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Fragmentation of Rabbit Skeletal Muscle Calsequestrin: Spectral and Ion Binding Properties of the Carboxyl-Terminal Region†

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ABSTRACT: Rabbit skeletal muscle calsequestrin was fragmented by using trypsin in the presence and absence of calcium. Calcium ion was found to protect calsequestrin from proteolysis, and the peptides produced in the presence of calcium were stable to further digestion. Peptides produced in the presence or absence of calcium had a decreased helical content but maintained their ability to bind calcium. The amino acid sequence of a 59-residue carboxyl-terminal tryptic peptide was determined by automated Edman degradation and carboxypeptidase Y digestion of carboxyl-terminal tryptic, chymotryptic, and cyanogen bromide peptides. This peptide is highly acidic (Asp + Glu = 42%, Lys + Arg = 0), and it bound a total of 15 calcium ions per mole of peptide (K_d = 8.5 mM). The intrinsic tryptophan fluorescence of the peptide was enhanced by 10% upon binding Ca^{2+} with the dissociation constant of 1 mM. Analyses of the circular dichroism spectra of the peptide showed that it was primarily in a random-coil conformation with little helical (2%) and moderate β-structure (25%) regardless of the calcium concentration. This peptide also bound 7 mol of terbium per mole of peptide with high affinity (K_d = 7.5 μM).

Calsequestrin is a calcium binding protein found in the terminal cisternae of sarcoplasmic reticulum (MacLennan & Wong, 1971). It has an important physiological function in that it sequesters calcium ions within the sarcoplasmic reticulum when muscle is relaxed [see MacLennan et al. (1983) for a review]. Calsequestrin binds 40–50 mol of calcium per mole of protein with a dissociation constant of 1 mM under physiological conditions (MacLennan & Wong, 1971; Ikemoto et al., 1972, 1974; Ostwald & MacLennan, 1974). Calsequestrin is also found in heart muscle (Cala & Jones, 1983; Campbell et al., 1983), where it likely plays a crucial role in sequestrating calcium in cardiac sarcoplasmic reticulum. Physicochemical and biochemical properties of calsequestrin have been extensively examined by many workers, i.e., circular dichroism (Ikemoto et al., 1972, 1974; Ostwald & MacLennan, 1974; Cozens & Reithmeier, 1984), fluorescence spectroscopy (Ikemoto et al., 1972, 1974; Ohnishi & Reithmeier, 1987), ultracentrifugation (Cozens & Reithmeier, 1984), NMR spectroscopy (Aaron et al., 1984), Raman spectroscopy (Williams & Beeler, 1986), and crystallization (Maurer et al., 1985; Williams & Beeler, 1986).

One of the unique properties of calsequestrin is that it contains high amounts of acidic amino acid residues (MacLennan & Wong, 1971). The calcium binding mechanism of calsequestrin is not yet understood. Many calcium binding proteins have been isolated, and their calcium binding mechanisms have been elucidated, i.e., EF hand (Kretsinger & Nockolds, 1973; Reid & Hodges, 1980), γ-carboxyglutamic acid containing proteins (Furie et al., 1979; Bajaj et al., 1982), β-hydroxyaspartic acid containing proteins (Drakenberg et al., 1983), and phosphoproteins (Lee et al., 1977; Bennick et al., 1981). However, the calcium binding mechanism of calse-

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questrin probably does not fall into any of those categories. Since there are large numbers of acidic amino acid residues in the protein, Ostwald and MacLennan (1974) have predicted that the positively charged calcium ion binds to the negatively charged amino acid residues in the calsequestrin molecule.

The amino acid sequence of rabbit skeletal calsequestrin has recently been determined (Fliegel et al., 1987), and there was no indication of a regular distribution of acidic residues. In fact, a 17 amino acid stretch near the amino terminus of the protein (residues 14–30) did not contain any acidic amino acid residues (Reithmeier & Cozens, 1982; MacLennan et al., 1983; Fliegel et al., 1987). The amino-terminal region is relatively hydrophobic, containing only eight acidic residues out of the first 41 residues, suggesting that this region binds less calcium ions compared with the rest of the calsequestrin molecule. In contrast, the carboxyl-terminal region of calsequestrin is highly acidic (Fliegel et al., 1987).

We wish to determine directly the calcium binding properties of various regions of the calsequestrin molecule. Our approach was to fragment calsequestrin under various conditions, to study the effect of fragmentation on calcium binding and protein conformation and then to purify various peptides and to measure their calcium binding capacity and conformation. In this study, we have focused on the carboxyl-terminal region of calsequestrin since it is highly acidic and therefore likely to bind a significant amount of calcium. Carboxyl-terminal peptides were isolated from tryptic, chymotryptic, and cyanogen bromide digests and sequenced by automated Edman degradation and carboxypeptidase Y digestion. The calcium binding properties of the carboxyl-terminal tryptic peptide, which was the largest peptide, were examined by using circular dichroism, fluorescence spectroscopy, and gel filtration.

It has been shown that lanthanides, which are spectroscopically active, can substitute for calcium binding (Brittain et al., 1976; Dockter, 1983). The intact calsequestrin has been shown to bind up to 30 terbium ions per molecule of protein with an affinity of 7 μM (Ohnishi & Reithmeier, 1987). Terbium binding increases the α-helical content of calsequestrin from 16% to 30%, which suggests a similar conformational change in calsequestrin as induced by calcium. Terbium was used as a high-affinity calcium analogue to examine the metal binding properties of the carboxyl-terminal peptide.

Materials and Methods

Calsequestrin Purification. Calsequestrin was prepared from fast skeletal muscle of New Zealand white rabbit according to ammonium sulfate precipitation method (Slupsky et al., 1987). All the preparations were carried out at 4 °C unless stated otherwise.

