Characterization of a calsequestrin-like protein from sea-urchin eggs

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Following our studies on the identification of a calsequestrin-like protein (CSLP) from sea-urchin eggs [Oberdorf, Lebeche, Head & Kaminer (1988) *J. Biol. Chem.* **263**, 6806–6809], we have characterized its Ca^{2+} -binding properties and identified it as a glycoprotein. The molecule binds 23 mol of Ca^{2+} /mol of protein, as determined by equilibrium dialysis. This is in the range reported for cardiac calsequestrin but is about half the binding capacity of striated muscle calsequestrin. The affinities of the CSLP for Ca^{2+} are decreased by increasing KCl concentrations (20–250 mM) and the presence of Mg^{2+} (3 mM) in the medium: the half-maximal binding values varied from 1.62 to 5.77 mM. Hill coefficients indicated mild co-operativity in the Ca^{2+} binding. Ca^{2+} (1–8 mM)-induced u.v. difference spectra and intrinsic fluorescence changes suggest a net exposure of aromatic residues to an aqueous environment. C.d. measurements showed minor Ca^{2+} -induced changes in α -helical and β -sheet content of less than 10%. These spectral changes are distinctly different from those found in muscle calsequestrin. Immunoblotting studies showed that the CSLP is distinct from calreticulin, a low-affinity Ca^{2+} -binding protein.

INTRODUCTION

At fertilization, a measurable increase in the concentration of cytoplasmic Ca^{2+} in the egg (Steinhardt *et al.*, 1977; Gilkey *et al.*, 1978; Eisen *et al.*, 1984; Poenie *et al.*, 1985) is probably due, at least in part, to release of Ca^{2+} from an internal store, the endoplasmic reticulum (ER) (Jaffe, 1983). A number of studies implicate this organelle as a cytoplasmic Ca^{2+} regulator. Microsomes derived from the ER of sea-urchin eggs take up Ca^{2+} in an ATP-dependent manner (Inoue & Yoshioka, 1982; Oberdorf *et al.*, 1986) and release it on exposure to InsP_3 (Clapper & Lee, 1985) or cyclic ADP-ribose (Dargee *et al.*, 1990). ER containing cortical lawn preparations also release Ca^{2+} on exposure to InsP_3 (Oberdorf *et al.*, 1986; Payan *et al.*, 1986). InsP_3 , which also releases Ca^{2+} in intact sea-urchin eggs (Whitaker & Irvine, 1984; Turner *et al.*, 1987), was known earlier to induce Ca^{2+} release (Berridge & Irvine, 1984) from the ER of a number of cell types (Streb *et al.*, 1984; Berridge, 1987).

Little is known on the protein constituents of the ER of the egg involved in Ca^{2+} uptake, storage and release, in contrast with the sarcoplasmic reticulum (SR), a specialized form of the ER in muscle. In the muscle organelle a number of proteins have been well characterized, as for example the Ca^{2+} / Mg^{2+} ATPase (MacLennan *et al.*, 1985), which pumps Ca^{2+} into the SR (Ebashi *et al.*, 1969), calsequestrin, which presumably stores the Ca^{2+} in striated (MacLennan *et al.*, 1983; Fliegel *et al.*, 1987) and cardiac muscle (Mitchell *et al.*, 1988), and the Ca^{2+} -releasing channel which has been defined as a ryanodine receptor (Imagawa *et al.*, 1987; Lai *et al.*, 1989; Takeshima *et al.*, 1989; Wengneknecht *et al.*, 1989). We have purified and partially characterized a calsequestrin-like protein (CSLP) from the sea-urchin egg (Oberdorf *et al.*, 1988). It has an apparent molecular mass of 58 kDa and resembles muscle calsequestrin in its metachromatic blue staining with 'Stains-all' of electrophoretic gels, amino acid composition and Ca^{2+} binding. It also cross-reacts with cardiac calsequestrin antibody. Antibodies against CSLP, however, do not cross-react with calsequestrin from skeletal and cardiac

muscle. We subsequently showed by immunolocalization that the CSLP is present in the ER of the egg and gets redistributed during mitosis and cell division in the first cell-cycle embryo (Henson *et al.*, 1989). A dynamic distribution of the ER was also observed by immunolocalization of the CSLP during oogenesis (Henson *et al.*, 1990). CSLPs have apparently also been identified in nervous tissue (Choi & Clegg, 1990; Villa *et al.*, 1990), in plant cells (Krause *et al.*, 1989), in liver (Damiani *et al.*, 1988, 1989) and in two mammalian cell lines, pancreas and liver (Volpe *et al.*, 1988; Hashimoto *et al.*, 1988). However, other studies suggest that the protein expressed in liver corresponds to calreticulin (Treves *et al.*, 1990).

