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OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA ISOLATED FROM HUMAN FIBROBLASTS

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SUMMARY

Mitochondria were isolated from several strains of human fibroblasts in order to evaluate their potential for use in screening for inherited defects in mitochondrial function. The mitochondria were purified 10-fold from whole fibroblasts as determined by the increase in the specific activity of cytochrome oxidase. Polarographic studies of respiration at 26 °C, in mitochondria isolated by enzymatic digestion, revealed that these mitochondria contained an intact electron-transport system and three sites for the oxidative generation of ATP. Further, they showed tight coupling of respiration with phosphorylation. It appears that fibroblasts will be suitable for use in studying individuals suspected of having inherited abnormalities of mitochondrial function.

INTRODUCTION

Fibroblasts may be readily cultured from most individuals and comprise the typical cellular outgrowth from explants of skin[1]. In culture, these cells maintain both the chromosome complement and the metabolic uniqueness of their donor[2–4]. The use of such cultures to investigate the molecular basis of inherited diseases is well-known[5].

There has been a report of hypermetabolism related to a defect in mitochondrial respiratory control[6]. The screening of individuals for such defects in respiratory control and capacity for oxidative phosphorylation requires the isolation of mitochondria from affected cells. Fibroblasts, rather than biopsy specimens or peripheral blood cells, are useful because they may be grown under regulated conditions and provide a continuous supply of uniform cells.

We now report the isolation of mitochondria, with intact systems of electron transport and oxidative phosphorylation, from human fibroblasts.

ISOLATION OF MITOCHONDRIA

Human fibroblasts were cultured in medium 199, supplemented with 20% fetal bovine serum, in an atmosphere of air $-CO_2$ (97:3, v/v)[1], in cylindrical roller vessels revolving at 0.1 revolution per min. Each vessel contained media sufficient

to cover 28% of the glass surface. The vessels were seeded with 10^7 cells and cultured for 1 week. By the 5th day of culture, the pH had fallen below 6.8 and was raised to 7.0 by the addition of $NaHCO_3$.

Mitochondria were prepared by scraping monolayer cultures from two roller vessels (40 mg of cellular protein) into 60 ml of an "isolation buffer" consisting of 0.27 M mannitol, 0.1 mM EDTA, 0.05% bovine serum albumin, and 10 mM Tris–HCl (pH 7.3). After centrifugation at 500 × *g* for 2 min, the resultant pellet was suspended in 20 ml of isolation buffer containing 45 μl of a 100 μg/ml solution of Protease VI (Sigma Chemical Co.); a modification of the method of Chance and Hagihara[7]. This mixture was incubated for 7 min at 0 °C then gently and briefly homogenized in a tight-fitting Teflon homogenizer. The homogenate was then mixed with an equal volume of the same buffer and centrifuged at $770 \times g$ for 7 min. The supernatant fraction was decanted before centrifugation at $11\,100 \times g$ for 7 min. The pellet obtained from the high-speed centrifugation was used without further washing and represents the mitochondria used in these studies.

To establish that our method of isolation resulted in the selective elimination of nonmitochondrial protein, we compared the specific activity of cytochrome oxidase in mitochondria with that of whole cells. Several methods of cell disruption were examined and the results shown in Table I. Shaking with glass beads resulted in a homogenous mixture of broken cells from which mitochondria could not be extracted. Cells homogenized to disruption without preliminary Protease treatment, yielded purified mitochondrial preparations as judged by the marker enzyme, cytochrome oxidase[8], but further examination revealed the mitochondria to be uncoupled. Mitochondria prepared by enzymatic digestion were coupled (see below) and showed a cytochrome oxidase activity of about 200 nmoles of cytochrome *c* oxidized per mg of protein per min, as compared to 20 for whole cells, a 10-fold purification.

TABLE I

CYTOCHROME OXIDASE SPECIFIC ACITIVTY IN MITOCHONDRIA AND WHOLE CELLS

The values reported in this table were obtained from a single fibroblast line. Analysis of six other fibroblast lines with respect to this same mitochondrial parameter reveals a specific activity range of 185–241 when homogenized to disruption, and 175–245 after Protease extraction. Whole-cell analysis of three other fibroblast lines shows a range in specific activities from 19.1 to 24.2.

Preparative method	*Specific activity**	
	Mitochondria	*Whole cells***
Homogenization***	225.5	23.1
Protease VI	240.3	–

* nmoles cytochrome *c* oxidized per mg protein per min.

** Whole cells were solubilized in deoxycholate[9].

*** Refers to disruption by a mill homogenizer (A. H. Thomas Co.).

ANALYSIS OF OXIDATIVE PHOSPHORYLATION

To study oxidative phosphorylation, mitochondria were suspended at a concentration of 1.2–1.8 mg protein per ml in air-saturated "respiratory buffer" consisting of 0.25 M mannitol, 0.2 mM EDTA, 1 mM $MgCl_2$, 10 mM KCl, and 10 mM potassium phosphate (pH 7.2). A Gilson Oxygraph fitted with a Clark electrode was used to measure the respiratory rate[10].

Typical patterns of oxygen consumption are shown in Fig. 1. Respiration in the presence of excess substrate and P_i was clearly regulated by the availability of ADP. ADP/O values, calculated as described by Estabrook[10] were close to the values expected for mitochondrial systems with three sites for oxidative phosphorylation.

