Biochimica et Biophysica Acta, 1099 (1992) 118–122

BBABIO 43562

Isoforms of cytochrome *c* oxidase in tissues and cell lines of the mouse

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(Received 15 July 1991)

Key words: Cytochrome *c* oxidase; Isoforms; (Mouse tissues, cell lines)

The subunit pattern of immunopurified cytochrome *c* oxidase from cultured mouse cells and mature tissues of the mouse was investigated by electrophoretic analysis. In mature tissues two forms of cytochrome *c* oxidase could clearly be identified on the basis of differences in mobility or staining intensity of subunits VIa and VIII. One form was present in muscle and heart, and the other in liver, kidney and spleen. In lung both forms were found. In the thymus, subunit VIII showed the characteristics of subunit VIII found in muscle and heart, whereas subunit VIa resembled subunit VIa found in liver. This suggest the existence of a third cytochrome *c* oxidase isoform. The subunits of cytochrome *c* oxidase from cultured cell lines showed no differences between the various cell lines and resembled those of mature mouse liver tissue. The cytochrome *c* oxidase isoform from cultured proliferating cells might therefore be the same as the one found in liver. Alternatively, it might represent either a normaliy occurring fetal isoform, or a form specific for poorly differentiated cultured cells.

Introduction

Cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, is composed of several subunits. In mammalian cells, three of the thirteen subunits are encoded by the mitochondrial genome [1]. These three subunits constitute the functional core of the enzyme. The function of the other ten subunits, which are encoded by the nuclear genome, remains to be established. In bovine tissues the occurrence of two different forms of three of the nuclearly coded subunits (VIa, VIIa and VIII) has been demonstrated; in rat and in human tissues isoforms of at least two cytochrome *c* oxidase subunits (VIa and VIII in the rat, and VIa and VIIa in man) have been identified [2–4]. Surprisingly, in man, subunit VIII does not occur in isoforms. Thus, the composition of cytochrome *c* oxidase isoforms is not the same for every mammalian species. The differences in amino-acid sequences of subunit isoforms are usually reflected in differences in mobility on gels, in staining intensity, or in immunoreactivity. The isoforms of cytochrome *c* oxidase are classified as either heart-type or liver-type forms on the basis of the degree of homology between the amino-acid sequence of corresponding subunits in bovine liver and bovine heart. Since these tissues mainly consist of cells of mesodermal embryonic origin, the existence of different cytochrome *c* oxidase isoforms implies that the subunit composition of the enzyme changes during embryonic or perinatal development.

Proliferating cultured cells are generally considered to have a low degree of differentiation; in this respect they resemble embryonic cells and tissues. Due to the limited amount of material available, not much attention has so far been paid to the subunit composition of cytochrome *c* oxidase in cultured cells. In fact, most studies are limited to cytochrome *c* oxidase isolated from liver, kidney, heart or muscle. These tissues can be obtained in relatively large amounts and have a high content of mitochondria, which offers the opportunity to purify the enzyme to an extent and in amounts allowing direct identification of the subunits by analysis of their amino-acid sequence. When the amount of available material is limited, immunoprecipitation can be used to isolate the enzyme. Subsequent analysis of

Abbreviations: PBS, phosphate-buffered saline; TPCK, 1-(*p*-tosyl)-1-lysine chloromethyl ketone; DME medium, Dulbecco's modified Eagle medium.

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the mobility of the subunits on gels can then serve as a first indication of differences between isoforms of cytochrome *c* oxidase.

In the present study we have used this approach to compare the cytochrome *c* oxidase subunits from several tissues and cell lines of the mouse. The aim of this study was several-fold. Firstly, the occurrence and especially the type of cytochrome *c* oxidase isoforms in the mouse have not yet been investigated. Secondly, we wished to include cytochrome *c* oxidase from tissues, such as spleen and thymus, which have not yet been analysed in mammalian species. Thirdly, we were interested to see whether or not possible differences between cytochrome *c* oxidase subunits or the specific activity of the enzyme in various mouse tissues would be reflected in cultured cells with different degrees of mesodermal differentiation.

Materials and Methods

Cell lines and tissues. P19 EC, a mouse embryo carcinoma stem cell line, was used as a model for undifferentiated, omnipotent stem cells [5]. P19 MES represents a stable mesodermal cell line derived from P19 EC [6]; it served as a model for early mesodermal cells. Since fibroblasts and myoblasts are of mesodermal embryonic origin, an established fibroblast cell line, 3T3, and primary cultures of fibroblasts and myoblasts were studied also. P19 EC, P19 MES, and 3T3 cells were cultured on gelatin-coated flasks in DME medium supplemented with 10% heat-inactivated fetal calf serum. Primary mouse fibroblasts and myoblasts were cultured in DME medium supplemented with 20% heat-inactivated fetal calf serum, 2% chicken-embryo extract and 100 IU penicillin/ml, 100 μg streptomycin/ml. All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

The cultures were trypsinised and, after inactivation of trypsine by the addition of complete culture medium, the cells were collected by centrifugation. The cells were washed twice with PBS (140 mM NaCl, 27 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM EDTA). The cells were resuspended in PBS supplemented with 10% glycerol, counted in a counting chamber, and adjusted to a concentration of about $20 \cdot 10^6$/ml. The cell suspension was divided into the aliquots needed for various analytical purposes and stored at −80°C.

