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PURIFICATION AND PROPERTIES OF STEROID 17α-HYDROXYLASE FROM CALF TESTIS

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Summary—Steroid 17α-hydroxylase has emerged as a key enzyme in steroidogenic cells: (i) it represents the branch point between the 17-deoxy (mineralo) and the 17-hydroxy (gluco) corticosteroid pathways in the adrenal cortex; (ii) the corresponding specific cytochrome (P-$450_{17\alpha}$) is highly dependent upon hormonal regulation; and (iii) the enzyme also catalyzes the steroid 17–20 lyase reaction, leading to the major androgens in the testis. As a prerequisite to the study of its regulation in intact cell, 17α-hydroxylase was purified from calf testis microsomal preparations. Following five chromatographic steps, the enzyme was obtained as an apparently homogeneous protein of $M_r = 57$ kDa upon gel electrophoresis. The procedure yielded a recovery of about 10% as judged by cytochrome P-450 assay. Whereas 17α-hydroxylase specific activity was about 30-fold enriched during the purification, that of the C17–20 lyase was increased by about 6-fold, strongly suggesting that its organelle environment may modulate the enzymatic activity. The purified enzyme yielded a 20 N-terminal amino-acid sequence showing a complete homology with that of its adrenal counterpart and a polyclonal antibody raised against our preparation revealed a 57 kDa protein band in bovine adrenocortical microsomal extracts, upon immunoblotting experiments. It was thus concluded that bovine 17α-hydroxylase activity is supported by highly similar if not identical enzymatic proteins in both testis and adrenal cortex tissues. The purified P-$450_{17\alpha}$ preparation is now being used in reconstitution experiments which suggest that microsomal components may contribute to a different expression of the enzyme specificity in its native testis or adrenocortical intracellular environment, respectively.

INTRODUCTION

17α-Hydroxylase-C17–20 lyase is a key regulatory enzyme in the adrenocortical steroidogenic pathways, since it represents a branch point between mineralocorticoids (corticosterone) biosynthesis on the one hand and cortisol and sex hormone production on the other [1].

Purified preparations from pig testes [2, 3] and pig adrenals [4, 5] have been shown to be identical and to exhibit similar activities, i.e. (i) 17α-hydroxylation of pregnenolone and progesterone and (ii) the 17-hydroxy products being substrates of a C17–20 lyase activity. The enzyme has been purified from guinea pig adrenal microsomes [6] and has been shown to possess both activities; the ratio of the 17α-hydroxylase over the lyase activities was similar to that of the testicular pig preparation. Bumpus and Dus [7] have reported the purification of a steroid 17α-hydroxylase from bovine adrenocortical microsomes, but this enzyme lost its 17α-hydroxylase activity during the preparation and exhibited side-chain cleavage activity with cholesterol as substrate.

The primary sequence of bovine microsomal cytochrome P-$450_{17\alpha}$ has been obtained [8]. The N-terminal amino-acid sequence of the pig adrenal enzyme appears 75% homologous to that of its bovine counterpart, whereas that of the guinea pig [9] is 53% homologous. The bovine adrenal preparation of Bumpus and Dus [7] showed an apparent M_r of 50 kDa, whereas an M_r of 56 kDa was expected from its amino-acid composition.

With the aim of examining the factors responsible for the regulation of the dual activities of the cytochrome in the bovine adrenocortical cell, the 17α-hydroxylase was purified from calf testis. Characterization of the molecular and catalytic properties are reported in the present communication.

MATERIALS AND METHODS

Materials

The steroid was from Fluka, NADPH was from Boehringer Mannheim and [7-

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^{3}H]pregnenolone (22.6 Ci/mmol) was from New England Nuclear. [^{3}H-17α-OH]pregnenolone was prepared by enzymatic hydroxylation of [^{3}H]pregnenolone.

Renex 690 (equivalent to Emulgen 913) was a generous gift from Seppic (France). DEAE–cellulose and phosphocellulose were from Whatman, DEAE–Sepharose and CM–Sepharose were from Pharmacia and hydroxylapatite was from Biorad. Trilostane was a generous gift from Sterling Winthrop Co.

Buffer A: potassium phosphate buffer (× mM, pH 7.4) containing glycerol (20%, v/v), EDTA (0.1 mM), DTT (0.1 mM) and Renex 690 (0.2% w/v).

Buffer B: buffer A (20 mM) containing cholate (0.4%, w/v) instead of Renex 690.

