DIFFERENCES IN THE MITOCHONDRIALLY SYNTHESIZED SUBUNITS OF HUMAN AND MOUSE CYTOCHROME *c* OXIDASE

Alec J. JEFFREYS* and Ian W. CRAIG**

Genetics Laboratory, Department of Biochemistry, South Parks Road, Oxford OX1 3QU, England

Received 14 March 1977

1. Introduction

Ten or so proteins synthesized in mammalian mitochondria in vivo can be resolved by electrophoresis in polyacrylamide gels, after solubilization with sodium dodecyl sulphate [1]. Using interspecific differences in the electrophoretic pattern of these proteins, we analysed the nature of the mitochondrially made proteins which are synthesized in human–mouse somatic cell hybrids. The observed patterns of these proteins in such cells suggested that interspecific variations in mitochondrially synthesized proteins are determined extrachromosomally and therefore possibly by mitochondrial DNA [2].

We have attempted to discover the functions of some of these products of mammalian mitochondrial protein synthesis, and now report that one of them, which shows considerable interspecific variation, appears to be associated with the cytochrome *c* oxidase (EC 1.9.3.1) complex of mammalian mitochondria.

2. Materials and methods

Mitochondria were prepared from human hearts and from the pooled hearts, livers, kidneys and spleens of mice, essentially by the method of Crane, Glenn and Green [3]. Cytochrome *c* oxidase was partially purified from mitochondria by the selective solubilization of enzyme with KCl and deoxycholate followed by ammonium sulphate fractionation [4]; further purification was effected by ammonium sulphate fractionation in the presence of cholate [5]. Mouse cytochrome *c* oxidase was further purified by ion-exchange chromatography on DEAE-cellulose [6,7]; attempts further to purify the human enzyme by this method resulted in a considerable loss of activity in test samples and were avoided.

Human cell-lines used were D98/AH-2 [8] and HeLa B [9]; the mouse cell-line used was PG19, a 6-thioguanine resistant derivative of the C57, black 6J melanoma, provided by Jon Jonasson, Dept. of Pathology, Oxford. Cell culture methods, the preparation of cells labelled with [^{35}S]methionine in the presence of various protein synthesis inhibitors and the purification of labelled mitochondria from HeLa B cells are described elsewhere [1]. Cytochrome *c* oxidase was purified from labelled mitochondria, essentially by the method of Jacobs et al. [6,7].

Cytochrome *c* oxidase activity was assayed by the method of Smith [10]. Human enzyme samples were incubated at 37°C in 10 mM K-phosphate, pH 7.0, containing 15 μM horse heart cytochrome *c* (type III, Sigma, London) which had been freshly reduced with hydrogen over a palladium–asbestos catalyst and the decrease in absorbance at 550 nm was monitored spectrophotometrically. Mouse cytochrome *c* oxidase activity was similarly assayed in 60 mM K-phosphate, pH 7.0. Spectra of soluble cytochrome *c* oxidase preparations, diluted to 1 mg protein/ml with 50 mM Tris–HCl, pH 8.0, were recorded at room temperature on a Unicam SP-800 scanning spectrophotometer. Protein was assayed by the method of Lowry et al. [11].

* Present address: Department of Medical Enzymology and Molecular Biology, Laboratory for Biochemistry, Jan Swammerdam Instituut, University of Amsterdam, Amsterdam, Netherlands

** To whom communications should be addressed

Procedures for sodium dodecyl sulphate–polyacrylamide slab-gel electrophoresis and autoradiography are described elsewhere [1].

3. Results and discussion

Cytochrome *c* oxidase was purified from human heart mitochondria and from mouse mitochondria, essentially using techniques developed for the purification of the beef heart enzyme [4,5]. The final soluble enzyme preparations had cytochrome absorption spectra similar to those reported for cytochrome *c* oxidase from beef heart [12] and rat liver [6,7] (fig.1). Both preparations appeared to be free from contamination with cytochromes *b*, *c* or c_1. The specific haem a content of both preparations, determined from the reduced cytochrome absorption spectra (table 1), compared favourably with that of highly purified beef heart cytochrome *c* oxidase (11 nmol haem a/mg protein [13]). Both human and mouse cytochrome *c* oxidase preparations showed increased specific activity of ferrocytochrome *c* oxidation over starting mitochondria (table 1). The relatively low specific activity of the human enzyme was, in part, due to a substantial (70%) inactivation of the cytochrome *c* oxidase activity during ammonium sulphate fractionation in the presence of cholate; little inactivation of the mouse enzyme occurred during this purification stage.

