THE BIOSYNTHESIS OF THE PROTEIN AND LIPID COMPONENTS OF THE INNER AND OUTER MEMBRANES OF RAT LIVER MITOCHONDRIA

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Several groups have studied the time sequence of *in vivo* labeling of various submitochondrial protein fractions in an attempt to understand the processes involved in mitochondrial biogenesis. Beattie, Basford, and Koritz (1966) reported that at short time intervals after injection of radioactive leucine, the specific activities of the water-soluble proteins and the fraction containing cytochrome *c* from rat liver and kidney mitochondria were significantly lower than the specific activity of the unfractionated mitochondria, while at longer times their specific activity was higher. These results suggested that certain mitochondrial proteins might be synthesized outside the mitochondria and subsequently incorporated into the mitochondrial structure. In a similar study, Truman (1963) reported that particles associated with mitochondrial membranes, obtained by three different fractionation procedures, were the site of most rapid incorporation of labeled amino acids *in vivo*.

These previous fractionations were based for the most part on the differential solubilities of the various mitochondrial proteins and hence no conclusions with respect to the assembly of mitochondria in morphological terms could be drawn. With the

advent of methods for fractionating mitochondria into morphologically meaningful fractions, it has become possible to study mitochondrial biogenesis on a morphological basis. In the present study, mitochondria were fractionated into inner and outer membrane components at various times after injection of (^{14}C) leucine and (^{14}C) glycerol. The data indicate that the proteins and lecithin of the outer membrane are maximally labeled within 5 min. while the proteins and lecithin of the intact inner membrane-matrix fraction do not reach a maximum until 10-15 min. after injection. The insoluble proteins of the inner membrane are also labeled before the soluble proteins of the "matrix" fraction.

These results suggest that during mitochondrial biogenesis, the outer membrane may be the first mitochondrial component synthesized.

EXPERIMENTAL SECTION

Twenty microcuries of uniformly labeled (^{14}C) L-leucine were injected intravenously into male rats weighing 150-200 gms and the animals were killed at 5, 10, 15, 30 and 45 minutes after injection. Liver mitochondria were prepared in 0.25 M sucrose adjusted to pH 7.4 with Tris, washed 4 times and separated into three fractions; inner membrane plus matrix, outer membrane and a soluble fraction by the digitonin method of Schnaitman and Greenawalt (1968). The completeness of the separation was monitored by enzyme markers (Beattie, 1968a).

The inner membrane was resuspended in 0.1 M phosphate buffer pH 7.4, and sonified for 30 sec. in a Branson sonifier at a setting of 2.5 amps, and then centrifuged for 60 min. 100,000 x g. Over 90% of the isocitric dehydrogenase activity was released into the supernatant (matrix fraction) by this method while 63% of the succinic dehydrogenase was sedimented.

Microsomal contamination of the mitochondria and all submitochondrial fractions was determined by mixing radioactive post-mitochondrial supernatant with unlabeled mitochondria as well as by use of the microsomal marker enzymes, glucose-6-phosphatase and TPNH-cytochrome c reductase. The results by all methods indicated that the mitochondria were 4% and the outer membrane 8% contaminated by microsomes, while the other fractions had lower amounts of contamination. Since the radioactive specific activity of the microsomes is nearly 8 times that of the mitochondria, a small microsomal contamination in terms of protein would result in a significant contribution to the radioactivity of a fraction. For each mitochondrial preparation, the radioactivity of the microsomes was also determined. Using this value and the percentage of microsomal contamination for each fraction, the observed specific activity for each fraction was corrected and the corrected results thus obtained were used in plotting the data.

