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Effect of Calcium Ion on S-100, a Protein of the Nervous System*

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ABSTRACT: A specific protein of the nervous system, S-100, appears to exist in multiple forms when subjected to acrylamide gel electrophoresis in the presence of either Ca^{2+} or 8 M urea, but only in a single form in their absence. Calcium ion causes a limited conformational change in S-100 leading to a more unfolded structure in a region probably containing its single tryptophan, several of its tyrosine and phenylalanine, and two of its three cysteine residues. These effects are not seen with Mg^{2+}. Monovalent cations antagonize the effect of Ca^{2+}, K^+ being more effective than Na^+. At physiological levels of K^+ and Na^+ the effects of Ca^{2+} occur also in its physiological range.

A protein, called S-100 since it was soluble in saturated $(NH_4)_2SO_4$ at pH 7, has been shown to be specific to the nervous system and to be present in an immunologically similar form in the nervous systems of all vertebrates (Moore, 1965; Levine and Moore, 1965; Kessler *et al.*, 1968; Moore *et al.*, 1968). It was characterized by its high mobility during starch or acrylamide gel electrophoresis as a consequence of its high content of glutamic and aspartic acid residues and its small size.

Hyden and McEwen (1966), Vincendon *et al.* (1967), and Gombos *et al.* (1966) found that S-100 showed immunologically similar, multiple forms when electrophoresed, either in pure form or from brain extracts, on acrylamide gel. We attempted to answer the question: Do the multiple forms of S-100 represent similar proteins differing in primary structure? Although this question has not been answered unequivocally, the results of the first experiments led to the bulk of the work in this paper which demonstrates that S-100 shows interesting specific interactions with calcium ion which might give a clue to the function of S-100 in the nervous system.

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Methods

The protein was prepared from beef brain as described previously (Moore, 1965). It gave a single band when electrophoresed on starch gel, using the discontinuous system of Poulik (1957) and on acrylamide gel in Tris-glycine buffer (without Ca^{2+}) as described below. It gave a single peak when chromatographed on DEAE-cellulose, DEAE-Sephadex, G-100 Sephadex, and hydroxylapatite, and when centrifuged through a sucrose density gradient (see below). When subjected to double diffusion in Agarose against its antiserum, a single precipitin band was formed. It was kept at $-20°$ as a lyophilized powder and solutions were prepared fresh as needed. The molecular weight, estimated by sucrose density gradient centrifugation or chromatography on G-100 Sephadex, was 24,000. To provide a basis for determining concentrations, a sample of S-100 was dried *in vacuo* to constant weight; a 1-mg/ml solution of the dried protein in 60 mM KCl–20 mM Tris-Cl (pH 8.3) gave an absorbance of 0.344 at 280 mμ in a 1-cm cell which corresponded to a molar extinction of 8260 $mole^{-1}$ cm^{-1}.

Centrifugation in sucrose density gradients was done by the method of Martin and Ames (1961) using an International B-60 centrifuge with an SB-405 rotor spun at 60,000 rpm at 2° for 16 hr. Molecular weight standards were crystalline bovine serum albumin, cytochrome C, and ovalbumin. Chromatography on G-100 Sephadex was done with a 0.6 × 200 cm column pumped at a constant flow rate of 4 ml/hr. Standard proteins (bovine albumin, cytochrome C, ovalbumin, chymotrypsin, and glutamic dehydrogenase) were run as molecular weight markers.

Tryptophan content was determined by titration with *N*-bromosuccinimide at pH 4 in 8 M urea by the method of Spande and Witkop (1967). Tryptophan and tryosine were also estimated by spectrophotometry in 6 M Gu-HCl (Edelhoch, 1967). In addition, tyrosine was measured by iodination (Steiner, 1966).

Acrylamide Gel Electrophoresis. The Hoefer Scientific Instruments apparatus was used. The buffer composition was identical in all parts, since S-100 moved as a single band with the front in a discontinuous system. After a prerun of 60 min at 2 mA/tube, the reservoir buffer was renewed and electrophoresis at 1 mA/tube was carried out toward the anode until a bromophenol blue marker moved 10 cm. The gels were polymerized by adding 0.1 ml of a freshly prepared 7% solution of ammonium persulfate to 20 ml of a 7.5% acrylamide solution also containing 0.37% of *N,N'*-methylenebisacrylamide, 15 μl of *N,N,N',N'*-tetramethylethylenediamine–Tris-glycine buffer (pH 8.1), 60 mM with respect to glycine. When ions were added, these were present at the same concentrations in the gels and the reservoir buffer as well as in the protein sample. When electrophoresis was carried out with urea, this was present in the gels at a final concentration of 8 M. The gels were stained for protein with 0.5% Amido Black in 7% acetic acid and destained by washing in 7% acetic acid. To rerun individual bands, 1-mm segments were cut from unstained gels (by comparison with a stained gel). Each segment was placed on top of a 0.5 cm length of stacking gel (2.5% acrylamide), which had been polymerized on top of a regular running gel (7.5% acrylamide) as described above, and covered with buffer. The gels were run 5 min at 0.5 mA/tube and then about 40 min at 1 mA/tube until the marker dye (bromophenol blue) moved 10 cm from the running gel.

To determine immunological similarity of bands of S-100 separated on acrylamide gels, the following method was used. After electrophoresis, a section of the unstained gel 3 cm long, estimated to contain all the S-100 bands, from results of previous experiments, was cut into 14 equal segments. Agarose plates were poured from 0.5% Agarose dissolved in 0.15 M NaCl–0.02 M potassium phosphate (pH 7.2). A center hole and another hole 13 mm apart were cut; 20 μl of anti-S-100 and 20 μl of 0.2 mg/ml of S-100, respectively, were pipetted into each of the holes, and seven of the gel segments were laid serially in a circle of 13-mm radius around the center hole. Thus, two plates were prepared for each gel. The plates were incubated in a moist chamber at room temperature for 72 hr.

Calcium Binding. Equilibrium dialysis was done in 4-mm i.d. Pyrex tubes ground flat at one end. Each tube was fitted with a piece of wet dialysis tubing stretched over the ground end and held by a polyethylene band. The S-100 was contained in 0.1 ml of buffer inside the tube which was dipped into 1 ml of buffer containing various concentrations of ^{45}Ca. Dialysis was carried out at 25° with shaking in a water bath for 24 hr. It was shown that equilibrium was attained in less than 20 hr. Samples were taken from inside and outside the bag, dissolved in NCS reagent (Nuclear-Chicago), and counted in scintllation fluid using a Packard Tri-Carb counter. The S-100 concentration inside the tube was determined after the dialysis by measuring the absorbance at 280 mμ.

