THE SYNTHESIS OF PROTEOLIPID PROTEIN BY ISOLATED RAT LIVER MITOCHONDRIA

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SUMMARY

About 15% of the total (^{3}H)leucine incorporated into protein by isolated rat liver mitochondria in vitro could be extracted by chloroform:methanol. This incorporation was inhibited by chloramphenicol and carbomycin, both specific inhibitors of mitochondrial protein synthesis. SDS-gel electrophoresis of the mitochondrial membrane revealed 6-7 labeled bands. Label in the proteolipid fraction was present mainly in a band of 40,000 molecular weight. Several labeled bands observed in gels of the mitochondrial membrane were not removed or changed by extraction with chloroform:methanol suggesting that some, but not all, of the proteins synthesized by rat liver mitochondria are proteolipids.

During the biogenesis of mitochondria, proteins synthesized at two different sites in the cell are integrated into a functional unit. Although the vast majority of mitochondrial proteins are synthesized in the cytoplasm and transported into the mitochondria in a subsequent step (1), the proteins synthesized within the mitochondria are essential for proper assembly of certain enzyme complexes of the inner membrane (2-6). The proteins which are synthesized on mitochondrial ribosomes have not been purified and characterized chemically, although they are among the most insoluble proteins of the membrane (7). Recently, however, Tzagoloff and Akai (8) reported that all the products of mitochondrial protein synthesis were soluble in chloroform: methanol and hence were proteolipids. The major product was a low molecular weight peptide extracted with neutral chloroform: methanol. In contrast, Murray and Linnane (9) reported that

proteolipids were, indeed, synthesized by yeast mitochondria in vitro, but that these were not the sole products of mitochondrial protein synthesis. In the present study, the synthesis of proteins soluble in chloroform:methanol by isolated rat liver mitochondria is reported.

METHODS

Rat liver mitochondria were isolated in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.8, and 1.0 mM EDTA under sterile conditions by previously described methods (10) which yield a mitochondrial fraction which is 3% contaminated with microsomal protein. Amino acid incorporation was determined in a medium containing 50 mM Bicine, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM potassium phosphate, pH 7.6, 90 mM KCl, 2 mM ATP, 5 mM phosphoenolpyruvate, 10 µg/ml of pyruvate kinase, 22.5 µg of an amino acid mixture minus leucine (11), 12 mg of mitochondrial protein, and (^{3}H)leucine in a final volume of 5 ml. After 30 min of incubation at 30°, 10 mM unlabeled L-leucine was added and the mitochondria reisolated at 12,000 x g. The mitochondrial pellet was washed twice in isolation medium containing unlabeled leucine. The pellet was resuspended to a concentration of 2 mg/ml, sonicated for 10 sec at maximum output on a Branson sonifier and then centrifuged for 30 min at 100,000 x g. The pellet containing submitochondrial particles (SMP) were then extracted by either of two methods.

Method 1

The SMP pellet containing 5 mg of protein was suspended to 1 ml in isolation medium and extracted with 9 ml of chloroform: methanol (2:1 v/v) for 30 minutes at room temperature. The extracts were centrifuged at maximum speed in a clinical centrifuge. The lower layer was washed once with 5 ml of water and three times with 10 ml of chloroform:methanol:water (3:48:47) (12).

Method 2

The SMP pellet containing 5 mg of protein was suspended in one ml of isolation medium and extracted with 10 ml of 90% methanol. The pellet after centrifugation at 1000 x g was then extracted with 2 ml of chloroform:methanol (2:1 v/v) and incubated at 50° for 30 minutes. The mixtures were centrifuged at 1000 x g and the residues extracted a second time with chloroform: methanol. The residues and extracts were dried under a stream of nitrogen at 50° and dissolved in 1% SDS for counting or in the medium used for gel electrophoresis (4). Polyacrylamide gel electrophoresis was performed on 9 cm gel as described by Tzagoloff (4).

RESULTS AND DISCUSSION

Less than 10% of the protein of the submitochondrial particles and 16% of the radioactivity were extracted by neutral chloroform:methanol after an incubation of liver mitochondria *in vitro* with (^{3}H)leucine (Table 1, Method 1). The loss of total radioactivity observed after this method of extraction resulted partly from the difficulty in recovering all of the residue protein when the chloroform:methanol extracts were centrifuged, and alternately from the possible loss of radioactive proteins into the washes of the lipid fraction. Extraction with methanol prior to chloroform:methanol (Method 2) removed about 25% of the total radioactivity. These counts may represent free leucine not present in peptide linkage (8) or polypeptides which are soluble in methanol. As a result, the chloroform:methanol extract obtained by this method of extraction contained only 8% of the total radioactivity. The specific activity of the chloroform:methanol extract, after both extraction procedures, was double that of the submitochondrial particles (Table 1) or four times greater than that of the intact mitochondria (Table 2).