Microsomes preparations

All subsequent operations were conducted at 4°C. Calf tests were homogenized in Tris–HCl buffer (5 mM, pH 7.4) containing sucrose (275 mM) with a Teflon homogenizer. The homogenate was centrifuged at 800 *g* for 15 min, then at 10,000*g* for 15 min to eliminate the nuclear and mitochondrial pellet, and the resulting supernatant was spun at 100,000*g* for 45 min. The pellet was suspended in phosphate buffer (100 mM, pH 7.4) containing glycerol (20% v/v), EDTA (1 mM) and DTT (1 mM); the concentration of proteins was adjusted to 4 mg/ml and sodium cholate was added (0.6% w/v) over 30 min for protein solubilization. After centrifugation at 100,000*g* for 45 min, the resulting supernatant was diluted with 2 vol aqueous glycerol (20%, v/v). The purification procedure described below was usually started with about 600 mg of microsomal proteins.

Chromatographic steps

The diluted supernatant was applied to a DEAE–cellulose (2.6 × 20 cm) column equilibrated with buffer B and eluted with the same buffer. Cytochrome P-450$_{17\alpha}$ was not retained on this column. The active fractions were applied on a hydroxylapatite column (4 × 6 cm) equilibrated with buffer A (20 mM). The column was washed with the same buffer and eluted by a linear phosphate gradient (20–300 mM) in buffer A. Fractions containing enzymatic activity were pooled and dialyzed overnight against buffer A (10 mM). The dialyzed fractions were applied to a CM–Sepharose CL. 6B column (1.5 × 8 cm) equilibrated with buffer A (10 mM) and washed with the same buffer. Cytochrome P-450$_{17\alpha}$ was not retained on this column and was immediately loaded on a DEAE–sepharose (1.5 × 8 cm) column equilibrated with buffer A (10 mM). After washing with the same buffer, elution was performed with a phosphate gradient (10–200 mM) in buffer A. Cytochrome P-450$_{17\alpha}$ was eluted at a phosphate concentration < 100 mM.

Fractions containing enzymatic activities were pooled and transferred onto a phosphocellulose column (0.9 × 5 cm) equilibrated with buffer A (50 mM); after washing with the same buffer, elution was performed with buffer a (300 mM).

Antiserum against cytochrome P-450$_{17\alpha}$ was raised in rabbits.

Blotting procedure and immunodetection of cytochrome P-450$_{17\alpha}$

Following electrophoresis [10] the proteins were transferred onto nitrocellulose sheets using a Biorad electro-blot apparatus (60 V, 15 h) and a 25 mM Tris–192 mM glycine buffer in 20% (v/v) methanol at pH 8.3 [11, 12]. The tracks were soaked in 20 mM Tris–HCl buffer (pH 7.5) containing 500 mM NaCl (TBS buffer) and gelatine (3%, w/v) for 30 min at room temperature then washed 3 times with TBS buffer. Incubation with anti-cytochrome P-450 antibody in 10 ml TBS buffer was carried out for 6 h and the tracks were then thoroughly washed with TBS. The immunocomplexes were revealed using a horseradish peroxidase kit from Biorad, following the manufacturer's indications.

Enzymatic activities

Assays of steroid 17α- and 21-hydroxylases and C17–20 lyase activities were determined aerobically at 30°C. The assay system was composed of 2.5 nmol of labeled substrate ([^{3}H]pregnenolone or [^{3}H-17α-OH]pregnenolone; [^{3}H]progesterone or [^{3}H-17α-OH]progesterone), glucose-6-phosphate (2.5 μmol), glucose-6-phosphate dehydrogenase (0.6 U), $MgCl_2$ (1.5 μmol) NADPH (120 nmol) and the fraction to be tested in a final volume of 500 μl of 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA. Incubations were 15 min for hydroxylase and 30 min for lyase activity assay, respectively. The reactions were stopped by addition of methanol (1 ml) and chloroform (1 ml). The lower phase was evaporated to dryness and the steroids were separated by silica gel TLC in chloroform–ethyl acetate

Table 1. Example of results obtained during purification of calf testis 17-hydroxylase

	Proteins (mg)	CO (nmol)	nmol heme (mg protein)	Sp. act. (nmol/min/mg protein)		
				17-hydroxylase[a]	Lyase[b]	Hydroxylase/lyase
Microsomes	1000	420	0.4	2.4	0.4	6
Cholate extract	780					
DEAE-cellulose	427	374	0.9	4.4		
Hydroxylapatite	130	200	1.5	10		
CM-Sepharose	78	136	1.9		13.4	
DEAE-Sepharose	2.4	83	3.5			
Phosphocellulose	3.5	36	10	78	2.5	31

[a]17α-Hydroxylation of pregnenolone.
[b]Transformation of 17-OH pregnenolone to dehydroepiandrosterone.

(1:1, v/v), using authentic pregnenolone, 17α-OH pregnenolone as well as 17α-OH progesterone, 11-deoxycortisol and dehydroepiandrosterone as internal standards.

