Synthesis of Actin-like Protein in Rat Liver Mitochondria

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Abstract. Isolated rat liver mitochondria failed to exhibit in vitro incorporation of [^{14}C]-amino acids into actin-like protein. The use of a pulse-labelling technique demonstrated the appearance of [^{14}C]-actin-like protein in the mitochondria of control, cycloheximide-free rats. The actin-like protein was identified by the method of affinity binding on DNAse1-sepharose and by electrophoresis on polyacrylamide gel with sodium dodecyl sulphate. It was shown that mitochondrial actin-like protein is not included among the nine polypeptides synthesized in mitochondria during cycloheximide-induced blockade of cytoplasmic protein synthesis. It was shown that actin-like protein was not desorbed from mitochondria by repeated washing with isotonic sucrose-mannitol medium. The results obtained indicate that the actin-like protein is biosynthesised in the cytoplasmic compartment.

Key words: Actin — Liver mitochondria — Protein synthesis

Introduction

The cytoplasmic compartment as well as certain cellular organelles contain contractile proteins which, in many respects, resemble those of muscle (Clarke and Spudich 1977). The principal constituent of this group is actin (Korn 1978). The majority of the other proteins belonging to this group are able to alter the aggregate state of actin or to participate in the assembly of microfilaments into more complex macromolecular structures, visible in a diversity of cell types as "stress-fibres" (Weeds 1982).

An actin-like protein has also been discovered in the mitochondria of non-muscle tissues (Ohnishi and Ohnishi 1962; Neifakh and Kazakova 1963). A comparative study of the primary structure of mitochondrial actin-like protein, employing a peptide mapping technique, has revealed its close similarity to that of actin from skeletal and, especially, smooth muscle (Stozharov 1983). Possessing as it does the appropriate structural-functional properties, the actin-like protein may be involved in the morphological transformation of mitochondria and the control of membrane-bound enzyme activity (Bartley et al. 1969).

The mechanism of biogenesis of actin-like protein is unclear. Mitochondria are formed by a coordinated interaction between two genetic systems: the nucleo-

cytoplasmic and the mitochondrial genetic system (Ades 1982). It has been established that the nuclei of many cell types contain an actin-like protein which differs in several respects from cytoplasmic actin (Bremer et al. 1981). Although transfer of actin from the cytoplasmic compartment to the nucleus is known to occur in certain instances, there is nevertheless strong evidence to suggest that authentic nuclear actin is not a cytoplasmic contamination product, but instead it constitutes a separate variety of this protein (Wehland et al. 1980; Nakayasu and Ueda 1983).

In view of this and the well-known autonomy of mitochondria in the synthesis of a number of mitochondrial polypeptides, we have investigated the mechanism of biosynthesis of actin-like protein in rat liver mitochondria.

Materials and Methods

Partial hepatectomy of male rats was performed according to the method of Higgins and Anderson (Pedersen et al. 1978). Animals were used for experiments on the third day following operation.

Hepatic mitochondria were isolated by differential centrifugation in 220 mmol.l^{-1} mannitol, 70 mmol.l^{-1} sucrose, 2 mmol.l^{-1} HEPES-KOH, pH 7.4, bovine serum albumin 0.5 g/l (H-medium) (Pedersen et al. 1978). Following two washings with H-medium the mitochondrial fraction (25 mg protein) was suspended in 5 ml of solution containing 0.15 mol.l^{-1} sucrose, 35 mmol.l^{-1} KCl, 5 mmol.l^{-1} $MgCl_2$, 2 mmol.l^{-1} ADP, 5 mmol.l^{-1} succinate, 20 mmol.l^{-1} KH_2PO_4, cycloheximide 50 μg/ml, 35 mmol.l^{-1} Tris-HCl, pH 7.4. After preincubation at 30°C for 5 min, 9.3 MBq of a mixture of [^{14}C]-amino acids (protein hydrolysate) was added to the reaction mixture. A 250 μl solution of puromycine (50 μg/ml) was added to the mitochondrial suspension 40 min after introduction of the label, and incubation was continued for a further 5 min. The mitochondria were separated from the incubation medium by centrifugation at 12,000 × g for 15 min. The mitochondrial pellet was then washed three times with H-medium and treated with 20 volumes of G-buffer, composed of 0.2 mmol.l^{-1} $CaCl_2$, 0.2 mmol.l^{-1} dithiothreitol, 0.2 mmol.l^{-1} ATP, 2 mmol.l^{-1} Tris-HCl, pH 7.9. Formamide was added to give a final concentration of 10% and 20 min later the mitochondrial fragments were precipitated by centrifugation at 100,000 × g for 60 min. The clear supernatant was concentrated by ultrafiltration using PM-10 membranes (Amicon, Holland) for use in subsequent affinity chromatography.

