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Comparison of S100b Protein with Calmodulin: Interactions with Melittin and Microtubule-Associated τ Proteins and Inhibition of Phosphorylation of τ Proteins by Protein Kinase C[†]

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ABSTRACT: To gauge similarities between S100b protein and calmodulin, interactions were observed between S100b and melittin and between S100b and τ, the microtubule-associated proteins. The interaction of melittin with S100b protein in the presence and absence of calcium was studied by fluorescence polarization, UV difference spectroscopy, and sulfhydryl derivatization. Whether calcium was present or not in the solution, melittin and S100b form a complex of molar ratios up to 2:1. Further binding of melittin occurred, but it resulted in precipitation of S100b, as is true of the corresponding case of melittin binding to calmodulin. In the absence of calcium, the interaction of melittin and S100b shielded the tryptophan (Trp) of the former protein and exposed cysteine-84β (Cys-84β) of the latter protein, leaving the tyrosine-16β (Tyr-16β) of S100b unaffected. Calcium addition to the complex partially restored the exposure of Trp of melittin and caused changes in the environment of Tyr-16β (unlike the environmental changes induced for Tyr-16β by calcium in the absence of melittin). The conformational changes induced in S100b by interaction with melittin increased its affinity for calcium and offset the inhibition of calcium binding otherwise observed in the presence of potassium ions. This corroborated the previous finding that S100b affinity for calcium greatly depends on the protein conformation. The phenomena described above are similar to the interactions of melittin with calmodulin and thus suggest that S100b and calmodulin have a common structural domain not only that binds melittin but also that may interact with common target proteins. In support of this suggestion is our observation that S100b, as previously reported for calmodulin, binds to the microtubule-associated protein τ in the presence of calcium, a fact that could explain their common calcium-dependent effects on microtubule assembly. Complex formation of τ with S100b or calmodulin was confirmed when we discovered that both S100b and calmodulin inhibit the calcium/phospholipid-dependent phosphorylation of τ proteins by protein kinase C by interacting with the substrate rather than with the kinase itself.

S100b is a cytosolic, acidic protein that belongs to the calcium binding protein family of the "EF-hand" type (Isobe & Okuyama, 1978). While present mainly in glial cells of the central nervous system (Ghandour et al., 1981), it has also been found in several nonnervous tissues [for a review, see Molin et al. (1984)]. The biological function of S100b protein remains unproven, but it is clear that S100 proteins affect the in vitro assembly and disassembly of microtubules (Baudier et al., 1982; Donato, 1983; Endo & Hidaka, 1983), modulate the phosphorylation of a 19-kilodalton (19-kDa)[1] brain protein by protein kinase X (Qi & Kuo, 1984a,b), and inhibit the phosphorylation of a soluble 73-kDa brain protein (Patel et al., 1983).

Although S100 protein and calmodulin are clearly different proteins and have different distributions in the brain (Tabuchi et al., 1984), they are structurally related and share some physicochemical properties. They both bind to phenothiazine–Sepharose columns (Marshak et al., 1981) and to melittin–Sepharose columns (Kincaid & Coulson, 1985) in a calcium-dependent manner. Furthermore, the majority of brain proteins, including calcineurin, that bind to S100–Sepharose also bind to calmodulin–Sepharose and vice versa (Gopalakrishna et al., 1985), suggesting that both of the latter proteins might regulate some target proteins in common. For instance, a Ca^{2+}-dependent function mediated by calmodulin,

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[1] Abbreviations; Trp, tryptophan; Tyr, tyrosine; Cys, cysteine; FPLC, fast-protein liquid chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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the inhibition of microtubule assembly, has also been reported for S100 (Baudier et al., 1982; Donato, 1983; Endo & Hidaka, 1983), and calmodulin and S100 were both found to inhibit the calcium/phospholipid-dependent phosphorylation of an 87-kDa (Albert et al., 1984). To define further the similarities between S100b and calmodulin, their interactions with two other macromolecules were compared.

Complexes can be formed between calmodulin and melittin, a 26-residue polypeptide from bee venom, in the presence and absence of calcium (Comte et al., 1983; Maulet & Cox, 1983), and these complexes were reported to be good models in studies of calmodulin binding sites that are specific for target enzymes. Using melittin binding as a test for similarity between calmodulin and S100b, we demonstrated in the present work that mellitin does in fact interact with S100b protein as well as with calmodulin and that it probably does so in some ways similar to the ways it does with calmodulin. As a further and perhaps more physiologically relevant test for similarities between S100b and calmodulin, we used affinity columns of immobilized S100b or calmodulin to observe that both proteins interact with the microtubule-associated protein τ in a calcium-dependent manner, a fact that could explain their common calcium-dependent effects on microtubule assembly. Moreover, we discovered that in binding to τ, both S100b and calmodulin inhibit the phosphorylation of τ by the calcium/phospholipid-dependent protein kinase C. The phosphorylation of τ (Lindwall & Cole, 1984) has taken on new interest recently, because of a possible connection with Alzheimer's disease (Grundke-Iqbal et al., 1986).

Experimental Procedures

Materials. Bovine brain S100b was purified as previously described (Baudier et al., 1983a), and its concentration was measured by UV assuming an $\epsilon_{280nm} = 3400\ M^{-1}\ cm^{-1}$ (Baudier & Gerard, 1983). The protein was homogeneous in polyacrylamide and urea–SDS–polyacrylamide gel electrophoresis (Baudier et al., 1983b), in FPLC on a Mono Q column (Pharmacia) (Baudier et al., 1986), and by spectroscopic criteria. The purity of melittin (Serva) was checked by reverse-phase chromatography on a FPLC-ProRPC column (Pharmacia). Melittin eluted mainly under a single peak (280 nm), but there were minor contaminants (<10%). Chromatographically purified melittin showed no difference from the commercial melittin when their interactions with S100b protein were compared by means of fluorescence polarization. Therefore, all the experiments presented here were obtained with the commercial melittin. To remove contaminating divalent cations, both S100b and melittin solutions were mixed with Chelex 100 resin (Bio-Rad) and filtered on Millipore filters. Proteins were then dialyzed against working buffer prior to use.