In the present study we report on quantification of Ca^{2+} binding by this CSLP and the associated conformational changes as determined by changes in u.v. difference spectra, intrinsic fluorescence and c.d. These changes are distinctly different from those reported for muscle calsequestrin. We have also identified the presence of two sugar moieties in the molecule. Attention will be drawn to similarities and differences between the CSLP and muscle calsequestrin on the one hand and calreticulin, a presumed Ca^{2+} -storage protein in non-muscle cells, on the other.

MATERIALS AND METHODS

Preparation of microsomal fractions

Sea-urchins (*Strongylocentrotus droebachiensis*) were obtained from Ocean Resources, Peaks Island, ME, U.S.A. Microsomal fractions from homogenized eggs were prepared and stored at -70°C until use, as previously described (Oberdorf *et al.*, 1986).

Purification of egg calsequestrin

Proteins were extracted from microsomal preparations in a buffer solution [10 mM-Tris/HCl, pH 8.5, 50 mM-KCl, 1 mM-phenylmethanesulphonyl fluoride (PMSF), 1 mM-dithiothreitol and 0.5% Nonidet P40]. After centrifugation at 100000 *g*, the supernatant was purified sequentially by DEAE-cellulose (DE-52) and hydroxyapatite chromatography in the presence of 0.1%

Abbreviations used: CSLP, calsequestrin-like protein; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; DMSO, dimethyl sulphoxide; PMSF, phenylmethanesulphonyl fluoride; BCA, bicinchoninic acid.

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Nonidet P40 as previously described (Oberdorf *et al.*, 1988). In some instances hydrogenated Triton X-100 was used because it has a markedly decreased absorption in the u.v. compared with Nonidet P40. Without detergents, the yield of protein from the columns was considerably decreased.

Ca²⁺ binding measured by equilibrium dialysis

Spectropor semimicro dialysis tubing (4 mm, 12–14 kDa molecular-mass cut-off) was sequentially boiled in glass-distilled water, in 0.4% NaHCO₃, and in 5 mM-EDTA, pH 7.0, and then stored in 5 mM-EDTA containing 0.2% NaN₃ at 4 °C. Before use, the tubing was extensively washed with deionized glass-distilled water. Protein samples (at approx. 0.5–0.56 mg/ml) were decalcified by first dialysing against 2 l of buffer (20 mM-Mops, pH 7.0, 1 mM-EGTA, 20–250 mM-KCl) for 36–48 h (changing the solution three to four times) and then against 2 l of the same buffer without EGTA for 24–36 h (changing the solution two to three times) at 4 °C to remove the EGTA. The protein samples (160 µl) were then dialysed at 4 °C for 24 h in capped polyethylene scintillation vials against 20 ml of the following equilibrium-dialysis solutions: 20 mM-Mops, pH 7.0, and various concentrations of KCl (20–25 mM), in the presence or absence of 3 mM-MgCl₂. These solutions contained, in addition, various concentrations of ⁴⁵Ca²⁺ at constant specific radioactivity (486 c.p.m./nmol) (Mitchell *et al.*, 1988). Additional blank samples (without protein) were dialysed to check when equilibrium had been reached. When equilibrium had been reached, duplicate 30 µl samples were taken (and weighed for accuracy) from both inside and outside of the dialysis bags, and radioactivity was measured with a liquid-scintillation counter (Packard Tri-Carb). For each set of conditions, two to three samples were measured in duplicate on two different protein preparations. Protein determinations were performed on samples, in duplicate, using the bicinchoninic acid (BCA) method (Smith *et al.*, 1985).

