-bound S100a vs. pH (Figure 5).

In both cases the fluorescence variations were reversible between pH 5 and pH 10. For the Ca^{2+}-free S100a protein variation in pH does not greatly affect the fluorescence quantum yield value. In contrast, in the presence of 4 mM Ca^{2+} an increase in the pH causes a marked variation in the Trp fluorescence quantum yield but does not affect the maximum of emission. There was no marked effect until pH 6.5, but the maximum effect is reached at pH 8.5. The shape of the titration curve indicates that the fluorescence is affected by some chemical groups which undergo changes in this pH range, characterized by a pK of about 7.3. The Trp quantum yield and decay time corresponding to the plateau, obtained with 4 mM Ca^{2+} at pH 8.3, are $\phi = 0.072$ and $\tau = 2.8$ ns, respectively, whereas at pH 7.3 the values are $\phi = 0.05$ and $\tau = 2.8$ ns (see Table I).

The effect of zinc titration of the S100a protein is also illustrated in Figure 4. Zinc binding on the S100a protein induces an increase in the Trp fluorescence quantum yield and decay time without any change in the maximum of emission. The fluorescence quantum yield and decay time corresponding to the plateau are $\phi = 0.054$ and $\tau = 2$ ns, respectively (see Table I). In contrast of the effect of calcium no pH effect on the quantum yield value for the Zn^{2+}-loaded protein was observed.

Mg^{2+} (5 mM) was shown to have no effect on the quantitative fluorescence parameters of the S100a protein.

In case of the S100b protein in the absence of divalent cations the Tyr fluorescence quantum yield is weak compared with most class A proteins ($\phi = 0.01$); the decay time is also very low ($\tau = 0.5$ ns). Denaturation in 6 M guanidinium chloride induces an increase in the quantum yield ($\phi = 0.038$) and in the lifetime of the excited state ($\tau = 1.4$ ns).

Calcium titration of the S100b protein was carried out as illustrated in Figure 6a. Calcium binding at pH 7.5 or 8.3 induces a decrease of 30% in the quantum yield of the Tyr fluorescence. The quantum yield corresponding to the plateau, obtained upon addition of 2 mM Ca^{2+}, is $\phi = 0.007$. In contrast the decay time increases up to 0.9 ns (see Table II).

The effect of zinc titration is shown in Figure 6b. Zinc binding induces a marked increase in the Tyr fluorescence intensity and decay time values: the quantum yield corresponding to the plateau is $\phi = 0.06$, and the decay time is $\tau = 2.1$ ns (see Table II).

(*4*) *Fluorescence Titration by Divalents Cations.* Fluorescence titration curves provide some information on the affinity and properties of ion binding sites.

In order to give an accurate interpretation of these data, we undertook a study of the ion binding sites of S100 proteins using the continuous flow dialysis method. As this work is in progress, only some preliminary results will be presented in this paper. Calcium binding studies of S100a and S100b proteins in the absence of monovalent ions pointed to the presence on both proteins of two types of calcium binding sites, the dissociation constant of the highest affinity sites being 2×10^{-5} M. In the presence of potassium (120 mM KCl) the affinity of these high-affinity sites was considerably reduced, as previously reported by Calissano et al. (1974), indicating an antagonistic effect of K^+ on Ca^{2+} binding.

Concerning zinc binding, flow dialysis experiments showed that S100a and S100b proteins have six to eight Zn^{2+} binding sites with dissociation constants ranging from 10^{-8} to 10^{-6} M.

