Characterization of Cardiac Calsequestrin†

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ABSTRACT: Calsequestrin, a calcium-binding protein found in the sarcoplasmic reticulum of muscle cells, was purified from rabbit and canine cardiac and skeletal muscle tissue. The amino acid compositions and amino-terminal sequences of skeletal and cardiac calsequestrin from rabbit and dog were determined. The amino acid composition of the cardiac form was very similar to the skeletal form. The amino-terminal sequence of the cardiac form was homologous to, but not identical with, the amino-terminal sequence of the skeletal form of the protein. Few species differences in the amino-terminal sequences were observed. The calcium-binding capacity of the cardiac form was half the capacity of the skeletal form although the affinities of the two forms of calsequestrin for Ca^{2+} were similar (K_d = 1 mM). Calcium binding to the cardiac form induced structural changes in the protein as determined by circular dichroism and intrinsic fluorescence spectroscopy. The α-helical content of cardiac calsequestrin increased from 3.5% to 10.9% upon binding calcium, while the intrinsic fluorescence of the protein increased 14%. Potassium ions also affected the conformation of cardiac calsequestrin.

Calsequestrin is a Ca^{2+}-binding protein found in the terminal cisternae of the sarcoplasmic reticulum of muscle cells (Meissner, 1975; Jorgensen et al., 1977, 1979, 1982a,b; Campbell et al., 1980; MacLennan et al., 1983; Saito et al., 1984). This protein has been purified from both skeletal muscle and cardiac muscle (MacLennan & Wong, 1971; Ikemoto et al., 1972a,b; MacLennan, 1974; Cala & Jones, 1983; Campbell et al., 1983; White et al., 1983; Mauer et al., 1985). Calsequestrin prepared from rabbit skeletal muscle has been extensively characterized (MacLennan et al., 1983). This protein has a molecular weight of ~40000 (Cauldwell et al., 1978; MacLennan et al., 1983; Cozens & Reithmeier, 1984) and has a very acidic amino acid composition (MacLennan & Wong, 1971; Ikemoto et al., 1974; MacLennan, 1974; Cauldwell et al., 1978; White et al., 1983). The complete amino acid sequence of calsequestrin from rabbit skeletal muscle was recently deduced from cDNA and peptide sequencing (Fliegel et al., 1987). Rabbit skeletal muscle calsequestrin binds 40–50 Ca^{2+} per molecule with an affinity of 1 mM under physiological conditions (MacLennan & Wong, 1971; Ikemoto et al., 1974; MacLennan, 1974; Ostwald et al., 1974; Cozens & Reithmeier, 1984; Mauer et al., 1985). The function of calsequestrin is to bind large amounts of Ca^{2+}, thereby lowering the level of free Ca^{2+} within the lumen of sarcoplasmic reticulum. Since calsequestrin is localized to the terminal cisternae, most of the bound Ca^{2+} within the sarcoplasmic reticulum would be localized to this region of the sarcoplasmic reticulum, the site of Ca^{2+} release. Whether calsequestrin plays a direct role in Ca^{2+} release, however, is not known. Skeletal calsequestrin undergoes dramatic conformational changes upon binding Ca^{2+} (Ikemoto et al., 1972, 1974; Ostwald et al., 1974; Cala & Jones, 1983; Aaron et al., 1984; Cozens & Reithmeier, 1984). Circular dichroism studies (Ikemoto et al., 1972, 1974; Ostwald et al., 1974; Michalak et al., 1980; Aaron et al., 1984) have shown that the α-helical content of the protein increases from 10–13% to 20–35% upon binding Ca^{2+}. There is also a doubling of the intrinsic fluorescence of the protein upon binding Ca^{2+} (Ikemoto et al., 1972, 1974), consistent with a burying of aromatic residues. This change in conformation is also observed in studies of the interaction of calsequestrin with phenylSepharose (Cala & Jones, 1983). Calsequestrin binds to this resin in the absence of Ca^{2+}, where a hydrophobic domain is exposed, and is eluted by Ca^{2+}, where the hydrophobic domain is buried. NMR studies have also shown that calsequestrin undergoes dramatic changes in its structure upon binding Ca^{2+} (Aaron et al., 1984). Other cations also bind to calsequestrin, inducing similar structural changes (Ikemoto et al., 1972, 1974; Ostwald et al., 1974; Aaron et al., 1984). K^+ lowers the affinity of Ca^{2+} for calsequestrin (Ikemoto et al., 1972, 1974; MacLennan, 1974; Aaron et al., 1984). Changes in the level of K^+ within the sarcoplasmic reticulum may therefore modulate the Ca^{2+}-binding properties of calsequestrin.

