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ISOLATION AND CHARACTERIZATION OF MITOCHONDRIA FROM HUMAN B LYMPHOBLASTOID CELL LINES

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Mitochondria were isolated from detergent-treated Epstein-Barr virus-transformed human lymphocytes to examine their potential use in the study of the functional expression of genetic disorders of the respiratory chain. The increase of cytochrome *c* oxidase activity in the mitochondrial fraction indicated a 6-fold purification of intact mitochondria. Polarographic and spectrophotometric studies revealed that the isolated mitochondria were functionally well preserved. Furthermore, the isolated mitochondria supported an active *in organello* protein synthesis, which was dependent on the presence of a respiratory substrate generating ATP and was essentially abolished by chloramphenicol or by a specific respiratory chain inhibitor, such as antimycin. Thus, B lymphoblastoid cell lines constitute a valuable source of mitochondria to investigate mitochondrial functions in patients affected by respiratory chain disorders.

Until now, mitochondrial disorders have been essentially regarded as neuromuscular diseases (1). As a consequence, investigation of respiratory chain (RC) defects is generally carried out on muscle biopsies (1). Use of cultured fibroblasts has also been proposed (2), but the consistency of the expression of RC defects in these cultured cells has been repeatedly questioned (2,3). Recently, observations have accumulated suggesting that, particularly in childhood, mitochondrial disorders are not restricted to neuromuscular diseases, but rather may affect any organ or tissue (4). A specific expression of a RC disorder in blood cells has thus been reported in young patients presenting with Pearson syndrome (5). Detailed molecular studies have shown that this disorder, which is often fatal, originated from rearrangements of the mitochondrial genome (mtDNA), affecting variable

Abbreviations: BLCL, B lymphoblastoid cell lines; BSA, bovine serum albumin; CAP, chloramphenicol; EBV, Epstein-Barr virus; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; MOPS, morpholinopropanesulphonic acid; PMSF, phenylmethane-sulphonyl fluoride; Prot, protein; SDS, sodium dodecyl sulfate; Tris, 2-amino-2(hydroxymethyl)1, 3, propanediol.

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proportions of the population of mtDNA (5). Rearranged mtDNA, accumulated in lymphocytes, bone marrow and gut, and was still present in cultured B lymphoblastoid cell lines (BLCL) (5). In contrast, the functional expression of this genetic disorder (enzyme function, translation ability of the mitochondria) has been poorly characterized. Such a study, when using BLCL as model, would require the isolation of the mitochondria. Unfortunately, although several methods have been proposed to isolate mitochondria from cells in culture (6,7,8,9), persistent difficulties are encountered to obtain satisfactory yields of functionally intact mitochondria from human BLCL. This has been mainly ascribed to the difficulty to break cells open without affecting the integrity of the mitochondrial membranes (9).

In this report we present the characterization of the functional properties of intact mitochondria isolated from cultured human BLCL, using a slightly modified method based on the use of digitonin to specifically destabilize the plasma membrane (10),

