chain. In the absence of this histidine and water molecule at the ligand binding site, very high ligand (N_3^-) combination rates have been reported for Mb^+ aplysia (Giacometti et al., 1981).

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Isolation and Spectral Studies on the Calcium Binding Properties of Bovine Brain S-100a Protein†

Rajam S. Mani and Cyril M. Kay*

ABSTRACT: The brain-specific S-100 protein is a mixture of two predominant isomers, S-100a and S-100b, which exist in brain tissue in almost equal amounts. The subunit compositions of S-100a and S-100b are $\alpha\beta$ and β_2, respectively. S-100a, isolated in the present study by using hydroxylapatite chromatography in its final purification, is homogeneous by the criteria of gel electrophoresis in the absence and presence of sodium dodecyl sulfate. The S-100a protein undergoes a conformational change upon binding calcium, as indicated by ultraviolet (UV) difference spectroscopy, circular dichroism (CD) studies in the aromatic and far-UV spectral regions, and fluorescence measurements. The binding affinity of Ca^{2+} to S-100a was studied at two pH values, 8.3 and 7.5. The effect of Ca^{2+} binding on the UV absorption difference spectrum and fluorescence emission spectrum was different at these two pH values. When the apoprotein at pH 8.3 was excited at 280 nm, the emission maximum was located at 335 nm. In the presence of Ca^{2+}, the emission maximum occurred around 339 nm and was accompanied by a nearly 60% increase in fluorescence intensity. Fluorescence titration of S-100a with Ca^{2+} indicated the presence of two calcium binding sites on the protein at pH 8.3 with K_d values of 5.5×10^{-5} M and 2.5×10^{-4} M, whereas at pH 7.5, the protein possesses only one Ca^{2+} binding site with a K_d of 1.2×10^{-4} M. The effect of K^+ on the protein was antagonistic to that of calcium. Although the affinity of both S-100 proteins for calcium is similar and pH dependent, subtle differences exist in the microenvironment of specific chromophores. Near-UV CD studies revealed that the environments around the tyrosine and phenylalanine residues are different in the two S-100 proteins. UV difference spectroscopy also suggests that the single tryptophan and the tyrosine chromophores in S-100a are blue shifted (i.e., exposed to the solvent) in the presence of Ca^{2+}, in contrast to the observed red shift noted with S-100b.

The S-100 protein is a nervous tissue specific protein widely distributed in the nervous systems of various vertebrates (Moore, 1965, 1972). This protein is found primarily in glial cells (Ludwin et al., 1976) and represents up to 0.2% of the total soluble brain protein. Recently, Gayner et al. (1980) have show the presence of S-100 protein in continuous cell lines of human malignant melanoma. The biological function of this protein is unknown; however, existing literature suggests 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 is actually a mixture of two predominant isomers, S-100a and S-100b with a subunit composition of $\alpha\beta$ and β_2, respectively (Isobe & Okuyama, 1981). 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 its sequence is similar to calcium binding proteins such as calmodulin (Cheung, 1980; Kasai et al., 1980), troponin C (Van Eerd & Takahashi, 1975), and parvalbumin (Perchere &

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Thatcher, 1977). The α-subunit in S-100a is distinguishable from the β-subunit specifically by the presence of a single tryptophan (Trp-90) in α, and both α- and β-subunits possess an acidic cluster of 12 amino acids with a high sequence homology with the "calcium binding sites" proposed by Kretsinger (1976).

S-100b protein undergoes a conformational change upon binding Ca^{2+}. CD[1] measurements indicated a loss in secondary structure as a consequence of Ca^{2+} binding (Mani et al., 1982, 1983). The effect of K^+ on the protein was antagonistic to Ca^{2+}, and the protein's affinity for calcium was significantly lowered by the presence of K^+. 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), the Ca^{2+} binding properties of S-100b were studied as a function of pH. At pH 8.5, S-100b protein bound two Ca^{2+} per monomer with K_d values of 6×10^{-5} M and 2×10^{-4} M, whereas at pH 7.5, the protein bound only one Ca^{2+} per monomer with a K_d of 2×10^{-4} M.

In the present study, we have employed CD, UV difference spectroscopy, and fluorescence measurements to explore the calcium binding properties of purified S-100a protein at two pH values, 8.3 and 7.5. The spectral data obtained on S-100a have enabled us to compare its calcium binding properties with those of its counterpart, S-100b, and the comparison has revealed some interesting similarities as well as differences between the two proteins, which may ultimately be related to their functional properties.

