Purification and properties of oestradiol 17 β -dehydrogenase extracted from cytoplasmic vesicles of porcine endometrial cells

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Porcine endometrial oestradiol-17 β dehydrogenase was solubilized from the particulate fraction of homogenates sedimenting between 1200 g and 10000 g by treatment with 0.4 % Brij 35 in neutral buffers. The extracts were processed by successive passage through DEAE-Sepharose, Amberlyte XAD-2 and Blue-Sepharose, and the enzyme was collected from the washed affinity matrix at 0.8 M of a 0–2 M-KCl gradient. A genuine oestrone reductase was eluted at 1.9 M-KCl. The dehydrogenase pool was resolved by butyl-Sepharose chromatography into a major (80 %) peak (EDH_M) eluted at 0.8 M-(NH₄)₂SO₄ and a very hydrophobic fraction (VHF) recovered at 0.1 M. EDH_M was further purified by filtration through Sephadex G-200 and cation-exchange chromatography on Mono S. Sephacryl 300 was used for VHF followed by Mono S. Enrichments from the homogenate amounted to 1074-fold for EDH_M and 632-fold for VHF. A single silver-stained band at 32 kDa is seen on SDS/PAGE of EDH_M, and VHF contains additional bands at 45 and 80 kDa. Polyclonal antibodies (G436) raised against EDH_M and the monoclonal antibody F1 raised against VHF recognize the single 32 kDa band in EDH_M and both the 32 kDa and 80 kDa bands in composite VHF. The 45 kDa band of VHF reacts with neither. Monoclonal antibody W1 raised against EDH_M only recognizes the 32 kDa peptide of EDH_M and VHF. The specific activity for oestradiol oxidation amounts to 4081 μ units/mg for EDH_M and to 2402 μ units/mg for VHF. Both possess a minimal (1/260) endogenous reductase activity and are devoid of 3 β , 3 α - and 20 α -dehydrogenases. We consider EDH_M to be authentic oestradiol-17 β dehydrogenase of porcine endometrium. The composite VHF could reflect the situation of the enzyme *in vivo* or result from aggregations occurring during processing.

INTRODUCTION

The 17 β -hydroxysteroid oxidoreductases are involved in the biosynthesis and metabolism of steroids. The soluble enzyme from the human placenta, which has been purified to homogeneity [1,2] and the amino acid sequence of which was deduced from cDNA [3], is the best known oestradiol 17 β -dehydrogenase (EDH; EC 1.1.1.62). It is possibly the only one justifying the systematic name oestradiol-17 β :NAD⁺ oxidoreductase. Most membrane-associated 17 β -dehydrogenase activities described, especially those of target organs, favour oxidation over reduction, unless the presumed single enzyme is indeed a mixture of two [4–7]. The dehydrogenation of oestradiol to oestrone in target organs has generally been ascribed to the microsomal fraction of homogenates [7], with two exceptions. Pollow *et al.* [8] reported on a mitochondrial enzyme in human endometrium. We found the highest EDH activity in a subfraction of lysosomes from porcine endometrial epithelium [5], suggesting an involvement of the enzyme in the termination of the hormonal stimulus. Its slightly alkaline pH optimum [6] indicated that the particles differed from classical lysosomes. We therefore reinvestigated the problem with improved techniques for homogenization and organelle separation and discovered an apparently specialized type of particle containing the EDH [9]. Its authenticity remains to be proven by immunological identification in cell sections at the electron-microscopic level. We describe the purification of the enzyme, its properties and the production of both poly- and mono-clonal antibodies.

