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THE TURNOVER OF THE PROTEIN OF THE INNER AND OUTER MITOCHONDRIAL MEMBRANE OF RAT LIVER

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SUMMARY

The relative turnover rate of proteins of the inner and outer mitochondrial membranes and of the microsomal fraction of rat liver have been estimated by a double isotope procedure. Outer mitochondrial membrane proteins turn over more rapidly than inner mitochondrial membrane proteins. Microsomal proteins have a turnover rate similar to that of the outer mitochondrial membrane.

INTRODUCTION

Electron microscopists have demonstrated that mitochondria have both an inner and an outer membrane. The independent regulation of the synthesis and degradation of these two membranes became of particular interest when the discovery of mitochondrial DNA led to the consideration of the existence of mitochondrial autonomy[1-3]. With the advent of appropriate techniques for the separation of these two membranes[4,5], it has become possible to study many problems posed by this consideration. The composition of the outer mitochondrial membrane bears many similarities to that of the membranes of the endoplasmic reticulum and is quite different from that of the inner mitochondrial membrane[4,5].

Incubation of isolated mitochondria in a medium that would support protein synthesis *in vitro* leads to the synthesis of inner mitochondrial membrane proteins without any demonstrable synthesis of those of the outer membrane[6]. The growth of yeast in the presence of chloramphenicol, a specific inhibitor of mitochondrial protein synthesis, results in dissociation of the formation of the inner and outer mitochondrial membranes[7].

Since there is reason to believe that the inner mitochondrial membrane, or a major subunit of it, may turn over as a unit[8], it was of interest to investigate separately the turnover of the protein of inner and outer mitochondrial membranes and those of the microsomes. The experimental plan for this investigation was based upon the double isotope procedure outlined by SCHIMKE *et al.*[9].

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MATERIALS AND METHODS

Rat liver mitochondria were prepared essentially by the procedure of PARSONS[4], except that the final mitochondrial pellet was washed 3 times more in the homogenization medium at 6000 × *g* for 10 min. The preparation of outer and inner mitochondrial membranes from these highly purified mitochondria was performed by the procedure of PARSONS[4]. A single sucrose gradient was used for the purification of the outer mitochondrial membrane. For the preparation of the microsomal membranes the supernatant from the initial mitochondrial centrifugation was centrifuged at 10000 × *g* for 20 min and the pellet was discarded. The supernatant from this last centrifugation was then centrifuged in the Spinco Ti 50 rotor in a Spinco L-2 ultracentrifuge at 100000 × *g* for 60 min. The pellet from this centrifugation was employed for enzyme assay and for the determination of the specific activity of microsomal protein. For the enzymic assay of the degree of cross contamination of the whole mitochondria by microsomal proteins and of the outer membrane by inner membrane proteins, assays of glucose-6-phosphatase at pH 6.5 by the procedure of DE DUVE *et al.*[10], and of cytochrome oxidase, according to RABINOWITZ *et al.*[11] were performed on the separated fractions. NADH and NADHP cytochrome *c* reductases were assayed at 37° essentially as described by SOTTOCASA *et al.*[5], except that 0.02–0.04 % deoxycholate was employed to 'open' the inner mitochondrial membrane fractions[12]. These last two enzymes were used to assess the cross contamination of inner membrane by outer membrane and of the latter by microsomes respectively. All cytochrome *c* based assays were performed at 37° on the Gilford recording spectrophotometer. Initial rates were employed to calculate activities. In most cases, enzymes were assayed at two different concentrations to ensure that activities were linearly related to enzyme concentration. A millimolar extinction coefficient of 19.1 was employed for the absorbance difference between reduced and oxidized cytochrome *c* at 550 mμ. Protein determinations were performed according to either LOWRY *et al.*[13] (for enzyme specific activity determinations) or by the biuret technique[14] (for specific radioactivity determinations). The preparation and purification of protein samples for radioactive assays were carried out by a procedure kindly furnished us by Dr. R. ZAK (unpublished). This consisted of the precipitation of protein from a suspension of the membrane fraction by 0.05 M $HClO_4$. The precipitate was washed successively with 10 ml of 0.5 M $HClO_4$, acetone, abs. ethanol, ethanol–ether (2:1, v/v) and 10 ml ether. The precipitate was extracted with 5 ml 0.5 M $HClO_4$ at 80° for 30 min to extract nucleic acids, centrifuged and again washed in the cold with $HClO_4$. After the addition of 4 ml of 5 M NaOH it was incubated on ice overnight. The volume was measured and an aliquot was taken for protein determination by the biuret technique[14]. A second aliquot containing no more than 5 mg of protein was precipitated with trichloroacetic acid (5 % final concentration) on ice for 30 min. The precipitate was collected by centrifugation, washed once with abs. ethanol, and dissolved in 0.5–0.7 ml of concentrated formic acid for counting in 15 ml of scintillation liquid (5 g 2,5-bis-2-(*tert.*-butylbenzoxazolyl)thiophene, 850 ml toluene, 150 ml methyl cellosolve). Radioactivity determinations were made in the Packard Tri-Carb liquid scintillation spectrometer and were corrected for quenching according to the channel's ratio method[15].