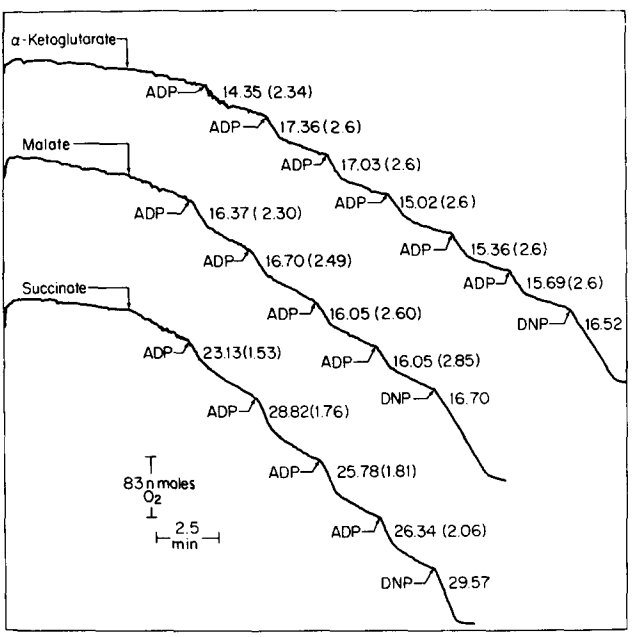

Fig. 1. Mitochondrial respiration in the presence of α-ketoglutarate, succinate or malate. The first number on each slope represents the State 3 respiratory rate, followed by the ADP/O ratio in parentheses. Mitochondria were isolated as described in the text. The mitochondrial pellet was suspended in aerated respiratory buffer at 26 °C, to a concentration of 1.5 mg of mitochondrial protein per ml of buffer. The reaction vessel contained 1.65 ml of that suspension. Reactants were added, as shown by the arrows, by means of a micropipette. All substrates were present at 3.3 mM. The first State 3 for α-ketoglutarate and succinate was achieved by the addition of 106.9 nmoles of ADP. At all other times, 160.35 nmoles of ADP were added. 2,4-Dinitrophenol (DNP; in ethanol) was added to $3 \cdot 10^{-5}$ M. A value of 260 nmoles/ml was used for the oxygen concentration at 26 °C (ref. 10). Protein was determined by the method of Lowry *et al.*[11].

Table II summarizes the results obtained from ten independently isolated fibroblast lines. Oxidation of malate produced an average ADP/O ratio of 2.57 which is within the range expected for the oxidation of NAD-linked substrates. The ratio was not enhanced by the addition of pyruvate to the assay mixture containing malate.

TABLE II

OXIDATIVE PHOSPHORYLATION IN FIBROBLAST MITOCHONDRIA

The values represent the mean ± standard error of the mean and were obtained from ten fibroblast lines by the methods described in Fig. 1. The number of observations is shown in parentheses. Total number of cell lines used with each substrate is: 4 with malate, 5 with succinate, and 7 with α-ketoglutarate.

Substrate	*ADP/O*	*Respiratory control index**	Q_{O_2}**		*DNP/S****
			State 3	*State 4*	
Malate	2.57 ± 0.09 (5)	3.40 ± 0.13	14.83 ± 0.64	4.73 ± 0.19	4.25 ± 0.56
Succinate	1.73 ± 0.05 (14)	3.83 ± 0.19	22.35 ± 0.86	5.96 ± 0.35	6.67 ± 1.60
α-Ketoglutarate	2.32 ± 0.06 (21)	4.01 ± 0.15	13.17 ± 0.52	3.45 ± 0.11	4.86 ± 0.56

* Respiratory control index, State 3, and State 4 as defined by Chance and Williams[12].
** nmoles O_2 consumed per mg protein per min.
*** Ratio of dinitrophenol-stimulated respiratory rate to substrate-stimulated rate (State 4).

Succinate oxidation was examined to determine if this pathway was intact in the isolated mitochondria. The average ADP/O ratio of 1.73 indicated that two phosphorylation sites were available during transport of succinate-donated electrons. The difference between the ADP/O ratios obtained with malate and succinate was approximately one and represented the NAD-linked phosphorylation site.

Finally, we examined the oxidation of α-ketoglutarate. The evaluation of oxidative phosphorylation with this substrate is complicated by a step of phosphorylation not linked to electron transport. We found an ADP/O ratio of 2.32 with α-ketoglutarate. Therefore, it was likely that substrate-level phosphorylation was not operative in these mitochondria.

Tight coupling between oxidation and phosphorylation is believed to be a characteristic of "normal" mitochondria. This behavior was evaluated in several ways. It is obvious from Fig. 1 that the availability of a phosphate acceptor (ADP) had an immediate effect on the rate of oxygen utilization. Respiratory control indices range from 3.4 for malate to 4.0 for α-ketoglutarate, values characteristic of tightly coupled systems. Further, the addition of the uncoupler dinitrophenol stimulated the respiratory rate to a value 4 to 6 times greater than the State 4 rate.

During the course of each assay, mitochondria were run through a minimum of four cycles of transition between State 4 and State 3 respiration (Fig. 1). In general, both the ADP/O ratio and the respiratory control increased with cycling. A similar improvement in respiratory control has been found in HeLa cell mitochondria and is thought to be an effect of ATP generated in the previous cycles[13].

The data presented here demonstrate that mitochondria isolated from cultured human fibroblasts contain three sites of oxidative phosphorylation. This result, as well as the consistency of the data obtained from cell strains derived from normal individuals, suggest that cultured fibroblasts will be suitable to screen for defects in oxidative phosphorylation in various inherited disease states.

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