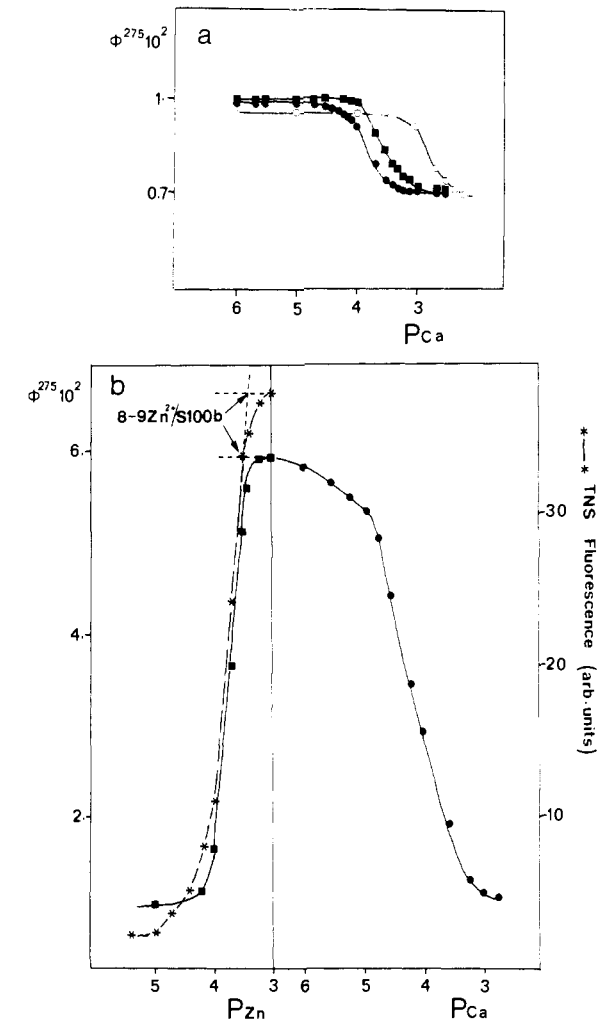

FIGURE 6: (a) Effect of Ca^{2+} on the intrinsic fluoresence quantum yield of the S100b protein (8×10^{-6} M in 100 mM Tris) (●) at pH 8.3, (■) at pH 7.5, and (○) in the presence of 120 mM KCl, pH 7.5. (b) Effect of Zn^{2+} on the intrinsic fluoresence quantum yield (ϕ^{275}) of the S100b protein (3.5×10^{-5} M) in 50 mM Tris at pH 7.5) (■). Similar experiments were performed by adding Zn^{2+} ions to the S100b protein 3.5×10^{-5} M) in the presence of TNS (*), the solution was excited at 265 nm, and the TNS fluorescence emission was measured at 340 nm. In the same figure is reported the effect of subsequent addition of Ca^{2+} on the fluorescence quantum yield of the eight Zn^{2+}-bound S100b (3.5×10^{-5} M in 50 mM Tris, pH 7.5) (●).

Zn^{2+} binding was not considerably affected by the presence of KCl.

Coming back to the fluorescence titrations induced by calcium binding, it is first to be noted that the rather low affinity of the sites with respect to the protein concentration used in the fluorescence measurement (10^{-5} to 5×10^{-5} M) does not allowed us to perform stoichiometric titration.

For the S100a protein, calcium binding induces a pH-dependent increase of the Trp quantum yield (see Figure 4) associated with a shift of the maximum of emission from 340 to 343 nm. At pH 7.3, the calcium titration curve is monophasic with an apparent dissociation constant determined at the transition midpoint of 2×10^{-4} M. At pH 8.3, the titration curve is biphasic. This could correspond to the titration of two kinds of Ca^{2+} binding sites with different affinities. The transition midpoint for the higher affinity site is 2×10^{-4} M. In the presence of 120 mM KCl at pH 8.3, the calcium titration curve is monophasic and shifted to higher Ca^{2+} concentration (Figure 4). The transition midpoint for 1.5×10^{-3} M Ca^{2+} indicates an important antagonistic effect of K^+ toward the calcium binding site titrated.

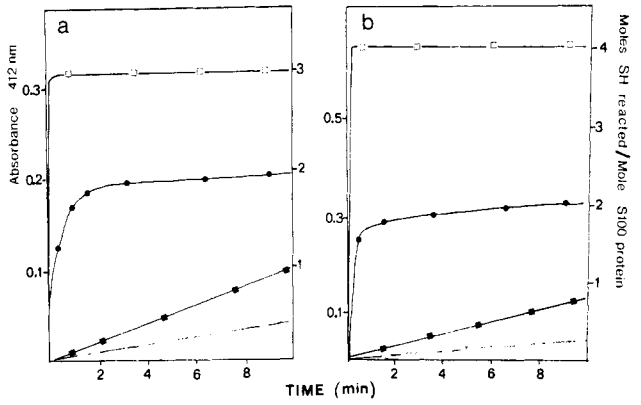

FIGURE 7: Reactivity of thiols (a) in the S100a protein (8×10^{-6} M in 100 mM Tris, pH 8.3) and (b) in the S100b protein (1.2×10^{-5} M in 100 mM Tris, pH 7.5) toward DTNB under various conditions: (○) free of divalent cations; (●) in the presence of calcium (4 mM); (■) in the presence of zinc (0.1 mM/S100a, 0.3 mM/S100b); (□) denatured in 6 M GdnCl.