U.v.-absorption difference spectroscopy

Before spectrophotometric measurements, protein samples (0.5 mg/ml) were dialysed against 2 l of a buffer containing 20 mM-Mops, 100 mM-KCl and 0.1 mM-EGTA, pH 7.0, at 4 °C for 24–36 h, with two to three changes of solution. After dialysis, the samples were clarified by centrifugation for 10 min at 16000 g in an Eppendorf microcentrifuge (model 5415). U.v.-absorption spectra of the protein solutions were scanned between 310 and 250 nm with a Perkin-Elmer Lambda 2 u.v./visible dual-beam spectrophotometer, using matched 10 mm light-path quartz cuvettes at a controlled temperature of 20 °C, since precipitation of the protein can occur at 4 °C in the presence of CaCl₂. Difference spectra were measured at given concentrations of CaCl₂ by adding small volumes of CaCl₂ (250 mM) sequentially to the sample cuvette and equal volumes of deionized water to the reference cuvette; the final dilution of the protein was less than 3%. For comparative purposes, the effect of Ca²⁺ on the u.v. difference spectra of muscle calsequestrin was also studied under identical conditions. The effect of increased concentration of KCl on the u.v. absorption was also studied. Difference spectra were also measured in the presence of Mg²⁺.

Intrinsic fluorescence spectroscopy

Purified CSLP samples (about 200 µg/2 ml) were dialysed against 1 litre of buffer solution (20 mM-Mops, 250 mM-KCl, 0.1 mM-EGTA, pH 7.0) for 36 h, at 4 °C, with three changes of solution. Intrinsic protein fluorescence was recorded using a Perkin-Elmer LS-5B luminescence spectrometer. The excitation wavelength was at 275 nm using a slit-width of 3 nm and emission

scans were from 250 to 450 nm with a 1 cm × 1 cm cuvette. The temperature was controlled at 20 °C. To titrate Ca²⁺-dependent fluorescence changes, increasing amounts of Ca²⁺ were added in a sequential fashion. The effect of Ca²⁺ on muscle calsequestrin was also studied by fluorescent measurements, for comparison, under identical conditions. Similarly, the effects of Mg²⁺ were determined.

C.d. spectroscopy

Protein samples were dialysed against 2 l of 10 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EGTA for 36 h at 4 °C, with three changes of solution. The c.d. spectra of the samples were recorded in an Aviv c.d. spectropolarimeter (model 60 DS) in a cell with a path-length of 0.2 cm at 20 °C scanning over wavelengths of 250–200 nm. The protein concentration was 0.3 mg/ml. The ellipticities were calculated using a mean residue weight of 109. The α -helical and β -sheet percentages were calculated as described by Siegel *et al.* (1980). The effect of 2 mM-free Ca²⁺ on these parameters was examined.

Detection of glycoproteins by SDS/PAGE

A fluorescent labelling technique was used (Eckhardt *et al.*, 1976) to determine whether sugar moieties, known to be present in muscle calsequestrin, were also present in the CSLP. Samples electrophoresed on SDS/polyacrylamide gels were fixed in the gels with ethanol (40%) and acetic acid (5%), and treated with periodic acid [0.7% (w/v) periodic acid in 5% acetic acid] for 2 h at room temperature. This oxidizes sugars generating aldehyde functions. Excess periodic acid was removed by agitating the gel for 1 h in 0.5% (w/v) sodium metabisulphite in 5% acetic acid. The resulting aldehydes were condensed with dansylhydrazine [2 mg of acidic dimethyl sulphoxide (DMSO)/ml (0.6 ml of HCl/l of DMSO) for 2 h at 60 °C] to form hydrazones. The latter was reduced to stable hydrazine derivatives with sodium borohydride (0.2 mg/ml of DMSO) for 30 min at room temperature. After being destained with 1% acetic acid, the fluorescent labelled glycoprotein was visualized with a long-wavelength u.v.-light lamp, and photographed under a yellow-orange filter. In control experiments, the periodic acid oxidation step was omitted.

Amino sugar analysis

Purified protein samples (200–400 µg) were hydrolysed with 4 M-HCl for 3 h at 100 °C (Hess *et al.*, 1988). The protein samples were freeze-dried in Eppendorf tubes. The tubes were placed in reactivials (Pierce) and 200 µl of HCl was added. Air was evacuated, the vials were sealed, and incubated at the temperature indicated above. The samples were dried by a stream of nitrogen and then processed for hexosamine analysis using a Beckman model 119Cl amino acid analyser with a 126 data system.

