Journal of Steroid Biochemistry, Vol. 11, pp. 1135 to 1140

0022-4731/79/0801-1135$02.00/0

OXIDATIVE PHOSPHORYLATION REACTIONS AND CHOLESTEROL HYDROXYLATION MECHANISMS IN HUMAN TERM PLACENTAL MITOCHONDRIA*

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(*Received* 9 *November* 1978)

SUMMARY

A simple and rapid method is described for the preparation of mitochondria of human term placenta. Phosphorylative oxidation properties were determined by polarography. Respiratory control values were higher than 3, showing that mitochondria are tightly coupled, but ADP/O ratios were lower than theoretical values due to ATPase activity. The activity of several specific mitochondrial marker enzymes was measured. In the outer membrane, rotenone-insensitive NADH-cytochrome c reductase and monoamine oxidase exhibited activitites of 331 nmol of cytochrome c reduced/min/mg protein and 26.7 nmol of tyramine consumed/min/mg protein respectively. No kynurenine hydroxylase activity was detected. In the inner membrane, activities of succinate cytochrome c reductase and cytochrome oxidase were 15.3 nmol of cytochrome c reduced/min/mg protein and 21.6 nmol O_2 utilized/min/mg protein respectively. Glutamate, malate and succinate oxidases had low activities of about 1 to 3 nmol O_2 used/min/mg protein. In the matrix, glutamate dehydrogenase activity was very slight.

Mitochondrial proteins were analyzed by polyacrylamide gel electrophoresis in the presence of phenol, urea and acetic acid. Mitochondria contained at least 20 components with mol. wt. ranging from 15,000 to 150,000. A component which probably contained cytochrome P450 subunits was characterized with a mol. wt. of 52,000. The concentration of the phosphorylating respiratory chain cytochromes was determined; the latter were in a similar range to that reported for adrenal cortex mitochondria, but their concentration was lower. The content of cytochrome P450 was lower than in other endocrine tissues. Oxygen consumption rate was stimulated by addition of (20α)-hydroxycholesterol and (22R)-hydroxycholesterol but not by (22S)-hydroxycholesterol. The placental cholesterol hydroxylating mechanism appeared to follow a pathway similar to that of adrenal cortex mitochondria.

INTRODUCTION

Reactions involved in oxidative phosphorylation have been shown to occur in mitochondria prepared from human term placenta [1]; the mitochondria oxidize various substrates such as glutamate, malate and succinate, but not β-hydroxybutyrate [2]. The electron transport is coupled with ATP formation but P/O ratios are lower than expected because of the high ATPase activity found even in intact mitochondria [3]. It has been postulated that the complete respiratory chain is present, although there appears to be no information available about the concentrations of cytochromes *b*, c_1, *c* and $a + a_3$ in placental mitochondria. Since electron microscopy has shown that isolated placental mitochondria have a double membrane of the type seen in liver mitochondria [4], the possibility that specific enzyme markers may be found in placental mitochondria should be considered, since these contain high levels of monoamine oxidase [5] and glycerophosphate dehydrogenase [6]. However the activity of other mitochondrial enzymes has not been completely determined. The structure of mitochondrial membranes is generally analyzed after protein separation by polyacrylamide gel electrophoresis but, until now, such analysis has not been performed with human term placental mitochondria.

Mitochondria from human placenta contain not only the normal electron transport system but also a second electron transport system terminating with cytochrome P450 and functional in steroid biosynthesis [7, 8] Placental progesterone biosynthesis from cholesterol takes place in the mitochondria in several steps. By analogy with the mechanisms described for adrenal cortex mitochondria [9, 10], cholesterol in placental mitochondria is assumed to undergo its first hydroxylation in position 20 or 22, followed by a second hydroxylation to form (20α,22R)-dihydroxycholesterol. However, this has never actually been confirmed. The aim of this work was to develop a simple technique for the preparation of active human term placental mitochondria. Isolated organelles were used in experiments designed to obtain new information about their structure and functions. Respiratory

* Part of a dissertation for a Doctor of Science.