A number of experiments were performed using [^{3}H]-leucine as a label. In this case the incubation medium contained 50 mmol.l^{-1} KCl, 5 mmol.l^{-1} $MgCl_2$, 1 mmol.l^{-1} EDTA, 5 mmol.l^{-1} succinate, 2 mmol.l^{-1} ADP, 20 mmol.l^{-1} KH_2PO_4, cycloheximide 50 μg/ml, 50 mmol.l^{-1} Tris-HCl, pH 7.4, supplemented with a synthetic mixture of amino acids (Truman and Korner 1962). Incorporation of the label into proteins was initiated by the addition of 3.7 MBq [^{3}H]-leucine. Incubation was continued for 30 min at 37°C and the reaction was stopped by the addition of a 5 mmol.l^{-1} solution of unlabelled amino acid. After the addition of puromycine (50 μg/ml) the mitochondria were precipitated by centrifugation at 12,000 × g, washed three times with H-medium, and the soluble proteins were extracted with a 10% solution of formamide in G-buffer.

A pulse-labelling approach was adopted in studying protein synthesis in vivo. A mixture of [^{14}C]-amino acids (5.6 or 9.3 MBq) was injected intraperitoneally into hepatectomized rats and the animals were sacrificed by decapitation 10 or 20 min later. Where appropriate, cycloheximide (1.5 mg/kg) was injected intraperitoneally 1.5 h prior to the administration of [^{14}C]-amino acids. Mitochondria (100 mg protein) isolated in H-medium in the above-mentioned manner were extracted

with a 10% solution of formamide in G-buffer. Insoluble matter was removed by ultracentrifugation and the supernatant was preserved.

Isolation of the actin-like protein was performed by affinity chromatography on immobilized DNAse1 (EC 3.1.4.5) according to the method of Zechel (1980). The clear mitochondrial supernatant was applied to a DNAse1-column prepared from 1.0 g lyophilized BrCN-activated sepharose 4B. The column was washed with a 10% solution of formamide in G-buffer to remove unbound proteins, and non-specifically bound components were then removed by washing with a solution of 0.2 $mol.l^{-1}$ NH_4Cl in G-buffer. The actin-like protein was eluted with a 40% solution of formamide in G-buffer. The optical absorbance of the eluate was measured at 280 nm using a "Multi-Chromatocord" (FRG). Samples were assayed using a SBS-2 liquid scintillation counter (USSR).

Protein electrophoresis was performed on a linear gradient (8—20 %) of polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS), according to the method of Laemmli (1970). The density of gels stained with 0.1% Coomassie brillant blue R-250 was measured using a gel scanner at 550 nm with an integrator. Following protein electrophoresis, sections of polyacrylamide gel were prepared, incubated in 0.2 ml of 1% SDS solution at 50°C for 12 hours, and the radioactivity of the eluate was determined using a liquid scintillation counter.

Actin from rabbit skeletal muscle was isolated according to the method of Spudich and Watt (1971). The protein concentration was determined according to the method of Lowry (1951), using bovine serum albumin as standard.

Chemical reagents used for the preparation of buffer were of analytical grade. Cycloheximide and DNAse1 were obtained from Calbiochem (USA), SDS and puromycine from Serva (FRG), HEPES from Sigma Chemical Co. (USA), BrCN-activated sepharose 4B from Pharmacia (Sweden), formamide from Merck (FRG), Coomassie brilliant blue R-250 from Ferak (W. Berlin), [^{14}C]-amino acids from ÚVVVR (Czechoslovakia) and [^{3}H]-leucine from Isotop (USSR).

