Bimane- and Acrylodan-Labeled S100 Proteins. Role of Cysteines-85α and -84β in the Conformation and Calcium Binding Properties of S100$\alpha\alpha$ and S100b ($\beta\beta$) Proteins†

Jacques Baudier,* Nicole Glasser, and Guy Duportail
Laboratoire de Physique, U.E.R. des Sciences Pharmaceutique, Unitê Associêe au CNRS 491, Universitê Louis Pasteur, 67048 Strasbourg Cedex, France

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ABSTRACT: Bovine brain S100$\alpha\alpha$, S100a ($\alpha\beta$), and S100b ($\beta\beta$) protein dimers were labeled with the sulfydryl-specific fluorescent probes monobromo(trimethylammonio)bimane (bimane) and 6-acryloyl-2-(dimethylamino)naphthalene (acrylodan) at cysteines-85α and -84β. The conformation and fluorescence properties of the S100 proteins derived were studied by means of anion-exchange chromatography on a Mono Q column using a fast protein chromatography system and fluorescence intensity, maximum emission wavelength, and polarization measurements. Spectroscopic studies on the intrinsic absorption and fluorescence properties of S100$\alpha\alpha$ and S100b proteins chemically modified on cysteines-85α and -84β with iodoacetamide completed this study. Several arguments suggest that the alkylated S100 proteins undergo conformational changes that are mainly characterized by the destabilization of the quaternary protein structure, which provokes a slow dimer-monomer equilibrium at high protein concentrations and induces total subunit dissociation at low ones. Calcium binding studies on bimane-S100$\alpha\alpha$ and -S100b proteins showed that alkylated proteins had a much higher calcium binding affinity than native protein and that the antagonistic effect of KCl on calcium binding was much less pronounced. These results confirmed our previous observations that the affinities of calcium binding sites IIα and IIβ in S100 proteins are highly dependent on protein conformation [Baudier, J., & Gerard, D. (1986) *J. Biol. Chem. 261*, 8204-8212].

The highly acidic water-soluble protein, S100, has long been considered as specific to the nervous system (Moore, 1965) where it is largely present mainly in the cytosol of glial cells (Tabuchi et al., 1983). Recently, S100 has also been found in various peripheral cells of neuroectodermal as well as nonneuroectodermal origin [for review see Molin et al. (1984)]. The intracellular location of S100 protein is generally admitted, but several recent studies have also shown that cells synthesize and secrete S100 proteins (Ishikawa et al., 1983; Kato et al., 1983a,b; Shashoua et al., 1984), and a membrane-bound form of S100 protein has been characterized (Haglid et al., 1974; Hyden & Ronnback, 1985; Isobe et al., 1984). S100 protein, purified from bovine brain, is a mixture of at least three similar components, S100$\alpha\alpha$, S100a, and S100b, which are noncovalent dimers with subunit compositions $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$, respectively. (Isobe et al., 1977; Masure et al., 1984). A fourth species, S100a′, has also been identified but is not yet fully characterized (Baudier et al., 1983). Immunohistochemical studies have reported different regional distributions of S100$\alpha\alpha$, S100a, and S100b proteins in the brain and peripheral tissues and differences in the distribution of the α and β subunits between cell types in nerve tissues (Molin et al., 1984; Kimura et al., 1984; Isobe et al., 1984). The exact reasons for this heterogeneity and the functions of these proteins in the brain are still unknown. However, it has been suggested that S100 proteins might be involved in the maturation and differentiation of glial cells (Labourdette & Mandel, 1978) and regulation of protein phosphorylation (Patel et al., 1983; Qi & Kuo, 1984), and S100 protein has been reported as interacting with synaptosomal and artificial lipid membranes (Calissano et al., 1974) as with the microtubule's proteins both in vitro (Baudier et al., 1982a; Donato, 1983; Endo & Hidaka, 1983) and in brain extracts (Hesketh & Baudier, 1986). The amino acid sequence of the α and β subunits revealed the structural relationship of S100 with the calcium binding proteins of the EF-hand type (Isobe & Okuyama, 1978, 1981). Flow dialysis measurements of calcium binding to bovine brain S100$\alpha\alpha$, S100a, and S100b proteins, in 20 mM Tris-HCl[1] buffer, pH 8.3, showed that S100 proteins bind specifically four Ca^{2+} equiv per mole of protein dimer with $K_a^{Ca} = 10^4$-10^5 M^{-1} (Baudier et al., 1986). The specific calcium binding sites had therefore been asigned to typical amino acid sequences on the α and β subunits. Potassium strongly antagonizes calcium binding on two calcium binding sites responsible for most of the Ca^{2+}-induced conformational changes in S100 proteins, probably sites IIα and IIβ (Baudier et al., 1986). S100b had also been shown to bind zinc with higher affinity than calcium ($K_a^{Zn} = 10^6$-10^8 M^{-1}) whereas S100a and S100$\alpha\alpha$ have only low-affinity zinc binding sites. Zinc binding on the higher affinity sites on S100b regulates calcium binding to S100b by increasing the protein affinity for calcium and decreasing the antagonistic effect of potassium on calcium binding (Baudier et al., 1986). This was not observed with S100a protein, but incubation of S100a protein with calcium and KCl induces time- and temperature-dependent conformational changes in the protein structure, which provokes subunit exchanges between S100a protein dimers and decreases the antagonistic effect of KCl on calcium binding (Baudier & Gerard, 1986). These results strongly suggest that S100 protein affinities for calcium depend greatly on the quaternary

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* Correspondence should be addressed to this author at the Department of Biochemistry, University of California, Berkeley, CA 94720.

[1] Abbreviations: Cys, cysteine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; FPLC, fast protein liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNS, 2-(*p*-toluidino)-naphthalene 6-sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ELISA, enzyme-linked immunosorbent assay; GdmCl, guanidinium chloride.

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and tertiary protein structures.

In the search for a possible function for brain S100 protein, particular attention has been paid to the state of its highly reactive free sulfhydryl groups. Their contribution to electrophoretic band multiplicity (Calissano et al., 1969), reactivity of the protein with antibodies (Dannies & Levine, 1971), and conformation (Rusca & Calissano, 1970) has been described. In the present study we have labeled Cys-85α and Cys-84β of S100$\alpha\alpha$, S100a, and S100b proteins, which are exposed upon calcium binding (Baudier & Gerard, 1983, 1986) with the fluorescent probe molecules bimane (Kosower et al., 1979) and acrylodan (Prendergast et al., 1983) and iodoacetamide. The alkylation of cysteines-85α and -84β induces drastic conformational changes in the S100 protein structure that greatly affect the calcium binding properties of these proteins.