Female Sprague–Dawley rats, weighing between 220 and 225 g were employed in all experiments. In the first experiment, each rat was injected intraperitoneally

with 100 μC of L-[^{3}H]leucine-L-[4,5-$^3H_2$]leucine (2.0 C/mmole) 6 days before sacrifice, followed by 18.2 μC of L-[^{14}C]leucine (uniformly labeled, 160 mC/mmole) 2 days before sacrifice. 2 days after this last injection, animals were killed after an overnight fast, and the various membrane fractions were prepared from the livers of 10 such animals. A second experiment was performed with a group of 14 animals, each of which was injected with 17.8 μC of [^{14}C]leucine 6 days before sacrifice, followed by 100 μC of [^{3}H]leucine at 2 days before sacrifice.

RESULTS

From the data presented in Table I it will be seen that the washed mitochondrial pellet contains less than 2 % of microsomal protein as assayed from the presence of glucose-6-phosphatase activity in this fraction. In addition, the pure outer mitochondrial membrane fraction on sedimenting between the 23.2 and the 37.7 % sucrose in the discontinuous gradient centrifugation of PARSONS *et al.*[4] contained about 13 % of inner mitochondrial membrane protein as measured by cytochrome oxidase specific activity.

TABLE I

ENZYMIC DETERMINATION OF PURITY OF MEMBRANE FRACTIONS

For cytochrome *c* based assays, a millimolar extinction coefficient at 550 mμ (difference between oxidized and reduced cytochrome *c*) of 19.1 was used. Numbers in parentheses refer to the number of different membrane preparations assayed. In these cases data are expressed as the means of the determinations.

Mitochondria and microsomes	*Glucose-6-phosphatase (μg P_i liberated per 16 min per mg protein)*	*Contamination index (A/B) × 100*
Mitochondria (A)	2.3	1.84
Microsomes (B)	125.0	—
Mitochondrial membrane fractions	*Cytochrome oxidase (μmoles cytochrome c oxidized per min per mg protein)*	(D/C) × 100
Inner membrane (C) (3)	1.92	—
Outer membrane (D) (3)	0.25	13.2
	*Antimycin-resistant NADH–cytochrome c reductase*** *(μmoles cytochrome c reduced per min per mg protein)*	(C/D) × 100
Inner membrane (C) (2)*	0.133	2.34
Outer membrane (D) (2)	5.70	—
Microsomes (E)	1.63	—
	NADPH–cytochrome c reductase (μmoles cytochrome c reduced per min per mg protein)	(D/E) × 100
Outer membrane (D) (3)	0.032	26.0
Microsomes (E) (2)	0.123	—

* In this assay, inner mitochondrial fractions were treated with deoxycholate to achieve a final concentration of 0.02–0.04 %.

** Antimycin was used in a concentration of 1–4 μg/ml.

It is worth noting that the inner mitochondrial membrane fraction prepared by the procedure of PARSONS *et al.*[4] still contains a substantial proportion of matrix proteins represented by the malic dehydrogenase marker[4], so that in assessing the turnover of this fraction, account must be taken of the fact that it is a combination of inner membrane and matrix. From the distribution of antimycin-resistant NADH–cytochrome *c* reductase between the mitochondrial fractions, the inner membrane fractions appear to be well stripped of outer membrane, which accounts for less than 3 % of the inner membrane fraction protein. The relative activity of this enzyme in the outer membrane and the microsomes is consistent with the data previously reported by PARSONS *et al.*[4] and SOTTOCASA *et al.*[5]. Assuming that the pure outer membrane of rat liver mitochondria[4,5] is devoid of NADPH–cytochrome *c* reductase, our outer mitochondrial membrane fractions were contaminated by no more than 26 % of microsomal protein. Although the original mitochondria were minimally contaminated by microsomes, the contaminating microsomal membranes would be expected to be maximally concentrated in the density fraction containing the outer mitochondrial membrane. In any event, taking account of the contamination of this latter fraction by inner mitochondrial membrane and microsomal proteins, it is still clear that at least 60% of the protein of this fraction was truly outer mitochondrial membrane.