Digestion of Calsequestrin by Trypsin. Purified calsequestrin dissolved in 50 mM NH_4CO_3, pH 8.6, was incubated at room temperature for up to 3 h with 1% N^{α}-(*p*-tosyl)-L-phenylalanine chloromethyl ketone (TPCK) treated trypsin (by weight of the protein), in the presence of either 4 mM $CaCl_2$ or 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Some digestions were also performed in the presence of 0.4 M KCl to prevent precipitation of calsequestrin. Aliquots were removed at various times and pipetted into a solution containing either soybean trypsin inhibitor (final concentration of 2%) or phenylmethanesulfonyl fluoride (final concentration of 1 mM). The samples were analyzed by gel electrophoresis with either 6 M urea/16% acrylamide or 0.1% sodium dodecyl sulfate/16% acrylamide, reverse-phase high-performance liquid chromatography (HPLC), and circular dichroism spectroscopy.

Peptide Purification. Purified calsequestrin dissolved in 50 mM NH_4HCO_3, pH 8.6, was incubated with 1% TPCK-treated trypsin (by weight of the protein) at room temperature for 3 h. It was applied to a Sephacryl S-200 gel filtration column (2.6 cm × 100 cm) in 15 mM NH_4HCO_3, pH 8.3, and 3-mL fractions were collected. The peptide content of each fraction was monitored by the absorbance at 215 nm and polyacrylamide gel electrophoresis in urea. Appropriate fractions were combined and freeze-dried for further examination. Chymotrypsin digestion was performed in 50 mM NH_4HCO_3, pH 8.6, with 1% N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone (TLCK) treated chymotrypsin (by weight of the protein) in the presence of 1 mM $CaCl_2$ at room temperature for 3 h. Cyanogen bromide digestion was done in 70% formic acid with 1.5 mg of cyanogen bromide (Eastman Kodak) per milligram of protein. These digests were applied to a Sephacryl S-200 gel filtration column under the same conditions applied to the trypsin digest. The carboxyl-terminal tryptic peptide could be purified in one step. It was the first peak to be eluted from the Sephacryl S-200 column (V_e = 255 mL). Similarly, the chymotryptic fragment eluted as a single peak (V_e = 270 mL) from the same column. The cyanogen bromide peptide eluted in the third peak of the S-200 column (V_e = 340 mL) and was further purified by using DEAE A-25 and a linear gradient of 0–0.5 M KCl in 15 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5. It eluted as the first peak at a KCl concentration of 0.15 M from the ion-exchange column.

High-Performance Liquid Chromatography (HPLC). HPLC was performed on a Waters HPLC system equipped with two 6000-A pumps and a Model 660 solvent programmer with a Waters μBondapak C18 reversed-phase column (4.1 × 30 cm). Peptide solutions of 0.1–1 mL were injected with a Waters U6K injector. The separation was performed with a gradient of 0–60% acetonitrile in 15 mM NH_4HCO_3, pH 8.3. Peaks were detected by UV absorption at 215 and 280 nm using Waters Model 450 variable-wavelength and Model 440 absorption detectors, respectively.

Gel Electrophoresis. Polyacrylamide gel electrophoreses were performed with 16% acrylamide, 0.2% *N*,*N*-methylenebis(acrylamide), 0.03% ammonium persulfate, 0.18% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 6 M urea, 0.025 M Tris, and 0.08 M glycine, pH 8.6, or by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis according to Laemmli (1970).

Protein Assay. The protein concentrations of samples were determined by using amino acid analysis assuming a molecular weight of calsequestrin of 40 000.

Amino Acid Analysis. Protein was hydrolyzed using 6 N HCl at 110 °C for 20 h. For the determination of cysteine content, the protein was performic acid oxidized in the presence of 88% formic acid and then hydrolyzed with 6 N HCl at 110 °C for 20 h (Hirs, 1967). The protein was hydrolyzed with 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110 °C for 20 h for an estimate of the tryptophan content (Simpson et al., 1976). Amino acid analyses of the hydrolysates were determined by using a Dionex Model D-500 amino acid analyzer.

Peptide Sequencing. Amino acid sequence analyses were performed on an Applied Biosystems Model 470A protein sequencer. Phenylthiohydantoin (PTH)–amino acid derivatives were analyzed by on-line high-pressure liquid chromatography. Between 50 pmol and 3 nmol of peptide was subjected to sequence analysis. Some sequence determinations

were also performed by using a Beckman Model 890C spinning cup sequencer; 25–50 nmol of purified peptide was dissolved in 500 μL of water containing 2 mg of polybrene. Edman degradation was performed with a 0.5 M Quadrol program.

Carboxypeptidase Y Digestion. Intact calsequestrin (25 nmol) and carboxyl-terminal tryptic peptide of calsequestrin (25 nmol) dissolved in 600 μL of 50 mM sodium acetate buffer, pH 5.3, were digested with 20 μL of carboxypeptidase Y (1 mg/mL in water) Aliquots of 150 μL were taken at 30-min and 1-h incubation, and the reaction was stopped by adding 50 μL of 10% trichloroacetic acid. The sample was subjected to amino acid analysis.

Circular Dichroism. The circular dichroism spectra were recorded on a Jasco Model J-500 spectropolarimeter using cells with path lengths of 0.0103 or 0.0503 cm. Protein concentrations were between 0.5 and 1.8 mg/mL in 10 mM Tris-HCl, pH 7.5. Spectra were also recorded of samples that contained 0.1 or 0.4 M KCl. The ellipticities were calculated by using a value of 115 for the mean residue molecular weight, and the α-helical and β-structure contents were calculated according to Chen et al. (1974).

Fluorescence Spectroscopy. The fluorescence spectra were taken on a Perkin-Elmer MPF-44B spectrofluorometer at 20 °C with both emission and excitation slit widths of 8 nm using a 0.3 cm × 0.3 cm microcuvette. Terbium fluorescence spectra were measured at 545 nm either by exciting terbium directly at 222 nm or by energy transfer at 295 nm. Peptides (2.5–5 nmol) were dissolved in 2 mL of 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.5. Intrinsic protein fluorescence measurements were made by exciting the protein at 280 nm and monitoring the fluorescence at 350 nm. Samples were dissolved in 50 mM Tris-HCl, pH 7.5, at a peptide concentration of 1.25 μM.

