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Zn^{2+} BINDING TO CARDIAC CALSEQUESTRIN

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Zn^{2+} binding to canine cardiac calsequestrin was investigated using the Zn^{2+} specific fluorescence dye salicylcarbohydrazone (SACH), $^{65}Zn^{2+}$ overlay and Zn^{2+}-IDA chromatography. Cardiac calsequestrin binds ~200 moles of Zn^{2+}/mole of protein with the K_d=300 μM. Zn^{2+} binding to calsequestrin was further confirmed by $^{65}Zn^{2+}$ overlay and Zn^{2+}-dependent aggregation of the protein. However, calsequestrin did not bind to a Zn^{2+}-IDA-agarose column, indicating that histidine residues may not be involved in Zn^{2+} binding to the protein. Circular dichroism revealed only minor Zn^{2+}-dependent conformational changes in calsequestrin. We conclude that calsequestrin is a Ca^{2+}- and Zn^{2+}-binding protein and that Zn^{2+} may modulate the structure and function of the protein.

Calsequestrin (CS) is the major Ca^{2+}-binding protein in the SR of cardiac and skeletal muscle (1). It is found in the lumen of the terminal cisternae of the SR where it functions to localize Ca^{2+} near to the junctional face of the cisternae. From this location Ca^{2+} can be released into the cytosol *via* the ryanodine receptor, thereby initiating muscle contraction (2). Extensive studies have been carried out on the physicochemical properties of CS (1) using tryptophan fluorescence, circular dichroism (CD), Raman spectroscopy and ^1H NMR, and proteolytic digestion (1). The protein is highly acidic and has characteristic Ca^{2+} binding properties; notably a large capacity (about 40-50 moles of Ca^{2+}/mol of protein) and a moderate to low

Abbreviations used: CS, calsequestrin; SR, sarcoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IDA, iminodiacetate-substituted agarose; SACH, salicylcarbohydrazone.

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affinity (~1 mM). The C-terminal part of CS , covering amino acids 300-367, is exceptionally rich in acidic amino acids and it is responsible for the high capacity Ca^{2+} binding to the protein (3). As a result of its Ca^{2+} binding properties CS also exhibits several other additional biochemical properties, including staining blue with the cationic carbocyanine dye Stains-All, and having a pH-sensitive electrophoretic mobility on SDS-PAGE (1).

Several Ca^{2+} binding proteins including S100, calmodulin and calreticulin also bind Zn^{2+} suggesting that Zn^{2+} may modulate their structure and function (4, 5). In this paper we report that CS is also a Zn^{2+}-binding protein. Zn^{2+} binding to CS was established using the Zn^{2+}-sensitive, fluorescence dye SACH, by $^{65}Zn^{2+}$ overlay and by Zn^{2+}-dependent precipitation of the protein. CS failed, however, to interact with a Zn^{2+}-IDA (iminodiacetate-substituted) agarose suggesting that histidine residues may not be involved in Zn^{2+} binding to the protein.

EXPERIMENTAL PROCEDURES

CS was isolated from rat skeletal muscle or dog heart as described earlier (6). Zn^{2+}-dependent precipitation of CS was carried out for 16 h in a buffer containing 10 mM Mops, pH 7.1, 150 mM NaCl and different amounts of $ZnCl_2$ followed by centrifugation for 45 min at 100,000 xg. CS was identified in the pellets and supernatants by blue staining with Stains All (1). S100a was prepared from bovine brain as described earlier (7).

The Zn^{2+}-IDA-agarose chromatography of CS was carried out according to (8). Purified CS was applied to the Zn^{2+}-IDA-agarose column and elution was with a linear gradient of 0 to 50 mM imidazole. The peak containing CS was pooled, dialyzed and analyzed for immunoreactivity with specific antibodies.

Zn^{2+} binding to CS was determined using the fluorescence dye SACH and $^{65}Zn^{2+}$ overlay. SACH was synthesized by a one step organic Schiff base reaction (9). Zn^{2+} binding assay was carried out in a solution containing 50 μM SACH, 10 mM Mops, pH 7.0 and 150 mM KCl in the presence or absence of CS (18 nM). Samples were excited at 360 nm, and the emission spectrum was analyzed between 390 nm to 550 nm. $^{65}Zn^{2+}$ binding to CS on nitrocellulose membranes was carried out in the presence of 100 μM $^{65}ZnCl_2$ by the procedure described for the $^{45}Ca^{2+}$ overlay (10). SDS-PAGE was carried out as described by Laemmli (11). Protein was determined by the method of Lowry *et al.* (12).

CD spectra was recorded using a JARCO/J-520 spectropolarimeter. Spectra were analyzed between 320 nm and 195 nm with protein concentrations of 0.754 mg/ml in 50 mM Tris-HCl pH 7.0. The mean residue ellipticity (deg cm^2 $dmole^{-1}$) was calculated (13).

