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CHARACTERIZATION OF CALCIPHORIN, THE LOW MOLECULAR WEIGHT CALCIUM IONOPHORE, FROM RAT LIVER MITOCHONDRIA

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Calciphorin, the putative mitochondrial calcium ionophore from rat liver mitochondria, exhibits the inherent properties of the mitochondrial calcium transport system and is similar to the calf heart preparation reported earlier. The protein has a strong selectivity for Ca^{2+}, and has a K_d for Ca^{2+} of 56.5 ± 6.6 μM and 13.9 ± 2.1 μM in organic extraction and flow dialysis experiments, respectively. Reduction of the contaminating lipids from 23 ± 6.5 to 1.73 ± 0.4 moles per mole protein does not alter the affinities, Ca^{2+}/protein stoichiometry or selectivity for Ca^{2+}.

Calcium transport in mitochondrial membranes was first described almost three decades ago [1]. However, the transport system per se, still remains largely uncharacterized. It has been established that the influx of calcium into the mitochondria is electrophoretic and mediated through a uniport system located in the inner membrane. The search for the electrophoretic uniporter has been enthusiastically pursued by several groups of workers. However, most of the proteins that have been isolated are hydrophylic, extrinsic membrane proteins for e.g. the 67 kDa glycoprotein, isolated by Gomez-Puyou et al. [2] the 33 kDa glycoprotein isolated by Carafoli [3], and calvectin a 15 kDa protein recently reported by Panfili et al. [4].

Jeng and Shamoo [5–7] had earlier described the purification of calciphorin, a 3 kDa protein from calf heart inner mitochondrial membranes. This protein was the first to exhibit all the properties inherent in the mitochondrial transport system; hydrophobicity, high affinity for calcium, presence in the inner membrane, selectivity for calcium, and sensitivity to Ruthenium red and lanthanide. An earlier report by Sokolove and Brenza [8] described the isolation of a rat liver calciphorin fraction. That fraction was unable to bind calcium when delipidated. However, the methodology employed in that case, was significantly different from that reported for the calf heart preparation [7], and therefore the authenticity of the sample isolated as calciphorin is highly questionable.

Calciphorin was purified from rat liver mitochondria, prepared according to Shamoo et al. [9] following the procedure reported for its isolation from calf heart mitochondria by Jeng and Shamoo [5,6]. Fig. 1 shows the 280 nm absorbance profile of the fractions eluted from the Sephadex G-50 column. This profile and that reported by Jeng and Shamoo for the calf heart preparation are very similar. However, it is significantly different compared to that reported by Sokolove and Brenza [8]. As described in that paper, 50 mM KCl was included in the elution buffer. The presence of KCl has been reported to alter the deoxycholate micelle size [10]. This also resulted in major altera-

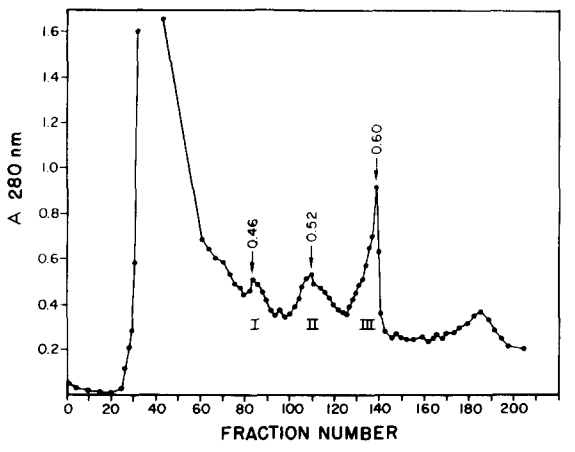

Fig. 1. Elution profile of the deoxycholate-solubilized inner mitochondrial membranes chromatographed on Sephadex G-50. The column was preequilibrated and eluted with 10 mM Tris-HCl, pH 7.8/0.02% NaN_3/1% potassium deoxycholate. The arrows indicate the column volume at which peaks I, II and III are eluted.