RESULTS AND DISCUSSION

In the time course of leucine incorporation into various submitochondrial fractions (Figure 1) the specific activity of the outer membrane attained its maximum 5 min. after injection. The proteins of the intact inner membrane-matrix did not become maximally labeled, however, until 15 min. after injection. The proteins of the soluble fraction continued to be labeled at significant rate for 30 min. after injection. When the inner membrane was further fractionated, (Figure 2) it was observed that the specific activity of the insoluble residue increased rapidly in the first 5 min. and continued to increase at a lower rate for 30 min. after injection. On the contrary, the specific activity of the proteins of the "matrix" fraction were significantly lower than that of the

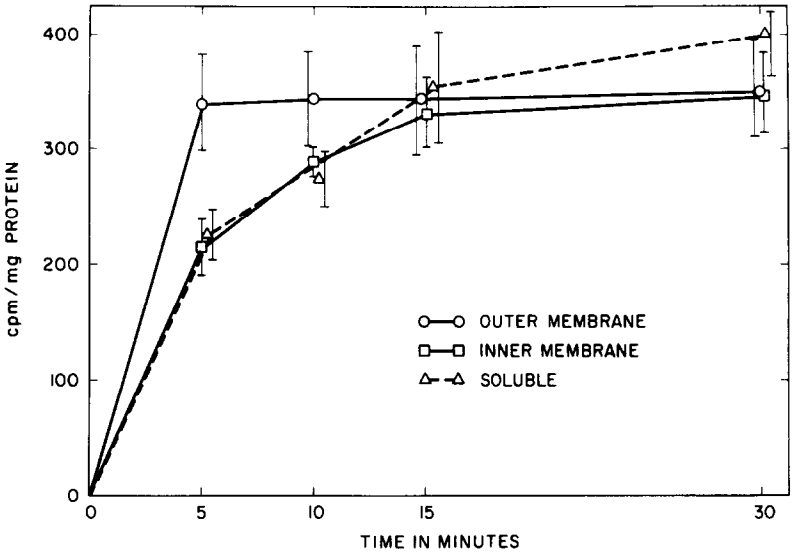

Fig. 1 - Time curve of incorporation of (^{14}C) leucine into submitochondrial fractions. Each point is the average of 5 or 6 animals.

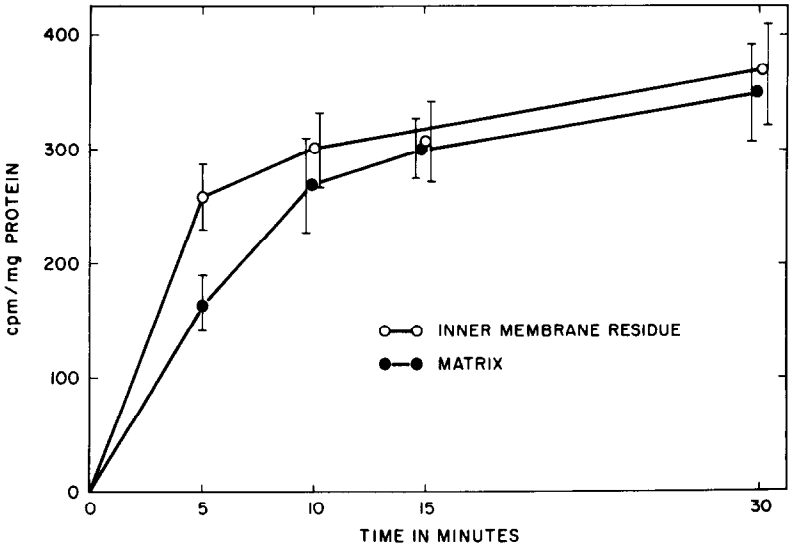

Fig. 2 - Time curve of incorporation of (^{14}C) leucine into subfractions of the inner membrane.

residue at 5 min. During the next 5 min, the specific activity of this fraction almost doubled. From 10 min. on the rate of incorporation into both inner membrane fractions increased at a similar rate until 30 min. after injection when the maximum was reached. At this time the proteins of all the submitochondrial fractions had essentially identical specific activities with the exception of the soluble fraction which was slightly higher than the remaining fractions.

The rates of lecithin and phosphatidyl ethanolamine synthesis in the inner and outer mitochondrial membrane were also determined at various times after injection of radioactive glycerol. Mitochondrial lipids were extracted with chloroform: methanol. The various phospholipids were separated by thin layer chromatography and counted directly. Lecithin of the outer membrane achieved a maximum specific activity 5 minutes after injection of glycerol, while the lecithin of the inner mebrane did not reach its maximum specific activity until 10 min. after injection (Figure 3). Phosphotidyl ethanolamine, on the contrary, was labeled much more slowly in both membranes, and did not become fully labeled until much later times.

