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THE PROTEINS OF THE OUTER MEMBRANE OF BEEF HEART MITOCHONDRIA

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SUMMARY

The outer membrane, liberated from beef heart mitochondria by freezing and thawing in a hypotonic medium, was isolated by sucrose density gradient centrifugation. Prepared in this way, the outer membrane was found to be free of microsomal contamination and almost totally devoid of inner membrane material. Both monoamine oxidase and rotenone-insensitive NADH–cytochrome *c* reductase were found in the outer membrane but not glucose-6-phosphatase or kynurenine hydroxylase. In all, at least 14 different polypeptides were present, with three of molecular weights 46000, 34000 and 27000 accounting for over half the protein.

INTRODUCTION

The outer membrane of rat liver mitochondria has been investigated extensively and the enzymic activities of this membrane have been fairly well characterized[1–4]. In contrast, the outer membrane of beef heart mitochondria has received little attention, even though the reduced number of microsomes in heart tissue makes it possible to reduce the contamination from this membrane to negligible proportions. Recently, however, a partial purification of the outer membrane has been obtained by sonication of beef heart mitochondria followed by repeated hypotonic and detergent washings of the residue[5]. Rotenone-insensitive NADH–cytochrome *c* reductase (EC 1.6.2.1) and monoamine oxidase (EC 1.4.3.4) were identified in this preparation but citric acid cycle enzymes were absent. Polyacrylamide gel electrophorograms of this outer membrane showed the predominance of a single polypeptide which it was suggested might be the major structural element of the outer membrane in beef heart mitochondria[5]. We have investigated this possibility further, using a new method of isolation of the outer membrane from beef heart mitochondria which yields a preparation almost totally free of contamination with inner membrane or microsomal fragments. We have characterized the different proteins in the membrane by enzymatic activity and also by gel electrophoresis.

MATERIALS AND METHODS

Preparation of mitochondria

Beef heart mitochondria, were prepared by the method of Crane *et al.*[6] as

described by Hatefi and Lester[7]. Beef liver mitochondria were prepared as described by Allmann *et al.*[8] Rat liver mitochondria were generously provided by Dr Southard.

Enzymic assays

Monoamine oxidase was assayed by the method of McCaman *et al.*[9] as modified by Allmann *et al.*[8].

A modification of the method of Mackler and Green[10], described by Smoly *et al.*[5] was used to measure rotenone-insensitive NADH–cytochrome *c* reductase activity. ATPase (EC 3.6.1.4) was assayed by the method described by Senior and Brooks[11] while glucose-6-phosphatase (EC 3.1.3.9) was assayed by the method of Harper[12]; inorganic phosphate released during an incubation time of 10 min was determined as described by Lindberg and Ernster[13]. Aconitase (EC 4.2.1.3) was detected according to Anfinsen[14] and fumarase (EC 4.2.1.2) by the method of Massey[15]. α-Ketoglutarate dehydrogenase (EC 1.2.4.2) was assayed as described by Bachmann *et al.*[16] and kynurenine hydroxylase (EC 1.14.1.2) by the method of Hayaishi[17], and succinate dehydrogenase was assayed by the method of Green and Ziegler[18].

Other methods

Protein concentrations were measured by the method of Folin and Ciocalteu[19]; total lipid was extracted as described by Folch *et al.*[20] and phospholipid concentrations were estimated according to Chen *et al.*[21]. Cytochrome *a*, *b*, c_1 and b_5 were determined spectrophotometrically by the method of King *et al.*[22] using a Cary 14 spectrophotometer. The following millimolar extinction coefficients were used[23,24]: cytochrome *a* (605–630 nm) = 16, cytochrome *b* (562–575 nm) = 22, cytochrome c_1 (553–540 nm) = 19.1, cytochrome b_5 (426–405 nm) = 160.

Flavin was assayed by the method of Blair *et al.*[25].

Electron microscopy was performed using the Hitachi 11E microscope. Specimens were prepared for negative staining with 1 % phosphotungstic acid (pH 7.2). Positively stained specimens were fixed by mixing with an equal volume of a solution of 4.0 % glutaraldehyde, 0.05 M potassium cacodylate, pH 7.4, and 0.25 M sucrose. The samples were further treated with osmium tetroxide and uranyl acetate, dehydrated and then embedded as described previously[26].

