INCORPORATION OF AMINO ACIDS INTO THE OUTER AND INNER MEMBRANE OF ISOLATED RAT LIVER MITOCHONDRIA [+]

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Numerous reports indicate that isolated mitochondria have the ability to incorporate a variety of amino acids. The identity of the proteins into which these amino acids are incorporated is largely unknown. However, there is evidence to suggest that the amino acids are associated almost entirely with the insoluble membrane fraction (Roodyn, 1962; Wintersberger, 1965; Bronsert et al., 1965).

As a means of localizing further the newly-synthesized protein we have made use of recently reported methods by which reasonably pure fractions of outer and inner membranes can be prepared from isolated mitochondria (Parsons et al., 1966a; Sottocasa et al., 1967; Schnaitman et al., 1966).

In this paper data will be presented which indicate that the bulk of amino acids incorporated by isolated rat liver mitochondria is associated with the inner membrane fraction.

EXPERIMENTAL

Rat liver mitochondria were prepared according to the method of Schneider (1948). The basic incubation medium contained: 0.25 M sucrose, 20 mM KCl, 15 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM ADP, 0.188 mg/ml amino acid mixture, 2 mM EDTA, 0.06 mg/ml streptomycin, 0.02% bovine serum albumin, 4-5 mg/ml mitochondrial protein, 0.3 uc/ml ^{14}C-l-leucine(U)(325 mc/mmole). The pH was 7.6. Final volume, 1 ml. The samples were shaken for 40 min at 32°C in open centrifuge tubes.

A slightly modified "swelling-shrinking" procedure combining

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the methods of Parsons et al. (1966a) and Sottocasa et al. (1967) was used to separate inner and outer membrane. Mitochondria were also fractionated by the method of Schnaitman et al. After incubation, 1.05 mg digitonin per 10 mg mitochondrial protein were added and the suspension maintained for 10 min at 0°C in a volume of 5 ml. The sucrose concentration was 0.25 M. After dilution to 80 ml with 0.25 M sucrose it was successively centrifuged for 10 min at 9,500xg, 20 min at 35,000xg and 1 hr at 144,000xg. The resulting pellets were resuspended in 0.1 M phosphate buffer for determination of enzyme activities and radioactivity.

Samples were prepared for analysis of radioactivity by the method of Simkin et al. (1957) and counted with an efficiency of about 35%. Protein was determined by the biuret method (Beisenherz et al., 1953). Succinate cytochrome c reductase was estimated by following the reduction of cytochrome c at 550 nm in the presence of 0.15 mM phenazine methosulphate (Arrigoni et al., 1962). Monoamine oxidase was estimated according to Tabor et al. (1954). Malate dehydrogenase and glutamate dehydrogenase were determined according to Bücher et al. (1964).

RESULTS AND DISCUSSION

The incubation conditions described were optimal for the incorporation of ^{14}C-l-leucine into isolated rat liver mitochondria. Control experiments were routinely carried out which established that bacteria and microsomes did not significantly contribute to amino acid incorporation. In all experiments succinate cytochrome c reductase (SDH) (Sottocasa et al.) and monoamine oxidase (MAO) activities (Schnaitman et al.) were used as markers for the inner and the outer membrane respectively. Data from a series of experiments in which mitochondria were fractionated by the "swelling-shrinking" method after incubation with ^{14}C-l-leucine are presented in Table I.

On the basis of the distribution of SDH and MAO activities it is seen that fraction LI (band B of Parsons et al.) contains the bulk of the outer membrane and fraction LII (band C of Parsons et al.) the bulk of the inner membrane. These observations regarding the distribution of inner and outer membrane thus confirm those of Parsons et al. It is apparent that within fraction LI outer membrane is contaminated by inner membrane and that within fraction LII inner membrane is contaminated by outer membrane. However, it is possible to calculate on the basis of enzyme activities the proportions of inner and outer membrane in fractions LI and LII by the following procedure:

TABLE I

Specific activities of succinate cytochrome c reductase and monoamine oxidase and specific radioactivities in different fractions of mitochondria prepared by the "swelling-shrinking" method, following incorporation of 14C-l-leucine.

Expmt.		1	2	3	4	5
MS	SDH	----	9.8	14.5	----	7.7
	MAO	----	803	224	----	595
	SR	----	112	44	----	117
LM	SDH	21.2	38.0	28.4	27.6	25.2
	MAO	1800	2240	1480	785	1570
	SR	81	238	126	248	180
LI	SDH	7.8	7.0	11.0	2.1	12.5
	MAO	3340	2960	3180	4310	3855
	SR	26	83	66	50	122
LII	SDH	29.6	18.3	40.4	24.8	23.0
	MAO	1320	1035	962	803	1210
	SR	102	240	238	361	244
LIII	SDH	40.2	25.4	24.7	18.8	27.1
	MAO	698	562	328	206	1050
	SR	104	168	85	88	192
SR of pure outer membr.		0.3	14	2	21	-18
SR of pure inner membr.		151	325	314	439	294
SR of outer membr. in % of SR of inner membr.		0.2	4.2	0.6	4.8	-6.0

Abbreviations: SDH succinate cytochrome c reductase, MAO monoamine oxidase, SR specific radioactivity of protein, MS mitochondrial suspension, LM input to density gradient, LI upper fraction of gradient, LII middle fraction of gradient, LIII pellet. Enzyme activities expressed in umoles/hr/mg protein, specific radioactivities in cts/min/mg protein.

$$p_{oLI} + p_{iLI} = 1.$$

$$p_{oLII} + p_{iLII} = 1.$$

where p_{oLI} and p_{oLII} represent that fraction of pure outer membrane in bands LI and LII respectively, p_{iLI} and p_{iLII} represent that fraction of pure inner membrane in bands LI and LII respectively.