Materials and Methods

S-100a protein was prepared from bovine brain by using the methodology described in our earlier publication for S-100b (Mani et al., 1983) with minor modifications. In order to improve the yield, the 40–80% ammonium sulfate fraction, after heat treatment, was applied to a DEAE-cellulose column. Final purification was achieved on a hydroxylapatite column (Bio-Rad), and the solvent system used was 50 mM Tris-HCl buffer, pH 8.0, and 0.2 M NaCl containing 1000 mL of a $(NH_4)_2SO_4$ gradient from 0 to 0.8 M.

Standard polyacrylamide gel electrophoresis was performed at pH 8.5 with the Tris–glycine buffer described by Schaub & Perry (1969). $NaDodSO_4$–polyacrylamide gel electrophoresis and molecular weight determination were carried out according to Weber & Osborn (1969). Amino acid analyses were performed on a Durrum D-500 amino acid analyzer (Dionex Corp.). Quantitative analyses of tryptophan were estimated by UV absorption in 0.1 N NaOH (Bencze & Schmid, 1957). Protein concentrations were established in the ultracentrifuge by employing the Rayleigh interference optical system, assuming 41 fringes equivalent to 10 mg/mL (Babul & Stellwagen, 1969). From a plot of the number of fringes vs. optical density, a value of 5.4 was established as the extinction coefficient, $E^{1\%}_{1cm,278nm}$, for this protein.

The absorption spectra of S-100a samples used were measured on either Perkin-Elmer λ 5 or Cary 118C recording spectrophotometers over the wavelength range 350–250 nm. Difference spectra were obtained on the Cary 118C instrument by using 1-cm path-length cells. Aliquots of concentrated perturbant, either $CaCl_2$ or KCl, 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 0.3–1.0 mg/mL. In most cases, the instrument was operated in the "Autoslit" mode and full-scale absorbance of 0.05. Scan speeds from 0.02 to 0.1 nm/s were employed and the spectra were measured at 25 °C with a Lauda thermoregulator. Sedimentation velocity experiments were carried out in a Beckman Spinco Model E ultracentrifuge employing the schlieren optical system with a rotor velocity of 60000 rpm and a temperature of 20 °C. A partial specific volume of 0.73 mL/g was assumed for the S-100a protein. The solvent system employed in the centrifuge work was 0.1 M Tris-HCl buffer, pH 8.3, and 2 mM $CaCl_2$.

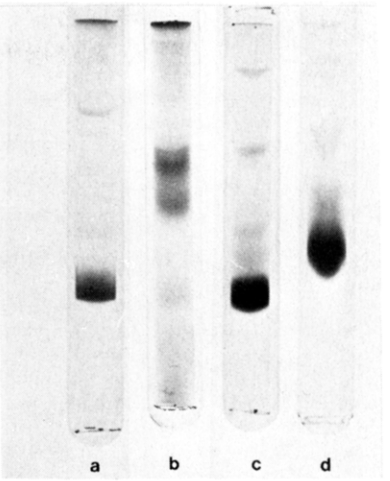

FIGURE 1: Electrophoresis of the purified S-100a protein in the following: (a) 15% $NaDodSO_4$ gels; (b) 6 M urea–$NaDodSO_4$ gels; (c and d) Tris–glycine buffer, pH 8.6 (10% gels), in (c) 0.1 mM EDTA and (d) 0.1 mM Ca^{2+}, respectively.

Circular dichroism measurements were made on a Jasco J500C instrument fitted with a DP500N data process unit. The instrument was routinely standardized with *d*-10-camphorsulfonic acid and pantoyl lactone. A Perkin-Elmer Model MPF-44B recording spectrofluorometer was used for fluorescence measurements. The sample compartment was water jacketed and connected with a Lauda thermoregulator, and measurements were conducted at 25 °C. The instrument was operated in the ratio mode with 5-nm bandwidths for excitation and emission slits. The OD_{280nm} of the sample was 0.10 or less. All the solvents used in the optical studies were routinely passed through a Chelex-100 column. The protein was initially dissolved in appropriate buffer in the presence of EDTA and, thereafter, was subjected to exhaustive dialysis with at least four changes against the solvent that had been passed through the Chelex-100 column.

