Calcium binding to polypeptides of rat liver and Zajdela hepatoma mitochondrial inner membranes

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Abstract Composition and amount of $^{45}Ca^{2+}$-binding proteins in the inner membrane fraction of rat liver and Zajdela hepatoma mitochondria were determined. In the inner membrane of liver mitochondria, three major $^{45}Ca^{2+}$-binding polypeptides: a protein of ~130 kDa (carbamoyl-phosphate synthetase), a glycoprotein of 43–44 kDa (previously considered as the calcium uniporter), and 29–30 kDa protein were found. These components were absent (130 kDa component) or relatively reduced (43–44 kDa and 29–30 kDa components) in the inner membrane of hepatoma mitochondria. Previously unknown low molecular mass polypeptides, having very high Ca^{2+}-binding ability, were found in the inner membrane of hepatoma mitochondria. One of them might be the natural Ca^{2+}-binding inhibitor of H^+-ATPase.

Key words: Liver cell; Hepatoma cell; Mitochondrion; Ca^{2+}-binding protein

1. Introduction

The interaction of mitochondria with Ca^{2+} ions plays an essential role in regulation of intracellular processes and is mediated by Ca^{2+}-binding proteins (see reviews [1,2]). Changes in the Ca^{2+} concentration in the mitochondrial matrix is a signal regulating energy-transducing system, in part the activity of NAD-linked dehydrogenases. Of particular interest are the Ca^{2+}-binding proteins of the inner mitochondrial membrane (IMM) where Ca^{2+}-transporting systems and the basic systems of energy transformation are located.

The redistribution of Ca^{2+} in IMM is mediated by the activity of the Ca^{2+} uniporter, the electroneutral antiporters $Ca^{2+}/2H^+$ and $Ca^{2+}/2Na^+$ and by the Ca^{2+}-induced permeability transition pore (PTP) opening [2]. Many attempts to identify the Ca^{2+} uniporter have been undertaken during the recent 20 years. Ca^{2+}-binding proteins with molecular mass of 3 kDa (calciphorin) [3], 20 kDa [4], 33 kDa [5] and 40–43 kDa (glycoproteins) [6] have been isolated, although the nature of Ca^{2+} uniporter still remains elusive. Important Ca^{2+}-binding components of IMM may be proteins that participate in PTP opening. It is known that the PTP opening is induced by Ca^{2+} accumulation in the mitochondrial matrix [2], and in patch-clamp experiments Ca^{2+} has been shown to induce the formation of large pores (megachannels) in IMM [7]. However, the Ca^{2+}-binding proteins participating in regulation or directly forming PTP have not been identified. Data on the influence of Ca^{2+} on the ADP/ATP antiporter and on an oxidative phosphorylation have been published [1,8,9]. In this context, the data on the isolation of the Ca^{2+}-binding inhibitor protein (CaBI) of H^+-ATPase [10], the discovery of calmodulin in mitochondria and its action on suppressing of H^+-ATPase activity [11], and also the description of the Ca^{2+}-binding properties of β-subunit of H^+-ATPase are of interest [12].

Investigations of Ca^{2+}-binding proteins have in general been carried out on mitochondria of liver and heart muscle. Data on Ca^{2+}-binding proteins of mitochondria of tumor cells are lacking, nevertheless there are a number of functional differences in the interaction of Ca^{2+} with mitochondria of normal and tumor cells. Thus it has been reported that oxidative phosphorylation and H^+-ATPase of tumor cells are highly sensitive to the inhibitory effect of Ca^{2+} [13], while the PTP opening in tumor mitochondria is relatively resistant to Ca^{2+} [14]. These functional differences can be connected with different composition and content of Ca^{2+}-binding proteins in mitochondria.

In the present study the composition of Ca^{2+}-binding proteins of mitochondrial inner membrane of normal and tumor cells has been compared. An estimation of Ca^{2+}-binding was carried out after polypeptide separation by gel electrophoresis, followed by Western blotting.