Materials and Methods

Materials

All chemicals were high-grade commercial products. Ultrapure water (Milli Q Instrument from Millipore Corp.) was used. Chelex 100 was from Bio-Rad. Bimane sold under the trademark of Thiolyte MQ was from Calbiochem, iodoacetamide was from Pierce Chemical, and acrylodan was a generous gift from Prof. F. Prendergast, Mayo Clinic Foundation, Rochester, MN. The Pharmacia FPLC apparatus was equipped with a high-precision P500 pump, a GP 250 gradient programmer, and a Mono Q^R 5/5 column. Bovine brain S100$\alpha\alpha$, S100a, and S100b were prepared as previously described (Baudier et al., 1982b, 1983, 1986). The proteins were shown to be homogeneous by 20% polyacrylamide gel electrophoresis (Baudier et al., 1983), FPLC on a Mono Q column (Baudier & Gerard, 1986), and spectroscopic criteria (Baudier et al., 1982b).

Methods

Fluorescence spectra were obtained on a Perkin-Elmer MPF-44A spectrofluorometer equipped with a Perkin-Elmer 7500 Professional computer.

Fluorescence polarization of bimane–S100 protein was measured on an SLM 8000 SC spectrofluoropolarimeter. Excitation wavelength was set at 365 nm and the emission wavelength at 480 ± 5 nm. Relaxation times (ρ) were deduced from the polarization values (P) and the mean fluorescence lifetime (τ) by using Perrin's equation:

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$$

where P_0 is the limiting polarization.

Fluorescence lifetimes were measured as previously described (Baudier & Gerard, 1983). Excitation was performed at 365 nm, and fluorescence was observed at 480 ± 5 nm.

Flow Dialysis Experiments. The flow dialysis cell was as described by Colowick and Womack (1969) with a lower chamber (0.1–0.2 mL) separated from the upper one (1.5 mL) by Spectrapor dialysis membranes (cutoff, 6000–8000). The upper chamber contained the protein in solution in the working buffer, and the lower chamber was perfused at a flow rate of 200 mL/h with the same buffer, using a Gilson peristaltic pump. A hydraulic buffer was used to minimize variations in flow rate, and both chambers were under constant magnetic stirring.

Calcium binding experiments were performed at room temperature, according to Colowick and Womack (1969) and Haiech et al. (1980). The upper chamber contained 500 μL of S100b protein solution plus 10–15 μM $^{45}CaCl_2$ in working buffer. The lower chamber was perfused with the same buffer, and fractions were collected every 50 s. The response time of the cell was within 80 s. The ligand ($CaCl_2$) was added as aliquots (5 μL) of concentrated $CaCl_2$ solution every five fractions. The last three fractions (1 mL each) were counted in 8 mL of Aquasol (NEN). The mean of these three values was used. The final chase-out was for 20 mM $CaCl_2$.

Each experiment gave a set of data (ν, x), where ν is the average number of moles of ligand bound per mole of protein and x is the free ligand concentration. The binding was then analzyed by means of the general Adair–Klotz equation by using the approach to Fletcher et al. (1970).

Interaction of S100 Protein with DTNB. We previously reported that Ca^{2+} binding to S100$\alpha\alpha$, S100a, and S100b proteins induces the exposure of two sulfhydryl groups to solvent readily accessible to Ellman's reagent (DTNB) that had been assigned to Cys-85α and Cys-84β (Baudier & Gerard, 1983, 1986). Since accessibility of sulfhydryl groups in protein to reagents is diffusion-controlled and the reaction rate depends on the reagent concentration, we reinvestigated the reaction rates of DTNB with sulfhydryl groups in S100$\alpha\alpha$, S100a, and S100b proteins at a given DTNB/S100 protein molar ratio, i.e., 15/1. In the absence of calcium, the reaction rates of DTNB with S100 proteins were very slow; addition of 1 mM Ca^{2+} to the S100 proteins resulted in a rapid increase in reactivity toward DTNB, amounting to two thiol groups reacting per S100 protein after a 10-min incubation. The slower reaction rate of DTNB with exposed thiol groups in S100a and S100b in the presence of calcium compared to that we previously reported (Baudier & Gerard, 1983) stems from the lower DTNB/S100 ratio used.

Interaction of S100 Protein with Bimane. The fluorescent probe monobromo(trimethylammonio)bimane or bimane, synthesized by Kosower et al. (1979), is a thiol-specific reagent, the fluorescence of which increases when it covalently binds to thiol groups of protein. Bimane, from a freshly prepared solution in water, was added at 5:1 molar excess to the S100 protein species (40–50 μM) in 20 mM Tris-HCl buffer, pH 8.3. The labeling reaction was monitored from the increase in bimane fluorescence at 460 nm for an excitation wavelength set at 385 nm (Baudier & Gerard, 1986). In the absence of calcium, the reaction rate of the sulfhydryl groups in S100$\alpha\alpha$, S100a, and S100b proteins with bimane was very slow. Addition of 1 mM Ca^{2+} induced a rapid increase in bimane fluorescence, which reached a plateau after 1–2 min of reaction. The extent of the fluorescence increase was identical for S100$\alpha\alpha$, S100a, and S100b proteins. The labeling reaction was stopped by addition of excess β-mercaptoethanol. The labeled proteins were separated from excess reagent and calcium by gel filtration on a Sephadex G-25 column in the presence of 1 mM EGTA. After extensive dialysis against 100 mM Tris buffer, pH 8.3, to remove EGTA the labeled proteins were obtained totally free of divalent ions by two successive runs on a Chelex 100 column and a new dialysis against the working buffer. The labeled proteins were stored at −20 °C and used within 1 week. The degree of labeling was determined by using DTNB. The number of sulfhydryl groups reacted with bimane was given by the difference between the total number of sulfydryl groups of unlabeled proteins and the number of DTNB-titratable remaining sulfhydryl groups after protein denaturation of 6 M guanidinium chloride. The stoichiometry for bimane labeling of S100 proteins was 2 ± 0.2 mol of bimane bound per mole of S100 protein dimer. The extinction coefficient of bimane in water at 358 nm is 4000 M^{-1} cm^{-1}. An ϵ_{385nm} of 3600 M^{-1} cm^{-1} was determined for

bimane bound to S100 proteins under conditions where all the bimane has reacted with the proteins (i.e., 0.5 molar ratio of bimane/S100 in buffer plus 2 mM Ca^{2+}). Therefore, bimane-labeled S100 protein concentrations were subsequently determined by using an ϵ_{385nm} of 7200 M^{-1} cm^{-1}, corresponding to 2 mol of bimane bound per mol of protein.