While there is a great deal of information concerning skeletal calsequestrin, there is a paucity of information concerning cardiac calsequestrin. The cardiac form of calsequestrin has an apparent molecular weight of 55000 as determined by sodium dodecyl sulfate gel electrophoresis using the Laemmli gel system and an apparent molecular weight of 44000 using a Weber and Osborn gel system (Campbell et al., 1983). The skeletal form of calsequestrin shows similar anomalous behavior on sodium dodecyl sulfate gel electrophoresis (Michalak et al., 1980). The cardiac protein, like the skeletal form, stains blue with Stains-All (Jones et al., 1979; Jones, 1981; Cala & Jones, 1983; Campbell et al., 1983). Both forms are glycoproteins; however, only the mobility of the cardiac form on sodium dodecyl sulfate gel electrophoresis is changed by endoglycosidase H digestion (Campbell et al., 1983). Both proteins bind large amounts of Ca^{2+}; however, the cardiac form appears to have a lower Ca^{2+}-binding capacity (Cala & Jones, 1983; Campbell et al., 1983). This may account for the lower capacity of cardiac sarcoplasmic reticulum vesicles for Ca^{2+} (Schwartz et al., 1979). The amino acid compositions of the skeletal and cardiac forms are very similar and indicate that cardiac calsequestrin is also a very acidic protein (Campbell et al., 1983). Antibodies raised against rat skeletal calsequestrin cross-react with canine cardiac calsequestrin (Campbell et al., 1983). Both forms of the protein

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bind to phenylSepharose in the absence of Ca^{2+} (Cala & Jones, 1983). In this paper, we present a characterization of canine cardiac calsequestrin. The amino acid composition of calsequestrin prepared from canine cardiac tissue was compared to those of canine skeletal calsequestrin as well as the rabbit skeletal and cardiac forms of this protein. The amino-terminal sequences of rabbit skeletal and cardiac calsequestrin and canine cardiac calsequestrin were determined. The Ca^{2+}-binding properties and the effect of Ca^{2+} binding on the structure of canine cardiac calsequestrin were studied by using $^{45}Ca^{2+}$ binding, circular dichroism, and fluorescence spectroscopy.

Materials and Methods

Purification of Calsequestrin. A rapid method for the purification of calsequestrin was developed in the laboratory of Dr. K. P. Campbell, University of Iowa. This method involved direct extraction of calsequestrin from whole muscle homogenates and eliminates the need to prepare a sarcoplasmic reticulum fraction. Fresh canine hearts were provided by T. Germaine of the Surgical–Medical Research Institute at the University of Alberta. No animals were sacrificed for these experiments; all dogs had been used for surgical training or other medical experiments. All procedures were carried out at 0–4 °C unless otherwise stated. The muscle was homogenized at high speed in a blender for 1 min in 4 volumes/g of tissue of 0.1 M potassium phosphate buffer, pH 7.1, containing 2.66 M $(NH_4)_2SO_4$ (65% saturation), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5 mM phenylmethanesulfonyl fluoride, and 0.5 mM benzamidine. The homogenate was spun at 13000*g* for 30 min using a Sorvall GSA or Beckman JA-10 rotor. The supernatant was filtered through cheesecloth and saved. The pellet was rehomogenized for 1 min using half the volume of buffer used originally. The homogenate was centrifuged as before, and the supernatant was filtered and combined with the first supernatant; 0.5 mL each of 0.1 M phenylmethanesulfonyl fluoride and 0.1 M benzamidine was added per 100 mL of supernatant; 150 g of solid $(NH_4)_2SO_4$ was added per liter of supernatant to achieve 85% saturation of $(NH_4)_2SO_4$, and the suspension was stirred for 30 min. The pH was adjusted to 4.7 using phosphoric acid and the suspension was stirred for at least 3 h in the cold room. The precipitate was collected by centrifugation at 13000*g* for 30 min. The pellet was dissolved in 80 mL of 0.1 M potassium phosphate buffer, pH 7.1, containing 1 mM EGTA, and dialyzed against the same buffer overnight and for 4 additional h after changing the buffer in the morning. The solution was clarified by centrifugation at 9500*g* for 10 min. The supernatant was applied to a 2.5 × 8 cm column of fresh DEAE-cellulose equilibrated with 0.1 M potassium phosphate, pH 7.1, containing 1 mM EGTA and 50 mM NaCl. The column was washed with 5 volumes of the initial buffer, and then a linear gradient of NaCl from 50 mM to 0.7 M was applied. Three-milliliter fractions were collected, and the protein content of each fraction was monitored by the absorption at 280 nm, by the Lowry protein assay, and by sodium dodecyl sulfate (SDS) and urea gel electrophoresis. The fractions containing calsequestrin were pooled and dialyzed against 15 mM NH_4HCO_3 and freeze-dried. An additional purification step involving the use of phenylSepharose was sometimes employed (Cala & Jones, 1983). PhenylSepharose chromatography was carried out at room temperature. Briefly, calsequestrin dissolved in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 500 mM NaCl, and 1 mM dithiothreitol was applied to a fresh phenylSepharose column equilibrated with the same buffer. Two milligrams of protein was applied per milliliter of resin. The column was washed with the same buffer until the absorption at 280 nm was at base-line levels. Calsequestrin was eluted by the same buffer containing 10 mM $CaCl_2$.