Intrinsic viscosity measurements were carried out in a Cannon-Mansing semimicroviscometer in a constant-temperature bath controlled at 25 ● 0.005°. Flow times in all experiments were reproducible to ±0.4 sec.

Optical rotatory dispersion measurements were made on a Cary 60 spectropolarimeter using a 1-cm path-length thermostated cell.

Fluorescence measurements were made in a 1-cm quartz cell with an Aminco-Bowman spectrofluorometer equipped with a high-stability xenon lamp, at an ambient temperature (22–25°). For fluorescence titrations the S-100 samples were generally 60 μg in 1 ml of buffer, and titrants ($CaCl_2$ or KOH solutions) were added in 1-μl portions from a constriction pipet. The primary monochromater was set at 290 mμ and the secondary at 360 mμ. The sensitivity of the instrument was adjusted before every reading with a tryptophan standard (in the same buffer) and checked immediately after. There was negligible turbidity at the Ca^{2+} and S-100 concentrations were used.

Difference spectra were obtained using a Cary 14 double-beam spectrophotometer with a thermostated cell compartment. In all experiments four matched cells of identical path length were used, and reagents added to the protein solution in the sample beam were compensated in the reference beam in a concentration identical with the reagent-treated protein sample beam. Difference spectra were recorded from 3200 to 2500 Å. Except when specifically stated, 2 g/l. of S-100 was used in a 20 mM Tris-HCl buffer (pH 8.3) containing 60 mM KCl.

Spectrophotometric Tyrosine Titration. The titration was done in 1-cm quartz cells in a Zeiss PMQ spectrophotometer with humidified N_2 blowing into the cell compartment. The S-100 was 0.38 mg/ml in 60 mM KCl with or without 2 mM Ca^{2+}, or with 6 M Gu-HCl. Absorbance changes were recorded at 245 and 295 mμ; data at the two wavelengths gave similar titration curves.

Denaturation Rate in Urea. The denaturation rate in urea was

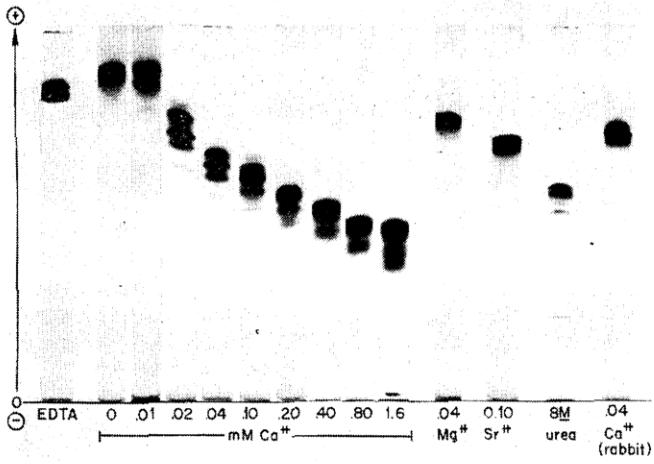

FIGURE 1: Polyacrylamide gel electrophoresis of 20 μg of S-100 on 0.25 × 10 cm 7.5% gels under the conditions described in Methods. The gels were run at 1 mA/tube until the marker dye (bromophenol blue) migrated 10 cm. All samples were beef S-100 except the one marked "[rabbit]."

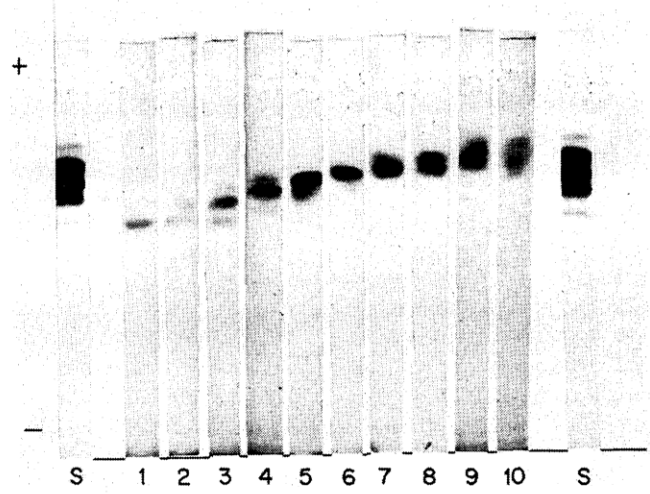

FIGURE 2: Reelectrophoresis of individual S-100 bands on polyacrylamide gel. Segments of an unstained gel containing bands of S-100 separated in the presence of 40 μM Ca^{2+} were cut out and rerun as described under Methods.

measured as rate of increase of fluorescence of the tryptophan of the protein in 8 M urea as described by Hopkins and Spikes (1968). The output of the Aminco-Bowman spectrofluorometer was connected to a recorder to determine the time course of fluorescence change. At zero time, 3 μl of a 20-mg/ml solution of S-100 in H_2O was added to 1 ml of 8 M urea in 60 mM imidazole chloride (pH 6.0) containing various Ca^{2+} concentrations. The temperature was kept at 22 ± 1°.

Rate of Enzyme Digestion. The S-100 was 5 mg/ml in 60 mM KCl–20 mM imidazole chloride (pH 7.5), with or without Ca^{2+}; at zero time trypsin or chymotypsin was added (as a 10-mg/ml solution in the same buffer) to a final concentration of 0.2 mg/ml. Incubation was at 25 ± 0.5° and 3-μl samples were taken at intervals of time into 3 μl of cold 1 N HCl to stop the digestion. Then 100 μl of ninhydrin reagent was added and the mixture was heated on a boiling-water bath. Finally, 0.5 ml of H_2O–isopropyl alcohol (1:1, v/v) was added and the absorbance was read at 570 mμ. Standards of leucine were assayed for ninhydrin color at the same time. Controls were incubated with S-100 and no enzyme, and with enzyme alone. For determination of rate of digestion of denatured S-100 a sample of 5 mg/ml of S-100 was heated 10 min in boiling H_2O.