Calcium Binding. $^{45}Ca^{2+}$ binding to calsequestrin or calsequestrin fragments was measured by using a Bio-Gel P-2 gel filtration column (1.0 cm × 30 cm) equilibrated with 10 mM Tris-HCl/100 mM KCl, pH 7.4, with 1–30 mM radioactive $CaCl_2$ (Hummel & Dreyer, 1962). Fractions of 0.5 mL were collected, and the radioactivity was measured with a Beckman Model LS 7800 liquid scintillation counter.

Terbium Binding. Terbium binding to the carboxyl-terminal tryptic peptide of calsequestrin was determined by equilibrium dialysis. One milliliter of peptide (0.125 mg/mL) was dialyzed for 20 h at room temperature against 100 mL of 25 mM PIPES, pH 6.8, containing various amounts of $TbCl_3$ (1 μM–50 mM). The amount of Tb^{3+} present in the solution was determined by using a dipicolinic acid assay (Barela & Sherry, 1976). The amount of bound terbium ion was plotted against free terbium concentration. Terbium binding was also measured by fluorescence spectroscopy. Titration of the carboxyl-terminal tryptic peptide (0.2 mg/mL) of calsequestrin was performed by using excitation at 222 nm and at 295 nm in 25 mM PIPES, pH 6.5, in the absence and presence of 0.1 M KCl. Luminescence was measured at 545 nm. The relative luminescence was plotted against the total $TbCl_3$ concentration.

Terbium binding data were analyzed with an Adair's equation [cited by Roberts (1977)] by nonlinear least-squares fittings using a BASIC program for the IBM PC:

$$\text{binding function } r = \frac{\sum_{i=1}^{n} iK_i \frac{[S]^i n!}{(n-i)!i!} \prod_{j=1}^{i} K_j}{1 + \sum_{i=1}^{n} \frac{[S]^i n!}{(n-i)!i!} \prod_{j=1}^{i} K_j}$$

where n is the number of ligand binding sites on the protein, i is the ith ($1 \leq i \leq n$) ligand binding site, [S] is the concentration of bound ligand, and K_j is the intrinsic association constant at the jth binding site.

RESULTS

Trypsin Digestion of Calsequestrin. The amino acid sequence of rabbit fast skeletal muscle calsequestrin contains 6 arginine and 24 lysine residues (Fliegel et al., 1987). A total of 31 tryptic peptides are therefore expected to be produced. Purified calsequestrin was incubated with trypsin at room temperature as described under Materials and Methods. The extent of digestion was monitored by gel electrophoresis with either 6 M urea/16% acrylamide or 0.1% SDS/16% acrylamide. A polyacrylamide gel with urea was stained by using Coomassie Brilliant Blue G-250, in 2.5% perchloric acid. As staining was completed within 15 min, small peptides with a mass of 1000 daltons could be detected. When gels were stained with Coomassie Brilliant Blue R-250, in 25% methanol and 10% acetic acid (Laemmli, 1970), those small peptides were extracted from the gel due to the long staining and destaining procedure.

The rate of digestion of calsequestrin was much slower in the presence of 4 mM calcium chloride as compared with the rate of digestion in the absence of calcium (in the presence of 1 mM EGTA) (Figure 1). In the absence of calcium, all of calsequestrin was cleaved after 1 min of incubation. However, in the presence of calcium, almost all of calsequestrin was left intact after 1 min of trypsin treatment. It is clear from this experiment that Ca^{2+} protects calsequestrin against trypsin digestion.

Not only is the rate of trypsin digestion different in the presence of Ca^{2+} but also the peptide pattern produced by trypsin digestion of calsequestrin was markedly different in the presence and absence of calcium. This can be seen by one-dimensional (Figure 1) and two-dimensional (not shown) gel electrophoresis and by HPLC analyses (not shown) of the tryptic digests. Tryptic cleavage of calsequestrin in the presence of Ca^{2+} produced a limited number of major peptides. These peptides were resistant to further trypsin digestion, producing a peptide pattern at 3 h of digestion that was similar to the pattern produced after only 1 min of trypsin treatment (Figure 1). While the peptide pattern remained constant in the presence of Ca^{2+}, the peptide pattern produced in the absence of Ca^{2+} changed dramatically over the 3-h digestion period (Figure 1). Many lysine and arginine residues either are protected by Ca^{2+} directly or are rendered inaccessible to trypsin due to burying induced by the bound Ca^{2+}. Trypsin digestion can be used to distinguish two conformations of calsequestrin. These experiments provide additional evidence that Ca^{2+} markedly affects the structure of calsequestrin.

A peptide pattern similar to that produced in the presence of calcium can also be produced by trypsin in the absence of Ca^{2+} at short times of digestion. The peptide bonds producing these peptides are readily cleaved by trypsin whether Ca^{2+} is bound to calsequestrin or not. These lysine and arginine residues are in regions of calsequestrin that are not sensitive to the presence of Ca^{2+}.

