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THE MEMBRANE-BOUND 17β-ESTRADIOL DEHYDROGENASE OF PORCINE ENDOMETRIAL CELLS: PURIFICATION, CHARACTERIZATION AND SUBCELLULAR LOCALIZATION

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Summary—The membrane-bound 17β-estradiol dehydrogenase of porcine endometrial cells was purified to homogeneity as judged by SDS–PAGE and silver staining of a single 32 kD band. A second, more hydrophobic product of the purification protocol contained additional bands at 45 and 80 kD. The 17β-estradiol dehydrogenase activities of both products exceeded those for 17-one reduction by more than 260-fold. Activities of 3α-, 3β- and 20α-dehydrogenases were absent in either fraction. Polyclonal and monoclonal antibodies raised against the 32 kD protein and the more hydrophobic product precipitated the enzymatic activity and reacted with the 32 and 80 kD bands, but not with the 45 kD band in Western blots. The subcellular localization of the enzyme was studied in sections of intact cells and of isolated organelles using gold sol coated with $F(ab')_2$ fragments of monoclonal antibody F1. Gold particles were found exclusively over cytoplasmic vesicles of 120–150 nm diameter with electron-dense contents.

INTRODUCTION

The 17β-estradiol dehydrogenase of porcine endometrial cells differs from the placental oxidoreductase [1, 2] and is most likely engaged in the termination of estradiol action after the nuclear passage of the hormone. The enzymatic activity was first assigned to a subpopulation of lysosomes [3], which—because of the slightly alkaline pH optimum for estradiol oxidation—could not be of the classical type. We have investigated this problem in some detail [4] and discovered apparently specialized cytoplasmic vesicles harboring the enzyme [5]. An exclusive localization of the enzyme in these vesicles should be ascertained by a complementary technique applicable to isolated organelles and to organelles *in situ*. We report here the isolation and characterization of the enzyme, the production of antibodies and localization studies at the electron-microscopic level.

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EXPERIMENTAL

All materials and auxiliary techniques used have been described in detail elsewhere [6–9].

Purification

The dehydrogenase was purified from luteal phase porcine uteri. All operations were carried out at 0–4°C. Tissue was collected at the local abattoir, chilled in ice, minced and homogenized with a constant tolerance shearing device [6] in 2 vol 10 mM piperazine, 10 mM glycylglycine, 20 mM NaCl, 1 mM NaN_3, pH 8.2. The homogenate was centrifuged for 15 min at 1800 ***g***. The supernatant was adjusted to 10 mM $MgCl_2$ and spun for 60 min at 11,000 ***g***. The sediment was suspended in 4 vol 20 mM potassium phosphate buffer, 2 mM NaN_3, pH 7.8 and recovered by centrifugation at 161,000 ***g*** for 2 h.

Of 12 detergents of different classes tested, nonionic Brij 35 proved to be the best for solubilizing stable enzymic activity. The washed 161,000 g_{av} pellets were resuspended in 50 mM potassium phosphate, 2 mM NaN_3, 0.4% Brij 35, pH 7.8 at a protein concentration 10 mg/ml. The suspension was stirred for 1 h and cleared by centrifugation at 161,000 g_{av} for 3 h.

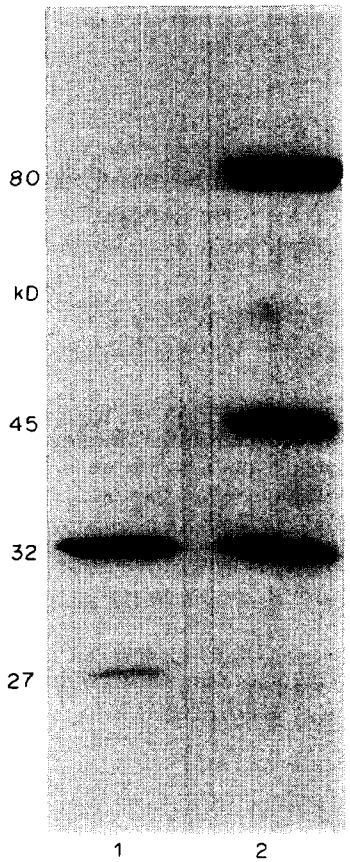

Fig. 1. SDS–PAGE survey of purified estradiol dehydrogenase. Samples of EDH_M (5 μg, lane 1) and VHF (10 μg, lane 2) were subjected to SDS–PAGE and silver stained [8, 9].

