Phosphatidylserine decarboxylase is located on the external side of the inner mitochondrial membrane

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It is shown that the trypsin-treatment of rat liver mitochondria, depleted of the outer membrane, causes a strong inactivation of phosphatidylserine decarboxylase. This inactivation is dependent on trypsin concentration and the time of digestion in a similar manner as the inactivation of cytochrome oxidase. Under these conditions only a moderate inactivation of succinate dehydrogenase is observed. Phosphatidylserine decarboxylase is thus localized in the outer leaflet of the inner mitochondria membrane or, at least, is accessible from the outer surface of the inner membrane.

Phosphatidylserine decarboxylase *Mitochondria* *Intramitochondrial localization* *Rat and mouse liver*

1. INTRODUCTION

The reaction utilizing CDP-ethanolamine and diacylglycerol as precursors, and the decarboxylation of phosphatidylserine are alternative pathways for the formation of phosphatidylethanolamine in animal cells [1]. The latter reaction, catalyzed by phosphatidylserine decarboxylase (EC 4.1.1.65), occurs in mitochondria [2,3] and its substrate, phosphatidylserine, has to be transported to these organelles from the endoplasmic reticulum, where it is synthesized [4], by means of phospholipid transfer protein(s) [5,6].

The intramitochondrial localization of phosphatidylserine decarboxylase is still controversial. Authors in [7] have shown the highest specific activity of this enzyme in the outer membrane fraction of mouse liver mitochondria, whereas in [8] evidence was provided for its localization in the inner membrane of rat liver mitochondria. Here, we first re-examined this problem using both mouse and rat liver mitochondria and confirmed the compartmentation proposed in [8]. Subsequently, we investigated the localization of phosphatidylserine decarboxylase in the transverse plane of the inner mitochondrial membrane by subjecting mitochondria depleted of the outer membrane (mitoplasts) to controlled proteolytic digestion, which has been widely used for studying the sidedness of enzymes in various biological membranes [9,10]. Along with phosphatidylserine decarboxylase we measured the inactivation of two respiratory enzymes, viz. cytochrome oxidase (EC 1.9.3.1) and succinate dehydrogenase (EC 1.3.99.1), localized on opposite sides of the inner mitochondrial membrane.

2. MATERIALS AND METHODS

Mitochondria from rat and mouse liver and microsomes, and the cytoplasmic fraction from rat liver were isolated by the conventional procedure [11]. The cytoplasmic fraction was made free of lipoproteins by sedimenting them at pH 5.1 [12]. Then, after adjusting pH to 7.4 with 1 M NaOH, Tris–HCl buffer was added to final concentration of 10 mM.

For studying intramitochondrial compartmentation of phosphatidylserine decarboxylase, outer membranes and mitoplasts were obtained using the

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swelling procedure [13]. When the localization of the enzyme in the inner membrane was investigated, mitoplasts were obtained as in [14] using 0.16 mg digitonin/mg mitochondrial protein.

Decarboxylation of phosphatidylserine was followed by measuring liberation of $^{14}CO_2$ from phosphatidylserine labelled in the carboxyl group of serine [2,15]. The main compartment of a Warburg vessel contained 250 mM sucrose–10 mM Tris–HCl (pH 7.4), mitochondria or submitochondrial fractions in amounts indicated in the legends, the cytoplasmic fraction (4.5 mg protein) and liposomes (0.3–0.6 μmol phospholipid phosphorus) containing about 4.5% phosphatidylserine (~15000 cpm of [^{14}C]phosphatidylserine) in a total volume of 1.5 ml. The center compartment contained a strip of filter paper soaked with 250 μl Hyamine 10-X hydroxide in methanol (10%, w/v). The side arm was filled with 0.5 ml 3 M $HClO_4$ that was poured into the main compartment to stop the reaction. Incubation was carried out under constant shaking at 37°C for 60 min. After the reaction was stopped, shaking was continued for 30 min in order to absorb all CO_2 that was liberated. Thereafter, the content of the center compartments of the vessels was quantitatively transferred into counting vials containing scintillation cocktail and counted for radioactivity.

The substrate for phosphatidylserine decarboxylase was prepared by base exchange reaction [4] as follows. Microsomes (200 mg protein) were incubated with 50 μCi DL-[1-^{14}C]serine (4.4 mCi/mmol) and 3 mM $CaCl_2$ in 10 ml of 60 mM imidazole buffer (pH 7.4) for 30 min at 30°C. The incubation was terminated by addition of ice-cold EDTA to a final concentration of 10 mM, and the mixture was allowed to stand for 15 min. Lipids were extracted as in [16]. The lipid material was washed successively with EDTA, HCl, water and KCl as described in [17]. After evaporation of the solvent, the lipids were suspended in 250 mM sucrose–10 mM Tris–HCl (pH 7.4) and subjected to sonication for 4 × 30 s using the MSE 60 W sonicator operated at maximum output.

Trypsin treatment was performed in 250 mM sucrose–10 mM Tris–HCl (pH 7.4) at 37°C. The reaction was stopped by addition of a large excess (4 mg/ml) of trypsin inhibitor with subsequent incubation for 2 min before placing the tubes in an ice-water bath. After digestion, the mitoplasts were used for enzyme assays without washing.