The following trivial names have been used: (20α)-hydroxycholesterol: 5-cholesten-3β,20-diol; (22R)-hydroxy cholesterol: (22R)-5-cholesten-3β,22-diol; (22S)-hydroxy cholesterol: (22S)-5-cholesten-3β,22-diol; (20α,22R)-dihydroxycholesterol: (22R)-5-cholesten-3β,20α,22-triol.

cytochrome content and mitochondrial marker enzyme activities were estimated. Mitochondrial proteins were analyzed by polyacrylamide gel electrophoresis.

The rate of oxygen binding to hydroxylated cholesterol derivatives in the presence of placental mitochondria was measured by polarography.

MATERIALS AND METHODS

Isolation of mitochondria and oxidative phosphorylation

Isolation of mitochondria. Mitochondria were prepared according to Olivera and Meigs[1], but tissue preparation was modified. The placentae, used within 0.5 h of the natural birth and kept at 0°C, were washed in Solution I (0.25 M sucrose, 0.15 M KCl, 1 mM EDTA adjusted to pH 7.4 by 1 M KOH) and cut with scissors. The preparation was homogenized with a Teflon Potter type homogeniser in Solution II (0.25 M sucrose, 1 mM EDTA adjusted to pH 7.4 by 1 M KOH) and centrifuged twice at 800 *g* and then at 5000 *g* for 10 min. The mitochondrial sediment was washed twice, and isolated at 5000 *g* with a minimum volume of Solution II. The mitochondrial suspension corresponding to the heavy particles contained 30–40 mg/ml of proteins measured by the method of Gornall *et al*[11].

Respiration and oxidative phosphorylation. Mitochondrial respiration and oxidative phosphorylation were studied polarographically by means of a GME oxygraph equipped with a vibrating platinum electrode. Assays were carried out at 25°C in a final volume of 1.6 ml of a mixture with the following composition: 15 mM tris–HCl (pH 7.4), 10 mM potassium phosphate (pH 7.4), 1.5 mM EDTA, 0.13 M sucrose, 2 mg/ml bovine serum albumin and 30 mM $MgCl_2$. Glutamate, malate and succinate (10 mM) were used as substrates with 8 mM ADP.

Mitochondrial enzymatic activities

The activities of several marker enzymes from the outer membrane, the inner membrane and the matrix, were determined under the conditions described in previous work [12].

Electrophoresis

Electrophoretic analyses were carried out after protein dispersion, using a mixture of 40% phenol, 24% urea, 20% acetic acid and 5% mercaptoethanol [13]. Gels (1 × 17 cm) had the following composition: 6% acrylamide, 0.16% methylene bisacrylamide, 34% urea, 28% acetic acid, 0.4% ammonium peroxodisulphate and 0.5% tetramethylenediamine. Electrophoresis was carried out at 4°C for 22 h at a voltage of 320 V. Proteins were then stained with a solution of 12.5% trichloacetic acid containing 0.05% Coomassie blue and destained with 10% trichloracetic acid. Densitometric tracings were performed at 580 nm in a Gilford gel spectrophotometer. Molecular weights were determined by using commercial marker proteins [14]: β-galactosidase (130,000), phosphorylase a (94,000), bovine serum albumin (68,000), creatine kinase (40,000), trypsin (23,300) and cytochrome *c* (11,700).

Phosphorylating respiratory chain cytochromes

Differential spectra of reduced and oxidized cytochromes were spectrophotometrically recorded between 535 and 630 nm. This permitted simultaneous assay of the four cytochromes *b*, c_1, *c* and *a*; at both specific wavelengths, correction allowed elimination for each cytochrome of interference due to the three other cytochromes [15].

Mixed-function oxidase

Cytochrome P450. The concentration of cytochrome P450 was determined by differential spectra according to Omura and Sato's method [16]. Mitochondrial proteins (about 2 mg) were suspended in 1 ml 0.1 M phosphate buffer, pH 7.0. A few sodium dithionite crystals were added to reduce possible traces of blood pigment. The content of one cuvette was then gassed with CO to form a P450–CO complex. The cytochrome P450 concentration was calculated according to the extinction coefficient difference between 450 and 490 nm, which is 91 cm/mM.

