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Purification and Characterization of Calsequestrin from Chicken Cerebellum

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SUMMARY. Chicken cerebellum microsomal fractions contain a protein tentatively identified as calsequestrin (CS) *(Volpe et al., Neuron 5, 713-721, 1990)*. Here we report, for the first time, the purification of cerebellum CS from whole tissue homogenate by DEAE-Cellulose chromatography and Ca^{2+}-dependent elution from phenyl-Sepharose. The purified cerebellum CS displays the shift and increase in intrinsic fluorescence characteristic of skeletal muscle CS, and is shown to be a high-capacity, low-affinity Ca^{2+} binding protein (K_d = 1 mM).

Redistribution of Ca^{2+} from intracellular, rapidly exchanging Ca^{2+} stores plays a key role in several cellular functions (1). The molecular components predicted to be present in Ca^{2+} stores of non-muscle cells are Ca^{2+} release channels, Ca^{2+} pump, and intralumenal, low-affinity Ca^{2+} binding proteins (1,2).

The identification of intralumenal Ca^{2+} binding proteins has received much attention in the last four years (2). In particular, the structural and functional analogy with calsequestrin (CS), the low-affinity, high-capacity Ca^{2+} binding protein of sarcoplasmic reticulum of striated muscle (3), has been investigated. It is now clear that there are at least two main classes of low-affinity Ca^{2+} binding proteins, i.e., proteins truly belonging to the CS family, based on their amino terminal aminoacid sequence (4-7) and characteristic behavior with phenyl-Sepharose resin (8-10), and calreticulins, which lack a Ca^{2+}-regulated hydrophobic site (10,11) and have a low degree of structural homology with CS (12-14).

CS has been tentatively identified in chicken cerebellum microsomal fractions (7). Here we report the purification and characterization of CS from chicken cerebellum, and definitively show that CS is expressed in neuronal cells, of the chicken at least. CS is proposed to be (one of) the intralumenal Ca^{2+} binding protein(s) of intracellular Ca^{2+} stores in Purkinje neurons, since CS immunoreactivity is restricted to these neurons (2,15-17).

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EXPERIMENTAL

Cerebellum CS was purified from whole tissue (35-70 g wet wt, or 100-200 cerebella) homogenates by an ammonium sulphate precipitation procedure followed by DEAE-Cellulose chromatography (4). Fractions containing CS, as indicated by Western blot (see below), were pooled and subjected to phenyl-Sepharose chromatography (8). Experiments were repeated on three separate preparations. CS from either chicken or rabbit fast-twitch skeletal muscle was purified as described above. Protein concentration was determined according to Lowry et al. (18).

SDS-slab gel electrophoresis was carried on 10% polyacrylamide gels (19). Slab gels were stained first with Coomassie Blue and then with Stains all (20). Nitrocellulose membranes (blots) were stained with Ponceau red (21) and immunodecorated with polyclonal antibodies specific for chicken skeletal muscle, as previously described (7).

The intrinsic fluorescence changes of cerebellum CS were measured as described (9) using 275 nm as the excitation wavelength.

^{45}Ca ligand overlay was carried out on purified CS (2-3 ug of protein) electroblotted on nitrocellulose membranes (21), in a medium containing 5 mM $MgSO_4$, 60 mM KCl, 5 mM imidazole, pH 7.4, and 0.01-4 mM $^{45}CaCl_2$ (spec. activity, 500 uCi/mmol). Individual nitrocellulose lanes were incubated at room temperature for 20 min. After being processed for autoradiography, CS bands were excised and counted for radioactivity. For each lane, background was obtained by counting a piece of nitrocellulose membrane having an area equivalent to that of CS.

^{45}Ca binding to purified cerebellum CS (about 0.25 mg of protein) was carried out by equilibrium dialysis (48 hr) in a medium containing 5 mM $MgSO_4$, 60 mM KCl, 5 mM imidazole, pH 7.4, and 2 mM $^{45}CaCl_2$. Protein concentration was determined on aliquots of dialysate.

