AN INSOLUBLE CA^{2+}-BINDING FACTOR FROM RAT LIVER MITOCHONDRIA

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SUMMARY: An insoluble factor capable of binding Ca^{2+} with high affinity has been obtained in 100-fold purification from water extracts of rat liver mitochondria. It contains a major protein species of MW ~ 67,000 ± 10%, 27 per cent phospholipid, free fatty acids, over 8 per cent hexosamines, and considerable chloroform-methanol insoluble phosphorus. It binds up to 70 nmoles of Ca^{2+} per mg protein at high-affinity sites and about 900 nmoles of Ca^{2+} at low affinity sites. Ca^{2+} binding is inhibited by La^{3+} and ruthenium red, but not by respiratory inhibitors or uncouplers. Dialysis in the presence of Ca^{2+} inactivates Ca^{2+} binding. The factor is compared with a soluble glycoprotein of ox liver mitochondria isolated by Sottocasa et al. (4).

INTRODUCTION

Intact rat liver mitochondria (RLM) contain respiration-independent high-affinity Ca^{2+} binding sites whose properties suggest they may be concerned in respiration-dependent Ca^{2+} transport (1,2). Extraction of intact RLM with distilled water yields a soluble, non-dialyzable, heat-labile factor containing high-affinity La^{3+}-sensitive Ca^{2+}-binding sites (3). Gel filtration of these extracts showed the molecular weight of this factor to lie between 150,000 - 200,000 (3).

In this communication we report the purification and some properties of this Ca^{2+} binding factor, which on exposure to ammonium sulfate becomes insoluble in aqueous systems. Its properties are contrasted with those of a soluble Ca^{2+}-binding glycoprotein obtained from ox liver mitochondria, described in the accompanying paper of Sottocasa et al. (4).

METHODS

RLM were isolated from 30 - 40 gm batches of liver and extracted with cold distilled water (1,2) at an initial concentration of 20 mg protein per ml. Powdered ammonium sulfate was dissolved gradually in the cold water extract with gentle mechanical stirring to a final concentration of 4 mg per ml; stirring was continued for another 3 hrs. The slightly cloudy suspension was then centrifuged at 12,000 g for 30 min. The fluffy, grayish-white

pellet was resuspended in 2 - 3 ml cold water and dialyzed overnight in the cold against 500 ml distilled water to remove excess ammonium sulfate. The material remaining insoluble, which contains the activity, was recovered by centrifugation, resuspended in a small volume of water, and sonicated for 15 seconds to yield a homogenous suspension.

Equilibrium dialysis across cellophane membranes was carried out in the apparatus described by Englund et al. (5). A 20 μl aliquot containing the factor protein was added to one compartment and 20 μl of a buffered $^{45}Ca^{2+}$ solution of known concentration and specific activity to the other. After equilibration across the membrane, which required 2 hours at 4°, the distribution of radioactivity between the two compartments was measured and the Ca^{2+}-binding data plotted according to Scatchard.

Sialic acid and hexosamine were determined essentially according to Molnar et al. (6) and neutral sugars by the phenol-H_2SO_4 method of Dubois et al. (7).

RESULTS

Precipitation of the Ca^{2+} binding factor with ammonium sulfate. No success attended efforts to purify the high-affinity Ca^{2+} binding activity present in water extracts of rat liver mitochondria (2) with conventional protein fractionation methods. Chromatography of the water extracts on Sephadex G-100 or G-200 columns, although useful for an indication of particle weight, was ineffective since most of the proteins appeared to have about the same Stokes radius. Fractionation with ammonium sulfate at pH 7.0 precipitated essentially all the activity below 10 per cent saturation, much lower than required to salt out most proteins. Further study showed that no Ca^{2+}-binding activity remained in the supernatant at 0.3 M ammonium sulfate. Since only about 1 - 2 per cent of the total protein appeared in the fraction precipitated with 0.3 M ammonium sulfate, the Ca^{2+} binding activity was purified some 50 - 100 fold on a protein basis.