Results

During final hydroxylapatite chromatography of the fraction enriched with respect to S-100 proteins, calmodulin is eluted first, followed by a protein of nearly M_r 60000. Subsequent to this high molecular weight protein, S-100 elutes, followed by the S-100 mixture and, finally, S-100b protein. The resolution obtained on this column depends to a certain extent on the initial loading level. For optimal separation of S-100a and S-100b proteins, one should apply about 200 mg of S-100 (mixture) proteins. S-100a isolated from the hydroxylapatite column was homogeneous according to polyacrylamide gel electrophoresis, in the presence as well as in the absence of $NaDodSO_4$ (Figure 1). A molecular weight of 10300 ± 500 was deduced from its mobilities in 15% $NaDodSO_4$ gels, with reference to the mobilities of several standard proteins. The

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TN-C, troponin C; TN-I, the inhibitory subunit of the troponin complex; TN-T, the tropomyosin binding subunit of the troponin complex; NMR, nuclear magnetic resonance.

Table I: Amino Acid Composition of Bovine Brain S-100a Protein[a]

amino acid	S-100a	PAPI-a[b]
Lys	8.3	8.2
His	3.5	3.6
Arg	1.0	0.7
Asp	12.2	12.4
Thr	3.1	3.6
Ser	3.3	5.1
Glu	17.2	19.0
Pro	0	0
Gly	6.2	5.8
Ala	7.6	7.3
Cys	ND[c]	1.5
Val	8.1	7.3
Met	2.8	2.9
Ile	2.8	2.9
Leu	10.2	10.2
Tyr	1.5	1.5
Phe	6.4	6.6
Trp[d]	0.7	0.7

[a] The values are based on molar ratio. [b] Taken from Isobe & Okuyama (1977). [c] ND, not determined. [d] Determined spectrophotometrically (3).

molecular weight of the standard proteins ranged from 11 000 to 40 000, and these were parvalbumin (M_r 11 200), calmodulin (M_r 16 500), cardiac TN-C (M_r 18 500), cardiac TN-I (M_r 27 000), and cardiac TN-T (M_r 36 000). In 15% $NaDodSO_4$ gels, S-100a appears as a single broad diffuse band (gel a). However, when run in the presence of 6 M urea and 15% $NaDodSO_4$ (gel b), the protein resolves itself into two well-defined bands. The faster moving component corresponds to the β-chain, and the slower one is the α-chain. The mobility of S-100a in polyacrylamide gels in Tris–glycine buffer is shown in gels c and d (Figure 1). The protein moves faster in the presence of EDTA. A decrease in mobility in the presence of Ca^{2+} could be due to a decrease in negative charge on the protein resulting from binding the cation. In this respect, S-100a behaves similarly to S-100b and calmodulin but different from TN-C. A comparison of the amino acid analysis of our S-100a preparation with that of Isobe et al. (1977), as summarized in Table I, reveals excellent agreement between the two protein preparations.

S-100 proteins (a and b) have a characteristic UV absorption spectrum, and it is very useful in identifying them as they emerge from the column. The fine structure seen in the UV absorption spectra of S-100 proteins is caused by larger amounts of phenylalanine relative to tyrosine (S-100b) or to tyrosine plus tryptophan, in the case of S-100a. The observed absorbance ratio, A_{278nm}/A_{260nm}, is nearly 1.0 for S-100b, whereas for S-100a, the ratio is approximately 1.5. Also, S-100a is characterized by the presence of a single tryptophan in the α-chain, and it appears as a shoulder around 290 nm in the UV absorption spectrum and is also apparent in the derivative spectrum (Figure 2). In the case of the S-100 mixture, the A_{278nm}/A_{260nm} ratio ranged from 1.2 to 1.3. In the analytical ultracentrifuge, the S-100a protein sedimented as a single symmetrical boundary and a $s_{20,w}$ value of 2.03 S was obtained for a 2 mg/mL protein concentration.