H1 histones were purified from bovine kidney as described previously (Pehrson & Cole, 1981). Calmodulin was prepared from bovine brain by the method of Isobe et al. (1977). Semipure τ protein was obtained from bovine brain essentially by the method I of Lindwall and Cole (1984). τ proteins migrated as seven distinct protein bands when analyzed in 12% polyacrylamide–SDS gels and as five bands in 9% polyacrylamide–SDS gels. The τ protein preparation was contaminated mainly by a low molecular weight protein (see Results). The concentration of semipure τ protein was empirically determined by using the Coomassie blue technique (Bradford, 1976) with bovine serum albumin as standard. It should, however, be noted that using this technique for pure τ underestimates the true concentration (unpublished results). Protein kinase C was purified from rat brain (Kikkawa et al., 1982) with modifications as described (Mochly-Rosen & Koshland, 1987) and stored at −20 °C in 50% glycerol, 10 mM Tris, 0.5 mM EDTA, and 0.5 mM EGTA, pH 7.4. For some experiments, enzyme was diluted 2–40-fold as noted, in 20 mM Tris, 1 mM EDTA, and 1 mM EGTA, pH7.4, before use.

Methods. Fluorescence spectra were obtained on a Perkin-Elmer MPF-44A spectrofluorometer. Fluorescence polarization was measured on an SLM-8000 SC spectropolarimeter. The excitation wavelength was 296 nm, and the emission was measured at 342 ± 5 nm. Difference absorption spectra were obtained on a Cary 219 spectrophotometer. The auto base line was recorded with protein solutions at the same concentration in the reference and sample cells. To study the interaction between melittin and S100b in the absence of calcium, we used double-compartmentalized cells. The auto base line was recorded with separated proteins into both cells. For measurements, proteins in the sample cell were subsequently mixed.

Flow dialysis binding experiments were conducted as previously described (Baudier et al., 1986). Sulfhydryl measurements were done as previously described (Baudier & Gerard, 1983) assuming a molar extinction coefficient of 1.36×10^4 at 412 nm for 5-nitro-2-thiobenzoate.

S100b and calmodulin affinity chromtography columns were prepared as previously described by Lee and Wolff (1984). Ten milligrams of each protein was coupled to 1.5 g of cyanogen bromide activated Sepharose 4B (Pharmacia) in the presence of 1 mM $CaCl_2$. The yield of the coupling reaction was approximately 90% and 80% for S100b and calmodulin, respectively. The elution of τ protein from the affinity column was monitored by the Coomassie blue protein assay (Bradford, 1976). Forty microliters of each fraction was mixed with 1.5 mL of the Bradford reagent (Bio-Rad, 1:10 dilution) and the absorbance read at 595 nm.

Phosphorylation of τ by protein kinase C was as described for histone phosphorylation (Mochly-Rosen & Koshland, 1987). Briefly, protein kinase C (10 μL) was incubated with 10 μL of phospholipid mixture (containing 1.4 mM brain phosphatidylserine and 39 μM diolein in 20 mM Tris, pH 7.4) and 5 μL of 20 mM $CaCl_2$, or in the presence of 20 μL of 20 mM Tris, 1 mM EDTA, and 1 mM EGTA, pH 7.4, for 15 min at 30 °C. The reaction was quenched by addition of 20 μL of dye buffer (0.265 M Tris, 8.4% SDS, 42% glycerol, 0.008% bromophenol blue, and 20% β-mercaptoethanol, pH 6.8), and samples were analyzed by polyacrylamide gel electrophoresis (Ames, 1974) followed by autoradiography. Diolein was from Sigma and L-α-phosphatidylserine from Avanti Polar Lipids.

Results

S100b–Melittin Complex: Stoichiometry. A complex was formed between S100b and melittin in the absence and in the presence of Ca^{2+}. Ca^{2+}-independent interaction of S100 protein in solution with larger proteins has already been reported (Qi et al., 1984; Gopalakrishna et al., 1985; Van Eldik et al., 1985; Donato, 1985). The stoichiometry of the S100b–melittin soluble complex was estimated by fluorescence polarization of the single Trp residue present in melittin. The Trp fluorescence polarization of melittin in 20 mM Tris buffer ± 1 mM Ca^{2+}, $P = 0.07 \pm 0.005$, is similar to that found for the melittin monomer by Faucon et al. (1979) and is compatible with rapid motions of the small polypeptide. In the presence of equimolar concentrations of S100b (two Tyr, no Trp), the Trp fluorescence polarization of melittin increased considerably to $P = 0.21 \pm 0.005$. This change of P can be attributed to a large decrease of the rotation rate of the Trp

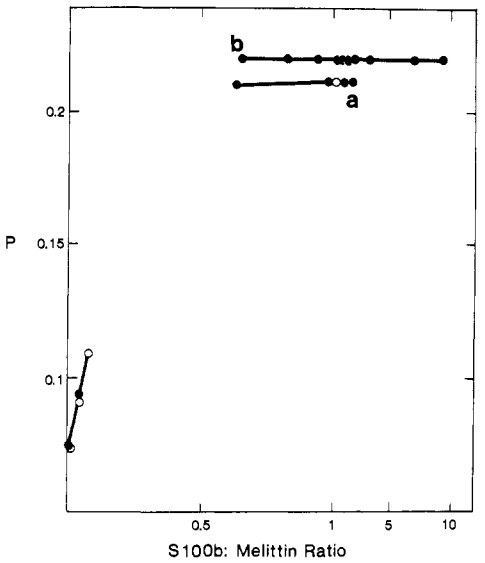

FIGURE 1: Degree of polarization (P) of melittin vs. the S100b:melittin molar ratio. λ_{exc} = 296 nm, λ_{em} = 345 ± 5 nm. The proteins were in 20 mM Tris buffer, pH 7.5, in the absence (●) or presence of 1 mM Ca^{2+} (○). (a) Increasing concentrations of S100b in 500 μL of buffer were added to a fixed concentration of melittin in 500 μL (final melittin concentration 13 μM). (b) Aliquots of concentrated melittin were added to S100b protein (final concentration 15 μM) in 1 mL of buffer. Experimental points where protein solutions showed abnormal light scattering or turbidity due to probable S100b denaturation are not reported.