2. Materials and methods

Zajdela hepatoma ascites cells were allowed to grow in the peritoneal cavity of male white (Wistar) rats. Cells were harvested 6–7 days after inoculation. Normal liver mitochondria were prepared by differential centrifugation, as previously described [15], hepatoma mitochondria were isolated as described by Moreadith and Fiskum [16]. The isolation medium contained 210 mM mannitol, 70 mM sucrose, and 5 mM HEPES (pH 7.2), and 1 mM EGTA. Mitochondria were twice washed with the isolation medium without EGTA. Additional purification of liver and hepatoma mitochondria was carried out in sucrose density gradient [17]. The outer membranes and mitoplasts were separated by the digitonin procedure [18]. Mitoplasts were washed twice and were sonicated on ice for 2 min at 20 s intervals. Unbroken mitoplasts were then sedimented at $3000 \times g$, followed by centrifugation of the supernatant at $140\,000 \times g$ during 1 h in order to obtain the inner membrane fraction. The procedure was previously described in more detail [15]. Calcium-binding ATPase inhibitor protein, namely, heat-stable fraction from hepatoma mitochondria was obtained according Yamada's procedure [10].

Electrophoresis was performed in SDS-polyacrylamide gel in Laemmli's system [19], containing 5% acrylamide in the concentrating gel and 7–20% linear acrylamide gel gradient in the resolving gel. The following molecular mass standards were used: phosphorylase B 94 kDa, bovine serum albumin 67 kDa, egg albumin 43 kDa, carboanhydrase 30 kDa, soybean trypsine inhibitor 20.1 kDa, bovine milk α-lactalbumin 14.4 kDa. Solubilized samples (5–8 μg protein) were layered onto the gel surface. After the electrophoresis, the gels were fixed and stained with Coomassie Brilliant Blue R-250. Electrophoresis of

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Abbreviations: IMM, inner mitochondrial membrane; PTP, permeability transition pore; LMM CaBP, low molecular mass Ca^{2+}-binding protein; CaBI, Ca^{2+}-binding inhibitor protein of H^+-ATPase

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low molecular mass Ca^{2+}-binding proteins (LMM CaBPs) was performed in Laemmli's system containing 4% acrylamide in the concentrating gel and 15% acrylamide in the resolving gel. Kaleidoscope polypeptide standards (Bio-Rad) were used as molecular mass markers: carbonic anhydrase 36.9 kDa, soybean trypsin inhibitor 27.6 kDa, lysozyme 15.3 kDa, aprotinin 8.7 kDa, and insulin 5.5 kDa. After electrophoresis the gels were silver stained. Proteolysis of LMM CaBP was performed in the presence of trypsin (2.0 mg/ml) during 30 min at 37°C. Ca^{2+}-binding to polypeptides of the inner membrane was examined after Western electrotransferring by Towbin's method [20]. Proteins were electro-blotted from gels to nitrocellulose membranes (0.2 μm) for 1.5 h at 75 V in 24 mM Tris base, 192 mM glycine buffer (pH 8.3), containing 20% methanol. The binding $^{45}Ca^{2+}$ by blotted polypeptides was performed according to Maruyama [21]. The nitrocellulose membrane was soaked in a solution containing 60 mM KCl, 5 mM $MgCl_2$, and 10 mM imidazole-HCl (pH 6.8–7.1). Buffer was exchanged 4 times an hour to wash away the electrode buffer. Then the membrane was incubated in the same buffer, containing 3 μM $^{45}Ca^{2+}$ (1 mCi/l) for 30 min. The membrane was rinsed twice with deionized water (mQ) in 200 ml. Autoradiographs of the $^{45}Ca^{2+}$-labeled proteins on the nitrocellulose membrane were obtained by exposure of dried membrane to Kodak XAR-5 film for 5 days.

Scanning of electrophoregrams and autoradiograms and determination of the areas of absorption bands were carried out using a SD-1M spectrophotometer-densitometer. It should be noted that in the range of registered values of optical density the linear dependence of the areas of absorbance bands of separated polypeptides to the amount of total protein was observed.