Previous studies on the time sequence of mitochondrial biogenesis had indicated an initial synthesis of the insoluble proteins of the mitochondria as a prerequisite for the subsequent integration of the soluble proteins into the mitochondrial structure. The results of chase experiments in rat liver slices (Beattie, 1968b) and Ascites tumor cells (Freeman *et al*, 1962) plus *in vivo* studies (Gonzalez-Cadavid and Campbell, 1967) have demonstrated that mitochondrial soluble proteins, including cytochrome *c*, are synthesized on extramitochondrial ribosomes and transferred into the mitochondria in a subsequent step.

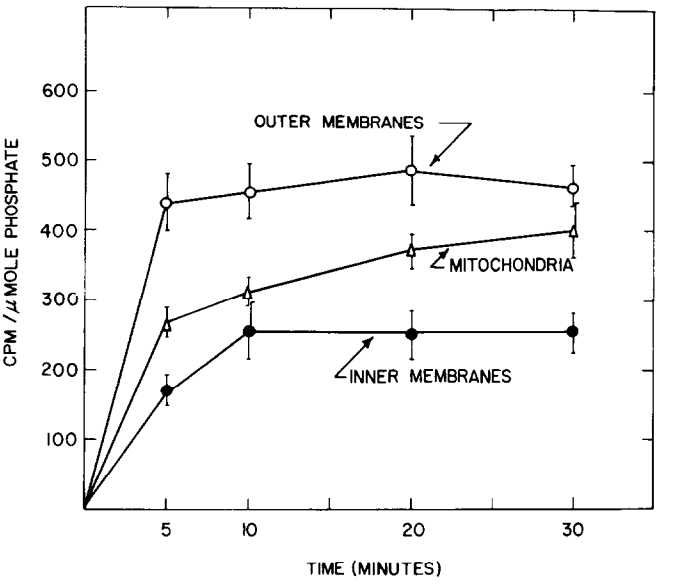

Fig. 3 - Time curve of incorporation of (^{14}C) glycerol into lecithin of submitochondrial fractions.

The nature of the insoluble protein initially synthesized was not clarified by the above studies. It has been demonstrated, however, that isolated mitochondria are capable of incorporating amino acids into certain insoluble proteins, mainly "structural" proteins of the inner membrane (Beattie et al, 1967)

The above studies have contributed no information, however, about the biosynthesis of the outer membrane with respect to the proteins of the inner membrane. Previous studies in several laboratories (Beattie et al, 1967; Neupert et al, 1968) have demonstrated that isolated mitochondria do not incorporate significant amounts of amino acids into the proteins of the outer membrane. The results of the present study have shown that the proteins of the outer membrane reached a maximum specific activity 5 min. after injection compared to 15 min. for the intact inner membrane-matrix and 30 min. for the soluble fraction. This suggests that the pro-

teins of the outer membrane may be among the first proteins synthesized during mitochondrial biogenesis. These results clearly demonstrate that the kinetics of labeling of the proteins of the outer membrane differs from that of the soluble proteins. It should be stressed that neither of these proteins fractions are labeled by isolated mitochondria.

The rate of labeling of lecithin, the major mitochondrial phospholipid, was similar to that for protein in the two membranes; i.e., that in the outer membrane reached its maximum specific activity 5 min. after injection while that of the inner membrane obtained maximum labeling 10 min. after injection. These results suggest the proteins are assembled into their appropriate membranes concurrently with phospholipid, perhaps as a lipo-protein complex.

The observation that both the proteins and lipid components of the outer membrane have the same labeling pattern *in vivo* relative to the inner membrane supports the interpretation that the outer membrane is the first mitochondrial component to be synthesized. Thus, mitochondrial biogenesis may involve an initial synthesis of the outer membrane as a prerequisite for the formation of the inner membrane which, in turn, might provide the structural elements necessary for formation of the matrix.

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