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ON THE MITOCHONDRIAL 17β-HYDROXYSTEROID DEHYDROGENASE FROM HUMAN ENDOMETRIUM AND ENDOMETRIAL CARCINOMA: CHARACTERIZATION AND INTRAMITOCHONDRIAL DISTRIBUTION

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SUMMARY

To determine intramitochondrial location of an endometrial 17β-hydroxysteroid dehydrogenase (17β-HSD), purified mitochondria from secretory endometrium were separated into inner and outer membranes, matrix and an intermembrane fraction.

The purity of each fraction was monitored by marker enzymes. A radiochemical assay was used for the determination of 17β-HSD activities. It was found that the 17β-HSD was mainly located in the outer membranes of the mitochondria. Kinetic parameters and substrate specificity of the enzyme were determined.

The conversion of estradiol (E_2) to estrone (E_1) by purified mitochondria was linear with time and protein concentration. The optimum temperature was approximately 40°C and the optimum pH 9·5. For the reduction of E_1 the optimum pH was 6·0. With NAD E_2 was oxidized approximately 15 times more rapidly than with NADP. The apparent K_m-values for E_2 (in the presence of NAD) were 13×10^{-6} M and 4×10^{-6} M in proliferative and secretory endometrium, respectively. The maximal velocity was highest in secretory endometrium. Testosterone and androstenedione could also serve as substrates. In normal endometrium they were interconverted at approximately 50% of the rate of E_2 and E_1. In endometrial carcinoma androstenedione was reduced twice as rapidly as estrone.

Sulfhydryl groups were shown to be essential for catalysis. The mitochondrial 17β-HSD was similar in character to the cytoplasmic, microsomal and nuclear enzymes described previously.

INTRODUCTION

Human endometrial tissue contains a 17β-hydroxysteroid dehydrogenase (17β-HSD) which catalyzes the oxidation of the 17β-hydroxy group of both C_{18} and C_{19} steroids [1–7]. This enzyme has been located by Pollow *et al.* [8–10] in the cytosol as well as in different particulate fractions (mitochondria, microsomes and nuclear fraction). Subfractionation of endometrial microsomes demonstrated the presence of the 17β-HSD in the smooth surfaced microsomes (ribosome-free membranes). Since the 17β-HSD was associated with the microsomal fraction, its presence in mitochondria could have been due to microsomal contamination. To exclude this possibility only highly purified mitochondrial preparations were used in the present experiments, which attempt to establish the intramitochondrial location of this enzyme. Furthermore the question arose whether the enzyme activity which is present in all subcellular fractions was in each case the same. Therefore the aim of the present investigation is also to report on the kinetic properties of the mitochondrial 17β-HSD.

MATERIALS AND METHODS

Non standard trivial names and abbreviations:

Estradiol-17β = E_2, estrone = E_1, 17β-hydroxysteroid dehydrogenase = 17β-HSD.

TEM–glycerol-buffer. Tris–HCl (10 mM), EDTA (1 mM), mercaptoethanol (12 mM), pH 7·0, 20% glycerol (v/v).

Steroids. [4-^{14}C]-androstenedione (60 mCi/mmol), [4-^{14}C]-testosterone (58 mCi/mmol), [4-^{14}C]-E_2 (53 mCi/mmol) and [4-^{14}C]-E_1 (58 mCi/mmol) were purchased from the Radiochemical Centre, (Amersham, England).

The radiochemical purities were verified by thin-layer chromatography on silica gel using benzene–methanol (19:1, v/v) and cyclohexane–ethylacetate (1:1, v/v). All reference steroids were obtained from commercial sources.

Chemicals. Ficoll: Pharmacia Fine Chemicals, Uppsala, Sweden. Lubrol WX: I.C.I. Organics Inc., Frankfurt.

NAD^+, $NADP^+$, NADH, α-oxoglutarate, succinate: Biochemica Boehringer, Mannheim.