Skeletal muscle calsequestrin was purified by using a similar procedure. The only difference was to precipitate calsequestrin with 200 g of $(NH_4)_2SO_4$ per liter of supernatant (to achieve 92% saturation) rather than 150 g. PhenylSepharose chromatography was not usually required to obtain a pure preparation of rabbit or dog skeletal muscle calsequestrin.

Amino Acid Analysis. Samples of calsequestrin were hydrolyzed under vacuum in 6 N HCl at 100 °C for 20 h. For the cysteine content determination, the protein was oxidized by performic acid and then hydrolyzed with 6 N HCl at 110 °C for 20 h (Hirs, 1967). For the determination of tryptophan, the protein was hydrolyzed in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110 °C for 20 h (Simpson et al., 1976). Hydrolysates were analyzed with a Dionex Model D-500 amino acid analyzer.

Protein Sequencing. Automated sequence analyses were performed on an Applied Biosystems Model 470A protein sequencer (Hewick et al., 1981). Phenylthiohydantoin–amino acid (PTH–amino acid) analyses were made either by the method of Somack (1980) on a Spectra Physics Model 8000B liquid chromatograph fitted with an Altex Ultrasphere 5-μm ODS column (4.6 × 250 mm), a Schoeffel SF770 detector, and a Wisp 710 automatic injector or by an on-line Applied Biosystems 120A PTH analyzer using the current protocol of Applied Biosystems on a Spheri-5 C18 column (2.1 × 220 mm). All sequencer chemicals were from Applied Biosystems. The amount of sample injected was varied from 0.1 to 3 nmol.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoreses were performed with 12% acrylamide in the separating gel and 4% acrylamide in the stacking gel containing 0.09% SDS according to Laemmli (1970), with 7.5% acrylamide containing 1% SDS according to Weber and Osborn (1969), or with 7.5% acrylamide containing 6 M urea, 0.025 M tris(hydroxymethyl)aminomethane (Tris), and 0.08 M glycine, pH 8.6. SDS gel electrophoreses were run using a Bio-Rad Mini Protean II mini slab gel system, and urea-containing gel electrophoreses were run using a Bio-Rad Protean vertical slab gel unit.

Circular Dichroism Spectroscopy. The circular dichroism spectra were recorded on a Jasco Model J-500 spectropolarimeter using a cell with a path length of 0.0103 cm. Proteins were dissolved in 10 mM Tris-HCl, pH 7.5, in the absence or presence of 0.1 or 0.4 M KCl. Protein concentrations (0.5 and 1.0 mg/mL) were calculated by using amino acid analysis assuming a molecular weight of 40000. The ellipticities were calculated by using a value of 115 for the mean residue molecular weight, and the α-helical, β-sheet, turn, and random-coil contents were calculated according to Chen et al. (1974).

Fluorescence Spectroscopy. Fluorescence spectra were taken on a Perkin-Elmer MPF-44B spectrofluorometer at 20 °C with both emission and excitation slit widths of 8 nm. The protein was dissolved in 25 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), pH 6.5, and a 1.0 × 1.0 cm cuvette was used. Intrinsic protein fluorescence was measured at 340 nm by exciting the protein at 280 nm.

Results and Discussion

Purification of Calsequestrin. The procedure employed for the purification of calsequestrin takes advantage of the high solubility of calsequestrin in solutions of ammonium sulfate. A crude muscle homogenate was extracted with 65% saturated

Table I: Amino Acid Compositions[a] of Skeletal and Cardiac Calsequestrins

amino acid	rabbit skeletal	rabbit cardiac	canine skeletal	canine cardiac
Asp	60.8	51.7	58.8	61.1
Thr	10.9	14.0	11.7	11.3
Ser	15.2	ND[d]	14.3	15.7
Glu	63.2	58.3	62.2	58.8
Pro	17.9	13.8	16.5	14.5
Gly	17.1	ND	16.7	19.2
Ala	26.3	26.4	28.8	21.0
Cys[b]	0.0	ND	0.0	1.2
Val	22.2	21.0	21.1	23.4
Met	5.1	5.6	6.1	5.5
Ile	17.6	18.0	16.4	17.5
Leu	34.1	30.3	35.2	31.7
Tyr	10.6	ND	11.4	10.2
Phe	18.9	17.5	20.5	21.8
His	5.2	6.5	4.7	4.8
Lys	26.9	25.2	23.6	28.9
Arg	7.5	7.6	7.2	8.5
Trp[c]	5.0	ND	5.2	5.3
no. of determinations	6	2	4	5

