Registry No. 16α-BAP, 84623-69-8; progesterone, 57-83-0; histidine, 71-00-1; methionine, 63-68-3; 11α-hydroxyprogesterone, 80-75-1; 11α-[2′-^{3}H]BAP, 84623-70-1; 11α-BAP, 36049-50-0.

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Spectral Studies on the Calcium Binding Properties of Bovine Brain S-100b Protein†

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ABSTRACT: The effect of Ca^{2+} binding on the circular dichroism (CD) and 270-MHz proton nuclear magnetic resonance (NMR) spectra of brain-specific S-100b calcium binding protein has been examined at two pH values, 8.5 and 7.5. At pH 8.5, S-100b protein binds two Ca^{2+} per monomer with K_d values of 6×10^{-5} and 2×10^{-4} M, whereas at pH 7.5, the protein binds only one Ca^{2+} per monomer with a K_d of 2×10^{-4} M. The presence of K^+ inhibits the binding of Ca^{2+} to the higher affinity site at pH 8.5, and the affinity for calcium is lowered to $K_d = 8.5 \times 10^{-4}$ M. Mg^{2+} has no effect on protein conformation. In the absence of Ca^{2+}, S-100b undergoes a conformational change when the protein is titrated from pH 8.6 to 6.0. Addition of Ca^{2+} perturbed the environment of tyrosine and phenylalanine residues as measured by ultraviolet difference spectroscopy and ^{1}H NMR. CD melt experiments and far-ultraviolet CD studies at alkaline pH and NMR experiments suggest that the protein is more stable in the presence of Ca^{2+}. The single tyrosine residue in the protein ionizes only after the protein is denatured by exposure to high pH.

The characterization of proteins specific to nervous tissue is essential for studying the structure and the function of the nervous system at the molecular level. The brain-specific S-100 protein is found primarily in glial cells (Moore, 1965) and represents up to 0.2% of the total soluble brain protein. The biological function of this protein is unknown; however, previous results suggest a role for it in the function or development of the nervous system (Hyden & Lange, 1970; Calissano & Bangham, 1971; Calissano et al., 1974). S-100 protein, which is a water-soluble, highly acidic calcium binding protein (Calissano et al., 1976), is actually a mixture of two components, S-100a and S-100b, with a subunit composition of $\alpha\beta$ and β_2, respectively (Isobe & Okuyama, 1981). S-100b exists as a dimer of 21 000 molecular weight in native solvents,

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and the two subunits are held together by noncovalent forces (Mani et al., 1982).

The amino acid sequences of S-100 proteins have been determined (Isobe & Okuyama, 1978, 1981). The S-100b protein polypeptide chain (β subunit) consists of 91 amino acid residues and contains a cluster of acidic amino acids that has a high sequence homology with the calcium binding proteins the parvalbumins (Pechêre et al., 1971) and troponin C (Collins et al., 1978; Van Eerd & Takahashi, 1976), suggesting an evolutionary relationship among them. The α subunit in S-100a consists of 93 amino acid residues, possesses an extensive sequence homology (58%) with that of the β subunit, and also shares an apparent calcium binding site in the C-terminal half of the molecule, implying a close evolutionary relationship between these subunits.

In our earlier work on the purified S-100b protein, we demonstrated a conformational change upon binding Ca^{2+}. There was a loss in protein secondary structure when Ca^{2+} was bound at pH 7.5. The effect of K^+ on the protein was antagonistic to Ca^{2+}, and the protein's affinity for calcium was lowered by the presence of K^+ (Mani et al., 1982). Since the binding of S-100 proteins to synaptosomal particles and liposomes depends on pH, as well as Ca^{2+} and K^+ concentration (Donato, 1976), it seemed important to study the Ca^{2+} binding properties of S-100b as a function of pH. Also, in an earlier study on the mixture of S-100 proteins, Calissano et al. (1974) had shown that a pH 8.3, S-100 protein binds 4 mol of Ca^{2+}, whereas at pH 7.6, only 2 mol of Ca^{2+} was bound per mol of protein.

In the present study, we have employed circular dichroism (CD),[1] NMR, and UV difference spectroscopy to study the binding of Ca^{2+} to purified S-100b protein at pH 8.5 in order to compare these results with our earlier findings at pH 7.5. The effect of K^+ on the binding of Ca^{2+} to S-100b at pH 8.5 was also studied.

In order to probe the ionization characteristics of the tyrosine residue in the protein, we carried out a spectrophotometric titration in the alkaline pH range. CD melt experiments in the absence and presence of Ca^{2+} were effected to study the thermal stability of this brain-specific protein. The additional spectral data obtained on S-100b have enabled us to compare its behavior with that of other calcium binding proteins, which in the final analysis might give a clue to understanding its possible functional role in the brain.