Sulfhydryl Measurements. Sulfhydryl groups in S-100 were titrated by the method of Boyer (1954). The reagent, *p*-hydroxymercuribenzoate, was made up to 1 mM concentration and standardized by titrating a known amount of mercaptosuccinic acid. The rate of reaction of sulfhydryl groups with DTNB[1] was determined as follows (Ellman, 1959). To 100 μl of buffer (either 60 mM KCl–20 mM Tris-Cl (pH 8.3) or 6 M Gu-HCl, pH 8.3) containing 40 μg of S-100 (1.67 nmoles) and various concentrations of Ca^{2+} was added 10 μl of buffer containing 20 nmoles of DTNB. The increase in absorbance at 412 mμ was recorded as a measure of the amount of free 5-nitro-2-thiobenzoate (RSH) produced, and therefore the amount of S-100 sulfhydryl derivatized according to the equation: (S-100)-SH + RSSR → (S-100) SSR + RSH. The extinction coefficient of RSH was the same in buffer and in 6 M Gu-HCl and was assumed to be 13,600 $mole^{-1}$ cm^{-1}.

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

Results

Acrylamide Gel Electrophoresis. Hyden and McEwen (1966), Vincendon *et al.* (1967), and Gombos *et al.* (1966) found that S-100 showed several bands when separated on acrylamide gel electrophoresis. We carried out a series of experiments under different conditions in a continuous buffer system (see Methods). The pure protein as well as the S-100 present in a total brain homogenate gave a single band when either no Ca^{2+} or 0.2 mM Tris-EDTA was present in the system. However, with no EDTA present, addition of Ca^{2+} gave a multiple-band pattern (Figure 1). Several bands were observable with as low as 0.01 mM Ca^{2+} and increased Ca^{2+} concentrations up to 0.1 mM showed a greater number of more completely resolved bands up to five in number. Higher concentrations from 0.2 to 1.6 mM showed a more diffuse pattern. The multiple-band patterns with Ca^{2+} appeared when electrophoresis was done at pH 7.5 as well as at 8.1. We carried out another series of experiments to ascertain the specificity of such effects and the possible involvement of the conformational state of the protein. Electrophoresis in the presence of three different Mg^{2+} concentrations (0.04, 0.1, and 0.5 mM) showed a single component indicating, as will be more extensively later demonstrated, the specificity of the Ca^{2+} effect. However, when the S-100 was run in gels containing 8 M urea with or without 0.1 mM Ca^{2+}, multiple bands were also seen. When regions containing each band were cut out and rerun as described under Methods, there was no evidence that the forms were interconvertible (Figure 2). The five bands produced when S-100 was electrophoresed in the presence of 0.04 mM Ca^{2+} were immunologically identical as measured by double diffusion in agar as described under Methods (Figure 3). That is, a band of identity was produced by all the segments of the gel containing the five separated bands of S-100. Finally, rabbit S-100 also gave several bands in the presence of Ca^{2+} (Figure 1).

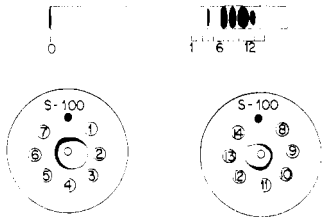

FIGURE 3: Immunological similarity of forms of S-100 obtained by polyacrylamide gel electrophoresis with 40 μM Ca^{2+}. Fourteen segments from an unstained gel were laid around a center well of anti-S-100 on an Agarose plate. The diffusion was allowed to proceed 72 hr at room temperature.

Sucrose Gradient and Sephadex Column Results. In order to determine whether or not the Ca^{2+} effect on electrophoretic patterns was due to aggregation or disaggregation of S-100, the protein was chromatographed on G-100 Sephadex with or without Ca^{2+}. No difference in pattern was observed indicating that the effect was not accompanied by change in molecular size. In addition, when S-100 was centrifuged in a sucrose density gradient (60,000 rpm for 16 hr), no effect of Ca^{2+} on sedimentation was observed. Sucrose (20%) did not have any effect on the increase in fluorescence observed with Ca^{2+} as discussed below, so that sucrose presumably did not inhibit the possible conformational change induced by Ca^{2+} as described below.

Optical Rotatory Dispersion and Intrinsic Viscosity Measurements. While our studies on the electrophoretic behavior of S-100 with Ca^{2+} and other ions were in progress, an investigation of the effect of Ca^{2+}, EDTA, and mercaptoethanol was published (Kessler *et al.*, 1968) which showed that the only detectable effect of such substances on S-100 consisted in protecting the protein from thermal denaturation as evaluated by quantitative complement fixation and by optical rotatory dispersion measurements. In addition, optical rotatory dispersion measurements did not show any direct effect of Ca^{2+} or EDTA on the secondary structure of the protein. We confirmed these optical rotatory dispersion results showing that different Ca^{2+} concentrations (0.1–2 mM) had no detectable effect on the optical rotatory dispersion pattern. In addition, intrinsic viscosity measurements of the protein did not show any consistent difference. For example, the intrinsic viscosity was unchanged ($[\eta]$ = 2.9 cc/g) when measurements were made in a solution of 20 mM Tris-Cl (pH 8.3) containing 60 mM KCl and 10 mg/ml of S-100 in presence or absence of 10 mM Ca^{2+}; measurements in the presence of the same buffer, containing 5 mM KCl and 5 mg/ml of protein gave an ($[\eta]$ = 3.4 cc/g) but no difference whether 5 mM Ca^{2+} was present or absent.

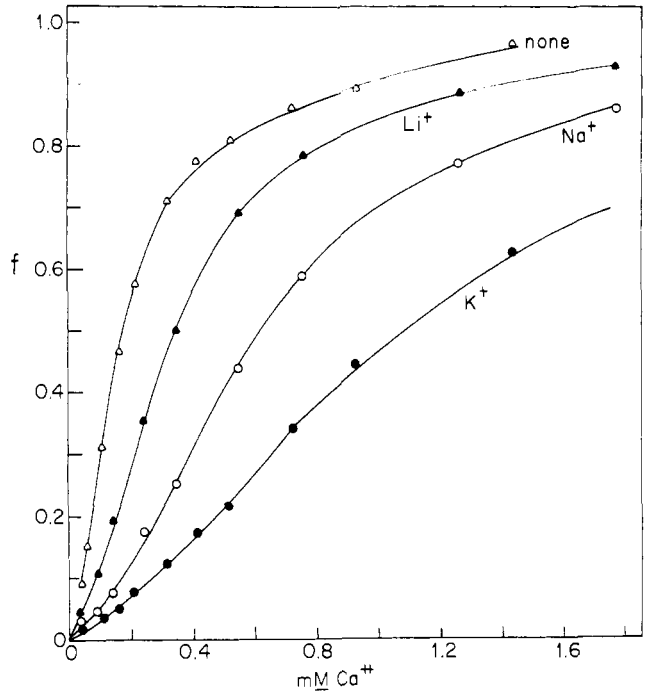

FIGURE 5: Fluorescence titration of S-100 with Ca^{2+}, under the conditions described in Methods, in the presence of no monovalent cation and of 60 mM concentrations of K^+, Na^+, and Li^+ f is defined in the text.