The purified mouse cytochrome *c* oxidase contained four prominent polypeptides which could be resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (some aggregated material at the top of the resolving gel was also observed) (fig.2). We suggest that these four major polypeptides represent subunits of mouse cytochrome *c* oxidase and have designated them I–IV in order of decreasing apparent molecular weight. Human heart cytochrome *c* oxidase was judged to be less pure than the preparation of mouse enzyme, on the basis of a lower specific haem a content (table 1). Nevertheless, it also possessed four prominent fast-migrating components as shown by polyacrylamide gel electrophoresis, together with an array of (mainly fainter) higher molecular weight polypeptides. From a comparison of the human enzyme preparation with cytochrome *c* oxidase isolated from mouse mitochondria and from labelled human cell mitochondria (see below), we suggest that the four low molecular weight polypeptides (I–IV in order of decreasing molecular weight) are subunits of human cytochrome *c* oxidase. Of these, II–IV apparently corresponded, with minor variations, to mouse subunits II–IV. In contrast, human and mouse subunits I had different electrophoretic mobilities.

We have previously shown that the products of mitochondrial protein synthesis in cultured mammalian cells can be labelled selectively by incubating cells with [^{35}S]methionine in the presence of emetine or cycloheximide (inhibitors of cytoplasmic protein

Table 1
Properties of purified human and mouse cytochrome *c* oxidase

	Human	Mouse
Final yield, mg/g mitochondrial protein	6	1.0
Increase in spec. act. over starting mitochondria	3.6	31
Spec. act. μmol ferrocytochrome *c* oxidized/min (initial rate) /mg protein at 37°C	1.8	27
Haem a content (nmoles/mg protein)[a]	7	12

[a] Determined from $\Delta\epsilon$ (609–634 nm) reduced (see fig.1), using the $\Delta\epsilon$ (605–630 nm) of 16.5 $mM^{-1}.cm^{-1}$ for purified beef heart cytochrome *c* oxidase [12]

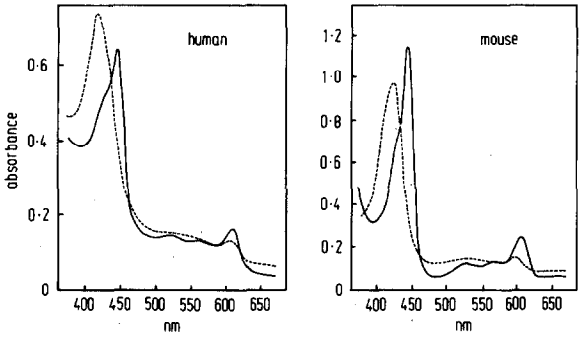

Fig.1. Absorption spectra of purified human and mouse cytochrome *c* oxidase. (- - - - -) Air-oxidized preparation; (——) Reduced preparation (15 min after addition of sodium dithionite). 1 mg protein/ml.