Materials and Methods

S-100b protein was prepared from bovine brain as previously described (Mani et al., 1982) with a minor modification. The solvent system used for the Sephacryl S-200 gel filtration column consisted of 0.1 M Tris-HCl buffer, pH 7.5, 2 mM EDTA, and 0.4 M NaCl. The objective was to improve the resolution and the recovery of the protein from the column. S-100b protein used in the present study was homogeneous according to the criteria set out in our previous publication, which included polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. In particular, in the absence of the surfactant, S-100a and S-100b should separate since their mobilities are different (Isobe & Okuyama, 1981). No such separation was noted in our protein preparation, suggesting that our S-100b is pure. In addition, there was no indication of any tryptophan in our S-100b preparation, as revealed by ultraviolet absorption and derivative spectra. Protein concentrations were routinely determined from absorbance measurements by using a value of 2.4 as the extinction coefficient, $E^{1\%,1cm}_{278nm}$, for this protein (Mani et al., 1982). Ultraviolet difference spectra were obtained on a Cary 118C recording spectrophotometer using a 1-cm-path-length cell. Aliquots of concentrated perturbant, i.e., $CaCl_2$, were added to the sample cell while an equivalent volume of water was added to the reference cell. Protein concentrations employed were in the range 0.7–1.5 mg/mL. In most cases, the instrument was operated in the "autoslit" mode, and full-scale absorbance was 0.05. Scan speeds from 0.02 to 0.1 mm/s were employed, and the spectra were measured at 25 °C with a thermoregulator using a thermostated cell assembly.

Circular dichroism measurements and melt experiments were carried out on a Cary Model 6001 CD attachment to a Cary 60 reecording spectropolarimeter, in accordance with previously described methodology (Mani et al., 1974). Spectrophotometric titrations were performed with a Beckman DU spectrophotometer, and the solvent system used was 10 mM Tris-HCl buffer, pH 8.0, with either 1 mM EDTA or 0.5 mM $CaCl_2$ included. Protein samples were titrated manually with KOH in a constantly stirred vessel. After the addition of base, an aliquot was removed, and the absorbance was read at 294 nm. The pH of the solution was measured in a Radiometer 62 pH meter after the aliquot was replaced in the titration vessel.

The ^1H NMR spectra were obtained by using a Bruker HXS-270 NMR spectrometer operating in the Fourier-transform mode and equipped with a Nicolet quadrature accessory. The ambient temperature of the samples was 299 K, and all samples were equilibrated 10–15 min prior to acquisition. The parameters used for the spectra were typically 4K data points, ±2300-Hz sweep width, 5000-Hz filter width (Bessel), and 8-μs radio-frequency pulse (~80°). The HDO resonance was suppressed with homonuclear decoupling. Chemical shift values are relative to the major resonance of DSS, which was measured separately. The protein solutions were dialyzed against 10 mM Tris, pH 7.5 or 8.5 (see text), in the presence of EDTA and then subjected to exhaustive dialysis against the same buffer in the absence of EDTA (Mani et al., 1982). The solution was then lyophilized to dryness and lyophilized once again from 1–2 mL of dithizoned D_2O. The sample was then made up to volume with dithizoned D_2O; protein concentrations were 0.1–0.5 mM, as determined by the UV absorbance (see previous text).

The pH measurements were made with an Ingold microelectrode (Model 6030-04) attached to a Radiometer 24 pH meter; the pH values quoted are those observed and are not corrected for the deuterium isotope effect on the glass electrode. Values above pH 11 were not corrected for Na^+ interference and thus cannot be considered more accurate than ±0.2 pH unit. Electrode standardization was achieved prior to each measurement by using Fischer standard buffers 4, 7, and 10. pH adjustments were made by the addition of small aliquots of 0.5 M Chelex-treated NaOD. The total volume change over the titrations was 11–20%, primarily due to loss of sample on the electrode. The sample pH was measured prior to acquisition, and the samples were equilibrated 10–15 min prior to acquisition. The histidine resonances were isolated from the rest of the spectrum by using the Hahn spin-echo pulse sequence (T2HSE; Campbell, 1975); these results are not shown.

[1] Abbreviations: NMR, nuclear magnetic resonance; TN-C, troponin C; CD, circular dichroism; UV, ultraviolet; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; ICABP, intestinal calcium binding protein; Mops, 3-(*N*-morpholino)propanesulfonic acid; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; T2HSE, T_2 Hahn spin-echo.

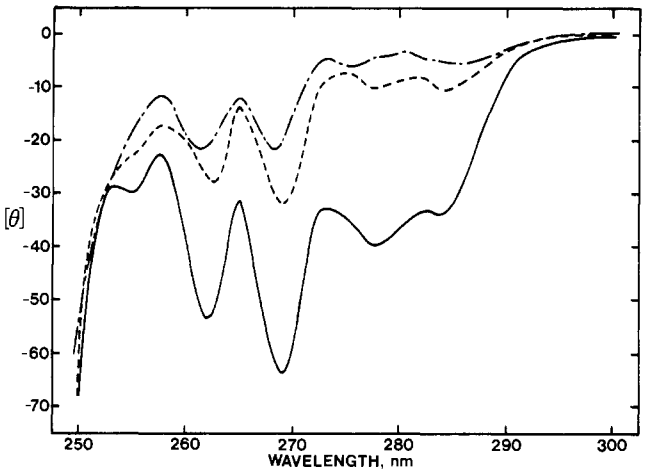

FIGURE 1: Aromatic CD spectra of S-100b in 0.1 M Tris, pH 8.5 (—), in 0.1 M Tris and 1 mM Ca^{2+}, pH 8.5 (-·-), and in 0.1 M Tris, 1 mM Ca^{2+}, and 60 mM KCl, pH 8.5 (---).