Calcium Binding. Binding of Ca^{2+} to S-100 was studied by equilibrium dialysis using ^{45}Ca. The results of four separate experiments at two concentrations of S-100, given as reciprocal plots in Figure 4, indicated that there were 8–10 binding sites with dissociation constants of 1 mM under the conditions

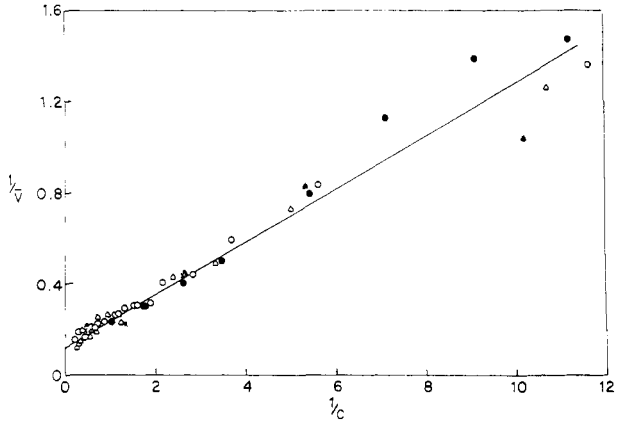

FIGURE 4: Reciprocal plot of data for binding of Ca^{2+} by S-100. The concentration of Ca^{2+} is c (millimolar) and the average number of Ca^{2+} ions bound per molecule of S-100 (mol wt 24,000) is $\bar{v}$. O, ●, and ▲ refer to three experiments at 1 mg/ml of S-100 and X refers to one experiment at 0.5 mg/ml.

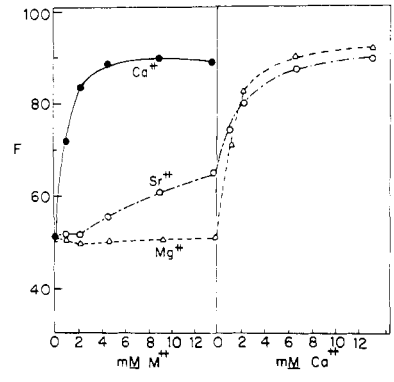

FIGURE 6: Fluorescence titration of S-100 with Ca^{2+}, Sr^{2+}, or Mg^{2+}. The S-100 was 60 μg/ml on 60 mM KCl–20 mM Tris-Cl (pH 8.3). F is the fluorometer reading.

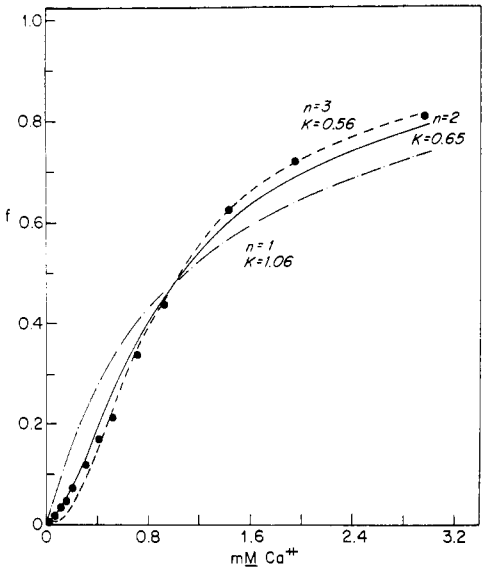

FIGURE 7: Fluorescence titration of S-100 (60 μg/ml in 60 mM KCl–20 mM Tris-Cl, pH 8.3) with Ca^{2+}. The symbol f is described in the text. The three lines are calculated for different numbers of involved Ca^{2+} binding sites (n = 1, 2, and 3) with the best dissociation constants. The filled circles represent the experimental data.

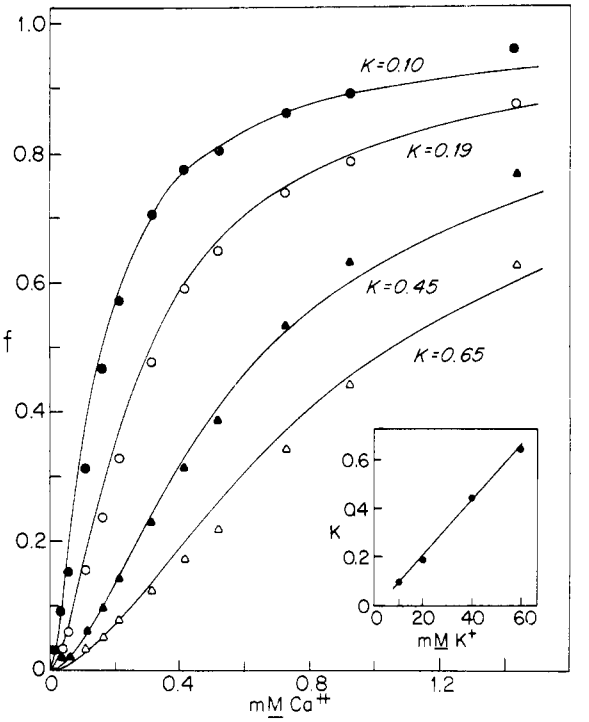

FIGURE 8: Fluorescence titration of S-100 (60 μg/ml in 20 mM Tris-Cl, pH 8.3) with different concentrations of KCl: △, 60 mM; ▲, 40 mM; ○, 20 mM; ●, 10 mM. The lines are calculated lines assuming two Ca^{2+} binding sites and dissociation constants shown. The inset shows the relationship between K^+ concentration and the dissociation constant, K.

used (pH 8.3, 60 mM K^+). It was shown that equilibrium was reached within the time period used (20–24 hr) when ^{45}Ca was added to either side of the membrane.