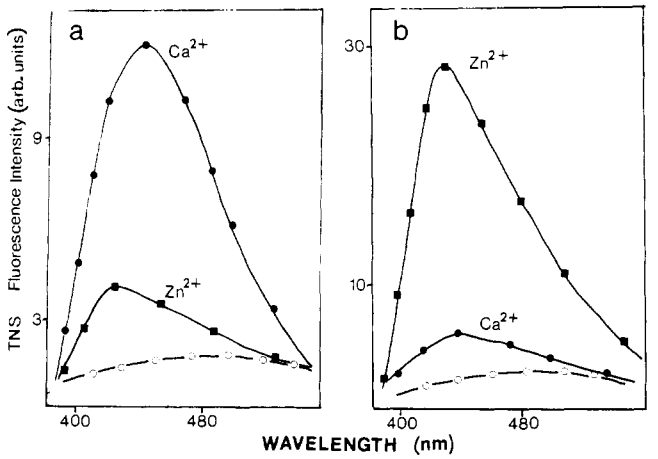

FIGURE 8: Fluorescence spectra of TNS bound to (a) S100a protein (8×10^{-6} M in 100 mM Tris, pH 8.3, with 20 μM TNS) and (b) the S100b protein (8×10^{-6} M in 100 mM Tris, pH 7.5, with 20 μM TNS) under various conditions: (○) protein freed of divalent cations; (●) in the presence of 4 mM Ca^{2+}; (■) in the presence of 0.1 mM Zn^{2+} (excitation at 365 nm).

In the absence of monovalent ions, Ca^{2+} binding to the S100b protein induced a decrease of the tyrosine fluorescence (Figure 6a). At pH 7.3 and 8.3, titration curves are monophasic with transition midpoints of 3×10^{-4} and 1.4×10^{-4} M, respectively. In the presence of 120 mM KCl the titration curve is shifted to higher Ca^{2+} concentrations with a transition midpoint at 1.5×10^{-3} M Ca^{2+} as for the S100a protein.

In Zn^{2+} binding experiments, the high affinity of the proteins for this ion ($K_D \simeq 10^{-8}$–10^{-6} M) allow stoichiometric titrations since the protein concentrations used are largely higher than 10-fold the K_D values.

Zn^{2+} titration curve of the S100a protein (Figure 4) showed a one-step transition plateauing at 4–5 mol of Zn^{2+} bound/mol of S100a protein.

Concerning the S100b protein, Zn^{2+} binding induced a high increase of tyrosine fluorescence and led to a monophasic transition curve, the plateau being obtained after binding of eight to nine Zn^{2+} per mol of S100b protein (Figure 6b).

For both proteins subsequent addition of Zn^{2+} induced sample precipitation.

In experiments where Ca^{2+} was subsequently added to the Zn^{2+}-bound S100 proteins, the following results were recorded. For the S100a protein, Ca^{2+} induced an additional increase of the Trp fluorescence with a shift of the maximum of emission to 341 nm (see Table I). The shape of the Ca^{2+} titration curve and the transition midpoint are identical with those recorded for Ca^{2+} binding on the Zn^{2+}-free protein (data not shown).

In the case of the S100b, subsequent addition of Ca^{2+} to the Zn^{2+}-bound S100b protein (eight Zn^{2+} per S100b) induced abolition of Tyr fluorescence. The back titration curve exhibits an apparent K_D (determined at the transition midpoint) of 5.5 $\times 10^{-5}$ M, much lower than the one observe for the Zn^{2+}-free S100b [K_D(app) = 3×10^{-4} M].

(*5*) *Reaction with Sulfhydryl Reagent.* When DTNB was added to the native and divalent ion free S100a or S100b protein, no SH groups proved to be accessible to the reagent, indicating that all the cysteine residues are enclosed (Figure 7). After denaturation by 6 M guanidinium chloride, all the sulfhydryl groups present in both proteins, i.e., three SH for S100a and four SH for S100b, were rapidly titrated.

Addition of calcium to the divalent cation free proteins markedly stimulated the reaction rate with DTNB. However, the effect remains limited to only two SH groups in both cases.

In the presence of zinc, the rate of reaction with DTNB is slower than with Ca^{2+} for the S100a protein, while no significant effect was observed with the S100b protein.

(*6*) *Interaction between TNS and the S100 Proteins.* It has been postulated that Ca^{2+} binding to the crude S100 protein induces a change in the protein conformation involving a partial exposure of one hydrophobic sequence to the solvent (Calissano et al., 1974). In the present paper we report a more systematic study on the different purified S100 proteins, in the absence or presence of divalent cations, using 6-(*p*-toluidino)naphthalene-2-sulfonate (TNS) as a hydrophobic probe. This probe is very weakly fluorescent in water but becomes strongly fluorescent when bound to hydrophobic sites of proteins (McClure & Edelman, 1966).