Table 1. Extraction of Labeled Mitochondrial Proteins by Chloroform:Methanol

	Specific Activity	Protein	Total Radio-activity	% of Total
	cpm/mg	mg	cpm	
Method 1				
Particles	92,600	5.0	463,000	100
C:M Extract	173,000	0.42	72,600	16
Residue	46,400	3.5	162,000	35
Method 2				
Particles	111,000	5.0	555,000	100
Methanol Extract	-	-	122,000	25
C:M Extract	269,000	0.3	80,800	7
Residue	63,800	4.4	281,000	68

Isolated mitochondria were incubated with 20 μC_1/ml of (^{3}H)leucine (50 C_1/mmole) and fractionated as described in Methods.

Incorporation into the proteolipid fraction was inhibited to the same extent as the intact mitochondria by chloramphenicol (70%) and carbomycin (50%), both known to be specific inhibitors of protein synthesis on mitochondrial ribosomes (13) (Table 2). These results suggest that the radioactivity present in the pro-

Table 2. The Effect of Inhibitors on Proteolipid Synthesis

Fraction:	cpm/mg		
	Intact Mito	C:M Extract	Residue
Control	15,400	65,000	20,200
+chloramphenicol	4,520	20,300	4,840
%Inhibition	71	69	76
+carbomycin	3,080	12,300	4,240
%Inhibition	80	81	79

Mitochondria were incubated with 5 μC_1/ml of (^{3}H)leucine with 100 µg/ml of chloramphenicol and 30 µg/ml of carbomycin.

teolipid fraction represents products of the mitochondrial system for protein synthesis. Furthermore, previous studies (13,15) have demonstrated that the products of amino acid incorporation by mitochondria *in vitro* are identical with those labeled *in vivo* in the presence of sufficient cycloheximide to block 95% cytoplasmic protein synthesis. Hence, amino acid incorporation *in vitro* is an accurate reflection of events occurring *in vivo*.

The proteins of the submitochondrial particles labeled *in vitro* separated after SDS-gel electrophoresis into seven peaks (Figure 1). The major radioactive protein(s) were present in that portion of the gel corresponding to a molecular weight of 40,000 (14) with a shoulder of molecular weight 48,000. Significant radioactivity was also present in peaks of molecular weights

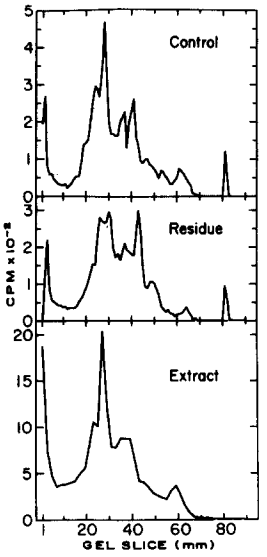

Figure 1

SDS-gel electrophoresis of 100 µg of submitochondrial particles (CONTROL), 50 µg of the washed chloroform:methanol extract (EXTRACT) and 100 µg of the residue after chloroform:methanol extraction. Specific activities of the fractions are presented in Table 1 (Method 1). Recovery from the gel 95%. The gels were cut into 1 mM slices and counted (8).

28,000 and 20,000, while minor labeling was observed into three peaks of lower molecular weight. The proteolipid fraction (Extract), prepared by Method 1, separated into a sharp peak of 40,000 molecular weight with a slight shoulder and two very broad peaks of lower molecular weight. In the residue after extraction with chloroform:methanol, labeling of the 40,000 molecular weight peak was diminished, while an almost complete loss of radioactivity in the two lowest molecular weight peaks was observed. The magnitude of labeling of the other peaks was unchanged by extraction with chloroform:methanol. Extraction with acidic chloroform:methanol also removed 20% of the counts, but the extract did not separate into clearly, defined peaks after gel electrophoresis.

These results suggest that a major product of rat liver mito-

chondrial protein synthesis is a proteolipid of 40,000 molecular weight, although labeled proteins of lower molecular weight were also extracted with chloroform:methanol. At no time, however, were polypeptides of molecular weight less than 10,000 observed after incubating mitochondria *in vitro* with (^{3}H)leucine. The proteolipids represent less than 20% of the total radioactivity incorporated into mitochondrial membranes *in vitro* suggesting that all proteins synthesized by mitochondria are not proteolipids. Furthermore, many labeled peaks observed after SDS-gel electrophoresis of submitochondrial particles were unchanged by extraction with chloroform:methanol in terms of total label in the peak or migration into the gel. In contrast, Tzagoloff and Akai (8) have reported that all of the products of mitochondrial protein synthesis in yeast are proteolipids, and that the major product is a low molecular weight polypeptide (7,800) extracted with chloroform:methanol. These discrepancies may result from a species difference or from the methods used to grow the yeast and label the mitochondrial proteins *in vivo*.

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