THE SIDENESS OF MITOCHONDRIAL MEMBRANES AND THEIR SUB-PARTICLES AS CHARACTERIZED BY PREPARATIVE FREE FLOW ELECTROPHORESIS

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1. Introduction

Within the past few years investigations have been carried out in order to show the sideness of submitochondrial particles obtained by sonication or sonication combined with digitonin treatment of mitochondria [1–7]. In the present study the different electrical surface charges of outside-out and inside-out inner membrane particles of rat liver mitochondria were used in order to directly characterize these particles, to separate them from one another, and to isolate a highly purified inside-out submitochondrial preparation free of any outer and outside-out inner membranes or microsomal vesicles.

2. Materials and methods

All the experiments were carried out at 4°. Rat liver mitochondria and inner mitochondrial membranes were prepared as described in [8] except that EDTA was omitted from the isolation and separation medium (5×10^2 μmhos). The inner membrane-matrix particles from the electrophoresis run were sonicated in isolation medium without sucrose in a Branson Sonifier B-12 at 130 W for 5 × 10 sec at 0°. Then they were spun down at 100,000 *g* for 10 min and washed twice with isolation medium in order to remove non-membrane-bound enzymes. Preparative free flow electrophoresis (FF3 electrophoresis) of these particles was performed as described in [8] with a separation medium without EDTA (95 V/cm, 100 mA, $t = 5°$, buffer flow 1.75 ml per hr/fraction). Protein concentration, malate dehydrogenase (MDH) and glutamate dehydrogenase (GluDH) activity were determined as in [8], as was succinate-cytochrome *c* reductase (SDH) activity, but 0.1 mg DOC was added to 2.5 ml test volume of the latter test for activation of the enzyme which was measured kinetically at 36°. The electron micrographs were taken with a JEM T-7 electron microscope after negative staining with 2.5% ammonium molybdate, pH 7.4 [9] or 2.5% phosphortungstate, pH 7.2. Embedded thin sections were prepared as in [8].

3. Results

In an earlier paper [8] it was shown that after a mild swelling-shrinking process of rat liver mitochondria the outer and the inner (outside-out) membrane particles could be separated very well from each other on the basis of their different surface charges. Outer mitochondrial membranes have the same surface charge as the intact mitochondria. Inner membranes enclosing the matrix show different electrical properties. Especially the inner membrane-matrix fraction from the electrophoresis runs was highly pure as was shown by enzyme tests and electron microscopy. The maximum fractions of this inner membrane-matrix peak were used in the present study for preparing submitochondrial particles by

