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Interaction of S-100 Protein with Cations and Liposomes†

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ABSTRACT: The interaction of the brain-specific protein S-100 with Ca^{2+}, K^+, and artificial lipid membranes (liposomes) was studied. The protein S-100 has two sets of Ca^{2+} binding sites with dissociation constants which, in 60 mM Tris-HCl buffer (pH 7.6 and 22°), are, respectively, $\simeq 5 \times 10^{-5}$ and 1×10^{-3} M. In the presence of K^+ the binding of Ca^{2+} to the high affinity sites induces a conformational change which causes an increase of the protein intrinsic fluorescence and makes the protein capable of interacting with 2-toluidinonaphthalene-6-sulfonic acid and with liposomes. The interaction with S-100 greatly facilitates the leak from liposomes of Rb^+ and Ca^{2+} but not of other solutes, such as D-glucose, γ-amino butyrate, and L-glutamate. It is proposed that when Ca^{2+} binds to the high affinity sites of S-100 it induces a conformational change which exposes some hydrophobic groups and thus makes the protein capable of interacting with liposomes and of changing their permeability to some cations.

The brain-specific protein S-100 has been recently shown to interact with lipid membranes (liposomes) and to induce an increase in their permeability to Rb^+ (Calissano and Bangham, 1971). Liposomes are liquid crystals formed by two or more lipid layers surrounding an aqueous phase (Bangham *et al.,* 1965). They have been used as a model of biological membranes to investigate the effect of several substances (antibiotics, proteins, etc.) on ion permeability. The effect induced by S-100 requires the presence of Ca^{2+} and varies with the composition of the lipid membrane, the largest effect being observed with negatively charged liposomes, consisting of phosphatidylserine and phosphatidylcholine. A simple hypothesis on the mechanism of this induced diffusion was based on the well-established interaction between S-100 and Ca^{2+} ions (Calissano *et al.,* 1969). The binding of this cation to the protein is accompanied by the exposure to the solvent of some hydrophobic amino acid residues. Ca^{2+} would facilitate the interaction between the negatively charged liposomes and the acidic protein S-100 both by electrostatic effects and by promoting the above-mentioned conformational changes which would make the protein more lipophilic.

The finding that a brain-specific protein, of still unknown function, exerts a calcium-dependent effect on membrane permeability seemed to deserve further study, in view of possible physiological implications.

Materials and Methods

Chemicals. Egg phosphatidylcholine and bovine brain phosphatidylserine, Na salts, were grade I products from Lipid Products Ltd., England, and used without further purification. The open ampoules were stored under nitrogen at −30° to minimize air oxidation. Stearlyamine was a kind gift from N. Miller (ARC, Babraham, England). 2-Toluidinonaphthalene-6-sulfonic acid, K salt, was purchased from Serva, Heidelberg, Germany. All common salts and solvents were reagent grade. KCl, $CaCl_2$, and RbCl for binding experiments and fluorescence studies were "Suprapur" reagents from Merck, Darmstadt, Germany. $^{86}RbCl$ and $^{45}CaCl_2$ were obtained from the Radiochemical Centre, Amersham, and had a specific activity of 2–10 mCi/mg.

S-100. The S-100 protein was prepared in pure form from beef brain as described by Moore (1965) and kept as a lyophilized powder at −30°. Protein concentration was measured by absorbancy at 280 nm assuming a molar extinction coefficient of 8260 mol^{-1} cm^{-1} (Calissano *et al.,* 1969). The protein was free of EDTA employed during the purification procedure (Levi *et al.,* 1974).

Liposome Preparation and Diffusion Assays. The method of liposome preparation was a modification of the original procedure of Johnson and Bangham (1969). Vesicles were prepared by adding 1.0 ml of 20 mM Tris-Cl buffer (pH 7.5), containing 60 mM KCl, 1 mM RbCl, plus 10–20

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μCi of ^{86}Rb, to a mixture of PS-PC[1] (1.5:1) previously evaporated to dryness. The suspension, after mechanical shaking for 5 min, was transferred to a 1-cm diameter tube and sonicated under a small stream of water-saturated nitrogen in a Kerry's ultrasonic cleaning bath type KS100 until it became clear and bluish. The temperature in the bath was maintained at 15° by circulating cold water.

After sonication, the liposome solution was left at room temperature for at least 2 hr under N_2 before use. The untrapped radioactivity was removed by gel filtration through a Sephadex G-25 fine column equilibrated with the same solution used to prepare the liposomes. Aliquots (0.5 ml) containing 2–3 μmol of phospholipids were then transferred into $^{8}/_{32}$ in. Visking dialysis bags to which the protein or the buffer solution was added to a final volume of 1 ml. The bags were allowed to incubate in tubes containing 10 ml of the same buffer; at time intervals the bags were transferred to another set of tubes and the incubation was continued. The incubation was carried out at 37° in a shaking bath. At the end of the experiment the labeled material remaining in the dialysis bags was counted. Radioactivity was counted in a Beckman LS-250 liquid scintillation system. Leak was expressed as the per cent of the total counts initially captured by the liposomes (Johnson and Bangham, 1969).

Ca^{2+} influx into the liposomes was measured as follows. Liposomes prepared under identical conditions as those used for ^{86}Rb leak experiments were incubated at 37° with 0.8 mM $CaCl_2$ plus ^{45}Ca in the absence or in the presence of the protein. At time intervals aliquots (generally 0.2 ml) of the incubation mixture were passed through a Sephadex G-25 fine column equilibrated with the same buffer used for the incubation and containing 1 mM EDTA. We found that this substance removed all the Ca^{2+} free in solution or bound to the external part of the liposome. The liposome peak was collected and phospholipid concentration as well as ^{45}Ca radioactivity were measured. The amount of radioactivity present was expressed as nanomoles of Ca^{2+} entered into the liposomes per micromole of phospholipid.