MATERIAL AND METHODS

Cell line. Epstein-Barr virus-transformed lymphocytes from normal individuals were grown in RPMI 1640 medium (GIBCO) supplemented with 10% foetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin at 37°C under standard conditions (11).
Isolation of mitochondria. Lymphoblastoid cells (10^8 cells, about 6.5 mg of protein) washed three times with medium A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS (pH 7.4), 1 g/l BSA), were resuspended in 1 ml of medium B (medium A *plus* 10 mM triethanolamine, 5% Percoll, 0.1 mg/ml digitonin). After 3 min incubation at 4°C, cells were disrupted with 7 strokes of a motor driven (500 rpm) tightly fitting glass-Teflon pestle. The homogenate was centrifuged twice at 2,500 *g* for 5 min and the resulting supernatant further centrifuged at 10,000 *g* for 5 min. The pellet of crude mitochondria (about 750 μg of protein) was resuspended in medium C (300 mM sucrose, 1 mM EGTA, 20 mM MOPS (pH 7.4), 1 g/l BSA, 1 mM PMSF). All steps were carried out at 4°C under sterile conditions.
Polarographic and spectrophotometric studies. Oxygen uptake was measured in medium D (300 mM mannitol, 10 mM KH_2PO_4 (pH 7.2), 10 mM KCl, 5 mM $MgCl_2$ and 1 g/l BSA) with a Clark oxygen electrode in a 300 μl-cell, magnetically stirred and thermostated at 37°C. Cytochrome *c* oxidase (EC 1.9.3.1.), succinate cytochrome *c* reductase, NADH and NADPH cytochrome *c* reductase, and lactate dehydrogenase (EC 1.1.1.27) activities were spectrophotometrically measured using standard procedures (12,13,14,). NADH-ubiquinone reductase (EC 1.6.99.5) was measured using decylubiquinone (2,3-dimethoxy-5-methyl-6-decyl-1, 4 benzoquinone) (15). Catalase activity (EC 1.11.106) was polarographically determined (14). Room temperature difference spectra (reduced *minus* oxidized) were recorded with a computerized UV-3000 Shimadzu spectrophotometer using one quartz cell (200 μl). The absolute spectrum of the oxidized sample was automatically substracted from the reduced spectra. Spectra performed at liquid nitrogen temperature (-196°C) were recorded in 80 μl cells. Baseline (oxidized *minus* oxidized) was substracted from difference spectra (reduced *minus* oxidized). Quantitative measurements of cytochromes were performed according to Chance (16). All studies were carried out in medium D.
Mitochondrial protein synthesis. Mitochondrial protein synthesis in intact cells (50 x 10^6 cells) was performed in 5 ml methionine-free RPMI 1640 medium, supplemented with 2%

foetal calf serum, 100 μg/ml cycloheximide and 300 μCi [^{35}S] methionine. After 2 h of labeling and a 30 min chase with unlabeled medium, mitochondria were isolated as described above. Alternatively, protein synthesis by isolated mitochondria (200 μg protein) was carried out in 100 μl of a medium modified from McKee *et al.* (17) containing 44 mM mannitol, 14 mM sucrose, 25 mM MOPS (pH 7.4), 90 mM KCl, 1 mM EDTA, 0.4 mM EGTA, 6 mM $MgSO_4$, 2.5 mM KH_2PO_4, 1mM PMSF, 1 g/l BSA, 100 μg/ml cycloheximide, 10 mM succinate, 20 mM glutamate, 4 mM ATP, 0.5 mM GTP, 0.2 mM amino acid mixture (*minus* methionine), 60 μCi [^{35}S] methionine. After 1 h incubation at 30°C in a magnetically stirred 1.5 ml micro test tube, mitochondria were pelleted and treated for SDS-urea-polyacrylamide gel electrophoresis. The [^{35}S] methionine incorporation was determined as described by Mans and Novelli (18). Background incorporation was determined by measuring the incorporation immediately after the addition of radioactivity. Bacterial contamination was determined by plating 50 μl of incubation medium containing mitochondria (2 mg/ml) on blood agar plates. After 48 h incubation at 37°C, the plates were examined for the presence of bacterial colonies.

Protein electrophoresis. Gels were a modification of the urea gels described by Ching *et al.* (19). The separating gel consisted of 15% polyacrylamide [1:50 bis(acrylamide)], 8 M urea in 375 mM Tris-HCl (pH 8.8), and 0.1% SDS. A stacking gel (1.5 cm) consisted of 5% acrylamide [1:37.5 bis(acrylamide)], 8 M urea in 125 mM Tris-HCl (pH 6.8), and 0.1% SDS. The samples were prepared in 62.5 mM Tris-HCl (pH 6.8), 4% SDS, 10% β-mercaptoethanol, 8M urea, 0.05% bromophenol blue. Gels were electrophoresed for about 5-6 hours for 20 cm gels (15 mA / gel), treated for fluorography with Entensify (New England Nuclear) and exposed to a Kodac X-OMAT AR film at -80°C for 5 days.

Protein determination and chemicals. Protein was determined by the method of Bradford (20) using BSA as a standard. The amino acid mixture *minus* methionine was from Promega. *In vivo* labeling grade [^{35}S] methionine was from Amersham. All other chemicals were analytical reagent grade from Sigma Chemical Company.