Interaction of S100b Protein with Acrylodan. Acrylodan [6-acryloyl-2-(dimethylamino)naphthalene] is a specific thiol reagent recently synthesized by Prendergast et al. (1983). The quantum yield of this probe is markedly enhanced after reaction with thiols, and the fluorescent derivatives are very sensitive to dipolar perturbation from their environment. The procedure used to label S100b protein with acrylodan was identical with that developed with bimane. Acrylodan, from a freshly prepared solution in dimethylformamide, was added to S100b protein solution (40–50 μM) in 20 mM Tris-HCl buffer, pH 7.5, at 5:1 molar excess to the protein. The labeling reaction was monitored from the increase in fluorescence at 490 nm for an excitation wavelength at 385 nm. After the addition of 2 mM Ca^{2+} to the protein solution, acrylodan fluorescence rose rapidly, corresponding to the reaction between the two sulfhydryl groups exposed and acrylodan. After 2–4 min of reaction, the fluorescence increase reached a plateau and the labeled reaction was stopped by addition of excess β-mercaptoethanol. The stoichiometry for acrylodan labeling of S100b was 1.9 ± 0.2 mol per mole. Acrylodan-labeled S100b protein concentration was determined by using an ϵ_{360nm} of 25 800 M^{-1} cm^{-1}, corresponding to 2 mol of acrylodan bound per mole of S100b protein (Prendergast et al., 1983).

Interaction of S100b and S100αα with Iodoacetamide. S100b protein was first dissolved in 20 mM Tris-HCl buffer, pH 8.4, to reach a concentration of 40 μM. This high pH value was chosen to avoid unspecific His labeling.

Iodoacetamide dissolved in water was added at 50:1 molar excess to the S100b protein solution in the presence of 2 mM Ca^{2+}. The reaction rate between sulfhydryl groups and iodoacetamide was monitored as follows: 200-μL aliquots of the protein solution were denatured in guanidinium chloride (final volume = 1 mL) at different incubation times and the free sulfhydryl groups titrated with DTNB. After 2, 9, 15, 22, and 25 min, the number of sulfhydryl groups that had reacted with iodoacetamide were respectively 0.27, 0.46, 0.66, 1.01, and 1.34 per mole. The apparent linearity of the reaction rate of iodoacetamide with the sulfhydryl groups proved the reaction to be diffusion-controlled. We checked that at the concentration used the reaction rate of iodoacetamide with the colored 5-nitro-2-thiobenzoate produced during the titration of the free thiol groups was very slow and did not interfere with this titration.

To increase the reaction rate between sulfhydryl groups in S100b and iodoacetamide and avoid possible unspecific His labeling, iodoacetamide was added at 500:1 molar excess to the S100b protein solution, and the reaction was stopped after 5 min by addition of excess β-mercaptoethanol. The S100b protein was then freed of calcium, as previously described for bimane–S100 protein. The stoichiometry for iodoacetamide labeling of S100b protein was 1.7 ± 0.1 mol per mole (average of two different experiments). An identical procedure was used to alkylate Cys-85α in S100$\alpha\alpha$ protein with iodoacetamide.

RESULTS

Conformational Properties of Bimane and Acrylodan S100 Proteins. In any labeling study, it is important to assess how far labeling affects the conformational properties of the protein. Our first step was therefore to compare the chromatographic behavior of bimane–S100 proteins with that of native S100 proteins. We also studied the steady-state and dynamic fluorescence properties of bimane- and acrylodan–S100$\alpha\alpha$ and –S100b protein to obtain further information on the location of the fluorophores in the protein structures.

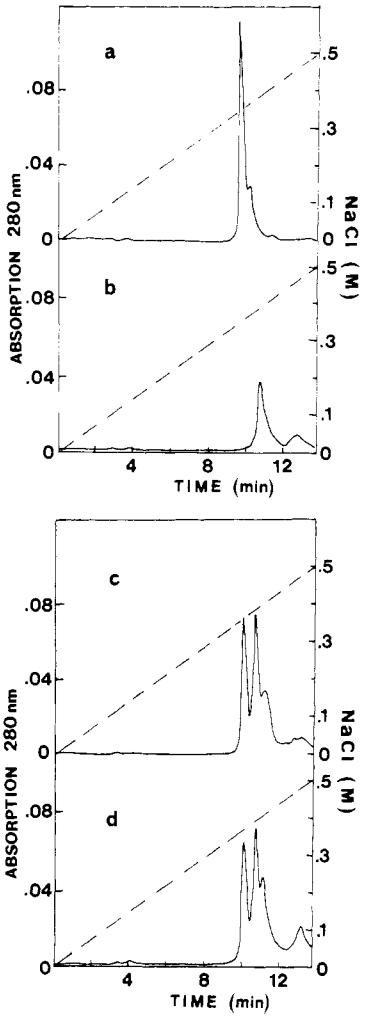

FIGURE 1: FPLC of bimane-labeled S100 proteins on a Mono Q column. The column was first equilibrated with 40 mM Tris-HCl buffer, pH 7.5. The protein sample (200 μL) was loaded on the column and the protein eluted with a linear salt gradient from 0 to 0.5 M NaCl at a flow rate of 1 mL/min. The elution profiles corresponded to the following runs: (a) bimane–S100$\alpha\alpha$ at a concentration of 12 μM; (b) bimane–S100b at a concentration of 14 μM; (c) bimane–S100a at a concentration of 27 μM; (d) mixture of bimane–S100$\alpha\alpha$ and bimane–S100b at concentrations of 12 and 19 μM, respectively. Identical elution profiles were obtained when 1 mM EGTA was included in all buffers.