Ca^{2+} Binding to Phosphatidylserine. The technique employed for these experiments was essentially the one used by Blaustein (1967) with some minor modifications. An aqueous solution (0.15 ml) containing 5 mM Tris-Cl (pH 7.6), 60 mM KCl, and varying concentrations of $CaCl_2$ (0.015 up to 1.0 mM) plus ^{45}Ca, was added to 0.15 ml of a 1:1 mixture of chloroform–methanol (v/v) containing 60 nmol of PS. The S-100 protein was present in the aqueous solution at a concentration of 3.5 nmol. The mixture was allowed to stand for 15 min with shaking at intervals of 3 min and then centrifuged at 3000 rpm for 10 min in a desk centrifuge. The two phases obtained after centrifugation were assayed for radioactivity. In the absence of phospholipids less than 1% of the ^{45}Ca was taken into the organic phase. All the lipids remained in the chloroform–methanol phase. Binding was expressed as moles of Ca^{2+} bound per mole of PS in the organic phase ($\bar{\nu}$). The S-100 partitioned in the aqueous phase.

Fluorescence Measurements. All titrations and fluorescence spectra were recorded with an Aminco Bowmann spectrofluorometer equipped with a high-stability xenon lamp scanning from 200 to 800 nm.

The intrinsic fluorescence of S-100 was studied with an exciting light of 280 nm and measuring emission at 355 nm unless otherwise specified. Titrations were carried out in a thermostated cuvet (1-cm light path, 22°) in 20 mM Tris-Cl (pH 8.3), with or without 60 mM KCl. Titrations were carried out by adding the titrant solution in 2-μl aliquots with a micropipet. Total dilution never exceeded 5–7% and relative fluorescence values were uniformly corrected for dilution. The optical density of the final titration mixture was always less than 0.1 at the exciting wavelengths.

Equilibrium Dialysis. Calcium binding activity was measured by equilibrium dialysis according to the following procedure. Dialysis tubing (Visking $^{8}/_{32}$) was cut into 14-cm lengths and heated two times in twice distilled water and once in the same buffer to be used in the experiment plus trace amounts of the radioactive cation. Samples (0.2 ml) containing the protein were added to small bags formed with the tubing, knotted to include an air bubble and vertically placed in a test tube filled with 10 ml of 60 mM Tris-Cl buffer (pH 7.6 or 8.25) and varying concentrations of Ca^{2+}. The total Ca^{2+} concentration was varied between 0.005 and 1.0 mM by the addition of $CaCl_2$. ^{45}Ca was kept constant in all experiments. Equilibrium was obtained in 18 hr at 25° ± 1.0 in a shaking bath. A relatively high ionic strength (0.06 M Tris-Cl) was used in order to reduce possible Donnan effects. The S-100 concentration was 0.5–1.0 mg/ml; a molecular weight of 24,000 was assumed (Calissano *et al.*, 1969). Binding data were analyzed according to Scatchard (1949).

The amount of S-100 "tightly" bound to the liposomes was initially measured with the microcomplement fixation technique (Moore *et al.*, 1968). In successive experiments, however, we found that addition of S-100 antiserum to the S-100 liposome complex resulted in a quantitative reproducible precipitation of the S-100 protein, the amount of which was calculated by reference to a standard obtained with the free protein. Since the determination of S-100 bound to liposomes obtained by the two methods agreed within the experimental error, the "precipitation" assay was employed in most of the experiments reported. Phospholipid concentrations were determined by estimation of inorganic phosphate according to the method of Fiske and SubbaRow (1925). Glass twice distilled water was used to prepare all solutions.

Results

Effect of S-100 on the Diffusion of ^{86}Rb out of Liposomes. The leak of ^{86}Rb out of liposomes, in the presence of 1.0 mM $CaCl_2$, was studied as a function of S-100 concentration and of the time of interaction between the protein and liposomes. As shown in Figure 1 the rate of leakage increases with S-100 concentration.

It is interesting that, under the experimental conditions we used, a substantial enhancement of the leak relative to the control is observed even at an S-100/phospholipid molar ratio as low as 10^{-3}; assuming that each sonicated liposome contains approximately 2000 phospholipid molecules (Johnson *et al.*, 1971), this would roughly correspond to an average of 1 or 2 molecules of S-100 per liposome.

The Ca^{2+} dependence of the effect of S-100 on the leak of Rb^{+} out of liposomes follows a sigmoid curve (Figure 2). In the absence of Ca^{2+}, S-100 exerts no effect. At concentrations of Ca^{2+} lower than 0.1 mM, the effect of S-100 is negligible, while in the 0.4–1.0 mM range it increases sharply; at concentrations greater than 1.5 mM, Ca^{2+} induces an instability of the liposomal membrane which is ac-

[1] Abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; SA, stearylamine; ANS, 8-anilino-1-naphthalenesulfonic acid; TNS, 2-toluidinonaphthalene-6-sulfonic acid.