RESULTS AND DISCUSSION

We first determined the optimal conditions for the disruption of the plasma membrane, using the latency of lactate dehydrogenase activity as an index of cell breakage. As freezing of cells also tends to damage energy linked functions of mitochondria (9), the use of membrane destabilizing agents associated with mechanical homogeneization (10) was the method of choice to disrupt the cells. Homogeneization in a relatively low osmoticum (0.1 M saccharose) with 10 mM triethanolamine-acetate buffer and 0.1 mg/ml digitonin released all the activity of the cytosolic lactate dehydrogenase. No additional activity was revealed upon treatment of the cell homogenate with a high concentration of a detergent (Tab.1; 2.5 mM lauryl maltoside). We simultaneously checked the integrity of the mitochondrial outer membrane by measuring its permeability to exogenous cytochrome *c*. In the absence of detergent, only a low cytochrome *c* oxydase activity could be detected (less than 3% of the total activity in presence of 2.5 mM lauryl maltoside), indicative of the intactness of the mitochondrial outer membranes.

Mitochondria were next isolated by differential centrifugations as described under Material and Methods. The distribution of different cellular markers among the various

Table 1. **Marker distribution in the fractions separated by differential centrifugation during the isolation of BLCL mitochondria.** Experimental conditions as described under Material and Methods. Enzyme assays were performed in the presence of detergent (2,5 mM lauryl maltoside). Data are expressed *per* fraction.

Fraction	Protein (mg)	LDH	Cyt *c* oxidase	Catalase	NADPH cyt *c* reductase
		(nmol acceptor or donor min^{-1})			
Homogenate	4.5	10,867 (10,867)[a]	430 (9)[a]	108,000	103
Supernatant S1 (3000 g)	2.75	9,500	330	56,000	68
Pellet P1	1.75	1,207	95	52,000	30
Supernatant S2 (10,000 g)	2.3	9,299	110	50,000	41
Mitochondrial pellet P2	0.4	200	217 (8)[a]	4,000	24

[a] Activity measured in the absence of lauryl maltoside.

fractions was studied (Tab. 1). Only 2% of the lactate dehydrogenase activity, a cytosolic marker (14), and 5% of the catalase activity, a marker for peroxisomes (21), were found in the mitochondria-enriched fraction P_2. In contrast, about 25% of the total NADPH-cytochrome *c* reductase activity, a marker for microsomes (13), was found in this fraction. The yield of intact mitochondria (more than 95% intact) was about 45 ± 8 % based on the initial cytochrome *c* oxidase content of the cells.

The spectral composition of mitochondria was then studied using low temperature (-196°C) differential spectrophotometry (Fig.1A). Reduction of the respiratory chain was first triggered by succinate in the presence of ATP and cyanide (Fig.1A, spectrum *a*). Under this condition, succinate brought about a full reduction of the respiratory chain. Quantification of the cytochromes at room temperature (Fig.1A, inset) indicated ratios roughly similar to those previously reported for mitochondria from other human tissues (22). When the preparation was maintained chemically reduced by dithionite (Fig.1A, spectrum *b*), no changes in the reduction level of cytochrome oxidase and cytochrome *c* were observed. In contrast a significantly higher reduction of the *b*-type cytochromes was measured (+ 50 %), consistent with the presence of cytochrome-*b* containing microsomes previously indicated by the measurement of the NADPH-cytochrome *c* reductase (Tab.1).

Polarographic studies showed that succinate was actively oxidized by these mitochondria (Fig.1B, trace *a* and *b*). In the absence of EDTA, ATP was exogenously hydrolysed to ADP (23), and respiratory controls (ranging from 6 to 9) could only be calculated by using rates measured in the presence of an inhibitor (oligomycin) of the ATPase *versus* the rates measured in the presence of an uncoupler (carbonyl cyanide *m*-chlorophenylhydrazone). After inhibition of succinate oxidation by malonate, a potent

