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THE ASSOCIATION OF CALMODULIN WITH SUBCELLULAR FRACTIONS ISOLATED FROM RAT LIVER

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SUMMARY: Calmodulin associated with rat liver mitochondria has been found to belong to a contaminant membranous fraction which contains different subcellular membranes. The concentration of calmodulin in this fraction is relatively high, about 1.6µg/mg protein, and can not be decreased with EGTA. The calmodulin-rich membranous fraction seems to contain cytoskeletal proteins which could be responsible for the binding of calmodulin.

Calmodulin is a calcium-binding protein which serves as a major intracellular receptor for Ca^{2+}, regulating several calcium-dependent enzymes and playing an important role in the control of other cellular functions. Studies on the cellular localization of calmodulin carried out with fixed tissue sections have shown that a large proportion of the total cell calmodulin is found in the cytosol, in part associated with glycogen particles, the remainder bound to different subcellular membranes (1). Immunofluorescence studies with cultured 3T3 fibroblasts have shown intense calmodulin staining around mitochondria (2), indicating that calmodulin might be associated with the outer mitochondrial membrane. The presence of calmodulin in mitochondria isolated from rat liver and beef heart has been reported (3). However, the purification level of mitochondria was

Abbreviations:

SDS, sodium dodecylsulfate; Hepes, N-2-Hydroxyethylpiperazine-N-2-ethane-sulfonic acid; PMSF, Phenylmethylsulfonyl fluoride; EDTA, Ethylendiamine-tetraacetic acid; EGTA, Ethyleneglycol-bis-(amino-ethylether) N,N,-tetraacetic acid.

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not specified so that the results are still open to question. In order to clarify the possible role of calmodulin in mitochondria, conclusive data on the presence of calmodulin in isolated mitochondria are necessary. In this work we show that calmodulin is indeed associated with crude mitochondrial fractions isolated from rat liver. However, after purification by isopycnic centrifugation on a Percoll gradient, pure mitochondria fractions contain only insignificant amounts of calmodulin. Almost all of the calmodulin is recovered in a mixed membrane fraction in which several subcellular organelles, including mitochondria, are found. The amount of calmodulin found in the mixed membrane fraction is rather high and can not be decreased with EGTA. This type of EGTA-resistant calmodulin-interaction has been previously reported in the case of the cytoskeleton structure (4,5). Our data strongly indicate that cytoskeletal proteins present in the mixed membrane fraction represent the binding site for calmodulin.

Materials and Methods

Female rats fasted overnight were used in this study. Liver mitochondria were prepared using a modification of the procedure described by Chan et al. (6). The homogenization medium contained 310mM Mannitol, 20mM Hepes-KOH, 1 mM PMSF and 0.5 mM EDTA, final pH 7.4. The mitochondrial pellet was washed three times with homogenization medium containing 0.5 mM EGTA instead of EDTA, (buffer A). In some experiments EDTA and EGTA were not added to the isolation medium. The crude mitochondrial fraction was then further purified on a Percoll gradient prepared in buffer A. A heavy (the purified mitochondria) and a light fraction were separated. These fractions were washed once with buffer A. The light fraction was then further purified by centrifugation on a linear sucrose gradient (0.85M-1.6M prepared in 20 mM Hepes-KOH pH 7.4). The supernatant, after separation of the crude mitochondrial fraction, was centrifuged first at 20,000gmax for 15 minutes to remove swollen mitochondria and subsequently at 105,000g max for 1hr to sediment a crude microsomal fraction.

Extraction of cytoskeletal proteins from the mixed-membrane fraction. 1) Alkaline extraction was carried out as previously described (7). The mixed-membrane fraction was centrifuged and resuspended in 250 mM sucrose at a concentration of 5 mg protein/ml. The membranes were then mixed with sufficient 1 N NaOH to achieve a pH of 10.15. After 60 minutes incubation on

ice, the suspension was centrifuged at 106,000 g for 60 min. The supernatant and pellet were saved for subsequent analysis.

Isolation and identification of calmodulin in the different subcellular fractions. The different membranous fractions isolated as described above were diluted with at least two volumes of buffer B (80mM Tris-Cl, 80 mM Imidazole, 6 mM $MgCl_2$, 0.2mM $CaCl_2$, pH 7.5 at 37°C). The samples were placed in a boiling water bath for 3 min.,then cooled down and centrifuged at 15,000gmax for 20 min. to sediment the denatured protein. Calmodulin in the supernatant was then purified on phenyl-sepharose column equilibrated with buffer C, (50mM Tris-Cl pH7.4, 1mM Mercaptoethanol, 0.1mM $CaCl_2$). Calmodulin was eluted with buffer C containing 1mM EGTA instead of CaCl2. Calmodulin was determined according to its ability to activate the calmodulin-deficient phosphodiesterase (8),or, in some cases, the Ca-ATPase activity of calmodulin-deficient erythrocyte ghosts(9).