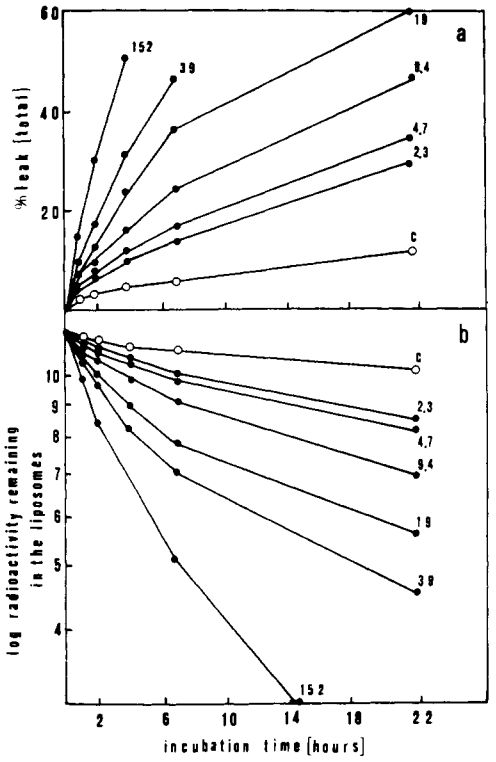

FIGURE 1: Effect of S-100 concentration on ^{86}Rb leak. Liposomes were prepared with PS-PC (1.5:1) as described under Materials and Methods in the presence of 5 mM Tris-Cl buffer (pH 7.48), 60 mM KCl, 1 mM RbCl, and trace amounts of ^{86}Rb. Each dialysis bag contained 3 μmol of phospholipid plus various amounts of S-100 in a final volume of 1 ml. Incubation was carried out at 37° in the same buffer used to prepare liposomes also containing 0.8 mM $CaCl_2$: (a) total leak of radioactivity expressed as per cent of the radioactivity initially present in the liposomes; (b) log of radioactivity remaining in the liposomes. The number on each line indicates the micromolar concentration of S-100. The c line refers to control experiments without S-100. Liposomes contained 3-4 × 10^4 cpm of ^{86}Rb/μmol of phospholipid.

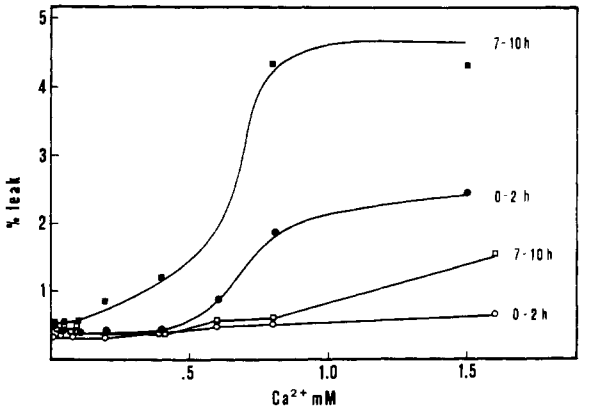

FIGURE 2: Dependence of the S-100 induced leak upon Ca^{2+} concentrations. Liposomes (PS-PC, 1.5:1) loaded with $^{86}RbCl$ were prepared as described under Materials and Methods with 20 mM Tris-Cl-60 mM KCl (pH 7.6). $CaCl_2$, at the concentrations indicated in the abscissa, was present both in the dialysis bag containing the liposomes and in the buffer of incubation (10 ml). The two curves refer to different incubation times in the absence (□,○), and in the presence (■,●) of 17 μM S-100.

companied by an increase of permeability (Papahadjopoulos and Bangham, 1966), so that an accurate evaluation of the specific effect of S-100 becomes impossible. It is important to point out that the interaction between S-100 and Ca^{2+} as evaluated by spectral methods (see Figures 6 and 7) in the presence of 60 mM K^+ also follows a sigmoid curve, the steep part of which occurs at the same Ca^{2+} concentrations as those corresponding to the steep portion of the curve shown in Figure 2.

Effect of S-100 on the Interaction between Ca^{2+} and Liposomes. The effect of S-100 on the interaction between liposomes and Ca^{2+} was studied in two sets of experiments. Attempts were first made to study the leak of Ca^{2+} out of liposomes under experimental conditions similar to those used for ^{86}Rb; however, the experiments were complicated by the binding of Ca^{2+} to the phosphatidylserine component of the liposomes (Papahadjopoulos and Bangham, 1966; Seimiya and Ohki, 1972). Thus, for instance, the preparation of liposomes starting from 35 μmol of phosphatidylserine and 17 μmol of phosphatidylcholine in the presence of 1.1 ml of buffer containing 1 μmol of Ca^{2+} results in an incorporation of approximately 0.5 μmol of Ca^{2+}. This amount of incorporated calcium is more than ten times the amount of Ca^{2+} which can be contained in the volume of buffer enclosed within liposomes (*ca.* 0.016 ml according to Johnson *et al.*, 1971). It seems probable, therefore, that most of the incorporated calcium is bound to the phospholipids forming the interior of the liposomes rather than dissolved in the buffer enclosed within them. Consequently the marked effect of S-100 on the release of Ca^{2+} from the liposomes, which we observed in experiments performed under identical conditions to those employed for the Rb^+ leak, could result not only from the leakage of the dissolved Ca^{2+} but also from the displacement of the cation bound to the phosphatidylserine residues.

This interpretation was supported by the experiments performed on the binding of Ca^{2+} to PS (see Materials and Methods) in the presence and absence of S-100. These studies demonstrated that in the presence of S-100, the amount of Ca^{2+} bound to phosphatidylserine is lower than in its absence, the competition exerted by the protein being most evident (from 50 to 20% inhibition) at the lower Ca^{2+} concentrations (0.01-0.2 mM), *i.e.* where a substantial fraction of the total Ca^{2+} is bound to S-100 (see Figures 5 and 6).

The effect of S-100 on the incorporation of Ca^{2+} into liposomes has also been studied. Liposomes (which did not contain ^{86}Rb) were suspended with and without S-100 in the usual incubation mixture containing 60 M KCl, 0.8 mM $CaCl_2$ and 1.5 μCi of ^{45}Ca; at time intervals aliquots were taken from the suspension and passed through a G-25 Sephadex column containing 1.0 mM EDTA. We found that this substance removes all the Ca^{2+} bound to the external part of the liposome. The amount of Ca^{2+} radioactivity eluted with the liposomes was taken as a measure of Ca^{2+} incorporation into liposomes. The results, reported in Figure 3, show that S-100 exerts a marked effect on the incorporation of Ca^{2+} into liposomes and that the effect increases with the time of incubation. Moreover, the effect of S-100 is much greater when potassium chloride in the incubation mixture is replaced by choline chloride; this can be due to the fact that K^+ competes for some of the Ca^{2+} binding sites (Figures 5 and 6) of S-100 while choline does not (S. Alemà *et al.*, unpublished data).

