ISOLATION AND CHARACTERIZATION OF A PEPTIDE SYNTHESIZED IN MITOCHONDRIA

Bernhard Kadenbach
Eidgenössische Technische Hochschule Zürich
Laboratorium für Biochemie, 8006 Zürich, Switzerland

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Summary: A radioactive peptide has been isolated from rat liver mitochondria after labeling with leucine-C^{14} *in vivo* in the presence of cycloheximide. It has the following properties: 1. It is extremely hydrophobic; 2. After extraction from the membrane, it is tightly bound to phosphatides, possibly by ionic forces; 3. It gives a ninhydrin positive reaction; 4. Only one labeled compound with a molecular weight of about 2000 was detected; 5. It is not hydrolyzable by conc. HCl, but is split by subtilisin; 6. It contains most of the main amino acids.

Numerous studies have demonstrated the capability of mitochondria to incorporate amino acids into a membrane bound protein. However, this protein has never been identified. All known mitochondrial proteins, including the membrane bound cytochromes, the "structural protein", and the mitoribosomal proteins, have been shown to be sythesized at the cytoribosomes (for review see (1)). Many attempts have been undertaken to isolate the protein which is synthesized by mitochondria (2-15). These investigations have been unsuccessful so far, because of the extreme and unusual properties of this compound. The present investigation demonstrates that the product of mitochondrial protein synthesis is a lipophilic low molecular weight peptide.

Materials and Methods. L-leucine-C^{14} (U) (250 mc/m mole) was obtained from The Radiochemical Centre, Amersham. The chemicals (all analytical grade) and silicagel plates were from Merck, Darmstadt. Sephadex LH-20 was obtained from Pharmacia, Uppsala, cycloheximide from Sigma, St.Louis. Chloramphenicol was applied as chloramphenicol-monosuccinate (Paraxin, Boehringer, Mannheim). Subtilisin A and papain (both cryst. puriss.) were from Serva, Heidelberg.

Male rats of 300 - 350 g body weight were used. Phosphatides were digested and phosphate estimated according to Schnitger et.al. (16). Mitochondria were isolated in a medium consisting of 0.25 M sucrose, 0.02 M triethanolamine-HCl, pH 7.2, 0.002 M EDTA, 0.01 M L-leucine. Protein fractions were isolated as described (17). Specific radioactivity was measured by the previous method (17), except that washing with ethanol-ether and ether was replaced by washing with H_2O. The dried samples were treated as described (17). The scintillator consisted of 60% toluene, 40% ethyleneglycolmonomethylether, and 6 g/l butyl-PBD (Ciba, Basel). An aliquot of the insoluble mitochondrial protein fraction was extracted with 20 volumes $CHCl_3$ - CH_3OH (2:1, v/v).

Identification of the product of mitochondrial protein synthesis as a chloroform-methanol soluble compound. Cycloheximide inhibits the incorporation of leucine-C14 into microsomal, cytoplasmic and soluble mitochondrial proteins of rat liver in vivo by up to 98% (18,19). Under these conditions the incorporation into insoluble mitochondrial proteins is inhibited only by 90%. The remaining incorporation into mitochondrial membrane proteins is furthermore inhibited by chloramphenicol (18) and therefore has been related to the mitochondrial protein synthetic system (14, 20-22). The cycloheximide-resistent incorporated radioactivity of the mitochondrial membrane can be extracted by $CHCl_3$ - CH_3OH (2:1, v/v) as shown in table 1. In some experiments we could extract up to 96% of the radioactivity. The table clearly shows that the synthesis of the labeled $CHCl_3$ - CH_3OH soluble compound is inhibited by chloramphenicol and therefore apparently represents the product of mitochondrial protein synthesis.

Isolation and characterization of the peptide. Mitochondria from 3 rats (one of which was injected with 18 uc leucine-C14 and cycloheximide as described in table 1) were dissolved in 5 ml 5% sodium dodecylsulfate, 5% $HSCH_2CH_2OH$, 10 mM γ-aminobutyric acid and 50 mM Tris-HCl, pH 8.5. All subsequent operations were performed at room temperature. The solution was extracted twice with 30 ml $CHCl_3$ - CH_3OH (2:1, v/v). Part of the lower phase was mixed with 0.2 volumes 0.73% NaCl. The resulting lower phase was evaporated to dryness, dissolved in $CHCl_3$ - CH_3OH - H_2O (65:25:4, v/v/v), and chromatographed on silicalgel thin layer

Table 1. Chloramphenicol-sensitive incorporation of leucine-C^{14} into a chloroform-methanol soluble compound of rat liver mitochondria in vivo in the presence of cycloheximide. Cycloheximide (50 mg/kg b.w., dissolved in 0.9% NaCl) and chloramphenicol where indicated (300 mg/kg b.w.) were injected i.p. into rats 5 min. prior to 20 uc leucine-C^{14}. After 100 min. a chase of 2 ml unlabeled L-leucine was injected. 25 min. later the rats were killed and mitochondria isolated from the livers.