All other chemicals and organic solvents were of reagent grade and came from E. Merck, Darmstadt, Germany.

Tissue preparation. The tissue was obtained either by curettage or from uteri removed at total hysterectomy.

Pieces of the tissue were washed in freshly prepared 0·9% NaCl solution and then 5 g portions of the tissue were homogenized in 5 ml of 0·25 M sucrose in TEM-glycerol-buffer in a glass teflon homogenizer (Braun Melsungen, Potter S) by 5 up and down movements of the Teflon pistle. The homogenate was then passed through 4 layers of cotton gauze and centrifuged for 15 min at 850 *g* to remove the nuclear fraction. The supernatant was centrifuged at 5500 *g* for 15 min. After removing the floating fat the supernatant was carefully decanted. The pellet was suspended in TEM–glycerol-buffer containing 0·25 M sucrose and re-centrifuged at 5500 *g* for 10 min. This procedure was repeated 1 to 3 times. The sediment obtained after the final centrifugation was designated as "washed mitochondria". The 5500 *g* supernatant was centrifuged at 12,000 *g*; the obtained pellet was not used in this study. Microsomes were sedimented from the 12,000 *g* supernatant at 105,000 *g* for 60 min (rotor 30, Beckman) and washed twice in TEM–glycerol-buffer.

Mitochondrial fractions were separated into mitochondria, peroxysomes and lysosome-rich fraction by Ficoll-gradient centrifugation (0–15%, w/v) as described by Brown [11]. These fractions were concentrated by sucrose gradient centrifugation (20–60%, w/v) (30,000 rev./min for 4 h in a rotor SW 65, Spinco, Beckman Instruments).

Outer membranes and mitoplasts (inner membranes and matrix) were prepared by osmotic lysis as described basically by Parsons *et al.* [12]: the washed mitochondrial pellet was suspended in 0·02 M phosphate buffer (pH 7·2), incubated at 4°C for 20 min and sedimented at 35,000 *g* for 20 min to obtain a pellet of lysed mitochondria. From the supernatant the intermembrane fraction was collected and concentrated by placing the solution in dialysis tubing, applying dry Sephadex G-200 over the bag and placing the bag in the cold (4°C) overnight. The pellet was suspended by homogenization in 2 Vol of 0·25 M sucrose (10 mg protein/ml 0·25 M sucrose) and subjected to ultrasonication at 0°C for 2 min (4 times for 30 s) with intervals for cooling every 30 s (Bronson Sonifier, 20 kcycles/s). The sonicated mitochondria were treated with non-ionic detergent Lubrol WX at a concentration of 0·05 mg/mg mitochondrial protein, incubated at 4°C for 20 min and fractionated by differential centrifugation [13]. The unbroken mitochondria were sedimented at 12,000 *g* for 15 min. The 12,000 *g* supernatant was collected and centrifuged at 105,000 *g* for 60 min to sediment the inner membrane fraction. The 105,000 *g* supernatant was further centrifuged at 155,000 *g* for 75 min to isolate the outer membrane fraction.

Incubation procedure. Standard reaction mixtures (total vol. 4·1 ml) contained 0·15 M phosphate buffer (pH 7·4), 0·1 μCi of [^{14}C]labelled steroids plus varying amounts of unlabelled steroids (added in 100 μl of propylene glycol), mitochondria and NAD (400 μM). Time and temperature of incubations are indicated in the legends. Reactions were started by addition of coenzyme and terminated by addition of 5 ml of ether–chloroform (3:1, v/v). The extracts of the reaction mixtures (3 × 5 ml of ether–chloroform) were pooled, evaporated under nitrogen and chromatographed on silica gel thin-layer plates (0·25 mm, F_{254}, E. Merck, Darmstadt, Germany) in the system benzene–methanol (19:1, v/v). Reference steroids were located by fluorescence absorption. Radioactivity of separated steroids was quantitated by a radio-chromatogram scanner (LB 2723, Berthold, Wildbad, Germany) equipped with a 2π counting device and count integrator.