(1) Chromatographic Behavior of Bimane–S100 Proteins on a Mono Q Column. Native S100$\alpha\alpha$, S100a, and S100b proteins can be separated by FPLC on a Mono Q column by using a linear salt gradient, under three distinct protein peaks, at 0.36, 0.39, and 0.41 NaCl, respectively[2] (Baudier & Gerard, 1986). Panels a and b of Figure 1 give the elution profiles of bimane–S100$\alpha\alpha$ and bimane–S100b, respectively, from a Mono Q column followed by their absorbance at 280 nm, where both aromatic residues and the fluorophore absorb. Bimane–S100$\alpha\alpha$ eluted mainly under a sharp symmetrical peak at 0.35 NaCl.[2] Bimane–S100b eluted under a main peak at 0.40 NaCl,[2] followed by a minor nonresolved peak at around 0.47 M NaCl. These results point out that bimane–S100$\alpha\alpha$

[2] Xerox copies of the original elution profiles have been provided to the referees for examination and will be sent to readers upon request.

and -S100b proteins behave almost identically to native protein when chromatographed on an anion-exchange column. Figure 1c shows the elution profile of bimane–S100a protein from the Mono Q column. The labeled protein eluted mainly under three absorbing peaks at 0.35, 0.38, and 0.40 ± 0.005 M NaCl,[2] followed by a small minor peak at around 0.47 M NaCl. Both peaks were pooled separately, dialyzed against buffer, and again subjected to chromatography on a Mono Q column. The protein peaks initially eluted at 0.35 and 0.40 M NaCl still eluted at the same salt concentration under single peaks, as did the bimane–S100$\alpha\alpha$ and -S100b proteins. On the contrary, the protein peak that initially eluted at 0.38 M NaCl eluted under three peaks as did the bimane–S100a protein (not shown). We concluded that the peak that eluted at 0.38 M NaCl might correspond to the bimane-labeled S100a ($\alpha\beta$) dimer and that α- and β-labeled subunits in S100a protein can exchange and associate like $\alpha\alpha$ and $\beta\beta$ dimers. This was confirmed when a mixture of bimane-labeled S100$\alpha\alpha$ and S100b was chromatographed on a Mono Q column (Figure 1d). Three main absorbing peaks eluted from the column at 0.35, 0.38, and 0.40 ± 0.005 M NaCl, as the result of a subunit exchange between bimane-labeled S100$\alpha\alpha$ and S100b protein dimers and the emergence of a bimane–S100$\alpha\beta$ species that eluted at 0.38 M NaCl.[2] The minor absorbing peak at 0.47 M NaCl that was routinely observed on the bimane–S100 chromatogram might correspond to dissociated labeled subunits since it exhibits bimane fluorescence properties (not shown). Further analysis is required for its exact characterization.

(*2*) *Fluorescence Properties of Bimane- and Acrylodan-Labeled S100 Proteins.* Fluorescence spectroscopy is an excellent tool for studying the conformational properties of fluorescent protein derivatives. Fluorescence intensity, maximum emission wavelength, and lifetime are influenced by properties of the close environment of the probe, such as polarity, solvation, etc. Fluorescence polarization can inform on the relative motion of the probe bound to the protein. This study focused mainly on the fluorescent properties of bimane and acrylodan S100b derivatives, both bimane and acrylodan adducts giving complementary information. Indeed, the fluorescence properties of bimane moieties are not very sensitive to dipolar perturbation from their environment, unlike acrylodan, which is a highly polarity sensitive fluorescent probe (Prendergast et al., 1983). However, the rather high fluorescence lifetime of the bimane derivative (present study) compared to that of the acrylodan (Prendergast et al., 1983) makes bimane a suitable fluorescent probe for accurate dynamic fluorescence measurements.

The fluorescence spectra of bimane–S100$\alpha\alpha$ and -S100b in Tris buffer exhibited a maximum emission wavelength at 465 nm (Figure 2). Upon the transition from the native state to the unfolded one, following denaturation in 6 M guanidinium chloride, bimane fluorescence intensity decreased markedly, and the fluorescence maximum emission shifted to 480 nm (not shown). The motions of the bimane groups in bimane–S100b were studied by steady-state fluorescence polarization. Table I gives the fluorescence polarization values and fluorescence lifetimes of native and denatured bimane–S100b, from which we calculated the rotational relaxation times ρ by using the Perrin equation (see Methods). The value ρ = 7 ns found for bimane groups in the native state of bimane–S100b protein is of the same order as the rotational relaxation time of the Trp residue in the unlabeled S100a ($\alpha\beta$) protein dimer (ρ = 6) (Baudier & Gerard, 1986) and suggests that the bimane moieties have no faster motion than that

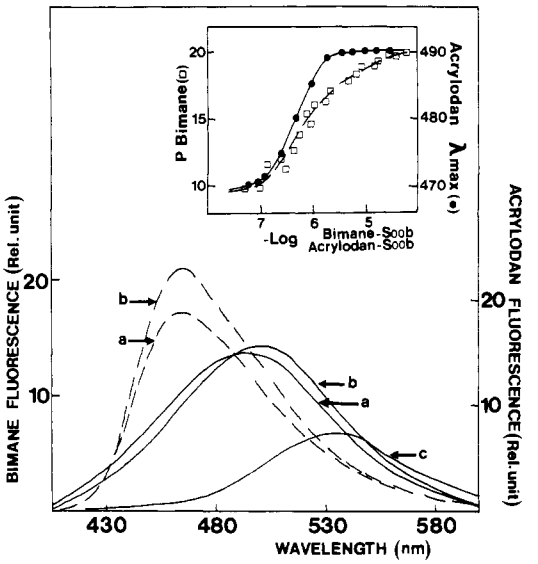

FIGURE 2: Fluorescence emission spectra of bimane–S100b (dashed line) and acrylodan–S100b (solid line). The proteins (15 μM) were in 20 mM Tris-HCl buffer, pH 7.5: plus 1 mM EGTA (curves a); plus 1 mM Ca^{2+} (curves b); or denatured in 6 M guanidinium chloride (curve c). λ_{ex} = 365 nm. The inset shows the effects of protein dilution on bimane–S100b fluorescence polarization (□) and acrylodan–S100b fluorescence maximum emission (●). The proteins were in 20 mM Tris-HCl buffer, pH 7.5 at 20 °C. λ_{ex} = 365 nm.