In the absence of divalent cations, the fluorescence of TNS when added to S100a and S100b proteins was negligible (see Figure 8).

After addition of calcium to the S100a protein in the presence of TNS, the intensity of emission of TNS is greatly enhanced, and the maximum of the TNS fluorescence spectrum shifts to 445 nm (Figure 8a). The curve of the TNS fluorescence as a function of a Ca^{2+} concentration is presented in Figure 4. This curve exhibits the same shape and the same transition midpoint at 2×10^{-4} M Ca^{2+} as the transition curve obtained by measuring the variation in Trp fluorescence. In contrast, no significant change in TNS fluorescence was observed for the Ca^{2+}-bound S100b protein (Figure 8b).

The results obtained after addition of Zn^{2+} to S100 proteins in the presence of TNS were opposite to those produced by addition of Ca^{2+}. No change in TNS fluorescence was observed when Zn^{2+} bound to S100a protein (Figure 8a) whereas a large increase in the TNS emission and a shift to 429 nm of the maximum of fluorescence spectrum were recorded for the Zn^{2+}-bound S100b protein (Figure 8b). It was noted that for S100b protein the titration curve of TNS fluorescence vs. Zn^{2+} concentration (Figure 6b) exhibits the same shape as do tyrosine fluorescence variations and that the maximum of hydrophobic change of the protein occurs after binding of eight to nine Zn^{2+} per S100b.

(*7*) *Heat Stability of S100a Protein.* We report in Table III the absorbance and fluorescence characteristics of S100a protein after it has been heated to 80 °C and cooled back to 20 °C in the absence and in the presence of 4 mM calcium. For comparison, the spectroscopic characteristics of the non-heated protein are also indicated.

In the absence of calcium all the spectroscopic parameters

Table III: Temperature Effect on the Absorption and Fluorescence Characteristics of S100a Protein

conditions[a]	20 °C				20 °C after heat treatment			
	ϵ_{280}[b] (M^{-1} cm^{-1})	λ_{max}^{295} (nm)	ϕ^{295}	τ (ns)	ϵ_{280}[b] (M^{-1} cm^{-1})	λ_{max}^{295} (nm)	ϕ^{295}	τ (ns)
free of cations	11 500	340	0.03	1.1	10 500	341	0.037	1.3
4 mM Ca^{2+}	10 700	343	0.072	2.8	10 700	343	0.046	2.1

[a] All protein solutions (S100a at 10^{-5} M) were in 100 mM Tris buffer, pH 8.3. [b] ϵ_{280} (M^{-1} cm^{-1}), molar extinction at 280 nm. For other symbols see footnote *a* of Table I.

and especially the absorbance properties are modified by the heat treatment at 80 °C. This indicates that the protein underwent an irreversible thermal denaturation in accordance with the results of Kessler et al. (1968) obtained by studying the immunological activity of the crude S100 protein.

In the presence of 4 mM calcium, the thermal treatment of the protein affects only the quantitative fluorescence parameters of the protein (ϕ^{295} and τ). These data indicate that heating of the Ca^{2+}-bound protein does not induce global structural changes but probably causes local disturbances which affect the Trp fluorescence quenching processes. This conclusion is in agreement with the results of Kessler et al. (1968), who noted that the antigenic activity of the Ca^{2+}-bound S100 protein is protected from thermal denaturation. Nevertheless the experiment indicates that a heat step in the purification procedure of S100 proteins should be avoided.

Discussion

Fluorescence Quenching Processes Occurring in S100a and S100b Proteins. In order to interpret the effect of the binding of ions on the structure of S100 proteins, it is initially necessary to analyze the emission quenching processes occurring in the ion-free proteins.

Dynamic quenching due to a diffusion-controlled encounter between the excited fluorophore and the quenching groups of the protein is evidenced by lifetime values being lower for the residue than for the model system (free L-tryptophan in water or free L-tyrosine in water).

Static quenching, related to a permanent contact between the chromophore and the quenching group, can be deduced from the ϕ/τ ratios since it induces a quantum yield decrease without modification of decay time values. The efficiency (ω) of this process can be estimated from ϕ/τ ratios of the protein and the monomer model system since the fraction ω of fluorescent residues subjected to this process is given by

$$\omega = 1 - \frac{(\phi/\tau)_p}{(\phi/\tau)_m}$$

where the subscripts p and m refer to the residues and the corresponding model system, respectively. The values of ω are given in Table I (S100a) and Table II (S100b).

Concerning S100a, both dynamic quenching and static quenching are very efficient. After denaturation by the chemical reagent, the Trp fluorescence is still susceptible to quenching, in particular dynamic quenching (τ = 2.1 ns), which could be explained by the fact that the Trp residue is not totally exposed to the solvent (λ_{max} 346 nm).