Because high concentrations of neutral salts inhibit high-affinity Ca^{2+} binding activity in both intact mitochondria and mitochondrial extracts, the precipitated material was suspended in a minimum volume of water and dialyzed against water for 16 hours at 0° prior to assay for binding activity. However, much of the salt-precipitated material failed to redissolve during dialysis. Assay of the dialyzed supernatant and insoluble fractions recovered after centrifugation showed that the Ca^{2+}-binding activity resided entirely in the insoluble residue. Figure 1 is a Scatchard plot of Ca^{2+} binding by the latter, showing the occurrence of both high-affinity and low-affinity binding sites. The dissociation constant of the high-affinity sites varied from K_D = 0.7 to 4.0 μM in different preparations and the number of

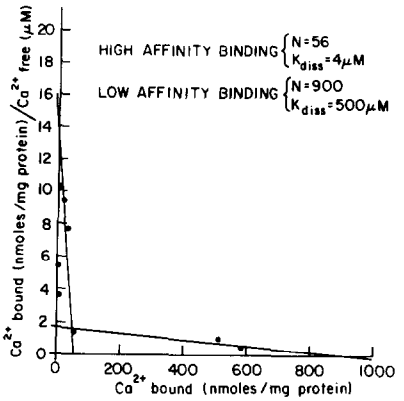

Figure 1. Scatchard plot of Ca^{2+} binding by insoluble factor. The protein concentration was 0.5 mg per ml.

high-affinity binding sites varied from 48 to 70 nmoles per mg. The low-affinity sites bound over 900 nmoles per mg protein. Ca^{2+} binding by the factor was inhibited by La^{3+} and ruthenium red, but uncoupling agents and respiratory inhibitors had no effect.

No success attended attempts to redissolve the Ca^{2+}-binding activity in aqueous media; the precipitation with salt had evidently converted the once-soluble factor into an insoluble form. Since this change precluded further attempts to purify the Ca^{2+} binding factor by standard fractionation methods, studies were carried out on the composition and properties of the insoluble material.

Disk gel electrophoresis. Electrophoresis of the Ca^{2+} binding factor dispersed in 1 per cent sodium dodecyl sulfate in 10 per cent polyacrylamide gel yielded a sharp, prominent major band after staining with Coomassie blue and four minor bands. The mobility of the major band corresponded to a polypeptide chain of molecular weight 67,000 ± 10%, assuming a normal content of amino acids with charged R-groups.

Carbohydrate content. Preparations of the salt-precipitated Ca^{2+}-binding material showed the presence of 40 - 45 nmoles total hexosamines per mg protein, about equal to the number of Ca^{2+} binding sites and equivalent to about 0.8 mg carbohydrate per mg protein. They also contained 1 - 1.2 nmoles total sialic acid per mg protein, corresponding to only 0.2 - 2.0 per cent of the total Ca^{2+} binding sites. Thus it is possible that sialic acid is not related to Ca^{2+} binding.

Phosphorus and lipid content. The dialyzed ammonium sulfate-precipitated fraction contained about 900 natoms total phosphorus per mg protein. Extraction with acetone solubilized about 40 per cent of the phosphorus which TLC

showed to be largely lysophosphatidylcholine in the amount of about 0.27 mg per mg protein. About 135 ngatoms of phosphorus per mg of protein could not be extracted with chloroform-methanol and is suggested to be phosphoprotein phosphorus. The acetone-soluble fraction also contained some free fatty acids, which were separated from the lysophosphatidylcholine by chromatography on Sephadex LH-20. The fatty acids were found by mass spectrometry of their trimethylsilyl derivatives to be a mixture of palmitic, stearic, oleic, and palmitoleic acids. (We are indebted to Dr. Catherine Fenselau for the mass spectrometry.).