Effect of cholesterol derivatives on the oxygen uptake rate. Oxygen uptake was measured in a thermostated (25°C) vessel (vol 1.6 ml) equiped with a vibrating electrode. The medium used contained 200 mM sucrose, 20 mM KCl, 10 mM potassium phosphate, 20 mM HEPES (pH 7.3), 1 mM EGTA, 1% bovine serum albumin (w/v) [9]. Other additions are mentioned in the legend to Fig. 2.

RESULTS

Respiration and oxidative phosphorylation

Experiments were performed with human placental mitochondria prepared as described under methods. When 8 mM ADP was added in the presence of glutamate, malate or succinate (10 mM), respiration was stimulated with a transition from State 4 to 3. Results obtained are given in Table 1; the respiratory rates in the resting state (E_4) and in active respiration (E_3) were higher when succinate or malate were used as substrates than with glutamate. Respiratory control ratios were respectively 3.67, 1.45 and 3.02 with glutamate, malate and succinate as substrates. The ADP-O ratios were 1.38, 0.87 and 0.60 with glutamate, malate and succinate respectively in the absence of NaF. These values were about 30% higher when 9 mM NaF was added. Oxygen uptake rate was unchanged after addition of β-hydroxybutyrate as substrate, in accordance with previous results [2].

Enzymatic activities

Mitochondrial enzyme activities are reported in Table 2. In the outer membrane, rotenone-insensitive

Table 1. Oxidative phosphorylation properties of human placental mitochondria

Mitochondrial activities	Substrates		
	Glutamate	Malate	Succinate
Respiration E_4	1.37 ± 0.39 (6)	2.44 ± 0.46 (5)	3.26 ± 0.40 (6)
Respiration E_3	5.08 ± 1.73 (6)	4.05 ± 1.47 (5)	10.4 ± 3.75 (6)
RC	3.67 ± 0.37 (6)	1.45 ± 0.43 (5)	3.02 ± 0.89 (6)
P/O	1.38 ± 0.59 (6)	0.87 ± 0.24 (5)	0.60 ± 0.16 (6)

Respiratory rates in resting state of respiration (E_4) and active respiration (E_3) are expressed in nmol O_2/min/mg mitochondrial proteins.

Values are expressed as Mean ± SEM, the number of experiments is given in parenthesis. RC = E_3/E_4. P/O = ratio of the mole of ADP added to atoms of extra oxygen consumed.

Table 2. Enzymatic activities of human placental mitochondria

Outer membrane	
Monoamine oxydase (b)	26.7 ± 5.1
Cynurenine hydroxylase (b)	≃0
Rotenone-insensitive NADH-cytochrome *c* reductase (a)	331 ± 27
Inner membrane	
Succinate cytochrome *c* reductase (a)	15.3 ± 3.7
Cytochrome oxydase (c)	21.6 ± 2.8
Glutamate oxydase (c)	1.15 ± 0.43
Succinate oxydase (c)	2.93 ± 0.79
Malate oxydase (c)	1.85 ± 0.42
Matrix	
Glutamate dehydrogenase (b)	2.01 ± 0.53

Activities are expressed in nmol of cytochrome *c* reduced (a), of substrate utilized (b) or oxygen consumed (c)/min/mg of mitochondrial proteins.

Values ± SEM are the mean of five experiments.