Results

On account of the high specificity with which actin binds to DNAse1, affinity chromatography may be regarded as an effective method for the isolation and purification of actin-like proteins (Zechel 1980). In a preliminary series of experiments we showed that washing of the affinity matrix with a 40% solution of formamide resulted in elution from the mitochondrial lysate of a protein which comigrated with skeletal muscle actin during electrophoresis with SDS (see Fig. 1) and which possessed the ability to inhibit pancreatic DNAse1 activity.

Fig. 2 illustrates the elution profile on DNAse1-sepharose and distribution of radioactivity of the soluble protein fractions obtained from rat liver mitochondria following in vitro incubation with a mixture of [^{14}C]-amino acids in the presence of cycloheximide. The majority of [^{14}C]-labelled proteins either failed to bind, or bound non-specifically, to the agarose matrix and passed through the column (fractions 1 and 2). On the basis of gel electrophoretic analysis, the mitochondrial actin-like protein eluted from the DNAse1-affinity matrix with a 40% formamide solution (fraction 3) was identified as being a polypeptide with an apparent molecular weight of 42,000 daltons. Radioactive label was not detected in this fraction.

It is evident that, under the experimental conditions employed here, incorpo-

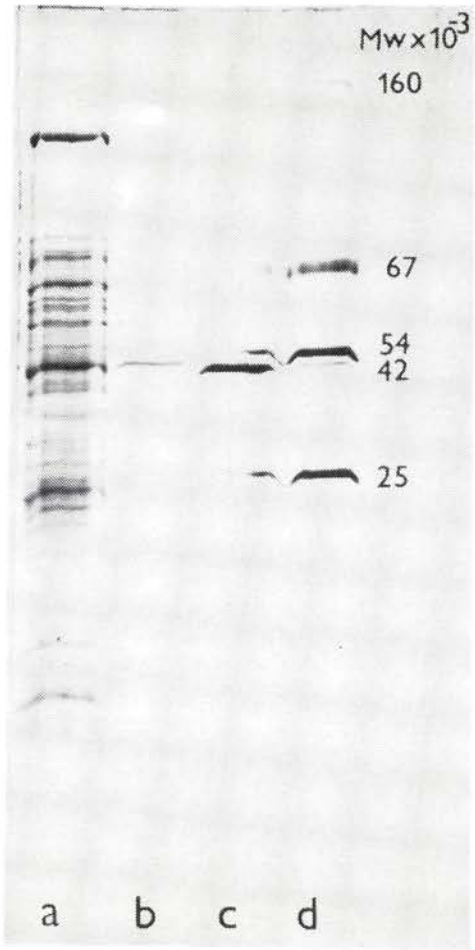

Fig. 1. Electrophoresis of fractions obtained following affinity chromatography of mitochondrial extract on DNAse1-sepharose. *a*) original mitochondrial extract; *b*) fraction obtained by elution of the affinity column with a 40% solution of formamide; *c*) actin from rabbit skeletal muscle; *d*) proteins from vesicular stomatitis virus (markers of molecular weight).

ration of radioactive label into mitochondrial actin-like protein did not occur. Consequently, biosynthesis of this protein would appear to take place on the cytoplasmic ribosome. This suggestion may be tested by pulse-labelling mitochondrial actin-like protein in vivo in the presence of cycloheximide, an inhibitor of ribosomal protein synthesis. However, despite its apparently simplicity, this approach introduces a number of difficulties which need to be avoided. The major problem is that of the time course of action of the protein synthesis inhibitor. As