Circular Dichroism Studies. Typical far-UV CD spectra of S-100a in 0.1 M Tris-HCl buffer, pH 8.3, in the absence and presence of Ca^{2+}, are shown in Figure 3. In the absence of Ca^{2+}, the $[\theta]_{222nm}$ is nearly $-16\,700 \pm 300$ deg·cm²·dmol⁻¹, while the addition of Ca^{2+} causes approximately a 7% decrease to $-15\,600 \pm 300$ deg·cm²·dmol⁻¹. Analysis of the CD data, according to the Chen et al. (1974) method, indicates a decrease in apparent α-helical content from 54 to 49%. The

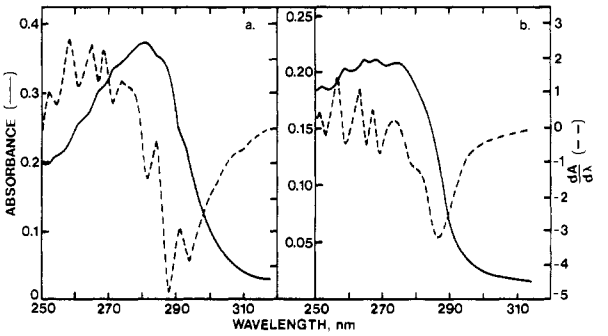

FIGURE 2: Ultraviolet absorption (—) and derivative spectra (---) of S-100a protein (a) and the S-100b protein (b) in 100 mM Tris (pH 7.5) at room temperature.

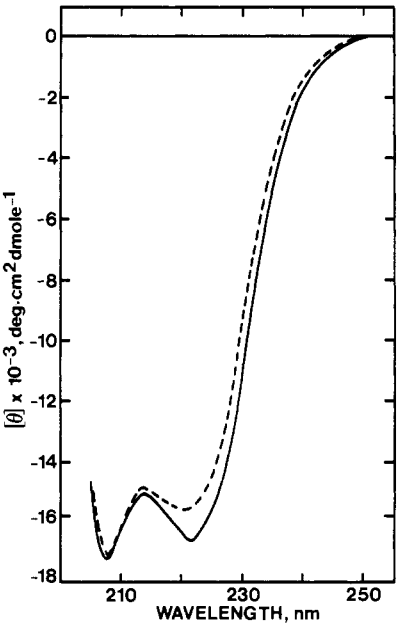

FIGURE 3: Far-ultraviolet CD spectra of S-100a in the absence of Ca^{2+} in 0.1 M Tris, pH 8.3 (—), and in 0.1 M Tris, pH 8.3, and 1.5 mM Ca^{2+} (---).

effect of Ca^{2+} on the conformation of S-100a is very similar to that of S-100b (Mani et al., 1982, 1983), but 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 apparent α-helix content was observed with calcium addition, whereas with S-100a, we have noted a decrease in α-helix. The drop in ellipticity $[\theta]_{222nm}$ at pH 8.3 is nearly 1100 deg·cm²·dmol⁻¹ while at pH 7.5, the observed decrease was only 700 deg·cm²·dmol⁻¹, clearly suggesting that the Ca^{2+} effect on S-100a is different at the two pH values studied. Since the maximum drop in ellipticity $[\theta]_{222nm}$ at pH 8.3 was only 1100 deg·cm²·dmol⁻¹, we were not able to obtain a reliable binding constant for Ca^{2+} by carrying out a calcium titration using this technique. However, fluorescence measurements (see fluorescence section) enabled us to determine K_d values for Ca^{2+} at these pH values.

The observed Ca^{2+}-induced conformational changes were irreversible in 0.1 M Tris buffer at pH 8.5. However, when dithiothreitol was included in the solvent system, the Ca^{2+} effect could be reversed by adding EDTA, suggesting the involvement of sulfhydryl groups. Our findings are consistent with the earlier observations of Calissano et al. (1976), who used gel electrophoresis to demonstrate that Ca^{2+} can induce intramolecular cross-linking of S-100 protein by disulfide bond formation. Mg^{2+} had no significant effect on the far-UV CD

