DIFFERENCES IN THE IN VITRO AMINO ACID LABELLING PATTERN OF MITOCHONDRIA FROM MELANOMA AND LIVER

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1. Introduction

It is well established that isolated mitochondria have the ability to incorporate amino acids into certain proteins [1–3]. These proteins are insoluble and integrated into the inner membrane [4, 5] whereas the soluble proteins, including malate dehydrogenase, catalase and cytochrome *c*, obtained by sonication of whole mitochondria are not labelled under the in vitro conditions [4, 6, 7]. Many of the findings obtained with rat liver have been confirmed with mitochondria from rat cerebral cortex, spinal cord [8], plant tissue [9] as well as from Krebs ascites tumor cells [10].

It has been shown by Sebald [11, 12], that the insoluble mitochondrial proteins of three very different tissues, such as neurospora, locust flight muscle and rat liver, exhibit when separated by electrophoresis on polyacrylamide, a very similar distribution of radioactivity. In all these patterns, a very slow moving band (band 4, see also [12]) was most highly labelled.

During our studies on the protein synthesis in a malignant hamster melanoma we observed that mitochondria from this tissue exhibit some peculiar features. Although they do contain cytochrome oxidase with about the same specific activity as that found in hamster or rat liver mitochondria [13] and seem to be intact with respect to oxidative phosphorylation [14] we found that in their electrophoretic pattern the band 4 mentioned above was markedly reduced [15]. Since the protein of this band seems to be a major product of the mitochondrial protein synthesis, it was of interest to discover if melanoma mitochondria are able to incorporate amino acids. If they exhibit a protein synthesis the further question would be whether the products are similar or different from that usually found in liver mitochondria. This communication deals with the incorporation rate of amino acids, the effect of some drugs and the distribution of radioactivity in the electrophoretic pattern of mitochondria from melanoma and liver.

2. Materials and methods

The amelanotic hamster melanoma used in our studies is the A-Mel 3 described by Fortner [16], which is serially transplanted subcutaneously into female Syrian hamsters, weighing 60–80 g. On the tenth day after transplantation, animals were killed by decapitation, the tumours and livers carefully dissected, washed and homogenized. Mitochondria were isolated by differential centrifugation as previously described [17] and washed twice with 0.03 M triethanolamine buffer pH 7.6, containing 0.1 M sucrose, 50 mM KCl, 2 mM EDTA, 10 mM $MgCl_2$, 20 mM KH_2PO_4.

Isolated mitochondria were incubated at pH 7.6 for 30 min at 37° in a medium containing: 0.1 M sucrose, 50 mM KCl, 30 mM triethanolamine, 2 mM EDTA, 10 mM $MgCl_2$, 20 mM KH_2PO_4, 4 mM ATP and an amino acid mixture of 0.3 mg per ml, free of leucine, isoleucine, and phenylalanine. Mitochondria (about 1 mg per ml) and 1 μCi per ml

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^{14}C-L-leucine (251 mCi/mmole), 1 μCi per ml ^{14}C-L-isoleucine (262 mCi/mmole) and 1 μCi per ml ^{14}C-L-phenylalanine (366 mCi/mmole) were added to the incubation medium. After incubation the mitochondria were washed three times with sucrose medium (0.25 M sucrose, 10 mM triethanolamine and 2 mM EDTA) containing unlabelled leucine, isoleucine and phenylalanine, and the specific radioactivity was determined according to Siekevitz [18]. For determining the effect of cycloheximide, chloramphenicol and proflavine on the in vitro incorporation, the drugs were added to give a final concentration of 0.18 mM cycloheximide, 5 mM chloramphenicol (Serva, Heidelberg, Germany) and 2×10^{-2} mM proflavine (Fluka, Buchs, S.G. Switzerland). To obtain soluble and insoluble proteins, the mitochondria were extracted twice with 0.1 M phosphate buffer pH 7.2, after sonicating 8 times for 30 sec and centrifuging for 60 min at 144,000 *g*. The pellet was regarded as insoluble membranous proteins, the supernatant as the soluble mitochondrial proteins. The proteins were precipitated with ethanol–ether (3:1) at a final concentration of 95%, washed twice with the same solvent, once with chloroform–methanol (2:1), once more with ethanol–ether (3:1) and finally with ether. The samples were then dissolved in phenol–acetic acid–water (2:1:1, v/v/v) at a concentration of 8 mg/ml.

Electrophoresis was performed in 7.5% (v/v) polyacrylamide gel, equilibrated with a medium containing phenol–acetic acid–water (2:1:1, v/v/v), as described by Braunitzer [19]. After staining the gels for 1 hr with 0.2% Amido black in 7.5% acetic acid and destaining in 10% acetic acid, densitometer tracings were recorded at 578 nm. The radioactivity in the gels was determined by liquid scintillation counting after slicing the gels into 1 mm thick pieces and depolymerising them with 30% H_2O_2.