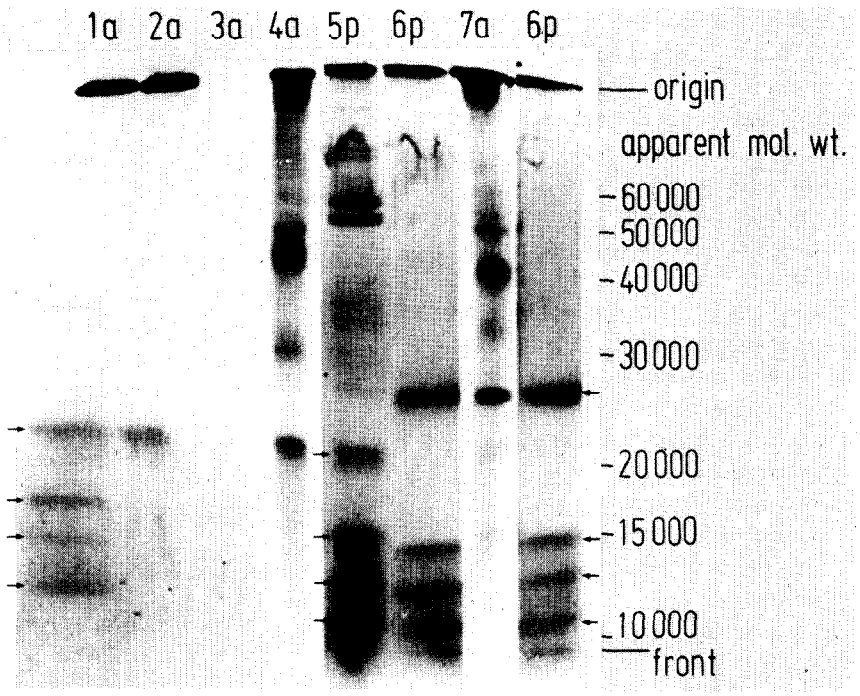

Fig.2. An electrophoretic comparison of cytochrome *c* oxidase subunits and products of mammalian mitochondrial protein synthesis. Samples were solubilized by heating to 100°C in the presence of sodium dodecyl sulphate plus 2-mercaptoethanol [1] and electrophoresed in a single 12% polyacrylamide slab-gel containing sodium dodecyl sulphate. The gel was stained with Coomassie Brilliant Blue and subsequently autoradiographed. The figure is a composite of a photograph of the gel stained for protein and the corresponding autoradiograph. The letters 'p' and 'a' following sample numbers refer respectively to photographs of the protein stain and to autoradiographs detecting radioactively labelled polypeptides. (1a) Cytochrome *c* oxidase (70 μg) purified from HeLa B cells labelled with [^{35}S]methionine in the absence of inhibitors; (2a) As 1a, cells labelled in the presence of 200 μg/ml cycloheximide; (3a) As 1a, cells labelled in the presence of 200 μg/ml cycloheximide plus 100 μg/ml chloramphenicol; (4a) Whole-cell extract of human cells (D98) labelled with [^{35}S]methionine for 3 h in the presence of 100 μg/ml emetine; 5p, 50 μg purified human heart cytochrome *c* oxidase; 6p, 50 μg purified mouse cytochrome *c* oxidase; (7a) Whole-cell extract of mouse cells (PG19) labelled with [^{35}S]methionine for 3 h in the presence of 100 μg/ml emetine. The determination of the apparent molecular weight calibration shown is described elsewhere [1]. The four putative subunits of cytochrome *c* oxidase are indicated by arrows, (→) for human cytochrome *c* oxidase subunits and (←) for those of the mouse enzyme.

synthesis) [1]. Typical electrophoretic profiles of the labelled products of human and mouse mitochondrial protein synthesis are shown in fig.2, in which mitochondrially made proteins (detected by autoradiography) are electrophoretically resolved alongside samples of purified human and mouse cytochrome *c* oxidase. Only one human mitochondrially synthesized protein was found to correspond in electrophoretic mobility to a putative enzyme subunit (subunit I). Similarly, only subunit I of mouse cytochrome *c* oxidase comigrated with a product of mouse mitochondrial protein synthesis. Similar observations were made in four independent electrophoretic comparisons of human and mouse cytochrome *c* oxidase and ^{35}S-labelled protein extracts.

Confirmation of the mitochondrial origin of subunit I of human cytochrome *c* oxidase was obtained by labelling a human cell-line (HeLa B) with [^{35}S]methionine, either in the absence of inhibitors, or in the presence of cycloheximide ± chloramphenicol (the latter is an inhibitor of mitochondrial protein synthesis). Labelled cells were subsequently allowed to recover for 3 h in medium lacking both inhibitors and [^{35}S]methionine, to permit labelled polypeptides to be assembled into their correct cellular sites [1]. Labelled mitochondria were isolated,