EXPERIMENTAL

Materials

Tritium-labelled steroids, [2,4,6,7-³H]oestradiol (specific radio-

activity 3.5 TBq/mmol), [2,4,6,7-³H]oestrone (3.6 TBq/mmol), [1,2,6,7-³H]testosterone (3.4 TBq/mmol), 5 α -[1,2,4,5,6,7-³H]dihydrotestosterone (4.4 TBq/mmol), 5 α -[9,11-³H]androstane-3 α ,17 β -diol (1.7 TBq/mmol), 5-[1,2-³H]androstene-3 β ,17 β -diol (2.1 TBq/mmol), [9,11-³H]androsterone (1.9 TBq/mmol) and 20 α -[1,2-³H]dihydroprogesterone (1.7 TBq/mmol), were bought from New England Nuclear; [1,2,6,7-³H]dehydroepiandrosterone (3.9 TBq/mmol) was obtained from Amersham International. H.p.l.c.-grade acetonitrile was obtained from Zinsser (Frankfurt, Germany). H.p.l.c. water was from Millipore Milli-Q System (Eschborn, Germany). Scintillator Hydroluma and reversed-phase extraction cartridges, RP18 100 mg bed, were bought from Baker (Gross-Gerau, Germany). Amberlite XAD-2 and α -amylase were from Serva; aldolase, catalase, ovalbumin, DEAE-Sepharose CL-6B fast flow, Blue-Sepharose CL-6B, butyl-Sepharose CL-4B, Sephadex G-200, Sephacryl S300 HR, Mono S HR 5/5 and protein G-Sepharose FF were obtained from Pharmacia. The following detergents were used: Brij 35 and Brij 58 from Serva; Lubrol PX, Lubrol WX, Triton X-100, 1-*O*-octyl β -D-glucopyranoside, *N*-decyl-*NN*-dimethyl-3-ammonio-1-propanesulphonate, *N*-tetradecyl-*NN*-dimethyl-3-ammonio-1-propanesulphonate, CHAPS and sodium deoxycholate from Sigma. Prestained molecular-mass standards (range 184–26 kDa) were bought from Sigma. All other chemicals were grade A and obtained from Merck. Concentrators Centricon-10 (10 kDa molecular-mass cutoff) were bought from Amicon.

Buffers

Buffer A consisted of 10 mM-piperazine, 10 mM-glycylglycine, 20 mM-NaCl and 1 mM-Na₃N₃, pH 8.2; buffer B contained 50 mM-potassium phosphate and 2 mM-Na₃N₃, pH 7.8; buffer C was 2 M-KCl in buffer B, pH 8.2; buffer D contained 20 mM-potassium phosphate, 2 mM-Na₃N₃ and 1.1 M-(NH₄)₂SO₄, pH 7.8; buffer E

Abbreviations used: EDH, oestradiol dehydrogenase; EDH_M, medium hydrophobic oestradiol dehydrogenase; VHF, very hydrophobic fraction containing oestradiol dehydrogenase activity; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

contained 20 mM-potassium phosphate and 2 mM- NaN_3 , pH 7.8; buffer F contained 20 mM-potassium phosphate, 2 mM- NaN_3 and 0.02% (w/v) Brij 35, pH 7.8; buffer G was 50 mM-KCl in buffer F; buffer H was 100 mM-KCl in buffer F; Buffer I was as buffer F but pH 7.0; buffer J was 1 M-KCl in buffer F, pH 7.0.

All operations were carried out at 0–4 °C unless otherwise stated.

Subcellular fractionation

Sow uteri were collected at the local abattoir, the horns immediately separated from ovaries and parametrium, sliced open and transferred to ice-cold buffer A. The chilled uteri were passed through a stainless-steel meat grinder (7 mesh) and homogenized with a constant-tolerance shearing device [10] at (1700 rev./min) for 5 min in 2 vol. of buffer A and the homogenates centrifuged at 1800 $g_{av.}$ for 15 min (Heraeus Cryofuge 6-6, rotor 5710). The supernatants were adjusted to 10 mM- MgCl_2 [11] and spun at 11000 $g_{av.}$ for 60 min (Beckman J2-21, JA10 rotor). The resulting pellets were stored frozen at –20 °C for 2–4 weeks. No loss of EDH activity during storage was observed.

