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CALMODULIN BINDING PROTEINS IN RAT LIVER MITOCHONDRIA

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Calmodulin binding proteins have been found in submitochondrial fractions obtained from highly purified rat liver mitochondria.The matrix fraction contains two major calmodulin binding proteins:one,having Mr of 145,000,apparently is carbamoyl-phosphate synthetase.Another has a Mr of 58,000 and has not been associated with enzyme activities.A major calmodulin binding protein of unknown function and having Mr of 32,000 has been found in the Triton X-100 solubilizate of the inner membrane.Minor amounts of two calmodulin binding proteins having Mr of about 37,000 and 56,000 have been found in the outer membrane.

Calmodulin is the most important intracellular calcium binding protein of eukaryotic cells (1).After tissue fractionation, calmodulin is found associated with both the soluble and the membranous fractions, the extent of binding to the latter being highly dependent on the Ca^{2+} concentration in the medium. Cytochemical studies carried out using fluorescently labelled calmodulin have shown that a large part of the intracellular calmodulin in 3T3 fibroblasts is associated with the outer membrane of mitochondria (2). It has been suggested that this calmodulin could regulate the movements of mitochondria through the interaction with the cytoskeleton associated with fibroblast mitochondria (3). Calmodulin has also been detected in the matrix fraction obtained from isolated beef heart mitochondria

Abbreviations: BSA, bovine serum albumin; SDS, sodium dodecylsulfate; Hepes, N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; EDTA, Ethylendiaminetetraacetic acid. EGTA, Ethyleneglycol-bis-(amino-ethylether) N,N,-tetraacetic acid.

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(4).The presence of calmodulin in mitochondria, which are often assumed to originate from bacteria,is of interest not only from a general biochemical point of view but also for the study of calmodulin in the evolutional process:the presence of calmodulin in bacteria has not been conclusively established as yet(5,6). Calmodulin binding proteins have also been detected in mitochondrial preparations(2,7),however,these studies were carried out on conventionally isolated,and thus probably contaminated mitochondria. The possibility that the calmodulin binding proteins originate from non-mitochondrial contaminants is thus certainly realistic.In this study the matter of the presence of calmoduling binding proteins in mitochondria has been investigated on highly purified preparations. Their distribution in the submitochondrial fractions has also been investigated.

METHODS AND MATERIALS

Rat liver mitochondria were prepared using a modification of the procedure described by Chan et al.(8). The homogenization medium contained 0.21 M Mannitol, 0.07 M Sucrose, 0.01 M Hepes-KOH,1mM PMSF,and 0.5 mM EDTA, final pH 7.4. The mitochondrial pellets were washed three times with the homogenization medium containing 0.5 mM EGTA and 0.5 mg BSA/ml (buffer A). Mitochondria were then further purified on a Percoll gradient (prepared in buffer A) as previously described (9). To remove the outer membrane, the mitochondria were swollen, shrunken and sonicated as described by Sottocasa et al. (10), except that 1 mM PMSF was included in the medium and the sonication time was 10 sec. After sonication the suspension was centrifuged at 27,000 gmax for 15 min to sediment the mitoplasts.The supernatant was recentrifuged at 140,000gmax for 1 h to sediment the crude outer membrane.The outer membrane was resuspended in a small volume of 5 mM Hepes-KOH, 10 mM KCl,1 mM $MgCl_2$, 0.5 mM PMSF, 2 mM DTT pH 7.4 (buffer B)and then further purified by centrifugation on a linear sucrose gradient (0.85M-1.6M,prepared in buffer B), as previously described (9). Fractions with high monoaminoxidase activity were pooled together, diluted with 300 mM NaCl, 10 mM Hepes-KOH pH 7.4 and centrifuged at 140,000gmax for 1 hr to sediment the purified outer membrane. The pellet was resuspended in a small volume of 300 mM NaCl, 10 mM Hepes-KOH pH 7.4 and stored at -20°C. The mitoplast fraction was washed once with buffer A and then suspended in 2 mM EDTA pH 8.5 to a final concentration of 20 mg/ml. The suspension was then sonified with a Branson sonifier for 3 min at 0°C (pulsed,50% duty cycle) and subsequently centrifuged 15 min at 15,000gmax to remove unbroken mitoplasts. The supernatant was removed and centrifuged at 140,000gmax for 1 hr to sediment the "inner membrane fraction". The inner membrane was resuspended in a small amount of 0.3 M NaCl, 0.01M Hepes-KOH