RESULTS

Homogenates of chicken cerebellum were fractionated on DEAE-Cellulose using a 0.05-1.0 M NaCl linear gradient. Fractions eluted between 0.2 and 0.6 M NaCl were electrophoresed and their Coomassie Blue and Stains all staining patterns are shown in Fig. 1, panels A and B, respectively. The protein profile was highly complex (panel A) and fractions 8-12 (lanes c-h in panel B) displayed several metachromatically blue-stained bands having an apparent molecular weight higher and lower than that of chicken skeletal muscle CS, i.e., 51 KDa (lane i, panel B; cfr. Ref. 7). Fractions 9-12 (lanes e-h), collected between 0.4 and 0.6 M NaCl, showed a polypeptide comigrating with skeletal muscle CS and having a characteristic deep bluish staining (22). Fig. 1, panel C, shows the Western blot of the fractions obtained from DEAE-Cellulose chromatography, stained with polyclonal antibodies specific for chicken skeletal muscle CS. Fractions 9-12 (lanes e-h) contained a polypeptide cross-reactive with the antibody.

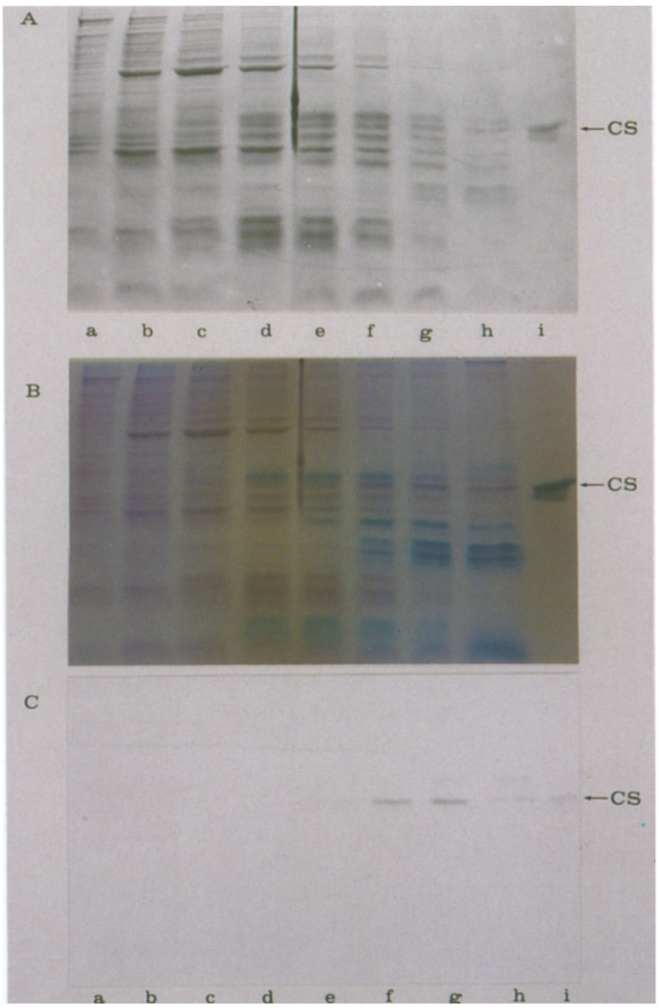

FIG. 1. *Chicken cerebellum fractions obtained from DEAE-Cellulose chromatography: Coomassie Blue, Stains all staining pattern and Western blot with antibodies specific for chicken skeletal muscle CS.* Panel A: Coomassie Blue staining pattern. Panel B: Stains all staining pattern. Panel C: Indirect immunoenzymatic staining pattern with antibodies specific for chicken skeletal muscle CS (5 µg/ml). Key to lanes: a-h, fractions 5-12 (15 µg of protein in a-h, and 10 µg of protein in a and h); i, chicken skeletal muscle CS (2 µg of protein).

The latter fractions were pooled, and applied to phenyl-Sepharose in the presence of 0.1 mM EGTA. About 65% of total protein applied did not bind to the column: Fig. 2A, lane a, shows the Ponceau-red staining protein pattern of the void volume. Upon addition of 10 mM $CaCl_2$, several fractions (# 3-9) were collected which contained a virtually pure polypeptide (lanes b-h) comigrating with chicken skeletal muscle CS (lane i). Fig. 2B shows that the purified protein