Endoglycosidase H treatment

The protein samples (10–15 µg) dissolved in 50 µl of 50 mM-sodium citrate buffer, pH 5.5, containing SDS (1.2 × weight of protein) were boiled for about 1 min, then cooled to 37 °C. PMSF (1 mM) and endoglycosidase H (0.1 unit/mg of protein) were added. The endoglycosidase was a cloned preparation obtained from Genzyme Corp., Boston, MA, U.S.A. The solution was incubated for about 20 h at 37 °C. The reaction was terminated by adding SDS (2%) sample buffer and boiling for 1 min. Control incubations excluded the enzyme (Campbell *et al.*, 1983; Trimble & Maley, 1984).

PAGE

The analysis of proteins by SDS/PAGE was routinely performed by using the discontinuous buffer system of Laemmli

(1970) with 10% (w/v) separating gel and 3% (w/v) stacking gel. All gels had an acrylamide/bisacrylamide ratio of 29:1.

Protein determination

Proteins were routinely determined by the BCA method (Smith *et al.*, 1985).

N-Terminal sequence

This was determined by the method of Matsudaira (1987).

RESULTS

Ca²⁺ binding measured by equilibrium dialysis

Fig. 1 illustrates the Ca²⁺ binding, expressed in nmol of Ca²⁺/mg of protein, on dialysing the protein against various concentrations of free Ca²⁺ (0.4–10 mM) under different salt conditions in 20 mM-Mops buffer at pH 7.0. The maximum binding was about 400 nmol of Ca²⁺/mg of protein corresponding to about 23 mol of Ca²⁺/mol of protein based on a molecular mass of 58 kDa. The half-binding maxima determined from the Hill (1910) equation were 1.62 mM, 3.64 mM, 4.81 mM and 5.77 mM in the presence of 20 mM-KCl, 100 mM-KCl, 100 mM-KCl plus 3 mM-MgCl₂ and 250 mM-KCl plus 3 mM-MgCl₂ respectively; the latter conditions approximate more closely to the ionic concentration in the egg. The increasing concentration of KCl and the presence of MgCl₂ lowers the affinity for Ca²⁺, the affinity being lowest in the ionic medium approximating the milieu in the sea-urchin egg. Hill coefficients under the above salt conditions were respectively 1.65, 1.83, 2.24 and 2.21. The first coefficient of 1.65 (obtained at low KCl) is hardly significantly different from unity ($P \approx 0.90$). The others are significantly different from unity ($P > 0.99$), indicating a mild degree of co-operativity considering that there are 23 Ca²⁺-binding sites in the protein molecule (Hill, 1910; Cornish-Bowden, 1979).

U.v. difference spectroscopy findings

The u.v.-absorption measurements were done on CSLP preparations which had been exposed to hydrogenated Triton X-100 rather than Nonidet P40 because the latter absorbs in the u.v.

Fig. 1. ⁴⁵Ca²⁺ binding of the CSLP

Ca²⁺ binding was measured by equilibrium dialysis. Protein samples (0.56 mg/ml) were dialysed as described in the Materials and methods section against a buffer solution containing 20 mM-Mops, pH 7.0, and one of the following: ●, 20 mM-KCl; ○, 100 mM-KCl; ▲, 100 mM-KCl and 3 mM-MgCl₂; △, 250 mM-KCl and 3 mM-MgCl₂. The curves were determined by a non-linear least-squares fitting procedure. Each point represents the mean $\pm$ S.E.M. of four to six determinations, using two different preparations of protein.

range. The CSLP showed a maximum absorbance at 278 nm and the difference spectrum between samples containing Ca²⁺ and those free of Ca²⁺ showed a small decrease in absorbance maximally at 278 nm (Fig. 2a), indicative of a net change in the environment of the aromatic residues due to a Ca²⁺-induced conformational change. The degree of change was a function of the Ca²⁺ concentration (1–8 mM) (Fig. 2b). For comparison, the difference spectrum of muscle calsequestrin (having been exposed to the same concentration of hydrogenated Triton X-100 as the CSLP and measured under identical conditions) showed, as expected, an increase in absorbance in the presence of Ca²⁺ (results not shown), as previously described by Ikemoto *et al.* (1974).