One of the sites that is sensitive to cleavage by trypsin in the presence or absence of Ca^{2+} was identified by HPLC analysis of the digests. Reversed-phase HPLC using 15 mM NH_4HCO_3, pH 8.3, with a 0–60% acetonitrile gradient was used to resolve the tryptic peptides. A peptide eluting at 15.7 min that absorbed strongly at 280 nm was produced by trypsin digestion in the presence or absence of Ca^{2+}. This peptide was purified and was found to correspond to the carboxyl-terminal tryptic peptide of calsequestrin. Trypsin cleavage at lysine-301 (Fliegel et al., 1987) occurs readily in the presence of Ca^{2+}

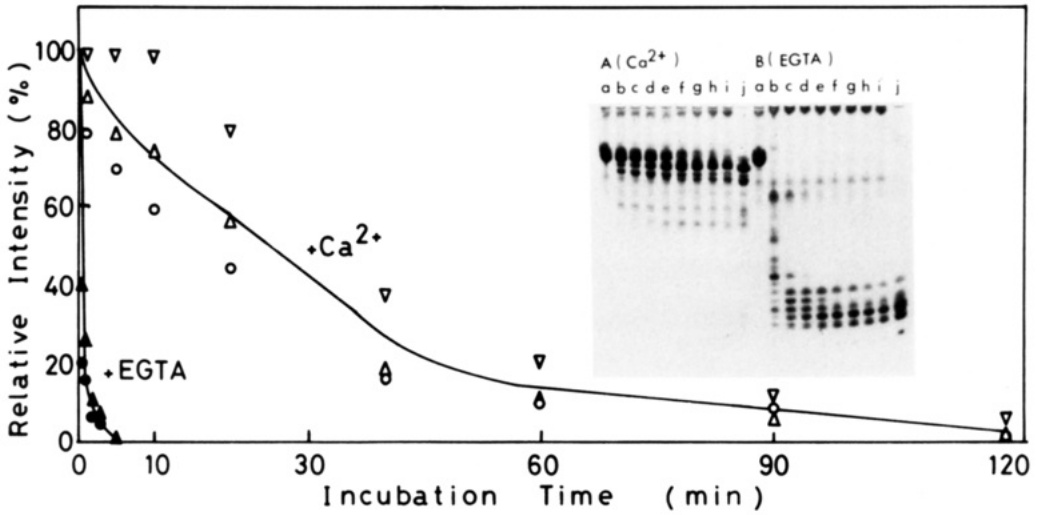

FIGURE 1: Time course of trypsin digestion of calsequestrin. Aliquots of a tryptic digest of calsequestrin were subjected to an electrophoresis with 16% acrylamide/6 M urea in 25 mM Tris/80 mM glycine, pH 8.6 (inset). The relative intensities of bands which were measured with a densitometer were plotted against incubation time with trypsin. Three determinations were plotted on the same graph. (○, △, and ▽) Incubated in the presence of 4 mM $CaCl_2$ in 50 mM NH_4HCO_3, pH 8.6. (● and ▲) Incubated in the presence of 1 mM EGTA in 50 mM NH_4HCO_3, pH 8.6. (a) 0 min; (b) 1 min; (c) 5 min; (d) 10 min; (e) 20 min; (f) 40 min; (g) 1 h; (h) 1.5 h; (i) 2 h; (j) 3 h.

and shows that this region of calsequestrin is accessible to trypsin in the presence and absence of Ca^{2+}.

The whole tryptic digest of calsequestrin cleaved in the presence of calcium eluted as several well-resolved peaks when subjected to gel filtration analysis in the presence or absence of Ca^{2+}. The presence of separate peaks indicates that the peptides produced in the presence of Ca^{2+} do not remain tightly associated with one another. The gel filtration pattern produced by the same peptides in the presence of EGTA was different however, from the pattern observed in the presence of Ca^{2+}. This may be due to conformational changes in individual peptides upon release of bound Ca^{2+}. The different elution pattern is unlikely due to dissociation of complexes since there was not a shift to smaller apparent molecular weight upon removal of Ca^{2+}. Ca^{2+} binding to calsequestrin therefore appears to be between neighboring carboxyl groups in the sequence and not between carboxyl groups that are widely separated in the sequence but close within the folded polypeptide.

Calcium Binding Properties of Peptide Digests. Calcium binding properties of the intact calsequestrin, and its total trypsin digest, were examined using $^{45}Ca^{2+}$ and a Bio-Gel P-2 gel filtration column. For the cleavage in the absence of Ca^{2+}, EGTA was not used since an EGTA–Ca^{2+} complex eluted at the void volume, disrupting the calcium binding measurement. The calcium binding capacity of intact calsequestrin was 950 nmol of calcium/mg of protein (Figure 2), which agrees well with previous measurements (MacLennan & Wong, 1971; Ikemoto et al., 1972, 1974; Ostwald & MacLennan, 1974; Cozens & Reithmeier, 1984). Intact calsequestrin was completely saturated with Ca^{2+} at 4 mM Ca^{2+} with a binding constant of 1 mM. There was no further increase in Ca^{2+} binding to calsequestrin up to 20 mM Ca^{2+} (Figure 2). This indicates that there is little or no nonspecific binding of Ca^{2+} to intact calsequestrin.

When the total tryptic digest was applied to a Bio-Gel P-2 column, the majority of the peptides as determined by the absorbance at 280 nm appeared in the void volume. These peptides bound Ca^{2+}, and, therefore, trypsin digestion did not destroy the Ca^{2+} binding sites. The Ca^{2+} binding of intact calsequestrin and its tryptic peptides generated in the presence and absence of Ca^{2+} was determined by using Ca^{2+} concentrations up to 30 mM (Figure 2). Surprisingly, after cleavage

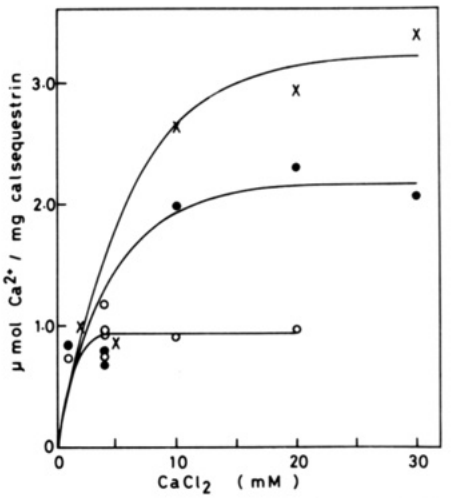

FIGURE 2: Calcium binding of calsequestrin. Calcium binding capacity determined in Figure 5 is plotted against calcium concentration. (○) Intact calsequestrin; (●) tryptic digest cleaved in the presence of 10 mM $CaCl_2$/50 mM NH_4HCO_3, pH 8.6; (×) tryptic digest cleaved in the absence of calcium (50 mM NH_4HCO_3, pH 8.6; no EGTA was used).