Mouse tissues (spleen, thymus, lung, kidney, liver, heart, and skeletal muscle (m. gastrocnemius)) were derived from young adult male Balb/c mice. The tissues were kept on ice and 10% (w/v) homogenates were prepared in PBS. The homogenates were divided into several portions and stored at −80°C.

Analytical assays. Phenylmethylsulfonyl fluoride and lauryl maltoside were added to the samples to final concentrations of 2.5 mM and 1.5%, respectively. The samples were left on ice for 30 min and centrifuged at 10000 × *g* in a microfuge. The supernatants were used for further analysis. The activity of cytochrome *c* oxidase was measured spectrophotometrically at 20°C in 30 mM phosphate buffer (pH 7.4) using 14 μM bovine heart cytochrome *c* as substrate [7]. The activity was expressed as the first-order reaction rate constant, *k*, per min per mg of protein. Citrate synthase activity was also measured spectrophotometrically at 20°C [8], the activity being expressed as the amount of product formed per min per mg of protein. The protein content of the samples was estimated using a modified Lowry method [9].

A polyclonal antiserum against cytochrome *c* oxidase from bovine heart was raised in rabbits. The specificity of this antiserum in immunoprecipitation and the isolation of the enzyme have been described before [4]. Cytochrome *c* oxidase was isolated from the supernatants by incubation overnight with the antiserum coupled to protein A-Sepharose 4B beads under rotation at 4°C. The beads were washed three times with 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5 M NaCl, and once with 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.05% lauryl maltoside. Finally they were incubated for 2 h at room temperature in sample buffer (4% sodium dodecylsulfate, 20% glycerol in 10 mM Tris-HCl (pH 6.8)). After centrifugation, the eluates were loaded onto polyacrylamide gels. Electrophoresis was performed as described by Schägger and Von Jagow [10], and the proteins were visualized by staining the gels with silver.

Results

Fig. 1 shows the mobility of the nuclearly coded subunits of cytochrome *c* oxidase in different mouse tissues. The presence of immunoglobulins in the upper region of the gels obscured the mitochondrially coded subunits I–III. The position of the largest of the nuclearly coded subunits, subunit IV, was identified by using the mobility of the subunits from purified bovine cytochrome *c* oxidase as a reference [4]. The other subunits are numbered according to their electrophoretic mobility, as proposed by Kadenbach and co-workers [11].

Subunit IV appeared frequently as a double band, the position of both bands being the same in all tissues investigated. In spleen, usually only the protein band with the highest mobility was visible. In rat, the two bands in the subunit IV region are explained by the presence of a proteolytic breakdown product of subunit IV [12]. As in rat tissues, the protein band with the highest mobility was absent or reduced in amount when TLCK, a specific proteinase inhibitor, was added to the homogenates. Subunits Va and Vb were difficult

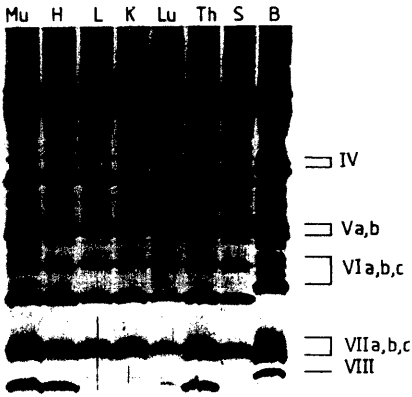

Fig. 1. Comparison of cytochrome *c* oxidase subunits in mouse tissues. Cytochrome *c* oxidase was isolated from various mouse tissues by immunoprecipitation and separated on SDS-polyacrylamide gels containing glycerol, according to Schägger and Von Jagow [10]. The gels were stained with silver, and the subunit nomenclature of Kadenbach [11] was used. The intensively stained bands in the upper part of the gel (above subunit IV) are derived from the immunoglobulins used for immunoprecipitation [4]. B: immunoprecipitated purified cytochrome *c* oxidase from bovine heart (reference); Mu: muscle; H: heart; L: liver; K: kidney; Lu: lung; Th: thymus; S: spleen.