Stock calcium solutions were prepared from anhydrous $CaCl_2$ by weight in the same buffer as the protein samples. The final concentrations were determined by titration with EDTA in 0.1 M NaOH, using murexide as the indicator, and by atomic absorption spectrophotometry. The total volume change over the Ca^{2+}/K^+ titrations was ≤4%.

Results

A typical aromatic CD spectrum of S-100b in 0.1 M Tris-HCl buffer, pH 8.5, is shown in Figure 1. The ellipticity of the protein is negative between 250 and 300 nm. The two bands at 284 and 278 nm can be assigned to the single tyrosine residue. The two well-resolved CD bands at 268.5 and 262 nm can be attributed unambiguously to the phenylalanine residues. It is obvious from the CD spectrum that binding of Ca^{2+} to S-100b results in the perturbation of the single tyrosine and one or more of the seven phenylalanine residues. For example, $\theta_{268.5nm}$ decreases from −65 to −20 deg·cm²·dmol⁻¹ upon the addition of Ca^{2+}. Figure 1 also illustrates the aromatic CD spectrum of s-100b in 0.1 M Tris-HCl buffer, pH 8.5, in the presence of 60 mM KCl. It is clear from the magnitude of the ellipticity value at 268.5 nm obtained in the presence of KCl that the Ca^{2+} effect is diminished by the presence of K^+. This finding at pH 8.5 is in agreement with our earlier aromatic CD studies on S-100b at pH 7.5 where we have shown that K^+ is antagonistic to the Ca^{2+} effect (Mani et al., 1982).

The interaction of Ca^{2+} with S-100b was studied by CD in the aromatic region in greater detail. The change in $\theta_{268.5nm}$ as a function of Ca^{2+} concentration is plotted in Figure 2. From the titration curve, it is evident that at pH 8.5 there exist two sets of Ca^{2+} binding sites, and the affinity of calcium for these two sites is $K_d = 6 \times 10^{-5}$ and 2×10^{-4} M, respectively. In addition, Figure 2 includes the Ca^{2+} titration curve of S-100b in 0.1 M Tris-HCl buffer–60 mM KCl at pH 8.5. In the presence of KCl, there is evidence for only one binding site with $K_d = 8.5 \times 10^{-4}$ M. From these results, one may conclude that the binding of Ca^{2+} to the higher affinity site is inhibited by the presence of 60 mM KCl, or alternatively, K^+ binding causes an increase in K_d for the tight Ca^{2+} binding site. On the other hand, Mg^{2+} had no significant effect on the aromatic CD spectrum, and the binding of Ca^{2+} to S-100b was not affected by the presence of Mg^{2+} (data not shown). In fact, Ca^{2+} titration studies in the presence of Mg^{2+} gave virtually the same K_d values for calcium, thus clearly indicating the Ca^{2+} effect to be specific in nature.

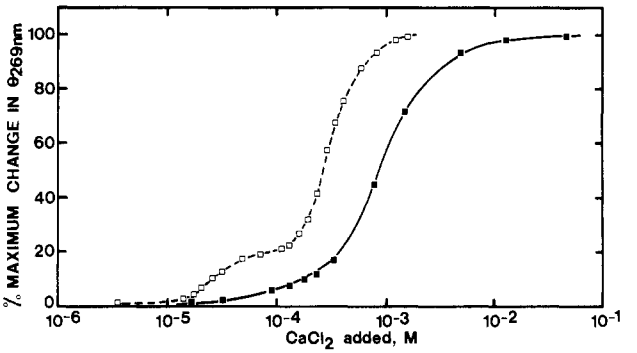

FIGURE 2: Percent change in ellipticity at 269 nm as a function of Ca^{2+} concentration for S-100b in 0.1 M Tris-HCl buffer, pH 8.5 (□), and in 0.1 M Tris-HCl buffer, pH 8.5, and 60 mM KCl (■).

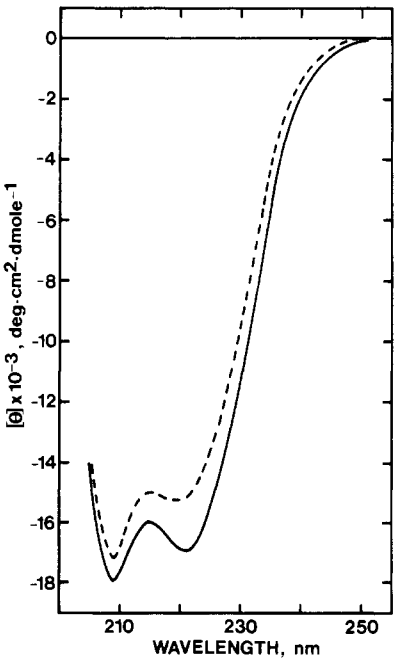

FIGURE 3: Far-ultraviolet CD spectra of S-100b in 0.1 M Tris, pH 8.5 (—), and in 0.1 M Tris and 1 mM Ca^{2+}, pH 8.5 (---).