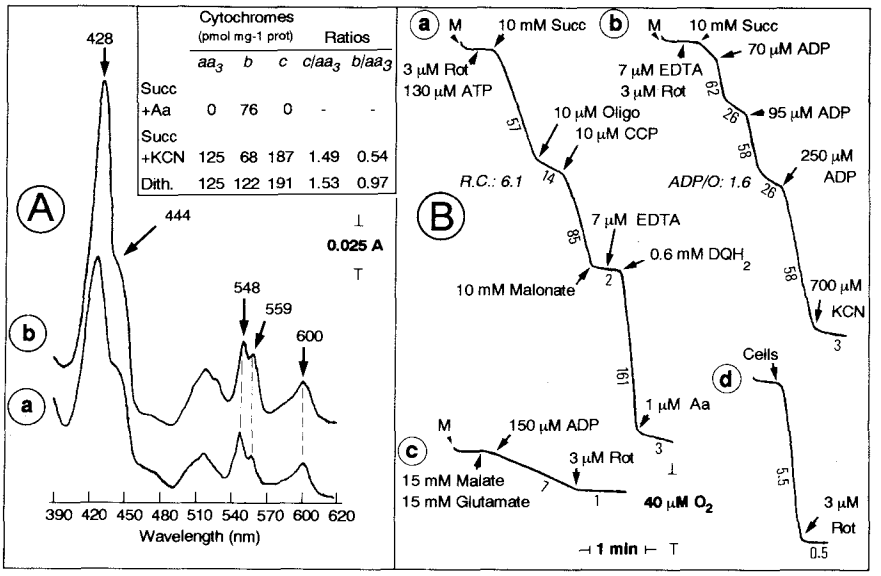

Figure 1. **Biochemical characterization of BLCL mitochondria.** ***A***: Low temperature (-196°C) difference spectra. Experimental conditions as described under Material and Methods. Spectrum *a*, mitochondria (3.5 mg prot) were supplemented with 10 mM succinate, 130 µM ATP, 700 µM cyanide; spectrum *b*, mitochondria (5 mg prot) were reduced by dithionite. *Inset*: cytochrome composition determined at room temperature. Values were means of three different experiments and were calculated according to Chance and Williams (16). ***B***: Oxidation of respiratory substrates by mitochondria (100 µg prot) and cell (2.7 mg) respiration. Numbers along the traces are nmol O_2 $min^{-1}mg^{-1}$ protein. Experimental conditions as described under Material and Methods. Aa: antimycin a; M: mitochondria; Oligo: oligomycin; Rot: rotenone; Succ: succinate.

competitive inhibitor of the succinate dehydrogenase (24), adding duroquinol in the presence of EDTA led to a rapid oxygen uptake (roughly twice the rate of succinate oxidation) which was fully inhibited by antimycin. In the presence of EDTA (Fig.1B, trace *b*), succinate oxidation was normally coupled with the phosphorylation process (ADP/O ratio value of 1.6 ± 0.2). As previously reported for mitochondria isolated from human lymphocytes (7), malate (*plus* glutamate) was not actively oxidized (Fig.1B, trace *c*). An identical result was obtained with pyruvate or α-ketoglutarate as substrate. However, cell respiration (Fig.1B, trace *d*) was very sensitive to rotenone, a specific inhibitor of Complex I. We attempted to measure Complex I activity using decylubiquinone, an analog of coenzyme Q_2 as electron acceptor (15), but no significant NADH oxidation (less than 5 nmol min^{-1} mg^{-1} prot) could be measured whatever the method used to permeabilize the mitochondria (osmotic shock, sonication, freeze-thawing). We therefore measured the NADH-cytochrome *c* reductase activity (12). Despite the high rate of rotenone-resistant activity (875 ± 150 nmol min^{-1} mg^{-1} prot), rotenone-sensitive NADH cytochrome *c* reductase activity (175 ± 37 nmol min^{-1} mg^{-1} prot) was roughly equal to the rate of the succinate cytochrome *c* reductase (185 ± 30 nmol min^{-1} mg^{-1} prot), an activity ratio previously observed in other human mitochondria (13).