The particle-free extracts were successively passed with upward flow through an on-line assembly of a DEAE-Sepharose CL-6B column (removal of inert proteins), an Amberlite XAD-2 column (removal of detergent) and a Blue-Sepharose column (adsorption of enzyme) followed by a wash with detergent-free 50 mM potassium phosphate buffer, pH 7.8. The enzymatic activity was eluted from Blue-Sepharose at 0.8 M of a linear 0 → 2 M KCl gradient. The 17β-dehydrogenase peak was well separated from that of estrone reductase eluting at 1.9 M KCl. The pooled dehydrogenase fractions were slowly adjusted to 1.1 M $(NH_4)_2SO_4$, a minor turbidity removed by centrifugation (10,000 *g*, 15 min) and the clear supernatant applied to Butyl-Sepharose. Elution was carried out by a linear 1.1 → 0 M $(NH_4)_2SO_4$ gradient. Approximately 80% of the enzymatic activity eluted at 0.9 M, the remainder was recovered at 0.1 M $(NH_4)_2SO_4$. The first peak of estradiol dehydrogenase activity eluted from Butyl-Sepharose was denoted EDH_M (M—for medium hydrophobic). It was purified by gel filtration through Sephadex G200 in 50 mM KCl, 20 mM potassium phosphate, 2 mM NaN_3, 0.02% Brij 35, pH 7.8. The symmetrical EDH_M peak was pooled and adjusted to 20 mM potassium phosphate, 2 mM NaN_3, pH 7.0 for final cation exchange chromatography on Mono S HR 5/5 in a Pharmacia FPLC apparatus. The second, very hydrophobic fraction (VHF) eluted from Butyl-Sepharose showed heterogeneity both in gel filtration on Sephacryl S300 HR and in subsequent Mono S chromatography (conditions as above). Total recoveries from 40 kg of uteri amounted to 1 mg of the uniform 32 kD protein (EDH_M) and 0.8 mg for VHF.

Antibodies

A polyclonal goat antiserum (G436) against EDH_M and a mouse monoclonal antibody (F1)