3. Results

Fig. 1 shows typical gel electrophoretic densitograms of proteins in the inner mitochondrial membranes from rat liver and Zajdela hepatoma cells. There are clear differences in the polypeptide patterns between liver and hepatoma mitochondria. In the inner membrane of liver mitochondria there are four zones of increased content of polypeptides with a M_r of 100–140 kDa, 40–60 kDa, 30–40 kDa and 15–20 kDa. Protein components of hepatoma mitochondria show a different pattern; the number of large components is distributed rather regularly in the range of 30–100 kDa; in addition, there is a separate polypeptide group in the range of 10–16 kDa (Fig. 1B).

Fig. 2 shows the pattern of Ca^{2+}-binding proteins using $^{45}Ca^{2+}$ radioautography after electrophoresis and blotting. Three Ca^{2+}-binding components can be seen in liver IMM (Fig. 2A): a Ca^{2+}-binding band I (~130 kDa), a narrow, intensive band III (43–44 kDa) and a wide band IV (29–30 kDa). It should be noted that there are two wide bands V and VI in the range of low molecular mass peptides (< 10 kDa). The several low-intensity bands can also be seen in Fig. 2A, however, the Ca^{2+}-binding with these components is weak and apparently non-selective.

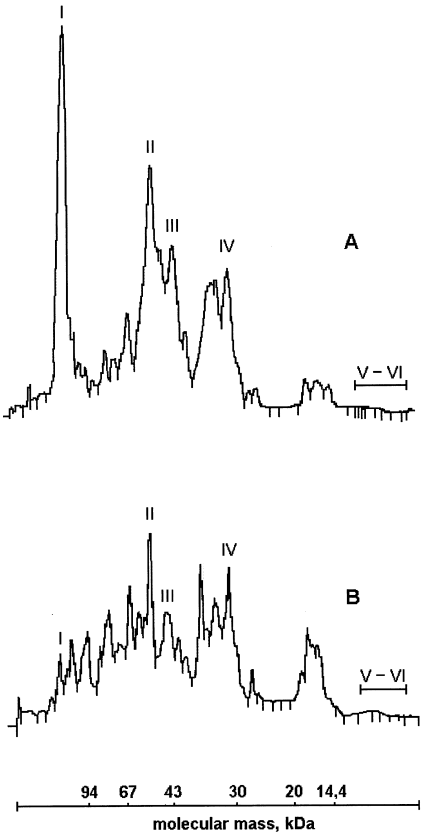

Fig. 1. The distribution of polypeptides on electrophoregrams of rat liver (A) and Zajdela hepatoma (B) IMM. Polypeptide bands having a high Ca^{2+}-binding ability: I–VI.

The Ca^{2+}-binding pattern of polypeptides in the IMM of hepatoma cells is significantly different. Fig. 2B shows that appreciable Ca^{2+}-binding in the range of 130 kDa is absent, there are two Ca^{2+}-binding band II and III with similar intensity, while the intensity of Ca^{2+}-binding in band III is much lower in comparison with liver mitochondria. A striking feature is the markedly high Ca^{2+}-binding of the low molecular mass peptides in bands V and VI in the case of hepatoma IMM.

The quantitative data describing the relative contents of polypeptides and their Ca^{2+}-binding capacity in preparations of IMM of liver and hepatoma are given in Table 1. As can be seen from the table repeated distinctions of Ca^{2+}-binding are observed to the I, III and V–VI components. The absence of appreciable Ca^{2+}-binding to 130 kDa protein in the hepatoma IMM indicates a low content of this component or its com-

Table 1
The contents of polypeptides and their Ca^{2+}-binding ability in IMM of liver and hepatoma mitochondria