to separate, but where both bands were visible their mobility was the same in all tissues. The mobilities of subunits VIb and VIc coincided frequently, as was the case for subunits VIIa and VIIb. The resolution of the subunits VII could be improved by using gels containing 6 M urea instead of 1.5 M glycerol (not shown). Differences in mobility for either subunits VIa-c or subunits VIIa-c were not observed between various tissues. However, as observed in other species [4], subunit VIa showed a tissue-specific staining intensity. Subunit VIII also clearly showed tissue-specific differences, not only in staining intensity, but also in mobility. The mobility in muscle, heart and thymus was higher than in the other tissues, whereas in ureum-containing gels the opposite was found (not shown). In lung tissue sometimes both bands in the subunit VIII region were found (Fig. 2). The poor staining of subunit VIII in liver, kidney, spleen, and occasionally in lung, made it necessary to load the gels with relatively high amounts of these samples. This explains the presence of several proteins that are not related to cytochrome *c* oxidase in the samples concerned on the gel shown in Fig. 2. The intensity of staining of subunit VIII proved to be inversely related to that of subunit VIa in muscle and heart (high intensity of VIII, low intensity of VIa) and in liver, kidney and spleen (low intensity of VIII, high intensity of VIa). In contrast, in

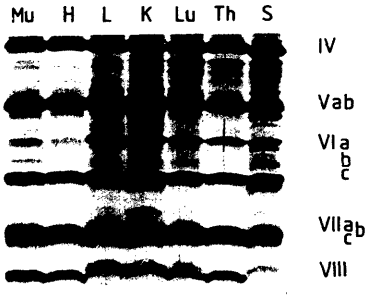

Fig. 2. Comparison of cytochrome *c* oxidase subunits in mouse tissues. Cytochrome *c* oxidase was isolated from various mouse tissues by immunoprecipitation and separated on SDS-polyacrylamide gels containing glycerol, according to Schägger and Von Jagow [10]. The gels were stained with silver and the subunit nomenclature of Kadenbach [11] was used. In this figure about 5-times more material from liver, kidney, spleen and lung was loaded onto the gels than in Fig. 1 to make subunit VIII from these tissues clearly visible. Mu: muscle; H: heart; L: liver; K: kidney; Lu: lung; Th: thymus; S: spleen.

thymus the two bands both showed a relatively high staining intensity. In lung, significant differences in the intensity of subunit VIa were not observed between samples in which mainly subunits VIII with heart-type or liver-type mobilities were present.

Fig. 3 shows the mobility of the subunits of cytochrome *c* oxidase from a number of cell lines of the mouse. There were no clear differences between any of the investigated cell lines with regard to the mobility of the subunits or their intensity of staining. Moreover, all cultured cell lines showed a subunit pattern which resembled that of liver.

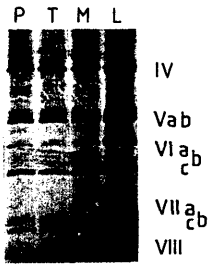

Fig. 3. Comparison of cytochrome *c* oxidase subunits in mouse cell lines. Cytochrome *c* oxidase was isolated by immunoprecipitation and separated on SDS-polyacrylamide gels containing glycerol according to Schägger and Von Jagow [10]. The gels were stained with silver and the subunit nomenclature of Kadenbach [11] was used. P: P19 EC; T: 3T3; M: primary myoblasts; L: liver.

TABLE I

Specific activities of cytochrome c oxidase and citrate synthase in tissues and cell lines of the mouse

The activity of cytochrome *c* oxidase (COX) and citrate synthase (CS) were measured according to [7] and [8]. The activities are expressed per mg protein, for COX as k/min and for CS as nmol/min. The last column shows the ratio between the COX activity and that of CS.

Tissue/ cell line	COX activity (k/min per mg)	CS activity (nmol/min per mg)	Ratio COX/CS
Heart	461	1680	0.27
Muscle	125	661	0.19
Liver	152	202	0.75
Kidney	277	356	0.78
Lung	45	178	0.25
Spleen	42	176	0.24
Thymus	53	532	0.10
P19 EC	25	174	0.14
P19 MES	27	204	0.13
3T3	29	225	0.13
Fibroblasts	23	158	0.15
Myoblasts	24	173	0.14

Table I shows the specific activity of cytochrome *c* oxidase in tissues and cell lines of the mouse as well as the specific activity of citrate synthase, a mitochondrial matrix enzyme. Large differences in specific activities of the two enzymes were found between the various tissues, not only regarding their concentration but also with respect to their ratio. The activities of the two enzymes in cultured cell lines revealed no such differences between cells with different degrees of mesodermal differentiation. On the contrary, the specific activity of the two mitochondrial enzymes was about the same in every tested cell line. Moreover, the specific cytochrome *c* oxidase activity was generally much lower in cultured cells than in mature tissues.

