THE IN VITRO INCORPORATION OF C^{14}-AMINO ACIDS INTO THE CONTRACTILE PROTEIN OF INTACT LAMB HEART MITOCHONDRIA.[1]

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Introduction

Although the incorporation of labeled amino acids into the protein of isolated mitochondria from various sources has been well documented, there is still little evidence that the incorporation represents the synthesis of any specific protein. Roodyn, Suttie and Work (1962) isolated purified catalase, malate dehydrogenase and cytochrome c from rat liver mitochondria which were labeled with C^{14}-amino acids in vitro and found no significant incorporation into any of these proteins. Further experiments, (Roodyn, 1962; Truman, 1964) have led to the conclusion that the major site of incorporation of amino acids in vitro is into insoluble lipoprotein probably derived from the mitochondrial membrane.

Preliminary evidence is presented here indicating that lamb heart mitochondria are capable of effecting the in vitro incorporation of C^{14}-amino acids into a specific protein. This protein can be extracted from the mitochondria with 0.6M KCl and has physical properties and ATPase activity suggestive of the contractile proteir

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of mitochondria, as described previously (Ohnishi and Ohnishi, 1962; Neifakh and Kazakova, 1963; Lehninger, 1964; Blair et al., 1964).

Materials and Methods

The methods for obtaining 3x-washed lamb heart mitochondria, the incubation conditions, the washing and plating of the labeled proteins and the estimation of the radioactivity of the samples was described previously (Kalf, 1964).

Contractile protein was extracted from the mitochondria by the method of Ohnishi and Ohnishi (1962). ATPase activity was assayed by incubating samples of the contractile protein for 10 minutes at 37° in 0.2M histidine buffer (0.15M in KCl), pH 6.0, 5mM $CaCl_2$, and 5mM ATP. The reaction was stopped with $HClO_4$ and inorganic phosphate was determined by the method of Fiske and SubbaRow.

Gel filtration of the contractile protein was carried out on a Sephadex G-200 column according to the method of Smoller and Fineberg (1964) for the purification of mouse myosin.

Results and Discussion

When it was observed that a portion of the total radioactivity of intact heart mitochondria labeled *in vitro* could be extracted with 0.6M KCl and was associated with a protein having ATPase activity, preliminary experiments were carried out to determine whether the protein being labeled was the actomyosin-like contractile protein of the mitochondrion and whether the characteristics of the labeling of the specific protein were similar to those of the intact mitochondrion. The results of such experiments suggested that the labeling of this protein was subject to

the same conditions as is the labeling of intact mitochondria (Kalf, 1963, 1964). In order to determine whether the ATPase activity of this labeled protein was affected by those inhibitors of mitochondrial incorporation, experiments were carried out and the results can be seen in Table I. The data in experiments 1 and 2 clearly show that the amount of ATPase activity which can be quantitatively extracted with KCl is reduced upon the addition of the usual inhibitors affecting mitochondrial incorporation as well as by the omission of Mg^{+2} from the incubation medium. That there is an apparent requirement for messenger RNA in order for the both incorporation of radioactivity and the production of ATPase is evident from the fact that low levels of actinomycin D suppress both activities.

TABLE I

The Effect of Various Inhibitors on Incorporation of C^{14} Amino Acids into Contractile Protein and its ATPase Activity

The reaction mixture consisted of the following additions per ml: α-Ketoglutaric acid (5.0 µmoles), ADP (0.6 µmole), AMP (0.6 µmole), KH_2PO_4 (15 µmoles), $MgCl_2$ (0.01 M) EDTA (0.008 M) KCl (50 µmole), Tris buffer, pH 7.4, (45 µmole), uniformly labeled L-14_C amino acids from Chlorella protein (containing 300,000 c.p.m.), and 2 mg of intact mitochondrial protein, pH 7.4. Generally, the total volume was 20 ml. Incubated 1 hr. at 37°. ATPase was quantitatively extracted and assayed as described in the Methods section.

Conditions	ATPase Activity µmoles Pi/0.2 ml. KCl extract		Specific Activity c.p.m./mg Contractile Protein
	I	II	III
Complete system	0.99	0.94	405
+ Puromycin(10µg/ml)	0.60	0.72	191
+ Actinomycin D(20µg/ml)	0.50	0.35	246
+ Dinitrophenol($3x10^{-4}$M)	0.09	-	-
+ RNase (10µg/ml)	-	0.56	-
- Mg^{+2}	-	0.40	-

The ATPase activity of the contractile protein of mitochondria may be latent and can be stimulated by dinitrophenol and inhibited by oligomycin (Lehninger, 1964). Our results indicate a qualitative resemblance of the labeled material to mitochondrial actomyosin with respect to ATPase activity. Dinitrophenol ($3x10^{-4}$ M) resulted in an 11% stimulation whereas oligomycin (10 µg/ml) inhibited the ATPase activity by 55%. An obligatory requirement for calcium was also noted. Furthermore, fibers extracted from labeled mitochondria with KCl showed the phenomenon of superprecipitation at an ionic strength below 0.1 and at pH 6.3 in the presence of 1mM $MgCl_2$ and 1mM ATP. The precipitated material contained the radioactivity of the extract.