Stability of Ca^{2+} binding factor. The Ca^{2+} binding activity of water extracts of mitochondria is heat-labile but survives freezing and thawing (3). However, the binding of added Ca^{2+} to the factor in water extracts of rat liver mitochondria undergoes a rapid decline during the equilibrium dialysis assay. Data in Fig. 2 show that in the absence of mitochondrial extract approximately two hours at 4^{o} are required to bring about substantially complete equilibration of $^{45}Ca^{2+}$ across the cellophane membranes in the cells employed. With the extract present in one compartment, the major portion of the Ca^{2+} added to the other compartment moved into the compartment containing the extract within 30 - 60 min; however, by 120 min, the time required for complete equilibration of Ca^{2+} in the control dialysis (extract absent), the concentration of Ca^{2+} became nearly equal in the two compartments.

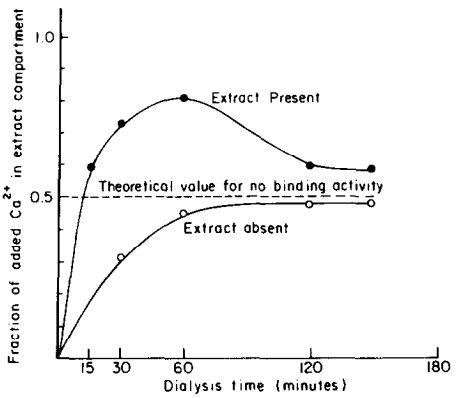

Figure 2. Time course of equilibrium dialysis. The Ca^{2+} concentration was 1 μM, added to one compartment and the protein concentration 1 mg per ml, added to the other compartment. The medium was 2.0 mM Tris chloride pH 7.4 in both compartments (0^{o}).

DISCUSSION

The purified but insoluble Ca^{2+} binding factor from rat liver mitochondria described in this paper may now be compared to the soluble Ca^{2+} binding

glycoprotein of ox liver described in the accompanying paper by Sottocasa et al. (4).

Both factors give biphasic Scatchard plots, the high-affinity leg showing K_D = 0.75 - 4 μM for the insoluble factor and $K_D \tilde{=}$ 0.1 μM for the soluble glycoprotein. The number of high-affinity Ca^{2+} binding sites is between 48 and 60 nmoles per mg protein for the insoluble factor and about 70 nmoles per mg for the soluble glycoprotein. Both factors contain phospholipids and appreciable amounts of carbohydrates. The insoluble factor contains significant amounts of lysophosphatidylcholine and free fatty acids, both known to be formed rapidly during mitochondrial swelling (8). Both preparations show single protein bands by gel electrophoresis but the molecular weight of the protein moiety of the soluble factor from ox liver is about 42,500 and that from the insoluble factor of rat liver about 67,000. Moreover, the yields of the two factors are quite similar, amounting to approximately 0.5 - 1.0 per cent of the total mitochondrial protein. The insoluble Ca^{2+}-binding factor of rat liver contained almost 130 ngatoms per mg of protein of a form of phosphorus insoluble in chloroform-methanol, presumably phosphoprotein phosphorus. There are striking resemblances between the properties of both the soluble and insoluble Ca^{2+} binding factors with the estrogen-induced glycolipophosphoprotein of toad serum (9).

The inactivation of the Ca^{2+} binding factor by dialysis of mitochondrial extracts in the presence of Ca^{2+} is also distinctive. Either it undergoes rapid inactivation or on contact with Ca^{2+} it loses its Ca^{2+}-binding group in a low-molecular weight, dialyzable form which can equilibrate across the cellophane membrane within the 2-hour equilibrium dialysis period.

It is possible that the soluble glycoprotein of Sottocasa et al. (4) arises from a different mitochondrial compartment than that described in this paper. The soluble factor was obtained by a procedure which extracts intermembrane proteins (10). On the other hand, the insoluble factor described in this paper was obtained by distilled water extraction, which removes significant amounts of protein from the matrix as well as from the intermembrane space (11), and is also known to remove some membrane proteins which may then become insoluble in dilute salt solutions (12). An extramitochondrial origin or contribution (e.g. from adhering microsomes) has not been excluded but is unlikely. It is also possible that the insoluble Ca^{2+} binding factor described in this paper is a denatured or aggregated form of the soluble glycoprotein of Sottocasa et al. (4). Whether either factor plays a role in respiration-supported transport of Ca^{2+} across the membrane remains to be determined.

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