3. Results and discussion

Isolated mitochondria of melanoma readily incorporate amino acids at a rate more than 4 times higher than that of liver mitochondria (table 1). Cycloheximide, a specific cytoplasmic protein synthesis inhibitor for eukariotic cells, has no effect on the ^{14}C-amino acid incorporation into melanoma mitochondria, whereas some inhibition is observed for liver mitochondria (table 1). This fact is probably due to microsomal contamination of our liver mitochondria.

Table 1
Effect of antibiotics on amino acid incorporation into isolated mitochondria from melanoma and liver.

	Melanoma (cpm/mg protein)	Liver (cpm/mg protein)
Control	11,300	2,780
Cycloheximide (0.18 mM)	12,400	2,380
Chloramphenicol (5.0 mM)	5,500	1,720
Proflavine (0.01 mM)	12,200	1,920

Mitochondria obtained from five tumours and five livers were incubated in air for 30 min at 37° in a metabolic shaker in an incubation medium containing ^{14}C-leucine, isoleucine and phenylalanine, as described under Materials and methods. Each ml of incubation mixture contained 1 μCi of ^{14}C amino acid mixture. The final concentration of antibiotics is stated in the brackets.

The inhibitory effect of chloramphenicol in melanoma as well as in liver mitochondria, shown in table 1, demonstrates that the amino acid incorporation is actually due to genuine mitochondrial protein synthesis. A 50% inhibition was achieved in melanoma and a 38% in liver mitochondria. Since in earlier experiments [15, 20] we have observed a proflavine sensitive protein synthesis in the melanoma, we argued that this protein synthesis might be partially associated with the mitochondria. On the basis of these findings we investigated the influence of proflavine on in vitro amino acid incorporation into isolated mitochondria from melanoma. As it is shown in table 1 no inhibition in melanoma mitochondria is found, but unexpectedly a 30% inhibition occurs in liver. This effect might be due to an influence of the synthesis of new m-RNA, assuming that this RNA species is less stable in liver than in melanoma mitochondria. Generally the effect of the three antibiotics on the in vitro labelling do not

Table 2
Specific and total radioactivity of soluble and insoluble mitochondrial proteins from melanoma and liver.

	Melanoma		Liver	
	(cpm/mg)	(total cpm)	(cpm/mg)	(total cpm)
Mitochondria	11,300	340,000	2,780	83,500
Insoluble mitochondrial protein[a]	19,400	232,000	4,500	74,500
Soluble mitochondrial protein[a]	4,600	83,000	400	5,350

[a] Insoluble and soluble mitochondrial proteins were obtained by centrifugation (1 hr at 144,000 *g*) after sonication in 0.1 M phosphate buffer, pH 7.2, as described in Materials and methods.

provide much evidence for differences between melanoma and liver.

However, differences can be detected in the specific and total radioactivity of the soluble and insoluble mitochondrial proteins from both sources (table 2). By sonication in phosphate buffer about one quarter of the total radioactivity incorporated and about 60% of the protein of melanoma mitochondria is solubilized. In liver mitochondria, 45% of the protein and only 6% of the total radioactivity is solubilized by this treatment. This 6% value is even higher in comparison to that reported by other authors [5, 11] and is probably due to the microsomal contribution to the incorporated radioactivity. This obvious difference in the distribution of radioactivity between the soluble and insoluble mitochondrial proteins could be explained in several ways. There could be a reduction of the forces which bind the proteins to the mitochondrial membrane involving lipid components. In other words a change or a deficiency in mitochondrial lipids may cause the difference in solubility between the in vitro labelled proteins of melanoma and of liver. On the other hand, it could be that proteins are synthesized by melanoma mitochondria which differ from that made by liver mitochondria.

In seeking confirmation of one or either possibility, the soluble and insoluble proteins from both sources were electrophoresed on polyacrylamide gels. If the differences are mainly based on the binding forces, we would expect a similar labelling pattern and only a change in the ratio of the soluble and insoluble label. It has to be mentioned at this point that mitochondria from three different sources (e.g. neurospora, locust flight muscle and rat liver), although differing in enzyme and electrophoretic pattern, exhibit a very similar radioactivity distribution in the gels. This indicates that the products of mitochondrial protein synthesis in these sources are similar at least with respect to their molecular weight. The observation we made for melanoma mitochondria clearly indicates that the labelling pattern is altered in comparison to that of liver (fig. 1). There are at least three bands (at 2.5 cm, 3.3 cm and 4.0 cm) which are radioactive in the melanoma pattern but unlabelled in the liver. These labelled proteins seem to be easily solubilized by sonication, since the largest part of their radioactivity is found in the soluble protein fraction (fig. 1C). In addition some more radioactivity is solubilized which is associated with the band at 0.8 cm. The degree of solubilization and distribution of radioactivity found in melanoma is obviously distinguishable from that observed for liver (fig. 1 C and F). However, a further difference concerns the insoluble proteins. In the liver pattern (fig. 1E) the bands at 1.0, 1.4 and 1.5 cm are highly labelled, a finding which is in good agreement with that observed by other authors [5, 21]. In the melanoma pattern (fig. 1B) high radioactivity is also found in the bands at 1.4 and 1.5 cm, but in addition the band at 2.5 is highly labelled. On the other hand