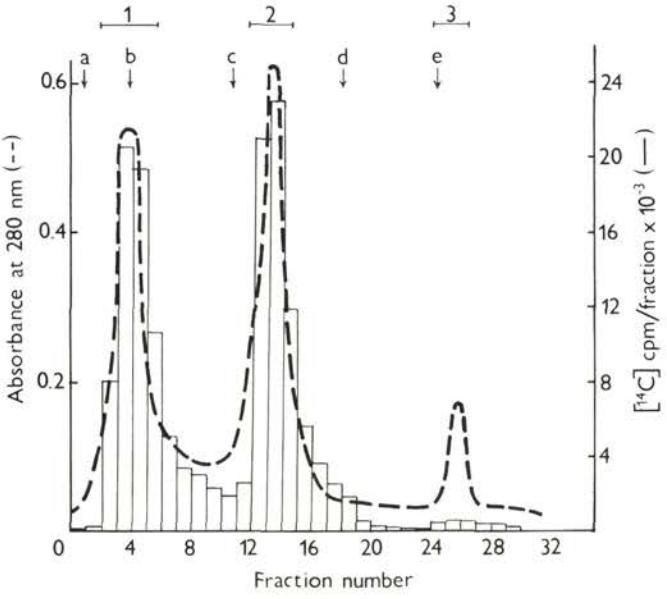

Fig. 2. Elution profile and the distribution of radioactivity in fractions, obtained by affinity chromatography on DNAse1-sepharose, of mitochondrial proteins labelled in vitro with a mixture of [^{14}C]-amino acids in the presence of cycloheximide. Arrows indicate: *a*) application of the mitochondrial extract; *b*) removal of sorbent from unbound proteins with a 10% formamide solution (fraction 1); *c*) removal of nonspecifically bound proteins with a 0.2 mol.l^{-1} NH_4Cl solution (fraction 2); *d*) removal of salts from the column with a 10% formamide solution; *e*) elution of actin-like protein with a 40% formamide solution (fraction 3). Volume of the fractions — 0.75 ml. Elution rate — 9 ml/h. For determination of radioactivity, 0.5 ml of each fraction was used.

shown by Ashour and Traube (1981), cells treated over a prolonged period of time with cycloheximide usually display a reduction in mitochondrial polypeptide synthesis. In view of this, they adopted an exposure time to the inhibitor not exceeding 1.5 hours. However, pretreatment for any shorter period than this is undesirable, since peak effect of cycloheximide is reached 1—2 hours after its intraperitoneal administration.

Figure 3 illustrates the elution profile and distribution of radioactivity, following affinity chromatography on DNAse1-sepharose, of mitochondrial protein fraction obtained from control and cycloheximide-pretreated hepatectomized rats. It is evident that fractions of actin-like protein from control rats display a significant degree of radioactive label incorporation. Pretreatment with cycloheximide not only decreased the total amount of radioactivity in mitochondrial proteins but also decreased the radioactivity of the eluted fraction containing the actin-like protein.

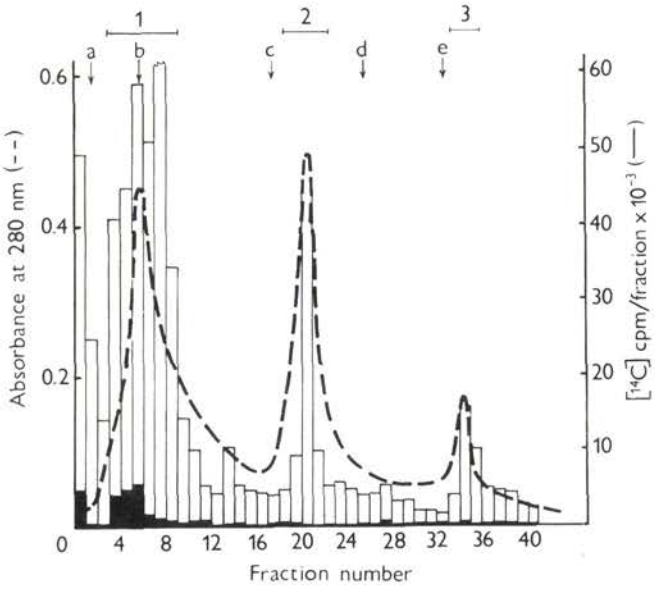

Fig. 3. Elution profile and the distribution of radioactivity in fractions obtained by affinity chromatography on DNAse1-sepharose of mitochondrial proteins labelled in vivo with a mixture of [^{14}C]-amino acids; control (□) and cycloheximide (■) treated animals. The interval between the time of administration of the label to the animal and the time of death was 10 min. For explanation of other symbols, see Fig. 2.