	Control	+ Chloramphenicol	
	cpm / mg protein		%inhibition
Soluble mitochondrial protein	29	21	28
Insoluble mitochondrial protein	890	382	57
Chloroform-methanol extracted insoluble mitochondrial protein	69	30	49
Chloroform- methanol extract	821	352	57

plates as shown in figure 1. The radioactivity run together with most of the separated phosphatides and with two unknown compounds. No separation of the label from the phosphatides was

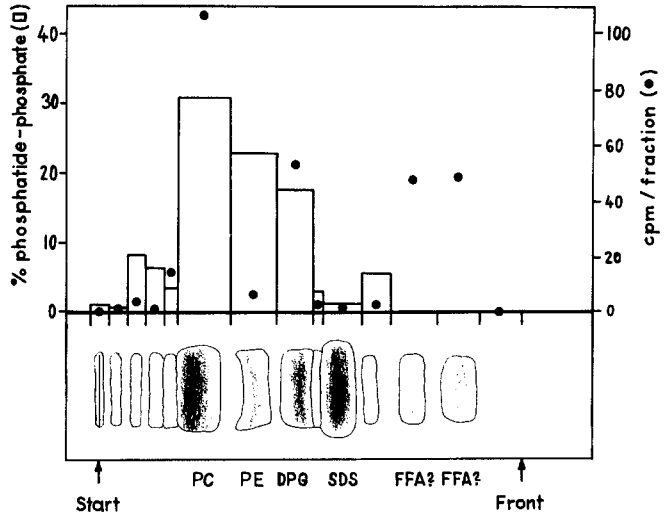

Figure 1. Thin layer chromatography of the chloroform-methanol soluble fraction of rat liver mitochondria on silica gel. The plate was eluted with $CHCl_3$ - CH_3OH - H_2O (65:25:4, v/v/v). The spots were stained with iodine vapor. Each spot was divided into equal parts and scratched out separately. One part was directly given into the scintillation vials and counted with 0.05 ml 10% KCN to remove the iodine color. The other part was used for phosphate determination. PC = phophatidylcholine, PE = phosphatidylethanolamine, DPG = cardiolipin, SDS = sodium dodecylsulfate, FFA = free fatty acid.

possible under these conditions, which suggested a binding by ionic forces.

The $CHCl_3$-extract was evaporated to dryness and dissolved in about 1 ml $CHCl_3$ - CH_3OH - 25% NH_3 - $HSCH_2CH_2OH$ (80:25:5:0.1, v/v/v/v), and chromatographed on a silicagel column (1.5 x 22 cm) using the same solvent as eluant (a white nonradioactive material remained insoluble). The radioactivity eluted together with the phosphatides in one peak. The combined samples of the radioactive peak were evaporated to dryness, dissolved in 0.5 ml $CHCl_3$ - CH_3OH - 20% NH_3 - $HSCH_2CH_2OH$ (2:2:1:0.05, v/v/v/v) and chromatographed on a Sephadex LH-20 column as shown in figure 2. Most of the radioactivity again was eluted together with the phosphatides, however, a small portion of the label appeared as low molecular

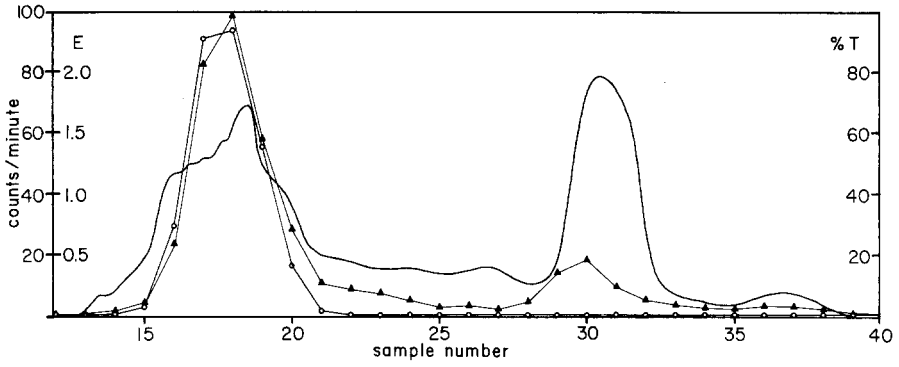

Figure 2. Chromatography of phospholipid extract on Sephadex LH-20. Column: 1.2 x 60 cm. Eluant: $CHCl_3$ - CH_3OH - $HSCH_2CH_2OH$ - 20% NH_3 (2:2:0.05:1, v/v/v/v). Flow speed: 7 ml/h. Samples of 1.9 ml were collected. ——— : transmission at 313 nm, o———o : absorbance of phosphatide-phosphate (0.02 ml/sample), v———v : radioactivity of 0.05 ml/sample.