The S100b data presented in Table II show that a mean of one Tyr residue (out of two) emits (ω = 0.5). The denaturation mainly induces a partial removal of dynamic quenching.

pH Effect on the Fluorescence of S100a. We reported under Results that the tryptophan fluorescence quantum yield of the Ca^{2+}-loaded S100a is affected by interaction with chemical groups, the quenching properties of which were highly pH dependent with a p*K* of 7.3. The shape of the quenching titration curve (see Figure 5) is quite similar to the one obtained by Shinitzky & Goldman (1967) in studies of the Trp fluorescence quenching by protonated His residues in peptides and proteins. Since S100a is a histidine-rich protein (seven His residues), we could conclude that these residues are responsible for the pH-dependent quenching of tryptophan. Moreover, analyses of the decay time and ω value (see Table I) indicate that this process is due solely to variation in static quenching (ω = 0.66 at pH 7.3; ω = 0.45 at pH 8.3). This suggests that Ca^{2+} binding on S100a may induce a close contact between Trp and His residues exposed to the solvent. In this condition, the His residues can be protonated and form with Trp a quenching complex by charge transfer.

Ca^{2+} and Zn^{2+} Binding Induces Different Conformational Changes. Several results show that structural modification of S100a or S100b proteins after Ca^{2+} and Zn^{2+} binding is dependent on the nature of the ion.

(a) The first evidence for this fact is given by analysis of the differential UV absorption spectra. While both cations cause aromatic residues to be exposed to an aqueous solvent, it should be noted that the effect of Ca^{2+} is significantly higher than the Zn^{2+} effect in the case of S100a. On the contrary binding of Ca^{2+} or Zn^{2+} ions on S100b leads to similar changes in the environment of the aromatic residues.

(b) As for absorption, the fluorescence characteristics of S100 proteins are affected by Ca^{2+} and Zn^{2+} binding. However, it is quite obvious that these ions induce very different conformational changes.

In the case of S100a, Ca^{2+} binding causes a partial exposure of the Trp residue to the solvent which is associated with a large increase in quantum yield and decay time (ϕ = 0.072; τ = 2.8 ns) related to a decrease in the dynamic quenching. Moreover, it was shown that the quantum yield variation is highly dependent on the pH. Zinc binding induces a lower, pH-independent increase in the quantitative fluorescence characteristics (ϕ = 0.054; τ = 2 ns) without any exposure of the Trp residue.

In the case of S100b, Ca^{2+} and Zn^{2+} binding has opposite effects on the fluorescence intensity, namely, a quantum yield decrease for Ca^{2+} and an increase for Zn^{2+}. Moreover, the physical mechanism involved in the fluorescence variations is quite different. While Ca^{2+} and Zn^{2+} both lead to an enhancement of decay time values (τ varies from 0.5 to 0.9 and 2.1 ns upon binding of Ca^{2+} and Zn^{2+}, respectively), the static quenching (generally due to hydrogen-bond formation between the hydroxyl of tyrosine and CONH or COO^- groups) is increased upon Ca^{2+} binding (ω = 0.8) whereas it is greatly reduced upon Zn^{2+} binding (ω = 0.3). This last result indicates that the fluorescence enhancement induced by zinc could be related to a removal of the static quenching groups from the environment of Tyr residues (17β) after local structural changes.

(c) Reaction with sulfhydryl reagent has shown that for both S100a and S100b proteins, all the SH groups are embedded within the protein structure and that calcium binding induces a conformational change in these proteins leading to the ex-