It is possible that the enhancement of Ca^{2+} incorporation into liposomes could be partly due to the formation of an S-100-Ca^{2+}-liposome complex, rather than to an effect of S-100 on the flux of Ca^{2+} into the liposomes. However, under our experimental conditions, the maximum amount of Ca^{2+} which can be bound to the S-100 present (see Figures 5 and 6) is no more than 5-10% of the total while the

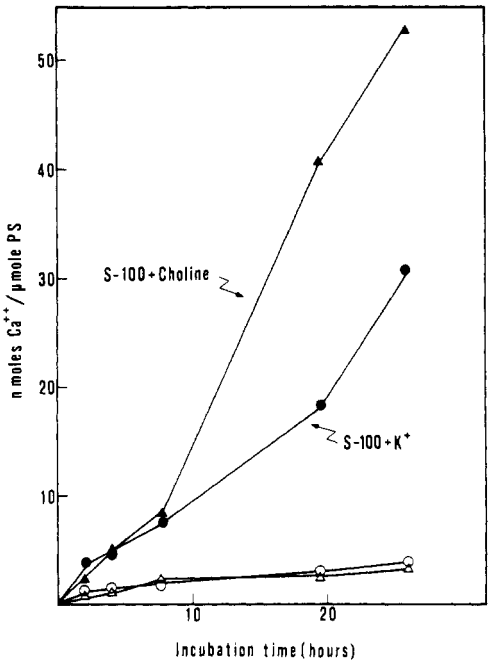

FIGURE 3: Ca^{2+} influx into liposomes in the presence and absence of S-100. Liposomes were prepared as described under Materials and Methods with 20 mM Tris-Cl, 60 mM KCl (○,●), or choline cloride (△,▲) (pH 7.7). The two batches of sonicated liposomes prepared in the presence of KCl or of choline chloride were divided in two aliquots and incubated with 0.8 mM $CaCl_2$ plus ^{45}Ca (3.5 μCi/ml) in the absence (open symbols) and presence (dark symbols) of 35 μM S-100. At time intervals aliquots (0.25 ml) of the incubation mixture were passed through a G-25 fine Sephadex column equilibrated with the same buffer salts and containing 1 mM EDTA. The liposome peak was collected and PS concentration as well as ^{45}Ca radioactivity measured. The amount of Ca^{2+} present is expressed as nanomoles of Ca^{2+}/micromole of PS.

amount of Ca^{2+} found in the liposomes exceeds 20% of the total.

Specificity of the S-100 Effect on the Leak of Solutes out of Liposomes. To test whether the effect of S-100 was specific for Rb^+ and Ca^{2+}, liposomes were prepared containing several labeled anions, cations, and nonionic solutes. ^{86}Rb was also included in all tests as a reference, in order to take into account the slight variations of permeability observed among different liposomal preparations. The results, reported in Table I, show that S-100 has no effect on the leak of D-glucose, γ-aminobutyrate, L-glutamate, and several other amino acids, either in the presence or absence of Ca^{2+}, under conditions where it exerts a marked effect on the leak of Rb^+. The leak of choline is affected by S-100, though less than the leak of Rb^+, K^+, and Na^+. Papahadjopoulos (1971) has recently shown that pure phosphatidylserine vesicles discriminate between monovalent cations, the rate of leak being ten times greater for K^+ than for Na^+. In this connection, it would be interesting to test whether the effect of S-100 would be different for K^+ and Na^+ if tested on liposomes made exclusively of phosphatidylserine.

Binding of S-100 to Liposomes. Samples were taken at various times from the reaction mixture for standard ^{86}Rb leak experiments (see previous section) and chromatographed on G-100 Sephadex columns. The effluent was analyzed for phospholipids and for S-100 by an immunoprecipitation reaction (see Materials and Methods).

As shown in Figure 4, in experiments carried out in the presence of Ca^{2+}, some of the S-100 emerged from the column with the liposomes. This amount increased with the time of incubation, over 24 hr; on the contrary, in the absence of Ca^{2+} and in the presence of EDTA, no S-100 could be detected in the fractions which contained the liposomes (Figure 4a). It is interesting that the time required for the

TABLE I: Effect of S-100 on Leak of Anions, Cations, and Nonionic Solutes.[a]

Solute	% Leak/hr				Fold Increase over Control
	Control + EDTA	S-100 + EDTA	Control + Ca^{2+}	S-100 + Ca^{2+}	
Na^+ (38.5 mM)	0.37	0.92	0.47	1.66	3.5
K^+ (38.5 mM)	0.17	0.59	0.23	1.28	5.5
Rb^- (1.0 mM)	0.28	0.79	0.8	5.6	7.0
Glucose (5.0 mM)	65.5	66.5	68.5	69.0	0
Rb^+ (1.0 mM)			0.9	2.7	3.0
Choline (5.0 mM)			1.3	2.5	1.9
Rb^+ (1.0 mM)	0.49	0.87	0.69	3.5	5.1
Glutamate (1.0 mM)	3.22	3.25	3.50	3.8	0.08
Rb^+ (1.0 mM)			0.8	2.5	3.1
GABA (5.0 mM)			34.8	33.8	0
Rb^+ (1.0 mM)	1.11	1.38	0.54	3.56	6.5
Amino acids mixture (1.0 mM)	11.2	12.1	9.34	11.5	0.012

[a] Liposomes were prepared with PS–PC (1.5:1) as described under Materials and Methods in the presence of 5 mM Tris-Cl buffer (pH 7.5), 1.0 mM RbCl, 60 mM KCl (except the experiment with both Na^+ and K^+), 10 μCi of $^{86}Rb^+$ plus the solute to be tested at the concentration indicated in the table, and 10.0 μCi of the same solute labeled on ^3H or ^{14}C. The S-100 concentration varied between 17 and 34 μM. Leak is expressed as per cent per hour of the total counts (between 10^4 and 5×10^4 cpm in the various experimental conditions) initially present in the liposomes. The last column on the right reports the increased leak induced by S-100 over controls in the presence of 0.8 mM Ca^{2+}. The values of per cent leak were calculated after the first hour of preincubation in order to allow equilibration of the S-100 liposomes mixture. The differences between controls and S-100 within each experiment did not change for at least an incubation time of 3 hr.