To rule out the possibility that spectral changes were due to protein aggregation, the u.v.-absorption spectra of the protein solutions were scanned between 450 nm and 250 nm, in the presence of increasing concentrations of Ca²⁺ over different periods of time. There was no light scattering observed in the longer wavelength range (especially in the region 340–360 nm where specific absorption of protein solution is absent), indicating absence of detectable protein aggregation. Furthermore, we studied the effect of KCl, which does not cause aggregation of muscle calsequestrin and the CSLP and is known to cause spectral changes of muscle calsequestrin similar to those produced by Ca²⁺ (Ikemoto *et al.*, 1974). Fig. 2(c) shows a small decrease in the absorbance maximum of the CSLP at 279 nm induced by 0.1 M-KCl. Fig. 2(d) shows a dependence of the u.v. difference spectra on the concentration of KCl (0.1–0.5 M). Hence K⁺ and Ca²⁺ induced similar changes in the u.v. absorption of the CSLP and these changes are not due to protein aggregation.

In contrast with Ca²⁺, Mg²⁺ caused a slight increase in the u.v. difference spectrum of the CSLP (Fig. 2e) and the degree of change was also dependent on the concentration of Mg²⁺, reaching a maximum at a concentration of 5 mM-MgCl₂ (Fig. 2f).

Intrinsic fluorescence spectroscopy findings

Fig. 3 illustrates a progressive small decrease in the intrinsic fluorescence peak at 340 nm with increasing concentrations of Ca²⁺, indicative of a change in the environment of tyrosine and tryptophan due to Ca²⁺-induced conformational changes. Corresponding concentrations of Mg²⁺ produced about half the change produced by Ca²⁺. For comparison, muscle calsequestrin, studied under identical conditions mentioned above, showed, as expected, an increase in fluorescence in the presence of Ca²⁺ and a shift towards the blue (results not shown) as previously described in Ikemoto *et al.* (1974).

Ca²⁺ induced only a small and insignificant decrease in the absorption spectrum at the excitation wavelength (275 nm). A significant change at this wavelength would be indicative of light scattering. Hence the Ca²⁺-induced changes in the fluorescence spectra are not caused by scattering due to protein aggregation.

C.d. spectroscopy findings

Fig. 4 illustrates the c.d. spectra. Calculations showed that the CSLP, in the presence of 1 mM-EGTA, showed a 16.1% α -helical content and 46.7% β -sheet formation. In the presence of 2 mM-free Ca²⁺ the α -helical percentage changed to 14.7 and the β -sheet percentage to 47.6; thus minor changes, less than 10%, which may not be significant, were observed in the presence of CaCl₂. Similar minor changes occurred in the presence of 0.1 M-KCl.

Identification of CSLP as a glycoprotein and enzymic deglycosylation

The presence of sugars in the CSLP was revealed by the

Fig. 2. U.v. difference spectra of the CSLP produced by (a) Ca^{2+} , (c) K^{+} and (e) Mg^{2+} , and changes in u.v. difference spectra of the CSLP (b) as a function of free Ca^{2+} concentration, (d) as a function of K^{+} concentration and (f) as a function of Mg^{2+} concentration

(a) The absorbance difference spectrum was measured in the absence versus the presence of 5 mM free CaCl_2 . The buffer solution contained 20 mM-Mops, pH 7.0, 100 mM-KCl and 0.1 mM-EGTA. The protein concentration was 0.42 mg. The absorbance is decreased maximally at 278 nm. (b) The changes in absorbance at 278 nm were measured as a function of increasing concentrations (1–8 mM) of Ca^{2+} under the same conditions as in (a). (c) The u.v. difference spectrum was measured in the absence versus presence of 0.1 M-KCl. The protein concentration was 0.52 mg/ml in 20 mM-Mops buffer, pH 7.0. The maximum decrease in the absorbance is at 279 nm. (d) Changes in u.v. difference spectra at 279 nm of CSLP as a function of K^{+} concentration. The conditions were the same as in (c). (e) The absorbance difference spectrum was measured in the absence versus presence of 2 mM- MgCl_2 . The buffer solution contained 20 mM-Mops, pH 7.0, 100 mM-KCl and 0.1 mM-EGTA. Protein concentration was 0.48 mg/ml. (f) The change in absorbance at 276 nm was measured as a function of increasing concentration of Mg^{2+} , under the same ionic conditions as in (e).