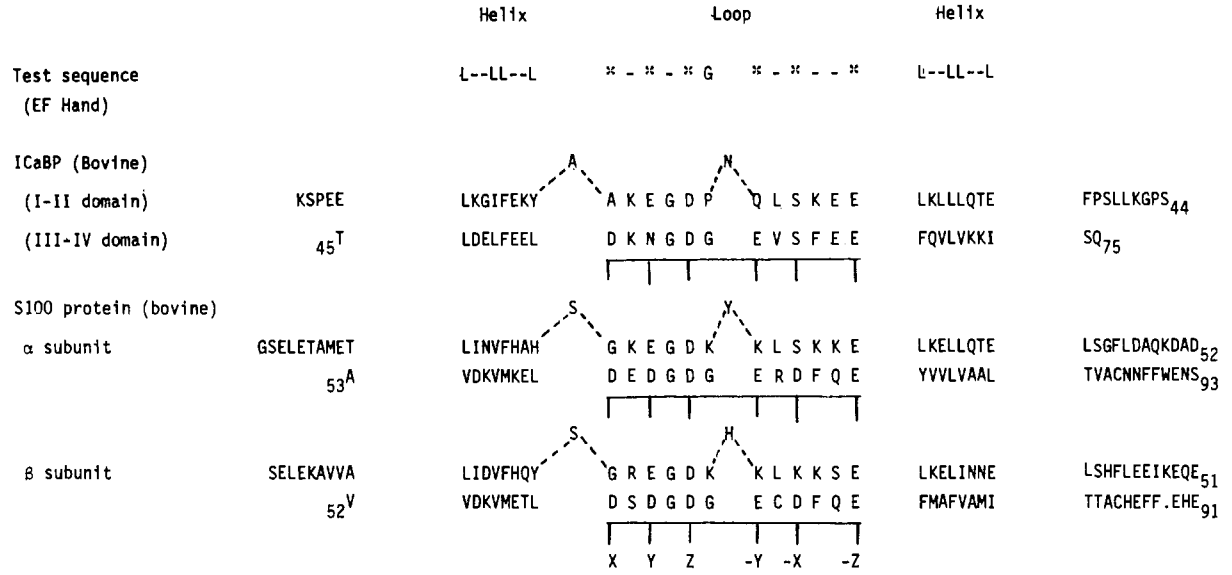

FIGURE 9: Comparison between the test sequence proposed by Tufty & Kretsinger (1975) for the EF hand structure and the typical and rearranged Ca^{2+} binding domains of the ICaBP and S100 proteins. In the test sequence, the asterisk indicates an oxygen-containing residue (D, E, N, Q, S, and T) corresponding to the Ca^{2+} coordinating ligands named X, Y, Z, –Y, –X, and –Z in the typical calcium binding sites (underlined sequences). L indicates a hydrophobic residue (L, V, I, F, and M), and G is a Gly residue. The amino acid residues are identified by single letter notation: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

posure of two SH groups which are highly reactive. If we consider that each identical subunit is affected by calcium binding in the case of S100b protein, each β subunit should expose one SH group. For S100a if it is concluded consequently that a β subunit exposes one SH group, the α subunit can expose only its single cysteine (85α). It is to be noted that this conclusion is in agreement with the exposure of the Trp residue (90α) which is located in the same region of the subunit. In view of these results and the subunit sequences described by Isobe & Okuyama (1978, 1981) (see Figure 9) it is reasonable to assume that Ca^{2+} binding to a β subunit as to a α subunit results in the exposure of cysteine-84β, the other cysteine-68β involved in the Ca^{2+} binding site remaining untitratable. This hypothesis, in good agreement with the recent finding of Haglid et al. (1982) for crude S100, would lead to the idea that calcium binding on α or β subunits induces the same conformational change in the part of the C-terminal chains next to the calcium binding site. On the contrary, zinc does not seem to greatly affect this part of the polypeptide chain.

Hydrophobic Properties of Ion-Bound Proteins. Interaction of S100 proteins with TNS upon Ca^{2+} or Zn^{2+} binding has enabled the characterization of a fundamental difference in physicochemical behavior between S100a and S100b. While the binding of calcium, but not of zinc, on S100a provokes the exposure of hydrophobic sequences to the solvent, the hydrophobicity of S100b will be affected by Zn^{2+} binding as well. This particular behavior has been useful to develop a rapid separation procedure of the S100a and S100b proteins (Baudier et al., 1982).

In the case of S100a, the ability to bind TNS originates from the same Ca^{2+}-induced conformational change as the variation in tryptophan fluorescence. The apparent dissociation constants for Ca^{2+} interaction determined by monitoring the TNS fluorescence and the enhancement of Trp emission are effectively similar (see Figure 4). Since Calissano et al. (1974) have previously reported that only 1 mol of TNS is bound per mol of Ca^{2+}-bound S100 protein and since we have shown that S100b, which is composed of two β subunits, does not expose any hydrophobic sequences upon Ca^{2+} binding, it could be reasoned that the hydrophobic region exposed by S100a is located on the α subunit. Moreover, we have previously shown that Ca^{2+} binding on S100a gives rise to the exposure of the single Trp residue (90α), responsible for the intrinsic fluorescence of the protein, and that of cysteine-85α. These two residues are located on the same hydrophobic sequence (residue 74α to residue 90α) close to the calcium binding site (see Figure 9). We can therefore conclude that the major structural modification upon Ca^{2+} binding is the exposure of the C-terminal hydrophobic sequence of the α subunit on which TNS will bind.