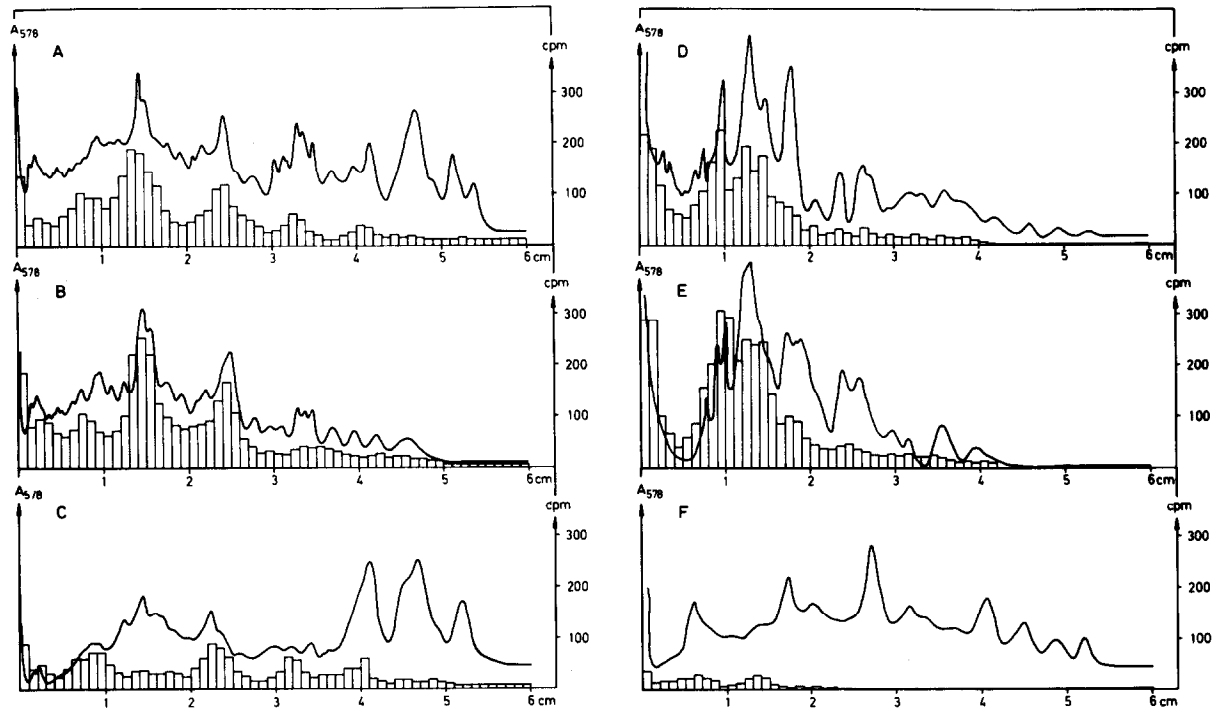

Fig. 1. Densitometer tracings of Amido black stained mitochondrial proteins from melanoma and liver after gel electrophoresis (smooth curve) and distribution of radioactivity over the pherograms (edged broken lines). (A) whole mitochondria from melanoma, (B) insoluble mitochondrial proteins from melanoma, (C) soluble mitochondrial proteins from melanoma, (D) whole mitochondria from liver, (E) insoluble mitochondrial protein from liver, (F) soluble mitochondrial proteins from liver. The direction of migration is left to right. For electrophoretic conditions see also Materials and methods.

the band at 1.0 cm, which is most highly labelled in the liver pattern, exhibits only low radioactivity in the melanoma pattern. In the light of these results together with the conclusions made by other investigators [12] we may assume that the protein of this band (at 1.0 cm) is the major product of mitochondrial protein synthesis and that it is strongly associated with the mitochondrial membrane. We might further conclude from our results that melanoma mitochondria are altered at least with respect to this particular protein band. The observed osmotic lability of melanoma mitochondria might be a further support to our assumption that this particular protein is involved in the formation of an intact inner membrane.

In conclusion we can say that in melanoma mitochondria, proteins are labelled in vitro which are clearly distinguishable from that labelled in liver mitochondria. The origin and the function of these proteins is unclear and we can only speculate on a possible correlation of these proteins with the virus-like particles, which we have been able to detect in our melanoma [22].

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