Cytochrome *c* oxidase is preferentially synthesized in the rough endoplasmic reticulum–mitochondrion complex in rat liver

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(Received 28 June 1982/Accepted 3 September 1982)

The specific activity and content of cytochrome oxidase in the rough endoplasmic reticulum–mitochondrion complex are higher than in the mitochondrial fraction. Radiolabelling studies with the use of hepatocytes and isolated microsomal and rough endoplasmic reticulum–mitochondrion fractions, followed by immunoprecipitation with anti-(cytochrome oxidase) antibody, reveal that the nuclear-coded cytoplasmic subunits of cytochrome oxidase are preferentially synthesized in the latter fraction. The results have a bearing on the mechanism of transport of these subunits into mitochondria.

The RER of the hepatocyte is a heterogeneous component and sediments from cell homogenates at centrifugal forces ranging from 100 to 100 000 *g*. The RER subfraction in close association with mitochondria (RERM) sediments along with the nuclear fraction and is well characterized in electron micrographs (Drochmans *et al.*, 1975; Wanson *et al.*, 1975; Shore & Tata, 1977; Pickett *et al.*, 1980; Meier *et al.*, 1981). RER in RERM complex is seen as stacks of bilamellar sheets in contact with mitochondria (Shore & Tata, 1977) or as single cisternae in intimate association with mitochondria (Meier *et al.*, 1981), as is observed to be the case with liver sections, but quite unlike the vesicular light-microsomal fraction that sediments from the post-mitochondrial supernatant at 100 000 *g*. Meier *et al.* (1978, 1981) have suggested that the RERM fraction may facilitate the assembly of an endoplasmic-reticulum protein such as cytochrome *P*-450, where the haem synthesized in the mitochondrion would be readily available to the apo-(cytochrome *P*-450) synthesized on the associated endoplasmic reticulum. The other consideration is that the RERM complex may serve as a preferential site for the synthesis and transport of nuclear-coded mitochondrial proteins. These comprise proteins made entirely on cytoplasmic free or bound polyribosomes and complex proteins requiring the assembly of subunits made on mitochondrial and cytoplasmic polyribosomes (Shore & Tata, 1977). Cytochrome oxidase is a typical example of the

latter, and the present study is designed to examine this aspect.

Experimental

Rat liver was fractionated into RERM, mitochondria and light-microsomal fractions as described by Meier *et al.* (1981). Briefly, livers were perfused *in situ* with 0.9% NaCl, washed with 1.15% (w/v) KCl and then homogenized in 0.35 M-sucrose in 2.5 mM-magnesium acetate/10 mM-Tris/acetate buffer, pH 7.4. The crude nuclear fraction was further processed to isolate the RERM fraction. Protein synthesis with isolated light-microsomal and RERM fractions was performed as described by Weinstein (1968) by using [³⁵S]methionine as the labelled precursor and chloramphenicol (5 μ M) to inhibit protein synthesis on mitochondrial ribosomes. After incubation at 37°C for 15 min, the reaction was stopped by the addition of cycloheximide (100 μ g/ml), and the mixture was solubilized and processed for immunoprecipitation. The washed immunoprecipitate was dissociated, transferred to filter-paper discs, washed with 10% (w/v) trichloroacetic acid and diethyl ether and then used for radioactivity measurements. A portion of the incubation mixture was also directly processed on filter-paper discs to measure synthesis of trichloroacetic acid-precipitable total protein (Parimoo & Padmanaban, 1980).

Hepatocytes from 1.5 g of liver slices, prepared by the procedure of Fry *et al.* (1976), were suspended in 2 ml of balanced Hanks solution containing ¹⁴C-labelled *Chlorella* protein hydrolysate (25 μ Ci/ml). Labelling of the cytoplasmic subunits of cytochrome oxidase was performed in the presence

Abbreviations used: RER, rough endoplasmic reticulum; RERM, rough endoplasmic reticulum–mitochondrion complex.