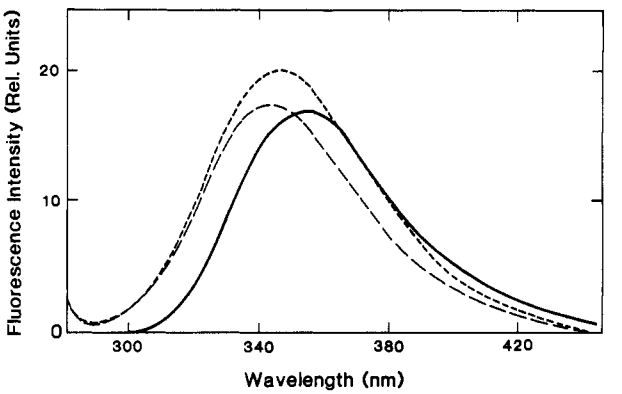

FIGURE 2: Emission fluorescence spectra of melittin. λ_{exc} = 296 nm. Melittin at a concentration of 29 μM was in 20 mM Tris buffer, pH 7.5 (solid line), plus 30 μM S100b (dashed line), or plus 30 μM S100b and 1 mM Ca^{2+} (dotted line).

residue in melittin. Since it occurred at constant viscosity and temperature, it has to be related to formation of a complex between melittin and S100b. Mellitin is so tightly bound to S100b protein that its only motions probably correspond to the overall motions of the complex. This is compatible with the fact that the Trp fluorescence polarization value of the complex, P = 0.21, is higher than that found for the single Trp (90α) residue in the homologous Trp-containing S100a ($\alpha\beta$) protein dimer (P = 0.16) (Baudier & Gerard, 1986).

Since melittin is an amphipathic peptide, we studied the changes in Trp fluorescence polarization in titration experiments in a wide range of S100b:melittin ratios. Two different titrations were performed (Figure 1). First, as increasing amounts of S100b in 500 μL 20 mM Tris buffer, pH 7.5, were mixed with a fixed concentration (27 μM) of melittin in 500 μL of 20 mM Tris buffer, pH 7.5, in the absence or presence of 1 mM Ca^{2+}, we observed rapid increases of Trp fluorescence polarization which reached a plateau near an S100b:melittin molar ratio of 0.5. This suggests that S100b and melittin form a 1:2 complex of high affinity. However, in the case of S100b:melittin ratios between 0.1 and 0.5, samples were slightly turbid, and the exact shapes of the curves cannot be observed reliably. This formation of turbidity with excess melittin resembles the situation when melittin binds to calmodulin in the absence of calcium (Maulet & Cox, 1983). Perhaps high ratios of melittin to S100b resulted in intermolecular cross-linking to produce large insoluble complexes. When titration experiments were performed by adding aliquots of concentrated melittin solution (100 μM in 20 mM Tris buffer, pH 7.5) to S100b at a concentration of 13 μM, melittin binding to S100b proved (Figure 1) to be stoichiometric for the first melittin bound per S100b (S100b:melittin ratio = 1) so that the dissociation constant of the interaction is evidently in the micromolar range or lower. Although for the binding of the first melittin to S100b no turbidity could be observed, with a lower S100b:melittin ratio we observed an increase of the excitation light scattering and the sample became turbid at a S100b:melittin molar ratio of 0.5. Light scattering was also observed at a S100b:melittin molar ratio of 0.5 when the interaction was studied by means of UV absorption spectroscopy (see below). There was no difference in the results whether Ca^{2+} (1 mM) was present or not. Therefore, all the following experiments were performed at a S100b:melittin molar ratio of 1.

S100b–Melittin Interaction: Conformational Changes. The fluorescence of Trp revealed that complex formation with S100b caused conformational changes in melittin that were different in the presence of calcium than they were in its absence. At an excitation wavelength of 295 nm, Trp is selectively excited (Figure 2). As previously reported (Quay & Condie, 1983), the maximum of fluorescence at 353 ± 0.5 nm indicates that the melittin monomer in solution has its unique Trp residue fully exposed to the aqueous environment. In the presence of equimolar concentrations of S100b and the absence of calcium, we observed a blue shift in the maximum of fluorescence to 344 ± 0.5 nm. Since S100b has no Trp, this shift indicates that Trp of melittin moved to a less polar environment upon interaction with S100b protein. The total fluorescence intensity was not markedly changed upon complex formation, but the Trp fluorescence quantum yield decreased slightly since the interaction of melittin with S100b resulted in an increase in absorbance at 295 nm (see below). In the presence of 1 mM Ca^{2+}, the maximum of fluorescence was shifted back to 346 ± 0.5 nm, indicating that Ca^{2+}-induced conformational changes in the S100b–melittin complex partially restored the exposure of the Trp of melittin to solvent. This partial restoration still left the Trp in a less polar environment than it had in uncomplexed melittin. Trp fluorescence quantum yield significantly increased as suggested by the enhancement of the fluorescence intensity associated with a decrease in Trp absorbance at 295 nm (see below).