In order to demonstrate unequivocally that the radioactivity and the ATPase activity resided in the same protein, the extracted material was subjected to gel filtration on Sephadex G-200 (Fig.1).

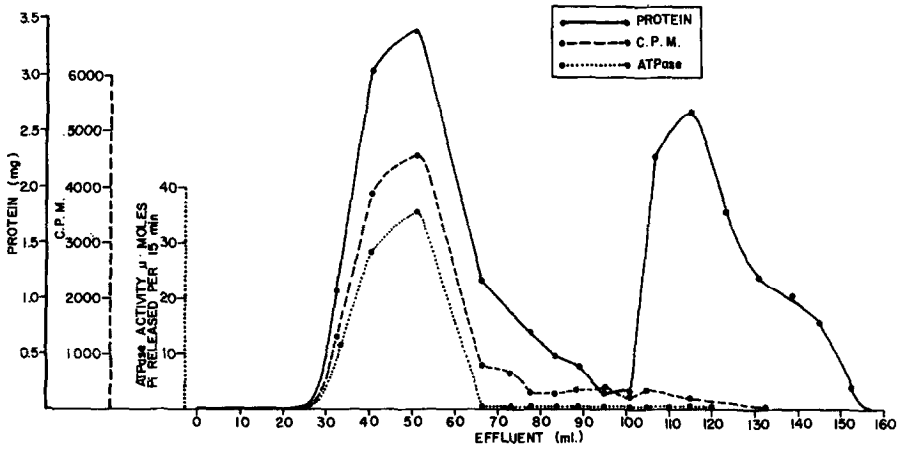

Fig.1 Gel filtration elution profile of mitochondrial contractile protein preparation. A 21x2.2 cm. column of Sephadex G-200 was charged with 20 ml. of KCl extract containing 18 mg. labeled protein (approx. 15,000 total c.p.m.) and 750 units of ATPase activity. One unit of ATPase activity is that amount of enzyme which released 0.1 µmole Pi per 15 minutes. The column was previously equilibrated with 0.5M KCl containing 1mM Tris-HCl buffer, pH 7.0. Chromatography was carried out at 4° at a flow rate of approximately 10 ml/hr.

Labeled mitochondria from a large scale incubation were washed thoroughly with 0.25 M sucrose and extracted at 0° for 3 hrs. with 0.6M KCl. After centrifugation of the extract at 20,000 x g for 30 min. the supernatant fluid was decanted and was assayed for ATPase activity, protein concentration and radioactivity. The KCl extract was then layered on the column and the chromatogram was developed under the conditions described in the legend to Fig.1.

As expected, the large actomyosin molecule is apparently excluded from the interstitial volume of the gel and is rapidly eluted. The elution pattern (Fig.1) shows an initial major peak with the ATPase activity and radioactivity followed by material with no enzyme activity and only a trace of radioactivity. The fractions included under the major peak contained 55% of the protein with which the column was charged and 98.5% of the enzyme units, representing approximately a two-fold purification of the ATPase activity over the pre-column preparation. In addition, 84% of the radioactivity was recovered in this peak.

In order to ascertain whether the radioactivity found in the contractile protein was the result of legitimate incorporation into peptide linkage or represented some form of artifactitious labeling as a result of non-specific acylation reactions (Moldave et al.,1959; Zioudrou and Fruton, 1959), a fluorodinitrobenzene experiment was performed according to the method of Sanger (1952). The presence of a substantial proportion of the radioactivity in the dinitrophenyl amino acid fraction would indicate the occurrence of such an acylation reaction.

Labeled contractile protein (4.2 mg), precipitated from the fractions of the major peak represented by an effluent volume of 40-50 ml (Fig.1), was treated with fluorodinitrobenezene. The dinitrophenyl protein was hydrolyzed and extracted with ether. Out

of a total radioactivity in the protein of 5550 c.p.m., only 183 c.p.m. or 3% of the total radioactivity was found in the ether layer (dinitrophenyl amino acid fraction), implying that only a very small percentage of the C^{14}-amino acid was present in N-terminal amino acid or in an acylated form. The remainder of the radioactivity was found associated with the free amino acids in the aqueous phase of the hydrolyzate.

These studies provide evidence for the ability of lamb heart sarcosomes to effect the *in vitro* incorporation of amino acids into a specific protein which has a physiological role in mitochondrial function. Since the radioactivity and the ATPase activity appear to reside in the same protein and since both activities are affected in the same manner by inhibitors of protein synthesis such as puromycin and actinomycin D mitochondria appear to be capable of the synthesis of the actomyosin-like contractile protein of the mitochondrial membrane. However, the unequivocal demonstration of the *net* synthesis of this protein awaits the results of experiments now in progress.

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