Typical far-ultraviolet CD spectra of S-100b in 0.1 M Tris-HCl buffer at pH 8.5 in the absence and presence of Ca^{2+} are shown in Figure 3. In the absence of Ca^{2+}, the $[\theta]_{222nm}$ is −17 000 ± 300 deg·cm²·dmol⁻¹ while the addition of Ca^{2+} causes approximately a 10% decrease to −15 200 ± 300 deg·cm²·dmol⁻¹. Analysis of the CD data according to the Chen et al. (1974) method indicates a decrease in the apparent α-helical content from 55 to 47%. The effect of Ca^{2+} on the conformation of this protein is very different from its effect on other calcium binding proteins such as TN-C, calmodulin, and parvalbumin (Murray & Kay, 1972; Walsh et al., 1979). With the latter proteins, an increase in the apparent α-helix content was observed with calcium addition whereas with S-100b we have noted a decrease in α-helix content at pH 8.5. It should also be noted that the drop in ellipticity at pH 8.5 is nearly 1800 deg·cm²·dmol⁻¹ whereas at pH 7.5 the noted decrease was only 1300 deg·cm²·dmol⁻¹. This additional decrease in ellipticity of about 500 deg·cm²·dmol⁻¹ at pH 8.5 may be attributed to the higher affinity calcium binding site being exposed at pH 8.5.

Ca^{2+} titration in the far-UV CD region (Figure 4) indicated the existence of two sets of calcium binding sites at pH 8.5 with K_d values of 6×10^{-5} and 2.5×10^{-4} M, in agreement with the aromatic CD Ca^{2+} titration data. Ca^{2+} titration in

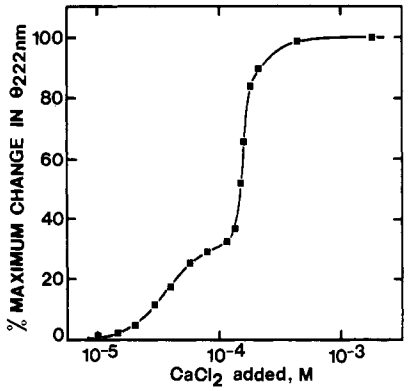

FIGURE 4: Percent change in ellipticity at 222 nm as a function of Ca^{2+} concentration for S-100b in 0.1 M Tris-HCl buffer, pH 8.5.

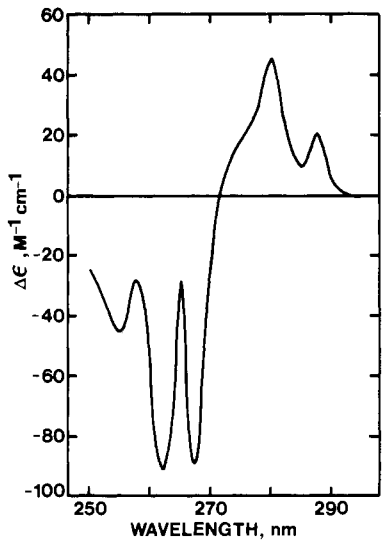

FIGURE 5: UV difference spectra of S-100b produced by Ca^{2+}. Chelex-treated S-100 in 0.1 M Tris, pH 8.5, was used. The data were corrected for dilution and are expressed as the difference in molar absorption, $\Delta\epsilon$. The temperature was 25 °C.

the presence of KCl gave evidence for only one binding site (data not shown). Mg^{2+} had no significant effect on the far-UV CD spectrum of S-100b, and the binding of Ca^{2+} to the protein was not influenced by the presence of Mg^{2+} (data not shown).

Ultraviolet Difference Spectroscopy. The difference in the absorption properties of S-100 protein between 250 and 300 nm induced by Ca^{2+} is shown in Figure 5. The difference peaks at 287 and 280 nm arise from the perturbation of the single tyrosyl chromophore. It is evident from the figure that one or more phenylalanine chromophores are also perturbed when calcium is bound. This lends further support to similar inferences drawn from the near-UV CD data. The sign of the tyrosyl difference peak (i.e., a "red shift") indicates that the chromophore is in a less polar environment in the presence of Ca^{2+} (Donovan, 1969).

Alternatively, the perturbations may be due to local charge effects upon binding calcium. The free negative charge on the carboxyl groups would be reduced by the binding of calcium, and this binding might result in changes in the geometry of the binding site and reorientation of the phenolic group. Observations to this effect have been noted in the past with skeletal and cardiac TN-C (Hincke et al., 1978) and porcine intestinal calcium binding protein (Dorrington et al., 1978). When one compares the UV difference spectrum generated at pH 8.5 with the pH 7.5 difference spectrum (Mani et al., 1982), differences are obvious. At pH 8.5, the