Solubilization and purification of EDH

The frozen pellets were thawed and resuspended with a constant-tolerance shearing device in 2 vol. of buffer B. The suspension was centrifuged for 2 h at 161 000 $g_{av.}$ in Ti45 rotors (Beckman ultracentrifuge L2-65B). The supernatants (containing about 1% of the total EDH activity) were discarded. Of ten detergents of different classes tested, non-ionic Brij 35 proved to be the best for solubilizing stable enzyme activity. The washed 161 000 $g_{av.}$ pellets were resuspended in buffer B/0.4% Brij 35 at a protein concentration 10 mg/ml. The suspensions were stirred for 1 h and cleared by centrifugation for 3 h at 161 000 $g_{av.}$. The supernatant was subjected to a succession of chromatographic operations as outlined in the flow-sheet of Fig. 1.

SDS/PAGE and silver staining

The samples were denatured in 1.5% (w/v) SDS/0.02 M- Na_2EDTA /0.1 M-thioglycolic acid/10% (w/v) glycerol for 5 min at 95 °C before application to the gels. The 1.5 mm slab gels were cast as described by Laemmli [12] using stacking gels of 5% (w/v) acrylamide/2.5% (w/v) bisacrylamide in 0.125 M-Tris/HCl, pH 6.8 and a modified separating gel of linear gradient of 5% (w/v) acrylamide/2.5% (w/v) bisacrylamide to 18% acrylamide/5% bisacrylamide in 0.375 M-Tris/HCl, pH 8.8. The gels were run at 90 V for 15 h at 7 °C in 0.05 M-Tris/0.384 M-glycine/0.2% (w/v) SDS, pH 8.8, fixed overnight at room temperature in 50% (v/v) methanol/12% (v/v) acetic acid/0.02% (v/v) formaldehyde/0.01% (v/v) glutaraldehyde and silver stained [13].

Production of antibodies

A polyclonal antibody (G436) was raised in a goat. The animal received $3 \times 50 \mu\text{g}$ of antigen (EDH_M) in monthly intervals with complete (first injection) or incomplete Freund's adjuvant by subcutaneous injection of 10 aliquots of 0.1 ml.

For monoclonal antibody production, Balb/c mice were immunized with $10 \mu\text{g}$ of antigen (EDH_M or VHF) in $30 \mu\text{l}$ of complete Freund's adjuvant, injected into the spleen, followed by intraperitoneal injections of $30 \mu\text{g}$ of antigen in $200 \mu\text{l}$ of incomplete adjuvant every second week. Antibody titres were measured by the immunodot technique [14] and as follows: $40 \mu\text{l}$ of serum was incubated with $40 \mu\text{l}$ of supernatant and $120 \mu\text{l}$ of

Fig. 1. Flow-sheet of purification of EDH

Fig. 2. Elution profile from Blue-Sepharose

...., A_{280} ; ●, EDH; △, Oestrone reductase; ----, KCl gradient; bar, fractions pooled for next step.

0.1 M-sodium phosphate buffer, pH 7.8, at 4 °C, overnight in Eppendorf cups with overhead tumbling. Protein G-Sepharose (100 μl of a 30% suspension in phosphate buffer) was added and the suspension incubated for 2 h at 4 °C with overhead tumbling. The beads were collected by centrifugation, washed three times with phosphate buffer and the activity of EDH was measured in the pellet.

Mice with the highest titre of relevant antibodies were boosted with $20 \mu\text{g}$ (150 μl) of antigen in phosphate-buffered saline (PBS; 154 mM-NaCl and 10 mM-sodium phosphate, pH 7.4) on 3 consecutive days before fusion. Spleen cells (10^8) were fused with an equal amount of NS0 myeloma cells using poly(ethylene glycol) 1500 [15]. The fused cells were dispensed into the wells of 96-well plates at a density of 30 000/0.2 ml together with 20 units of interleukin 6 and approx. 1700 peritoneal macrophages added per well. Hybridomas were selected in hypoxanthine, aminopterin and thymidine medium [16].