pH 7.4. The supernatant obtained after sedimenting the inner membrane represents the "matrix fraction". All the fractions were stored at -20°C. Rat heart mitochondria were prepared using the overall procedure described for rat liver except that the homogenization was carried out using a Polytron homogenizer as previously described (11). Brown fat mitochondria were prepared from cold adapted rats as described above for rat liver.

Affinity chromatography of mitochondrial fractions using a Sepharose 4B- calmodulin column. The coupling of calmodulin to Sepharose 4B was carried out as previosuly described (12). A calmodulin-Sepharose column, 2 ml bed volume,was equilibrated with 0.3 M NaCl, 0.01 M Hepes-KOH, 0.05 mM $CaCl_2$,0.5% Triton x-100,pH7.2. The inner and outer membrane fractions, at a concentration of 5 mg/ml,were solubilized with 1.5% Triton X-100 at 0 °C for 20 min. The solubilized membranes were centrifuged at 140,000gmax for 30 min. The supernatant was removed and diluted 1:1 with 0.3 M NaCl,0.01 M Hepes-KOH, 0.2 mM $CaCl_2$ pH 7.2. To the soluble matrix fraction $CaCl_2$ and NaCl were added to a final concentration of 0.2mM and 0.3M, respectively. The sample were then applied on the column and eluted using five column volumes of 0.3M NaCl,0.01M Hepes-KOH,0.05mM$CaCl_2$, 0.5% Triton X-100 (not added to the matrix fraction) final pH 7.4. The calmodulin interacting proteins were then eluted with the same medium containing 0.3 mM EGTA instead of $CaCl_2$. The protein content of the different fractions was analyzed by SDS-polyacryalmide gel electrophoresis. The affinity column was washed well with a medium containing 2M guanidine chloride, 0.5% Triton X-100, 1 mM EDTA,and finally stored in 0.3 M NaCl, 0.01 M Hepes-KOH, 0.2 mM $CaCl_2$, 0.02% Na-azide.

Other procedures. Polyacrylamide gel electrophoresis in SDS was carried out using the Laemmli procedure (13).The acrylamide concentration was 7.5% when matrix fractions were analyzed,and 12% when the solubilized inner and outer membrane were analyzed. Gels were stained for protein with 0.2% Coomassie blue R-250 . Cytochrome oxidase activity was measured polarographycally using a Clark oxygen electrode. Monoaminooxidase activity was assayed spectrophotometrically as described by Schnaitmann et al (14). The β-hydroxybutxrate dehydrogenase was measured as described by Bock et al. (15) in the presence of mitochondrial phospholipids. Acid phosphatase was assayed as described by Trouet (17). Brain calmodulin was prepared as previously described (12). The phosphate carrier was isolated from rat liver mitochondria using the procedure described by Kolbe et al. (18). Protein determination was carried out using a modified Lowry procedure (19). All reagents used were analytical grade.

RESULTS AND DISCUSSION

A main concern of this study has been the preparation of mitochondria with only minimal contamination by other subcellular membranes. For this reason mitochondria were routinely washed three times and then passed through a Percoll gradient. After this treatment the pellet was found to be completely free of microsomal and plasma membrane contaminations.The contamination by lysosomes was reduced to about one forth of that found in

crude mitochondrial fraction,as could be judged from the level of acid phosphatase activity (not shown). Complete removal of the lysosomal contamination could be achieved by treating the mitochondria with low concentrations of digitonin. However, this procedure was not used routinely,since digitonin interacted with the outer mitochondrial membrane,thus altering its density and making its separation from the inner membrane on a density gradient difficult. The monoaminoxidase acitivity of the purified outer and inner membrane fractions was 309 and 3 nmoles benzaldehyde produced/mg prot./min,respectively. The cytochrome oxidase activity was 40 and 1950 nmol oxygen reduced/mg protein/min,respectively. These values indicate that the level of cross contamination between the two mitochondrial membranes was minimal.