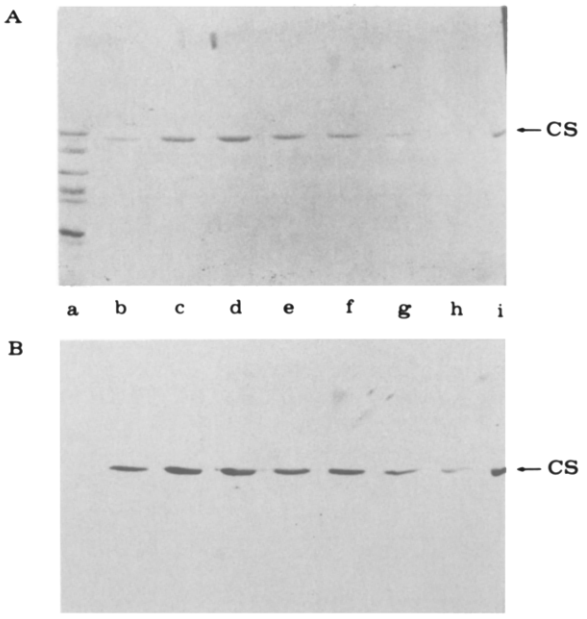

FIG. 2. *Chicken cerebellum fractions obtained from phenyl-Sepharose chromatography: Ponceau Red staining pattern and Western blot with antibodies specific for chicken skeletal muscle CS.* Panel A: Ponceau Red staining pattern. Panel B: Indirect immunoenzymatic staining pattern with antibodies specific for chicken skeletal muscle CS (5 µg/ml). Key to lanes: a, void volume (20 µg); b-h, 100 µl of fractions 3-9 eluted upon addition of 10 mM $CaCl_2$ (2.5, 5, 7, 2, 2, 1, and 0.5 µg of protein, respectively); i, chicken skeletal muscle CS (3 µg of protein).

was indeed CS, as indicated by Western blot with polyclonal antibodies specific for chicken skeletal muscle CS. The total amount of purified protein (fractions 3-9) was 6 ug protein/g wet wt, on average.

CS from fast-twitch skeletal muscle displays a characteristic fluorescent emission spectrum, if excited at 275 nm, both in the presence and in the absence of Ca^{2+} (9,20). Fig. 3A shows that cerebellum CS, in the presence of 1 mM EGTA, had a broad emission spectrum with a maximum at 345 nm. Upon addition of 2 mM $CaCl_2$, there was, as for skeletal muscle CS, a peak shift to 336 nm and a 50% increase of intrinsic fluorescence. The relationship between Ca^{2+} concentration and fluorescence changes is shown in Fig. 3B: the half-maximal increase of fluorescence was attained at 0.31 mM Ca^{2+} (cfr. Ref. 9).

Fig. 4A shows that the purified cerebellum CS was a Ca^{2+} binding protein, as judged by ^{45}Ca ligand overlay. The binding capacity (B_{max}) of cerebellum CS was measured by equilibrium dialysis in the presence of 2 mM $CaCl_2$, and found to be 220 nmoles Ca^{2+}/mg of

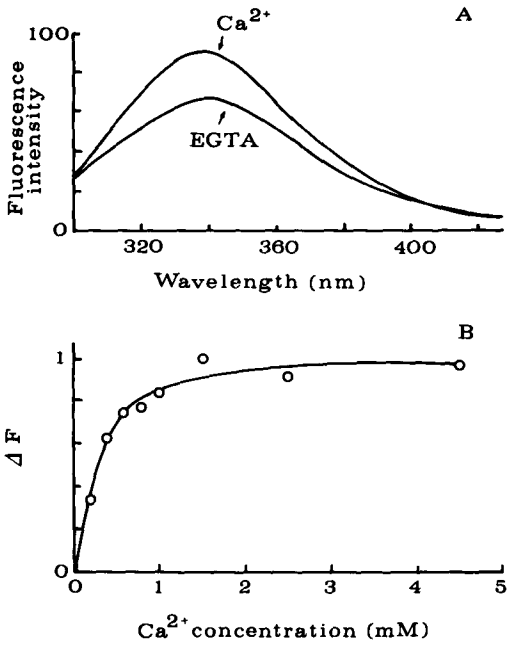

FIG. 3. *Ca^{2+}-dependence of intrinsic fluorescence changes of chicken cerebellum CS (15 µg of protein).* Panel A: Using an excitation wavelength of 275 nm, the emission spectrum was recorded. A second fluorescent emission spectrum was recorded after addition of 2 mM $CaCl_2$. Panel B: Experiments were carried out as described in panel A, except that 0.2-2 mM $CaCl_2$ increments were sequentially added to the cuvette containing 1 mM EGTA and 1 mM $CaCl_2$. The relative increase of fluorescence intensity at 336 nM is plotted as a function of free Ca^{2+} concentration. Computer fitting was performed using commercially available software (NFIT, Island Products).

protein, i.e., comparable to that reported for chicken skeletal muscle CS (3,23,24).