Discussion

The results show that isoforms of cytochrome *c* oxidase do also exist in mouse. As in rat [2,12], subunit VIa and VIII showed tissue-specific differences, which can be roughly classified as either heart-type (heart, skeletal muscle) or liver-type (other tissues). In thymus and sometimes also in lung, we found a heart-type subunit VIII. Kadenbach and co-workers recently demonstrated [13] that cytochrome *c* oxidase from brown fat tissue of the rat contains a heart-type subunit VIII, but a liver-type subunit VIa. The thymus might represent another tissue in which a 'mixed' type of cytochrome *c* oxidase is present, since the staining intensity of its subunit VIa resembled the liver-type, although heterogeneity in the thymus might explain this observation. A liver-type subunit VIII in thymocytes with a low staining intensity might be masked by a muscle type subunit present in, for example, supportive tissue of the thymus. Tissue heterogeneity does clearly explain the presence of a muscle-type subunit VIII in lung tissue.

No differences were observed for any of the subunits of cytochrome *c* oxidase from different cultured cells. Even primary myoblasts (of muscle origin) showed a subunit pattern which resembled that of liver. The specific cytochrome *c* oxidase and citrate synthase activities were also the same in all cell lines tested. The activities of these mitochondrial enzymes appeared to be directly related to the size and the protein content of the cells. The same relationship between cell size and mitochondrial content was recently described for the concentration of mitochondrial DNA in cultured mouse cell lines [14]. This is in sharp contrast to the situation in mature tissues, where not only differences in the specific activities of cytochrome *c* oxidase and citrate synthase were found, but also tissue-specific ratios. This might reflect the well-known differences in metabolic demands and pathways between various tissues. These differences are apparently absent between various cultured cell lines.

Taken together, it appears that proliferating cultured cells contain a basic, low mitochondrial content, and a basic cytochrome *c* oxidase isoform. Cytochrome *c* oxidase isoforms (or specific activities) associated with a differentiated function are thus absent in cultured cells, regardless of their degree of differentiation. It remains to be established whether the cytochrome *c* oxidase isoform found in mouse cell lines represents the liver-type, or a (fetal) isoform specific for cultured proliferating cells.

Our results do not exclude the possibility that different developmentally regulated isoforms of cytochrome *c* oxidase occur during embryonic development. The results do, however, imply that cultured cell lines derived from patients with a tissue-specific cytochrome *c* oxidase deficiency are not always suitable for studies on the nature of the defect. If the deficiency is based on the absence of a tissue-specific isoform, it is possible that this will be not be reflected in cultured cells.

Acknowledgements

Part of this study was made possible by a fellowship to Coby Van den Bogert of the Royal Netherlands Academy of Arts and Sciences. The work was also supported by the Netherlands Organization for Scientific Research (NWO) under the auspices of the Netherlands Foundation for Chemical Research (SON). Ruud Wolterman (Department of Neurology, University of Amsterdam) is thanked for his help in obtaining and culturing primary mouse fibroblasts and myoblasts.

References

1 Chomyn, A. and Attardi, G. (1987) in Current Topics in Bioenergetics, Vol. 15 (Lee, C.P., ed.), pp. 295–329, Academic Press, London.
2 Lomax, M.I. and Grossman, L.I. (1989) Trends Biochem. Sci. 14, 501–504.
3 Kennaway, N.G., Carrero-Valenzuela, R.D., Ewart, G., Balan, V.K., Lightowlers, R., Zhang, Y.-Z., Powell, B.R., Capaldi, R. and Buist, N.R.M. (1990) Pediatr. Res. 28, 529–535.
4 Van Kuilenburg, A.B.P., Dekker, H.L., Van den Bogert, C., Nieboer, P., Van Gelder, B.F. and Muijsers, A.O. (1991) Eur. J. Biochem. 199, 615–622.
5 Mc Burney, M.M. and Rogers, B.J. (1982) Dev. Biol. 89, 503–508.
6 Mummery, C.L., Feijen, A., Mooler.aar, W.H., Van den Brink, C.E. and De Laat, S.W. (1986) Exp. Cell Res. 165, 229–242.
7 Borst, P., Ruttenberg, G.J.C.M. and Kroon, A.M. (1967) Biochim. Biophys. Acta 149, 140–148.
8 Srere, P.A. (1969) Methods Enzymol. 13, 3–26.
9 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
10 Schägger, H. and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379.
11 Merle, P. and Kadenbach, B. (1980) Eur. J. Biochem. 105, 499–507.
12 Merle, P., Jarausch, J., Trapp, M., Scherka, R. and Kadenbach, B. (1981) Biochim. Biophys. Acta 669, 222–230.
13 Kadenbach, B., Stroh, A., Becker, A., Eckerskorn, C. and Lottspeich, F. (1990) Biochim. Biophys. Acta 1015, 368–372.
14 Shay, J.W., Pierce, D.J. and Werbin, H. (1990) J. Biol. Chem. 265, 14802–14807.