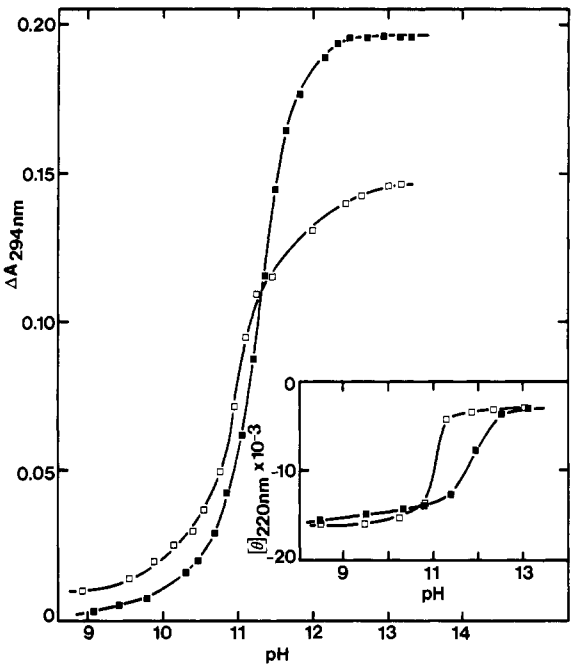

FIGURE 6: Spectrophotometric titration of S-100b in 10 mM Tris, pH 8.5, and 1 mM EDTA (□) and in 10 mM Tris, pH 8.5, and 0.5 mM Ca^{2+} (■). The insert shows the θ_{220nm} values as a function of pH, with the same symbolism for the two solvent systems.

magnitude of the tyrosine red shift ($\Delta\epsilon$) 287 and 280 nm is only 20 and 45 mol^{-1} cm^{-1}, respectively, compared to values of 90 and 150 mol^{-1} cm^{-1} at pH 7.5. This suggests that at pH 8.5 the tyrosine residue in the apoprotein is in a relatively less nonpolar environment. The $\Delta\epsilon$ value of −90 mol^{-1} cm^{-1} obtained at 268.5 and 262 nm, due to the perturbation of phenylalanine residues, is also significantly greater than the $\Delta\epsilon$ values of −40 to −50 mol^{-1} cm^{-1} observed at pH 7.5. From these results, one may conclude that the microenvironments of the tyrosine and phenylalanine residues are different at these two pH values and thus are further perturbed by the binding of a second calcium ion.

Effect of pH on S-100b Protein. Far-UV CD measurements indicated a loss of secondary structure when the S-100b protein, in the absence of Ca^{2+}, was titrated from pH 8.6 to 6.0. $[\theta]_{222nm}$ decreased by about 1300–1600 deg·cm^2·$dmol^{-1}$, which is well outside the range of experimental error (±300 deg·cm^2·$dmol^{-1}$). However, $[\theta]_{222nm}$ decreased by only 600–900 deg·cm^2·$dmol^{-1}$ when the protein was titrated in the presence of Ca^{2+} (data not shown). Thus, the protein is more susceptible to pH changes in the absence of Ca^{2+}.

The degree of ionization of the tyrosine residue in S-100b protein as a function of pH is illustrated in Figure 6. Loss in protein secondary structure as the pH was raised was monitored by CD measurements in the far-ultraviolet region (insert). It is obvious from the CD measurements (insert, Figure 6) that S-100b loses more than 50% of its secondary structure once pH 11.0 is exceeded. This would imply that the tyrosine residue in the native protein is not exposed to the solvent and titrates only after the protein is denatured. It should be noted that in the presence of Ca^{2+} the protein loses its secondary structure at pH 11.5 as opposed to pH 11.0 in the absence of Ca^{2+} (Figure 6). In both the presence and the absence of Ca^{2+}, the titration curve was not reversible once pH 11.0 was exceeded, implying that the protein undergoes irreversible denaturation, as revealed by CD measurements. Hence, one should not give any credence to the apparent pK_a values suggested by the titration curves except to conclude that

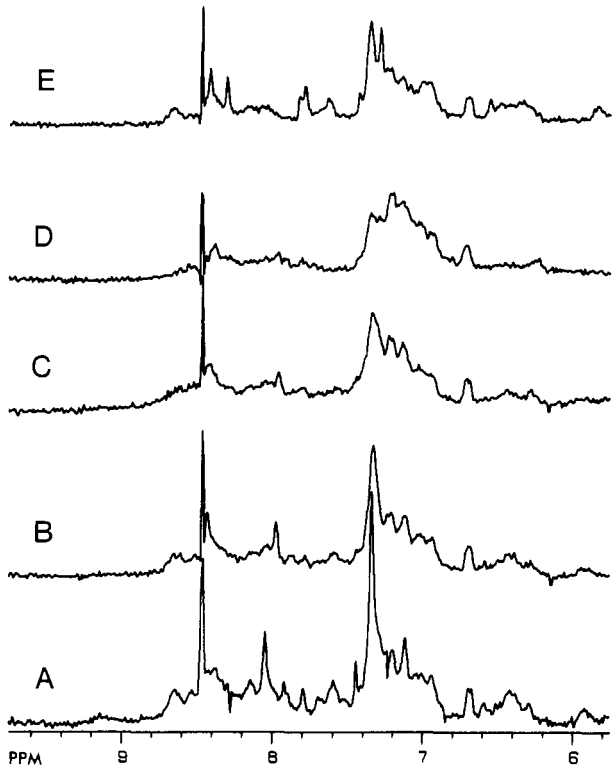

FIGURE 7: Titration of apo-S-100b with calcium, followed by the addition of KCl. The Ca^{2+}:S-100b ratios are (A) 0.00, (B) 0.58, (C) 1.25, and (D and E) 2.09. The KCl concentrations are (A–D) 0 and (E) 70 mM. The protein concentration was 0.21 mM, and the pH was ca. 7.5. The pH of the $CaCl_2$ solution was 7.48, and the KCl was added as a solid. A least-squares analysis of the initial slope of the binding curve (intensity at 7.33 ppm vs. $[Ca^{2+}]$; data not shown) indicated that the intensity of that resonance at 7.33 ppm in (A) was slightly higher than that expected for the apoprotein. This was likely due to the fact that this was the only sample which was obtained without saturation of the HDO resonance, and concomitant partial saturation of protein resonances via cross-relaxation pathways, during data acquisition.