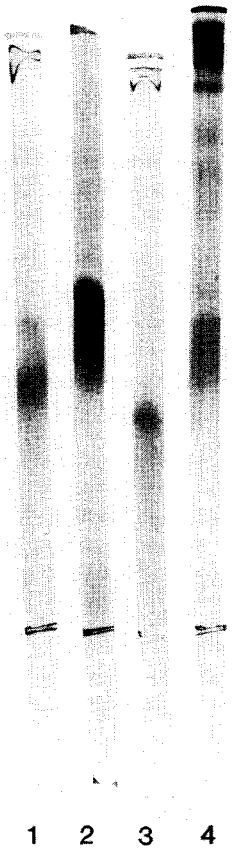

Fig. 2. Sodium dodecyl sulphate-polyacrylamide gels of isolated calciphorin. All the gels contained 8 M urea, 10% acrylamide and 0.1% SDS. Gels 1, 2, and 4 are isolated calciphorin, prior to delipidation, while gel 3 shows the migration of the delipidated protein. The solvent front is indicated for each gel. The R_f values for standard proteins were trypsin inhibitor (mol. wt. 21 300) 0.32 ± 0.01; cytochrome *c* (mol. wt. 11 700) 0.48 ± 0.14; and Insulin-1 (mol. wt. 3400) 0.80 ± 0.02; Insulin-2 (mol. wt. 2300) 0.87 ± 0.02. The R_f values for calciphorin and the calculated molecular weights are given in Table I.

tions in the elution profile of the calciphorin preparation from the Sephadex G-50 columns. The fraction collected as calciphorin by this procedure failed to bind calcium when delipidated, and also did not resolve well on SDS-urea gels.

Fractions forming Peaks I, II, and III were dialysed against 10 mM Tris-HCl, pH 7.8 and then concentrated by ultrafiltration using an Amicon DM5 membrane. Each fraction was then tested for calcium binding. SDS-urea gel electrophoresis was also performed. Peak II (fractions 99–125) has been identified as calciphorin on the basis of the properties that are described in this paper. Fig. 2 shows the SDS-urea gel electrophoresis patterns of crude and delipidated calciphorin. Gels 1, 2 and 4 show calciphorin as obtained after gel filtration and dialysis. Gel 3 is the delipidated protein. As seen from the gel, the crude calciphorin fractions contain a single major component with an R_f of 0.56 ± 0.15 while delipidated calciphorin has a major component at an R_f of 0.63. The only major contaminant observed on some gels is probably cytochrome *c*, which is eluted just before calciphorin from the Sephadex G-50 column.

The molecular weight of calciphorin, calculated from these gels is given in Table I. The values obtained (about 8350, for the crude and 6300 for the delipidated protein) are significantly higher than that reported by Jeng and Shamoo [5,6], or our subsequent calf heart preparations (Table I). We do not have any definite explanation for this discrepancy. However, since the amount of associated lipids seems to alter the electrophoretic mobility, the value could be a function of the contaminating lipids. Also, molecular weight differences may be due to the fact that molecular weight determination from SDS gels is very rough, especially for low molecular weight, hydrophobic proteins. Also, the R_f values probably change with the shape of the protein.

TABLE I

COMPARISON OF THE PROPERTIES OF CALF HEART AND RAT LIVER MITOCHONDRIAL CALCIPHORIN PREPARATIONS

Molecular weights were calculated from SDS-urea gels run according to the method of Swank and Munkres [16], as described for Fig. 2. Phospholipid phosphorus was estimated by the method of Ames and Dubin [17]. 5–10 μM calciphorin was used in the calcium binding studies. Organic extraction was performed according to Jeng and Shamoo [7]. Aqueous phase contained 5 mM Tris-HCl, pH 7.8, 0.2 mM picric acid and various concentrations of ^{45}Ca. The flow dialysis was performed according to the method of Colowick and Womack [18]. The medium contained 25 mM Tris-HCl, pH 7.4. The data were plotted in the form of Scatchard plots in order to calculate K_d and number of binding sites. n.d., not done.