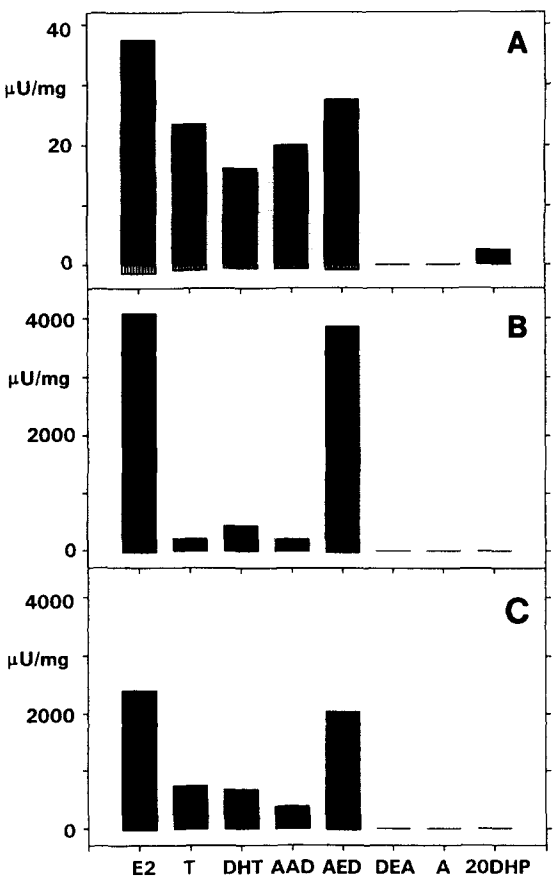

Fig. 2. Substrate specifity for oxidation and reduction in porcine endometrial cells. Crude membrane fraction (A), purified EDH_M (B) and VHF (C) were analyzed with substrates: estradiol (E2), testosterone (T), 5α-dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (AAD), 5-androstene-3β,17β-diol (AED), dehydroepiandrosterone (DEA), androsterone (A), 20α-dihydroprogesterone (20DHP). Oxidation (■) was measured in 0.1 M potassium phosphate buffer, pH 7.8 with NAD^+, the reduction (▥) at pH 6.6 with NADPH [4].

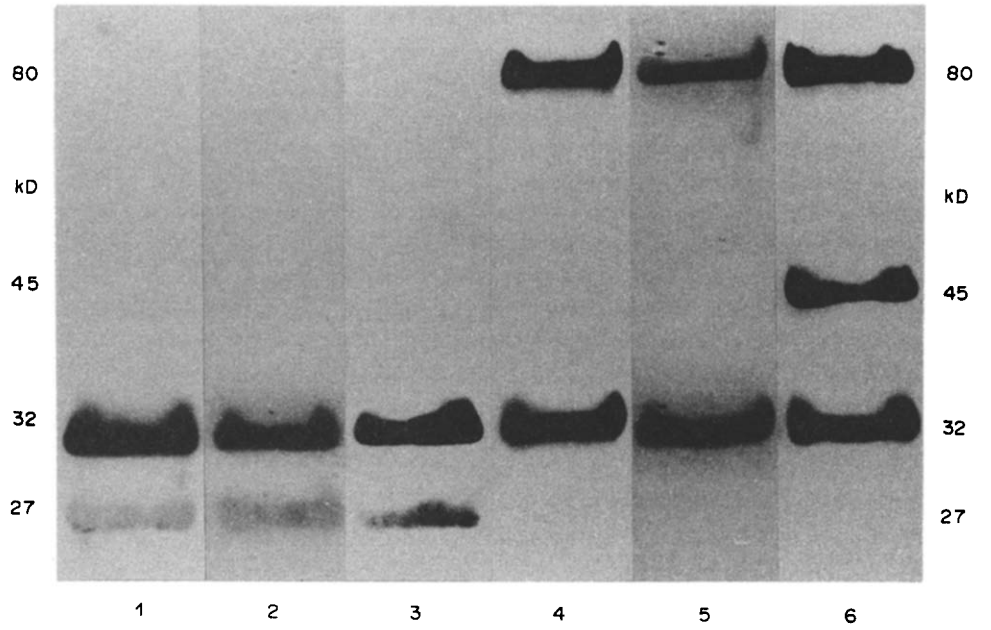

Fig. 3. Immunoblotting of purified estradiol dehydrogenase fractions. Samples of EDH_M (5 μg, lanes 1–3) and of VHF (10 μg, lanes 4–6) were resolved on SDS–PAGE, blotted, immunolabeled with Fab′-peroxidase conjugates of the monoclonal antibody F1 (lanes 1 and 4) and polyclonal G436 antibodies (lanes 2 and 5) or stained with India ink (lanes 3 and 6).

against VHF were prepared. The Fab′ fragments of antibodies were conjugated with horseradish peroxidase [7].

Electron microscopy

For electron microscopy, endometrium cells were collected by curettage [3], immediately fixed in 0.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h at 4°C, washed with phosphate buffer and dehydrated for embedding in hydrophilic methacrylate resin LR-Gold (London Resin Co.). Particles of 10 nm colloidal gold were coated with $F(ab')_2$ fragments of mouse mab F1 using 0.5% bovine serum albumin (BSA) as an expander. Sections of 70–80 nm were cut with a Reichert-Jung Ultracut, mounted on pioloform-coated gold grids for postembedding immunoreaction: preincubation with PBS containing 0.05% Tween 20, 0.5% BSA (PBSTB buffer) + 0.1 M glycine for 30 min at room temperature, 3 washes with PBSTB, overnight incubation at 4°C by floating on $F(ab')_2$-Au solution. Controls were performed by preincubations with $F(ab')_2$ fragments of F1. The sections were washed with 3 changes of PBSTB and jet-washed with H_2O before viewing in a Philips EM 301.

