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PURIFICATION AND CHARACTERIZATION OF CALMODULIN FROM RAT LIVER MITOCHONDRIA

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Mitochondrial calmodulin of rat liver was purified and classified. It co-migrated with bovine brain calmodulin in non-denaturing polyacrylamide gel electrophoresis, SDS-polyacrylamide gel electrophoresis and isoelectric focusing. The mitochondrial calmodulin activated Ca^{2+}-dependent phosphodiesterase of bovine brain in the presence of Ca^{2+}. About 80% of the mitochondrial calmodulin was proved to be of cytosol origin. It was easily detached by washing with buffer containing EGTA. The other 20% was intramitochondrial calmodulin; half of it was in the matrix space, and half in the membrane.

INTRODUCTION

The heat-stable, acidic and multifunctional Ca^{2+}-binding protein, calmodulin, modulates the activities of many Ca^{2+}-dependent enzymes such as Ca^{2+}-dependent phosphodiesterase (1,2), adenylate cyclase (3), NAD-kinase (4), erythrocyte Ca^{2+}-ATPase (5), myosin light chain kinase (6). Many communications suggest that calmodulin is ubiquitous in eukaryotes (7, 8,9), but the findings are ambiguous for prokaryotes (10).

Mitochondria are considered as a kind of parasitic organelle in eukaryotic cells. Clarification of the presence and properties of mitochondrial calmodulin would be relevant to the comparative study of calmodulin in the evolutional process. Recent reports showed that isolated liver mitochondria contained about 8% of the total cellular calmodulin (11), and that calmodulin binding sites existed on the mitochondrial surface (12).

In this communication, we report the purification and characterization of mitochondrial calmodulin and its distribution in mitochondria.

The abbreviation used is; EGTA, ethylene glycol bis(β-aminoethylether) N,N,N',N'-tetraacetic acid.

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MATERIALS AND METHODS

Chemical

Phenyl-methyl-sulfonyl-fluoride (PMSF) was purchased from Sigma Chemicals (St Louis MO).

Purification of Ca^{2+}-dependent phosphodiesterase

Ca^{2+}-dependent phosphodiesterase was purified by the method of Itano et al.(13).

Phosphodiesterase assay

The enzyme activity was measured by coupling the reaction to 5'-nucleotidase (13), and measurement of the inorganic phosphate produced was according to the method described by Ames (14).

Purification of bovine brain calmodulin

Bovine brain calmodulin was purified with 2-chloro-10(3-aminopropyl)-phenothiazine-Sepharose 4B affinity chromatography by the method of Jamieson and Vanaman (15).

Purification of mitochondrial calmodulin from rat liver

Mitochondria were isolated from rat liver by the modified method of Schnaitman and Greenawalt (16), except that the isolation medium contained 250mM Sucrose, 10mM Tris-HCl(pH 7.4), 1mM β-mercaptoethanol, 0.1mM PMSF, and 1mM $CaCl_2$. These mitochondria were named Ca^{2+}-mitochondria (Fraction A). The supernatant after removing Ca^{2+}-mitochondria was collected as a cytosol fraction (Fraction B). Ca^{2+}-mitochndria were washed three times with EGTA-buffer that contained 250mM Sucrose, 10mM Tris-HCl(pH 7.4), 1mM β-mercaptoethanol, 0.1mM PMSF, and 10mM EGTA. The EGTA-washed mitochondria were named EGTA-mitochondria. The EGTA-mitochondria had 10 volumes of hypotonic buffer containing 10mM Tris-HCl(pH 7.4), 1mM β-mercaptoethanol, 0.1mM PMSF, and 1mM $CaCl_2$ added, and were then homogenized (Fraction C). The homogenate was ultrasonicated at 19.5KHz 1mA for 15sec with a type T-A 4280 Ultrasonicator (Kaijo Denki, Japan). The sonicated homogenate was centrifuged at 7000xg for 15 min to remove unbroken mitochondria, The supernatant was centrifuged at 105000xg for 60 min. The supernatant was a mitochondrial matrix fraction (Fraction D) that was proved to have minimal contamination of membrane components. The pellet was dissolved with 5 volumes of the hypotonic buffer and was homogenized. This homogenate was named the mitochondrial membrane fraction (Fraction E). Ten ml of each of these fractions (A to E) was heat-treated in a boiling water bath for 3 min, and the denatured proteins were removed by centrifugation at 12000xg for 30 min. The calmodulin contents of five heat-treated supernatants were measured by phosphodiesterase assay. Calmodulins from three fractions (B,D,E) were purified by the same method of that for bovine brain calmodulin.

Electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was performed according to the method of Davis (17), and SDS-polyacrylamide gel electrophoresis was done by the method of Laemmli (18).