Properties	Rat liver		Calf heart [a]		Calf Heart [b]	
	Crude	Delip.	Crude	Delip.	Crude	Delip.
Mobility	0.56 ± 0.015	0.63	0.63 ± 0.014	0.54	0.68	
Mol. wt. from gels	8341	6308	6308	3466	3861	1904
Mol. wt. from A.A. Anal.	4619	–	–	–	2884	2884
Lipid/protein	23 ± 6.5	1.73 ± 0.4	17 ± 3.22	1.24 ± 0.47	150	0.1
Binding flow dialysis						
K_d HA site [c]	13.9 ± 2.1	11.46 ± 1.93	37.4 ± 9.5	14.4 ± 2.2	9.5	5.2
Ca^{2+}/protein	1.34 ± 0.51	0.72 ± 0.17	0.1 ± 0.30	0.86 ± 0.54	0.52	1.0
Binding-organic extraction						
K_d HA site [c]	56.5 ± 6.6	44.7 ± 4.8	50.3	45.0	n.d.	42
Ca^{2+}/protein	0.34 ± 0.08	0.14 ± 0.05	0.31	0.22	n.d.	0.39

[a] Calf heart calciphorin recently isolated.

[b] Calf heart calciphorin as reported by Jeng and Shamoo [7–9].

[c] K_d HA site is the affinity constant obtained from the high-affinity sites. Values are expressed ± S.E. for a minimum of three experiments with different preparations.

Calciphorin is extractable into organic solvents like methylene chloride, or chloroform/methanol mixtures, suggesting that it is strongly hydrophobic. There are approx. 25 moles lipid per mole protein in the 'crude' preparation (Table I). This amount is considerably less than that reported earlier by Jeng and Shamoo [6] for calf heart mitochondrial protein. However, subsequent calf heart preparations in our laboratory have yielded calciphorin preparations with lower lipid/protein ratios (17 ± 3.22 mole lipid/mole calciphorin). These are shown in Table I. Analysis of the lipids in TLC show that the main lipids associated with the isolated calciphorin are phosphatidylcholine, phosphatidylethanolamine and cardiolipin (data not shown). Deoxycholate which is present in this fraction can also be resolved on the same TLC system (chloroform/methanol/water, 65 : 25 : 4, v/v) and detected with chromerge. Residual amounts of digitonin can also be visualized. A ninhydrin positive spot can be seen just above the origin by thin-layer chromatography on silica gel. No lipids are detected in this region. Some ninhydrin positive material can be detected in the deoxycholate region of the plate. This suggests that some of the protein partitions with the detergent in the TLC system.

In order to rule out any involvement of the lipid moiety associated with the isolated calciphorin preparation in its calcium binding characteristics, we delipidated the protein using Sephadex LH-20 as described by Jeng and Shamoo [6]. The lipid/protein ratios obtained after this procedure are given in Table I. LH-20 chromatography yielded samples with a 20-fold decrease in phospholipid content. These delipidated calciphorin samples also contain very low or undetectable amounts of deoxycholate (The TLC system described here can detect a minimum of 1–2 μg of deoxycholate). Recent experiments on delipidating calf-heart calciphorin in the laboratory also yielded similar results.

Calciphorin, exhibits two classes of binding sites for calcium. We have only considered the high-af-

finity site since the binding characteristics of the low-affinity sites is almost negligible ($K_d > 200$ μM). Table I gives the values for K_d and the Ca^{2+}/protein stoichiometry obtained for various calciphorin preparations in a flow dialysis system. In each case, the same sample was used before and after delipidation. As can be seen from the data, there is no significant difference in the affinity for calcium of the delipidated protein (K_d 11.46 $\pm$ 1.93 μM), as compared to that obtained prior to delipidation (K_d 13.9 $\pm$ 2.1 μM). The stoichiometry of calcium bound per protein is also similar in both preparations. In some cases there was a slight reduction in the number of calcium bound per protein. This may be a consequence of denaturation of the protein. However, there is no correlation between the lipid content of the calciphorin sample and either the affinity or stoichiometry for calcium.