RESULTS AND DISCUSSION

Purity and molecular weight

On SDS–PAGE under reducing conditions, a single 32 kD, silver-stained band, was seen with EDH_M (Fig. 1). A second band of 27 kD appeared after storage at 4°C for 2 weeks without loss of dehydrogenase activity. The SDS–PAGE of VHF revealed the presence of bands at 32, 45 and 80 kD. The staining pattern was unchanged after 2 weeks of storage at 4°C. The molecular weight of EDH_M was estimated by gel filtration as 75 kD. After crosslinking with dimethyl pimelidate, a 70 kD band was seen in SDS–PAGE as compared to the single 32 kD band of not crosslinked EDH_M. Gel filtration of VHF gave a 190 kD peak with a 270 kD shoulder. A 240 kD band was seen in SDS–PAGE after crosslinking. The molecular weight (32 kD) of purified, homogeneous EDH_M is similar to that of two other membrane-bound dehydrogenases from mare placenta microsomes (33 kD [12]) and from porcine Leydig cells (36 kD [13]). The molecular weight of the soluble oxidoreductase of human placenta (34 kD) is in the same range in spite of the marked differences in enzymatic properties [1].

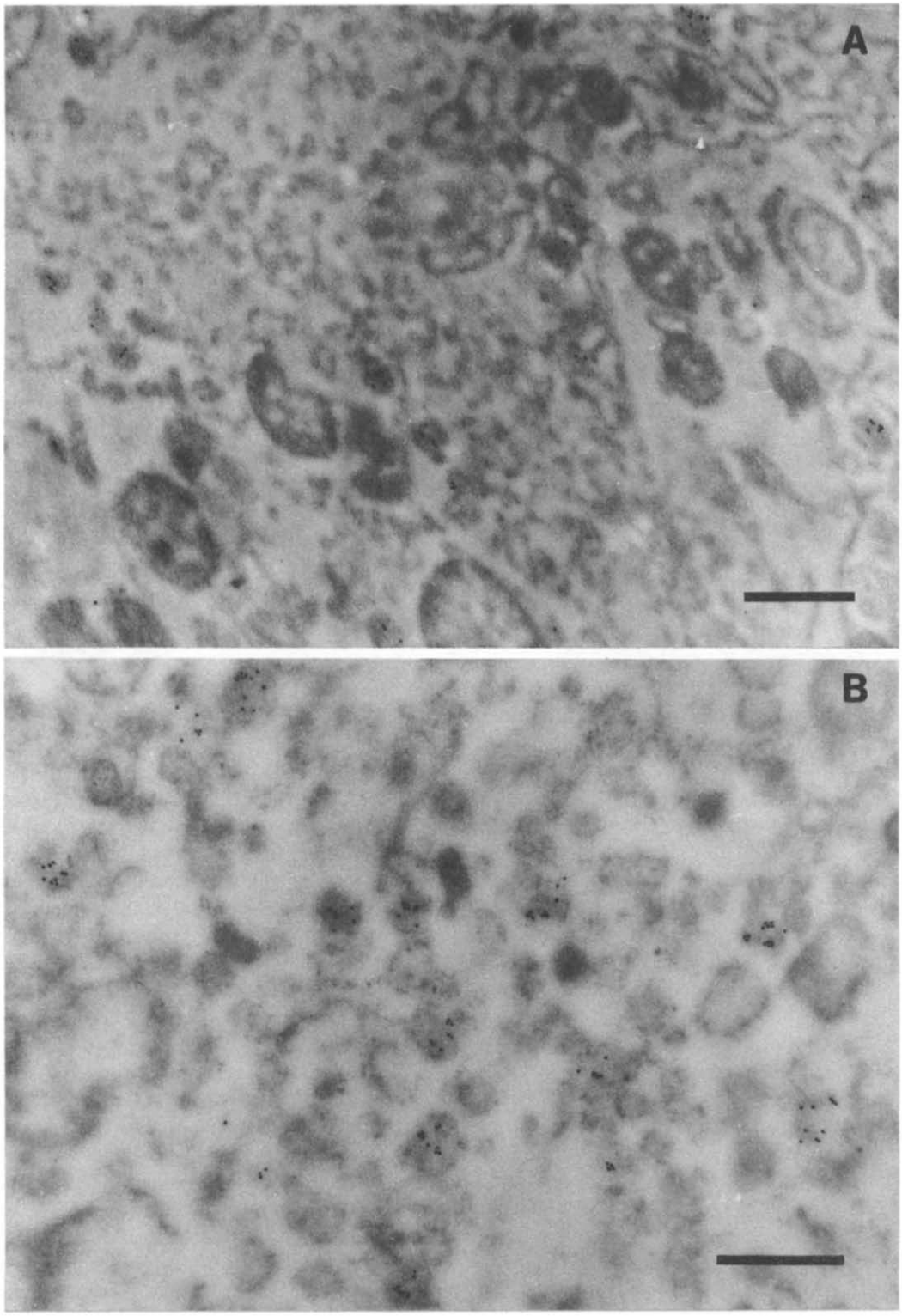

Fig. 4. *In situ* immunogold labeling of 17β-estradiol dehydrogenase. LR-gold sections were incubated with 10 nm gold particles coated with $F(ab')_2$ of mab F1: (A) luteal phase endometrial cells, (B) isolated vesicles [5]. Sections were counterstained with OsO_4. Bar 500 nm.

Enzymic properties

The oxidation of estradiol by both EDH_M and VHF is NAD^+ dependent, K_m for $EDH_M = 7 \times 10^{-7}$ M and for VHF $= 2 \times 10^{-6}$ M. The substrate specificities of crude membrane preparations and of the purified fractions are compared in Fig. 2. Specific activities for estradiol are: 4082 μU/mg protein for EDH_M and 2402 μU/mg protein for VHF. The VHF showed higher activity with testosterone than the EDH_M (728 vs 206 μU/mg). Oxidation was preferred