Identification of cytoplasmically synthesized mitochondrial [^{14}C]-actin-like protein was achieved by electrophoretic separation of the eluted fractions on polyacrylamide gel in the presence of SDS. The polypeptide present in the fraction eluted with 40% formamide solution had an apparent molecular weight of 42,000 daltons and comigrated with skeletal muscle actin. The radioactivity levels of this fraction obtained from control and cycloheximide-pretreated rats were 116 and 10 c.p.m., respectively.

An alternative approach to elucidating the site of the actin-like protein synthesis would be to analyse products of the mitochondrial translation system. In the present series of experiments, rat liver mitochondrial proteins, labelled in vivo against a background of pretreatment with cycloheximide, were fractionated electrophoretically and the levels of radioactivity of the polypeptides were determined after their elution from the gel. Fig. 4 illustrates the separation of rat liver mitochondrial proteins. The experiments show that only 9 of the polypeptides present were labelled (Fig. 5). The majority of them had molecular weights of less than 50,000 daltons. This is consistent with the findings of other investigators who

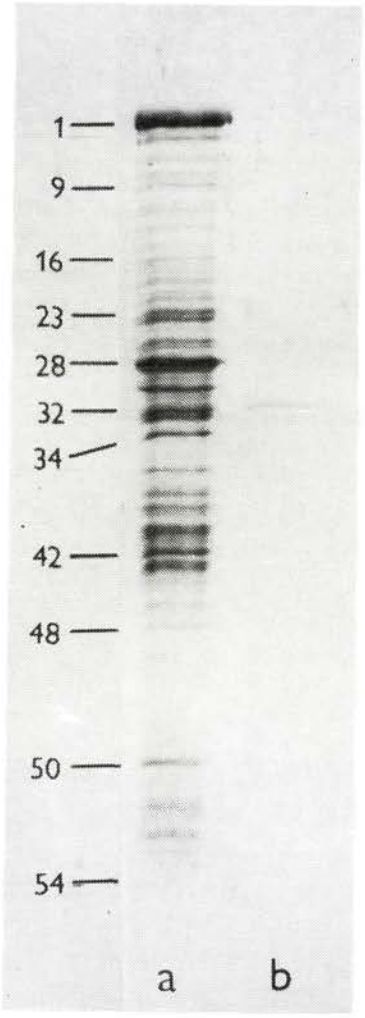

Fig. 4. Electrophoretic separation of mitochondrial proteins labelled in vivo with a mixture of [^{14}C]-amino acids during pretreatment of animals with cycloheximide. *a*) mitochondrial lysate; *b*) mitochondrial actin-like protein isolated by affinity chromatography on DNAse1-sepharose. 1—54 — numbers of fractions used for subsequent radioactivity counting.

have similarly discovered approximately the same range of molecular weights in mitochondrial translation products (Ashour and Tribe 1981). A comparison of the electrophoretic motility of the actin-like protein with the profile of radioactively-labelled mitochondrial polypeptides indicates that in the protein fraction

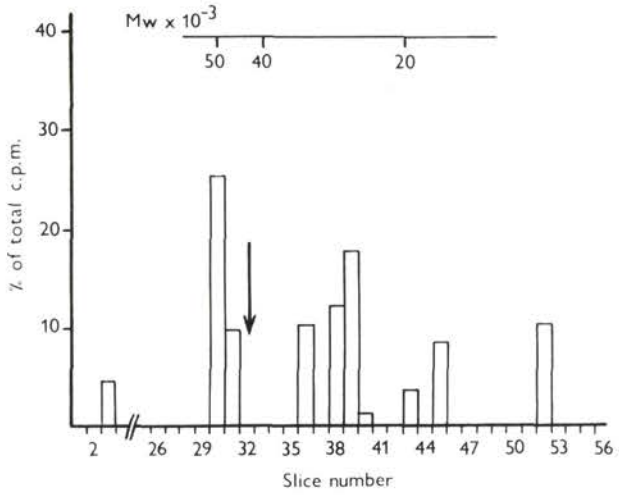

Fig. 5. Distribution of radioactivity following polyacrylamide gel electrophoresis in fractions of liver mitochondrial proteins labelled in vivo during pretreatment of animals with cycloheximide. Fractions numbers correspond to those in Fig. 4. The combined radioactivity of all the fractions (excluding background radioactivity) was taken to be 100%. The position of the actin-like protein is indicated by the arrow.