either the tyrosine residue is buried in the interior of the protein molecule or, alternatively, intramolecular interactions exist between the phenolic hydroxyl group of the tyrosine residue and a carboxyl group of either an aspartic acid or a glutamic acid, as suggested by recent fluorescence studies with the homologous intestinal calcium binding protein (O'Neil et al., 1982). Moreover, recently Morero & Weber (1982) have proposed that a hydrogen bond between the tyrosine residue and a carboxyl ion acceptor group might exist in the case of a mixture of S-100 proteins, based on the UV difference spectrum generated between pH 7.3 and 3.5.

CD Melt Experiments. The thermal stability of S-100b protein in the presence and absence of Ca^{2+} was monitored by measuring the ellipticity, $[\theta]$, value at 221 nm as a function of temperature in 50 mM Mops buffer, pH 7.0 (results not shown). In the absence of Ca^{2+}, there was an approximately 17% loss in protein secondary structure when the temperature was raised from 10 to 70 °C. The loss was quite linear, and no sharp transition could be detected. In the presence of Ca^{2+}, the loss in secondary structure in going from 10 to 70 °C was only 10%, and in this case, also, no sharp transition temperature could be observed. The observed thermal unfolding of the protein was reversible in the minus and plus Ca^{2+} states. The thermal stability conclusions are also borne out by CD and spectrophotometric titration of the protein in the alkaline pH range (pH 8–13), where the protein was found to be more stable in the presence of Ca^{2+}.

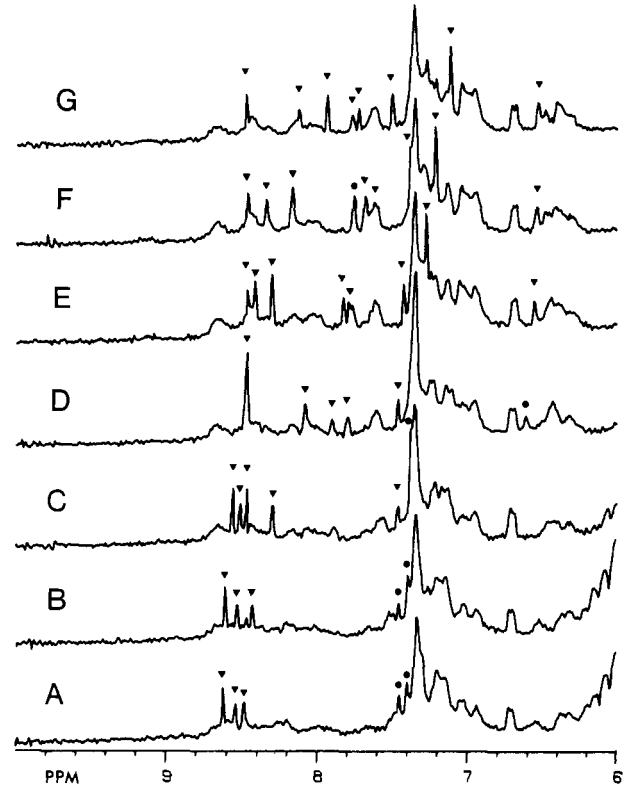

FIGURE 8: pH titration of apo-S-100b. The buffer was 10 mM Pipes, and the sample was made up to 60 mM NaCl through the addition of dithizoned NaCl in D_2O. The protein concentration was 0.2 mM. The pH values are (A) 5.58, (B) 5.92, (C) 6.55, (D) 7.09, (E) 7.62, (F) 7.94, and (G) 8.49. The resonances marked (▼) were isolated by the T2HSE technique; those marked (●) were not so clearly resolved by that technique but appear to follow the logical shift pattern of the (▼) resonances. For the T2HSE series, P1 = 18 μs, P2 = 9 μs, and D1 = 20 ms.