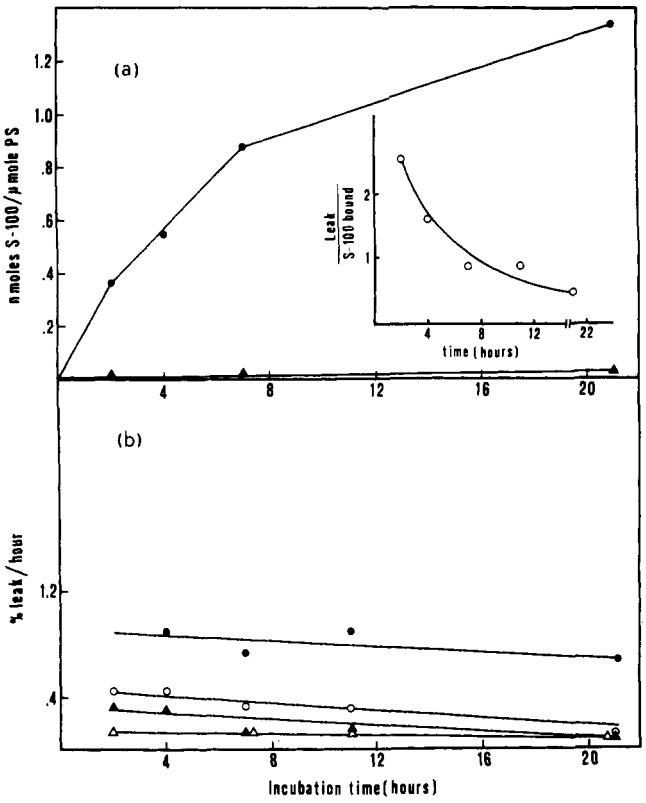

FIGURE 4: Correlation between binding of S-100 to liposomes and induced leak. Liposomes prepared as described under Materials and Methods and loaded with ^{86}Rb were incubated in the absence or in the presence of S-100 (17 μM) with 0.1 mM EDTA or 0.8 mM Ca^{2+}. At time intervals aliquots of the S-100 liposomes suspension were passed through a 1 × 30 cm G-100 Sephadex column equilibrated with the same buffer and salts used for the incubation; 1.0-ml fractions were collected with a flow rate of 20 ml/hr. The peak of liposomes was assayed for PS content and for S-100 concentration. (a) Binding of S-100 incubated with EDTA (△) and with Ca^{2+} (○). The inset reports the ratio between the induced leak/hour and the amount of S-100 bound to liposomes (nanomoles of S-100/micromole PS). (b) Effect of S-100 (17 μM) on ^{86}Rb diffusion: (●) S-100-Ca^{2+}; (○) S-100-EDTA; (▲) controls with Ca^{2+} and without S-100; (△) controls with EDTA and without S-100.

binding of S-100 to liposomes in a manner appreciable by the present method is much longer than the time of incubation sufficient to obtain a maximum effect of S-100 on the leak of ^{86}Rb (Figure 4b). Considering that the present method measures as bound only the portion of S-100 which does not dissociate from the liposomes during chromatography on Sephadex G-100, the discrepancy between the time course of S-100 binding and the time course of the S-100 effect on Rb^+ leak (see inset of Figure 4a) could be explained assuming that S-100 affects the leak even before it has bound to the liposomes tightly enough to resist chromatography. Another possible explanation is that the very first S-100 molecule which binds to a liposome is capable of determining a maximum effect on the leak. However, some caution should be used in the quantitative interpretation of these data, because the immunochemical method used to assay S-100 was calibrated on the free protein and its results may give incorrect absolute values when applied, as in the present case, to S-100 bound to liposomes.

Data on the reversible binding to liposomes of specifically labeled S-100 will be reported elsewhere.

Binding of Ca^{2+} and Rb^+ to S-100. The binding of Ca^{2+} to S-100 has been previously studied by equilibrium dialysis

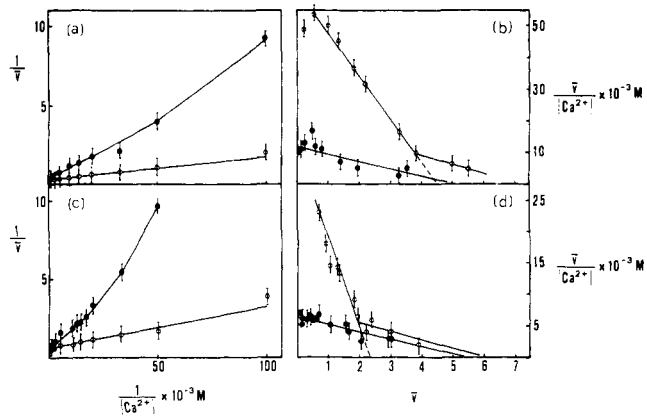

FIGURE 5: Studies of Ca^{2+} binding to S-100. Equilibrium dialysis experiments of ^{45}Ca binding to S-100 were performed in 60 mM Tris-Cl at pH 8.3 (a,b) and pH 7.6 (c,d) in the absence (○) and in the presence (●) of 60 mM KCl. The left part of the figure shows a double reciprocal plot, the right part a Scatchard plot. Each point is the average of three different experiments; $\bar{\nu}$ = moles of Ca^{2+} bound/mole of S-100 assuming a mol wt of 24,000. The S-100 concentration was 42 μM.

in the presence of 60 mM KCl at pH 8.3 (Calissano *et al.*, 1969). Moreover, it was shown that the fluorescence of S-100 increases in the presence of Ca^{2+} and that this effect is counteracted by monovalent cations. It seemed, therefore, important to extend these studies in order to assess the mutual effects of monovalent cations and Ca^{2+} on binding to S-100 and to repeat all experiments at pH 7.6, *i.e.*, under the conditions used to study the interaction of S-100 with liposomes. The results shown in Figures 5c and 5d show that at pH 7.6 in the absence of K^+, each S-100 molecule (mol wt 24,000) has two high affinity (apparent dissociation constant = 0.07 mM) and about five low affinity (apparent dissociation constant = 1.0 mM) Ca^{2+} binding sites. In the presence of 60 mM KCl, the binding of Ca^{2+} to the high affinity sites becomes cooperative (Figure 5a,b). Similar results were obtained at pH 8.3 except that the number of high affinity sites seems to be 4, instead of 2, per molecule (see Figure 5a,b).