Isoelectric focusing

Analytical electrofocusing was carried out by the method of Bazari and Clarke (19).

Measurement of protein concentration

Protein was determined by the method of Lowry et al. (20) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Table 1 indicates the calmodulin distribution in mitochondria. The total amount of cytosol calmodulin obtained from 400g of rat liver was 7100 μg and that of Ca^{2+}-mitochondria was 514μg (7.2%). By washing with the EGTA-buffer, about 80% of calmodulin of Ca^{2+}-mitochondria was detached. The

Table 1. Distribution of Mitochondrial Calmodulin

Fraction Name	Calmodulin μg (%)
Ca^{2+}-mitochondria	514 (100)
EGTA-mitochondria	118 (23)
Matrix Fraction	62 (12)
Membrane Fraction	42 (8)

The amounts of calmodulins in each of the fractions from 400g of rat liver were measured by the phosphodiesterase activation assay described in "Materials and Methods".

washed-out calmodulin was considered to be of cytosol origin and to be loosely bound to the outer membrane of mitochondria. The remaining 20% was intra-mitochondrial; about half of it existed as a soluble form in the matrix space, and the other half as a bound form in the membrane.

Figure 1 shows the non-denaturing polyacrylamide gel electrophoresis of the four calmodulins (a), and the densitometric pattern of the matrix calmodulin (b) that is considered to be intra-mitochondrial. In the presence of Ca^{2+}, there was almost no contaminant protein, but in the absence of Ca^{2+} (with EGTA), minor contaminant proteins (about 10%) appeared. This is difficult to explain but in the communications by Kretsinger et al.(21) and Dedman et al.(12), similar phenomena were reported in the SDS-polyacrylamide gel electrophoresis. In non-denaturing polyacrylamide gel electrophoresis, calmodulin shows a characteristic decreased mobility in the presence of Ca^{2+} (22). Troponin C and leiotonin C show increased mobility in the presence of Ca^{2+} (23,24), and the mobilities of most of the other proteins are not affected by the presence or absence of Ca^{2+}. Calmodulins from rat liver mitochondria co-migrated with bovine brain calmodulin in the presence and absence of Ca^{2+}. In SDS-polyacrylamide gel electrophoresis, calmodulin migrates faster in the presence of Ca^{2+} than in the absence of Ca^{2+}. Calmodulins from bovine brain and rat liver mitochondria migrated in the same manner (not shown). These results indicates that the molecular properties of mitochondrial calmodulin are very similar to those of bovine brain calmodulin.

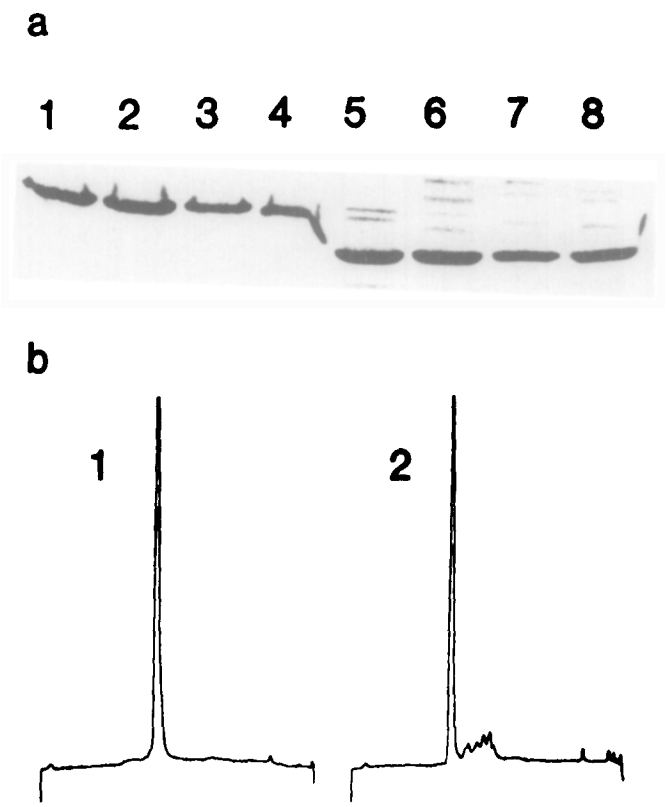

Fig. 1 a: Comparison of calmodulins in non-denaturing polyacrylamide gel electrophoresis.
(1-4): in the presence of 20mM Ca^{2+}
(5-8): in the presence of 20mM EGTA
(1,5): 50μg of bovine brain calmodulin
(2,6): 50μg of rat liver cytosol calmodulin
(3,7): 25μg of mitochondrial matrix calmodulin
(4,8): 25μg of mitochondrial membrane calmodulin
b: Densitometric pattern of matrix calmodulin in the presence of Ca^{2+} (1), and EGTA (2).