Gel electrophoresis

Samples were prepared for polyacrylamide gel electrophoresis by dissolving in a medium of 1 % sodium dodecyl sulfate and 1 % β-mercaptoethanol. Complete solubilization was achieved by heating to 100 °C for 1 min. All samples were dialyzed overnight at room temperature, against an aqueous solution of 1 % sodium dodecyl sulfate and 1 % mercaptoethanol before being applied to the gels. Gel electrophoresis was performed according to the method of Shapiro *et al.*[27]. 5 % polyacrylamide gels were loaded with 50–60 μg of protein and the gels were fixed and stained with either Coomassie blue or toluidine blue. Densitometric measurements of protein gels were performed with a Gilford linear scanning attachment to a Beckman DU spectrometer using 5 mm × 10 cm quartz cuvettes. Quantitative estimates of the amount of protein in each band were made by graphically integrating the trace obtained by scanning the length of the gel at 550 nm. The approximate molecular weights of the different proteins was estimated by comparing their migration on the gel with those of a number of standard proteins[28].

Preparation and purification of the outer membrane of beef heart mitochondria

Heavy beef heart mitochondria were suspended in a medium of 10 mM Tris chloride buffer, pH 7.5, and 1 mM EDTA (100 ml of mitochondrial paste were suspended in 1 l buffer). The suspension was homogenized thoroughly using a teflon glass homogenizer and the outer membrane liberated by freezing the suspension with dry ice-acetone and then thawing in a water bath at 25° C. After freezing and thawing three times, the resulting solution was homogenized and then centrifuged at 10 000 rev./min for 10 min in a Spinco No. 21 head. The supernatant from this spin (approx. 800 ml) was decanted and its volume reduced to about one-third by dialyzing against carbowax 6000 at 4 °C for 3–5 h. Aliquots of this solution (10 ml) were layered onto a discontinuous sucrose gradient (made from 5 ml each of 1.1 M, 0.8 M, 0.6 M and 0.4 M, pH 7.5) and centrifuged at 25 000 rev./min for 60 min. The 0.6 M sucrose layer which had a whitish opalescent appearance was collected with a disposable pipette, diluted three times with 10 mM Tris chloride buffer, pH 7.6, and centrifuged at 30 000 rev./min in a No. 30 rotor for 60 min. The protein material sedimented as a dark pellet above which was a fluffy white layer. This latter layer was collected and suspended in 10 mM Tris chloride buffer, pH 7.6, and centrifuged at 40 000 rev./min for 60 min in a Spinco No. 40 rotor. The dark brown pellet was retained and suspended in 10 mM Tris chloride buffer, pH 7.6. The final suspension, which contained approximately 50 mg of protein, was a highly purified preparation of the outer membrane.

RESULTS

Electron microscopic appearance of the outer membrane

The appearance of the outer membrane preparation is shown in the Figs 1 and 2. The characteristic "flattened balloon-like" or "folded bag" appearance of many of the outer membrane vesicles[1] makes them readily discernible from mitochondrial inner membrane or microsomal fragments.

Composition of the outer membrane

The lipid, flavin and cytochrome compositions of the outer membrane preparations are shown in Table I, and the different enzymic activities which were assayed are listed in Table II. Both the phospholipid and total lipid content per mg protein were found to be higher than the corresponding values for whole mitochondria or ETPH, in agreement with the observations of Smoly *et al.*[5]. Also there was an enrichment of flavoproteins in the outer membrane. The very small amounts of cytochrome *a*, cytochrome *b* and cytochrome c_1 indicated a small amount of contamination with inner membrane. This conclusion was confirmed by electron microscopy.

The absence of α-ketoglutarate dehydrogenase, aconitase and fumarase activities indicated the total absence of contamination from citric acid cycle enzymes while the absence of cytochrome b_5 and glucose-6-phosphatase activities eliminated the possibility of microsomal contamination. Both monoamine oxidase and rotenone-insensitive NADH–cytochrome *c* reductase were concentrated in the outer membrane. The activity of monoamine oxidase in our preparation of heart mitochondrial outer membrane (39.1 nmoles/min per mg) was much lower than that reported for the outer membrane of liver[2,3,29] of kidney[30] mitochondria. These variations may reflect the difference between mitochondria from different organs rather than the differences

between mitochondria from different species. The similarity in the activities of this enzyme in beef liver and rat liver mitochondria argue for this interpretation. The activity in beef liver mitochondria was 6–8 nmoles/mg per min (Table I) while the activity in rat liver mitochondria was 5–6 nmoles/mg per min[29]. Other differences between the liver and heart mitochondrial outer membranes are apparent. Most noticeable is the absence of kynurenine hydroxylase activity in the heart preparation,