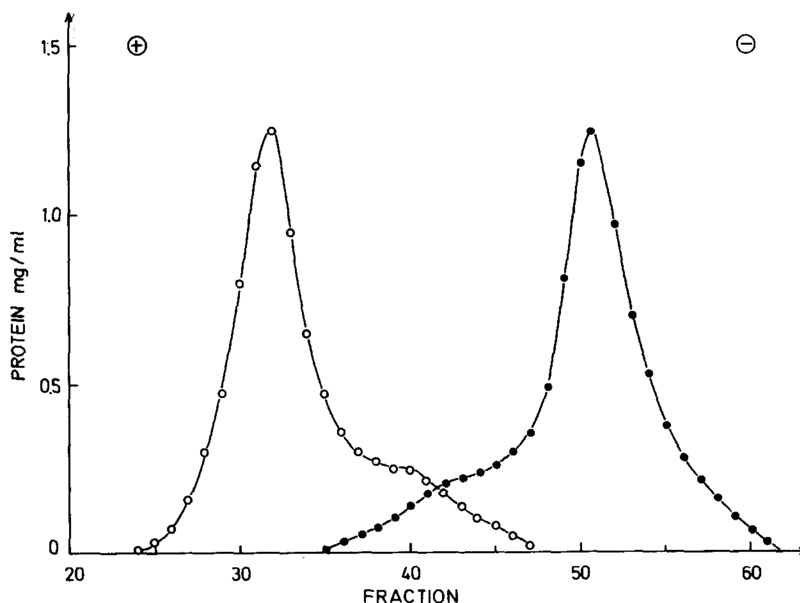


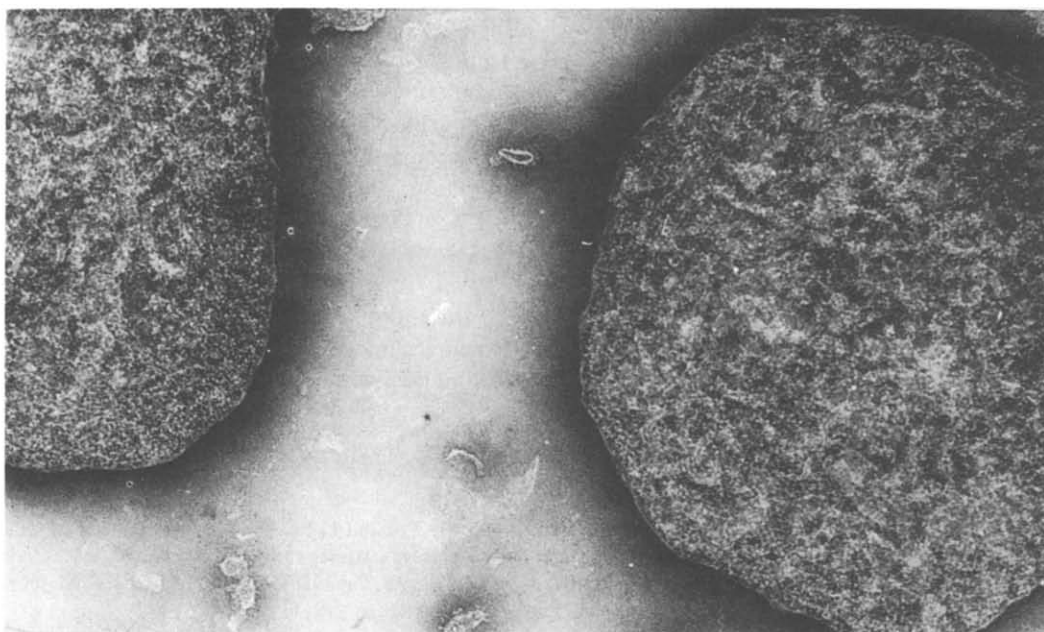
Fig. 1. Protein distribution in FF3 runs of: (a) mitochondrial inner membrane-matrix o—o—o; (b) sonicated inner membrane-matrix •—•—•.

Table 1
Some specific activities in the electrophoresis peaks of fig. 1.

Enzyme	Specific activity (mμmole/min/mg protein)	
	Fractions 26–34 inner membrane-matrix particles (outside-out)	Fractions 49–56 inner membrane particles (inside-out)
SDH	0.93	1.5
GluDH	1.6	0.023
MDH	3.6	1.1

sonication. No attempts were yet made to prepare digitonin submitochondrial particles since digitonin interferes with the electrophoresis method used. Fig. 1 shows the protein distribution in the electrophoresis runs of the inner membrane-matrix and the sonicated inner membrane-matrix. It is obvious that the electrophoretic mobility of the particles produced from the inner membrane-matrix fraction by sonication differs completely from that of the original material. The enzyme SDH, a tightly bound marker for the inner mitochondrial membrane, shows a higher specific activity in the submitochondrial particles (table 1), whereas GluDH and to a large extent MDH, markers for the mitochondrial matrix, have been lost. This is evidence that the inner membrane, at least in respect to the enzyme SHD, is itself unaltered, whereas its surface properties have been remarkably changed. This can be seen in the electron micrographs of the negatively stained particles (fig. 2). On the surface of the inner membrane-matrix particles (fractions 26–34) no subunits can be detected (outside-out membrane), whereas the surface of the sonicated particles (fractions 49–56) is regularly covered with Fernández-Moráns subunits (inside-out membrane) [10]. In addition, this fraction appears absolutely pure and no outer membrane fragments or microsomal membranes can be detected by negative staining (and thin sectioning). From the negatively stained preparation, however, no conclusions can be drawn as to whether the inside out particles are really vesiculated or not.

When inner membrane-matrix particles are sonicated for only 1–2 × 10 sec, unaltered original material and submitochondrial particles can be seen in the electrophoresis profile, well separated from each other.