and cytochrome *c* oxidase prepared by the procedure of Jacobs et al. [6,7]. As only limited material was available, the final preparations were relatively impure (each contained 4 nmol haem a/mg protein) but were free from contamination with cytochrome *b*, *c* or c_1. Electrophoresis and autoradiography of cytochrome *c* oxidase prepared from cells labelled in the absence of inhibitors revealed many protein components, including the four prominent low molecular weight polypeptides (subunits I–IV) (fig.2). The relative faintness of subunit III on autoradiographs is probably due to its low methionine content (unpublished observations). In contrast, when cytochrome *c* oxidase prepared from cells whose cytoplasmic protein synthesis has been blocked with cycloheximide was examined, only one labelled polypeptide corresponding to cytochrome *c* oxidase subunit I was observed (additional material which did not penetrate the resolving gel was also detected). This suggests that subunits II–IV of cytochrome *c* oxidase are synthesized in the cytoplasm and that subunit I is made in mitochondria. The mitochondrial origin of subunit I was further supported by the inhibition of its labelling in the presence of chloramphenicol (fig.2).

Complexes with cytochrome *c* oxidase activity isolated from yeast and *Neurospora* have been shown to contain a mixture of cytoplasmically and mitochondrially synthesized components [14–16], although it is not certain that the mitochondrially made subunits are essential for enzyme activity [17,18]. Our work suggests that in mammalian mitochondria the cytochrome *c* oxidase complex is similarly constructed from a mixture of polypeptides synthesized in the cytoplasm and in mitochondria. Also, it extends previous observations of interspecific differences in the products of mitochondrial protein synthesis. The mitochondrially synthesized and presumably homologous subunits I of human and mouse cytochrome *c* oxidase show a considerable difference in electrophoretic mobility corresponding to a difference of 5500 in apparent molecular weight. The cause of this variation is not known, although species-specific differences in glycosylation or other postsynthetic modifications appear not to be responsible ([1] and unpublished work on cytochrome *c* oxidase subunits). Despite such variations, we conclude that at least one of the proteins made in mammalian mitochondria may play a physiological role similar to that of counterparts synthesized by Protist mitochondria.

Acknowledgements

A. J. J. was the recipient of a Christopher Welch Scholarship. The research was partially supported by the Medical Research Council, NATO and the Cancer Research Campaign.

References

[1] Jeffreys, A. J. and Craig, I. W. (1976) Eur. J. Biochem. 68, 301–311.
[2] Jeffreys, A. J. and Craig, I. W. (1976) Nature 259, 690–692.
[3] Crane, F. L., Glenn, J. L. and Green, D. E. (1956) Biochim. Biophys. Acta 22, 475–487.
[4] Fowler, L. R., Richardson, S. H. and Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170–173.
[5] Tzagoloff, A. and MacLennan, D. H. (1965) Biochim. Biophys. Acta 99, 476–485.
[6] Jacobs, E. E., Andrews, E. C., Cunningham, W. and Crane, F. L. (1966) Biochem. Biophys. Res. Commun. 25, 87–95.
[7] Jacobs, E. E., Kirkpatrick, F. H., Andrews, E. C., Cunningham, W. and Crane, F. L. (1966) Biochem. Biophys. Res. Commun. 25, 96–104.
[8] Szybalski, W., Szybalska, E. H. and Ragni, G. (1962) Nat. Cancer Inst. Monogr. 7, 75–89.
[9] Siegel, R. L., Jeffreys, A. J., Sly, W. and Craig, I. W. (1976) Exp. Cell Res. 102, 298–310.
[10] Smith, L. (1955) Meth. Biochem. Anal. 2, 427–434.
[11] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[12] Griffiths, D. E. and Wharton, D. C. (1961) J. Biol. Chem. 236, 1850–1856.
[13] Tzagoloff, A., Rubin, M. S. and Sierra, M. F. (1973) Biochim. Biophys. Acta 301, 71–104.
[14] Mason, T. L. and Schatz, G. (1973) J. Biol. Chem. 248, 1355–1360.
[15] Rubin, M. S. and Tzagoloff, A. (1973) J. Biol. Chem. 248, 4275–4279.
[16] Sebald, W., Machleidt, W. and Otto, J. (1973) Eur. J. Biochem. 38, 311–324.
[17] Phan, S. H. and Mahler, H. R. (1976) J. Biol. Chem. 251, 257–269.
[18] Phan, S. H. and Mahler, H. R. (1976) J. Biol. Chem. 251, 270–276.