Calmodulin binding proteins were identified by passing the solubilized mitochondrial fractions through a Sepharose 4B-calmodulin affinity column and eluting them sequentially with calcium and EGTA containing buffers. Fig.1 shows the SDS- poly acrylamide gel pattern of the rat liver mitochondria matrix and of the fractions eluted from the calmodulin affinity chromatography column. Two major polypeptides, having Mr around 145,000 and 58,000 had calcium- dependent interaction with calmodulin and were eluted with the EGTA buffer (fractions C-I). Control experiments have shown that no protein was retained by Sepharose 4B not coupled to calmodulin. The 145,000 Mr protein is very likely to be the carbamoyl-phosphate synthetase,one of the enzymes involved in the biosynthesis of urea in liver mitochondria (20). This enzyme is regulated by a specific cofactor, acetylglutamate,requires MgATP and free Mg ions and is inhibited by calcium (21).The urea cycle ,or at least its mitochondrial portion,is a typical liver biosynthetic pathway,

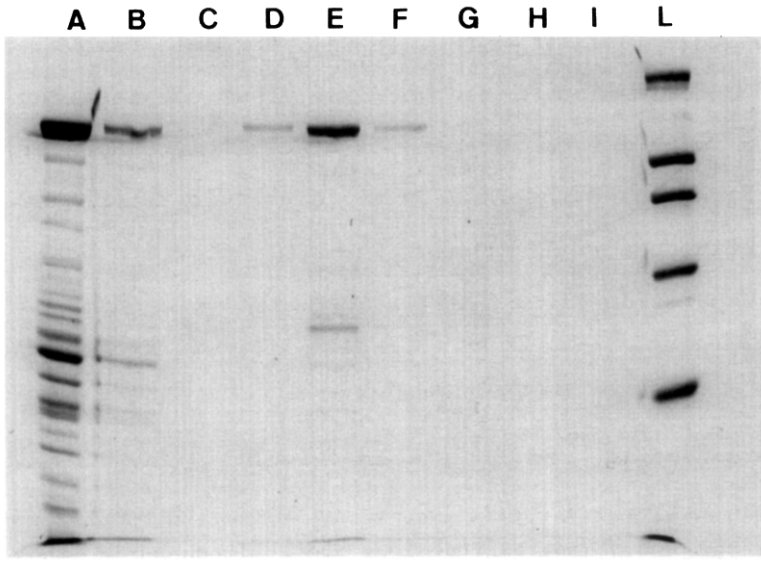

Fig.1 SDS-Polyacrylamide gel electrophoresis pattern of the mitochondrial matrix proteins eluted from the calmodulin affinity column. A. Matrix before affinity column B. Proteins eluted with the Ca^{2+} containing buffer. C-I.Fractions corresponding to the peak of proteins eluted with the EGTA containing buffer. L. High Mr standard from BioRad.(200,000-116,250-92,500 66,200-45,000).

not found in other tissues(small intestine,kidney and brain are partial exeptions). As can be seen from Fig.2, the matrix fractions isolated from rat heart and brown fat mitochondria do not posses the 145,000 Mr band,nor do they show calmodulin binding proteins in this Mr region. The 145,000 Mr component of the rat liver mitochondrial matrix is known to represent about 20% of the total matrix proteins(the amount of protein eluted with EGTA is only about 5% of the total matrix proteins). Therefore, the 145,000 Mr fraction seems to be present in two forms (or contains two proteins),one which shows Ca^{2+}-dependent interaction with calmodulin and another which does not. One possible explanation for these results is that the mitochondrial carbamoyl-phosphate synthetase consists of two isozymes. Another possibility is that partial denatura- tion of the enzyme has occured, producing two different molecular forms. These