With reference to S100b, the enhancement of both TNS and tyrosine fluorescence upon zinc binding is coupled (see Figure 6b) and probably reflects the same conformational change. Since each β subunit is characterized by two hydrophobic sequences, one in the N-terminal (residues 4–14) and the other in the C-terminal regions (residues 74–85), it was important to determine which part of the protein is involved in this conformational change. It is clear that the enhancement of TNS fluorescence cannot be due to the exposure of the C-terminal hydrophobic sequence involving cysteine-84β since no SH groups are titratable upon Zn^{2+} binding. Thus it may be envisaged that it is only the N-terminal hydrophobic sequence which can be exposed. This assumption is in agreement with the enhanced fluorescence of Tyr (17β) residues due to a structural change occurring in the same region.

Finally, we previously reported that Ca^{2+} binding to α or β subunits may induce the same conformational change in the C-terminal part of the polypeptide chains comprising cysteines-85α and -84β. In the case of S100a this hypothesis is confirmed by evidencing the exposure to solvent of the (74α–90α) hydrophobic sequence. In the case of S100b no such behavior is recorded. One of the characteristics of the α subunit is the presence of the single tryptophan (90α) which is absent in the β subunit. Thus, it should be possible that this residue may contribute to the different hydrophobic behavior between α and β subunits in the presence of calcium. Furthermore, the fact that the hydrophobicity of the S100a protein is not affected by Zn^{2+} binding indicates that the behavior of

the β subunit is modified by interaction with the α subunit.

Calcium Binding Sites. The family of intracellular calcium binding proteins such as parvalbumin, troponin C, calmodulin, intestinal CaBP, and the S100 proteins has evolved from a four-domain Ca^{2+} binding ancestor. Only calmodulin and skeletal troponin C have retained this type of structure and bind four Ca^{2+} ions per protein molecule. Other members of the family have lost the Ca^{2+} binding ability of one or more of the domains. The tertiary structure of the Ca^{2+} binding sites has been predicted from the study of the Ca^{2+} binding domain of carp parvalbumin (Tufty & Kretsinger, 1975). It is composed of a loop of 12 residues, flanked by two α helices (see Figure 9). In this loop, the six Ca^{2+} coordinating ligands are named X, Y, Z, -Y, -Z, and -X, the nature of the ligands being responsible of the properties of the calcium binding sites (Reid & Hodges, 1980). The helix–loop–helix is termed an "EF hand". The S100a and S100b proteins are characterized by only two typical Ca^{2+} binding domains in the COOH-terminal part of the α and β polypeptide chains (see Figure 9). It should be noted that the calcium binding loops of the C-terminal domain of the α and β subunit share the same amino acid in the position corresponding to the Ca^{2+} ligand which might be responsible for identical properties. These are extremely similar to the EF hand prototype found in parvalbumin. Recently, Szebenyi et al. (1981) on the basis of an X-ray crystallographic study of bovine intestinal CaBP reported a variant of the Ca^{2+} binding site, the ICaBP I–II domains (see Figure 9), which differ from the typical EF hand by a longer and rearranged Ca^{2+} binding loop. The α and β subunits of S100 proteins which belong to the same branch of the calcium binding protein family as the ICaBP (Demaille et al., 1980) also exhibit such unconventional Ca^{2+} binding sites in the N-terminal part of the polypeptide chains corresponding to the ICaBP I–II domain and could be considered as putative Ca^{2+} binding sites. However, these putative Ca^{2+} binding sites are more complex than those of the ICaBP since thay are characterized by a high content of basic amino acid in the loop which could be responsible for a lowered affinity.

The experimental confirmation of the presence of two kinds of calcium binding sites predicted by analysis of the amino acid sequence was obtained. Spectroscopic measurements and more quantitatively, flow dialysis experiments (J. Baudier, J. Haiech, and D. Gérard, unpublished results) reveal the presence of two rather high-affinity calcium binding domains ($K_D = 2 \times 10^{-5}$ M) per S100 protein corresponding probably to the two typical Ca^{2+} binding sites predicted by Isobe & Okuyama (1978, 1981). In addition, other calcium binding sites of lower affinities (six according to the preliminary flow dialysis results) were also evidenced.

From the conformational changes study of S100 proteins, it can be assumed that for both S100a and S100b proteins the major structural modifications are the result of calcium binding to the higher affinity sites as previously proposed by Calissano et al. (1974). This is demonstrated by the fact that the conformational changes occur for both proteins in a calcium concentration range (2×10^{-4} M) in agreement with the titration of the higher affinity sites detected by flow dialysis experiments ($K_D = 2 \times 10^{-5}$ M). Moreover, as suggested by flow dialysis experiments Ca^{2+} binding to these sites is highly antagonized by K^+ at physiological concentration (120 mM KCl).