As given in Table I, the K_d values obtained from organic extraction experiments of delipidated calciphorin (44.7 $\pm$ 4.8 μM) and non-delipidated calciphorin (56.5 $\pm$ 6.6 μM) are also very similar. The stoichiometry of calcium/protein is also similar for both preparations. Here again there is no

TABLE II

AMINO ACID ANALYSIS OF CALCIPHORIN FROM RAT LIVER MITOCHONDRIA

Amino acid composition was determined by the method of Spackman et al. [19]. 0.1 mg of protein sample was lyophilized and boiled at 110°C for 24 h in 6 M constant boiling HCl, under vacuum. The values reported are mole residue per mole calciphorin. n.d., not detected.

Residue	Rat liver	Calf heart [a]
Asp	3.0	2
Thr	2.0	4
Ser	1.0	4
Glu	3.0	4
Pro	2.0	–
Gly	4.0	7
Ala	5.0	3
Cys	n.d.	n.d.
Val	2.0	1
Met	1.0	1
Iso	2.0	1
Leu	4.0	2
Tyr	1.0	1
Phe	1.0	1
His	1.0	2
Lys	3.0	1
Arg	2.0	1

[a] According to Jeng and Shamoo.

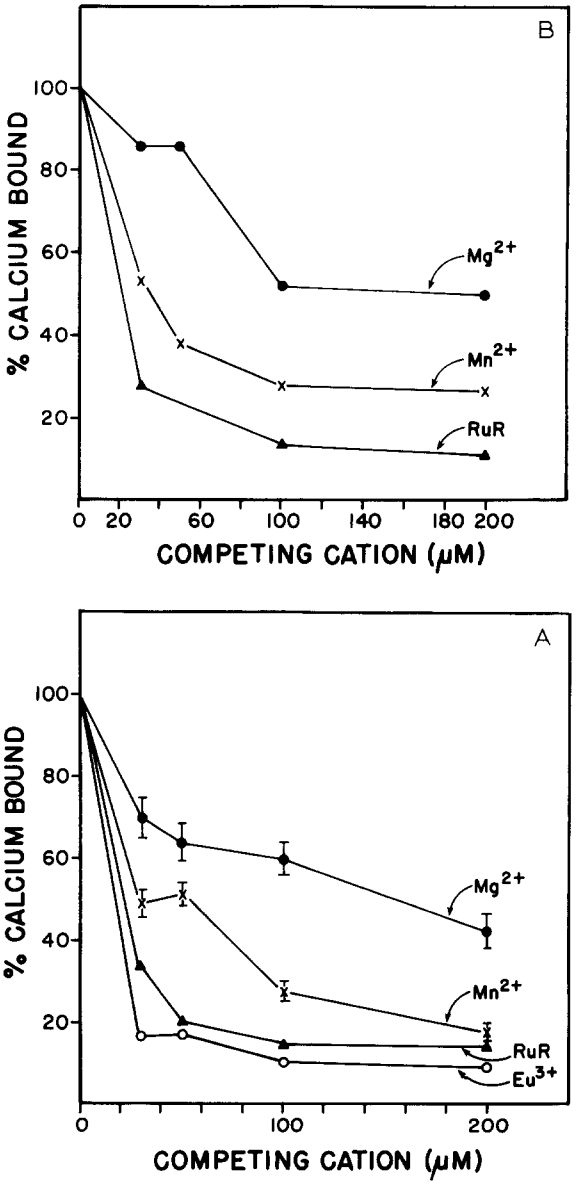

Fig. 3. Divalent cation selectivity of 'crude' calciphorin (A) and delipidated calciphorin (B). The selectivity was determined by the inhibition of the protein mediated Ca^{2+} extraction by various divalent cations and Ruthenium red (RuR), into a methylene chloride phase. The organic (1.0 ml) phase contained between 10–20 μg of protein. The aqueous phase (0.5 ml) contained 5 mM Tris, pH 7.4, 50 μM $^{45}CaCl_2$, 200 μM picric acid, and the indicated concentrations of the cations.