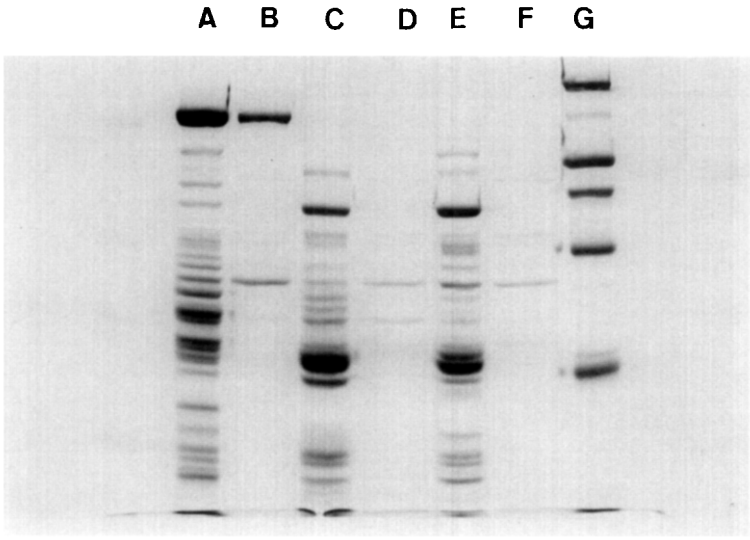

Fig.2 Comparison of matrix fractions from mitochondria isolated from different tissues. A. Liver matrix before fractionation on calmodulin affinity column. B. Liver matrix proteins eluted with the EGTA containing buffer. C. Heart matrix before fractionation on calmodulin affinity column. D. Heart matrix proteins eluted with the EGTA containing buffer. E. Brown fat matrix before fractionation on calmodulin affinity column. F. Brown fat matrix proteins eluted with the EGTA containing buffer. G. High Mr standards(see fig.1).

possibilities are currently being investigated,as is the possibility that calmodulin endogenously present in the matrix plays a role in the regulation of the synthetase.

Fig.2 shows that a calmodulin binding protein with Mr around 58,000 is present in the three types of matrix fractions investigated . This protein most likely correspond to that recently reported to be present in the matrix of beef heart mitochondria (7).

The possible presence of calmodulin binding proteins in the inner and outer mitochondrial membranes has been investigated after solubilization of the membranes with 1.5% Triton X-100.As shown in Fig.3 the inner mitochondrial membrane contains several polypeptides which show Ca^{2+}-dependent interaction with the calmodulin affinity column (EGTA fractions C-I).The most prominent has a Mr around 32,000 (the total proteins eluted with