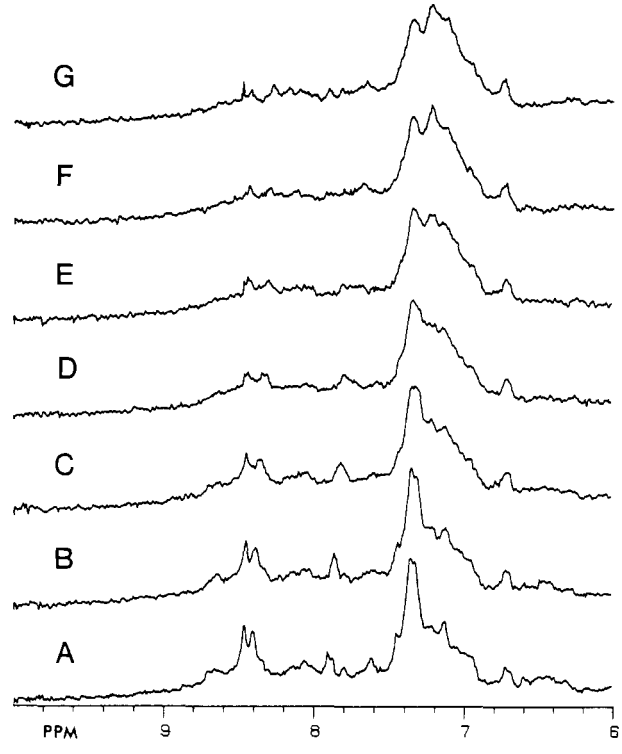

FIGURE 9: Titration of apo-S-100b with calcium at pH 8.23. The buffer was 10 mM Tris, and the protein concentration was 0.12 mM. The Ca^{2+}:S-100b ratios were (A) 0.00, (B) 0.53, (C) 1.04, (D) 1.58, (E) 2.10, (F) 3.21, and (G) 4.37. The stock calcium solution was made up in the same buffer at pH 7.45.

NMR Studies. The ^1H NMR results are presented in Figures 7–9. They all present the aromatic region of the spectrum and contain resonances which corresponds to the aromatic ring protons of seven phenylalanines, five histidines, one tyrosine, and an undetermined number of backbone amide proton resonances which do not exchange rapidly with the deuterated buffer at room temperture. From comparison of these spectra with those of other calcium binding proteins (Seamon et al., 1977; Levine et al., 1977; Hincke et al., 1981; J. G. Shelling et al., unpublished experiments), one can attribute (Figure 7A) the prominent resonance at 7.3 ppm as corresponding to the majority of the phenylalanine nuclei, the resonance in the range of 6.8–7.4 ppm to histidine C4 and phenylalanine nuclei, the resonance at 6.7 ppm to the 3,5-protons of the single tyrosine (this resonance is much clearer in Figure 8), the unfield-shifted resonances in the range of 6.2–6.6 ppm to phenylalanine protons, and the resonances in the range of 7.5–9.0 ppm to histidine C2 protons and amide NH's with the exception of the formic acid (contaminating) resonance at 8.44 ppm.

An attempt to further assign the tyrosine 3,5-proton resonance by using the laser photochemical induced dynamic nuclear polarization method was unsuccessful because the results, which depend upon exposure of the amino acid, were negative. Several of the histidine C2 resonances have been resolved more clearly by using the Hahn spin-echo method, and these will be discussed later.

Figure 7 shows the results of titration of the apoprotein at pH 7.5 with calcium and the effect of the subsequent addition of KCl. The addition of calcium up to a Ca^{2+}:S-100b ratio of 2:1 causes striking perturbation of the broad phenylalanine envelope, as well as the upfield-shifted phenylalanine resonances, and appears to broaden out many other spectral peaks. A least-squares analysis of the change in intensity of the phenylalanyl resonance at 7.32 ppm vs. added calcium, assuming one metal binding site per monomer, gave a dissociation constant of 2×10^{-4} M. This agrees well with the near- and far-UV CD measurements outlined previously. The addition of KCl was antagonistic and resulted in the resonances becoming sharper again and more clearly resolved.

Titration of the apoprotein at pH 7.5 with calcium was also performed in the presence of 63 mM KCl (data not shown). The initial addition of KCl resulted in some broadening and splitting of the resonance at 7.3 ppm, as well as other changes in the appearance of the spectrum, particularly in the upfield-shifted phenylalanines and in the histidine/backbone amide region from 9.0 to 7.5 ppm, but the changes are much less than those induced by the addition of calcium first. Further addition of calcium caused only minor changes in the spectrum. The final spectra, irrespective of which cation was added first, were very similar.

Figure 8 shows the titration of the five histidines in apo-S-100b in 60 mM NaCl. The initial pH was 7.09 and was raised to 8.49 by the addition of NaOD. The final concentration of Na^+ added was 7 mM. The pH was then restored to 7.09 by the addition of DCl, and the spectrum was very similar to that of the original sample. Thus, the effects of this small amount of Na^+ were negligible. The experiment was initiated in 60 mM NaCl as the resonance tend to be more clearly resolved in the presence of salt. The histidine resonances titrate over the complete pH range of 5.6–8.5, titrating slowly up to about pH 6.8 and then shifting to a much larger degree as one goes to higher pH. This is an indication that the influence of pH is more than that expected from a simple one-proton titration curve and most likely that a structural change was being felt by these residues. This agrees well with the far-UV CD results outlined previously.

Titration of the five histidines in calcium-saturated S-100b was also performed in the presence of 60 mM NaCl (data not shown). The sample pH was adjusted in the same manner as outlined for the apoprotein with the same result; thus, the effect of the small amount of Na^+ was again negligible. In this case, it was apparent that the same sensitivity to pH over the range 5.6–8.6 was obtained, indicative of the influence of more than a simple one-proton titration on the histidine resonances. However, the spectral changes were not as large as those observed in the absence of calcium.