[a] Residues per molecule assuming a molecular weight of 40 000. [b] Determined as cysteic acid after performic acid oxidation. [c] Determined after being hydrolyzed with 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. [d] Not determined.

ammonium sulfate which precipitated most muscle proteins. Calsequestrin could be precipitated from this solution by lowering the pH and increasing the concentration of ammonium sulfate. Pure cardiac calsequestrin could be obtained after DEAE-cellulose and phenylSepharose chromatography at a yield of 2 mg from a single fresh dog heart weighing about 250 g. When frozen dog hearts were used, a lower yield of 1.4 mg per heart was obtained. In addition, proteolysis of calsequestrin was a problem if frozen heart preparations were used. One milligram of calsequestrin was isolated from 15 frozen rabbit hearts weighing a total of 86 g. Pure skeletal muscle calsequestrin could be isolated from dog and rabbit skeletal muscle after DEAE-cellulose chromatography. The yield was approximately 50 mg from 250 g of fresh muscle. In some preparations, an additional step involving chromatography on phenylSepharose was employed (Cala & Jones, 1983). The yield of calsequestrin was, however, only 25% of the applied protein.

Amino Acid Composition of Calsequestrin. Table I presents the amino acid compositions of calsequestrin purified from rabbit and canine skeletal and cardiac muscles. The proteins were purified with a similar procedure, and the hydrolysis and analysis conditions were identical. It is apparent that the compositions are very similar. Cardiac calsequestrin, like the skeletal protein, has a high content of acidic amino acid residues. Analysis for cysteic acid showed that the skeletal form of calsequestrin lacked cysteine residues. The canine cardiac protein, however, contained 1.3 mol of cysteine per molecule as determined by amino acid analysis for cysteic acid. The presence of a free sulfhydryl group in cardiac calsequestrin was confirmed by the observation that canine cardiac calsequestrin bound to a sulfhydryl affinity column [i.e., Pharmacia activated thiol-Sepharose or [[[*p*-(chloromercuri)benzamido]ethylene]amino]Sepharose 4B] while the rabbit skeletal form was not retained by such columns. Cardiac calsequestrin could be eluted from the sulfhydryl column by reducing agents such as β-mercaptoethanol or dithiothreitol. In addition, cardiac calsequestrin formed dimers visible on sodium dodecyl sulfate gels if reducing agents were not included in the sample buffer.

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Rabbit Skeletal   Glu-Glu-Gly-Leu-Asp-Phe-Pro-Glu-Tyr-Asp-Gly-Val-Asp-Arg-Val-15
Canine Skeletal   Glu-Glu-Gly-Leu-Asp-Phe-Pro-Glu-Tyr-Asp-Gly-Val-Asp-Arg-Val-
Canine Cardiac    Glu-Glu-Gly-Leu-ASN-Phe-Pro-THR-Tyr-Asp-Gly-LYS-Asp-Arg-Val-
Rabbit Cardiac    Glu-Glu-Gly-Leu-ASN-Phe-Pro-THR-Tyr-Asp-Gly-LYS-Asp-Arg-Val-

Rabbit Skeletal   Ile-Asn-Val-Asn-Ala-Lys-Asn-Tyr-Lys-Asn-Val-Phe-Lys-Lys-Tyr-30
Canine Skeletal   VAL-Asn-Val-Asn-Ala-Lys-Asn-Tyr-Lys-Asn-Val-Phe-Lys-Lys-Tyr-
Canine Cardiac    VAL-SER-LEU-THR-GLU-Lys-Asn-PHE-Lys-GLN-Val-LEU-Lys-Lys-Tyr-
Rabbit Cardiac    VAL-SER-LEU-SER-GLU-Lys-Asn-PHE-Lys-GLN-SER-LEU-Lys-Lys-Tyr-

Rabbit Skeletal   Glu-Val-Leu-Ala-Leu-Leu-Tyr-His-Glu-Pro-Pro- X - X - X -Lys-45
Canine Skeletal   Glu-Val-Leu-Ala-Leu-Leu-Tyr-His-Glu-Pro-Pro- X - X -Asp-Lys-
Canine Cardiac    ASP-Val-Leu- X -Leu- X - X - X -Glu- X -VAL- X - X - X -Lys-

Rabbit Skeletal   Ala-Ser-Gln-Arg-Gln-Phe-Glu-Met-Glu-Glu-Leu-Ile- X -Glu-
Canine Skeletal   Ala-Ser-Gln-Arg-Gln-Phe-ASP-Met-Glu-Glu-Leu-
Canine Cardiac    X  -ALA-Gln-LYS-Gln-Phe-Glu-
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FIGURE 1: Amino-terminal sequences of skeletal and cardiac calsequestrin. Calsequestrin was purified from rabbit skeletal and cardiac muscle and canine skeletal and cardiac muscle and subjected to automated Edman degradation using an Applied Biosystems protein sequencer. PTH derivatives were identified by high-performance liquid chromatography (HPLC) analysis. Residues in capital letters are not identical with the rabbit skeletal calsequestrin sequence.