The binding of ^{86}Rb to S-100 was studied by equilibrium dialysis under conditions analogous to those used for Ca^{2+}. The binding of Rb^+, as a function of the concentration of free ion, follows a sigmoid curve. The concentration of Rb^+ giving half-saturation is *ca.* 7.0 mM. A more refined analysis of the data was considered not meaningful, considering the rather large experimental errors.

Fluorescence Studies. The interaction between Ca^{2+}, K^+, and S-100 was also studied by measuring the increase of the fluorescence of S-100 caused by the addition of calcium.

As shown in Figure 6a, at pH 8.3 and in the absence of potassium, the increase of fluorescence of S-100 proceeds in parallel with Ca^{2+} binding as if the four high affinity binding sites for calcium on each S-100 molecule (see Figure 5) were independent. The correspondence between the curve for the fluorescence increase and that for the binding could be explained by either of the two following hypotheses: (a) the binding of Ca^{2+} to any of the four binding sites exerts the same effect on the fluorescence of the protein or (b) fluorescence is affected by the binding to only one or more of the sites, but all sites have the same affinity for Ca^{2+}.

Similarly, at pH 7.6, the increase of fluorescence as a function of Ca^{2+} concentration parallels the binding of calcium to the high affinity sites; at this pH, however, the high

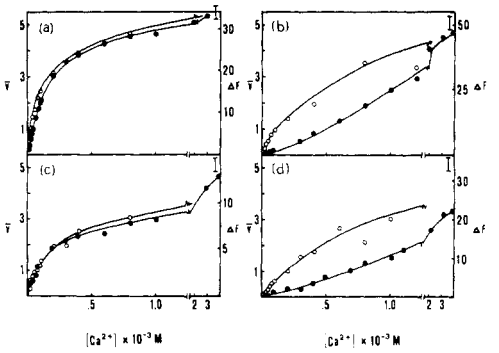

FIGURE 6: Correlation between Ca^{2+} binding and fluorescence of S-100: Ca^{2+} binding (○); fluorescence increase (●) at pH 8.3 (a,b) and pH 7.6 (c,d). The left part of the figure refers to experiments without KCl and the right part to experiments in the presence of 60 mM KCl. The fluorescence increase (ΔF) is equal to the fluorescence (in arbitrary units) of the protein at a given Ca^{2+} concentration minus the fluorescence at zero Ca^{2+} concentration. The symbol $\bar{\nu}$ is defined in Figure 5.

affinity sites seem to be $\simeq 2$ instead of $\simeq 4$ (see Figure 5c,d).

The data obtained at pH 8.3 in the presence of 60 mM KCl (see Figures 5 and 6b) agree with those previously published (Calissano *et al.*, 1969). A comparison between these data and those obtained in the absence of KCl suggests that K^+ competitively interacts with the high affinity sites for Ca^{2+}, *i.e.* with those which affect the fluorescence of the protein. On the other hand, the apparent binding constant for the low affinity sites is not affected.

As previously reported (Calissano *et al.*, 1969) in the presence of K^+ the dependence of the fluorescence increment on calcium concentration follows a sigmoid course; this had been interpreted assuming a cooperative binding between two or three sites (Calissano *et al.*, 1969). It is noteworthy that, in the absence of potassium, the cooperativity is lost (see Figure 6a,c). It seems likely therefore that the capacity of displaying site-site interactions requires the binding of potassium to the protein. This conclusion was confirmed by a Hill plot of the fluorescence data showing n values of 0.7 and 1.7, respectively, in the absence and presence of 60 mM KCl.

At pH 7.6 in the presence of KCl (Figure 6d) the situation is similar to that observed at pH 8.3, except that the apparent dissociation constant for binding is slightly greater than that at pH 8.3.

Interaction between TNS[1] *and S-100.* It has been postulated that the binding of Ca^{2+} to S-100 induces a change in the protein conformation which involves a partial exposure of hydrophobic residues to the solvent (Calissano *et al.*, 1969). This hypothesis was supported by the observation that ANS,[1] which is often used as a probe for hydrophobic regions of proteins, interacts with S-100 and its fluorescence (Moore, 1972) increases in the presence of Ca^{2+}. No quantitative analysis of the data, however, was attempted. In the present paper a more systematic study has been carried out using TNS as a probe.

The results are reported in Figure 7. As shown in Figure 7a, the fluorescence of TNS in the presence of S-100 is negligible unless Ca^{2+} is added to the reaction mixture. Ca^{2+} increases the quantum yield and causes a blue shift of the emission peak. In the absence of S-100 Ca^{2+} has no effect on the fluorescence of TNS. The increase of the dye fluorescence was then used to measure the affinity of S-100 for TNS in the presence of 5.0 mM $CaCl_2$. The binding follows a classical hyperbolic course and the dissociation constant is about 0.05 mM. A continuous variation analysis showed that 1 mol of TNS binds per mol of protein (Figure 7b inset) indicating that TNS probably binds to a specific region of the protein. Finally, the effect of Ca^{2+} on the interaction was studied both in the presence and absence of 60 mM KCl. As shown in Figure 7c, in the presence of KCl the calcium concentration dependence of the protein-bound TNS follows a sigmoid curve analogous to that observed for the fluorescence of the protein tryptophanyl residue (see Figure 6). The dissociation constants for calcium measured by TNS fluorescence and by the protein fluorescence method are very similar. This suggests that the same conformational changes are responsible for the effect of Ca^{2+} and K^+ ions on both the intrinsic protein fluorescence and the bound dye fluorescence and confirms the hypothesis that in the presence of calcium a hydrophobic site of the protein become accessible to the dye. The slight discrepancy of the dissociation constants of S-100 for Ca^{2+} measured by the two methods is probably due to the slight shift in the equilibrium induced by the binding of TNS to the S-100–Ca^{2+} complex.