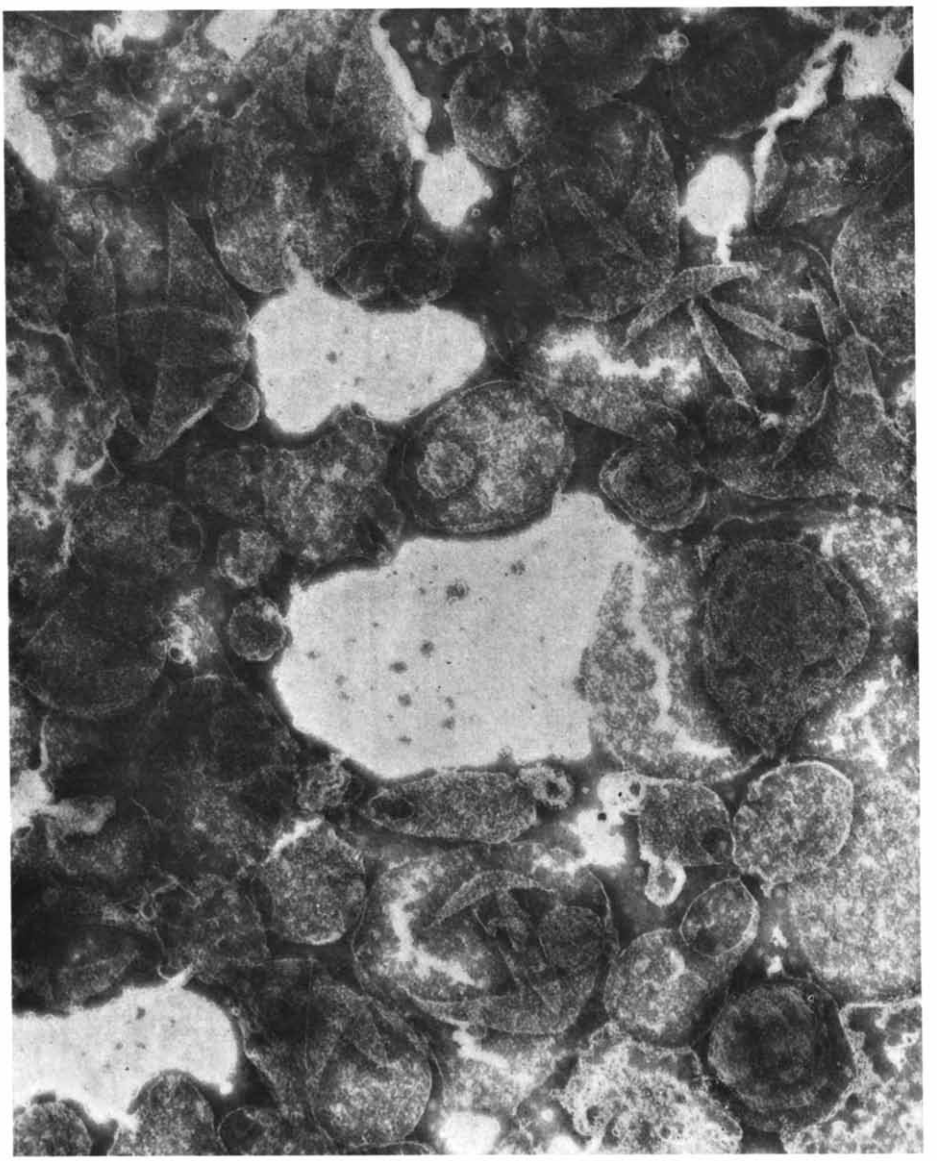

Fig. 1. The outer membrane of beef heart mitochondria negatively stained with 1% phosphotungstic acid (pH 7.2). × 50000.