The products of the reactions were further identified by preparation of derivates. Acetylation and chloroacetylation of steroids was carried out according to the methods of Zaffaroni and Burton [14] and Connell and Eik-Nes [15] respectively. The chromic acid technique described by Lieberman *et al.* [16] was used for oxidation reactions.

Assay of marker enzymes. Succinate dehydrogenase activity was determined by the method of Arrigoni and Singer [17]. Monoamine oxidase and adenylate kinase activities were assayed by the methods of Schnaitman *et al.* [18]. Glutamate dehydrogenase activity was determined by the method of Bergmeyer [19]. NADPH-cytochrome-*c*-reductase was determined according to Omura and Takesue [20].

Protein assay. Protein concentration was estimated by the method of Lowry *et al.* [21] using bovine serum albumin as standard.

Electron microscopy. Suitable aliquots of mitochondrial subfractions were fixed with glutaraldehyde in cacodylate buffer (pH 7·3) according to the method of Karnovsky [22].

Subsequently, all samples were dehydrated by passing through a graded series of alcohol and embedded in epoxy resin according to the method of Spurr [23].

Ultrathin sections were cut with a LKB-microtome, stained in uranyl and lead citrate solution and examined by a Siemens Elmiskop 1 A electron microscope.

RESULTS

The activity of the NADPH-cytochrome-*c*-reductase (marker enzyme of microsomes) in the mitochondrial fraction could be reduced considerably by several washings, while in the microsomal fraction the activity stayed nearly the same under the same conditions. The final microsomal contamination of the mitochondria (after 4 washings) was 2·5% (Table 1).

The data in the Table 2 show the distribution of the 17β-HSD activity and that of the marker enzymes succinate dehydrogenase (for inner membranes),

Table 1. Specific activity of NADPH-cytochrome-*c*-reductase (nmol/mg protein/30 min) in human endometrial mitochondria after several washings and in microsomes

Subcellular fraction	No. of washes	NADPH-cytochrome-c-reductase	Microsomal contamination %
Mitochondria	2	7.1	21
	3	2.5	7.7
	4	0.8	2.5
Microsomes	2	32.4	-

Table 2. Specific activities (nmol/mg protein/30 min) of marker enzymes and 17β-HSD in purified subfractions of mitochondria from human secretory endometrium, for details see under "Methods".

n.m. = not measurable.

	Succinate-DH	Monoamine-oxidase	Adenylate-kinase	Glutamate-DH	17β-HSD
Washed mitochondria	9.5	2.3	8.7	15.8	7.7
Outer membranes	2.7	10.4	12.3	n.m.	21.8
Inner membranes	27.3	n.m.	n.m.	2.3	3.6
Intermembrane fraction	n.m.	n.m.	43.2	1.8	n.m.
Matrix	n.m.	n.m.	n.m.	22.7	n.m.

monoamine oxidase (for outer membranes), adenylate kinase (for intermembrane fraction) and glutamate dehydrogenase (for matrix) in subfractions of washed mitochondria from human secretory endometrium.

These results suggest that the mitochondrial 17β-HSD is mainly associated with the outer membranes of the organelle. The soluble subfraction which contained the matrix was not capable of converting estradiol to estrone.

These results indicate that during the procedure used to subfractionate the mitochondria the 17β-HSD remained firmly attached to the membrane system.

Electron microscopy. Figure 1a shows a micrograph obtained after staining the 105,000 *g* sediment of the