Components (No.)	M_r (kDa)	Ca^{2+}-binding			The contents of polypeptides (%)	
		Percentage of total		Ratio hepatoma/liver	Liver	Hepatoma
		liver	hepatoma			
I	~130	15.2 ± 4.3	0.9 ± 0.1	0.05	12.0 ± 2.3	1.9 ± 0.1
II	50–52	1.7 ± 0.3	3.2 ± 0.5	1.98	2.1 ± 0.3	5.6 ± 0.5
III	43–44	12.8 ± 2.1	3.1 ± 0.2	0.24	7.4 ± 2.4	2.7 ± 1.5
IV	29–30	6.3 ± 1.8	4.4 ± 1.7	0.69	4.8 ± 0.4	3.5 ± 0.7
V–VI	< 10	6.6 ± 1.5	51.5 ± 9.3	7.80	–	0.07 ± 0.01

The average data obtained from three experiments are shown as percentage of the total contents of polypeptides or of the sum of Ca^{2+}-binding to IMM polypeptides. The experimental conditions are described in Section 2.

plete absence. The Ca^{2+}-binding to component III is several times higher in liver preparations of IMM and correlates with the increased content of this polypeptide. The Ca^{2+}-binding to components II and IV does not differ much. Notably, there is a significant increase in the Ca^{2+}-binding to the low molecular mass components V–VI (< 10 kDa). The Ca^{2+}-binding of these components amounts to 50% of total $^{45}Ca^{2+}$-binding in spite of the extremely low amount of protein in this low molecular mass fraction. It should be emphasized that non-protein compounds could also be present in the region of low molecular mass fractions. However, the staining of gels for nucleic acids did not show any presence of these macromolecules, and the staining for lipids did not show any appreciable differences in IMM of liver and hepatoma preparations.

Additional experiments were undertaken to determine the M_r value of low molecular mass Ca^{2+}-binding proteins and to confirm their polypeptide nature. LMM CaBP (bands V–VI, Fig. 2B) was extracted from electrophoretic gel after separation of hepatoma IMM. The extract was concentrated and subjected to electrophoresis in 15% SDS acrylamide gel.

It was found that LMM CaBP was able to bind Ca^{2+} after repeated electrophoresis and blotting, but only one Ca^{2+}-binding band was observed in 15% gel (Fig. 3B, E). According to Kaleidoscope polypeptide standards M_r of LMM CaBP is about 6.3–6.6 kDa. Ca^{2+}-binding and protein content were strongly decreased after proteolytic digestion of LMM CaBP (Fig. 3C, F). It should be noted that similar Ca^{2+}-binding band was found (Fig. 3D, G) in the case of electrophoresis and blotting of natural Ca^{2+}-binding inhibitor of H^+-ATPase prepared according to Yamada's procedure [10].

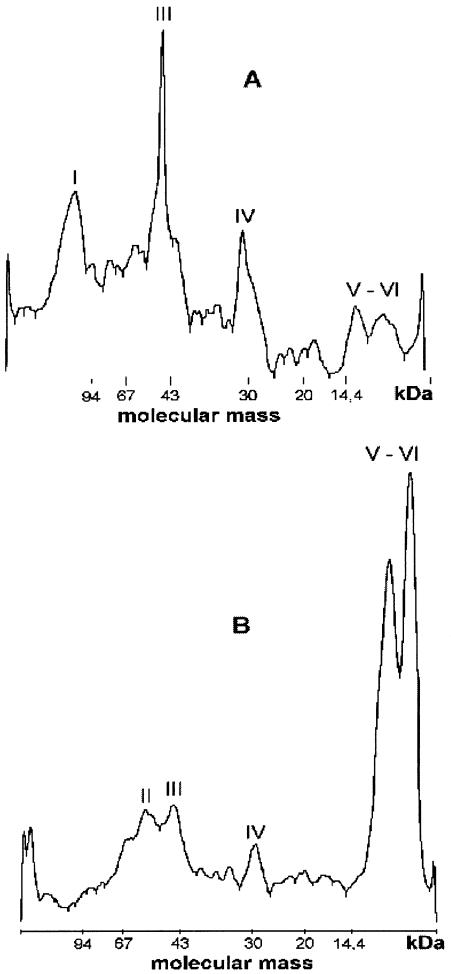

Fig. 2. The binding of $^{45}Ca^{2+}$ to polypeptides of rat liver (A) and Zajdela hepatoma (B) IMM. The experimental conditions are described in Section 2.