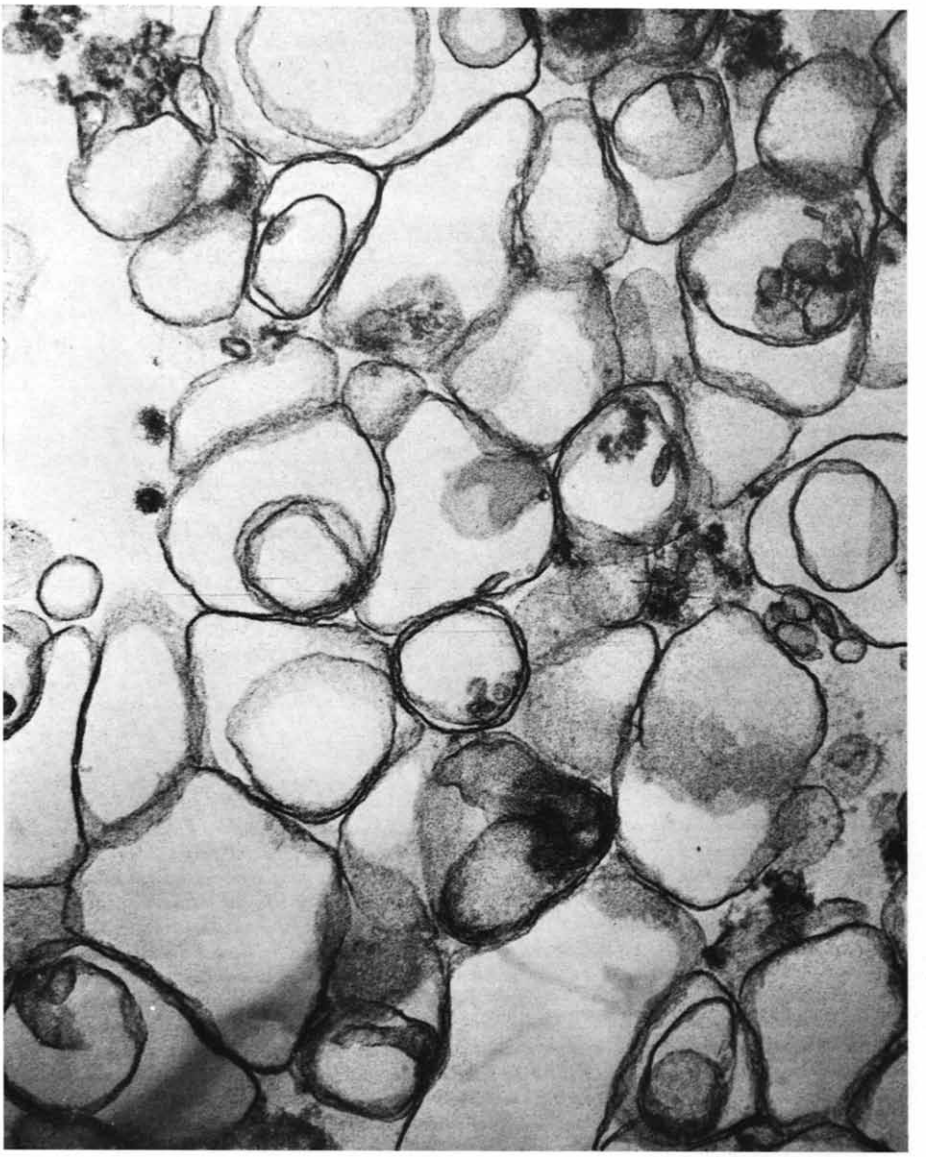

Fig. 2. An OsO_4-stained thin section micrograph of the outer membrane of beef heart mitochondria. × 48000.

an activity which has been reported to be present in the liver mitochondrial outer membrane[31].

Gel electrophoresis

The spectrophotometric scan of 5% polyacrylamide gels loaded with outer membrane is shown in Fig. 3. Fourteen staining bands were apparent, with polypeptides of molecular weights 46000. 34000 and 27000 adsorbing over half of the stain (Table III).