Fluorescence Measurements. In order to determine if Ca^{2+} induced a conformational change in S-100 involving aromatic residues, the following experiments involving fluorescence and spectral measurements were carried out. The amino acid analysis indicated three tyrosines per mole. Tryptophan content, determined by *N*-bromosuccinimide oxidation, was a single tryptophan residue per molecule when done in the presence or absence of Ca^{2+} or in 6 M Gu-HCl.

Binding of Ca^{2+} had a large effect on the fluorescence of the tryptophan of S-100 as shown in Figure 5. The fluorescence was more sensitive to low concentrations of Ca^{2+} in the absence of monovalent cations, while K^+, Na^+, and Li^+ ions inhibited the Ca^{2+} enhancement of fluorescence, the effects being in the order $K^+ > Na^+ > Li^+$. The Ca^{2+} enhancing effect was reversible, since addition of EDTA brought the fluorescence back to the value obtained without Ca^{2+}. The fluorescence enhancing effect was specific for Ca^{2+} (Figure 6), there being no enhancement with Mg^{2+} and slight with Sr^{2+}. Furthermore, the presence of Mg^{2+} did not seem to alter the effect of Ca^{2+}, indicating a lack of competition.

When a fluoresence titration of S-100 with increasing amounts of Ca^{2+} was carried out (in 60 mM KCl, pH 8.3), an S-shaped curve was obtained (Figure 7) suggesting that more than one Ca^{2+} binding site was involved in the fluorescence effect and that there was a cooperative binding effect. The relative fluorescence change, f, plotted in Figure 7 was defined as $(F - F_0)/(F_{max} - F_0)$, where F was the fluorescence reading at a certain calcium concentration, c, F_0 was the fluorescence reading at $c = 0$, and $F_{max} - F_0$ was estimated by plotting $1/(F - F_0)$ *vs.* $1/c$ at high c values (above 2 mM); extrapolation to $1/c = 0$ gave the value of $1/(F_{max} - F_0)$. The solid line in Figure 7 was calculated on the assumption that there were two consecutive binding sites involved in the fluorescence enhancing effect, with a dissociation constant, K, of 0.65 mM. With such an assumption, it can be shown that $1/f = 1 + K/c + K^2/c^2$. This assumption of two binding sites gave a much better fit to the data than the assumption of a single site, while the assumption of three sites also gave a reasonably good fit (see Discussion). Varying the concentration of K^+ apparently changed the binding constant for Ca^{2+} (Figure 8). The solid lines in the figure were calculated for two binding sites with the dissociation constants shown. There was an approximately linear relationship between K^+ concentration and the dissociation constant for Ca^{2+} (inset, Figure 8).

Finally, the fluorescence enhancement by Ca^{2+} was influenced by pH (Figure 9). There was no effect at pH 6 and a maximum effect between pH 8 and 10. The pH *vs.* fluorescence curve was reversible below pH 10 with and without Ca^{2+}. The amount of Ca^{2+} added in Figure 9 (4 mM) produced nearly the maximal effect at all pH values; that is, additions of more Ca^{2+} at any pH did not increase fluorescence appreciably. The shapes of the pH *vs.* fluorescence curves suggested that the fluorescence was affected by some group titrating with a pK of about 7.2 which had a greater effect in the presence of Ca^{2+} than in its absence. The effect of 8 M urea (Table I) seemed to be at least partially reversible, since although the fluorescence of S-100 in urea was not enhanced by Ca^{2+}, yet when S-100 was dissolved in 8 M urea and then the solution was diluted to a urea concentration of 0.032 M, the fluorescence fell and the Ca^{2+} enhancement of fluorescence was at least partially restored. There was no significant effect of Ca^{2+}

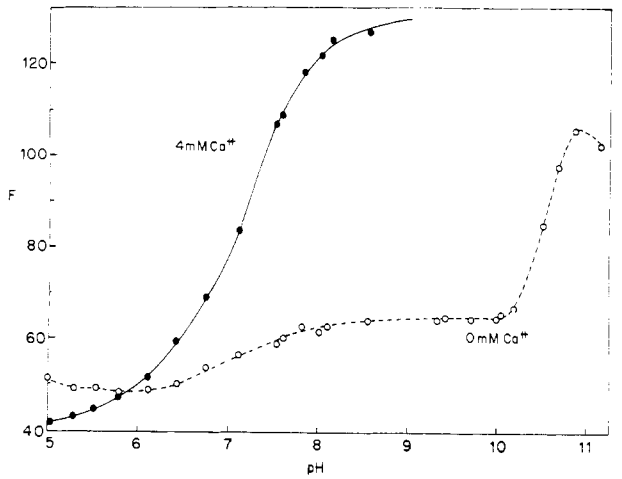

FIGURE 9: Fluorescence pH titration of S-100 (60 μg/ml) in the absence and presence of 4 mM Ca^{2+}. *F* is the arbitrary fluorometer reading.

(0.25 or 0.75 mM Ca^{2+} in 60 mM KCl–20 mM Tris-Cl at either pH 7.2 or 8.3) on the tryptophan fluorescence of the following pure proteins: bovine serum albumin, pepsin, glutamic dehydrogenase, alcohol dehydrogenase, trypsin, egg albumin, and chymotrypsin. Also, a similar fluorescence enhancement effect of Ca^{2+} on pure preparations of S-100 from human, hog, and rabbit brains was observed.

Difference Spectra. The possible effect of Ca^{2+} on tyrosine and phenylalanine, as well as tryptophan, chromophores was investigated by measuring difference spectra. Figure 10 shows the difference spectra of S-100 when Ca^{2+} or K^+ ions were added to the sample cell compared with nontreated protein in the reference cell. The effect of 4 mM Ca^{2+} was greater and opposite to the effect of 60 mM K^+, the first producing a blue and the second a red shift. This change evoked by K^+ confirmed its antagonism to the Ca^{2+} effect on fluorescence enhancement described above. Addition of 4 mM Ca^{2+} brought about a blue shift apparently involving tyrosine (at 2860 Å) and phenylalanine residues (at 2780 and 2720 Å) as well as the tryptophan (at 2930 Å). The effect of 4 mM Ca^{2+} on the tyrosine difference

TABLE I: Effect of Urea and Ca^{2+} on Fluorescence of S-100.[a]

S-100 Solvent	Dilution Solvent	$-Ca^{2+}$ *F*	$+Ca^{2+}$ (10 mM) *F*	ΔCa^{2+}
B	B	39.7	76.0	36.3
U	B	44.2	67.1	22.9
U	U	88.7	92.2	3.5

[a] S-100 was dissolved to a concentration of 20 mg/ml in either 8 M urea buffer (U) or buffer alone (B). Three microliters of each of these S-100 solutions were added to 1 ml of U or B and the fluorescence was measured. Finally, 10 μl of 1 M $CaCl_2$ was added to the diluted S-100 solutions and the fluorescence was read again. The buffer was 0 mM Tris-Cl–100 mM NaCl (pH 8.2). The fluorescence values are in arbitrary units (fluorometer readings).