Table I: Rotational Relaxation Times of Bimane–S100 Proteins (ρ) in 20 mM Tris-HCl, pH 7.5 (20 °C)[a]

conditions	polarization degree P (±0.005)	lifetime τ (ns) (±0.2)	relaxation time ρ (ns) (±0.2)
bimane–S100b, 15 μM	0.185	10	7
bimane–S100b, 0.2 μM	0.12	10.5	3.7
bimane–S100b, 6 M GdmCl	0.12	3.2	1.13
bimane–S100$\alpha\alpha$, 15 μM	0.22		
bimane–S100$\alpha\alpha$, 0.08 μM	0.11		

[a] A value of 0.41 was taken as the fundamental polarization degree P_0. This value was obtained for the proteins in 80% poly(propylene glycol) at -20 °C.

imposed on them by the rotation of the protein dimer itself. The large increase in mobility of the bimane groups in guanidinium chloride (ρ = 1.1 ns) corresponds to complete subunit dissociation and unfolding of the β polypeptide chains. Denaturation of S100b derivatives therefore resulted in measurable changes in all dynamic fluorescence properties of the bimane moieties. These groups are rigidly attached to the protein in the native state, whereas in the unfolded state they are mobile and probably exposed to solvent. The results of the fluorescence polarization experiments with native bimane–S100b protein can be correlated with the observation of gel filtration chromatography (not shown) and, to a lesser extent, with the results of anion-exchange chromatography (Figure 1), which seem to indicate that bimane–S100b proteins exist as dimers at a 10 μM concentration range. However, the probable dissociation of the labeled S100 proteins into subunits in Tris buffer was observed at a low protein concentration. The inset of Figure 2 shows the effect of dilution on the polarization of fluorescence of bimane–S100b. At protein concentrations below 2×10^{-7} M, fluorescence polarization is lower than for protein in 6 M guanidinium chloride, but the fluorescence lifetime remains unchanged (Table I). The rotational relaxation time (ρ = 3.7 ns) indicates that the bimane fluorophore motion is much faster than in concentrated protein solution, probably due to subunit disso-

ciation. The effect of protein dilution on the decrease in fluorescence polarization of bimane was also observed with the bimane–S100$\alpha\alpha$ derivative (Table I).

The fluorescence spectrum of acrylodan–S100b exhibited a fluorescence maximum at 490 nm, and its half-width is 3850 cm^{-1} (Figure 2). As for bimane-labeled protein, the acrylodan fluorescence intensity markedly decreased upon transition from the native state to the unfolded one following denaturation in 6 M guanidinium chloride, and the fluorescence maximum wavelength shifted to 582 nm associated with a decrease in the half-width to 2700 cm^{-1} (Figure 2). The detailed interpretation of the changes in either fluorescence intensity or maximum emission wavelength is difficult, but it is generally admitted that these two parameters are sensitive to different features of the probe environment: the maximum emission wavelength reflects the overall dipolar character of this environment, whereas the intensity, related to the quantum yield, is influenced rather by particular deactivating processes. Prendergast et al. (1983) have carried out a study of the fluorescence properties of the acrylodan–mercaptoethanol adduct, using solvents of different polarities. A decrease in solvent polarity results in an increase in fluorescence intensity and a decrease in the maximum emission wavelength. These results extrapolated to acrylodan–S100b indicate that when S100b derivatives are unfolded, the fluorescence probes are transferred from a highly hydrophobic environment in the native state to a much more polar environment (the aqueous solvent) in the unfolded state. The fluorescence properties of native acrylodan–S100b were similar to those of the acrylodan–mercaptoethanol adduct in ethanol, which suggests that acrylodan moieties in native protein are almost completely shielded from the aqueous solvent by the protein structure. This tallies well with the rather high fluorescence spectrum half-width in native acrylodan–S100b indicative of incomplete dipolar relaxation of the solvent around the chromophore (Prendergast et al., 1983). Let us recall that this difference in the fluorophore environment between the native and unfolded states of S100b derivatives also emerges from fluorescence lifetime measurements on bimane–S100b. The native S100b adduct gave 10 ns against only 3.2 ns for the unfolded state (Table I). Finally, the effect of protein dilution on S100b derivatives' conformation was also observed with acrylodan–S100b (Figure 2, inset). The fluorescence maximum wavelength of acrylodan bound to S100b shifted from 490 to 475 nm upon dilution (which parallels the decrease in bimane fluorescence polarization). This indicates that the fluorophores moved to a less polar environment and that consequently the dilution effect on subunit dissociation was not accompanied by subunit unfolding as in guanidinium chloride.

Conformational Properties of Acetamide Derivatives of S100$\alpha\alpha$ and S100b Protein. For a more precise study of the conformational changes in S100 protein associated with the alkylation of cysteines-85α and -84β, acetamide derivatives of S100$\alpha\alpha$ and S100b were prepared. The spectroscopic and hydrophobic properties of acetamide derivatives were compared to those of native proteins.

Figure 3 shows the UV absorption and fluorescence spectra of S100$\alpha\alpha$ and acetamide–S100$\alpha\alpha$. In the absorption spectrum of acetamide–S100$\alpha\alpha$ we observe the disappearance of the shoulder at 290 nm and of the absorbance peak at 284 nm that characterize apo-S100$\alpha\alpha$ protein. The red shift in the tryptophan fluorescence maximum at 345 ± 1 nm indicates the exposure of this residue to solvent. Identical absorption and fluorescence spectra are obtained with S100$\alpha\alpha$ protein