weight compound free from phosphatides. If the phosphatide peak from this column is chromatographed under exactly the same conditions, again about 15% of the label is removed from the phosphatide peak. This partial dissociation of the labeled compound from the phosphatides has been consistently obtained. In another experiment up to 80% of the label could be separated from the phosphatides during one chromatographic run under slightly changed conditions. The combined labeled fractions which were free from phosphatides were evaporated to dryness, dissolved in 0.2 ml $CHCl_3$ - CH_3OH - 100% CH_3COOH (10:10:1, v/v/v) and chromatographed on Sephadex LH-20 as shown in figure 3. The radioacti-

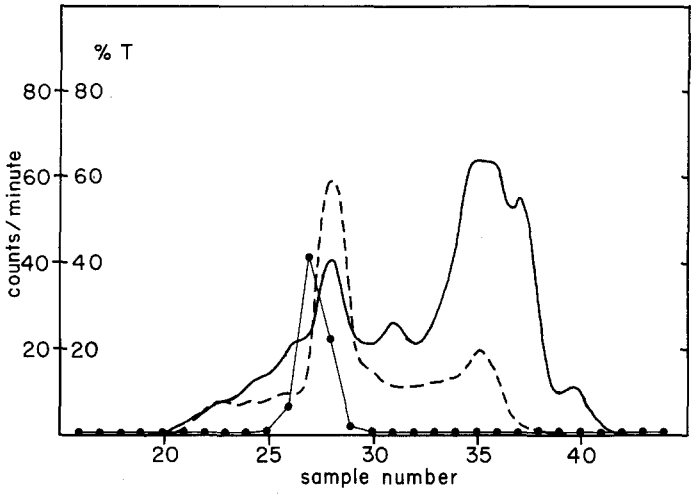

Figure 3. Chromatography of phosphatide-free labeled fractions as obtained in figure 2 on Sephadex LH-20. Column: 0.6 x 145 cm. Flow speed: 1.85 ml/h. Samples of 0.5 ml were collected. Eluant: $CHCl_3$ - CH_3OH - 100% CH_3COOH (10:10:1, v/v/v). ——— : transmission at 253 nm, - - - - : transmission at 313 nm, o———o : radioactivity of 0.05 ml.

vity was eluted as a single peak at a position equivalent to a molecular weight of about 2000 (calibrated with polyethyleneglycol). The labeled peak was further purified by repeated chromatography under the same conditions.

The purified labeled compound was subjected to thin layer chromatography on silicagel with $CHCl_3$ - CH_3OH (1:1, v/v) as eluant. Staining with ninhydrin revealed only one spot, which contained all of the radioactivity (R_f = 0.66). This again seemed to indicate that only one labeled compound is synthesized in the mitochondria.

In an attempt to hydrolyze the labeled compound, it was treated under various conditions: 6 M HCl; 12 M HCl - dioxan (1:1, v/v); 12 M HCl - 100% CH_3COOH (1:1, v/v). The samples, sealed under vacuum were heated at 110° for up to 19 days. One sample was heated at 200° for 22 hours. Under these conditions the labeled compound remained virtually unchanged. The 19 days treated sample (HCl - CH_3COOH) was evaporated to dryness and extracted with a mixture of $CHCl_3$ - CH_3OH - H_2O (2:1:1, v/v/v). More than 80% of the label appeared in the $CHCl_3$-phase. After evaporation about 0.1 mg of a white powder was obtained with a

total radioactivity of about 400 counts/min. The compound is soluble in chloroform and methanol, but insoluble in H_2O and aceton. Thin layer chromatography of about 1/10 of this compound under the above described conditions gave only one ninhydrin positive spot, which contained all radioactivity and had the same R_f of 0.66 as was obtained before the hydrolytic treatment.

After various attempts to hydrolyze the compound, finally the treatment with 2 x 1 ug subtilisin A for 1 hour at 37° in a medium containing 0.2 mM EDTA, 1 mM cysteine, 0.15 M NaCl, 0.02 M Tris-HCl, pH 8.0 dissolved most of the compound. After additional incubation with 1 ug papain for 1 hour under the same conditions, the turbid solution was evaporated to dryness, hydrolyzed with 6 M HCl for 20 hours and subjected to amino acid chromatography. In a preliminary run the following amino acids have been identified: Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Tyr, Phe. Lys, Arg, Try, His and Cys have not been determined.

Discussion. The isolated mitochondrial peptide has many properties in common with the phosphatides: 1. It is insoluble in water or acetone, but soluble in chloroform or methanol. 2. The molecular weight of about 2000 is only twice that of the phosphatides. 3. It contains a free amino group, which could form ionic bonds in a lipophilic milieu. On the other hand the compound represents chemically a peptide, containing most of the main amino acids. Its chemical behaviour, however, is quite unusual since it is not hydrolyzed by conc. HCl. It seems that the extremely hydrophobic surface of the molecule prevents proton attack.

Various investigators have observed several radioactive bands using polyacrylamide gel electrophoresis to separate the specifically labeled mitochondrial membrane proteins (5,10,12-14). We could find only one labeled compound after the peptide has been separated from the phosphatides. However, we cannot exclude the existence of other labeled peptides with very similar properties.

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