Figure 1 presents the amino-terminal sequences of calsequestrin from rabbit skeletal and cardiac tissue as well as canine skeletal and cardiac tissue. It is apparent that all forms of calsequestrin are highly homologous but not identical. Optimal alignments of these sequences did not require the introduction of any gaps into any of the sequences. Amino acids not identical with the amino-terminal sequence of rabbit skeletal calsequestrin are in capital letters. Only two amino acid residue differences were found between rabbit and canine skeletal muscle calsequestrin within the 53 amino acid common sequence determined for both (96% homology). Rabbit skeletal muscle calsequestrin has an isoleucine residue at position 16 while canine skeletal muscle calsequestrin has a valine residue. The glutamate residue at position 52 of rabbit skeletal calsequestrin was replaced by an aspartate residue in canine skeletal calsequestrin. These changes may be considered as conservative since the nature of the amino acids is not changed and single base changes can account for the differences. Similarly, the amino-terminal sequences of rabbit and canine cardiac calsequestrin are highly homologous (93% homology). A conservative change was found at residue 19, where a serine residue in rabbit cardiac calsequestrin replaces the threonine residue in the canine form. Interestingly, this position was occupied by asparagine residues in both rabbit and canine skeletal muscle calsequestrin. Rabbit cardiac calsequestrin has a serine residue at position 26 while all other forms of the protein have a valine residue at this position.

It is apparent that cardiac and skeletal calsequestrins are homologous molecules. Within the first 46 common residues of the canine forms, 28 amino acid residues are identical (61% homology). Although most of the amino acid differences are conservative, some are not. For example, valine-12 in the skeletal form is replaced by lysine in the cardiac form. Rabbit skeletal and cardiac calsequestrins are also homologous to one another. Out of the first 30 residues, 18 are identical (60% homology).

Few of the glutamate residues are amidated in the amino-terminal sequence of calsequestrin while about half of the aspartate residues are amidated. Glutamate residues likely play an essential role in Ca^{2+} binding. There is no evidence

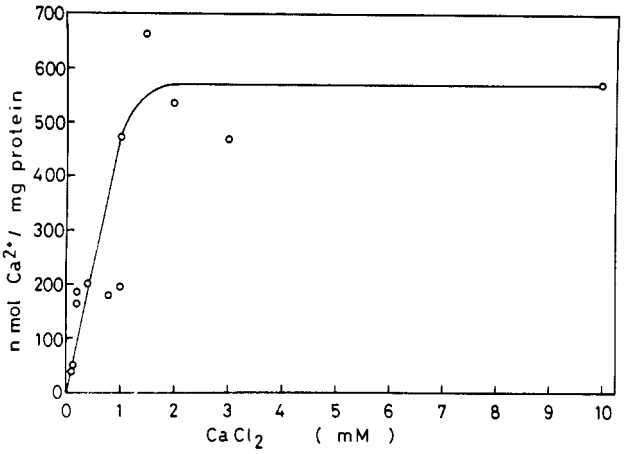

FIGURE 2: $^{45}Ca^{2+}$ binding of cardiac calsequestrin by equilibrium dialysis. 0.5 mL of calsequestrin at 1.0 mg/mL was dialyzed against 100 mL of 100 mM KCl and 10 mM Tris-HCl, pH 7.5, containing various concentrations of Ca^{2+} at room temperature for 18 h. Duplicate aliquots were taken for protein determination and liquid scintillation counting.

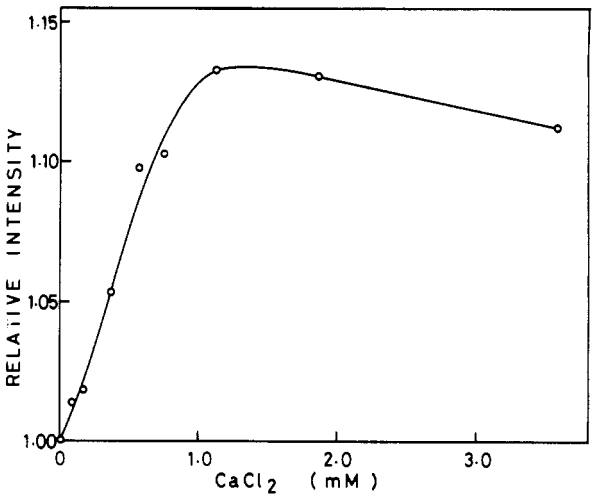

FIGURE 3: Effect of Ca^{2+} on the intrinsic fluorescence of cardiac calsequestrin. The protein was excited at 280 nm, and the fluorescence was measured at 340 nm. The protein concentration was 38 μg/mL in 100 mM KCl and 10 mM Tris-HCl, pH 7.5, and the titration was carried out at 20 °C.

for a repeating distribution of acidic residues within the amino-terminal sequence of calsequestrin.