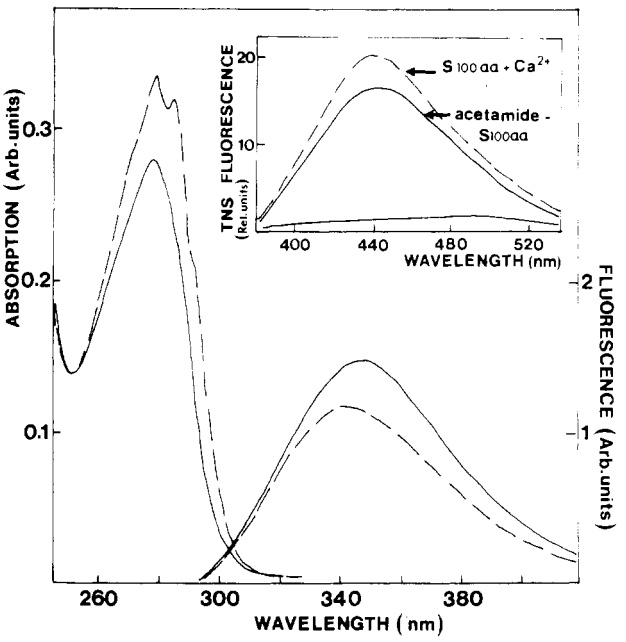

FIGURE 3: Absorption and emission spectra (λ_{ex} = 295 nm) of apo-S100$\alpha\alpha$ protein (dashed line) and acetamide–apo-S100$\alpha\alpha$ protein (solid line) in 20 mM Tris-HCl buffer, pH 7.5. The inset shows the TNS fluorescence spectra (λ_{ex} = 365 nm) at a concentration of 25 μM in 20 mM Tris-HCl buffer, pH 7.5, in the presence of S100$\alpha\alpha$ (15 μM), minus (—) or plus (--) 1 mM Ca^{2+}, and acetamide–S100$\alpha\alpha$ (15 μM) minus Ca^{2+}. Note that addition of excess EGTA to acetamide–S100$\alpha\alpha$ apoprotein induced no changes in the spectral properties of the protein nor in TNS fluorescence, suggesting that it was indeed calcium free.

saturated with calcium (Baudier & Gerard, 1986). This suggests that the alkylation of Cys-85α induced conformational changes in the S100$\alpha\alpha$ protein structure that resemble those induced upon calcium binding to nonalkylated S100$\alpha\alpha$ protein, i.e., the exposure of most of the hydrophobic aromatic amino acids to solvent (Baudier & Gerard, 1986). This was confirmed when we compared UV differential spectra of S100$\alpha\alpha$ and acetamide–S100$\alpha\alpha$ both denatured in guanidinium chloride against native proteins in buffer. The negative difference spectrum was much less pronounced for acetamide S100$\alpha\alpha$, indicating that most of the aromatic residues were already exposed to solvent (not shown). We also compared the hydrophobic properties of S100$\alpha\alpha$, in the absence and in the presence of calcium, with those of acetamide–S100$\alpha\alpha$ in the absence of calcium. In this study, TNS was used a hydrophobic fluorescence probe (McClure & Edelman, 1966). In the absence of calcium, the fluorescence of TNS, when added to apo-S100$\alpha\alpha$, was negligible (Figure 3, inset). Adding calcium induced a large increase in TNS fluorescence intensity, with a fluorescence maximum at 440 ± 1 nm, compatible with the exposure of hydrophobic domains to solvent. When TNS was added to acetamide–S100$\alpha\alpha$ in the absence of calcium, the TNS fluorescence spectrum was similar to that with Ca^{2+}-bound S100$\alpha\alpha$ protein (Figure 3, inset), which tallies with our previous suggestion that acetamide–S100$\alpha\alpha$ and Ca^{2+}-bound S100$\alpha\alpha$ have similar conformations (this will be developed under Discussion). Note also that calcium binding to acetamide–S100$\alpha\alpha$ only induced minor and hardly detectable changes in the intrinsic UV spectral properties of the protein and induced only a slight increase in TNS fluorescence (not shown).

Alkylation of Cys-84β in S100b protein with iodoacetamide also induced conformational changes in the protein structure that resulted in spectroscopic properties different from those of native S100b protein. This is clear in the aromatic ab-

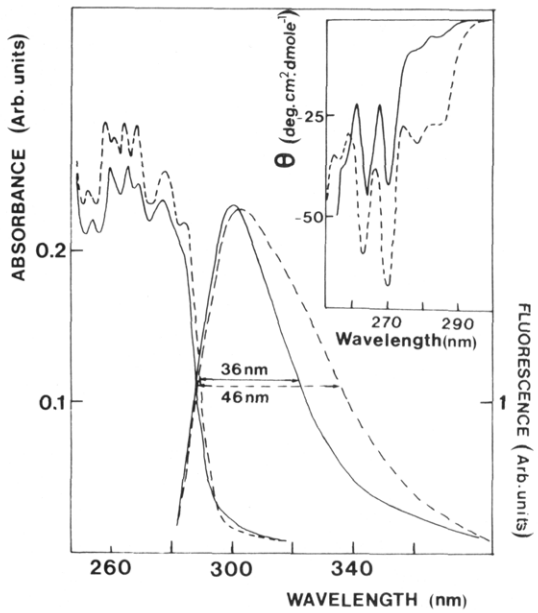

FIGURE 4: Absorption and emission spectra (λ_{ex} = 275 nm) of S100b protein (dashed line) and acetamide–S100b protein (solid line) in 20 mM Tris-HCl buffer, pH 7.5. The inset shows the near-UV circular dichroism spectra of S100b and acetamide–S100b.

Table II: Macroscopic Constants Describing Ca^{2+} Binding to Bimane–S100 Protein Dimers in 20 mM Tris Buffer, pH 7.5

conditions[a]	K_1 (10^{-5} M^{-1})	K_2 (10^{-5} M^{-1})	K_3 (10^{-5} M^{-1})	K_4 (10^{-5} M^{-1})
bimane–S100$\alpha\alpha$	12.2	9.2	4.3	0.46
bimane–S100$\alpha\alpha$ + 120 mM KCl	1.61	2.13	1.83	0.18
bimane–S100b	5.69	1.17	0.19	0.10

[a] See legend of Figure 5.

sorption and circular dichroic spectra of the acetamide–S100b derivative (Figure 4). Both spectra have the same shape as the Ca^{2+} or Zn^{2+} (4 Zn^{2+}/S100b) S100b conformers (Baudier et al., 1986). However, analysis of the tyrosine fluorescence spectrum of alkylated S100b reveals that it closely resembled that of the Zn^{2+}–S100b conformer; i.e., the tyrosine fluorescence quantum yield was about twice that of apo-S100b protein, and the emission spectrum had a minimum spectral half-width of 36 nm. In the presence of calcium, the tyrosine fluorescence quantum yield of native S100b decreased and the spectral half-width increased maredly, from 46 ± 1 to 56 ± 1 nm. It has been previously suggested that this corresponds to a Ca^{2+}-induced hydrogen bond between the tyrosine phenolic groups and a dicarboxylic acid residue (Lux et al., 1985).