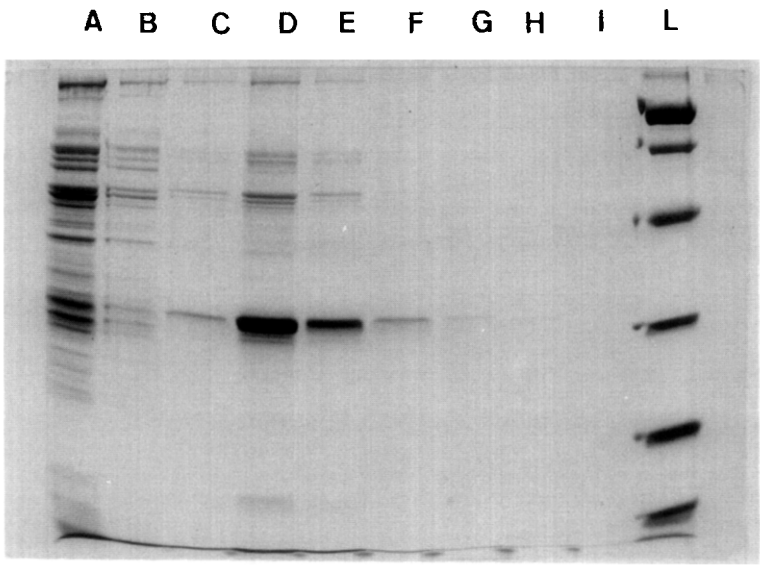

Fig.3 SDS-Polyacrylamide gel electrophoresis pattern of the Triton X-100 solubilized inner membrane proteins eluted from the calmodulin affinity column. A. Triton X-100 solubilized inner membrane before fractionation on the calmodulin affinty column. B. Proteins eluted with the Ca^{2+} containing buffer. C-I Fractions corresponding to the peak of proteins eluted with the EGTA containing buffer. L. Low Mr standard from BioRad. (92,500-66,200-45,000 31,000-21,500-14,400.)

EGTA represent about 0.15% of the inner membrane proteins).It is well known that the inner membrane of mitochondria contains several proteins with Mr around 32,000,among them the β-hydroxybutyrate dehydrogenase and the phosphate carrier. β-hydroxybutyrate dehydrogenase activity was not found in the fraction eluted with EGTA from the affinity column (not shown). The phosphate carrier also seems an unlikely candidate since the purified rat liver phosphate carrier does not bind to the calmodulin affinity column (not shown).

The Triton X-100 solubilized outer mitochondrial membrane contains marginal amounts of calmodulin-binding proteins. As shown in Fig. 4 some bands, particularly two with Mr around 37,000 and 56,000,can be visualized in the EGTA-eluted fractions. However,we have calculated that the protein eluted in the EGTA fractions represent less than 0.01% of the total outer membrane proteins and the possibility that they originate from impurities

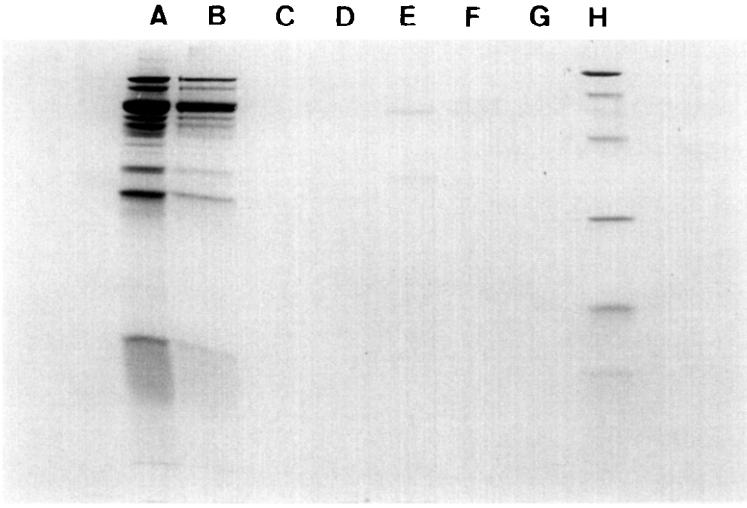

Fig. 4 SDS-Polyacrylmide gel electrophoresis pattern of the Triton X-100 solubilized outer membrane proteins eluted from the calmodulin affinity column. A. Triton X-100 solubilized outer membrane before fractionation on the calmodulin affinity column. B. Proteins eluted with the Ca^{2+} containing buffer. C-G. Fractions corresponding to the peak of proteins eluted with the EGTA containing buffer. H. Low Mr standards(see fig.3).

of the outer membrane preparation can thus not be ruled out. The lack of prominent calmodulin binding proteins in the outer membrane is somewhat surprising since the present study was stimulated by the finding that in intact cells calmodulin is concentrated around the mitochondria(2).It is possible, however,that components which are associated with the outer mitochondrial membrane in vivo (cytoskeleton?) become lost during the isolation of the mitochondrial fraction. This interesting possibility is now being investigated.

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