RESULTS AND DISCUSSION

Purified rat skeletal muscle CS and canine cardiac CS were tested for $^{65}Zn^{2+}$ binding by overlay technique. Figure 1 shows $^{45}Ca^{2+}$ (**upper panel**) and $^{65}Zn^{2+}$ (**lower panel**) binding to the purified CS as compared to calreticulin, another Ca^{2+} binding protein of the endo(sarco)plasmic reticulum (1). Skeletal muscle CS (Fig. 1, **lane 1**) and cardiac muscle CS (Fig. 1, **lane 3**) bound $^{45}Ca^{2+}$ and $^{65}Zn^{2+}$ under these

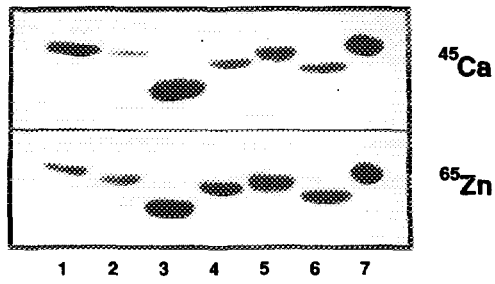

Figure 1. $^{65}Zn^{2+}$ to Calsequestrin and Calreticulin. Proteins were transferred to nitrocellulose membrane and incubated with $^{45}Ca^{2+}$ **(upper panel)**, or $^{65}Zn^{2+}$ **(lower panel)**. **Lane 1**, purified skeletal muscle CS ; **lane 2**, pig pancreatic calreticulin; **lane 3**, canine cardiac CS ; **lane 4**, human liver calreticulin; **lane 5**, bovine liver calreticulin; **lane 6**, rat liver calreticulin; **lane 7**, rat skeletal muscle SR vesicles.

conditions. Purified pig, human, bovine and rat calreticulins (Fig. 1, **lanes 2, 4, 5,** and **6**) also bound $^{65}Zn^{2+}$ under overlay conditions. $^{45}Ca^{2+}$ and $^{65}Zn^{2+}$ overlays were also carried out with purified skeletal muscle SR proteins and showed CS to be one of the major $^{45}Ca^{2+}$ and $^{65}Zn^{2+}$ binding protein in this membrane preparation (Fig. 1, **lane 7**). Picello *et al.* (14) did not detect any Zn^{2+} binding to CS as measured by $^{65}Zn^{2+}$ overlay. These discrepancies may be due to different conditions used by these

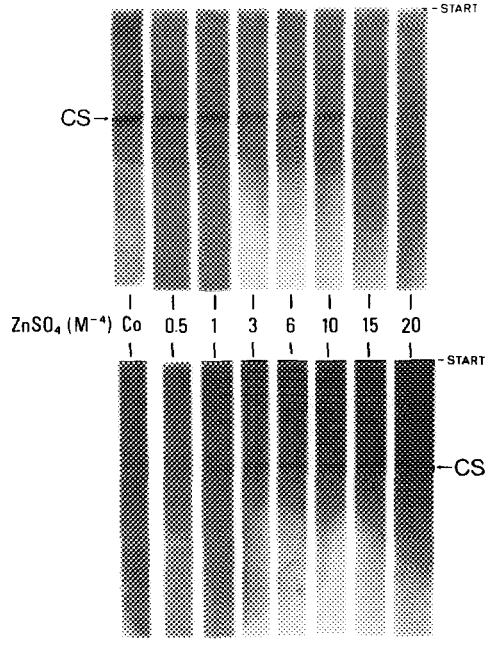

Figure 2. Zn^{2+}-Dependent Precipitation of Calsequestrin. Purified CS was incubated in the presence of increasing concentration of Zn^{2+} followed by centrifugation and SDS-PAGE. The protein was identified by blue staining with "Stains-All". **Upper panel**, supernatant; **lower panel**, pellet after Zn^{2+}-dependent precipitation. **CS**, calsequestrin.

authors for $^{65}Zn^{2+}$ overlay. Zn^{2+} binding to CS was further documented by the Zn^{2+}-dependent precipitation of the protein. Figure 2 shows that CS was quantitatively precipitated in the presence of Zn^{2+} concentration above 200 μM (Fig. 2). This behavior of the protein is similar to Ca^{2+}-dependent aggregation and precipitation of CS described earlier (1). In order to analyze kinetics of Zn^{2+} binding to CS we have utilized a Zn^{2+}-sensitive fluorescence dye SACH (9). In order to establish if SACH can be used for determination of Zn^{2+} binding to CS we first investigated Zn^{2+} binding to the bovine brain S100a, a well known Ca^{2+}- and Zn^{2+}-binding protein (5). Analysis of Zn^{2+} binding to S100a using the Zn^{2+}-sensitive fluorescence dye SACH revealed that the protein bound 3.3 moles of Zn^{2+}/mole of protein with a K_d~29 μM. These values are in agreement with earlier observations (5). Zn^{2+} binding to CS was measured the same experimental conditions. Scatchard analysis of Zn^{2+} binding to cardiac CS revealed that the protein bound ~200 moles of Zn^{2+}/mole of protein with K_d~300 μM. We conclude that CS binds Zn^{2+} and that Zn^{2+} binding to the protein induces its aggregation and precipitation.