NADPH–cytochrome P-450-reductase

This was purified from bovine liver using the same procedure as from pig liver [13]. The preparation exhibited a sp. act. 40 μmol cytochrome *c* reduced/min/mg protein.

Amino-acid analysis and N-terminal sequence determination

Protein samples were hydrolyzed for 24 h in constantly boiling HCl containing 1% phenol (v/v), under reduced pressure at 110°C. The resulting mixture was analyzed using a Beckman 7300 amino-acid analyzer and ninhydrin as the detection reagent. Automated Edman degradation was performed using an Applied Biosystems Model 470 A gas-phase sequencer. Identification of amino-acid phenylthiohydantoin derivatives was performed using an on-line Applied Biosystem Model 120 A HPLC apparatus according to the protocol recommended by the manufacturer.

Sodium dodecyl sulfate (0.1%)–polyacrylamide (10%) slab gel electrophoresis (SDS–PAGE) was performed according to Laemmli [10]. The gels were stained for protein with Coomassie blue.

RESULTS

Purification of the enzyme

Following microsomal suspension (protein: 4 mg/ml) treatment with cholate (0.6%), the enzymatic activity was solubilized with an average yield of about 90%. Other detergents such as Renex 690, deoxycholate or Tween 20 were very ineffective. A summary of a typical preparation including the DEAE–cellulose, hydroxylapatite, CM–sepharose, DEAE–sepharose and phosphocellulose chromatographic steps is given in Table 1. The overall yield in 17α-hydroxylase activity was about 10% and the purification factor was about 32-fold. The ratio of the 17α-hydroxylase over the lyase activities was strikingly increased (by about 5-fold) as a result of the purification procedure.

Molecular properties of the 17α-hydroxylase preparation

When the final preparation was subjected to SDS–PAGE a single protein band was detected following Coomassie blue staining, as shown in Fig. 1. Following calibration with marker proteins, an apparent M_r of 57,000 ± 1000 was estimated.

The amino-acid composition obtained for our cytochrome $P\text{-}450_{17\alpha}$ preparation is given in Table 2. It is in good agreement with the composition deduced from nucleic acid analysis for the adrenal enzyme [8]. Sequence determinations of the first 20 N-terminal amino acids showed no difference between that of our testicular enzyme preparation and that deduced for the adrenal protein (Table 3).

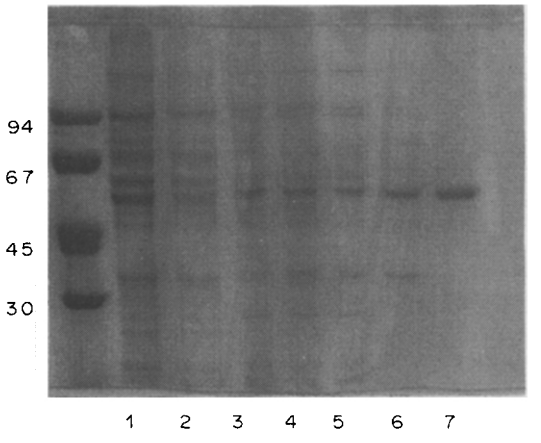

Fig. 1. PAGE of the purification steps: microsomes (1); cholate extracts (2); DEAE–cellulose (3); hydroxylapatite (4); CM–sepharose (5); DEAE–sepharose (6); phosphocellulose (7)

Table 2. Amino-acid composition of bovine cytochrome $P\text{-}450_{17\alpha}$

	This study	Ref. [7]	Ref. [8]
Ala	34	26	34
Ile	27[a]	25	37
Leu	68	51	71
Met	7	10	8
Val	27	27	27
Phe	28	26	27
Trp		3	7
Tyr	8	17	6
Asx	49	36	50
Glx	49	51	50
Arg	23	25	22
Lys	35	29	37
His	17	13	17
Ser	34	20	33
Thr	23	18	22
Gly	40	25	28
Pro	27	26	28
Cys	4	5	5

[a]Hydrolysis was performed for only 24 h, thus Ile–Ile bounds were not completely hydrolyzed giving a low value for ileu.