(a)

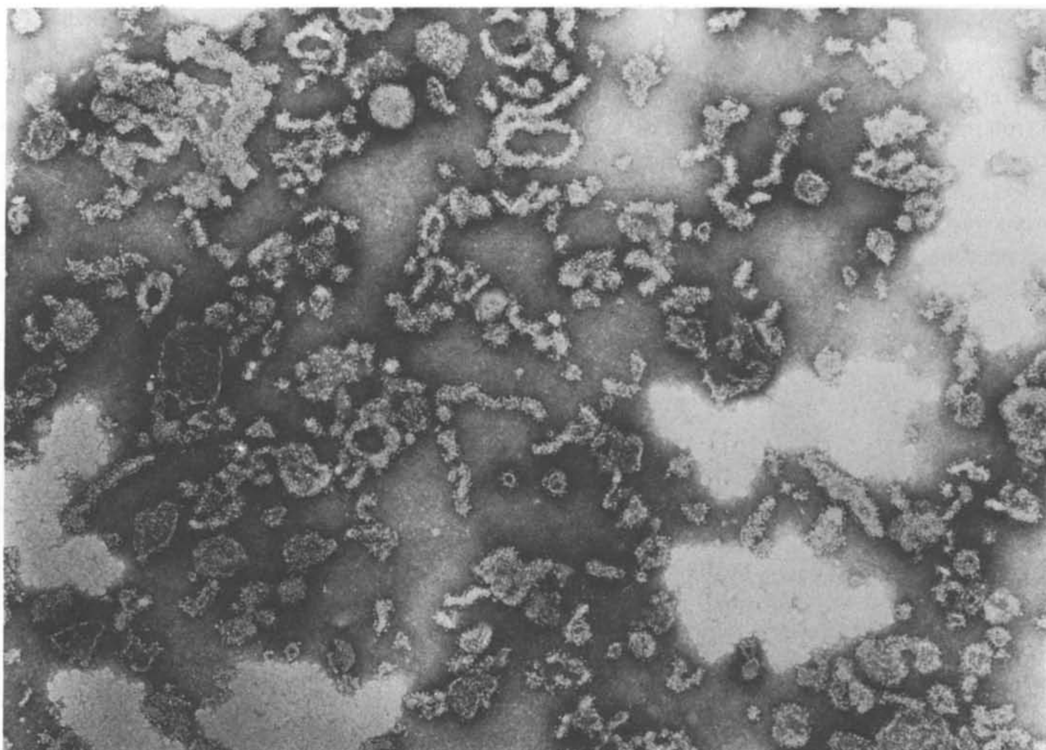

(b)

Fig. 2. Electron micrographs of negatively stained. (a) Mitochondrial inner membrane-matrix (fractions 26–34 from figs. 1 and 2). Magnification 12,000 × 4. (b) Sonicated inner membrane-matrix (fractions 49–56 from figs. 1 and 2). Magnification 24,000 × 2.

4. Discussion

From earlier investigations mentioned above [1–7] it has been accepted that the inner side of the inner membrane-matrix particles is covered with the elementary particles described first by Fernández-Morán [10], whereas the outer side of this membrane (and both sides of the outer mitochondrial membrane) do not show such conspicuous subunits. Sonicated submitochondrial particles show these elementary particles exclusively at the outside of the particles, i.e. the membrane should be inside-out. Some investigators have used transportation or binding studies with ions or substrates to show the sideness of the inner membrane in these particles [1–4], others have prepared specific antibodies against the two sides [6], or have determined the activities of side-specific enzymes of the inner membrane [5]. Freeze etching is another technique used in approaching this problem [7].

The criterium of the electrical charge of the two surfaces of the membrane used in this work provides direct evidence for the sideness of the membranes in submitochondrial particles and enables a separation of the different particles. Then the particles can be characterized enzymatically, immunologically and by electron microscopy. To what extent the lower electrophoretic mobility of the inside-out particles reflects the charge of the repeating units (ATPase) or of the cristal membrane itself, will be clarified by experiments under way, along with the question as to whether the particles are really vesiculated or closed. In addition, it would be of importance to know whether the outer mitochondrial membrane possesses two differently charged surfaces. Up till now there was no possibility of turning the outer membrane inside-out, or of perhaps detecting two electrically different surfaces in this membrane. However, even if polarity is a common feature of biological membranes, it does not necessarily have to be connected with morphological polarity.

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