The conclusions stated above were confirmed by UV difference spectra which also revealed effects of conformational changes on Tyr and Phe. Figure 3 shows the difference absorption spectrum of an equimolar mixture of S100b and melittin measured against separated proteins. The positive peak at 293 nm can be attributed to the Trp residue of melittin which moved to a less polar environment upon complex formation with S100b, in agreement with the blue shift observed in the fluorescence maximum (Figure 2). Assuming that at 293 nm S100b–Tyr absorption did not contribute to the ΔOD, we estimated an $\Delta\epsilon_{293}$ of 230 ± 20 M^{-1} cm^{-1} for melittin upon complex formation with S100b. In the absence of calcium, there were only a few changes in the Tyr and in the absorption regions below 270 nm. This means that the formation of the

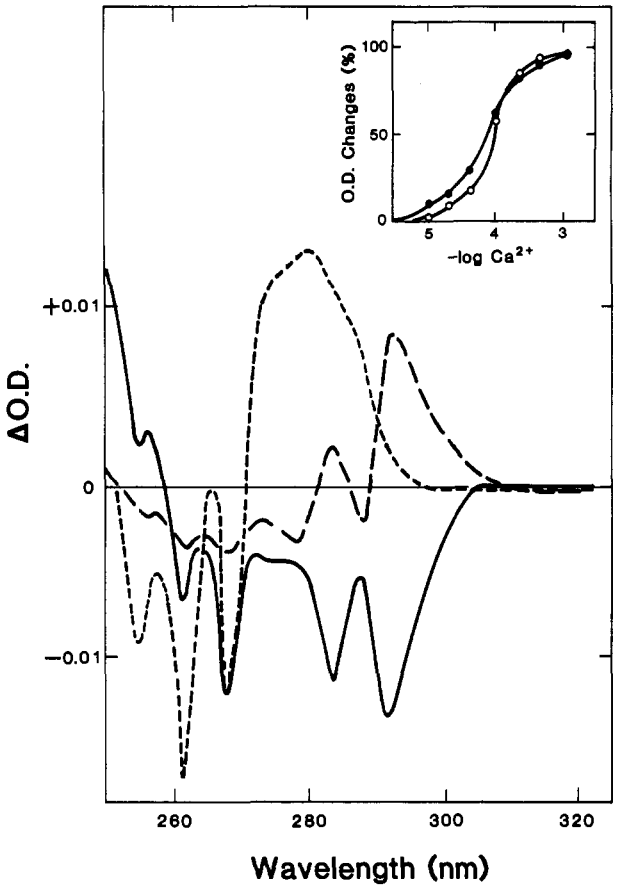

FIGURE 3: UV difference absorption spectra of melittin–S100b complex (see Methods and text for details). Buffer was 20 mM Tris-HCl, pH 7.5. (Dashed line) Mixture of 36 μM melittin and 40 μM S100b; (solid line) mixture of 60 μM melittin and 65 μM S100b plus 1 mM Ca^{2+}; (dotted line) S100b at a concentration of 61 μM plus 1 mM Ca^{2+}. Inset shows the percentage changes in ΔOD at 293 nm (●) and 269 nm (○) of the S100b–melittin complex upon calcium binding.

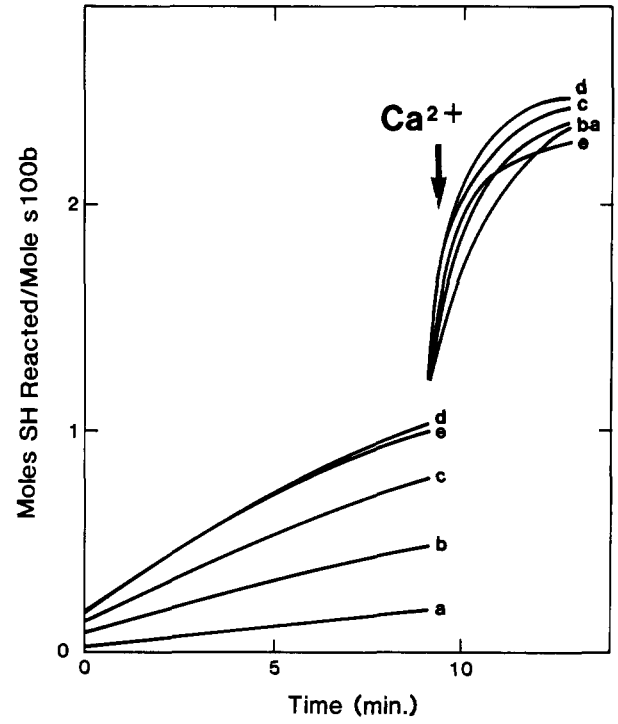

FIGURE 4: Reactivity of thiols in S100b protein toward DTNB. Buffer was 20 mM Tris, pH 7.5 (Curve a) S100b at a concentration of 22 μM; (curve b) plus 8 μM melittin; (curve c) plus 15 μM melittin; (curve d) plus 24 μM melittin; (curve e) plus 32 μM melittin. At the arrow, 1 mM^{2+} was added to the protein mixtures.

complex in the absence of Ca^{2+} did not induce important structural modifications in the environment of the tyrosines (16β) and phenylalanines in S100b. Figure 3 also compares the effect of Ca^{2+} on the UV difference spectrum of S100b protein in the absence and in the presence of equimolar concentrations of melittin. In the latter experiment, the control base line was recorded after the proteins were mixed in the absence of calcium in both reference and sample cells; calcium was added to the sample cells subsequently. (Note that the protein concentrations used in this experiment were twice those used for the melittin–S100b interactions studied in the absence of Ca^{2+}.) The difference spectrum for purified S100b in the presence of calcium has already been described (Baudier & Gerard, 1983). The difference spectrum of the 1:1 S100b–melittin mixture is characterized by a negative absorption peak at 293 nm, which can be interpreted as a partial restoration in the exposure of the Trp of melittin to the aqueous solvent when calcium binds to the S100b–melittin complex. The Tyr environment in S100b is also affected when Ca^{2+} binds to the complex but in a completely different way from the observed with S100b in the absence of melittin. A negative peak at 284 nm characterizes the calcium-saturated S100b–melittin complex, whereas a positive difference absorption was observed for the binding of calcium to S100b in the absence of melittin. The negative bands between 275 and 250 nm correspond to a higher exposure of the S100b–Phe residues to the solvent as also noticed for the protein in the absence of melittin. Figure 3 (inset) presents the calcium titration curves for the percentage change in ΔOD_{293nm} and ΔOD_{269nm} of the S100b–melittin complex. The midtransition point is $\sim 7 \times 10^{-5}$ M Ca^{2+}. The exposure of mellitin–Trp to solvent apparently paralleled the S100b–Phe absorption change. Note that in the absence of melittin the midtransition point for the exposure of S100b–Phe to solvent (ΔOD_{262nm}) is for 2×10^{-4} M (Baudier et al., 1986), so that S100b affinity for calcium seemed to increase upon complex formation with melittin (see below).