Molecular Weight Determination. The molecular weight of canine cardiac and skeletal muscle calsequestrins was monitored by using SDS containing polyacrylamide gel electrophoresis. According to the Laemmli gel system, the molecular weights of the proteins were 60 000 and 54 000 for canine skeletal and cardiac muscle calsequestrin, respectively, while according to the Weber and Osborn gel system both proteins had an apparent molecular weight of 44 000. The difference in the observed molecular weight using Laemmli and also Weber and Osborn gel systems is a prominent characteristic of calsequestrin and is due to the abnormal conformation of calsequestrin in sodium dodecyl sulfate and its sensitivity to pH (Cala & Jones, 1983; Campbell et al., 1983; Cozens & Reithmeier, 1984). Michalak et al. (1980) and Campbell et al. (1983) found that calsequestrin falls off the diagonal of a two-dimensional gel system consisting of a Weber and Osborn gel system in the first dimension and a Laemmli gel system in the second dimension.

Ca^{2+} Binding. The Ca^{2+}-binding properties of cardiac calsequestrin have not been well characterized. A single measurement (Campbell et al., 1983) made in 5 mM $CaCl_2$ containing 5 mM Tris-HCl, pH 7.5, and 100 mM KCl revealed that canine cardiac calsequestrin bound 302 nmol of Ca^{2+}/mg of protein, about half the amount of Ca^{2+} bound by rabbit skeletal calsequestrin under identical conditions. Figure 2 presents the Ca^{2+}-binding properties of canine calsequestrin as determined by equilibrium dialyses using $^{45}Ca^{2+}$. Canine cardiac calsequestrin bound a maximum of 550 nmol of Ca^{2+}/mg of protein with a binding constant of 0.4 mM in 100 mM KCl and 10 mM Tris-HCl, pH 7.5 (Figure 2). On the basis of a molecular weight of 40 000, this is equivalent to 18 mol of Ca^{2+} per molecule. The capacity of canine cardiac calsequestrin for Ca^{2+} is half the capacity of rabbit skeletal muscle calsequestrin which binds 40–50 mol of Ca^{2+} per molecule (MacLennan & Wong, 1971; Ikemoto et al., 1974; MacLennan, 1974; Ostwald et al., 1974; Cozens & Reithmeier, 1984; Mauer et al., 1985). The affinity for Ca^{2+} of cardiac calsequestrin (K_d = 0.4 mM) is, however, similar to the affinity of skeletal calsequestrin for Ca^{2+} (K_d = 1 mM; MacLennan & Wong, 1971; Ikemoto et al., 1974; MacLennan, 1974; Ostwald et al., 1974; Cozens & Reithmeier, 1984; Mauer et al., 1985). Skeletal muscle calsequestrin has a slightly higher content of acidic amino acids than cardiac calsequestrin based on amino acid analysis (Table I). Canine skeletal muscle calsequestrin contains 33.6% acidic amino acids and 8.6% basic amino acids, while canine calsequestrin contains 33.3% acidic amino acids and 10.4% basic amino acids. Calculated isoelectric points for those two proteins using Manabe's method (Manabe, 1982) are 3.74 and 3.82 for rabbit skeletal and cardiac muscle calsequestrin, respectively, and 3.68 and 3.80 for canine muscle calsequestrin, respectively, assuming that aspartic and glutamic acids are not amidated. These differences in amino acid compositions would not account for the dramatic reduction in Ca^{2+} binding by cardiac calsequestrin.

We have not accurately determined the isoelectric points for canine cardiac and skeletal muscle calsequestrin; however, isoelectric focusing by gel electrophoresis revealed that the cardiac form had a lower isoelectric point. Also, we have found that cardiac calsequestrin has a higher mobility toward the anode than skeletal muscle calsequestrin during gel electrophoresis in 6 M urea, 0.025 M Tris, and 0.08 M glycine, pH 8.6. These results show that cardiac calsequestrin is more, not less, acidic than skeletal calsequestrin. The number of acidic residues therefore does not correlate directly with the Ca^{2+}-binding capacity. Determination of the complete amino acid sequences of cardiac calsequestrin and its comparison to the sequence of skeletal calsequestrin (Fliegel et al., 1987) will be necessary to account for the differences in Ca^{2+}-binding properties.