NADH-cytochrome c reductase exhibited an activity of 331 nmol reduced cytochrome *c*/min/mg protein, and monoamine oxidase an unusual activity of 26.7 nmol consumed tyramine/min/mg. protein. No kynurenine hydroxylase activity was detected. The succinate cytochrome *c* reductase and many oxidases in the inner membrane were measured. The values were 15.3 nmol reduced cytochrome *c*/min/mg protein and 21.6 nmol O_2 utilized/min/mg protein for succinate cytochrome *c* reductase and cytochrome oxidase respectively. The other oxidases such as glutamate, malate and succinate oxidase had low levels ranging from 1 to 3 nmol O_2 used/min/mg protein. The activity of glutamate dehydrogenase, a matrix enzyme, was very low, about 2 nmol of oxidized substrate/min/mg protein. This finding agrees with previous results showing a notable difference in concen-

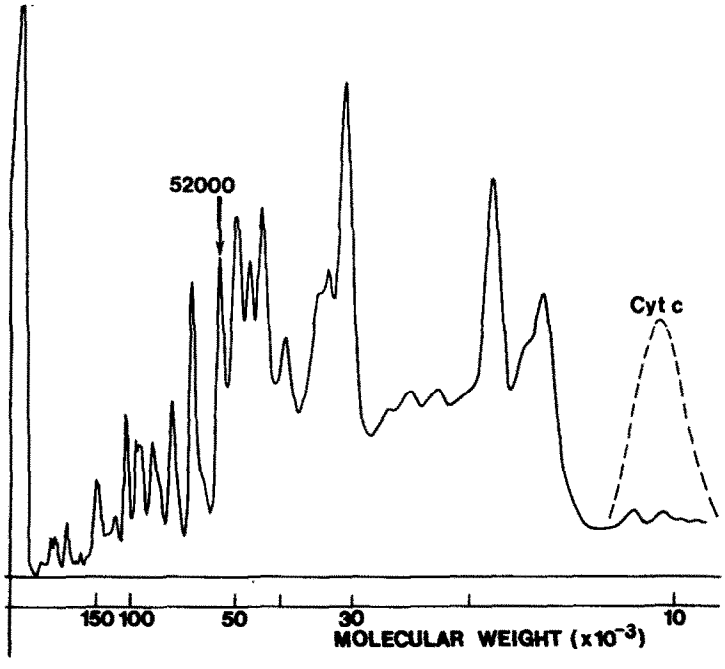

Fig. 1. Densitometric tracing of polyacrylamide gel electrophoresis of human term placental mitochondria. Cytochrome *c* (broken lines) was used as marker.

Table 3. Electron carriers in human placental mitochondria

Component	Peak band (nm)	Reference (nm)	Concentration of carriers	Concentration relative to cytochrome *a*
Cytochrome *b*	563	577	0.089 ± 0.018	0.63
Cytochrome c_1	554	540	0.071 ± 0.022	0.51
Cytochrome *c*	550	535	0.054 ± 0.021	0.38
Cytochrome *a*	605	630	0.140 ± 0.016	1.00
Cytochrome P450	450	480	0.110 ± 0.040	0.79

Concentrations of various cytochromes are expressed in nmol per mg of mitochondrial protein.
The values are the mean of six experiments ± SEM.

tration when this enzyme was isolated from placenta and from rat liver [17].

Electrophoresis of mitochondrial constituents

The method of electrophoresis used in these experiments resolved the mitochondrial proteins into more than 20 major constituents whose mol. wt. ranged from 15,000 to 150,000, as shown in the densitometric pattern (Fig. 1). Cytochrome *c*, used as an internal standard, made it possible to establish a very specific relative migration for each protein fraction. Under the present electrophoretic conditions, migration mainly depends on the size of particles, as proved with differential commercial marker proteins [14]. By comparison, it was possible to estimate the mol. wt. of the protein components, and most mitochondrial proteins ranged between 10,000 to 80,000. It is of interest to note the presence of large protein fractions ranging from 16,000 to 22,000, from 34,000 to 38,000 and from 43,000 to 62,000.

Electron carriers in human placenta mitochondria

Respiratory cytochromes and cytochrome P450 were estimated.

Respiratory cytochromes—The concentrations of the placental electron carriers are given in Table 3. Columns 2 and 3 show the peak absorption band and reference wavelength used to calculate the absorption of the different components. Column 4 shows the concentrations of respiratory cytochromes (*b*, c_1 *c* and *a*) in nmol/mg mitochondrial protein, and column 5, the relative concentrations of the various components in relation to cytochrome *a*.