Titration of SH groups with DTNB was useful for monitoring Ca^{2+}-induced conformational changes in S100b (Baudier & Gerard, 1983). Therefore, we used this method to monitor melittin-induced conformational changes in S100b. A constant DTNB:S100b molar ratio, i.e., 30, was used since accessibility of SH groups to reagent is diffusion controlled. The results presented in Figure 4 indicate that melittin binding induces conformational changes in S100b, resulting in the enhancement of the reaction between DTNB and SH groups, amounting to one SH titrated after 9-min reaction. This effect was maximal for one melittin added per mole of S100b. Since after addition of calcium to the S100b–melittin complex only two SH groups reacted with DTNB as was the case for S100b in the absence of melittin, one may suppose that it is also Cys-84β in S100b which is more accessible to DTNB after complex formation between S100b and melittin.

S100b–Melittin Interaction: Effects on Calcium Binding. The effect of melittin on the binding of Ca^{2+} to S100b was studied by flow dialysis in 20 mM Tris buffer, pH 7.5, plus 50 mM KCl. Figure 5 shows that melittin, at equimolar concentration with S100b, enhanced the affinity of the S100b protein for calcium, the effect being particularly pronounced for the first two Ca^{2+} bound per mole of S100b protein. In the presence of 10^{-4} M free calcium, two Ca^{2+} bound to the S100b–melittin complex, whereas only 0.5 bound to S100b in the absence of melittin. We estimated the K_D's for the binding of the first two Ca^{2+} as approximately 20 and 200 μM.

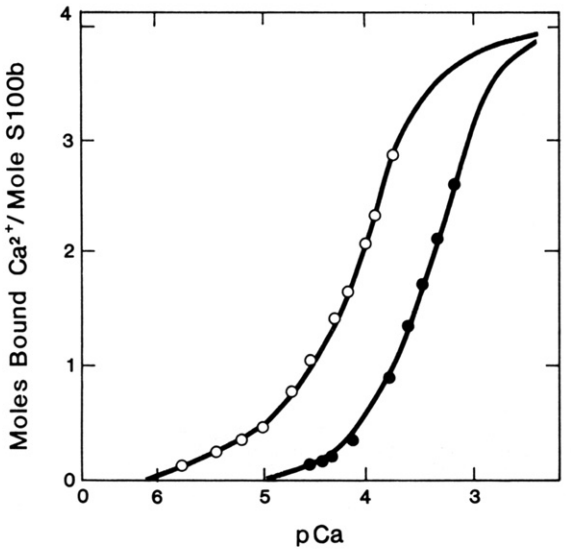

FIGURE 5: Ca^{2+} binding to S100b in the presence of melittin as determined by flow dialysis binding. The buffer used was 20 mM Tris, pH 7.5, plus 50 mM KCl. (●) S100b at a concentration of 50 μM; (○) plus 50 μM melittin. The binding curves were fitted to the experimental binding data assuming that at the maximum four Ca^{2+} bound per mole of S100b protein dimer.

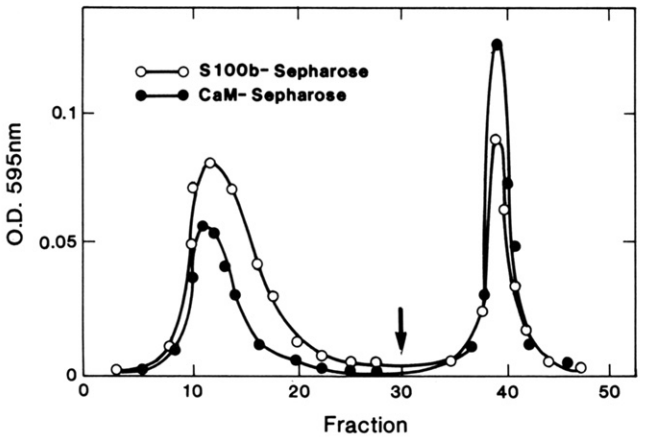

FIGURE 6: Affinity chromatography of τ protein on S100b and calmodulin–Sepharose 4B columns. The columns were first washed with 50 μM Tris buffer, pH 7.3, and 0.2 mM DTE (buffer A) containing 1 mM $CaCl_2$ and then equilibrated with buffer A containing 100 mM NaCl and 0.5 mM $CaCl_2$. Three milliliters of semipurified τ protein solution at 0.4 mg/mL in equilibrating buffer was applied to S100b or a calmodulin–Sepharose 4B column (1 × 5 cm). The columns were washed with equilibrating buffer, and then (see arrow) the τ protein eluted with buffer A containing 3 mM EGTA and 100 mM NaCl. Fractions (1 mL) were collected, and the elution profiles [(○) S100b column; (●) calmodulin column] were determined with Coomassie blue.