The relatively low Ca^{2+} affinity of this site, assumed to be the C-terminal typical calcium binding domain, as compared to the Ca^{2+} affinity for calmodulin and parvalbumin ($K_D = 10^{-8}$–10^{-6}), may be explained by the high content of acidic amino acids in positions corresponding to the calcium ligand of the loop (X, Asp-62; Y, Asp-64; Z, Asp-66; -Y, Glu-68; -X, Asp-70; -Z, Glu-73) (see Figure 9). Indeed, this is a particular feature not found in the other Ca^{2+} binding proteins, and according to the hypothesis of Reid & Hodges (1980) an acidic residue side chain in position -Y may compete with carbonyl oxygen of the peptide bound required for chelation of the cation in the -Y coordinate and be responsible for the relatively low Ca^{2+} affinity. Furthermore, the antagonistic effect of K^+ on the Ca^{2+} binding site may be explained by a competitive effect of K^+ on the Ca^{2+} binding site since Haiech et al. (1981) reported for calmodulin that the increase in the competitive effect of K^+ on the Ca^{2+} binding sites is a function of the number of carboxylates present in the loop.

The fluorometric studies of calcium binding to the S100a and S100b proteins in the presence of zinc showed that Ca^{2+} and Zn^{2+} ions could bind simultaneously to the proteins.

Concerning the S100a protein for which the fluorescence changes induced by successive ion addition (Zn^{2+} and Ca^{2+}) are additive without modification of the transition midpoint, it is obvious that the same Ca^{2+} binding sites were titrated and consequently that Ca^{2+} and Zn^{2+} binding sites are different and do not interfere with each other.

In the case of the S100b protein for which the effects of zinc and calcium on the tyrosine fluorescence were opposite, we noted an increase in the affinity of the Zn^{2+}-bound protein for calcium (K_D of 3×10^{-4} M decreases to $5.5\ 10^{-5}$ M). Such results have been corroborated by preliminary flow dialysis experiments and may be explained by structural changes of the Ca^{2+} binding loop induced by Zn^{2+} binding to the protein.

Zinc Binding Sites. We have provided the first evidence that Zn^{2+} ions can bind to S100 proteins. Flow dialysis studies have pointed out that the affinity of the protein for Zn^{2+} ranged between 10^{-8} and 10^{-6} M which is consistent with cellular concentration of Zn^{2+} in the brain (Fjerdingstad et al., 1974; Ebadi et al., 1981). To our knowledge, no models having been published for the Zn^{2+} binding sites of proteins. Nevertheless, histidine residues are known to be ubiquitous ligands for Zn^{2+} ions in proteins (Liljas et al., 1972; Holmes & Matthews, 1981; Iyer et al., 1981). The presence of several Zn^{2+} binding sites in S100 proteins is therefore supported by the high content of these residues in S100 proteins (seven His per mol of protein).

The stoichiometric fluorescence titration showed that four Zn^{2+} per S100a protein were needed for the total fluorescence change to occur whereas eight Zn^{2+} were necessary for the S100b protein. Noteworthy is also the fact that 8 mol of Zn^{2+}/mol of S100b is required for a total exposure of the hydrophobic sites. These results appear different from those obtained with the rat brain S100b for which the binding of only two Zn^{2+} ions is responsible for the hydrophobic change of the protein (J. Baudier, J. Haiech, G. Labourdette, and D. Gérard, unpublished results). Moreover, it should be noted that the hydrophobic change of the S100b protein in the presence of Zn^{2+} has also been found for the human protein (unpublished result) which may indicate that the hydrophobic behavior of the S100b protein in the presence of Zn^{2+} may be fundamental to understand the function of S100 proteins in the brain.

The properties of the S100a and S100b zinc binding sites will be developed in a subsequent paper devoted to the binding of Ca^{2+} and Zn^{2+} analyzed by flow dialysis experiments and optical properties of the proteins.

Concluding Remarks. Up to now no specific function has been attributed to the S100 proteins. In addition the reason

for the heterogeneity of these proteins (i.e., S100a and S100b) is unclear.

We reported in this paper different behaviors of the S100a and S100b proteins after ion binding. This suggests that the heterogeneity of S100 proteins could be correlated with a different biological function of each protein or with a different cellular or intracellular localization. One might expect a specific function of the different molecular forms of S100 protein related to the role of Ca^{2+} and Zn^{2+} in the brain.

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Registry No. Ca, 7440-70-2; Zn, 7440-66-6.

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