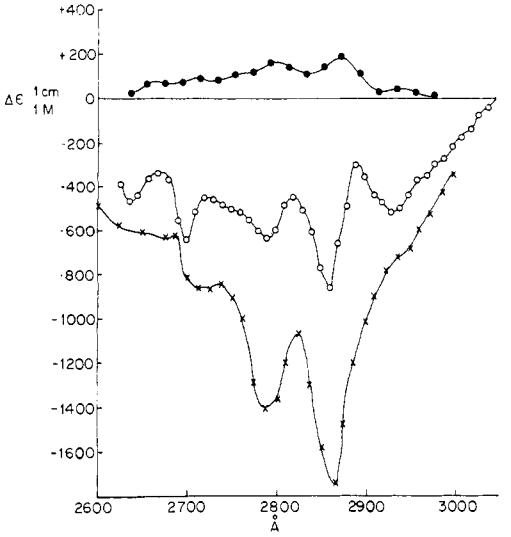

FIGURE 10: Difference spectra of S-100 in 20 mM Tris-Cl, pH 8.3. The reference cell contained S-100 in buffer and the sample cell contained S-100 in buffer plus: ●, KCl; ○, $CaCl_2$; X, 6 M Gu-HCl. The Δε was calculated assuming a molecular weight for S-100 of 24,000.

peak (at 2860 Å) seemed to produce slightly more than half the effect of 6 M Gu-HCl. The relative effect on the tryptophan peak (at 2930 Å) was difficult to judge since this peak was a shoulder on the tyrosine peak in Gu-HCl, but it appeared that Ca^{2+} produced nearly the effect of Gu-HCl. The effect of Ca^{2+} on the phenylalanine peak (at 2780 Å) also seemed to be slightly greater than half that of Gu-HCl. These results are consistent with the idea that Ca^{2+} caused a complete unmasking of the single tryptophan chromophore, but an unmasking of only a fraction of the total number of tyrosine and phenylalanine residues. There was no difference spectrum produced by adding 2 mM Mg^{2+} to S-100 under the same conditions, and there was no difference spectrum generated when Ca^{2+} was

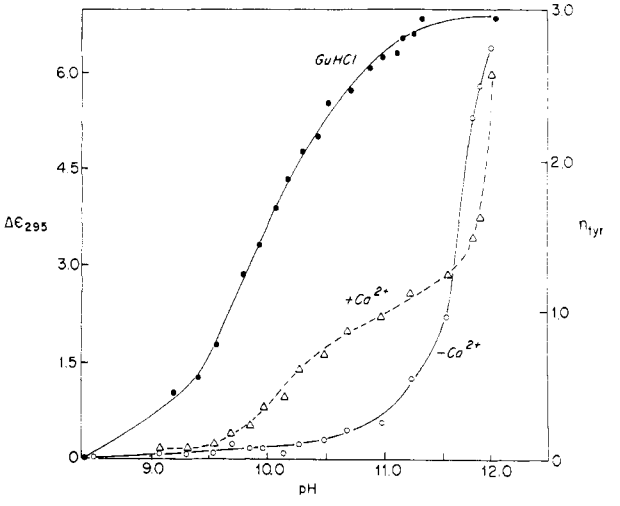

FIGURE 11: Tyrosine titration of S-100 (1 mg/ml). The conditions are: ●, 6 M Gu-HCl; ○, no Ca^{2+}; △, 4 mM Ca^{2+}. The values of Δε were calculated assuming mol wt 24,000 for S-100.

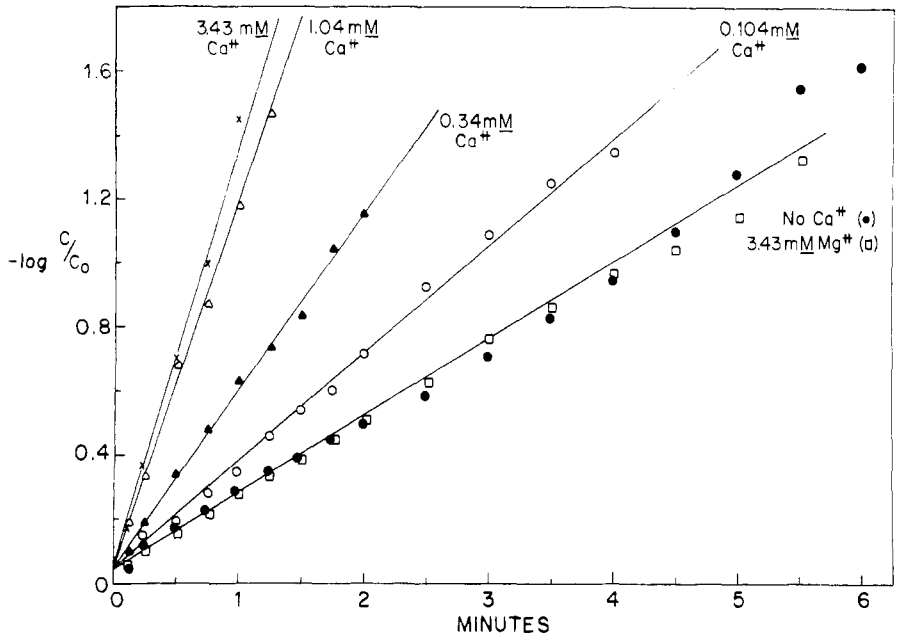

FIGURE 12: First-order reaction plots for denaturation of S-100 by 8 M urea as measured by increase of tryptophan fluorescence, in the presence of various concentrations of Ca^{2+} and Mg^{2+}. The S-100 was 60 μg/ml in 60 mM KCl–20 mM Tris-Cl (pH 8.3)–8 M urea.

added to bovine serum albumin under the same conditions as those under which S-100 was studied.