S100b and Calmodulin Bind to τ Protein. S100b or calmodulin binding to microtubule-associated τ proteins was studied by using affinity chromatography. The S100b– or calmodulin–Sepharose columns were first washed with buffer A containing 1 mM $CaCl_2$ but no NaCl and then equilibrated with buffer A containing 100 mM NaCl and 0.5 mM $CaCl_2$. Purified τ proteins were applied and eluted with 8 column volumes of equilibrating buffer and then with buffer A containing 3 mM EGTA and 100 mM NaCl. Figure 6 shows the profile of the proteins eluted from both columns. SDS gel electrophoresis of the protein peaks (Figure 7) showed that the τ proteins were retained on both S100b and calmodulin columns in the presence of Ca^{2+} and eluted with EGTA buffer. A major protein contaminant of low molecular weight (Figure 7, arrow) was retained on the calmodulin–Sepharose column

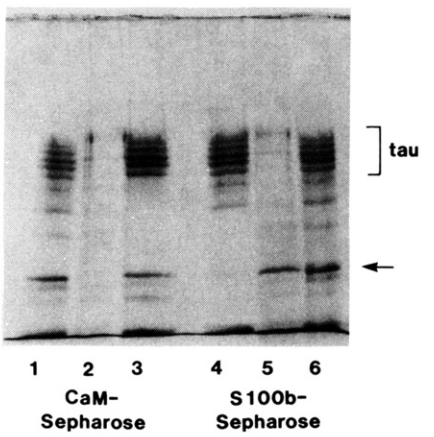

FIGURE 7: Electrophoretic analysis of proteins isolated from S100b and calmodulin–Sepharose 4B columns. Electrophoresis was performed in 0.1% SDS–9% polyacrylamide. Lanes 1 and 6, starting material, semipurified τ protein; lanes 2 and 5, fraction 12 from calmodulin and S100b–Sepharose columns, respectively; lanes 3 and 4, fraction 39 from calmodulin and S100b–Sepharose columns. Arrow indicates major contaminant.

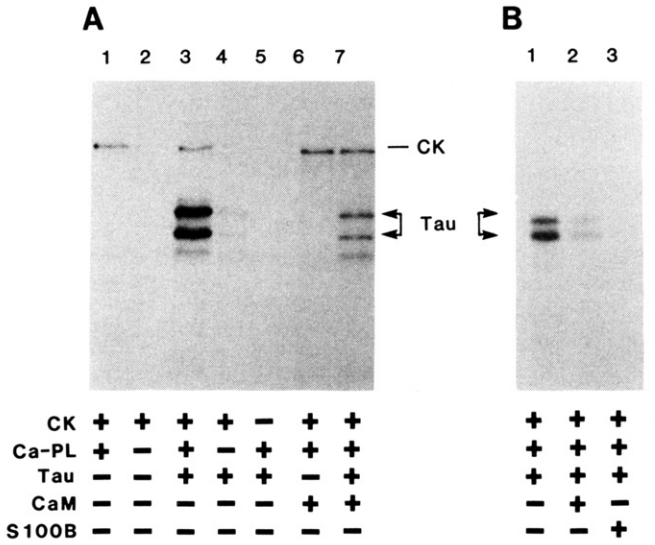

FIGURE 8: Ca^{2+}/phospholipid-dependent phosphorylation of τ I and τ III protein and its inhibition by calmodulin and S100b. Each lane of these autoradiograms represents a reaction mixture of protein kinase C in 20 mM Tris, pH 7.4, with the additions indicated below the autoradiograms. The additions were in the following final concentrations: total Ca^{2+}, 1.2 mM; phospholipids, 246 μM phosphatidylserine and 7 μM diolein; τ, 2 μM; calmodulin, 15 μM; S100b, 10 μM. In (A), protein kinase C diluted 2-fold was incubated for 15 at 30 °C; in (B), protein kinase C diluted 10-fold was incubated 5 min at 30 °C.

in the presence of Ca^{2+} but was unretained on the S100b column.

S100b and Calmodulin Inhibit τ Protein Phosphorylation by Protein Kinase C. In the course of our studies on the interactions between S100b, calmodulin, and τ protein, we observed that τ protein is an in vitro substrate for protein kinase C. Since S100 protein and calmodulin were previously found to inhibit the Ca^{2+} phospholipid-dependent phosphorylation of an endogenous substrate, called the 87-kDa protein (Albert el al., 1984), we investigated the effect of S100b and calmodulin on the phosphorylation of τ protein by protein kinase C. In this study, we used a purified τ protein preparation that mainly contained two τ protein species we called τ I and τ III (the preparation method will be described elsewhere). When purified τ I and τ III were incubated with protein kinase C, both τ proteins were phosphorylated to the

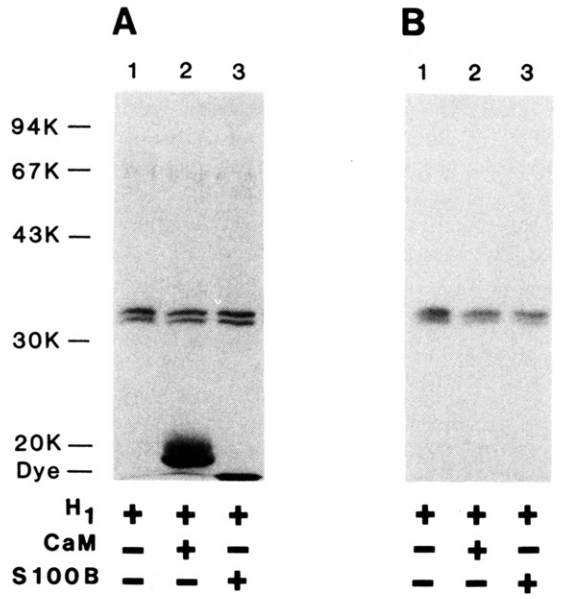

FIGURE 9: Effects of calmodulin and S100b on H1 histone phosphorylation by protein kinase C. The experimental conditions are similar to those in Figure 8B with H1 histone (2 μM) used as substrate for protein kinase C. (A) Coomassie blue stained gel; (B) autoradiogram. Molecular weight markers (in kilodaltons) are on the left margin.

same extent (see Figure 8A). As expected, this phosphorylation was almost completely dependent on the presence of calcium and phospholipid. No phosphorylation occurred in the absence of protein kinase C, indicating that the τ preparation did not contain contaminating kinase activities. Sufficient amounts of protein kinase C were used to allow observation of enzyme autophosphorylation (Figure 8A, arrow). The effect of calmodulin on the phosphorylation of τ protein is shown in Figure 8A,B. Calmodulin inhibited τ phosphorylation by ~80% but had no effect on protein kinase C autophosphorylation (compare Figure 8A, lanes 1 and 6). The ability of calmodulin to inhibit the Ca^{2+}/phospholipid-dependent phosphorylation of τ may then be compared with that of S100b protein (Figure 8B). Under the conditions used, S100b protein inhibited τ phosphorylation by nearly 100%. As with calmodulin, S100b had no effect on protein kinase C autophosphorylation (not shown).