Other procedures. Polyacrylamide gel electrophoresis in SDS was carried out using the Laemmli procedure (10). Glucose-6-phosphatase (in the presence of 10 mM tartrate) (11), and 5' nucleotidase (11) were used as marker enzymes of, respectively, endoplasmic reticulum and plasma membrane. Succinate-cytochrome c reductase (12) was used as mitochondrial marker. Brain calmodulin was prepared as previously described(13). Protein determination was carried out using a modified Lowry procedure (14). All reagents used were analytical grade.

Results and discussion

Purified mitochondria were prepared by isopycnic centrifugation of a crude mitochondrial fraction on a Percoll gradient. Two major bands were formed; a lower one, close to the bottom of the tube at a density of about 1.1 (g/ml), contained purified mitochondria. An upper band, in the density region around 1.03 (g/ml), contained about 5% of the applied protein and was characterized by the presence of different types of subcellular membranes. It was therefore defined as a "mixed-membrane" fraction. Table I shows that calmodulin is associated with the crude mitochondrial fraction. However, after Percoll gradient most of it was lost and it was recovered in the "mixed-membrane" fraction. After sucrose gradient centrifugation of the"mixed membrane" fraction, a calmodulin-rich fraction was obtained. As shown in Table I, this fraction was enriched in plasma membrane, but still contained microsomes and mitochondria.

Table I. Distribution of calmodulin and marker enzymes in subcellular fractions isolated from rat liver

Fraction	Calmodulin (ng/mg prot.)	Succinate-cyt.c reduct	5'-nucleo-tidase	Glucose-6-phosphatase
Mitochondria	31.7 (62)*	0.260	0.014	0.032
Percoll-mitochondria	4.9 (9.3)*	0.320	0.001	0.003
Mixed-membrane fract.	328	0.085	0.090	0.240
Mixed-membrane fract. after sucrose gradient	1647	0.069	0.271	0.265
Crude microsomes	145	0.003	0.114	0.416

The data in the Table are taken from a representative experiment. Experimental variation was within 10% of the data reported.
Marker enzyme activities are expressed as μmoles Pi released (or cytochrome c reduced)/minute/mg protein.
*Mitochondria were prepared in isolation medium which contained neither EDTA nor EGTA.

The concentration of calmodulin found in this membranous fraction was much higher than that found in the crude microsomal fraction (Table I) or in purified plasma membranes (15). The purified calmodulin obtained from this fraction had the same characteristics as brain calmodulin, as shown by its electrophoretic mobility (Fig.1) and by its ability to activate calmodulin-deficient phosphodiesterase (Fig.2). Furthermore, it could also stimulate the activity of another calmodulin-dependent enzyme, the Ca-ATPase of erythrocytes (data not shown). We found interesting that the calmodulin associated with the membranous fraction was still retained after extensive washing with EGTA (0.5 mM EGTA was always present in the buffer used for the isolation). At the moment there are only two examples of calmodulin dependent systems where calmodulin is associated in a Ca-insensitive manner. These are, 1), phosphorylase kinase, a key enzyme in glycogen metabolism, where 4 calmodulin subunits become associated with the enzyme in a EGTA resistant way (16) and, 2),

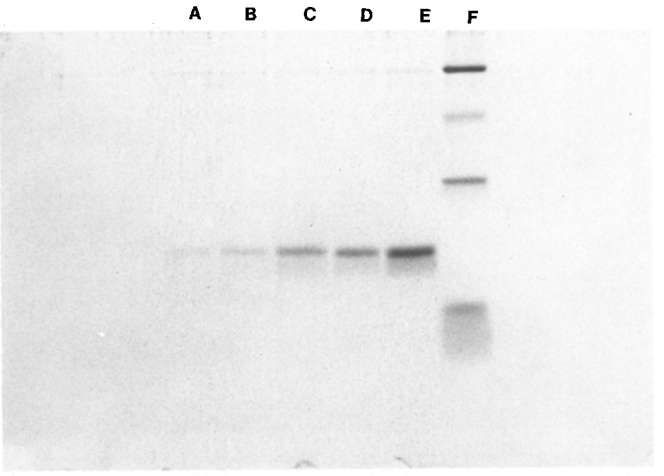

Fig.1 SDS-Polyacrylamide gel electrophoresis pattern of calmodulin isolated from the "mixed membrane" fraction. A-C: Calmodulin from "mixed membrane" fraction (0.5, 1 and 2 ng). D and E: brain calmodulin (2.7 and 5.4 ng). F: Low Mr standard (68,000-40,000-26,000-12,000). Proteins in the gel were stained using a modified siver staining technique (18).