Figure 9 illustrates the titration of apo-S-100b with calcium at pH 8.23. The broad phenylalanine/histidine C4 envelope is significantly perturbed by the addition of calcium, and the magnitude of this perturbation is maximized at a Ca^{2+}:S-100b ratio of 2:1 per monomer. As observed in Figure 7, the addition of calcium also results in a general broadening of resonances throughout the spectrum. The resulting plot of the intensity of the phenylalanine envelope as a function of $[Ca^{2+}]$ (not shown) lacked sufficient points in the critical region to accurately and independently determine the two binding constants observed in the CD studies, but a calculated binding curve using the two K_d's cited previously fit the observed data well. These results, therefore, agree well with the other spectroscopic evidence contained herein.

Discussion

Understanding the structure and the function of the central nervous system at the molecular level involves studying the chemistry of its constituent proteins. Brain-specific S-100b protein is unusual in certain aspects compared to other calcium binding proteins like ICABP, calmodulin, and TN-C. For example, S-100b exists as a dimer of 21 000 daltons in native solvents, whereas the above-listed calcium binding proteins exist as monomers. The calcium binding characteristics of S-100b are more complex than those of the other calcium binding proteins. The data presented in this study by near- and far-UV CD measurements clearly indicate the existence of two sets of calcium binding sites at pH 8.5 with K_d values of 6×10^{-5} and 2×10^{-4} M, whereas at pH 7.5, the protein binds only one Ca^{2+} per monomer with a K_d of 2×10^{-4} M. When one compares the amino acid sequence of S-100b with that of ICABP (Szebenyi et al., 1981) around the calcium binding "EF hand" test sequence, a characteristic feature of calcium binding proteins, it is obvious that S-100b contains three basic side chains, Lys-His-Lys, in positions corresponding to Pro-Asn-Gln in the calcium binding I–II loop of ICABP. As suggested by Calissano et al. (1974) and Szebenyi et al. (1981), titration of the His-25 residue in the calcium binding loop region may be responsible for exposing an additional calcium binding site ($K_d = 6 \times 10^{-5}$ M) at pH 8.5 by inducing a pH-dependent conformational change in the protein. Our observation, based on CD and NMR experiments which suggest that S-100b undergoes a conformational change when the protein is titrated from pH 8.0 to 6.0, especially in the absence of calcium, is consistent with the explanation given for the exposure of the extra calcium binding site at pH 8.5. The overall affinity of this site for calcium could conceivably be lower than that of ICABP, because of the presence of the additional basic side chains in the loop region of the protein molecule.

It is noteworthy that the extra site exposed at pH 8.5 is specific for calcium, since Mg^{2+} has no effect on S-100b protein conformation. Moreover, Ca^{2+} was able to induce a conformational change in the protein in the presence of Mg^{2+}.

However, the presence of K^+ inhibited the binding of Ca^{2+} to the higher affinity site exposed at pH 8.5. In the presence of K^+, the binding affinity for Ca^{2+} is virtually the same at both pH values studied (8.5 and 7.5). Hence, in our opinion, it is the low-affinity Ca^{2+} binding site which operates in the presence of K^+ that is the one of physiological significance. It is important to note here that the NMR studies presented concur with other CD work (Mani et al., 1982) in indicating that Ca^{2+} and K^+ display "antagonistic" effects with respect to their effect on the structure of S-100b. Regardless of which cation is added first, the final NMR spectrum is identical. Similarly, in the case of the TN-C molecule, it is the Ca^{2+}-specific low-affinity calcium binding site that is important from the physiological standpoint. However, differences do exist between these two systems. Mg^{2+} can compete with Ca^{2+} for the high-affinity site in TN-C, whereas with S-100b, Mg^{2+} has no effect on the higher affinity site exposed at pH 8.5; rather, it is K^+ which can antagonize calcium binding.

The single tyrosine residue in S-100b appears not to be exposed to the solvent and ionizes only after the protein undergoes denaturation. NMR studies (data not shown) show clearly that the protein unfolds as one tries to titrate the single tyrosine in either the presence or the absence of Ca^{2+}. This is evidenced by the shifting of the tyrosine protons to those chemical shift values typical of free tyrosine in solution. For the apoprotein, this is observed as one goes above pH 10.9, while in the presence of calcium, one needs to go to a higher pH (>11.4) to get denaturation, and in neither case was the denaturation reversible. These results agree well with the spectrophotometric pH titration data.

S-100b is very stable compared to other calcium binding proteins such as skeletal and cardiac TN-C. Work from our laboratory on the thermal stability of the above-mentioned calcium binding proteins (Mani et al., 1974; McCubbin et al., 1980) revealed that they lost nearly 60% of their secondary structure during CD melt experiments whereas with S-100b the loss was only 17% in the absence of calcium and 10% in its presence. NMR studies of S-100b at 70 °C and pH 7.8 (results not shown) in the presence of excess calcium ($[Ca^{2+}]$:[S-100b] = 5.09) also indicated only small structural changes due to heating, and these were completely reversible, aside from the loss of the histidine and most of the backbone NH resonances which was due to exchange with the deuterated buffer.

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Registry No. Calcium, 7440-70-2; L-tyrosine, 60-18-4; L-phenylalanine, 63-91-2; potassium, 7440-09-7.

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