of chloramphenicol (200 µg/ml). Labelling of the mitochondrial subunits was performed in digitonin-treated hepatocytes in the presence of cycloheximide (1 mM) as described by Kuzela *et al.* (1981). After labelling of the cells for 1 h at 37°C, the mitochondrial and RERM fractions were isolated, homogenized with 0.25 M-sucrose containing 0.5 mM-EDTA to strip the mitochondria of the associated RER (Meier *et al.*, 1981), and mitochondria were sedimented by centrifugation at 8000 g for 10 min. EDTA treatment was repeated once more, and then the mitochondria isolated from the RERM and mitochondrial fractions were processed for immunoprecipitation. All the immunoprecipitation data were corrected for non-specific precipitation, which accounted for about 10–15% of the radioactivity in the immunoprecipitate. Cytochrome $a+a_3$ content was measured spectrally (Williams, 1964). Cytochrome *c* oxidase activity was assayed by the procedure of Wharton & Tzagaloff (1967). Cytochrome *P*-450 was measured as described by Omura & Sato (1964). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

The results presented in Table 1 indicate that the specific activity and specific content of cytochrome *c* oxidase in the RERM fraction are substantially higher than in the mitochondrial fraction, indicating that the recovery of 65–70% of the total cytochrome *c* oxidase in the RERM fraction (Shore &

Tata, 1977; Meier *et al.*, 1981) is not merely due to the recovery of a higher proportion of total protein in this fraction.

The results on the labelling of cytochrome *c* oxidase in hepatocytes are presented in Table 2. The labelling of cytochrome *c* oxidase, the cytoplasmic subunits in particular, is nearly 2-fold higher in mitochondria from the RERM fraction than in those from the mitochondrial fraction, even though the labelling of total proteins is nearly 10–25% less in the former. The labelling of mitochondrial subunits of cytochrome *c* oxidase in the RERM fraction is only marginally higher than that of the mitochondrial fraction. This is perhaps due to the availability of a larger pool of the mitochondrial subunits and their slower turnover (Kolarov *et al.*, 1981).

The results presented in Table 3 indicate that the total protein-synthetic capacity of the isolated RERM fraction is nearly the same as that of the microsomal fraction, when the data are expressed in terms of cytochrome *P*-450, a typical microsomal marker in liver. This indicates that the RER associated with mitochondria in the RERM fraction is as active as the light-microsomal fraction in total protein synthesis. However, the RERM fraction shows 3–4-fold more synthesis of the cytoplasmic subunits of the oxidase than does the RER fraction.

Discussion

The association of a population of RER, manifesting preferential synthesis of cytochrome *c* oxi-

Table 1 Cytochrome $a+a_3$ content and cytochrome *c* oxidase activity of rat liver subfractions
The experimental details are given in the text. Abbreviation: N.D., not detectable.

Fraction	Protein content (mg/g wet wt. of liver)	Cytochrome $a+a_3$ content		Cytochrome <i>c</i> oxidase activity	
		(nmol/mg of protein)	(nmol/g wet wt. of liver)	(µmol of cytochrome <i>c</i> oxidized/min per mg of protein)	(µmol of cytochrome <i>c</i> oxidized/min per g of liver)
RERM	16.2	0.22	3.56	0.36	5.8
Mitochondria	10.5	0.12	1.25	0.25	2.6
Microsomes	14.0	N.D.	—	0.02	0.3

Table 2. Labelling of cytochrome *c* oxidase subunits in hepatocytes
For experimental details see the text.

Treatment	RERM		Mitochondrial fraction	
	Total (c.p.m./mg of protein)	Cytochrome <i>c</i> oxidase (% of total)	Total (c.p.m./mg of protein)	Cytochrome <i>c</i> oxidase (% of total)
No inhibitor	42 246	1.02	58 416	0.48
Chloramphenicol	30 394	1.04	32 640	0.52
Cycloheximide	17 704	1.21	19 708	0.98

Table 3. *Synthesis of cytoplasmic subunits of cytochrome c oxidase in homologous cell-free systems*

Protein synthesis with isolated light-microsomal and RERM fractions (5–8 mg of protein/ml) was performed in the presence of chloramphenicol. The cytochrome *P*-450 contents of the RER and RERM fractions were 0.78 and 0.14 nmol/mg of protein respectively. In some experiments purified mitochondria were added to the light-microsomal fraction to simulate the RERM fraction. However, addition of mitochondria did not significantly affect the incorporation of labelled amino acid into the light-microsomal fraction under the experimental conditions employed.