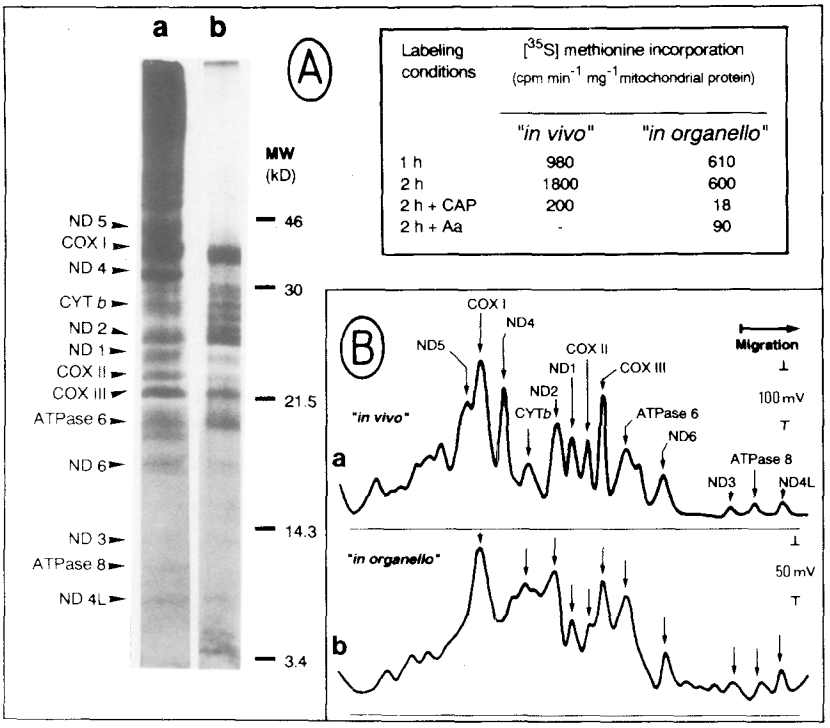

Figure 2. **Mitochondrial protein synthesis by BLCL mitochondria**. ***A***: Resolution of BLCL mitochondrial translation products by electrophoresis through SDS-urea-polyacrylamide gels. Pattern of products labeled with [^{35}S] methionine in the presence of cycloheximide in intact cells (*a*) or isolated mitochondria (*b*). Other experimental conditions as described under Material and Methods. Polypeptides were tentatively assigned according to Chomyn *et al.* (25). Mitochondrial protein was 50 μg for (*a*) and 100 μg for (*b*). *Inset*: Effect of incubation time, 200 μg/ml chloramphenicol (CAP) or 1 μM antimycin (Aa) on [^{35}S] methionine incorporation by intact cells *("in vivo")* and by isolated mitochondria *("in organello")*. ***B***, Densitometric profiles of mitochondrial translation products after 2 h labeling in intact cells (*a*) and isolated mitochondria (*b*). Experimental conditions as in **A**. Mitochondrial protein content was 50 μg.

Mitochondrial protein synthesis was next studied "*in vivo*" using intact cells (Fig. 2A, lane *a*), or "*in organello*" using isolated mitochondria (Fig. 2A, lane *b*). Tests for bacterial contamination of the incubation medium containing mitochondria did not reveal any bacterial colonies after 48 h. In the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis, both "*in vivo*" and "*in organello*" [^{35}S] methionine incorporations were found sensitive to chloramphenicol (89-97%), an inhibitor of mitochondrial protein synthesis (Fig. 2A, inset). Accordingly, no polypeptides were detectable after a 10 d exposure of the film (not shown). Antimycin, a respiratory chain inhibitor, inhibited 85% of the [^{35}S] methionine incorporation by isolated mitochondria (Fig. 2A, inset). Densitometric analysis of electrophoresis autoradiograms indicated that similar patterns of labeled polypeptides were obtained "*in vivo*" or "*in organello*" (Fig. 2B). In absence of specific

antibodies against mitochondrial polypeptides, the proteins were tentitatively assigned according to Chomyn *et al.* (25). Bands above 46 kD, and at least two polypeptides, possibly ND5 and ND4, were however not present or detectable when synthesis was performed "*in organello*".

CONCLUSION

This above procedure led to the quantitative release of intact and physiologically active mitochondria from human BLCL. The 6-fold increase of cytochrome *c* oxidase specific activity in the mitochondria-enriched fraction did not significantly differ from what has been reported for the isolation of rat liver mitochondria (26). This high yield of intact mitochondria allowed us to perform an exhaustive biochemical study of BLCL mitochondria, including polarographic tests, spectrophotometric investigations and "*in organello*" protein synthesis (about 600 to 700 x 10^6 cells required). Investigating properties of these isolated mitochondria did not reveal any unique characteristics. However, as previously described for human lymphocytes (7), isolated mitochondria from BLCL did not actively oxidize NAD^+-dependent substrates. Our results show for the first time that mitochondrial protein synthesis can be successfully studied using mitochondria isolated from human BLCL. The high rates of [^{35}S]methionine incorporation allowed for a relatively rapid analysis (5 d) of the translation products. This latter experiment could be of major interest for elucidating control mechanisms of mitochondrial genome expression and of nucleo-mitochondrial interactions in patient BLCL. Thus, BLCL may constitute a valuable source of mitochondria for the investigation of mitochondrial functions in patients with potential RC defects.

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