of calsequestrin with trypsin in the absence of calcium, the binding capacity was increased to 3 times that of intact calsequestrin while the capacity of the tryptic peptides of calsequestrin produced in the presence of calcium was twice that of intact calsequestrin. The affinity of the tryptic peptides for Ca^{2+} was decreased, however, relative to the intact molecule (Figure 2). Peptides produced in the presence of 4 mM calcium were saturated at 10 mM Ca^{2+}, with a binding constant of about 3 mM Ca^{2+}, while digests which were produced in the absence of calcium were saturated at 20 mM Ca^{2+} with a binding constant of about 4 mM Ca^{2+} (Figure 2).

The increase in the binding capacity of the peptides may be due to two factors. Trypsin digestion produces a new carboxyl group at the carboxyl terminus of each tryptic peptide. These new carboxyl groups may participate in Ca^{2+} binding, resulting in an increased Ca^{2+} binding. In addition, trypsin cleavage may also expose carboxyl groups previously buried as salt bridges inside the intact molecule. This indicates that not all carboxyl groups or potential Ca^{2+} ligands participate in Ca^{2+} binding in intact calsequestrin.

Table I: Effect of Trypsin Digestion on the Conformation of Calsequestrin

buffer[a]	content[b]	digestion time (min) 0	10	30	60	180
1 mM EGTA	α	13	2	2	2	3
	β	39	33	31	30	28
	R	48	65	67	68	69
2 mM $CaCl_2$	α	30	1	1	1	1
	β	36	32	31	31	31
	R	34	67	68	68	68
0.4 M KCl/1 mM EGTA	α	6	5	1	0	3
	β	53	60	63	64	59
	R	41	35	36	36	36
0.4 M KCl/2 mM $CaCl_2$	α	27	25	24	22	22
	β	43	37	39	43	39
	R	30	38	37	39	39

[a] Trypsin digestion was carried out in 50 mM NH_4HCO_3, pH 8.6, containing 1 mM EGTA or 2 mM $CaCl_2$ in the presence or absence of 0.4 M KCl. At the indicated times, aliquots were removed, and the digestion was stopped with 1 mM phenylmethanesulfonyl fluoride. Aliquots were diluted 4-fold into 10 mM Tris–HCl, pH 7.5, containing the same levels of EGTA, $CaCl_2$, or KCl as the digestion medium. [b] α, α–helix; β, β–structure; R, random coil.

Circular Dichroism Spectra of Trypsin-Digested Calsequestrin. The effect of trypsin digestion of calsequestrin in the presence and absence of Ca^{2+} on the conformation of the protein was examined by circular dichroism. CD spectra of intact calsequestrin and trypsin digests prepared in the presence and absence of calcium were measured from 190 to 260 nm. In all cases, the spectra were changed by trypsin digeston in a time-dependent manner. An analysis of these spectra is shown in Table I. The contents of ordered structure in the digest were calculated by using the method of Chen et al. (1974) with a chain length of 10. Intact calsequestrin had an α-helical content of 13% in the absence of Ca^{2+}. Analysis of the CD spectra of apocalsequestrin revealed the presence of a significant amount of β-structure (39%) in agreement with recent Raman spectroscopy studies (Williams & Beeler, 1986). Addition of Ca^{2+} to 2 mM increased the α-helical content to 30% with little change in the β-structure.

Trypsin digestion of calsequestrin in the presence or absence of Ca^{2+} produced a rapid decrease in the helical content of the protein while the β-structure was maintained. The α-helical content in the digest which was cleaved in the presence of calcium decreased from 30% to 1% after 10 min of digestion and did not change over the following 3-h digestion period. A similar effect was also found if the digestion was carried out in the absence of Ca^{2+} although a small decrease in the β-structure took place over the 3-h digestion period.

In some cases, KCl was included in the digestion medium to prevent precipitation which was produced by addition of 4 mM $CaCl_2$. It may be possible that certain peptides are precipitated in the presence of Ca^{2+} and therefore do not contribute to the circular dichroism spectrum. No water-insoluble peptides were produced by trypsin digestion in the presence of Ca^{2+}; indeed, trypsin treatment of Ca^{2+}-precipitated calsequestrin resulted in solubilization of the precipitate. A marked difference in the conformation of the tryptic peptides was observed if digestion and the circular dichroism measurements were carried out in the presence of 0.4 M KCl. In the presence of 0.4 M KCl, the α-helical content of the tryptic digest prepared in the presence of Ca^{2+} was only decreased slightly from 27% to 22%. In contrast, the tryptic peptides prepared in the absence of Ca^{2+} lost all of their helical content even in the presence of 0.4 M KCl.

Fragmentation and Sequencing. Carboxyl-terminal peptides of rabbit skeletal muscle calsequestrin (Table II) produced by trypsin, chymotrypsin in the presence of 1 mM $CaCl_2$, and cyanogen bromide cleavage were purified by gel filtration and ion-exchange chromatography. The carboxyl-terminal tryptic peptide was identified since it contained no arginine or lysine residues (Table II). Carboxypeptidase Y was used to determine the carboxyl-terminal amino acid sequence of intact calsequestrin and tryptic peptide; 2.8 mol of aspartic acid and 1.3 mol of glutamic acid were generated per mole of protein after 60 min of digestion; 0.8 mol of leucine per mole of protein was also released; 2.1 mol of aspartic acid, 1.5 mol of glutamic acid, and 0.8 mol of leucine were released per mole of tryptic peptide under identical digestion conditions which confirms that this peptide was derived from the carboxyl-terminal region of the intact protein. This tryptic peptide was subjected to automated Edman degradation, and a total of 50 amino acid residues were sequenced (Figure 3). A low yield of aspartate was obtained at residue number 15.