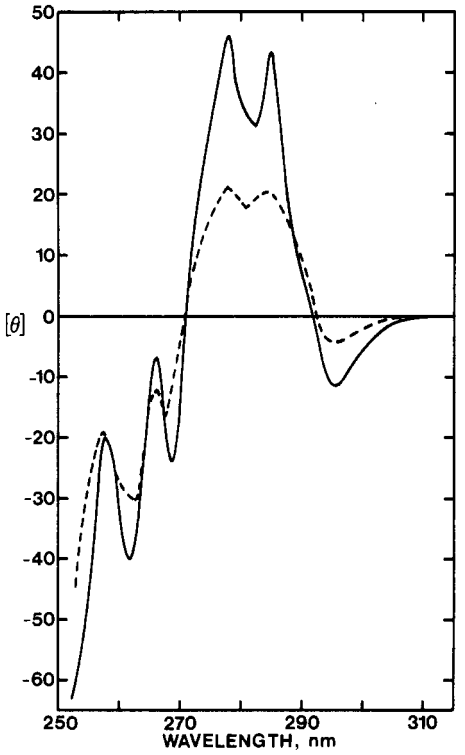

FIGURE 4: Aromatic CD spectra of S-100a in the absence of Ca^{2+} in 0.1 M Tris, pH 8.3 (—), and in 0.1 M Tris, pH 8.3, and 1.5 mM Ca^{2+} (---).

spectrum of S-100a, and the binding of Ca^{2+} to the protein was not influenced by the presence of Mg^{2+} (data not shown).

Figure 4 reveals the effect of Ca^{2+} on the aromatic CD spectrum of S-100a at pH 8.3. The negative band at 295 nm is due to tryptophan. The two bands at 284 and 277 nm can be assigned to the tyrosine residues while the two bands at 268.5 and 261.5 nm can be attributed to the phenylalanine residues. From the spectrum, it is obvious that the tryptophan band at 295 nm and the tyrosine residues in the 280-nm region are perturbed by the addition of Ca^{2+}. Ellipticity values at 268.5 and 261.5 nm are also affected.

Ultraviolet Difference Spectroscopy. The possible effect of Ca^{2+} on the tryptophan, tyrosine, and phenylalanine aromatic groups was investigated by measuring UV difference spectra. Figure 5 shows the difference spectra of S-100a when Ca^{2+} and K^+ were added to the sample cell, compared with nontreated protein in the reference cell. The difference peak at 292.5 nm is characteristic of a blue shift of the tryptophan absorption band. The double negative trough at 285 and 278 nm is characteristic of a blue shift of the tyrosine absorption bands, and these blue shifts associated with tryptophan and tyrosine residues are usually interpreted as arising from an increased exposure of these aromatic groups to solvent (Donovan, 1973). This behavior is reminiscent of similar findings with calmodulin (Klee, 1977). The observed sharpening of the fine structure of the absorption bands below 270 nm suggests that one or more of the phenylalanine residues are also perturbed in the presence of Ca^{2+}, and these findings are in agreement with our near-UV CD data, where we observed changes in the environment of tryptophan, tyrosine, and phenylalanine residues as a consequence of Ca^{2+} addition. Alternatively, the observed perturbations may be due to local charge effects upon binding calcium as discussed in our earlier papers on the S-100b system (Mani et al., 1982, 1983).

Calissano et al. (1974) have previously reported a change in the UV absorption spectrum of the S-100 protein mixture

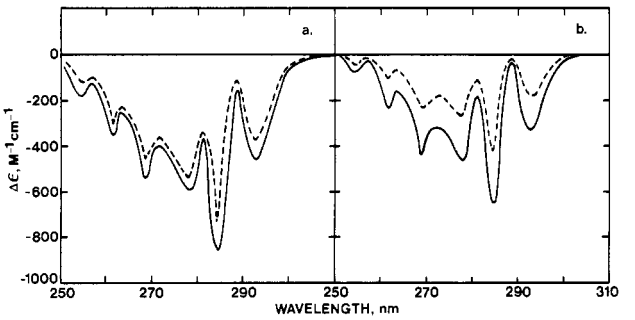

FIGURE 5: UV difference spectra of S-100a produced by Ca^{2+}. Chelex-treated S-100a samples in 0.1 M Tris at pH 8.3 (a) and 7.5 (b) were used. The data were corrected for dilution and are expressed as the difference in molar absorption, $\Delta\epsilon$. The temperature was 25 °C. (a) 2.0 mM $CaCl_2$, pH 8.3 (—); 2.0 mM $CaCl_2$ and 90 mM KCl, pH 8.3 (---). (b) 2.0 mM $CaCl_2$, pH 7.5 (—); 2.0 mM $CaCl_2$ and 90 mM KCl, pH 7.5 (---).