Figure 3 shows CD spectra for CS in the presence and absence of Zn^{2+}. The far UV CD spectrum of CS displays a minimum near 208 nm and a shoulder around 225

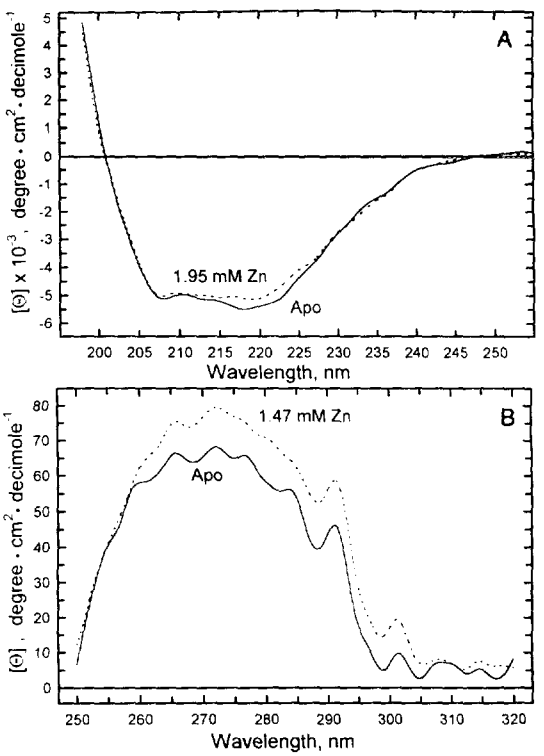

Figure 3. Zn^{2+}-Dependent Conformational Changes in Calsequestrin. In **A**, near UV CD spectra of CS in the absence (**Apo**) and presence of 1.95 mM Zn^{2+} (•••). In **B**; far UV CD spectra of CS in the absence (Apo) and presence of 1.47 mM Zn^{2+} (•••).

nm. In the presence of 1.95 mM Zn^{2+} there were only slight changes in the spectra. The aromatic CD spectrum of calsequestrin was characterized by a broad peak at 277 nm. In the presence of Zn^{2+} the near UV CD signal is quenched, although the overall shape of the spectrum is unaltered. Zn^{2+} produces only minor alterations in secondary structure of CS. This is in contrast to significant Ca^{2+}-dependent conformational changes observed in CS (6).

The Zn^{2+}-IDA-agarose column has been utilized for the identification and purification of Zn^{2+} binding proteins (9, 14). Free thiol groups and/or histidine residues in the proteins are believed to be involved in their interactions with Zn^{2+}-IDA-agarose (9). Canine cardiac CS does not have any cysteines and only 5 histidine residues dispersed throughout the entire amino acid sequence of the protein (15). We used Zn^{2+}-IDA-agarose chromatography to investigate whether these residues are involved in Zn^{2+} binding to CS. Figure 4 shows that calsequestrin did not interact with the Zn^{2+}-IDA. Since CS did not bind to Zn^{2+}-IDA-agarose we conclude that these histidine residues are not involved in Zn^{2+} binding to the protein. CS also does not have any consensus amino acid sequences such as "Zn^{2+}-fingers" known to be associated with Zn^{2+} binding sites in proteins (15). Zn^{2+} binding sites may be localized to the acidic C-terminal region of CS. This region of the protein contains a large number of negatively charged residues and binds Ca^{2+} with high capacity (3). A similar region of negatively charged residues is involved in Zn^{2+} binding to a 19-kDa Zn^{2+} binding protein identified in rat liver (17).

The physiological significance of Zn^{2+} binding to CS is not clear at present. Picello *et al.* (13) detected high amounts of Zn^{2+} localized to the terminal cisternae region of SR, a location also known to contain large quantities of CS (1). Additional Zn^{2+} binding proteins may also present in the muscle SR (14) suggesting that Zn^{2+} may play a significant role in the function of the SR membrane.

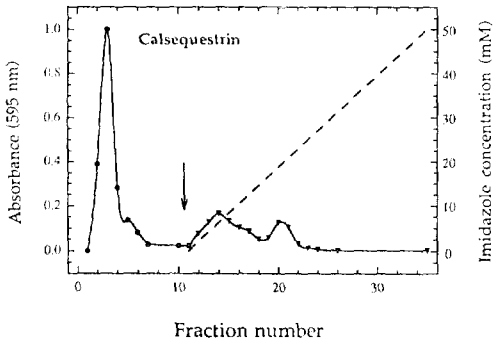

Figure 4. Zn^{2+}-Dependent Chromatography of Calsequestrin. Purified canine cardiac CS was loaded onto Zn^{2+}-IDA-agarose column followed by elution with a linear (0 - 50 mM) imidazole gradient. One-milliliter fractions were collected and assayed for protein content.

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