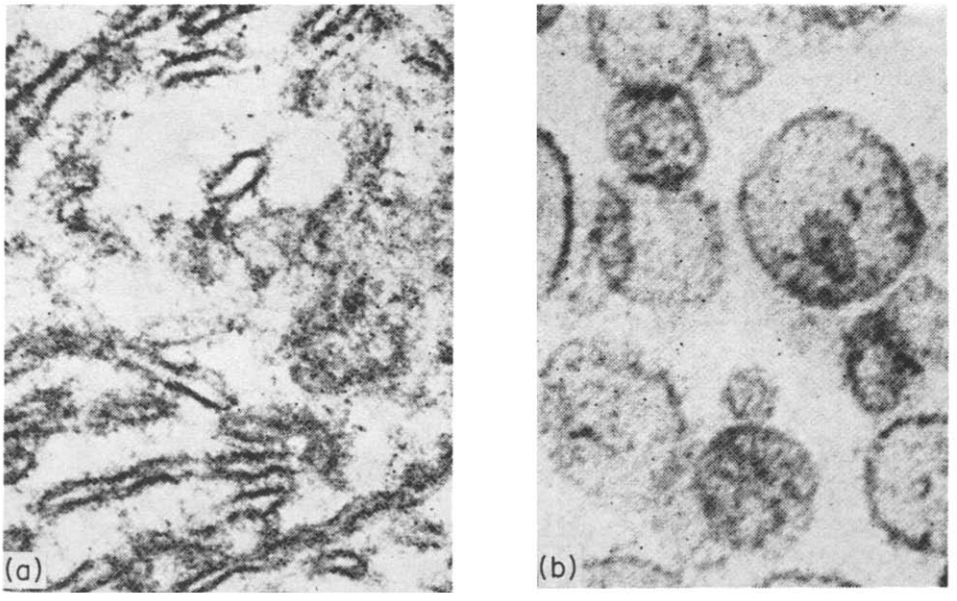

Fig. 1. Electron micrographs of a 105,000 *g* sediment (a, inner membranes) and 155,000 *g* sediment (b, outer membranes) of the mitochondria. Magnification: (a) × 116,000; (b) × 76,000.

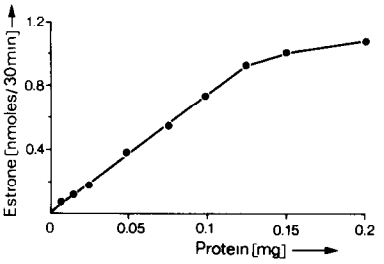

Fig. 2. Initial rates of E_2 oxidation as a function of different protein concentrations.

Each flask contained in 4·1 ml of reaction mixture (standard conditions): 10 μM [^{14}C]-E_2 (0·1 μCi, added in 100 μl of propylene glycol), 400 μM NAD, 0·15 M phosphate buffer (pH 7·4) and varying amounts of mitochondrial protein from secretory endometrium, time of incubation was 15 min at 37°C.

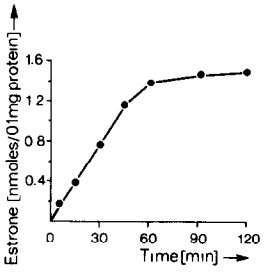

Fig. 3. E_2 oxidation as a function of time. Standard assay conditions as described under Fig. 2 (25 μg protein).

lysed mitochondria. It consists of inner membrane fragments of various size. Fig. 1b is a micrograph obtained after staining the 155,000 ***g*** sediment. It consists of small vesicles, bordered by a single membrane, characteristic of the outer mitochondrial membrane.

Effect of enzyme concentration and time (crude washed mitochondria). A linear relationship between the amount of protein used and the formation of estrone was observed in the range of 7·5–125 μg protein/ml. Enzyme assay was carried out in the presence of 25 μg of protein/ml (Fig. 2).

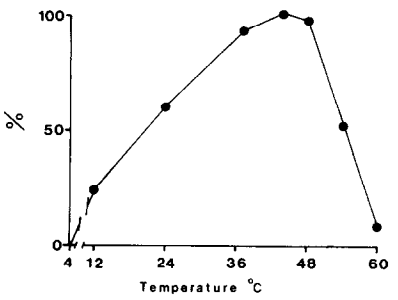

Fig. 4. Estradiol oxidation as a function of temperature. Standard assay conditions as described under Fig. 2 (25 μg protein). Enzyme activity is expressed as % of the highest value measured.