Now $p_{oLI} = \frac{E_{MAO\ LI}}{E_{MAO\ po}}$; $p_{iLI} = \frac{E_{SDH\ LI}}{E_{SDH\ pi}}$; $p_{oLII} = \frac{E_{MAO\ LII}}{E_{MAO\ po}}$; $p_{iLII} = \frac{E_{SDH\ LII}}{E_{SDH\ pi}}$;

where $E_{MAO\ LI}$, $E_{MAO\ LII}$, $E_{SDH\ LI}$, $E_{SDH\ LII}$ represent the activities of SDH and MAO in bands LI and LII respectively. $E_{MAO\ po}$ represents the specific activity of MAO in pure outer membrane and $E_{SDH\ pi}$ represents the specific activity of SDH in pure inner membrane.

The specific radioactivities of pure outer and inner membrane can be calculated by the following equations:

$$p_{oLI} \cdot SR_{po} + p_{iLI} \cdot SR_{pi} = SR_{LI}.$$

$$p_{oLII} \cdot SR_{po} + p_{iLII} \cdot SR_{pi} = SR_{LII}.$$

where SR_{po}, SR_{pi}, SR_{LI}, SR_{LII} represent the specific radioactivities of pure outer membrane, pure inner membrane, fraction LI and fraction LII respectively. The assumptions relevant to this calculation are discussed below.

The specific radioactivities of pure outer and pure inner membrane calculated in this way are shown in Table I. The specific radioactivity of pure outer membrane was always less than 5% of that of the pure inner membrane. Similar results to those described in Table I were obtained when ^{14}C-labelled arginine, isoleucine, phenylalanine or valine replaced ^{14}C-leucine.

TABLE II

Specific activities of succinate cytochrome c reductase and monoamine oxidase and specific radioactivities in different fractions of mitochondria prepared by the digitonin method, following incorporation of 14C-l-leucine.

Expmt.		1	2
MS	SDH	----	7.2
	MAO	----	644
	SR	79	65
9,500xg pellet	SDH	31.6	26.2
	MAO	548	711
	SR	194	70
35,000xg pellet	SDH	5.5	18.7
	MAO	568	1190
	SR	97	96
144,000xg pellet	SDH	2.0	12.6
	MAO	855	1750
	SR	41	41
SR of pure outer membr.		8	-70
SR of pure inner membr.		209	270
SR of outer membr. in % of SR of inner membr.		3.8	-26

Abbreviations and activities expressed as in Table I.

The results of experiments in which the mitochondria were fractionated by the digitonin method following incorporation of ^{14}C-l-leucine are shown in Table II. The distribution of outer and inner membranes in the various fractions as indicated by the activities of SDH and MAO is similar to that observed by Schnaitman et al. Calculation of the specific radioactivities of pure outer and pure inner

membrane by the method described above showed that again the specific radioactivity of pure outer membrane was less than 5% of that of the pure inner membrane. For these calculations the enzyme activities in the 35,000xg and the 144,000xg pellets were used (see below).

The method of calculation for the specific radioactivities of pure membrane fractions is valid provided the following assumptions are true: (a) SDH and MAO activities are specific for inner and outer membrane respectively, (b) the turnover times of inner and outer membrane are the same, (c) the membrane fractions are not contaminated with non-membraneous proteins.

There is considerable evidence that assumption (a) is true (Parsons et al., Sottocasa et al., Schnaitman et al.). Consistent with this was the observation that in the present experiments cytochrome oxidase activity gave similar distribution to SDH. There is little information available at present regarding assumption (b). Some information regarding assumption (c) can be obtained from a study of the distribution of the so called "soluble" matrix enzymes.

TABLE III

Specific activities of some "insoluble" and "soluble" enzymes in different fractions of mitochondria prepared by the "swelling-shrinking" method.

	SDH	MAO	MDH	GluDH
MS	8	549	220	201
LM	11	1260	41	61
LI	3	4460	37	46
LII	12	597	69	69
LIII	21	302	265	286

Abbreviations: MDH malate dehydrogenase, GluDH glutamate dehydrogenase. Other abbreviations as in Table I. Enzyme activities are expressed in umoles/hr/mg protein.

Data in Table III show that fraction LIII contains high specific activities of malate dehydrogenase (MDH) and glutamate dehydrogenase (GluDH). These specific activities are even higher than those found in the original unfractionated mitochondria. However, in both fractions LI and LII the specific activities of these two enzymes are low and similar. Also the data of Schnaitman et al. suggest that the 9,500xg pellet from the digitonin method contains considerable amounts of soluble proteins as indicated by a high proportion of MDH activity.

Of particular interest was the observation (Neupert, unpublished) that the RNA content of LIII fraction was unexpectedly low. This question of the distribution of RNA and its possible relationship

to the site of protein synthesis in isolated mitochondria is under investigation. What emerges then from this and the data presented in Table III, is that fraction LIII should not be used for calculations of specific radioactivities of outer and inner membrane.

It has been shown on the basis of electron microscopy, phospholipid analysis and distribution of electron transport enzymes that the outer and inner membranes of mitochondria are markedly different and the outer membrane and the smooth endoplasmic reticulum are markedly similar (Parsons et al., Sottocasa et al.). Also, in a recent report, Clark-Walker et al. (1966) observed that chloramphenicol, which strongly inhibits amino acid incorporation into isolated mitochondria (Wintersberger, 1965), blocks the formation of cristae without affecting the formation of outer membrane in glucose repressed yeast.

The results presented in this paper indicate that the protein synthesizing system of isolated rat liver mitochondria incorporates amino acids only into the inner membrane. These data therefore provide evidence for the suggestion (Parsons et al., 1966b) that the outer and inner membranes of isolated mitochondria are synthesized by quite different systems.

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