A carboxyl-terminal chymotryptic peptide which overlapped with the sequence of the tryptic peptide was purified (Table II), and the first 32 residues were sequenced (Figure 4). This peptide contained a number of sites that are potentially chymotrypsin sensitive; however, cleavage at these positions was restricted in the presence of 1 mM $CaCl_2$. This protection of

Table II: Amino Acid Compositions of Calsequestrin Peptides

	tryptic peptide analysis	tryptic peptide sequence	chymotryptic peptide analysis	chymotryptic peptide sequence	CNBr peptide analysis	CNBr peptide sequence
Asp	15.4	14	8.5	10	10.1	10
Asn		2		1		1
Thr	2.9	3	1.4	1	1.1	1
Ser	3.0	3	1.3	1	1.3	1
Glu	12.6	11	10.8	11	10.6	10
Gln		1		0		0
Pro	2.3	2	1.4	1	0.7	1
Gly	2.7	2	1.5	1	1.3	1
Ala	3.3	3	1.4	1	1.0	1
Cys	N.D.[a]	0	N.D.	0	N.D.	0
Val	3.2	5	1.4	1	0.8	1
Met	2.3	2	1.9	2		
Ile	2.4	3	1.4	1	1.2	1
Leu	6.2	5	4.5	4	3.9	4
Tyr						
Phe	0.7	1				
His						
Lys						
Arg						
Trp	N.D.	2	N.D.	1	N.D.	1

[a] N.D., not determined.

FIGURE 3: Carboxyl-terminal sequence of rabbit skeletal muscle calsequestrin. TP1 and T2-2, tryptic peptide; CT4-C, chymotryptic peptide generated in the presence of 4 mM $CaCl_2$; CNBr3-DE2 and CNBr2-DE2, cyanogen bromide peptides.

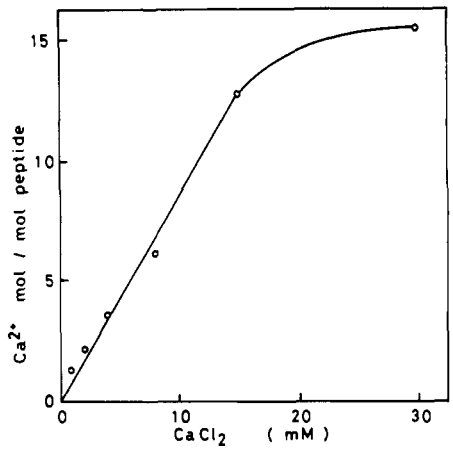

FIGURE 4: Calcium binding of the carboxyl-terminal tryptic peptide of calsequestrin. Calcium binding is plotted as a function of the free calcium concentration. Carboxyl-terminal peptide of rabbit skeletal calsequestrin (48 μmol) was applied to a column of Bio-Gel P-2 (1.0 cm × 30 cm) in the presence of 1–30 mM $CaCl_2$ with $^{45}Ca^{2+}$ (40 μCi/100 mL) in 10 mM Tris-HCl, pH 7.5, at room temperature. Elution of peptides was monitored at 215 and 280 nm. Aliquots of 50 μL were taken for measurement of radioactivity.

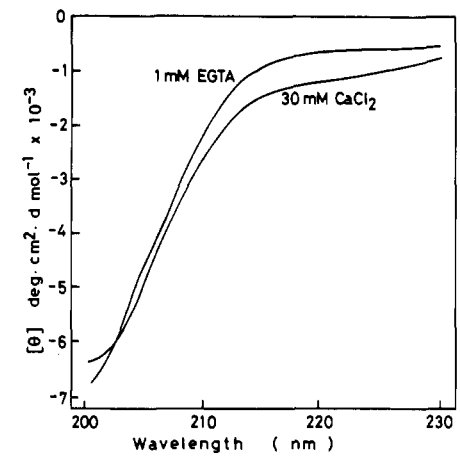

FIGURE 5: Circular dichroism spectra of the carboxyl-terminal tryptic peptide of calsequestrin. CD spectra were taken in the presence of 30 mM $CaCl_2$ or 1 mM EGTA in 0.1 M KCl/10 mM Tris-HCl, pH 7.5. The peptide concentration was 1 mg/mL.

calsequestrin against proteolytic digestion using chymotrypsin confirms the results described previously using trypsin.

A cyanogen bromide peptide containing no homoserine was purified (Table II) and was sequenced (Figure 3). This peptide had an overlapping sequence with the carboxyl-terminal tryptic and chymotryptic peptides and corresponds to the carboxyl terminus of calsequestrin. No further residues were detected after the terminal glutamic residue by automated Edman degradation. In addition, the amino acid composition of this cyanogen bromide peptide agreed well with that calculated from the sequence (Table II). The number of aspartic residues at the carboxyl-terminal region was difficult to establish. There may only be four aspartates rather than five. The amino acid sequence of a cDNA molecule corresponding to neonatal calsequestrin (Fliegel et al., 1987) was different at the carboxyl terminus, having four aspartate residues and a glutamate followed by eight additional aspartate residues. The mature form of calsequestrin may have arisen by differential splicing of the mRNA or perhaps by posttranslational removal of the terminal aspartate residues. It is also possible that loss of the terminal aspartate residues occurred during isolation of the protein.

It is clear that the carboxyl terminus of calsequestrin is very acidic. The tryptic peptide contains no histidine, lysine, or arginine residues. There are 14 aspartic and 11 glutamic residues with only 2 asparagines and a single glutamine residue. The tryptic peptide has a net negative charge of −25. In addition, the site of glycosylation is located at asparagine-15 of the tryptic peptide. This sequence is Asn-Val-Thr, consistent with the Asn-X-Thr/Ser sequence required for N-linked glycosylation. This is the only possible glycosylation site in the protein sequence (Fliegel et al., 1987), and no asparagine was detected by sequencing.