Effect of Ca^{2+} on the Conformation of Cardiac Calsequestrin. Rabbit skeletal muscle calsequestrin undergoes dramatic conformational changes upon binding Ca^{2+} (Ikemoto et al., 1972; Cala & Jones, 1983; Aaron et al., 1984; Cozens & Reithmeier, 1984). Similar changes were also observed with the canine cardiac form of the protein (Figures 3–5). The intrinsic fluorescence of canine cardiac calsequestrin was increased by 14% upon binding Ca^{2+} (Figure 3). This may be due to a change in the environment of aromatic residues, most particularly tryptophan residues, from a polar to a less polar environment. A 2-fold increase in the intrinsic fluorescence of rabbit skeletal muscle calsequestrin has been reported (Ikemoto et al., 1972, 1974). The conformational change for cardiac calsequestrin is less profound, or fewer aromatic residues are involved. The maximum fluorescence change was observed with 1.4 mM Ca^{2+}, and the average affinity for Ca^{2+} calculated at half-maximal fluorescence was 0.5 mM, in ex-

Table II: Secondary Structure of Canine Skeletal Muscle and Cardiac Calsequestrins

buffer[a]	skeletal muscle calsequestrin (%)				cardiac calsequestrin (%)			
	α	β	turn	random	α	β	turn	random
10 mM Tris-HCl, pH 7.5, plus	ND[b]	ND	ND	ND	6.6	65.8	3.6	24.0
1.0 mM EGTA	1.2	40.5	4.2	54.1	3.5	73.9	0.2	22.4
0.5 mM $CaCl_2$	27.8	32.0	2.6	37.6	10.9	69.0	2.0	18.1
0.1 M KCl	9.7	38.2	0.9	51.2	2.8	72.5	2.9	21.8
0.1 M KCl + 1.0 mM $CaCl_2$	26.7	28.4	7.4	37.5	2.2	71.5	6.6	19.7
0.4 M KCl	28.9	34.5	3.1	33.5	14.0	58.3	1.7	26.0
0.4 M KCl + 1.0 mM $CaCl_2$	28.5	33.0	0.6	37.9	ND	ND	ND	ND

[a] Sample was dissolved in 10 mM Tris-HCl, pH 7.5, in the absence or presence of 0.1 or 0.4 M KCl. A final concentration of 0.5 or 1.0 mM $CaCl_2$ was added just before the spectral measurement to avoid precipitation of calsequestrin–calcium complex. The protein concentration was between 0.5 and 1.0 mg/mL. Structural contents were calculated according to Chen et al. (1974) assuming a molecular weight for calsequestrin of 40 000 and a mean residue molecular weight of 115. [b] ND, not determined.

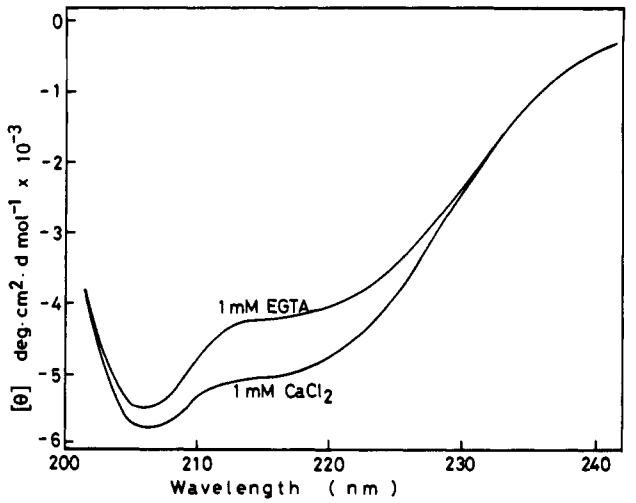

FIGURE 4: Circular dichroism spectra of cardiac calsequestrin in 1 mM EGTA, 10 mM Tris-HCl, pH 7.5, and in 1 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5.

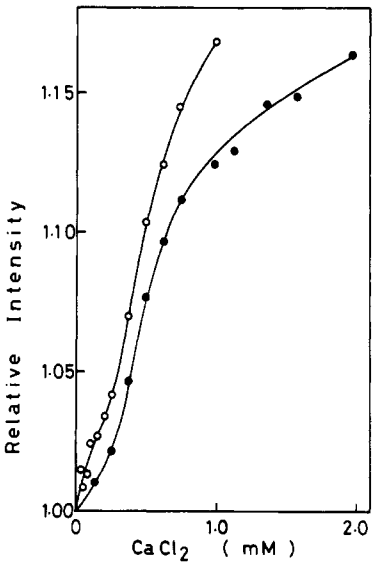

FIGURE 5: Effect of Ca^{2+} on the negative ellipticity of cardiac calsequestrin at 222 nm in 10 mM Tris-HCl, pH 7.5 (○), and in 100 mM KCl and 10 mM Tris-HCl, pH 7.5 (●). The protein concentration was 0.6 mg/mL. All measurements were made at 23 °C.

cellent agreement with the affinity measured by equilibrium dialysis. Quenching of fluorescence was observed at Ca^{2+} concentrations above 2 mM.