The formation of estrone was found to be linear up to 45 min (Fig. 3).

Effect of temperature on enzyme activity (crude washed mitochondria). Maximal activity of the mitochondrial 17β-HSD was obtained at 45°C incubation temperature (Fig. 4).

Effect of pH and different buffers on enzyme activity (crude washed mitochondria)

The 17β-HSD activity in different buffer systems and at different pH values is shown in Fig. 5. The pH optimum for the oxidation of estradiol-17β was 9·5. For the reduction of estrone in phosphate buffer the optimal pH was 6·0.

Inhibitors and activators

Enzymatic activity is completely inhibited by incubation with 10^{-3} M p-chloromercuribenzoate (Table 3). This inhibition suggests that the substrate is bound at or near essential sulfhydryl groups.

In the presence of cysteine enzyme activity increases, presumably by protecting the essential sulfhydryl groups of the enzyme.

17β-HSD activity was inhibited 82% by 10^{-3} M Cu^{2+}, 33% by 10^{-3} M Zn^{2+} and 15% by 10^{-3} M Mg^{2+}, but was unaffected by 10^{-5} M Mg^{2+}. The lability of the enzyme in the presence of certain heavy

Table 3. Effect of sulfhydryl groups blocking agents, cysteine and heavy metals on the mitochondrial 17β-HSD activity of human secretory endometrium; for details see under "Methods"

Additions	Concentration (mole/l)	Inhibition (%)	Amount estrone (μg)
No addition	-	-	2.7
p-chloromercuri-benzoate	10^{-5}	68	0.87
	10^{-3}	100	-
Iodacetamide	10^{-5}	22	2.1
	10^{-3}	78	0.6
Mg^{2+}	10^{-5}	-	2.9
	10^{-3}	15	2.3
Zn^{2+}	10^{-5}	11	2.4
	10^{-3}	33	1.8
Cu^{2+}	10^{-3}	82	0.48
Cysteine	10^{-3}	-	3.2
Zn^{2+} + EDTA	10^{-3}	15	2.3

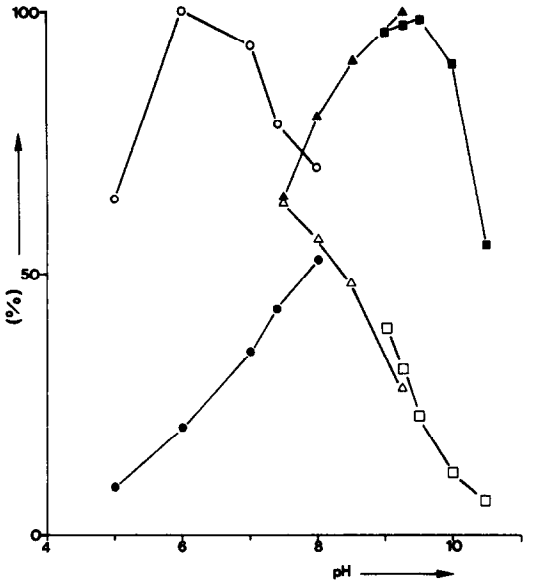

Fig. 5. The effect of pH and the influence of buffer solution on the enzyme activity of the 17β-HSD in human endometrium mitochondria of secretory phase. Standard assay conditions as described under Fig. 2 (25 μg protein). The buffers used were as follows (closed symbols oxidation of E_2, open symbols reduction of E_1):

● ○ 0·15 M Ringer-phosphate buffer
▲ △ 0·2 M Tris–HCl
■ □ 0·2 M Na-borat/NaOH

Enzyme activity is expressed as % of the highest value measured.

metal ions may be a reflection of the requirement of free sulfhydryl groups for enzyme activity.