Culture supernatants were screened for antibody production by solid-state e.i.s.a. [17] using anti-(mouse peroxidase)-antibodies (Sigma) as a detection system. The peroxidase reaction was performed using *p*-hydroxyphenylpropionic acid [17] and monitored with a Perkin-Elmer LS-3B fluorescence spectrometer ($\lambda_{ex.} = 320 \text{ nm}$, $\lambda_{em.} = 405 \text{ nm}$) equipped with a plate-reader

Fig. 3. Butyl-Sepharose chromatography

....., A_{280} ; ----, $(\text{NH}_4)_2\text{SO}_4$ gradient; ●, EDH activity. Short bar, EDH_M pool; long bar, VHF pool.

unit. The cells from e.i.s.a.-positive wells were expanded, cloned twice by limiting dilution at 0.3 cell per well and tested by Western blotting [18].

Two hybridomas were selected for antibody production: W1, raised against purified medium hydrophobic enzyme (EDH_M), and F1, raised against very hydrophobic fraction (VHF) of the EDH. Cloned cells were expanded and injected intraperitoneally ($10^7/0.2$ ml) into balb/c mice (primed by intraperitoneal injection of incomplete Freund's adjuvant 3 days earlier). The ascites fluids collected 2 weeks later were rich in monoclonal antibody

(2–4 mg/ml). They were diluted 5-fold with 20 mM-phosphate buffer, pH 7.0, and applied to protein G-Sepharose for IgG isolation [19]. Goat serum was fractionated with Na_2SO_4 [20] and the IgG fraction was also isolated by protein G-Sepharose chromatography. Fab'-horseradish peroxidase conjugates, cross-linked with sulphosuccinimidyl-4-(maleimidomethyl)-cyclohexane-1-carboxylate (Pierce) were prepared by using purified antibodies G436, W1 and F1 [21].

Western blotting and immunostaining

Western blotting from SDS/polyacrylamide gels on nitrocellulose membranes (Schleicher und Schüll) was performed as described previously [22]. The membranes were blocked with PBS/Tween 20 (0.05%, w/v) for 45 min at room temperature and incubated with 0.2 μg of immunoconjugates/ml for 14 h at 4 °C. After four washes with PBS/Tween, peroxidase staining was done [22]. Proteins were stained with India ink (Pelikan, diluted 1:1000 with PBS/Tween) for 1 h at room temperature.

Other methods

Enzyme activities were assayed with tritiated substrates. Products were analysed by h.p.l.c./flow-through counter as described [6]. Protein was determined as described by Lowry as modified by Markwell *et al.* [23].

RESULTS

Survey of purification

Batches of approx. 400 g of particulate fraction collected at

Fig. 4. Gel-filtration and cation-exchange chromatography of EDH_M

....., A_{280} ; ●, EDH activity. (a) Sephadex G-200 application of EDH_M eluate from Blue-Sepharose; molecular-mass markers: aldolase (158 kDa), α -amylase (97 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa). The pooled fractions (bar) were concentrated to 5 ml with simultaneous adjustment to buffer H (pH 7.0). (b) Cation-exchange chromatography on Mono S; elution with KCl gradient (----), buffer H $\rightarrow$ buffer I.

Fig. 5. Gel-filtration and cation-exchange chromatography of VHF

....., A_{280} ; ●, EDH activity. (a) Sephacryl S300 HR; all other conditions as in Fig. 4(a); the additional molecular-mass marker catalase was used (240 kDa); carbonic anhydrase was not. The pooled fraction (bar) was concentrated to 5 ml with simultaneous adjustment to buffer H. (b) Cation-exchange chromatography as in Fig. 4(b).