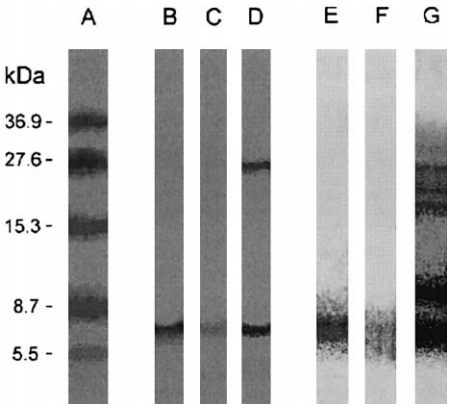

Fig. 3. Calcium binding to LMM CaBP and Yamada's CaBI. Kaleidoscope polypeptide standards (densitogram). B, E: LMM CaBP (from 25 μg protein of hepatoma IMM). C, F: Trypsin digested LMM CaBP. D, G: CaBI (heat-stable protein fraction). B, C, D: Autoradiograms. E, F, G: Silver stained proteins. The experimental conditions are described in Section 2.

4. Discussion

In the present paper the existence of several Ca^{2+}-binding components in IMM of rat liver and Zajdela hepatoma cells has been demonstrated. The observed Ca^{2+}-binding was specific since the binding of $^{45}Ca^{2+}$ to polypeptides was carried out using rather low concentration of Ca^{2+} in the medium (3 μM) and washing of nitrocellulose was carried out in solutions devoid of Ca^{2+}. Essential differences in composition of Ca^{2+}-binding polypeptides of IMM of liver and hepatoma were detected, suggesting differences in the interaction of Ca^{2+} with liver and hepatoma mitochondria.

The component with the M_r ~130 kDa was earlier identified as carbamoyl-phosphate synthetase in rat liver mitochondria [22]. The absence of carbamoyl-phosphate synthetase in hepatoma IMM observed in our experiments is in accordance with remarkably low specific activity of the enzyme in hepatoma cells [23]. Selective interaction of the rat liver enzyme with cardiolipin and other anionic phospholipids has been reported [24] and this interaction could be responsible for the enzyme binding with IMM and/or with Ca^{2+}. The observed carbamoyl-phosphate synthetase binding with Ca^{2+} indicates a possibility of regulating the activity of this enzyme by Ca^{2+} ions.

The Ca^{2+}-binding component of 43–44 kDa has been isolated earlier in our laboratory from liver mitochondria and its ability to carry out selective transport of Ca^{2+} through bilayer lipid membrane was demonstrated [6,25].

The nature of 50–52 kDa and 29–30 kDa Ca^{2+}-binding components is not known. However, it should be noted in this connection that the 50–52 kDa polypeptide could be β-subunit of H^+-ATPase, since it recently was reported that Ca^{2+} was able to bind with this subunit [12]. Earlier, in the work of Kottke et al. [26] it was shown that Ca^{2+}-binding

polypeptide of 30 kDa is located in the contact sites of outer and inner membranes of mitochondria. Recently it was shown that the mitochondrial ADP/ATP translocase can be reversibly converted into large channel by Ca^{2+} [27] and this translocase is also enriched in the contact sites. These data suggest that the observed 30 kDa Ca^{2+}-binding component is localized in the contact sites and may directly participate in the formation of Ca^{2+} regulated membrane contacts and/or transmembrane pores in analogy with annexins [28].

As demonstrated by our data the most interesting result is the higher affinity Ca^{2+}-binding of LMM CaBP of hepatoma mitochondria. The nature and functional role of these components remains to be elucidated. However, it may be suggested that among these components there is the Ca^{2+}-binding natural inhibitor of H^+-ATPase (CaBI), isolated earlier from heart mitochondria and recently detected in some other cells [10,29]. The assumption of the increased CaBI contents in hepatoma IMM supports our earlier data on strong inhibition of oxidative phosphorylation and H^+-ATPase activity in tumors by Ca^{2+} ions [13,30].

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