Calcium Binding Properties of Bimane-Labeled S100$\alpha\alpha$ and S100b Protein. Calcium binding was studied with the flow dialysis technique. The calcium binding isotherms for bimane–S100$\alpha\alpha$ and –S100b in 20 mM Tris-HCl buffer, pH 7.5, were compared to those obtained with native proteins (Figure 5). At pH 7.5, the labeled protein bound 4 mol of Ca^{2+} per mole of protein, and there was no sign of the lower affinity Ca^{2+} binding sites observed with native S100 proteins at the same pH (Baudier et al., 1986). The binding constants describing calcium binding on bimane-labeled S100 proteins are reported in Table II. They should be considered only as estimations, since binding experiments had been performed at a single protein concentration. The S100 proteins chemically modified with bimane had a much higher affinity for calcium than did native proteins. This was especially pronounced for bimane–S100$\alpha\alpha$ which, even in the presence of 120 mM KCl, had a higher affinity for calcium than the native

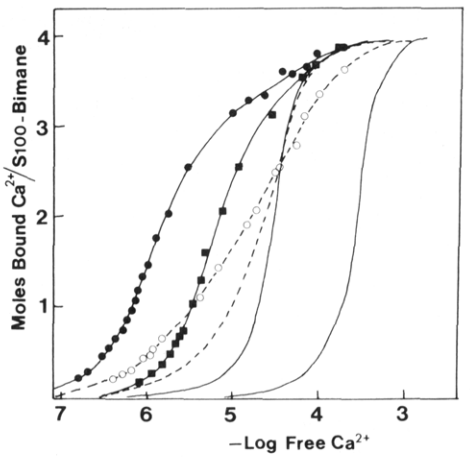

FIGURE 5: Binding isotherms of Ca^{2+} to bimane–S100 proteins. Bimane–S100$\alpha\alpha$ (filled symbols) and –S100b (open symbol) at a concentration of 35 μM in 20 mM Tris-HCl buffer, pH 7.5, were titrated by calcium in the absence (● and ○) or presence of 120 mM KCl (■). The binding curves are computer-fitted curves assuming that at the maximum 4 mol of calcium bound per mole of protein. The binding curves for native S100$\alpha\alpha$ (solid line) and S100b protein (dashed line) are from Baudier et al. (1986).

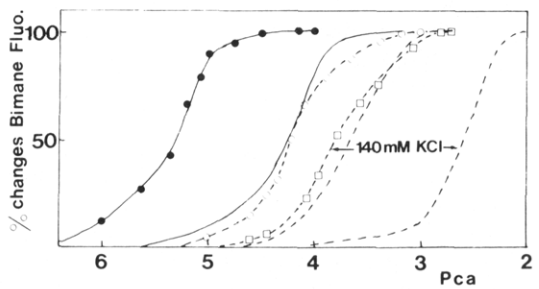

FIGURE 6: Titration profiles for percentage changes in fluorescence of bimane–S100$\alpha\alpha$ (filled symbol) and bimane–S100b (open symbols) upon calcium binding. λ_{ex} = 365 nm. Bimane–S100$\alpha\alpha$ (8.7 μM) and bimane–S100b (15 μM) were used in 20 mM Tris-HCl buffer, pH 7.5, in the absence (● and ○) or presence of 140 mM KCl (□). The curves without symbols correspond to the percentage changes in intrinsic Trp fluorescence for S100$\alpha\alpha$ (solid line) or Tyr fluorescence for S1000b (dashed line) in the same conditions.

protein in the absence of KCl (Figure 5). Further unequivocal evidence for the higher affinity of bimane–S100 proteins for calcium was obtained by calcium titration of the bimane fluorescence changes upon calcium binding to labeled proteins (see below).

Effects of Calcium on the Fluorescence Properties of Bimane- and Acrylodan-Labeled S100 Proteins. The fluorescence properties of S100 derivatives in the absence of divalent cations have already been described. There was no change in bimane maximum emission when Ca^{2+} was added to labeled S100$\alpha\alpha$ or S100b protein, although a significant 15–20% increase in bimane fluorescence was recorded, reflecting conformational changes in the protein structure upon Ca^{2+} binding (Figure 2). The fluorescence lifetimes and fluorescence polarization values of bimane–S100b did not change in the presence of calcium (not shown). Calcium binding to acrylodan–S100b protein induced a 5-nm red shift in maximum emission wavelength to 495 nm, combined with a decrease in the spectral half-width to 3450 cm^{-1} (Figure 2). This indicates an enhanced dipolar relaxation rate of the fluorophore environment which might correspond to partial exposure to the solvent. However, the fluorophores still seem to be enclosed in a rather nonpolar environment.

Figure 6 compares calcium titration curves of bimane fluorescence in labeled S100$\alpha\alpha$ and S100b proteins to the

intrinsic fluorescence titration curves of unmodified proteins. In agreement with the flow dialysis binding data, we observed for the alkylated proteins an apparent increase of the affinity of calcium by a factor of at least 10, and for the bimane–S100b protein, an important decrease in the antagonistic effect of potassium on calcium binding. Calcium titrations of the percentage increase in bimane fluorescence (ΔF), for both S100$\alpha\alpha$ and S100b labeled proteins, were also performed at protein concentrations identical with those in flow dialysis experiments, and the changes in bimane fluorescence were analyzed as a function of calcium saturation, using the flow dialysis binding data. This demonstrated that in S100$\alpha\alpha$ and S100b, it is the two first calcium ions bound that are responsible for most of the increase in bimane fluorescence. For the S100$\alpha\alpha$–2Ca^{2+} complex we found ΔF = 95%, and for the S100b–2Ca^{2+} complex, ΔF = 75%.