Finally, we studied the effect of calmodulin and S100b on the phosphorylation of H1 histone, an effective substrate for kinase C (Iwasa et al., 1980). In the presence of excess calmodulin and S100b compared to H1 histone concentration, no significiant inhibition could be observed (Figure 9A,B). This last result agreed with the results obtained by McDonald and Walsh (1985) on the lack of effect of calmodulin and S100 protein on HIII histone phosphorylation by protein kinase C.

Discussion

Interactions of S100b with Melittin. It was reported recently that in the presence of calcium, S100 protein binds to melittin immobilized on Sepharose (Kincaid & Coulson, 1985). In the absence of calcium, melittin in solution formed a high-affinity complex (K_D in the micromolar range) with S100b, and this is comparable to that found with calmodulin (Comte et al., 1983; Maulet & Cox, 1983). An apparent stoichiometry of two melittins per S100b protein dimer characterized the soluble complex, but an increase in light scattering of the protein mixture for S100b:melittin molar ratios lower than 0.5 indicated that additional binding caused the formation of an insoluble complex. Again, this behavior was like that observed with calmodulin–melittin complexes (Maulet & Cox, 1983). The Ca^{2+} dependence of S100b binding to the mellitin–Sepharose column seems to be explained by a calcium-induced increase in the affinity of the complex, and this, too, parallels the corresponding case for calmodulin (Maulet & Cox, 1983).

Upon equimolar complex formation between S100b and melittin, spectroscopic studies proved that in the presence or absence of calcium, the Trp of melittin is placed in a hydrophobic environment with a maximum fluorescence emission shifted from 353 nm to 346 or 344 nm, respectively. Such a shift has also been observed when melittin interacts with calmodulin (Maulet & Cox, 1983). Moreover, the decrease in exposure of Trp in melittin when it interacts with calmodulin was confirmed by NMR studies (Seeholzer et al., 1986). It is intriguing that the changes in the fluorescence maximum of Trp upon complex formation between melittin and proteins are similar to those found for melittin when it forms tetramers (Quay & Condie, 1983). In the latter case, the Trp fluorescence maximum shift was correlated with the enhancement of the α-helical content of melittin tetramer (Bello et al., 1982). If the Trp fluorescence maximum shift caused by complex formation between S100b and melittin likewise represents an enhancement of α-helical content of melittin, this would be another similarity between S100b and calmodulin, since interactions between melittin and calmodulin result in the enhancement of the α-helical content of melittin (Maulet & Cox, 1983).

In the absence of Ca^{2+}, the increase of reactivity of S100b–Cys-84β with DTNB upon complex formation with melittin indicates that melittin-induced conformational changes in S100b involve mainly the C-terminal part of the β chains, whereas the N-terminal part is apparently not affected, since no change attributable to the Try-16β of S100b was observed in the UV difference spectrum. In the presence of calcium, the S100b–melittin complex underwent conformational changes that perturb both S100b and melittin conformations. At the level of Try-16β of S100b, these changes differ from those induced by calcium with S100b alone, but they were similar in the case of Cys-84β and Phe residues which become more exposed to the solvent.

The observed increase in S100b affinity for calcium in the presence of melittin, which has again also been reported for calmodulin (Maulet & Cox, 1983), can be explained by conformational changes in S100b structure upon complex formation with melittin. In S100b, it is the calcium binding site IIβ that is antagonized by KCl and is strongly dependent on protein conformation (Baudier et al., 1986; Baudier & Gerard, 1986). This has been confirmed by ^1H NMR experiments and Tb^{3+} binding studies (unpublished results). Since melittin interactions with S100b modify preferentially the C-terminal part of the β chains where site IIβ is located, one may suppose that it is mainly the affinity of site IIβ for calcium that increases upon formation of the complex.

The overall similarity between the fluorescence properties of melittin in S100b and calmodulin complexes and the physicochemical properties of both complexes indicates that melittin may interact in a similar way with both proteins. It was suggested by others (Comte et al., 1983; Maulet & Cox, 1983) that the site on calmodulin where melittin binds is the same site to which target enzymes are bound. This site in calmodulin also binds such hydrophobic drugs as phenothiazine (Levin & Weiss, 1978), and it is significant that S100 also binds phenothiazine (Marshak et al., 1985; Donato, 1984). It is reasonable, then, to postulate that the melittin binding

domain on S100b might be more or less structurally related to the corresponding domain on calmodulin. The possibility of physiological significance in the analogous binding domains of S100 and calmodulin gains support from their common binding of proteins of more general occurrence than the bee venom polypeptide melittin. Along with the binding of other brain proteins, our discovery of the binding of τ by both S100b and calmodulin provides such support.