(fraction 32) radioactivity is absent. This may serve as further evidence in favour of a cytoplasmic site for the biosynthesis of the actin-like protein.

The phenomenon of the nonspecific adsorbtion of cytoplasmically synthesized actin-like protein onto mitochondrial membranes represents a possible cause of error in interpreting the experimental data. In an attempt to exclude this possibility, a study was made of the effect of successive washing of mitochondria with isotonic H-medium on mitochondrial actin-like content. Following isolation, mitochondria were subjected to repeated cycles of centrifugation and resuspension and their actin-like protein content was determined in the manner described previously (Stozharov 1983). The findings are summarized in Table 1. It is evident that as many as 5 repeated washes of the mitochondria with H-medium did not significantly alter their actin-like protein content, the level of which remained fairly constant. This finding might indirectly indicate that the mitochondrial actin-like protein isolated by affinity chromatography is an intrinsic component of these organelles, rather than a contaminant of cytoplasmic origin, adsorbed onto mitochondrial membranes in a nonspecific manner during the process of fractionation of the liver homogenate.

Table 1. The content of actin-like protein in rat liver mitochondria during their repeated washing with an isotonic isolation medium (% of total protein).

No. of washes	Amount of actin-like protein
1	2.07 ± 0.05 (4)
2	2.07 ± 0.10 (4)
3	1.95 ± 0.05 (4)
4	2.37 ± 0.21 (4)
5	2.07 ± 0.13 (4)

Notes: Figures in brackets indicate numbers of experiments. The value for the first wash corresponds to the level of actin-like protein in the mitochondrial fraction obtained after the initial centrifugation of the organelles at $9800 \times g$ for 15 min (see Materials and Methods). Each subsequent wash consisted of resuspension of the mitochondrial pellet in 8 ml H-medium and centrifugation at $9800 \times g$ for 10 min.

Discussion

It is well established that the mitochondrion is an organelle capable of autonomous synthesis of certain macromolecules (Kolarov et al. 1981). Despite a relatively small coding capacity, mitochondrial DNA is capable of coding the nucleotide sequences of several RNAs and a limited number of polyptides which account for 5—10% of total mitochondrial proteins. For example, the DNA of yeast mitochondria codes three subunits of cytochrome oxidase, one subunit of cytochrome b, two subunits of ATPase complex and one polypeptide associated with the small subunit of the mitochondrial ribosome (Ades 1982). The coding capacity of human mitochondrial DNA is somewhat greater, coding for the synthesis of 13 polypeptides (Anderson et al. 1981). The majority of these polypeptides are hydrophobic subunits of the respiratory chain complex which are immediately surrounded by the lipid bilayer of the mitochondrial membrane.

In our experiments, using a method of affinity chromatography on DNAse1-sepharose we failed to detect in vitro synthesis of the mitochondrial actin-like protein. In vivo incorporation of radioactive label into the protein during pretreatment of animals with cycloheximide was also absent. At same time, a relatively high level of radioactivity was detected in the actin-like protein fraction isolated from the mitochondria of control (untreated) animals. Consequently, on the basis of the findings described here, it would appear that mitochondrial actin is a product of cytoplasmic protein synthesis.

It has been shown that only two molecular forms (β and γ) of the actin-like protein are present in liver cells (Vanderkerckhove and Weber 1981). Our experiments indicate that the biosynthesis of mitochondrial actin occurs on the cytoplasmic ribosome. In view of these and the above-mentioned findings, we

suggest that hepatic mitochondria make use of a cytoplasmic pool of actin in performing their functions, a portion of the actin being transported into the intra-mitochondrial compartment.

In conclusion, our results support the view that the mitochondrial actin-like protein like the majority of mitochondrial components, is not synthesized in these organelles, but is transported there as a product of cytoplasmic biosynthesis.

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Received June 27, 1984/Accepted September 25, 1985