Fraction	Total protein synthesis (c.p.m./nmol of cytochrome <i>P</i> -450)	Cytochrome <i>c</i> oxidase synthesis	
		(c.p.m.)	(% of total)
RERM	1.68×10^6	5926	0.35
Light-microsomal	1.80×10^6	1820	0.10

dase subunits, with mitochondria, raises questions about the mechanism of transport of nuclear-coded mitochondrial proteins synthesized on cytoplasmic ribosomes. Although Ades & Butow (1980) and Kellems *et al.* (1975) have presented evidence for the synthesis, in yeast, of such proteins on a special population of cytoplasmic ribosomes attached to mitochondria, many such proteins have indeed been shown to be synthesized as precursors on free polyribosomes, released into cytosol and taken up by mitochondria with concomitant processing of the precursor to the mature form (Schatz, 1979; Raymond & Shore, 1979). In our laboratory it has been shown that individual precursors for the cytoplasmic subunits of rat liver cytochrome *c* oxidase can be detected in heterologous cell-free systems (Parimoo & Padmanaban, 1980). Schmelzer & Heinrich (1980) have shown the specific processing of the precursor to subunit IV by the post-translational addition of mitochondrial extracts to the heterologous cell-free system. However, it has not been possible to detect functional precursors in the liver cytosol of the intact animal (Parimoo & Padmanaban, 1980), tissue slices, hepatocytes or homologous cell-free systems, even at short periods of labelling (S. Parimoo & G. Padmanaban, unpublished work). Northemann *et al.* (1981) have shown that the cytochrome *c* oxidase subunit-IV mRNA is distributed between free and bound polyribosomes. In liver the bound polyribosomes constitute a major proportion of total polyribosomes, on taking into account the population sedimenting in the RERM fraction. It thus appears possible that in rat liver the nuclear-coded subunits of cytochrome *c* oxidase are primarily synthesized on RER in close association with mitochondria, in which case the release of subunits into the cytosol and their uptake by mitochondria may not be the major mechanism of transport of these subunits. It is possible that the synthesis of the nuclear-coded cytochrome *c* oxidase subunits is initiated in the light-microsomal fraction, and such populations may specifically associate with mitochondria, followed by vectorial transport into the organelles.

Thanks are due to the Council of Scientific and Industrial Research, New Delhi, for financial assistance.

References

- Ades, I. Z. & Butow, R. A. (1980) *J. Biol. Chem.* **255**, 9918–9924
- Drochmans, P., Wanson, J.-C. & Mosselmans, R. (1975) *J. Cell Biol.* **66**, 1–22
- Fry, J. R., Jones, C. A., Wiebkin, P., Belleman, P. & Bridges, J. W. (1976) *Anal. Biochem.* **71**, 341–350
- Kellems, R. E., Allison, V. F. & Butow, R. A. (1975) *J. Cell Biol.* **65**, 1–14
- Kolarov, J., Wielburski, A., Mendel-Hartvig, I. B. & Nelson, B. D. (1981) *Biochim. Biophys. Acta* **652**, 334–345
- Kuzela, S., Wielburski, A. & Nelson, B. D. (1981) *FEBS Lett.* **135**, 89–92
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Meier, P. J., Spycher, M. A. & Meyer, U. A. (1978) *Exp. Cell Res.* **111**, 479–483
- Meier, P. J., Spycher, M. A. & Meyer, U. A. (1981) *Biochim. Biophys. Acta* **646**, 283–297
- Northemann, W., Schmelzer, E. & Heinrich, P. C. (1981) *Eur. J. Biochem.* **119**, 203–208
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378
- Parimoo, S. & Padmanaban, G. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1673–1679
- Pickett, C. B., Montisano, D., Eisner, D. & Cascarano, J. (1980) *Exp. Cell Res.* **128**, 343–352
- Raymond, Y. & Shore, G. C. (1979) *J. Biol. Chem.* **254**, 9335–9338
- Schatz, G. (1979) *FEBS Lett.* **103**, 203–211
- Schmelzer, E. & Heinrich, P. C. (1980) *J. Biol. Chem.* **255**, 7503–7506
- Shore, G. C. & Tata, J. R. (1977) *J. Cell Biol.* **72**, 714–725
- Wanson, J.-C., Drochmans, P., May, C., Penasse, W. & Popowski, A. (1975) *J. Cell Biol.* **66**, 23–41
- Weinstein, I. B. (1968) *Methods Enzymol.* **128**, 782–787
- Wharton, D. C. & Tzagoloff, A. (1967) *Methods Enzymol.* **10**, 245–250
- Williams, T. N. C. (1964) *Arch. Biochem. Biophys.* **107**, 537–543