Interactions of S100b and Calmodulin with Purified τ Proteins. In 1972, Weisenberg reported that exclusion of Ca^{2+} was necessary for in vitro polymerization of microtubules from brain microtubule proteins and suggested that Ca^{2+} may play an important role in regulating microtubule assembly (Weisenberg, 1972). Nishida (1977) subsequently reported that Ca^{2+} sensitivity of microtubule assembly in vitro was modulated by a Ca^{2+}-sensitizing factor, and calmodulin has been suggested to have this role (Marcum et al., 1978; Nishida et al., 1979). Lee and Wolff recently demonstrated that calmodulin binds to both microtubule-associated proteins MAP_2 and τ in a Ca^{2+}-dependent manner and that this interaction was inhibited by trifluoroperazine (Lee & Wolff, 1984). These authors suggested that calmodulin exerts its effect on microtubule assembly through the formation of a Ca^{2+}–calmodulin–MAP_2 complex or the corresponding τ complex. An interaction between Ca^{2+}–calmodulin and τ proteins was directly confirmed here using purified τ proteins.

Recently, we reported that S100 protein, like calmodulin, induced total disassembly of microtubules at multimolar Ca^{2+} concentrations and did so with even higher efficiency than did calmodulin (Baudier et al., 1982). Subsequently, Endo and Hidaka reported inhibition of microtubule assembly by S100 protein in a dose-dependent manner in the presence of 20 μM Ca^{2+} (Endo & Hidaka, 1983). These authors also demonstrated by affinity chromatography that phosphocellulose-purified tubulin failed to bind to S100–Sepharose columns whether Ca^{2+} was present or not, suggesting that S100 protein might bind to microtubule components other than tubulin. The present experiments showed that S100b immobilized on Sepharose interacts in a strictly Ca^{2+}-dependent manner with τ proteins, as does calmodulin. This result indicates that S100b might also exert its effect on microtuble assembly through the formation of a Ca^{2+}–S100b–τ complex, analogous to the complex suggested for calmodulin (Lee & Wolff, 1984). S100b protein in solution would have only two calcium binding sites saturated in the conditions used in our experiment (100 mM NaCl–500 μM Ca^{2+}), of the four present on the protein dimer (Baudier et al., 1986a). This might indicate that the S100b–$2Ca^{2+}$ complex is sufficient for the interaction of S100b protein with τ proteins. However, it is also possible that coupling S100b protein to cyanogen bromide–Sepharose in its calcium-bound state induced irreversible conformational changes that increased the overall affinity of the protein for calcium. Alternatively, it is also possible that low-affinity interactions between S100b protein and τ proteins led to conformational changes in S100b protein, similar to those observed when it interacts with melittin, which would in turn increase its Ca^{2+} affinity.

The observation that S100b and calmodulin inhibited phosphorylation of τ by the Ca^{2+}/phospholipid-dependent protein kinase C confirmed the interaction of these two calcium binding proteins with τ. The observed inhibition evidently involved the interaction of S100b and calmodulin with the substrate τ rather than with the kinase itself since calmodulin and S100b inhibited neither the autophosphorylation of kinase C nor the phosphorylation of H1 histone, an effective substrate for kinase C. To learn whether or not S100 or calmodulin functions in a regulatory role in vivo in τ phosphorylation (Lindwall & Cole, 1984) in the normal state or in pathological conditions such as Alzheimer's disease (Grundke-Iqbal et al., 1986) will require further work, but the similarities in binding properties of the two proteins make it reasonable to search for clues to the natural function of S100, the abundant brain, calcium-binding protein, among the functions of calmodulin as the latter are progressively delineated.

Registry No. Calcium, 7440-70-2; protein kinase C, 9026-43-1; melittin, 37231-28-0.

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Immunoaffinity Purification and Fluorescence Studies of Human Adenosine Deaminase†

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ABSTRACT: Human thymus adenosine deaminase was isolated by using a monoclonal antibody affinity column. The highly purified enzyme produced by this rapid, efficient procedure had a molecular weight of 44 000. Quenching of the intrinsic protein fluorescence by small molecules was used to probe the accessibility of tryptophan residues in the enzyme and enzyme–inhibitor complexes. The fluorescence emission spectrum of human adenosine deaminase at 295-nm excitation had a maximum at about 335 nm and a quantum yield of 0.03. Addition of polar fluorescence quenchers, iodide and acrylamide, shifted the peak to the blue, and the hydrophobic quencher trichloroethanol shifted the peak to the red, indicating that the emission spectrum is heterogeneous. The fluorescence quenching parameters obtained for these quenchers reveal that the tryptophan environments in the protein are relatively hydrophobic. Binding of both ground-state and transition-state analogue inhibitors caused decreases in the fluorescence intensity of the enzyme, suggesting that one or more tryptophans may be near the active site. The kinetics of the fluorescence decrease were consistent with a slow conformational alteration in the transition-state inhibitor complexes. Fluorescence quenching experiments using polar and nonpolar quenchers were also carried out for the enzyme–inhibitor complexes. The quenching parameters for all enzyme–inhibitor complexes differed from those for the uncomplexed enzyme, suggesting that inhibitor binding causes changes in the conformation of adenosine deaminase. For comparison, parallel quenching studies were performed for calf adenosine deaminase in the absence and presence of inhibitors. While significant structural differences between adenosine deaminase from the two sources were evident, our data indicate that both enzymes undergo conformational changes on binding ground-state and transition-state inhibitors.

Adenosine deaminase (EC 3.5.4.4), a purine salvage pathway enzyme, catalyzes the deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. The enzyme is widely distributed in nature, and the highest activity in man is found in lymphoid tissues (Brady & O'Donovan, 1965). Adenosine deaminase is necessary for a functional immune system. An inherited deficiency of this enzyme in its most extreme form is associated with severe combined immunodeficiency disease (SCID;[1] Giblett et al., 1972). SCID is typically associated with severe lymphopenia as well as

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[1] Abbreviations: ADA, adenosine deaminase; ELISA, enzyme-linked immunoabsorbant assay; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; DHMPR, 1,6-dihydro-6-(hydroxymethyl)purine riboside; PAS, protein A–Sepharose CL-4B; PBS, phosphate-buffered saline; SCID, severe combined immunodeficiency disease; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

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