Kinetic parameters

K_m-values and maximal velocities (substrate estradiol) of the mitochondrial 17β-HSD from proliferative, secretory and neoplastic endometrium (with NAD and NADP as cofactor) are presented in Table 4. Kinetic parameters from proliferative and neoplastic endometrium were very similar. K_m-values from secretory endometrium were lower and maximal velocities higher than in proliferative and neoplastic endometrium. In all tissues estradiol was oxidized approximately 15 times more rapidly with NAD than with NADP.

Substrate specificity studies (crude washed mitochondria)

Aliquots of the mitochondrial 17β-HSD preparations from proliferative, secretory and neoplastic endometrium were incubated with estradiol-17β, estrone, testosterone or 4-androstene-3,17-dione. The relative amounts of oxidation or reduction products of these steroids were estimated (Table 5).

Testosterone was oxidized by all samples at less than 75% of the rate of estradiol-17β. Androstene-3,17-dione was reduced at approximately 50% or less than the rate of estrone when mitochondrial preparations of normal human endometrium were used,

Table 4. Kinetic constants of the mitochondrial 17β-HSD from normal and neoplastic human endometrium

Reactants		K_m	v
Variable	Constant	(μM)	(nmoles/10 min/mg protein)
Proliferative endometrium			
Estradiol	NAD	13.4	1.4
Estradiol	NADP	12.8	0.09
Secretory endometrium			
Estradiol	NAD	3.6	5.2
Estradiol	NADP	3.2	0.35
Undiff. endometrial carcinoma			
Estradiol	NAD	13.1	1.0
Estradiol	NADP	12.5	0.07

Table 5. Substrate specificity of the mitochondrial 17β-HSD of human endometrium and endometrial carcinoma

		17 β - HSD (nmoles/mg protein / 30 min)		
Substrate (10 μM)	Product	Proliferative	Secretory phase	Carcinoma
Estradiol-17β	estrone	2.5	7.7	1.8
Estrone	estradiol-17β	1.5	3.7	0.7
Testosterone	4-androstene-3,17-dione	1.6	5.8	1.1
4-androstene-3,17-dione	testosterone	0.8	1.8	1.5

while it was hydrogenated more than twice as rapidly as estrone in mitochondria from endometrial carcinoma.

DISCUSSION

The present and preceding studies [8–10] have shown that the 17β-HSD is ubiquitous in the cell of the human endometrium similar to the 17β-enzyme of human placenta. It is present in the cytoplasm of the cell, in the endoplasmic reticulum, in the nucleus and in the outer membranes of the mitochondria.

Kinetic properties of the mitochondrial 17β-HSD were similar to those of the soluble, microsomal and nuclear enzymes. These enzymes have been shown to be pyridine nucleotide linked enzymes which can use either NAD or NADP as cofactor. With the latter the initial reaction rate is about 1/15 that achieved with NAD. Similar results were obtained by others for the 17β-HSD from placental tissue [24–28].

The mitochondrial, microsomal, nuclear and soluble 17β-HSD from human endometrium possess sulfhydryl groups which are required for enzymatic activity. Heavy metal inactivation is characteristic of these enzymes. The pH optimum for the oxidation of estradiol-17β was consistently close to 9·5 and for the reduction of estrone close to 6·0. Studies of substrate specificity showed that the most reactive substrates were the C_{18} steroids with an aromatic A ring. The enzyme has a greater affinity for estradiol-17β than for estrone. The non-aromatic steroids (testosterone and 4-androstene-3,17-dione) could also serve as substrates but the reaction was usually slower than with estradiol-17β or estrone. One exception to this rule was the reduction of androstenedione by mitochondria of endometrial carcinoma which was more rapid than the reduction of estrone. The apparent K_m-values for estradiol-17β were found to be different when mitochondrial preparations from different phases of the cycle and of endometrial carcinoma were used. These phenomena cannot be explained at present.

From the kinetic similarities it may be speculated that the attachment of the enzyme protein to various subcellular structures does not influence the active centre. The ubiquitous location of the 17β-HSD in the endometrial target cell indicates a central role of this enzyme in the metabolism of C_{18} and C_{19} steroids.

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