Calcium Binding. Since it is likely that acidic residues are involved in Ca^{2+} binding, we determined directly the Ca^{2+} binding properties of the carboxyl-terminal tryptic peptide. The calcium binding capacity of the carboxyl-terminal tryptic peptide of calsequestrin was examined using $^{45}Ca^{2+}$ and a Bio-Gel P-2 gel filtration column in 10 mM Tris-HCl/100 mM KCl, pH 7.5, in the presence of from 1 to 30 mM $CaCl_2$ (Figure 4). With 30 mM $CaCl_2$, the peptide was saturated by 15 mol of calcium ions bound per mole of peptide. This portion of calsequestrin may bind fewer Ca^{2+} in the intact protein since fragmentation increased the total amount of Ca^{2+} bound by the peptide (Figure 2). The dissociation constant (K_d) of the peptide for calcium ion was 8.5 mM calculated from the half-maximum of the binding capacity. Intact calsequestrin has a calcium binding capacity of 40 mol/mol of protein in the presence of 100 mM KCl, with a binding constant of 1 mM (MacLennan & Wong, 1971; Ikemoto et al., 1972, 1974; Ostwald & MacLennan, 1974; Cozens & Reithmeier, 1984). The intact protein was saturated with calcium at a Ca^{2+} concentration of 3 mM, whereas the carboxyl-terminal tryptic peptide bound only two calcium ions at this Ca^{2+} concentration. This implies that after the digestion of calsequestrin using trypsin, the carboxyl-terminal peptide, which is the largest fragment, and is highly negatively charged, has a decreased calcium binding affinity.

Circular Dichroism Spectra. The effect of Ca^{2+} on the conformation of the carboxyl-terminal tryptic peptide of calsequestrin was investigated using circular dichroism spectroscopy (Figure 5). There was little change observed on the CD spectrum even in the presence of 30 mM $CaCl_2$. The α-helix (2%), β-structure (26%), β-turn (27%), and random-coil (45%) contents in 30 mM $CaCl_2$ were calculated according to Chen et al. (1974). In the presence of 1 mM EGTA, they were 1%, 24%, 29%, and 46%, respectively. This suggests that there was no major conformational change induced in the carboxyl-terminal tryptic peptide even after binding 15 calcium ions per mole of peptide. Similar results were obtained with the carboxyl-terminal peptides obtained by chymotrypsin and CNBr cleavages (data not shown). This result implies that the binding of calcium ion to the carboxyl-terminal tryptic peptide has no major effect on the structure of the peptide.

Fluorescence Spectra. The carboxyl-terminal tryptic peptide contains two tryptophan residues at positions 23 and 41 (Figure 3). We therefore determined the effect of calcium binding on the fluorescence properties of this peptide. The spectra were measured with an emission at 350 nm and excitation of aromatic residues at 280 nm. The emission spectra were enhanced maximally by 10% at a calcium concentration of 5 mM and above. The dissociation constant calculated from the half-maximum of the enhancement is 1.0 mM in the presence of 100 mM KCl. These changes are less profound than those found with the intact protein that undergoes a 2-fold enhancement in intrinsic fluorescence upon binding Ca^{2+} (Ikemoto et al., 1974).

Terbium Binding. Since Ca^{2+} binds to calsequestrin with a low affinity and fragmentation causes a 10-fold decrease in

affinity, it is therefore difficult to distinguish specific from nonspecific binding. We therefore examined the ligand binding properties of the carboxyl-terminal peptide using Tb^{3+}, a high-affinity Ca^{2+} analogue (Dockter, 1983). Terbium ion has been reported to bind to ~30 sites on intact calsequestrin with an affinity of 7 μM, and Ca^{2+} was found to displace and compete with Tb^{3+} binding (Ohnishi & Reithmeier, 1987). The Tb^{3+} binding properties of the carboxyl-terminal tryptic peptide of calsequestrin were studied by equilibrium dialysis and fluorescence spectroscopy. The amount of terbium binding to the carboxyl-terminal tryptic peptide was measured by equilibrium dialysis and a dipicolinic acid assay. Tb^{3+} binding was measured in 25 mM PIPES, pH 6.8, while Ca^{2+} binding was performed in 10 mM Tris-HCl, pH 7.4. This small difference in pH does not affect Ca^{2+} binding (MacLennan & Wong, 1971). This direct binding measurement of terbium to the peptide showed that the maximum number of terbium binding sites is 7 mol/mol of protein with an affinity of 7.5 μM. No further increase in Tb^{3+} binding was seen up to 50 μM Tb^{3+}, indicating that there was little or no nonspecific binding of Tb^{3+} to the carboxyl-terminal peptide. The affinity of the peptide for Tb^{3+} is similar to the affinity of intact calsequestrin for Tb^{3+} (Ohnishi & Reithmeier, 1987). Since calsequestrin binds ~30 Tb^{3+} ions per molecule, the carboxyl-terminal peptide does not contain all the Tb^{3+} binding sites.

A dramatic enhancement of terbium fluorescence at 545 nm was observed upon binding of the metal to the carboxyl-terminal peptide, both by exiciting the bound metal directly at 222 nm and by energy transfer from tryptophan by exciting the protein at 295 nm (data not shown). This suggests that terbium ions bind in close proximity to aromatic residues, especially Trp-23 or Trp-41 (Figure 3). The fluorescence terbium binding data were analyzed with Adair's equation (see Materials and Methods). The number of ligand binding sites was first assumed as two, and the most probable binding constants were calculated; then the number was increased in succession up to seven binding sites. The binding constants for the peptide in 100 mM KCl/25 mM PIPES, pH 6.5, were calculated from the fluorescence data (exciting terbium directly at 222 nm) to be 208, 286, 67, 100, 50, 33, and 5 μM for the seven terbium binding sites. Similar affinities were calculated in the absence of KCl and by exciting the protein at 295 nm. It is clear that the Tb^{3+} binding sites do not have an equal affinity for the metal. An examination of the sequence also reveals that the Tb^{3+} binding sites are not identical in terms of structure as there is no repeating sequence within the calsequestrin molecule (Fliegel et al., 1987).