Cytochrome P450—This cytochrome was found to be present in mitochondria at a concentration of 0.11 nmol/mg protein as previously reported, and in a molar ratio to cytochrome *c* of 0.79.

Oxygen uptake with hydroxycholesterol derivates

The effects were examined of cholesterol and of hydroxycholesterol derivatives like (20α)-hydroxycholesterol, (22R)-hydroxycholesterol and (22S)-hydroxycholesterol on the oxygen consumption rate. Placental mitochondria demonstrated a significant oxygen uptake with 10 mM malate, even in the presence of 1.5 μM rotenone, 5 μM antimycin A or 1 mM cyanide as inhibitors (Fig. 2). Sterol-stimulated oxygen consumption in the presence of malate is shown in this

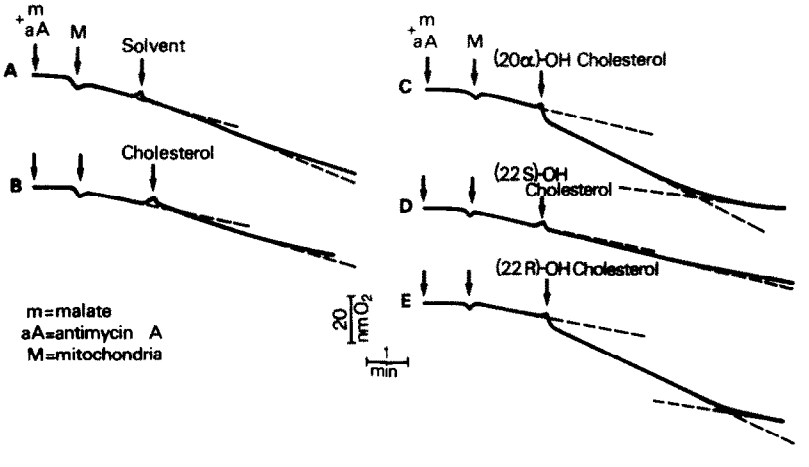

Fig. 2. Effect of adding sterols on rate of oxygen uptake by human placental mitochondria. Mitochondria (3 mg protein) were added to 1.5 ml medium, as indicated under methods. Final concentrations of products added: 10 mM succinate, 5 μM antimycin A, 10 mM malate, 20 μM (20α)-hydroxycholesterol, 20 μM (22R)-hydroxycholesterol and 20 μM (20α, 22R)-dihydroxycholesterol. Sterols were added in ethanolic solutions. The oxygen consumption rate is indicated in nmol O_2/min/mg protein.

Table 4. Effect of hydroxycholesterol on oxygen consumption

Sterol	OH Sterol (μM)	O_2 uptake rate	P.100
(22R)-hydroxycholesterol	20	0.57	100
(20α)-hydroxycholesterol	20	0.33	57.9
(22S)-hydroxycholesterol	20	0.12	21.0
Cholesterol	20	0.03	<6.0

P.100: Percentage of variation in oxygen consumption compared to (22R)-hydroxycholesterol, used as control.

Oxygen consumption rate is expressed in nmol/min/mg protein, after subtracting the presterol rate (supported by 10 mM succinate, 10 mM malate and 5 μm antimycin A).

figure, which also indicates that (20α)-hydroxycholesterol stimulated oxygen consumption less than (22R)-hydroxycholesterol. It is of interest that the addition of (22S)-hydroxycholesterol or cholesterol had a slightly stimulating effect on oxygen uptake. Sterol stimulation of oxygen consumption was always quick and oxygen consumption rates were 0.52, 0.33, 0.12 and 0.03 nmol O_2/min/mg protein for (22R)-hydroxycholesterol, (20α)-hydroxycholesterol, (22S)-hydroxycholesterol and cholesterol respectively (Table 4). The bursts of accelerated respiration were transient with these sterol concentrations and were reinitiated by adding more sterol.

DISCUSSION AND CONCLUSION

Heavy mitochondria from human term placenta were prepared according to Olivera and Meigs[1] but the first stages of the tissue preparation preceding homogenization were simplified. The effect of this was to improve respiratory control ratio of the organelles and above all their hydroxylating properties.