11 000 *g* from homogenates of 25–30 kg of uteri were used. Their Brij 35 extracts were passed successively with upward flow through an on-line assembly of a column (5 cm × 34 cm) of DEAE-Sepharose CL-65, a column (5 cm × 18 cm) of Amberlite XAD-2 and a column (5 cm × 24 cm) of Blue-Sepharose CL-6B (all equilibrated with buffer B and equipped with multiport bottom and top adaptors) at a flow rate of 180 ml/h. The anion-exchanger binds approx. 35% of the applied protein but no enzyme activity. The elution profile from the (detached) Blue-Sepharose column (Fig. 2) shows a single peak of EDH eluted at 0.8 M of a 0–2 M-KCl gradient (75 ml/h) together with a minor oestrone reductase activity (cosubstrate NADPH; pH optimum 6.6). The 260:1 proportion of dehydrogenase/reductase in this peak was observed throughout the purification process, indicating that EDH has a low inherent capacity for catalysing the reverse process. The second oestrone reductase peak eluted at 1.9 M-KCl, in contrast, is devoid of EDH activity and represents a different enzyme.

The pooled EDH fractions were diluted with one-third vol. of cold water, and solid $(\text{NH}_4)_2\text{SO}_4$ was added with stirring to a final concentration of 1.1 M. The slightly turbid solution was cleared by centrifugation and then applied at a rate of 120 ml/h to an (upward flow) column (5 cm × 5 cm) of butyl-Sepharose 4B, equilibrated with buffer D. The column was washed with 1 vol. of buffer D, turned upside down and eluted from the top with an $(\text{NH}_4)_2\text{SO}_4$ -negative gradient at a flow rate of 60 ml/h, 0–100% buffer E over 12 h. Approx. 80% of the EDH activity was eluted in a sharp peak at 0.7 M- $(\text{NH}_4)_2\text{SO}_4$, approx. 4% at 0.3 M and approx. 16% at 0.1 M (Fig. 3). The first peak was designated EDH_M (M for medium hydrophobic), the second

peak was discarded and the third one named VHF, for very hydrophobic fraction.

The volumes of the pooled fractions were decreased to 1.5 ml in Centricon-10 with simultaneous adjustment to buffer G (EDH_M) and buffer H (VHF). EDH_M was then further purified by gel filtration on a Sephadex G-200 column (2.5 cm × 73 cm, upward flow at 10 ml/h). The EDH_M activity was eluted with an apparent molecular mass of 75 kDa (Fig. 4a). The pooled fractions were concentrated with Centricon-10 to 5 ml in buffer 1 and applied at 0.25 ml/min to a Mono S HR 5/5 column operating in the f.p.l.c. apparatus from Pharmacia. The EDH activity was recovered in a single peak (Fig. 4b).

The VHF eluate was passed through a Sephacryl S300 HR column (2.5 cm × 71 cm, upward flow at 10 ml/h). The EDH activity was eluted in a non-symmetrical 190 kDa peak with a 270 kDa shoulder (Fig. 5a). The pooled fractions were concentrated in buffer 1 and processed on Mono S as above. EDH activity was eluted in two portions: a major followed by a minor activity peak (Fig. 5b) which were pooled separately.

Reducing SDS/PAGE of the final products (Fig. 6) revealed that freshly prepared EDH_M is a 32 kDa protein, apparently contaminated with a 27 kDa polypeptide. After storage at 4 °C for 12 days, the intensities of the bands were reversed, whereas the enzyme activity of the solution remained unchanged. The transition from 32 to 27 kDa is probably caused by traces of peptidases in the preparation. VHF contains three polypeptides of 80, 45 and 32 kDa. This pattern as well as the enzyme activity remain stable after storage. Lane 5 of Fig. 7, taken from the minor Mono S peak, reveals less 45 kDa protein compared with the main peak.

Fig. 6. SDS/PAGE survey of purified EDH

Samples (10 µg) of EDH_M and VHF were subjected to SDS/PAGE and silver staining. Either fresh samples [EDH_M (lane 1), VHF major Mono S peak (lane 3), minor Mono S peak (lane 5)] or samples after storage at 4 °C for 12 days [EDH_M (lane 2), VHF (lane 4)] were analysed. Molecular-mass standards (kDa) are indicated.