Discussion

Calcium binding to S100 proteins elicits structural changes that markedly increase the reactivity of thiol groups for DTNB and the thiol-specific fluorescence probes bimane and acrylodan. The reactivity studies indicate that two sulfhydryl groups react with the thiol reagents, and they have been assigned to Cys-85α and Cys-84β. The dependence for the reactivity of Cys-85α and Cys-84β in the presence of calcium against DTNB and iodoacetamide concentrations (see Materials and Methods) suggests that with these charged reagents the reaction is diffusion-controlled, unlike with the apolar fluorescence probes bimane and acrylodan. This might indicate that Cys-85α and Cys-84β are not in full contact with the solvent but probably lie in a hydrophobic structure on the protein surface. This hypothesis is supported by the spectral properties of acrylodan–S100b in the absence and presence of calcium, which show that the fluorescence probe is not in direct contact with the aqueous solvent. From sequence analogies with other Ca^{2+} binding proteins one may suppose the Cys-85α and Cys-84β, which are close to the calcium binding loops of sites IIα and IIβ, in hydrophobic sequences, are involved in α helix structures (helix D) known to be essential in forming calcium binding domains (Babu et al., 1985; Herzberg & James, 1985). In apo-S100 proteins these α helix structures are buried within the hydrophobic protein matrix. This has also been found for the helix D in the crystal structure of turkey skeletal muscle troponin C, and it has been suggested that the stabilization associated with this arrangement is partly responsible for the reduced calcium affinity of site II (Herzberg & James, 1985). It is all the more plausible that in the presence of calcium helix D in S100 proteins is exposed to solvent, which may explain the increase for the reactivity of Cys-85α and -84β against thiol reagents. Upon alkylation of Cys-85α and -84β, S100 proteins undergo conformational changes that apparently destabilize the quaternary protein structure, thus allowing subunit exchanges between protein dimers. A slow dimer–monomer equilibrium was also observed in concentrated S100 protein solutions at acidic pH and with S100a protein after calcium incubation (Baudier & Gerard, 1986). The effects of acidic pH and calcium on the spectroscopic and hydrophobic properties of S100$\alpha\alpha$ were very similar to those observed with S100$\alpha\alpha$ upon alkylation of Cys-85α with iodoacetamide. All these data indicate that alkylation of Cys-85α in S100$\alpha\alpha$ and S100a proteins induces conformational changes in the protein structures identical with those induced upon Ca^{2+} binding or by acidic pH. The alkylation of Cys-84β in S100b protein dimer induces conformational changes that resemble those induced upon Zn^{2+} binding to the four highest affinity Zn^{2+} binding sites in native S100b protein.

Calcium binding studies in bimane–S100$\alpha\alpha$ and –S100b proteins prove that the alkylation proteins have much higher calcium binding affinities than native proteins. The increase in the bimane–S100b affinity for calcium is on the same order as that for the Zn^{2+}-bound S100b (4 Zn^{2+}/S100b) (Baudier et al., 1986), which tallies with the identical conformational changes induced by both effectors. The increase in bimane–S100$\alpha\alpha$ protein affinity for calcium might be explained by the fact that the alkylated protein adopts a "Ca^{2+}-bound" conformation, characterized by a slow dimer–monomer equilibrium at high protein concentrations and probably subunit dissociation at low protein concentrations.

The increase in the alkylated S100 protein's affinity for calcium confirms that Ca^{2+} binding properties of S100 proteins depend greatly on protein conformation and fits in with the hypothesis of Gariepy and Hodges (1983) that the calcium affinity of an EF-hand site remains largely a function of the tertiary structure it adopts as part of a protein. We previously observed that, in S100a protein, the affinity of site IIα for calcium was highly dependent on protein conformation (Baudier & Gerard, 1986) and that it was mostly sites IIα and IIβ in S100 proteins that were strongly antagonized by KCl (Baudier et al., 1986). Since the alkylation of Cys-85α and -84β also decreases the antagonistic effect of KCl on calcium binding to S100 proteins, one may suppose that it is essentially the affinity of sites IIα and IIβ for calcium that increases upon cysteine alkylation. This is supported by the close proximity of Cys-85α and -84β to sites IIα and IIβ and by the fact that it is mostly the first two Ca^{2+} bound per protein dimer that increase bimane–S100$\alpha\alpha$ and –S100b fluorescence. In native apo-S100 proteins, the strong interactions between subunits are probably responsible for the lower protein affinities for calcium. The antagonistic effect of KCl on calcium binding to native S100 proteins might also stem partly from the fact that KCl (and ionic strength in general) increases hydrophobic interactions between subunits which rendered S100 proteins more compact (Mani & Kay, 1984) and the Ca^{2+} binding sites less accessible to Ca^{2+}.

Conformational changes in α and β subunits upon alkylation of Cys-85α and -84β with bimane were also observed through the different antigenic properties studied by an ELISA method using polyvalent rabbit S100 antibodies detecting epitopes in the β-chain but also in the α-chain. It was proved that native α-chain is approximately 100% more antigenic than α-bimane. However, native β-chain is approximately 100% less antigenic than β-bimane (A. Aurell, K. Haglid, and J. Baudier, unpublished results). These different antigenic properties of α and β subunits upon cysteine alkylation would give further interesting information on the conformation of the proteins if studied with monoclonal antibodies detecting single epitopes on the different α and β subunits.

In the brain, S100 proteins are predominantly located in the cytosol of glial cells (Tabuchi et al., 1983), but numerous works have reported that S100 proteins are also present in neurons (Haglid et al., 1974, 1976; Hyden & Ronnback, 1975; Isobe et al., 1984). The neuronal S100 protein fraction exists mainly as a membrane-bound form (Haglid et al., 1974, 1976; Shtark et al., 1981), and cellular distribution of S100 subunits in the human brain shows that neurons contain only the α subunit (Isobe et al., 1984). The conformational changes observed in S100$\alpha\alpha$ upon alkylation of Cys-85α, which resemble those observed upon Ca^{2+} binding, may have a physiological importance. It is possible that, in its membrane-bound form, S100$\alpha\alpha$ takes on conformations similar to those observed with alkylated S100 protein. In these associations, the

structural and ion binding properties of the protein, as deduced from its behavior in aqueous solutions, should be viewed with care if extrapolated to in vivo situations. Finally, the fluorescence properties of bimane–S100 proteins allowed us to accurately monitor the dynamic properties of the labeled proteins. The high quantum yield and fluorescence lifetime of bimane bound to S100 proteins prove that bimane fluorescence is a convenient probe for monitoring the rotational relaxation time of the proteins. We feel it will be very useful in studying the interactions of S100 proteins with other macromolecules, such as microtubule proteins (Baudier et al., 1982; Donato, 1983) or artificial and biological membranes (Calissano et al., 1974). The fluorescence S100 proteins should also prove useful as molecular cytochemical probes. Experiments on these possible applications of labeled S100 proteins are currently in progress.

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Registry No. DTNB, 69-78-3; Ca, 7440-70-2; ICH_2CONH_2, 144-48-9; L-Cys, 52-90-4; acrylodan, 86636-92-2; bimane, 71418-45-6.

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