Spectrophotometric Titration of Tyrosine in S-100. The curves shown in Figure 11 were obtained when S-100 was titrated with NaOH in the absence or presence of Ca^{2+}, and in 6 M Gu-HCl as a reference for S-100 with fully exposed tyrosine residues. The curve in Gu-HCl showed three tyrosines titrating with p*K*'s of about 9.9. None of the tyrosines, in the absence of Ca^{2+}, titrated until there seemed to be a sudden conformational change at pH 11.5. However, in the presence of Ca^{2+}, one tyrosine titrated at pH 10.1 while the other two were buried until the conformational change at pH 11.5. In agreement with the results from fluorescence and difference spectral measurements, Mg^{2+} had no effect on titration of tyrosine.

Rate of Denaturation. The effect of Ca^{2+} on the rate of denaturation of S-100 in 8 M urea was determined by measuring the rate of increase of tryptophan fluorescence of the protein due to its unfolding in urea. The denaturation rate was measured at pH 6.0 since at that pH the fluorescence of S-100 in the absence of urea was not affected by the presence of Ca^{2+} (see Figure 9). First-order rate plots for increase of fluorescence intensity after S-100 was added to 8 M urea containing various concentrations of Ca^{2+} are shown in Figure 12. The rate was increased five to six times when 3.43 mM Ca^{2+} was present compared with the rate in the absence of Ca^{2+}. This result was also consistent with the idea that Ca^{2+} had an effect on the environment of the tryptophan residue, probably inducing a looser or more unfolded structure in that region. Furthermore, in harmony with the observations on fluorescence and difference spectra, Mg^{2+} had no effect on rate of urea denaturation.

Digestion by Proteolytic Enzyme. As another measure of the effect of Ca^{2+} on exposure of specific residues of S-100, the rates of digestion of the protein by the proteolytic enzymes, trypsin and chymotrypsin, in the presence and absence of Ca^{2+} were determined (Figure 13). A large increase in the rate of digestion by chymotrypsin was induced by Ca^{2+}, but not by Mg^{2+}. As a control, the rate of digestion of the denatured protein (heated at 100° for 10 min) was high and only slightly affected by Ca^{2+}. As another control, the rate of digestion of either native or heat-denatured bovine albumin was not affected by adding Ca^{2+}. The rate of digestion of S-100 by trypsin was found to be slightly increased by the presence of Ca^{2+}, but the effect was only about one-third that seen with chymotrypsin. Since chymotrypsin is specific for peptide bonds adjacent to aromatic residues, this result is also consistent with the idea that a specific region (or regions) of the S-100 molecule, containing the tryptophan and a portion of the tyrosine and phenylalanine residues, is involved in the Ca^{2+} effect.

Reaction with Sulfhydryl Reagents. The rate of reaction of *p*-mercuribenzoate with S-100 was not affected significantly by Ca^{2+}, and the number of sulfhydryl groups, determined by *p*-mercuribenzoate titration in buffer or in 8 M urea, was three per molecule.

When DTNB was added to S-100 in 6 M Gu-HCl (Figure 14), all three sulfhydryls were rapidly derivatized, but when the reagent was added to S-100 in buffer without Ca^{2+}, the rate was extremely slow. The presence of various concentrations of Ca^{2+} stimulated markedly the reaction rate with DTNB, but the effect was limited to only two of the three sulfhydryls. There was only a small effect of Mg^{2+} on the rate.

In order to test the reversibility of the effect of Ca^{2+}, S-100 was allowed to stand 30 min in 1.51 mM Ca^{2+} in 60 mM KCl–20 mM Tris-Cl (pH 8.3). To a portion of the Ca^{2+}-treated S-100 was added EDTA to a final concentration of 1.99 mM and the reactivity of the sulfhydryl groups with DTNB was tested for the two samples. Treatment with EDTA seemed to reverse completely the Ca^{2+} effect on exposure of the sulfhydryls. As a control, it was shown that Ca^{2+} had no effect on the rate of reaction of DTNB with the sulfhydryls of bovine serum albumin or mercaptosuccinate.

Discussion

It is clear from the data that Ca^{2+} binding induces a confor-

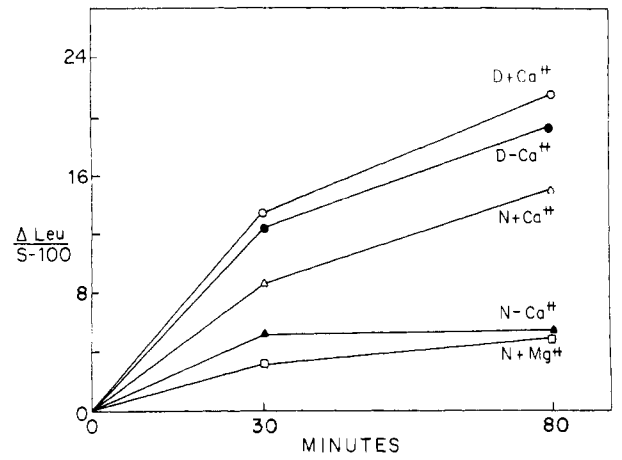

FIGURE 13: Digestion of S-100 by chymotrypsin as described under Methods. ΔLeu/S-100 is the increase in ninhydrin color after digestion divided by the concentration of S-100 in arbitrary units. D and N refer to heat-denatured and native S-100 as described in Methods. The Ca^{2+} concentration was 3 mM.