as a result of Ca^{2+} addition. Recently, Baudier et al. (1983) have also noted a similar effect of Ca^{2+} on S-100a at pH 8.3. However, in our present investigation we have studied the effect of Ca^{2+} on S-100a at two pH values, i.e., 8.3 and 7.5, since the binding of Ca^{2+} to S-100 proteins is a pH-dependent phenomenon, as cited earlier in the introduction. In addition, we have looked at the effect of K^+ on the Ca^{2+}-induced difference spectrum. When one compares the UV difference spectrum generated at pH 8.3 with the pH 7.5 difference spectrum, differences are obvious. At pH 8.3, the magnitude of the $\Delta\epsilon$ ($mol^{-1}\cdot cm^{-1}$) values for the tryptophan at 292.5 nm and tyrosine at 285 and 278 nm is −460, −860, and −600, respectively, compared to values of −330, −650, and −450 at pH 7.5. This suggests that at pH 8.3, tryptophan and the tyrosine residues are relatively more exposed to the solvent. The $\Delta\epsilon$ values of −540 and −360 at 268.5 and 262 nm, due to the perturbation of phenylalanine residues, are also significantly greater than the $\Delta\epsilon$ values of −440 and −230 observed at pH 7.5. From these results, one may conclude that the microenvironment of the single tryptophan, and the tyrosine and phenylalanine residues, is different at these two pH values and this is further perturbed by the binding of a second calcium ion, as evidenced by fluorescence titration with Ca^{2+} (see below). The effect of K^+ on the protein was found to be antagonistic to Ca^{2+}. In the presence of K^+, the single tryptophan and the tyrosine and phenylalanine residues move to a less polar environment at both pH values studied.

Fluorescence Studies. S-100a, which has one tryptophan and three tyrosine residues, has its emission peak centered at 335 ± 1 nm with a shoulder around 306 ± 1 nm when the apoprotein is excited at 280 nm (Figure 6). The emission peak at 335 nm is due to tryptophan which is partly buried, and the shoulder around 306 nm is due to one or more tyrosine residues. Addition of Ca^{2+} at pH 8.3 caused an approximate 60% increase in fluorescence intensity, and the position of the emission maximum in the presence of Ca^{2+} was at 339 ± 1 nm (i.e., a "red shift"), suggesting that the tryptophan residue is now more exposed to the solvent, in agreement with our difference spectral data. Recently, Baudier et al. (1983) have also observed a red shift in the emission maximum of tryptophan, indicating that it is exposed to the solvent in the presence of Ca^{2+}. A plot of the percentage change in relative fluorescence intensity at 335 nm vs. calcium added is indicated in Figure 7, and from the fluorescence Ca^{2+} titration data, it is possible to identify the existence of two sets of calcium binding sites on the protein at pH 8.3, with K_d values of 5.5×10^{-5} M and 2.5×10^{-4} M. At pH 7.5, there was an indication of only one binding site with a K_d of 1.2×10^{-4} M, or

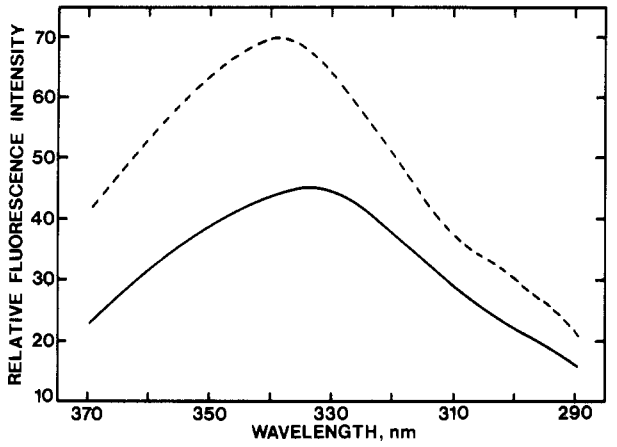

FIGURE 6: Fluorescence emission spectra of S-100a in 0.1 M Tris-HCl buffer, pH 8.3, at 25 °C in the absence of Ca^{2+} (—) and in 0.1 M Tris-HCl buffer, pH 8.3, containing 1.5 mM $CaCl_2$ (---) at 25 °C.