Discussion

The function of the brain-specific protein S-100 is still unknown. Recently, it has been shown that this protein interacts with liposomes prepared with a high mole fraction of phosphatidylserine, causing them to become 7–10 times more permeable to Rb^+ but not to glucose, when Ca^{2+} is

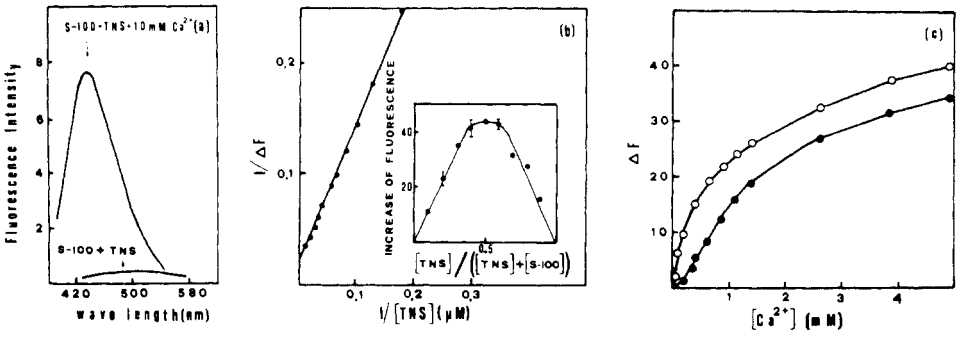

FIGURE 7: Effect of S-100 on TNS fluorescence. (a) Emission spectra of a solution of 20 mM Tris-Cl buffer (pH 8.3), TNS (15 μM), and S-100 (18 μM) in the presence and absence of 10 mM Ca^{2+}. The exciting light at 365-nm emission was measured at 445 nm. (b) Reciprocal plot of TNS fluorescence as a function of its concentration. All solutions contained S-100 (4.2 μM) and $CaCl_2$ (5.0 mM). The inset of b shows the stoichiometry of the binding of TNS determined by a continuous variation analysis. Concentrations of TNS and S-100 were varied over a tenfold range while the sum of the concentrations had the fixed value 20 μM in the presence of 5.0 mM $CaCl_2$. (c) TNS fluorescence increase as a function of Ca^{2+} concentrations in the absence (○) and in the presence (●) of 60 mM KCl. All solutions contained S-100 (8.0 μM) and TNS (15 μM).

present (Calissano and Bangham, 1971). Previous studies demonstrated that Ca^{2+} interacts with S-100 inducing an increase in the quantum yield of the protein fluorescence (Calissano *et al.*, 1969) and that K^+ ions counteract this effect. Present studies allow us to correlate these multiple interactions and provide some suggestions on the possible mechanism by which S-100, in the presence of Ca^{2+}, increases the permeability of model membranes to monovalent cations.

In the absence of Ca^{2+} S-100 does not bind to liposomes (Figure 3) and does not exert any relevant effect on the leak of Rb^+ from PS–PC liposomes (Figure 3). In the presence of Ca^{2+}, instead, S-100 binds to liposomes and greatly increases their permeability to Rb^+ (see Figures 1, 2, and 4). These results indicate that Ca^{2+} is required not only for the effect on permeability but also for the binding of S-100 to liposomes. A comparison of the time course of the two processes (Figure 4a, inset) shows that the increase of permeability occurs promptly and reaches a maximum value after a few hours of incubation, while the extent of binding of S-100 to liposomes continues to increase over more than 24 hr. When interpreting these data it is important to consider that the method used by us measures only "tight binding" (*i.e.*, the amount of S-100 which remains bound to the liposomes after chromatography on Sephadex G-100); it is, therefore, possible that the interaction between S-100 and liposomes in the presence of Ca^{2+} occurs in two steps; the first rapidly reversible step would be connected with the effect on permeability while the second step would require longer periods of time and would correspond to the slow "tight" binding assayed by our method. This sequence of events agrees with the scheme for lipid–bilayer protein interaction proposed by Kimelberg and Papahadjopoulos (1971a). Preliminary experiments (Calissano *et al.*, 1972a,b) show that if the S-100 "tightly bound" to liposomes is rechromatographed, after different times of incubation, a progressively larger portion of it is eluted as free protein. These findings also indicate that the tight binding is reversible with a much slower rate.

The effect of S-100 on liposomal permeability is strongly dependent upon the chemical composition of liposomes (Calissano and Bangham, 1971); the tight binding of S-100 is also dependent upon the chemical composition of the liposomes. Thus, nonsonicated SA–PC[1] (1.5:1) liposomes bind a much larger amount of S-100 than nonsonicated PS–PC liposomes, 1.5:1 (Calissano, 1973; Calissano *et al.*, 1972a,b) however, the effect of S-100 on Rb^+ leak is much greater with the latter than with the former. It seems therefore that binding of S-100 to liposomes "per se" does not necessarily affect their permeability to cations. The latter effect presumably requires a particular type of interaction. It is noteworthy to mention that other proteins (Kimelberg and Papahadjopoulos, 1971b; Calissano *et al.*, 1972b), including the brain-specific protein 14-3-2 (Calissano and Bangham, 1971), affect liposome permeability. The link between Ca^{2+}, S-100 and a lipid membrane described in this study, however, appears to be specific and quite unique for the effects observed (leak experiments) and the mechanism possibly underlying such effects (binding experiments).