Rabbit skeletal muscle calsequestrin is known to undergo profound changes in its circular dichroism spectrum upon binding calcium, consistent with an increase in α-helical content from 10–15% for the apoprotein to 20–34% for the calcium-saturated form (Ikemoto et al., 1972, 1974; Ostwald et al., 1974; Aaron et al., 1984). We therefore wished to examine the effect of Ca^{2+} and K^+ on the circular dichroism spectrum of canine cardiac calsequestrin. In order to eliminate any species differences, the circular dichroism properties of canine skeletal calsequestrin are also reported.

The α-helical content of canine skeletal muscle calsequestrin in 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM Tris-HCl, pH 7.5, was 1% (Table II). It was increased to 27.8% in the presence of 1 mM $CaCl_2$ with a decrease in the β-sheet and random-coil content. This change in structure is similar to that observed with rabbit skeletal muscle calsequestrin (Ikemoto et al., 1972, 1974; Ostwald, 1974). The α-helical content of canine skeletal muscle calsequestrin was also increased by KCl (Table II). The helical content was increased to 9.7% in the presence of 0.1 M KCl and to 28.9% in 0.4 M KCl. Addition of $CaCl_2$ to 1 mM to calsequestrin in 0.1 M KCl increased the helical content of the protein to 26.7%, while a similar addition of $CaCl_2$ to the sample in 0.4 M KCl did not result in a further increase in the helical content.

Analysis of the circular dichroism spectrum (Figure 4) of canine cardiac calsequestrin in 1 mM EGTA and 10 mM Tris-HCl, pH 7.5, revealed that the protein contained 3.5% α-helix, 73.9% β-sheet, 0.2% β-turn, and 22.4% random coil (Table II). These values are similar to those found for the protein in 10 mM Tris-HCl, pH 7.5, without EGTA (Table II). The most significant difference between the structural contents of canine skeletal and cardiac calsequestrin is that the cardiac form has a higher percentage of β-sheet structure. Both forms of calsequestrin have a low helical content in the absence of Ca^{2+} or K^+ with a significant amount of random coil.

$CaCl_2$ induced a dramatic change in the circular dichroism spectrum of canine cardiac calsequestrin, producing an increase in the negative ellipticity of the protein (Figure 4). This corresponds to an increase in the α-helical content of the protein. Analysis of the spectrum of cardiac calsequestrin in 0.5 mM $CaCl_2$ indicated that the α-helical content had increased to 10.9%, while the β-sheet content was 69%, β-turn 2%, and random coil 18.1% (Table II). The α-helical content of canine cardiac calsequestrin was decreased slightly to 2.8% by 0.1 M KCl and increased to 14% by 0.4 M KCl (Table II). The α-helical content of canine cardiac calsequestrin with $CaCl_2$ or 0.4 M KCl was about half of the content of the skeletal form. This implies that cardiac calsequestrin undergoes much less profound changes in its structure upon binding ligands. The structural differences between skeletal and cardiac muscle calsequestrin may reflect the difference in calcium-binding capacities between the two proteins.

The change in ellipticity at 222 nm of cardiac calsequestrin was followed as a function of $CaCl_2$ concentration in the

presence and absence of 0.1 M KCl (Figure 5). Ca^{2+} increased the negative ellipticity of cardiac calsequestrin at 222 nm by 16% at 1 mM $CaCl_2$. Further additions of $CaCl_2$ resulted in precipitation of the protein. The half-maximal change occurred at 0.5 mM $CaCl_2$, similar to the binding constant determined in Figures 2 and 3. A Ca^{2+} titration performed in the presence of 0.1 M KCl also caused a 16% increase in the negative ellipticity at 222 nm (Figure 5). The curve was displaced to the right in the presence of 0.1 M KCl. This indicates that K^+ ions decrease the affinity of cardiac calsequestrin for calcium; 100 mM KCl decreases the affinity of skeletal calsequestrin for Ca^{2+} by 10-fold (Ikemoto et al., 1972, 1974; MacLennan, 1974; Aaron et al., 1984). Potassium ions may play an important modulating role in the affinity of calsequestrin for Ca^{2+}, although the effect of K^+ on the Ca^{2+}-binding properties of cardiac calsequestrin is less than the effect of K^+ on skeletal muscle calsequestrin.

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