correlation between the lipid moiety associated with calciphorin and the binding characteristics. Table I also compares the K_d and Ca^{2+}/protein of rat liver calciphorin, calf heart calciphorin described by Jeng and Shamoo [5–7], and recent calf heart calciphorin preparations in the laboratory. As can be seen in the data presented, the calcium-binding characteristics of calciphorin are largely comparable in the rat liver and calf heart preparations.

Fig. 3 shows the effect of various concentrations of ions; viz., Mn^{2+}, Mg^{2+}, Eu^{3+}, and Ruthenium red on the amount of calcium bound by non-delipidated and delipidated calciphorin. In both cases, Ruthenium red completely inhibits binding. The pattern of competition for calcium by Mn^{2+} and Mg^{2+} are similar in both cases. Eu^{3+}, which was tried for the non-delipidated preparation, competed with calcium more successfully than Mn^{2+}.

The amino acid analysis (mole amino acid per mole calciphorin) is shown in Table II. The values obtained for the calf heart preparation [7] are also given. As can be seen, there is some variation between the two preparations in terms of the amino acid composition. The rat liver preparation has a total of 38 residues, with a minimum molecular weight of 4619. This is considerably lower than that seen with the crude preparation on SDS-urea gels. The gel may represent an aggregated state of the protein. Of the total amino acids, hydrophobic residues make up 44.7%; acidic 15.8%; basic 15.8%; and others, 21%.

Table I summarizes the results presented in this paper obtained with rat liver calciphorin. Also given are the data as reported earlier for the calf heart preparation by Jeng and Shamoo [6,7] and those subsequently obtained in the laboratory for similar calf heart preparations. There are a few significant differences within the calf heart preparations e.g. lipid/protein ratio and calcium binding in the flow dialysis system. We do not have any explanations for these differences at the present time. However, the results are largley comparable. The calcium binding and electrophoretic properties of rat liver calciphorin can also be compared with those of the calf heart preparation. The data suggest that the preparations are very similar in their properties, in spite of the variations in their molecular weights. The percentage yield at various steps in the isolation procedure and that of purified calciphorin (0.2 ± 0.15 per mg mitochondria for rat heart and 0.31 per mg mitochondria for calf heart), and the affinities for calcium in delipidated and non-delipidated preparations are comparable in the protein from the two mitochondrial sources.

The results presented in this paper give evidence for the presence of a low molecular weight Ca^{2+} binding protein in rat liver mitochondrial inner membranes. The protein was obtained using similar isolation procedures and is similar in its properties to calciphorin isolated from the calf heart preparation (Table I). Prior to delipidation, the protein is very hydrophobic and can be extracted into a number of organic solvents. Even after delipidation, it does not loose its hydrophobicity, even though there is less than a mole of lipid associated with it. The protein is rendered a little more polar and is soluble in methanol or chloroform/methanol (1 : 1, v/v). The hydrophobicity of the protein, thus, conforms with its possible role as a ion carrier in the mitochondrial membrane.

As was the case with calf heart calciphorin a large amount of lipids remain associated with the isolated rat liver calciphorin. Recent studies from this laboratory [11] have shown that lipids exhibit strong Ca^{2+} binding characteristics, fulfilling most of the criteria listed above. Tyson et al. [12] have shown that cardiolipin can function as an ionophore in a Pressman cell. Ca^{2+}-phospholipid interactions have also been reported by other workers. Cardiolipin and phosphatidic acid have been demonstrated to induce Ca^{2+} influx [13,14] in platelets and synaptosomes. In our preparation, a specific role for the protein has been established by the high-affinity binding exhibited with the delipidated protein. It is clear that the amount of lipid associated with the preparation at any time does not correlate with either its binding characteristics or the Ca^{2+}/protein stoichiometry. However, the binding is very susceptible to variations in pH [7]. The binding of Ca^{2+} by cardiolipin on the other hand has been shown to remain constant over a pH range of 5.0 to 8.4 [12].