fluorescence of the protein band after treatment of SDS/polyacrylamide gels by the method of Eckhardt *et al.* (1976) described in the Materials and methods section. Fig. 5 shows fluorescence bands on SDS/polyacrylamide gels indicating the presence of sugars in CSLP and in known glycoproteins, cardiac calsequestrin and ovalbumin. Amino sugar analysis revealed the presence of eight to nine glucoses/mol of protein and probably seven to eight mannoses/mol. The uncertainty in the identification of the latter is because its peak in the chromatogram did not correspond exactly to the peak of the mannose standard. Digestion of the protein with endoglycosidase H, however, did not cause a detectable decrease in the apparent molecular mass of the CSLP on SDS/polyacrylamide gels, a commonly used

criterion for hydrolysis of sugars in glycoproteins (Campbell *et al.*, 1983).

N-Terminal sequence

Fig. 6 gives the N-terminal sequence of the CSLP from the sea-urchin egg and a comparison is made with muscle calsequestrin and calreticulin.

Of the 25 amino acids in the N-terminal sequences presented, CSLP contains nine acidic residues (seven glutamates and two aspartates), somewhat more than in calsequestrin and calreticulin. The majority of them (six) are clustered within the first nine residues. Similar clustering is observed in skeletal-muscle

Fig. 3. Effect of Ca^{2+} on the intrinsic fluorescence of the CSLP

The protein sample was excited at 275 nm using a slit-width of 3 nm. Emission scans were from 250 nm to 450 nm using a 5 nm slit-width. The solution contained 0.1 mg of the CSLP/ml, 100 mM-KCl, 0.1 mM-EGTA and 20 mM-Mops, pH 7.0. Increasing amounts of Ca^{2+} were added sequentially. A, 0; B, 1 mM; C, 2 mM; D, 3 mM; E, 5 mM; F, 8 mM.

Fig. 4. C.d. spectra of the CSLP

C.d. spectra were recorded in the presence of 1 mM-EGTA (○) or 2 mM-free Ca^{2+} (●) in 10 mM-Tris/HCl, pH 7.5. The protein concentration was 0.3 mg/ml.

Fig. 5. SDS/polyacrylamide gels indicating the presence of glycoproteins

Protein samples (6 μg) were applied to 10% (w/v) SDS/polyacrylamide gels and electrophoresed (Laemmli, 1970). (a) Gel stained with dansylhydrazine. Fluorescent bands indicate the presence of carbohydrates in the proteins. Parallel controls, omitting the periodic oxidation step, showed no fluorescence. Calmodulin, a non-glycosylated protein, treated in the same manner did not stain with dansylhydrazine (not shown). (b) A corresponding gel was stained with Coomassie Blue. Lane 1, ovalbumin (obtained from Sigma); lane 2, CSLP; lane 3, canine cardiac calsequestrin (the lower-molecular-mass band is probably a degradation product caused by protein storage).

(1) EVEVEIEEDVAVLTDAAFAEYVAEN

(2) EEELDFPEYDGVDRVINVNAKNYKN

(3) EEGLNFPTYDGKDRVVSLTEKNFKQ

(4) DPAIYFKEQFLDGDGAWTNRMVESKH

(5) EPVVYFKEQFLDGDGWTERWIESKH

Fig. 6. Comparison of the *N*-terminal sequence of the CSLP from sea-urchin egg with muscle calsequestrin and calreticulin

(1) CSLP from sea-urchin; (2) calsequestrin from skeletal muscle (Fliegel *et al.*, 1987); (3) calsequestrin from cardiac muscle (Scott *et al.*, 1988); (4) calreticulin from plasmacytoma cells (Smith & Koch, 1989); (5) calreticulin from skeletal muscle (Fliegel *et al.*, 1989).

calsequestrin (six within the first ten residues). Such clustering is not observed in the other examples cited.

The *N*-terminal sequences of all these putative Ca^{2+} -storage proteins start with an acidic residue. Overall, there is no obvious similarity in the *N*-terminal sequence of CSLP compared with that of calsequestrin and calreticulin.