over reduction for each substrate tested. The proportion of oxidation/reduction for estradiol changed from 65 in the crude membrane fractions to 260 in EDH and VHF. This is a reflection of the separation of two authentic enzymes (17-ketoreductase and 17β-dehydrogenase) by the purification protocol. Activities of 3β-, 3α- or 20α-dehydrogenases were absent in both EDH_M and VHF. The 45 kD protein of the VHF fraction, therefore, is not a copurified 3β-dehydrogenase [10], and the membrane-bound enzyme carries no 20α activity unlike the soluble dehydrogenase [11].

Immunoblotting

Both G436 and F1 antibodies recognize the 32 kD EDH_M band (and the degraded protein at 27 kD) after Western blotting (Fig. 3). Of the three silver-stained bands of VHF, those at 32 and 80 kD are immunostained by F1 and G436, but not the 45 kD band. Bands of the same molecular weights were immunoreactive in crude membrane fractions and in purified material. The relationships between immunostained 32 and 80 kD proteins of VHF and the 32 kD of EDH_M remain to be clarified. The 45 kD protein of VHF could be an impurity or an integral membrane constituent.

Immunoelectronmicroscopy

The monoclonal antibody F1 raised against VHF was used in subcellular immunogold localization studies. Gold particles were found over 120–150 nm cytoplasmic vesicles with electron-dense contents [Fig. 4(A)]. Their morphology resembled that of the dehydrogenase-rich organelles isolated from prepuberal endometrial cells [5]. However, only some of them reacted with immunogold in sections of the isolated vesicles [Fig. 4(B)], indicating a high degree of specialization. The organelle fraction [5] was not contaminated with microsomes and mitochondria, both of which have been proposed as sites of residence for 17β-dehydrogenases by less stringent cell biological criteria [14, 15].

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