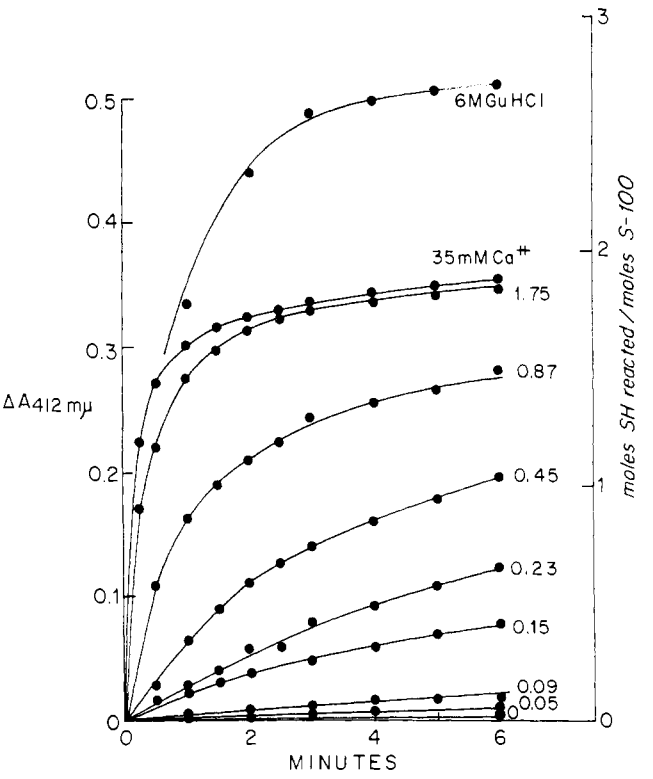

FIGURE 14: Effect of various concentrations of Ca^{2+} and 6 M Gu-HCl on rate of reaction of sulfhydryl groups of S-100 with DTNB as described in Methods. The moles of SH reacted per mole of S-100 was calculated assuming an extinction coefficient of 13,600 $mole^{-1}$ cm^{-1} for the product of the reaction and a value of mol wt 24,000 for S-100. The S-100 was 4 mg/ml in 60 mM KCl–20 mM Tris-Cl (pH 8.3).

mational change in S-100, which probably does not lead to a large change in shape of the molecule, since it is not disclosed by viscosity measurements. Furthermore, the secondary structure is not directly affected to any great degree by Ca^{2+} since there is no effect on optical rotatory dispersion spectra. The fluorescence, difference spectral, and spectrophotometric titration data indicated that the conformational change involves the single tryptophan residue and a portion of the tyrosine and phenylalanine residues in a manner that leads to greater exposure of the affected aromatic groups. The urea denaturation and chymotrypsin digestion data support this idea. Furthermore, two of the three cysteine residues also are affected by Ca^{2+} binding, so that they are transformed from a state of nearly complete unreactivity toward DTNB, a sulfhydryl reagent, to one of high reactivity.

The data for Ca^{2+} effect on tryptophan fluorescence were consistent with the assumption that two consecutive Ca^{2+} binding sites, with dissociation constants of 0.65 in 60 mM K^+ (pH 8.3) were involved in the conformational change. The dissociation constant for Ca^{2+} calculated from the equilibrium dialysis experiments was 1 mM which roughly agrees with this assumption. The assumption of three binding sites involved in the fluorescence change seems to fit the fluorescence data equally well, but the dissociation constant (0.56) is further from the value of 1 mM obtained from the binding experiments. The assumption of one site did not fit the data. Although binding constants were not calculated from the difference spectral data, the Ca^{2+} concentrations necessary to cause changes in the chromophores measured by this method were in general agreement with the fluorescence data. The same was true of the data on sulfhydryl reactivity. It cannot be decided from the data whether or not the same Ca^{2+} binding sites are involved in the changes in tryptophan, tyrosine, phenylalanine, and cysteine residues. The simplest explanation would, of course, be that only 2 of the 8–10 Ca^{2+} binding sites were concerned with all of the effects measured, involving a conformational change in only one region of the S-100 molecule.

The basis for the appearance of multiple bands on acrylamide gel electrophoresis in the presence of Ca^{2+} is not known. It is clear from the agar diffusion experiment that all the bands are immunologically similar, at least by agar diffusion against antiserum, and therefore, are presumably analogous proteins having the same function whatever that may be. The fact that the bands are stable when reelectrophoresed shows that they are not at all (or at least not readily) interconvertible. It seems unlikely that they are stable conformational isomers produced by Ca^{2+}, since the conformational effects of Ca^{2+} measured by the other methods described seem to occur rapidly and to be reversible.

The simplest explanation fitting the data would be that there are small differences in primary structure which are hidden in the absence of Ca^{2+} and exposed in its presence, and that these differences, when revealed, result in forms of S-100 with differing electrophoretic mobilities. Such differences in primary structure would also be revealed when the protein is completely unfolded in a denaturing solvent such as 8 M urea, which would explain the multiple forms seen under this condition.

It is not possible to tell whether the effect of Ca^{2+} leading to multiple forms of S-100 on acrylamide gel electrophoresis and its effects on the environment of aromatic and cysteine residues are related. The maximum number and the clearest separation of bands occurs at 0.04–0.1 mM Ca^{2+} at pH 8.3 in the absence of monovalent cations (Figure 1). This corresponds roughly to the range of Ca^{2+} concentrations where the fluorescence *vs.* Ca^{2+} curve is steepest under the same condi-

tions (in Figure 5, the second and third points on the curve for the effect of Ca^{2+} in the absence of monovalent cations are for Ca^{2+} concentrations at 0.04 and 0.1 mM, respectively). This correspondence can be taken as presumptive evidence that the electrophoretic effects and the conformational change are related in mechanism.

A tentative picture of the effect of Ca^{2+} on S-100 therefore can be drawn as follows: there are eight to ten binding sites for Ca^{2+} at least two of which are specific for Ca^{2+} and, when occupied, are responsible for a local unfolding or opening of the tertiary structure. This conformational change results in exposure of the tryptophan, several of the tyrosine and phenylalanine, and two of the three cysteine residues. The conformational change induced by Ca^{2+} also exposes a region of the molecule (which could be the same region containing the tryptophan residue) which has a variable primary structure.

Since some functions of the nervous system are accompanied by local changes in ionic concentrations of Ca^{2+}, Na^+, and K^+ and since S-100 is specific to the nervous system, it is possible that these conformational changes induced by Ca^{2+} may be related to its function. This idea is strengthened by the fact that the effect is specific for Ca^{2+}, no change being seen with Mg^{2+}, and that Na^+ and K^+ effects differ in degree. Although in the absence of K^+ or Na^+ S-100 is extremely sensitive to low Ca^{2+} concentrations, at physiological levels of monovalent cations (*e.g.*, 60 mM K^+) the conformational change occurs in the range of Ca^{2+} concentrations which probably exist in the nervous system (0.1–4 mM). If there are multiple forms of S-100 differing in primary sequence, such differences could be either masked or exposed depending upon relative concentrations of Ca^{2+}, Na^+, and K^+.

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