Thus, a suggestion about one aspect of the interaction between the protein and liposomes comes from the requirement of Ca^{2+} for the S-100-induced leak and the known effect of Ca^{2+} on S-100 conformation. It is tempting to think that the two phenomena are related. Chemical and spectral studies have shown that some aromatic amino acid side chains and two cysteinyl residues are exposed to the solvent upon binding of Ca^{2+} to S-100. Moreover, ANS, which is known to interact with hydrophobic portions of proteins, binds to S-100 only in the presence of Ca^{2+}, undergoing a characteristic spectral shift and quantum yield increase (Moore, 1972). The present detailed study with TNS has confirmed these results and moreover has shown that the dye forms a 1:1 complex with S-100 (Figure 7b, inset). This suggests that the dye interacts with a limited specific area of the protein surface which is made available by the Ca^{2+}-induced conformational change. On the basis of all these data, it seems reasonable to think that the exposure of nonpolar or semipolar groups induced by Ca^{2+} favors the interaction of S-100 with liposomes and, possibly, the partial penetration of the protein into the bilayer. Preliminary studies show that in the presence of liposomes and Ca^{2+}, a specific portion of S-100 is protected from digestion by proteolytic enzymes (Calissano *et al.*, 1972a).

Having obtained some indications about the nature of the Ca^{2+}-induced binding of S-100 to liposomes, the mechanism by which this interaction specifically increases the permeability of liposomes to monovalent cation should be considered. Present results show that, in the presence of K^+ (or Rb^+), the binding of Ca^{2+} to the high affinity sites of S-100 becomes cooperative, so that, at low Ca^{2+} concentrations, less Ca^{2+} is bound in the presence than in the absence of K^+. It is noteworthy that it is the binding of Ca^{2+} to these sites which apparently causes the conformational changes responsible for the increase of the protein fluorescence, the binding of TNS, and the capacity to interact with liposomes.

Taking all the above data into account, the Ca^{2+}-dependent effect of S-100 on the leak of Rb^+ from liposomes can be tentatively explained as follows. Ca^{2+} binds to the high affinity sites of S-100 inducing a conformational change which exposes some hydrophobic groups and thus makes the protein capable of penetrating into the liposomal membrane. Having penetrated into the membrane, which is initially poor in Ca^{2+} and rich in Rb^+, S-100 loses its Ca^{2+} ions and binds Rb^+. This ion exchange brings about a conformational change which makes the protein surface less hydrophobic and thus promotes the detachment of the Rb^+–S-100 complex from the liposomes; since the solution surrounding the liposomes contains an excess of Ca^{2+}, Rb^+ would then dissociate from the protein, while Ca^{2+} would bind to it making it again capable of interacting with the liposomes. The previously described functional link between Rb^+ and Ca^{2+} binding to S-100 would play an important role in this process.

Alternatively, it is possible that the S-100–Ca^{2+} complex, which can interact with the liposomes through the exposed hydrophobic groups of the protein, modifies the structure of the lipid membrane making it more permeable to Rb^+. Although it is not presently possible to establish the relevance of the present study for the understanding of the physiological role of S-100, it is interesting that 5–7% of the total S-100 present in mammalian brain is strongly bound to the membrane (Rusca *et al.*, 1972).

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A Spectroscopic Study of Bovine Lactoferrin†

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ABSTRACT: Bovine lactoferrin was examined spectroscopically in order to determine the constituents of the iron-binding site and the relationship of iron binding to conformation. In aqueous solutions of metal-free lactoferrin, one or two more tryptophan residues were accessible to the perturbants sucrose, ethylene glycol, glycerol, and dimethyl sulfoxide than in similar solutions of iron(III)-lactoferrin. At pH 11, six more tyrosine residues were ionized in lactoferrin than in iron(III)-lactoferrin. Difference absorption spectra indicated a shift of tyrosine and tryptophan residues from a nonpolar to a more polar environment in the presence of guanidine · HCl and aqueous acid. The shift was seen with both forms of the protein, but higher concentrations of denaturants were required when iron was present. The circular dichroism (CD) spectrum of iron(III)-lactoferrin was compared with that of the metal-free protein. Small changes in the ellipticities of the ultraviolet bands near 295, 291, 275, and 254 nm were seen and additional bands appeared at 455 and 330–310 nm when iron was bound. Below 240 nm, the CD spectra of the two forms were identical. The results suggest that tryptophan residues may not be bound directly to iron, but tyrosine residues probably are, and iron binding stabilizes the spectroscopically observed native conformation of lactoferrin.

Lactoferrin is a nonheme, iron-binding protein originally isolated from milk (Groves, 1960), but since identified in a variety of mammalian secretions (Masson *et al.*, 1966). It is similar to the serum protein, transferrin, and the egg-white protein, ovotransferrin, which is also called conalbumin. Each of these proteins consists of a single polypeptide chain which contains two specific metal binding sites.

The iron-binding characteristics of human transferrin and chicken ovotransferrin have been extensively studied. Feeney and Komatsu (1966) reviewed the early studies; more recent reports include those of Aisen *et al.* (1967), Lehrer (1969), Tan and Woodworth (1969), and Nagy and Lehrer (1972). Teuwissen *et al.* (1972) studied the role of tyrosine residues in iron binding by human lactoferrin. Aisen and Leibman (1972) compared the absorption and electron paramagnetic resonance spectra of human and bovine lactoferrin with those of human transferrin.

In each of these proteins, iron binding appears to occur at chemically equivalent, noninteracting sites (Aisen and Leibman, 1972), and includes the binding of one small anion, normally bicarbonate, with each atom of iron. This iron–bicarbonate–protein complex develops a salmon color with an absorption maximum between 470 and 460 nm.

The presence of two or three residues of tyrosine per iron-binding site in each of the transferrin-like proteins has been indicated by electrometric and spectrophotometric titrations (Tan and Woodworth, 1969; Teuwissen *et al.*, 1972; Wishnia *et al.*, 1961). Chemical modification and spectral studies including electron paramagnetic resonance, fluorescence, and circular dichroism (CD) have implicated histidine and tryptophan as well as tyrosine in the binding sites (Aasa *et al.*, 1963; Lehrer, 1969; Phillips and Azari, 1972; Tan, 1971).

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