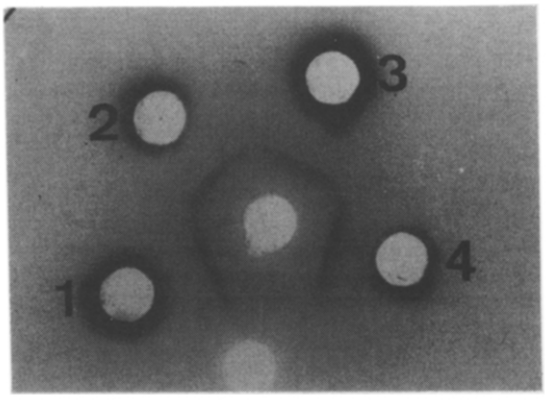

Fig. 2. Double-diffusion in agarose. Center well—anti-$P\text{-}450_{17\alpha}$ serum to testicular enzyme (5 μl); well 1—microsomes from testis (120 μg); well 2—purified testicular enzyme (12 μg); well 3—microsomes from adrenals (120 μg); well 4—purified adrenal enzyme (12 μg).

Spectral properties

Purified cytochrome $P\text{-}450_{17\alpha}$ showed absolute spectra of oxidized form with maxima at 571, 535 and 419 nm, which are characteristic of a low-spin hemoprotein without bound substrate. The reduced form showed a single peak at 448 nm (data not shown).

Upon addition of pregnenolone to the enzyme solution a high-spin transition (peak at 393 nm) was observed. This substrate-induced difference spectra allowed the determination of a K_s values (Table 4).

Immunological properties

Antibodies raised against the testicular enzyme showed a classical line of identity with the purified testicular and adrenal enzymes, testicular and adrenal microsomes (Fig. 2). Immunodetection after gel electrophoresis showed that the microsomes from testis as well as the purified enzyme gave a single band with the same R_f of migration (Fig. 3).

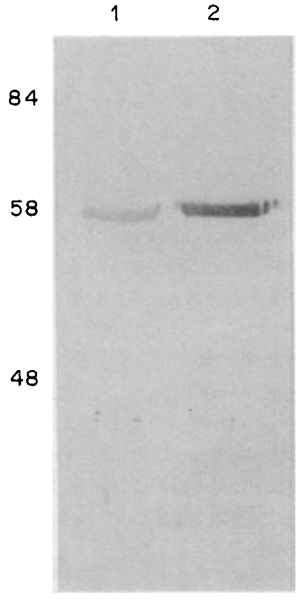

Fig. 3. Immunoblotting: (1) testicular microsomes; (2) purified testicular cytochrome $P\text{-}450_{17\alpha}$.

Table 3. NH_2-terminal sequence of cytochrome $P\text{-}450_{17\alpha}$ from porcine and bovine testis and adrenals

(1)	Met	Trp	Leu	Leu	Leu	Ala	Val	Phe	Leu	Leu	Thr	Leu	Ala	Tyr	Leu	Phe	Trp	Pro	Lys	Thr
(2)	Met	Trp	Leu	Leu	Leu	Ala	Val	Phe	Leu	Leu	Thr	Leu	Ala	Tyr	Leu	Phe	Trp	Pro	Lys	Thr
(3)	Met	Trp	Val	Leu	Leu	Val	Phe	Phe	Leu	Leu	Thr	Leu	Thr	Tyr	Leu	Phe				
(4)	Met	Trp	Val	Leu	Leu	Val	Phe	Phe	Leu	Leu	Ser	Leu	Thr	Tyr	Leu	Phe				

(1) Testicular bovine, this study; (2) adrenal bovine, Ref. [8]; (3) adrenal pig, Ref. [5]; (4) testicular pig, Ref. [3].

Table 4. Activity of purified 17-hydroxylase with different substrates

	Sp. act. (nmol/min/mg protein)			
Substrate	17-Hydroxylase	Lyase	K_s (μM)	K_m (μM)
Pregnenolone	78 ± 4		0.3	0.35
17α-OH Pregnenolone		2 ± 0.3		0.55
Progesterone	91 ± 5			3.80
17α-OH Progesterone		0		—

Enzymatic activities

The enzymatic activities with 4-ene and 5-ene substrates are reported in Table 4. As shown in this table, no lyase activity was observed with 17α-OH progesterone as substrate.

DISCUSSION

Cytochrome $P\text{-}450_{17\alpha}$ has been purified to homogeneity from calf testis by several chromatographic steps. The purified enzyme shows both 17α-hydroxylase and lyase activities; the ratio hydroxylase/lyase increases from the microsomes to the purified enzyme as was previously observed with pig [3] and guinea pig [6] testicular enzymes. The role of a single polypeptide chain in catalyzing both reactions has been confirmed by the expression of bovine cytochrome $P\text{-}450_{17\alpha}$ gene (cDNA) in COS-1 cells [14].

Changes in the ratio hydroxylase/lyase between adrenal and testes probably result from the interaction of cytochrome with the environment of the endoplasmic reticulum. The influence of phospholipids was observed and lyase activity was favored by the presence of testicular phosholipids [15] but the exact nature of the phospholipid (or phospholid associated) factor is not known.

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