Recent experiments in our laboratory using fluorescence lifetime measurements with Eu^{3+} as a

probe [15] have shown that the Ca^{2+}-binding species isolated from either rat liver or calf heart mitochondria can be differentiated from detergent or lipids in terms of its binding properties. Also, delipidation does not alter the fluorescence characteristics (emission pattern or lifetime). Very recent studies in the laboratory (Jayaweera et al., unpublished observations) have shown that delipidated calciphorin exhibits cation selective increases in the conductance of black lipid membrane. Reconstitution into lipid vesicles will provide more information on the nature of the transport.

The studies presented in this paper suggest that calciphorin, a probable candidate for the Ca^{2+} carrier involved in the influx, can be isolated from both rat liver mitochondria and calf heart mitochondria. The protein isolated from both sources are comparable in terms of Ca^{2+}-binding affinities and characteristics and probably represent the respective electrophoretic uptake system in these two mitochondria types.

References

1 Slater, E.C. and Cleland, K.W. (1953) Biochem. J. 55, 566
2 Gomez-Puyou, A., Tuena de Gomez-Puyou, M., Becker, G. and Lehninger, A.L. (1972) Biochem. Biophys. Res. Commun. 47, 814–819
3 Carafoli, E. (1975) Mol. Cell. Biochem. 8, 133–140
4 Panfili, E., Sandri, G. and Sottocasa, G.L. (1982) in EBEC reports, Vol. II, LBTM-CNRS Ed., pp. 485–486
5 Jeng, A.Y., Ryan, T.E. and Shamoo, A.E. (1978) Proc. Natl. Acad. Sci., U.S.A. 75, 2125–2129
6 Jeng, A.Y. and Shamoo, A.E. (1980) J. Biol. Chem. 255, 6897–6903
7 Jeng, A.Y. and Shamoo, A.E. (1980) J. Biol. Chem. 255, 6904–6912
8 Sokolove, P.M. and Brenza, J.M. (1983) Arch. Biochem. Biophys. 221, 404–416
9 Shamoo, A.E., Brenza, J.M. and Sokolove, P.M. (1980) in Calcium Binding Proteins Structure and Function (Siegel, F.L., Carafoli, E., Kretsinger, R.H., MacLennan, D.H. and Wasserman, R.H., eds.), pp. 131–138, Elsevier/North-Holland, Amsterdam
10 Small, D.M. (1971) in The Bile Acids: Chemistry, Physiology and Metabolism (Padmanabhan, P.N. and Kritchevsky, D., eds.), Vol. 1, pp. 249–355, Plenum Press, New York
11 Sokolove, P.M., Brenza, J.M. and Shamoo, A.E. (1983) Biochim. Biophys. Acta, 732, 41–47
12 Tyson, C.A., Vande Zande, H. and Green, D.E. (1976) J. Biol. Chem. 251, 1326–1332
13 Ikeda, Y., Kikuchi, M., Toyama, K., Watanabe, K. and Ando, Y. (1979) Thromb. Haemostas. 41, 779–786
14 Harris, R.A., Schmidt, J., Hitzemann, B.A. and Hitzemann, R.J. (1981) Science 212, 1290–1291
15 Hermann, T.R., Jayaweera, A.R. and Shamoo, A.E. (1983) Biophys. J. 41, 187a
16 Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462–477
17 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769–775
18 Colowick, S.P. and Womack, F.C. (1969) J. Biol. Chem. 244, 774–777
19 Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190–1206