TABLE I

THE ENZYMIC ACTIVITIES OF THE OUTER MEMBRANE OF BEEF HEART MITOCHONDRIA

Enzymes	*Specific activity in the outer membrane (nmoles/min per mg protein)*	*Specific activity in beef mitochondria (nmoles/min per mg protein)*	*Specific activity in other membranes (nmoles/min per mg protein)*
Monoamine oxidase	39.1	1.4	6–8 in bovine liver mitochondria
Rotenone-insensitive NADH–cytochrome *c* reductase	550	130	
ATPase	0	440	
Glucose-6-phosphatase	0	Trace	244 in bovine liver microsome
Aconitase	0	330	
Fumarase	0	1000	
α-Ketoglutarate dehydrogenase	Trace	130	
Kynurenine hydroxylase	0	0	10 in bovine liver mitochondria
Succinate dehydrogenase	Trace	920	

TABLE II

COMPOSITION OF THE OUTER MEMBRANE PREPARATIONS OF BEEF HEART MITOCHONDRIA

Components	*Concentration in the outer membrane (nmoles/mg protein)*	*Concentration in whole mitochondria (nmoles/mg protein)*
Cytochrome *a*	0–0.09	1.21
b	0.02–0.04	0.60
c_1	0.01–0.02	0.21
b_5	0	0
Flavins		
Acid extractable	0.58	0.37
Acid none-extractable	0.13	0.12
Total	0.71	0.59
	Percentage	*Percentage*
Phospholipids	30	25
Total lipids	40	30

The number of bands observed in the gel is similar to that reported by Schnaitman[4] for the outer membrane of rat liver mitochondria. However, the size of the major components is different. This might reflect another difference between liver and heart or may be a result of the different gel electrophoresis methods.

We have also considered the possibility that glycoproteins are present in the outer membrane. 5 % gels stained for carbohydrate with toluidine blue gave only one positively staining band which migrated with the lipid component. Thus we propose that the carbohydrate which is known to be present in the outer membrane[32,33] is in fact glycolipid. Preliminary experiments with lipid extracts support this contention.

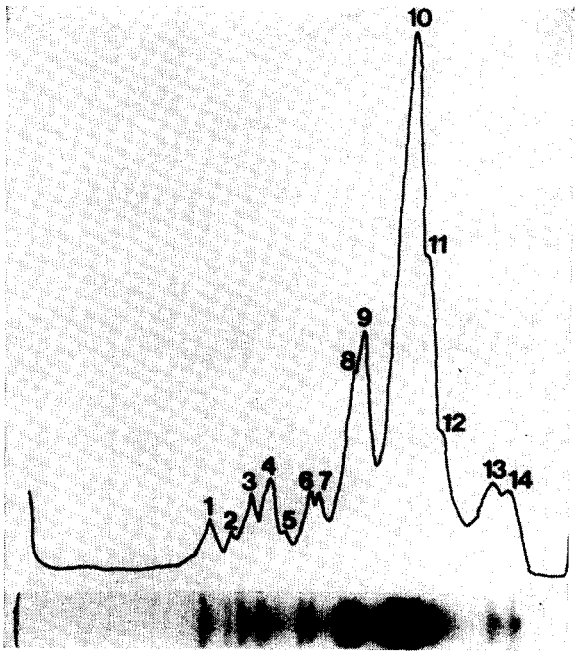

Fig. 3. The spectrophotometric scan of the 5% polyacrylamide gel of the outer membrane of beef heart mitochondria.

TABLE III

APPROXIMATE MOLECULAR WEIGHTS OF THE DIFFERENT POLYPEPTIDES IN THE OUTER MEMBRANE OF BEEF HEART MITOCHONDRIA AND THE RELATIVE AMOUNTS OF EACH, ESTIMATED FROM POLYACRYLAMIDE GELS

Band No.	*Mol. wt* $\times 10^{-3}$	%
1	220	2.5
2	160	1.9
3	140	3.8
4	110	4.5
5	100	1.9
6	75	3.8
7	70	3.8
8	55	9.6
9	46	11.8
10	34	25.5
11	27	15.2
12	21	7.0
13	15	4.5
14	12	4.1

DISCUSSION

The outer membrane of beef heart mitochondria has been something of an enigma. Separation of outer membrane from the inner membrane has proved difficult and previously described preparations have been contamined by inner membrane and

also by soluble enzymes which bind tightly to the membrane under the isolation procedures used[5,8]. These problems have been largely overcome in the present study and a highly purified membrane preparation has been isolated.

The contention of Smoly *et al.*[5] that the outer membrane has a single structural component and that the various enzymic activities are extrinsic to the membrane continuum has not been confirmed. It is clear that there are a number of proteins in the membrane in relatively high amounts. The question of which of these proteins are part of the membrane continuum and which are loosely attached to the surface of the membrane is under investigation.

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