cytoskeletal proteins (4,5). We can exclude that our preparation was contaminated by glycogen particles since the rats were fasted 16 hours before killing and, in addition, we have measured the glycogen content of the mixed membrane fraction and failed to

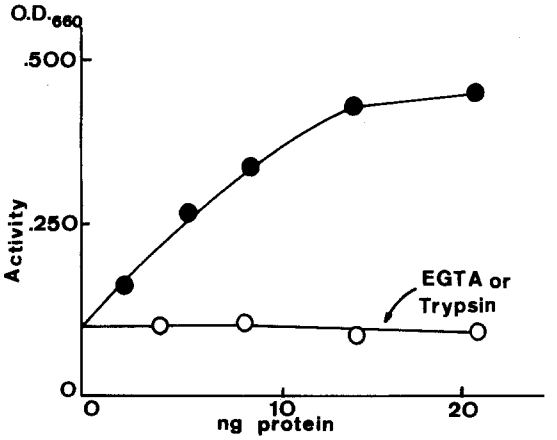

Fig.2 Activation of calmodulin-deficient phosphodiesterase by calmodulin isolated from the "mixed membrane" fraction. Phosphodiesterase activity was determined by measuring the Pi released in the two- step assay procedure described in reference 8. The O.D.changes in the phosphate assay are reported in the figure.

detect any (not shown). An attractive possibility is that the calmodulin-rich membranous fraction contains microfilamentous structures, which are known to be present in hepatocytes (6). Most cytoskeletal proteins, especially the one belonging to intermediate filaments, are known to be insoluble under physiological conditions. However, they appear to be soluble under certain conditions of low ionic strength and at extreme pH (lower than 3 or higher than 10) (17). In fact, it has been shown that exposure of rat liver plasma membrane to alkaline media results in loss of membrane-associated filaments (6). We have found that alkaline extraction of our calmodulin-rich membranes causes the release of about 28% of the protein and of most of the bound calmodulin. The concentration of calmodulin in this fraction was very high, about 3.5 µg/mg protein, (it should be noted that calmodulin is stable at pH 10). The electrophoretic analysis of the membranous fraction and the pH 10 extract revelead the presence of several polypeptides bands, with a major band having a Mr around 55 KDa (Fig.3), by lowering the amount of protein applied to the gel it was possible to resolve the major band into two bands having Mr of 56 and 54 KDa, (not shown). Since our membranous fraction contained also mitochondria, it was possible that some of the contaminants polypeptides derived from matrix proteins released by the rupture of mitochondria at alkaline pH. Therefore we tryed to separate the putative cytoskeletal proteins in the alkaline extract by increasing the ionic strength (addition of 60 mM NaCl) and by lowering the pH down to 6.8. The small flocculent masses which appeared were separated by centrifugation. The electrophoretic analysis of these proteins (Fig.3) shows that some minor bands have disappeared and that the band at 54 KDa is particularly decreased as compared to that at 56 KDa. A 56 KDa polypeptide was also

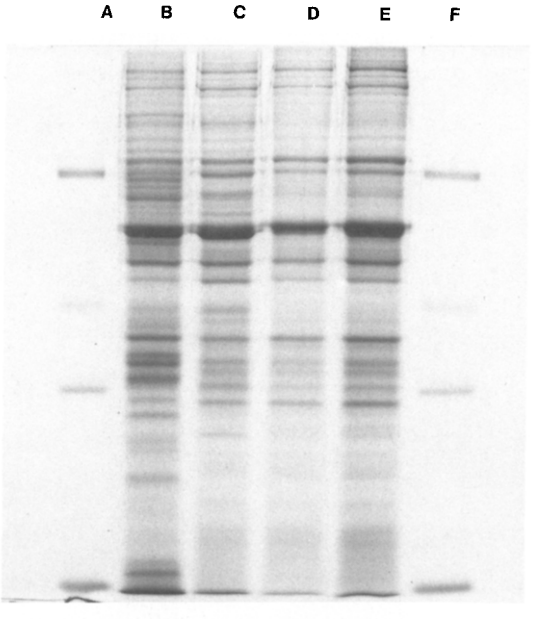

Fig.3 SDS-Polyacryalmide gel electrophoresis pattern of the calmodulin-rich membranous fraction and its alkaline extract. B: Calmodulin-rich membranes after sucrose gradient. C: Alkaline extract. D and E: proteins obtained after neutralization of the alkaline extract (15 and 30 µg). A and F: Low Mr standard (see fig.1). Proteins in the gel were stained with comassie blue.

previously recognized as beloging to intermediate filaments associated with rat liver plasma membrane (6). These data strongly indicate that cytoskeletal proteins associated with the calmodulin-rich membranous fraction are the binding site for calmodulin.

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