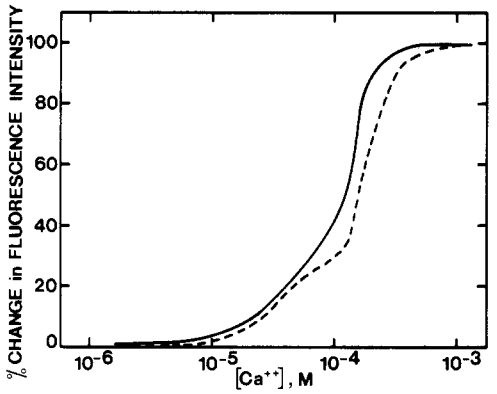

FIGURE 7: Percent change in fluorescence intensity at 335 nm as a function of Ca^{2+} concentration for S-100a in 0.1 M Tris-HCl buffer at pH 8.3 (---) and pH 7.5 (—). The temperature was maintained at 25 °C.

alternatively both sites have the same low affinity for Ca^{2+}. Also, the increase in relative fluorescence intensity at pH 7.5 was only about 25%, which is roughly 40% of the observed increase at pH 8.3. Similar observations have been made with the mixture of S-100 proteins by Calissano et al. (1974). The latter study indicated that the increase in fluorescence intensity due to Ca^{2+} addition at pH 7.6 was nearly 30% of the observed increase at pH 8.3. Equilibrium dialysis experiments at pH 8.3 using ^{45}Ca revealed the existence of four calcium binding sites per 21 000 molar mass ($g \cdot mol^{-1}$), whereas at pH 7.6 there was evidence for only two calcium binding sites per molecule (Calissano et al., 1974). With pure S-100b, we also observed two sets of calcium binding sites at pH 8.5 as opposed to one binding site at pH 7.5 (Mani et al., 1983). NMR experiments suggested that the S-100b protein bound two Ca^{2+} per monomer at pH 8.5, while at pH 7.5 it bound only one Ca^{2+} per monomer. Calcium binding ability of both S-100a and S-100b appears to depend on pH at around neutrality, and this behavior may be attributed to the titration of the His (25) residue located in the Ca^{2+} binding loop of the β-chain as elaborated in our previous paper (Mani et al., 1983).

Discussion

Brain-specific S-100 protein, which is a mixture of the two isomers S-100a and S-100b, must play an important role in the function of the central nervous system since it is present in fairly high concentration relative to other soluble acidic proteins. S-100a used in the present study was homogeneous when tested by polyacrylamide gel electrophoresis in the presence and absence of surfactant. Since the molecular weights of α- and β-subunits are 10 300 and 10 500, respectively, only one broad band was observed for S-100a on $NaDodSO_4$ gels. But this single band resolves itself into two bands upon polyacrylamide gel electrophoresis with 6 M urea and $NaDodSO_4$. Under these conditions, the β-chain moves faster than the α-chain. Similar observations have been made with S-100a by Isobe & Okuyama (1981). Recently, Baudier et al. (1983) have noticed another slow moving compartment on 6 M urea–$NaDodSO_4$ gels which they have termed α'. The amino acid composition and the tryptic digest pattern of α and α' are almost identical, and since the two chains have identical molar UV absorption spectra in the presence of 6 M guanidine hydrochloride, it is possible that α and α' represent different conformational states assumed by the same polypeptide chain.

S-100a protein is very similar to S-100b in certain aspects, and yet there are distinct differences between the two homologues. Both proteins (S-100a and S-100b) undergo a conformational change upon binding Ca^{2+}. Far-UV CD measurements at pH 8.3 indicated that the ellipticity at $[\theta]_{222nm}$ decreased by nearly 1100 $deg \cdot cm^2 \cdot dmol^{-1}$ for S-100a whereas for S-100b the decrease was 1800 $deg \cdot cm^2 \cdot dmol^{-1}$ (Mani et al., 1983). Near-UV CD measurements revealed that Ca^{2+} addition to S-100a perturbs the environment of tyrosine and phenylalanine residues, similar to our earlier observation with the S-100b system. However, the tyrosine peak positioned at 284 and 278 nm for S-100a is positive, while for S-100b, the ellipticity values at these wavelengths due to tyrosine were negative, indicating that the microenvironment around the tyrosine and phenylalanine residues is different in these two proteins.