DISCUSSION

The CSLP from the ER of sea-urchin eggs is a high-capacity low-affinity Ca^{2+} -binding protein. The binding of 23 mol of Ca^{2+} /mol of CSLP is in the range reported for cardiac calsequestrin (Slupsky *et al.*, 1987) but is about half the binding capacity of striated muscle calsequestrin (MacLennan *et al.*, 1983). The CSLP and calsequestrin molecules have a relatively high proportion of acidic residues (over 100/mol of protein), which are potential sites for Ca^{2+} binding. A CSLP in liver is reported as having a Ca^{2+} -binding capacity about twice that of the muscle protein (Damiani *et al.*, 1989). The affinities of the CSLP for Ca^{2+} under various ionic conditions (Fig. 1), lower than those found in muscle calsequestrin, decreased with increasing concentrations of KCl and in the presence of Mg^{2+} . These ions did not appear to influence the number of binding sites for Ca^{2+} . How possible changes in the concentration of KCl and/or MgCl_2 within the ER might influence the affinity of calsequestrin for Ca^{2+} during various functional states of the cell is not known. The mild positive co-operativity in Ca^{2+} binding, as determined by the Hill coefficients, presumably makes the protein more efficient, particularly at salt concentrations approximating physiological values, for Ca^{2+} storage and release. Mild co-operativity in Ca^{2+} binding has been reported also for calsequestrin in cardiac muscle (Mitchell *et al.*, 1988) and in striated muscle (Ikemoto *et al.*, 1974).

The Ca^{2+} -induced spectral changes of the CSLP are small, but opposite in direction compared with those of muscle calsequestrin. The possibility that the small changes were due to Ca^{2+} -induced aggregation of CSLP were ruled out by the lack of light scattering at the longer wavelengths. Furthermore, KCl, which does not cause protein aggregation, also caused spectral changes similar to those caused by Ca^{2+} .

Likewise, Ca^{2+} induced a small decrease in fluorescent emission of the CSLP (Fig. 3) compared with an increase in emission of muscle calsequestrin (results not shown) similar to that reported by others. The insignificant change at the excitation wavelength of 275 nm in the presence of Ca^{2+} again ruled out protein aggregation as a possible cause of the changes in intrinsic fluorescence. These spectral findings suggest Ca^{2+} -induced con-

formational changes in the molecules, causing a net exposure of aromatic residues of the CSLP to an aqueous environment and a net internalization of these calsequestrin residues into a hydrophobic environment (Freifelder, 1982). Since the CSLP molecule has 16 tyrosine residues (Oberdorf *et al.*, 1988) and three to four tryptophans determined in the present investigation (D. Lebeche & B. Kaminer, unpublished work), the specific exposure or burial of individual aromatic residues cannot be assessed by these measurements which reflect only a net effect. Similarly, the large number of phenylalanine residues [31/mol in CSLP and similar numbers in calsequestrin (Oberdorf *et al.*, 1988)] may contribute in some measure to changes in the 250–270 nm region of the u.v. spectra; however, the contribution is too difficult to analyse. The lack of detailed information on the three-dimensional structure of the CSLP and muscle calsequestrin makes it impossible to interpret the spectroscopic data in a more specific and precise way at this time.

Mg²⁺, in contrast with Ca²⁺, caused a slight increase in the u.v. difference spectra of the CSLP (Figs. 2e and 2f), suggesting conformational changes with a net result opposite to that produced by Ca²⁺. On the other hand, the fluorescence changes induced by Mg²⁺ (results not shown) were similar to, although to a lesser degree than, those produced by Ca²⁺. A possible explanation for this apparent discrepancy is a differential effect of Mg²⁺ on tyrosine and tryptophan residues, the latter dominating the fluorescence emission more than the u.v. absorption. The increase in the fluorescence emission of calsequestrin produced by Mg²⁺ is less than that caused by Ca²⁺ (Ikemoto *et al.*, 1972, 1974).

According to the c.d. spectra, the helical content (16.1%) of the CSLP is about the same as the 10–15% found in skeletal calsequestrin in the absence of Ca²⁺ (Ikemoto *et al.*, 1974; Ostwald *et al.*, 1974; Aaron *et al.*, 1984; Ohnishi & Reithmeier, 1987). Ca²⁺ induced a slight or insignificant decrease in the helical content of the CSLP compared with a rise to 20–40% in skeletal calsequestrin (Cozens & Reithmeier, 1984; Slupsky *et al.*, 1987; Ohnishi & Reithmeier, 1987). The β -sheet content (46.7%) of the CSLP is slightly more than the 40% found in skeletal calsequestrin in the absence of Ca²⁺ (Slupsky *et al.*, 1987; Ohnishi & Reithmeier, 1987). Ca²⁺ induces a slight increase in the β -sheet content of the CSLP to 47.6% compared with a slight decrease (32%) of β -sheet content in calsequestrin (Slupsky *et al.*, 1987). Although the Ca²⁺-induced changes in the c.d. of the CSLP are very small, and may be insignificant, they are once again opposite in direction compared with those found in calsequestrin.