Discussion

Calsequestrin, which consists of 360 amino acid residues (Fliegel et al., 1987), is not an EF-hand protein, as one EF-hand conformation requires about 28 residues to form a helix–loop–helix conformation (Kretsinger & Nockolds, 1973). On the basis of the size of calsequestrin, this protein could only contain a maximum of 14 EF hands, whereas calsequestrin binds more than 40 calcium ions per mole. Calsequestrin is not a Gla-containing protein, as no Gla has been detected after alkaline hydrolysis (data not shown). Calsequestrin can be phosphorylated in vitro (Varsanyl & Heilmeyer, 1979a,b; Campbell & Shamoo, 1980); however, the possible number of phosphorylation sites is too small to bind 40 calcium ions per mole of calsequestrin. The amino acid sequence homologies of the carboxyl-terminal peptide of calsequestrin to other calcium binding proteins, i.e., calmodulin–troponin C family (EF-hand protein), prothrombin family [γ-carboxyglutamic acid (Gla)-containing protein], phosphoproteins, and Ca^{2+}-ATPase in sarcoplasmic reticulum, were monitored by using a diagonal method (Gibbs & McIntyre, 1970). However, no homology has been detected to those proteins, indicating that calsequestrin is a unique calcium binding protein.

The binding of Ca^{2+} induces a conformational change in calsequestrin, making the protein more α-helical and more resistant to trypsin cleavage. In the presence of calcium, some of the arginine or lysine residues are protected directly by Ca^{2+} or are buried within the folded conformation so that they cannot be cleaved by the enzyme, while residues exposed on the surface can be cleaved by enzyme. If the protein is cleaved with trypsin, the peptides retain their ability to bind Ca^{2+}, however, with a lower affinity than the intact protein. It is clear that fragmentation has affected the calcium binding properties of calsequestrin. It is difficult, however, to distinguish specific from nonspecific binding in this system since calsequestrin binds a large amount of calcium with low affinity. The intact protein and the peptides do show saturable binding with binding constants in the range of the calcium concentration present within the sarcoplasmic reticulum. The carboxyl-terminal peptide binds a maximum of 15 Ca^{2+} with a 10-fold decrease in affinity relative to the intact protein, making a clear differentiation between specific and nonspecific binding even more difficult. The terbium binding studies indicate, however, that the carboxyl-terminal peptide binds Tb^{3+} with the same average affinity as the intact protein.

The capacity of the tryptic peptides for Ca^{2+} has increased, suggesting that not all potential Ca^{2+} ligands bind Ca^{2+} in the intact protein. These ligands are likely acidic residues that become buried by forming ionic bonds with lysine and arginine residues. This is supported by the finding that many of the lysine and arginine residues are not accessible to trypsin cleavage in the presence of calcium. The tryptic peptides do not remain associated in the presence of Ca^{2+}, suggesting that Ca^{2+} is not bridging different regions of the polypeptide but likely is binding to adjacent or single carboxyl groups. The peptides undergo modest changes in conformation in contrast to the intact protein. This may, however, be a consequence of the weaker binding constants and disruption of the native conformation of the protein.

A possible mechanism for calcium binding by calsequestrin is that glutamic and aspartic residues provide negative charges to interact with positively charged calcium ion (Ostwald & MacLennan, 1974). In metal binding, calcium ion, as well as sodium, potassium, and magnesium ions, can form octahedron-type complexes with six ligands from carbonyl, hydroxy, or carboxyl groups on proteins. In practically all cases of calcium binding proteins of which structures were determined by X-ray diffraction, calcium ion binds to carboxyl groups of aspartic and glutamic residues with oxygens of other carbonyl groups and hydroxy groups. The carboxyl-terminal tryptic peptide of calsequestrin is very unusual as it contains a high amount of glutamic and aspartic residues (Asp + Glu = 42.4%). In this regard, those acidic amino acid residues found in the carboxyl-terminal peptide of calsequestrin are likely to be involved in calcium binding. In this paper, we have shown directly that this region of calsequestrin is capable of binding 15 Ca^{2+}, 1 Ca^{2+} for every 2 negative charges. In addition, this peptide binds Tb^{3+} with the same average affinity as the intact protein, and like calsequestrin, the carboxyl-terminal peptide has a lower capacity for this metal when compared to calcium. The terbium binding studies have also shown that the metal binding sites on the carboxyl-terminal peptide have different binding constants.

Circular dichroism spectra showed that the carboxyl-terminal tryptic peptide of calsequestrin was primarily in a random-coil conformation regardless of the calcium concentration. A 10% increase in the intrinsic fluorescence of the peptide occurred upon Ca^{2+} binding. These studies indicate that although this peptide binds calcium ions, its conformation is hardly changed even after binding 15 calcium ions per mole of peptide. This might be due to only adjacent carboxyl groups participating in the binding of calcium ions. For example, two clusters of acidic amino acid residues (residue numbers 25–31 and 53–59) of the carboxyl-terminal region could form the higher affinity calcium binding sites, and pairs of acidic residues could form the lower affinity sites. However, the binding of calcium to the carboxyl-terminal tryptic peptide of calsequestrin will not be formed with the trans-extended chain manner as described by Wiener and Hood (1975), since α-helix or β-structure changes in the peptide were not induced after binding calcium. The binding of Ca^{2+} to the carboxyl-terminal region may cause conformational changes in other portions of the molecule. We are examining the calcium binding properties of other regions of calsequestrin in order to determine which regions of the protein undergo conformational changes upon binding calcium.

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Registry No. Ca, 7440-70-2; Tb, 7440-27-9.

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