Table II presents the isotope ratios of the proteins isolated from the two mitochondrial membrane fractions and of the microsomal proteins from the two separate experiments. It can be seen that in Expt. 1 the $^{14}C/^{3}H$ ratio of the outer mitochondrial membrane is substantially higher than that of the inner mitochondrial fraction protein. In Expt. 2, on the other hand, when the isotopes were administered in the reverse order, the $^{14}C/^{3}H$ ratio of the proteins of the outer mitochondrial membrane is sub-

TABLE II

SPECIFIC RADIOACTIVITY OF RAT LIVER MITOCHONDRIAL AND MICROSOMAL MEMBRANE FRACTIONS

In Expt. 1, 10 female Sprague–Dawley rats (220–225 g), were each injected intraperitoneally with 100 μC of L-[4,5-$^{3}H_2$] leucine (2.0 C/mmole). 4 days later the same rats each received an intraperitoneal injection of 18.2 μC uniformly labeled L-[^{14}C]leucine (160 mC/mmole), and were killed 2 days after this last injection. In Expt. 2, the plan of the experiment was identical except that the [^{14}C]-leucine and [^{3}H]leucine injections were reversed, and the dose of [^{14}C]leucine was 17.8 μC for each animal in this experiment. Membrane fractions were prepared, monitored for purity, and employed for the determination of specific radioactivity of their proteins as described in the text.

	Counts/min		*Specific activity (counts/min per mg protein)*		
	^{3}H	^{14}C	^{3}H	^{14}C	$^{14}C/^{3}H$
Expt. 1					
Outer membrane	408	905	107	238	2.21
Inner membrane and matrix	578	837	116	167	1.44
Microsomes	1212	2101	242	420	1.74
Expt. 2					
Outer membrane	79	104	46	60	1.31
Inner membrane and matrix	639	1200	128	240	1.87
Microsomes	393	547	88	122	1.39

stantially less than that of the inner mitochondrial fraction protein. The isotope ratios of the microsomal proteins are in both cases closer to those of the outer mitochondrial membrane than they are to the isotope ratios of the inner membrane proteins.

CONCLUSION

The isotope ratios of tritiated and [^{14}C]leucine incorporated into the proteins of the two separated mitochondrial membranes and of the microsomes suggest that the proteins of the two mitochondrial membranes have distinct turnover rates. In both experiments the evidence is strongly suggestive of a more rapid turnover of the outer mitochondrial membrane protein relative to that of the proteins of the inner membrane fraction. Similarly, the apparent turnover of microsomal proteins is more rapid than that of the proteins of the inner mitochondrial membrane. The inner mitochondrial membrane fraction contained a substantial portion of the matrix component of the mitochondria. It is evident that many matrix components have more rapid apparent turnover times than inner mitochondrial membrane components[16,17] Therefore the apparent relative turnover of the true inner membrane could be even slower than that reported for the fraction studied here, since it is contaminated by some proteins of the mitochondrial matrix, which have a rapid turnover. These data do not permit the exact calculation of the absolute turnover times of the proteins of the various fractions analyzed. However, the relative turnover rates of the respective membranes suggest quite clearly that, at least in the case of the proteins of these membranes, the apparent turnovers of the outer mitochondrial membrane and of the inner mitochondrial membrane are independent of one another. Indeed it also seems that the apparent turnover of microsomal proteins is distinct, certainly from that of the inner mitochondrial membrane, and perhaps also from that of the outer mitochondrial membrane. These conclusions are entirely consistent with the more specific studies recently reported by DRUYAN *et al.*[18,19], who showed that microsomal cytochrome b_5 had a more rapid turnover than outer mitochondrial membrane cytochrome b_5 and that both had more rapid apparent turnovers than the inner mitochondrial membrane hemoproteins, cytochromes *b* and *c*. This study reinforces the information already available suggesting that the synthesis and degradation of the inner and outer mitochondrial membranes are separately regulated. Despite this, however, there must clearly be some coordination between these two regulatory mechanisms, since there is no evidence, except in rather special circumstances, for redundancy *in vivo* of either inner or outer mitochondrial membrane.

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