Figure 2 shows the pattern of isoelectric focusing of calmodulins. The isoelectric points of calmodulins from various origins are about pH 4 (25). The mitochondrial calmodulin showed an isoelectric point of pH 3.9, almost the same as bovine brain one.

Figure 3 shows Ca^{2+}-calmodulin-dependent activation of phosphodiesterase. Ca^{2+}-dependent phosphodiesterase is activated by calmodulin in the presence of Ca^{2+} but not in the absence of Ca^{2+}. It may also be activated by certain phospholipids and fatty acids (26,27), but such activation is Ca^{2+}-independent. Therefore, to prove the Ca^{2+}-dependent activation of this enzyme is one method of identifying calmodulin. All of the purified calmodulins reported here

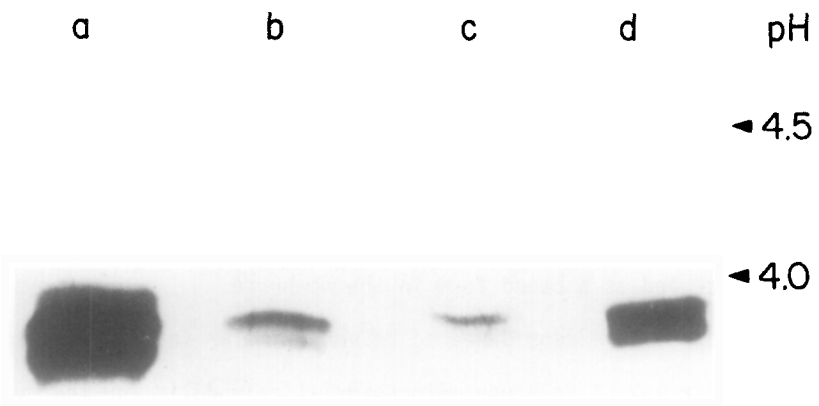

Fig. 2 Comparison of calmodulins in isoelectric focusing.
a: 50μg of bovine brain calmodulin
b: 15μg of mitochondrial membrane calmodulin
c: 10μg of mitochondrial matrix calmodulin
d: 40μg of rat liver cytosol calmodulin

activated the phosphodiesterase of bovine brain in the Ca^{2+}-dependent manner, and the maximum activation levels by these calmodulins were almost the same. The doses of calmodulins required for 50% activation of the enzyme were compared. Calmodulins from the cytosol fraction and from bovine brain were about 350ng, while those from the matrix fraction and the membrane fraction were about 550ng. The difference would indicate that the mitochondrial calmodulin differs from bovine brain or rat liver cytosol calmodulins.

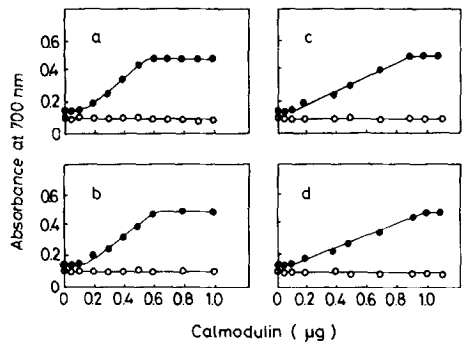

Fig. 3 Ca^{2+}-calmodulin-dependent activation of phosphodiesterase in the presence of Ca^{2+} (●) and EGTA (○). Absorbance at 700nm indicates the amount of inorganic phosphate produced and showing coloration by the reaction described in "Materials and Methods".
a: bovine brain calmodulin
b: rat liver cytosol calmodulin
c: mitochondrial matrix calmodulin
d: mitochondrial membrane calmodulin
The amounts of calmodulins required for 50% activation of the enzyme were as follows;
a: 360ng, b: 340ng, c: 550ng, d: 570ng

In conclusion, about 80% of the calmodulin of Ca^{2+}-mitochondria was easily detached by the first washing with the EGTA-buffer, proving it to be of cytosol origin. The second and third supernatants washed with the EGTA-buffer contained only traces or no calmodulin activity. Therefore, calmodulin from EGTA-mitochondria was intra-mitochondrial, and existed as a soluble form in the matrix space and as a bound form in the membrane.

As mitochondria are considered to be very similar in some ways to prokaryotes, it is relevant to study mitochondrial calmodulin for the evolutional analysis of calmodulin.

In this communication, we report its purification and some of its properties. The physiological significance of mitochondrial calmodulin is still unclear, but the co-existence of calmodulin and Ca^{2+}-sensitive enzymes such as glutamic dehydrogenase (28), isocitrate dehydrogenase (29), and succinate dehydrogenase (30), suggests that there would be certain Ca^{2+}-calmodulin-dependent proteins or enzymes modulating mitochondrial functions and configuration.

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