Rotenone-insensitive NADH–cytochrome *c* reductase was measured as in [18]. Succinate dehydrogenase was followed spectrophotometrically at 600 nm with phenazine methosulphate as the primary electron acceptor and 2,6-dichlorophenol-indophenol as the terminal electron acceptor as in [19]. Malate dehydrogenase (EC 1.1.1.37) was measured by the method in [20] after solubilizing the particles in 1% Lubrol WX. Cytochrome oxidase in mitoplasts was assayed by measuring polarographically the oxidation of external cytochrome *c* in the presence of ascorbate [21].

Protein was determined by the biuret method [22] after solubilization of the material with deoxycholate. Bovine serum albumin was used as standard.

[1-^{14}C]Serine was purchased from the Institute of Nuclear Research (Świerk). Digitonin was from Fisher Scientific (Fair Lawn NJ); trypsin (type III) from bovine pancreas and trypsin inhibitor (type I-S) from soybean were products of Sigma (St Louis MO).

3. RESULTS AND DISCUSSION

To ascertain that the transfer of phosphatidylserine from liposomes to mitochondrial membranes was not a limiting factor, the cytoplasmic fraction containing phosphatidylserine transfer protein(s) was always added.

As may be seen from table 1, phosphatidylserine decarboxylase activity in both rat and mouse liver mitochondria followed the distribution of succinate dehydrogenase, and not that of rotenone-insensitive NADH–cytochrome *c* reductase. Since the former enzyme is located exclusively in the inner membrane, and the latter is a marker of the outer mitochondrial membrane [18], this observation confirms the finding in [8] on the localization of phosphatidylserine decarboxylase in the inner membrane. A different localization proposed in [7] may be due to the fact that these authors omitted density gradient centrifugation of membrane fragments and therefore their outer membrane fraction was probably appreciably contaminated with inner membrane fragments. A possible consequence of this is that the specific activity of phosphatidylserine decarboxylase in the outer membrane fraction may have been even higher

Table 1

Phosphatidylserine decarboxylase activity in mitochondrial membranes

Fraction	Phosphatidylserine decarboxylase (nmol.h^{-1}.mg protein^{-1})		Succinate dehydrogenase ($-\Delta A_{600}$.min^{-1}.mg protein^{-1})		Rotenone-insensitive NADH–cytochrome *c* reductase (ΔA_{550}.min^{-1}.mg protein^{-1})	
	Rat	Mouse	Rat	Mouse	Rat	Mouse
Mitochondria	1.0	0.9	1.8	0.3	2.2	1.0
Mitoplasts	1.4	1.3	2.0	0.8	0.8	0.8
Outer membranes	0.02	0.17	0.2	0.04	47.1	6.9

Mitoplasts were obtained by the swelling procedure [13]. For measuring phosphatidylserine decarboxylase activity the medium contained 3 mg protein of mitochondria and submitochondrial fractions, except for the mouse outer membrane fraction where 0.63 mg protein was present, in a total volume of 1.5 ml

than in mitoplasts, the matrix of which accounts for a large fraction of protein.

Susceptibility of phosphatidylserine decarboxylase to proteolytic digestion was investigated in experiments in which mitoplasts were incubated either for a fixed time with increasing amounts of trypsin (fig.1A) or with a fixed amount of trypsin for various periods of time (fig.1B). Both types of experiments revealed the same feature, namely that phosphatidylserine decarboxylase was the most susceptible enzyme to trypsin digestion, closely followed by cytochrome oxidase, and that succinate dehydrogenase was by far more resistant. Since it is well established that cytochrome oxidase is accessible from the external side of the inner mitochondrial membrane [23], whereas succinate dehydrogenase is located at the internal side of this membrane [24,25], these experiments clearly point to the localization of phosphatidylserine decarboxylase in the external leaflet of the inner membrane, perhaps even more exposed than cytochrome oxidase.

Some inactivation of succinate dehydrogenase, especially after longer incubation or with higher amounts of trypsin, was probably due to a more profound digestion and rupture of the inner membrane in a portion of mitochondrial population. This was substantiated by the observation that malate dehydrogenase activity decreased in parallel (fig.1). Malate dehydrogenase could be destroyed by trypsin only when it leaked out to the external medium or when trypsin could get an access to the matrix compartment.

Localization of phosphatidylserine decarboxylase in the external leaflet of the inner mitochondrial membrane seems logical with regard to the fact that phosphatidylserine originates outside mitochondria. However, it cannot be excluded that the enzyme may also be accessible for its substrate from the internal side of the membrane.

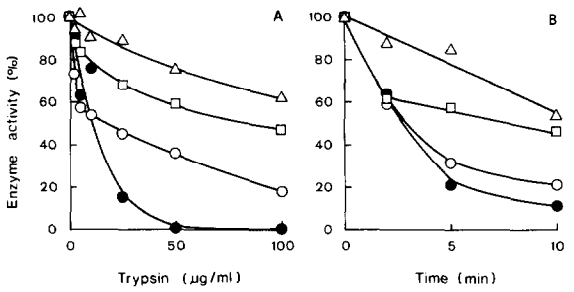

Fig.1. Effect of trypsin digestion of rat liver mitoplasts on their enzymic activities. Mitoplasts (3 mg protein/ml) obtained by digitonin treatment [14] were exposed to trypsin for the time indicated. The enzyme activities of samples to which trypsin inhibitor was added before trypsin was taken as 100%. All samples were assayed in duplicate and mean values are presented. (●) Phosphatidylserine decarboxylase; (○) cytochrome oxidase; (□) succinate dehydrogenase; (△) malate dehydrogenase. (A) Dependence on the amount of trypsin; incubation time 10 min; (B) dependence on the incubation time; trypsin 100 μg/ml.

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