Given the low yield of cerebellum CS, K_d of Ca^{2+} binding could not be measured by standard equilibrium dialysis techniques. The affinity of cerebellum CS for Ca^{2+} was, thus, determined by ^{45}Ca ligand overlay of purified CS electroblotted on nitrocellulose membranes. In preliminary ^{45}Ca ligand overlay experiments, it was ascertained that the affinity of skeletal muscle CS, purified from either rabbit or chicken, was around 1 mM (data not shown). In Fig. 4B, the amount of Ca^{2+} bound to cerebellum CS is plotted as a function of Ca^{2+} concentration. The best fit of the Hill equation to the experimental data indicates a K_d of 1.05 mM.

DISCUSSION

This paper reports the purification and characterization of CS from chicken cerebellum. In extension of previous results, i.e.,

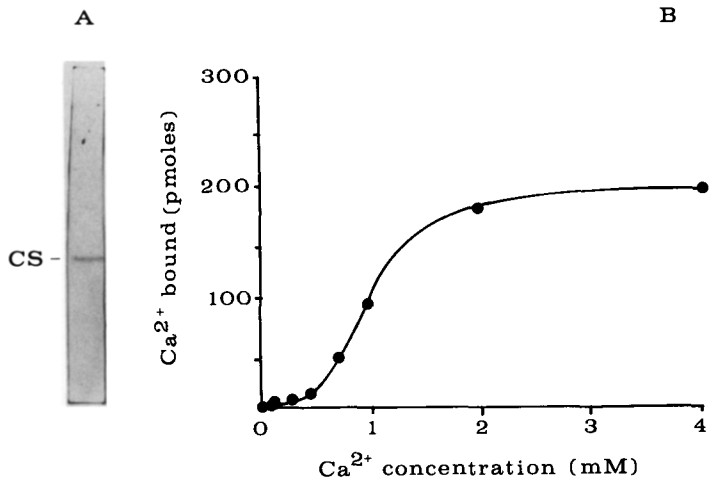

FIG. 4. *Ca^{2+} binding to chicken cerebellum CS.* Panel A: ^{45}Ca ligand overlay was carried out as described in EXPERIMENTAL on 3 µg of purified cerebellum CS. Panel B: $^{45}CaCl_2$ concentrations varied from 0.01 to 4 mM. Computer fitting was performed using NFIT. Assuming complete electrotranfer of protein, maximal capacity would be 67 nmoles Ca^{2+}/mg of protein: however, CS aggregation and CS interaction with the nitrocellulose matrix might lead to underestimates of B_{max}. Under the same experimental conditions, skeletal muscle CS from both rabbit and chicken displayed apparent B_{max} values that were 1/3-to-1/5 of those obtained by equilibrium dialysis (not shown).

immunological cross-reactivity, *Staph. aureus* V8 protease-peptide mapping and amino terminal aminoacid sequence (7), the cerebellum CS is definitively shown to be a member of the CS family of Ca^{2+} binding proteins on the basis of the following features:

1) CS was purified by Ca^{2+}-dependent elution from phenyl-Sepharose, thus indicating the presence of a Ca^{2+}-regulated hydrophobic site which, likely, internalizes upon Ca^{2+} binding (cfr. Ref. 8).

2) Upon Ca^{2+} binding, cerebellum CS displayed a characteristic shift and increase of intrinsic fluorescence, probably due to internalization of tryptophan residues (cfr. Ref. 9). The presence of a Ca^{2+}-regulated hydrophobic site seems to be the most invariant property of the CS family of Ca^{2+} binding proteins (24), whereas calreticulin, the other known intralumenal Ca^{2+} binding protein, invariably lacks such a site (10,11).

3) Finally, cerebellum CS was shown to be a low-affinity (K_d = 1 mM), high-capacity (220 nmoles Ca^{2+}/mg of protein) Ca^{2+} binding protein. Both K_d and capacity are in the range reported for the muscle CS isoform (3,23,24).

Immunogold labelling studies of chicken cerebellum Purkinje neurons with anti-CS antibodies, have demonstrated that CS is

concentrated over two types of subcellular structures: a) cisternae of the endoplasmic reticulum, including the smooth-surfaced cisternal stacks, i.e., the structures most enriched in the inositol 1,4,5-trisphosphate receptor (15,17,25); and b) a population of small vacuoles and tubules, about ten-fold more intensely labelled ("calciosomes" in Refs. 15-17). In this paper, we have thus provided additional evidence that an authentic CS is (one of) the intralumenal Ca^{2+} binding protein(s) of Ca^{2+} stores in chicken Purkinje neurons.

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