The β-chain, which is common to both S-100a and S-100b, has only one tyrosine residue, whereas the α-chain in S-100a has two tyrosine residues. The single tyrosine residue in position 16 in the β-chain is located far from the putative calcium binding site (residues 62–73), and the corresponding tyrosine residue in the α-chain is also removed from the Ca^{2+} binding loop, in position 26. The second tyrosine residue in the α-chain is in the helix region (i.e., residue 74), immediately following the calcium binding loop. There are seven phenylalanine residues in the β-chain, while the α-chain has only five phenylalanines. The two extra phenylalanines in the β-chain are in the helix region, i.e., residues 73 and 76, following the Ca^{2+} binding loop. The observed spectral differences between S-100a and S-100b with regard to the Ca^{2+} effect may be attributed to the unique tyrosine-74 in the α-chain and phenylalanine residues 73 and 76 in the β-chain, in view of the proximity of these aromatic residues to the Ca^{2+} binding region.

In the presence of Ca^{2+}, the microenvironments of the single tryptophan and one or more of the tyrosine and phenylalanine residues in S-100a are altered at the two pH values studied as revealed by UV difference spectroscopy. However, the extent of perturbation is different at these two pH values. The single tryptophan and the tyrosine chromophores are blue shifted in the presence of Ca^{2+}, thereby implying that they are exposed to the solvent and the magnitude of the blue shift at pH 8.3 is greater than that at pH 7.5. The effect of K^+ on the protein was antagonistic to Ca^{2+}. This effect of Ca^{2+} on S-100a is different from that observed with S-100b, where the tyrosine residue was red shifted by the addition of Ca^{2+}, suggesting that it moves to a more nonpolar environment.

The fluorescence emission spectrum for S-100a is typical of a protein containing tryptophan. The emission maximum for the apoprotein at 335 nm indicates that the tryptophan

residue is not fully exposed to the solvent. The observed red shift in the emission maximum upon Ca^{2+} addition implies that tryptophan now moves to a more polar environment, in agreement with our difference spectral observations, where the tryptophan residue was blue shifted in the presence of Ca^{2+}. Existence on S-100a of two sets of Ca^{2+} binding sites with K_d values of 5.5×10^{-5} M and 2.5×10^{-4} M at pH 8.3 and one binding site with a K_d of 1.2×10^{-4} M at pH 7.5 is very similar to the S-100b system (Mani et al., 1983). These results imply that the affinity of both proteins for Ca^{2+} is similar, as well as being pH dependent, and yet subtle differences exist in the microenvironment of specific chromophores. It is conceivable that these chromophores and the subtle differences in their response to Ca^{2+} may be exploited as specific probes in future structure–function studies on the S-100 proteins.

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Purification and Some Physicochemical Properties of Toxic-Shock Toxin†

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ABSTRACT: A procedure for the purification of a protein marker for the staphylococci isolated from toxic-shock syndrome patients has been developed. The purification procedure involves the removal of the toxic protein from culture supernatant fluids of toxic-shock syndrome associated *Staphylococcus aureus* strains FRI-1169 and FRI-1183 by batch absorption with CG-50 resin, ion-exchange chromatography on CM-Sepharose CL-6B, and gel permeation chromatography on Sephacryl S-200. The purified toxin is a simple protein with a molecular weight of 24 000 ± 500 as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The isoelectric point of the major band is 7.0 as determined by isoelectric focusing in polyacrylamide gels. The TS-toxin's reactivity with its specific antibody is not affected by tryptic digestion at pH 8.0 but is slowly reduced by treatment with pepsin at pH 4.5. The TS-toxin consists of 188 amino acid residues. Serine was shown to be the NH_2-terminal amino acid residue by end-group analysis. Initial studies indicated the protein was emetic; thus tentatively it was called staphylococcal enterotoxin F. In this paper it is called TS-toxin because the emetic action in monkeys has not been confirmed.

The staphylococci produce a number of toxic proteins that have been implicated in a variety of staphylococcal diseases, for example, gastric enteritis (Surgalla & Dack, 1955), scalded-skin syndrome (Melish & Glasgow, 1970), and toxic-shock syndrome (Todd et al., 1978). The signs and symptoms of the toxic-shock syndrome (Davis et al. 1980) are almost identical with those demonstrated in rhesus monkeys when staphylococcal enterotoxin B was injected intravenously (Beisel, 1972). Because of our interest in the role of the

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