As we reported earlier (Oberdorf *et al.*, 1988), both the CSLP and muscle calsequestrin bind to phenyl-Sepharose by hydrophobic interactions in the absence of Ca²⁺ and can be eluted by Ca²⁺. Thus Ca²⁺ appears to have a similar effect on both proteins regarding this particular hydrophobic interaction, but as already discussed, has different effects on the proteins as detected by spectroscopic changes. Also of interest is the comparison of CSLP with two high-affinity Ca²⁺-binding proteins, calmodulin and squid Ca²⁺-binding protein. The latter two proteins, in contrast with the CSLP, require the presence of Ca²⁺ to bind to hydrophobic sites in phenyl-Sepharose (Gopalakrishna & Anderson, 1983) and fluphenazine-Sepharose (Charbonneau & Cormier, 1979). But the effect of Ca²⁺ on the three proteins results, in general, in a similar u.v. spectral difference response in that calmodulin and squid Ca²⁺-binding protein also show a negative difference spectrum in the presence of Ca²⁺ (Sheldon & Head, 1988). CSLP, like calsequestrin, is a glycoprotein but at present there is insufficient information on the sugar moieties of these proteins for any comparison to be made. Having pointed out the similarities between the CSLP and calsequestrin in the

Introduction, we have now described a number of differences particularly with regard to the Ca²⁺-induced conformational changes. The *N*-terminal sequences of the CSLP and calsequestrin are also different, showing no similarity (Fig. 6).

Of relevance to this study are the characterizations of a protein from the ER which has been designated calreticulin and is regarded as a Ca²⁺-storage protein (Smith & Koch, 1989). It is similar to a liver protein previously named calregulin (Waisman *et al.*, 1985) and is closely related to the high-affinity Ca²⁺-binding protein from muscle SR whose complete sequence has now been deduced by cDNA techniques (Fliegel *et al.*, 1989). These proteins are now also designated calreticulin. It has also been identified in HL 60 cells (Krause *et al.*, 1990) and in smooth muscle and other tissues including liver and brain (Opas *et al.*, 1990; Treves *et al.*, 1990; Milner *et al.*, 1991). Calreticulin from muscle is highly acidic, binds 25 mol of Ca²⁺/mol of protein with low affinity and 1 mol/mol with high affinity (Ostwald *et al.*, 1974). In contrast with the CSLP which we have isolated from the ER, calreticulin is not a glycoprotein, is not eluted from phenyl-Sepharose in the presence of Ca²⁺ and does not undergo conformational changes on binding Ca²⁺ (Ostwald *et al.*, 1974; Waisman *et al.*, 1985; Krause *et al.*, 1990). Furthermore, the *N*-terminal sequence of the CSLP in no way resembles that of calreticulin (Fig. 6). Dr. Marek Michalak kindly provided us with a sample of calreticulin (prepared from dog pancreas) and its antibody (from goat antiserum) and we found no immunocross-reactivity between the CSLP and calreticulin. Immunostaining of transfer blots of extracts of microsomal fractions from sea-urchin eggs did not reveal the presence of calreticulin. Similarities and possible evolutionary relationships between the CSLP and any other high-capacity Ca²⁺-binding protein in the ER must await the sequencing of the molecule.

Calsequestrin has long been considered as a Ca²⁺-storage protein in the SR by virtue of its location, the presence of a structural link between it and the terminal cisternae (Franzini-Armstrong *et al.*, 1987) and its Ca²⁺-binding properties, but as yet no definitive assay has been devised to characterize it as a functional Ca²⁺-storage and -release protein. Likewise no-one has yet demonstrated that purported Ca²⁺-storage proteins within the ER function as such. Whether high-capacity low-affinity Ca²⁺-binding proteins in the SR and ER belong to the same family of proteins remains to be determined. How they might be regulated in their binding and release of Ca²⁺ in response to various physiological and biochemical events in the cells also remain outstanding and intriguing problems.

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