The enzymatic activities of these human placental mitochondria proved very similar to those of mitochondria isolated from other tissues like adrenal cortex [18]. The presence of high monoamine oxidase activity in the outer membrane of placental mitochondria has been confirmed [5], but the functional significance of this oxidase is not yet clear. The inner membrane of human placental mitochondria was found to contain the same enzymes as those characterized in the mitochondria of other types of tissue, but the activity of these enzymes was less intense. In line with previous finding [17], the activity of glutamate dehydrogenase from the matrix was also observed to be slight. Electrophoresis of human placental mitochondrial proteins revealed the presence of about 20 components with mol. wt. of 15,000 to 150,000. Similar results had previously been reported for adrenal cortex mitochondria analyzed under the same conditions [19]. In the present work, similarities with rat liver mitochondria were also noted, especially as regards those protein constituents with mol wt. between 43,000 and 52,000 and the fraction with a mol. wt. of 34,000 [14]. The well-characterized component with a mol. wt. of 52,000 was assumed to contain cytochrome P450 subunits, as previously observed after electrophoresis of bovine adrenal cortex mitochondria [19]. The structural and functional heterogeneity of placental mitochondria therefore seemed similar to that of other organelles.

Concentrations of respiratory chain cytochromes per mg protein were lower than those reported in mitochondria from other types of tissue, especially the adrenal cortex [20]. Mitochondrial fractions from human placenta contained a higher concentration of cytochrome P450/mg protein than microsomes (about 0.03 nmol/mg protein). The presence of cytochrome P450 could not therefore be imputed to microsomal contamination. However, the cytochrome P450 content was lower than in mitochondria isolated from other sources. In human placental mitochondria, the essential part played by cytochrome P450 is generally recognized to reside in cholesterol side chain cleavage, whereas in adrenal cortex mitochondria, this cytochrome affects both the cleavage mechanism and the 11β- and 18-hydroxylation processes.

Placental mitochondria were found capable of oxygen consumption, even in the presence of respiratory inhibitors, since with malate or succinate, oxygen consumption was reduced but not eliminated after addition of antimycin A and rotenone. This might be due to malic enzyme activity in the mitochondria leading to generation of NADPH, an essential cofactor in hydroxylating reactions [21]. No change was observed in the oxygen consumption rate in the presence of cholesterol, probably because cholesterol penetration of the mitochondria was only slight [22]. The oxygen consumption rates recorded for placental mitochondria after adding either (20α)-hydroxycholesterol or (22R)-hydroxycholesterol were similar to the rates found earlier for adrenal cortex mitochondria [23], i.e. they showed faster oxygen consumption after hydroxylate derivatives were added, with a higher speed in the presence of (22R)-hydroxycholesterol than (20α)-hydroxycholesterol, whereas the addition of (22S)-hydroxycholesterol hardly affected oxygen consumption at all. The data on sterol-stimulated oxygen consumption indirectly support the view that (20α)-hydroxycholesterol and (22R)-hydroxycholesterol can each serve as a substrate for oxidation of cytochrome P450-dependent placental mitochon-

dria. It may also be assumed that the cholesterol molecule is first hydroxylated in position 22 and then in position 20 before cleavage, thus forming pregnenolone. This seems to agree with the results of Burstein[24] which strongly suggest that cholesterol is attacked by oxygen in position C 22 and then in position C 20, forming the following sequence:

cholesterol → (22R)-22-hydroxycholesterol
→ (20R,22R)-20-22-dihydroxycholesterol
→ pregnenolone.

The pathway by which placental mitochondria appear to affect the cholesterol hydroxylating mechanism therefore seems to be very similar to that previously demonstrated for adrenal cortex mitochondria.

Acknowledgement—We are grateful to Dr Barrat, Gynaecology and Obstetrics Department, Hopital St. Antoine, Paris for providing us human placenta and to Miss Monique Basseville (CNRS) for her expert assistance.

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