Fig. 7. Immunoblots of isolated EDH_M and VHF

Freshly prepared EDH_M or VHF (10 µg each) were applied per lane. SDS/PAGE was carried out under reducing conditions followed by blotting on to nitrocellulose; lanes were stained with Fab'-peroxidase conjugates of antibodies G436 (lane 2), F1 (lane 3) and W1 (lane 4). Molecular-mass standards (kDa) are indicated. Lane 1, India ink staining.

The numerical record of the protocol (Table 1) emphasizes that yield was sacrificed for purity and that the total EDH content of uterine endometrial cells must be substantial. The most effective step was affinity chromatography on Blue-Sepharose. The stringent cutting of the major peak decreased the

Fig. 8. Screening of enrichment of EDH_M and VHF by monoclonal antibodies

SDS/PAGE Western blots were stained in parallel with Silver (Ag) and Fab'-peroxidase conjugates of mAbs F1 and W1. The amount of protein applied was 10 µg for silver staining and 80 µg for immunostaining. Molecular-mass standards (kDa) are indicated. Fractions, lane 1, crude membranes; lane 2, Blue-Sepharose; lane 3, butyl-Sepharose (EDH_M); lane 4, butyl-Sepharose (VHF).

80:17 proportion of dehydrogenase activity in EDH_M/VHF to 60:40 after the final cation-exchange chromatography. The five-step procedure described provides a 1074-fold purification of EDH_M and a 632-fold enrichment of EDH activity in the VHF.

Antibodies against EDH_M and against components of VHF

IgG (G436) raised against EDH_M in a goat, the monoclonal antibody (mAb) F1 raised against VHF and the mAb W1 raised against EDH_M react with native antigens (adsorption to protein G-Sepharose-attached IgG) and with denatured antigens in Western blots of SDS/PAGE under reducing conditions. All antibodies recognize the 32 kDa protein and its 27 kDa degradation product in EDH_M. They also react with the 32 kDa constituent of VHF. The 80 kDa VHF protein, however, is recognized only by G436 and mAb F1, not by mAb W1. None of the antibodies bind to the 45 kDa band of VHF (Fig. 7).

Western immunoblots with mAb F1 and mAb W1 of the crude membranes, the Blue-Sepharose fraction and the enzymically active peaks from butyl-Sepharose show that the purification of EDH_M and VHF can be monitored immunologically (Fig. 8).

Substrate specificities of EDH_M and VHF

The substrate specificities of the EDH_M polypeptide were compared with those of the composite VHF and the crude

Table 1. Record of purification

Purification step	Volume (ml)	Activity (µunits/ml)	Total activity (munits)	Protein (mg/ml)	Specific activity (µunits/mg)	Purification	
						Yield (%)	Fold
Homogenate	81 000	72	5832	19.1	4	100	1
11 000 g particles	1260	774	975	25.8	30	16.73	8
Supernatant	2900	17	51	1.8	10	0.87	3
Blue-Sepharose pool	375	65	24	0.7	90	0.42	24
Butyl-Sepharose (EDH _M)	85	94	8.0	0.285	330	0.14	87
Sephadex G-200	36	164	5.9	0.106	1543	0.10	406
Mono S	3	939	2.8	0.230	4081	0.05	1074
Butyl-Sepharose (VHF)	115	23	2.6	0.031	742	0.04	186
Sephacryl S300	35	74	2.6	0.049	1510	0.04	378
Mono S	2.5	517	1.3	0.215	2402	0.02	632

Table 2. Comparison of substrate specificities in crude membranes, EDH_M and VHF

Conditions for incubation and product analysis were as described in the Experimental section.

Conversion	Activity (μ units/mg of protein)		
	Crude membranes	EDH _M	VHF
Oestradiol $\rightarrow$ Oestrone	30.0	4081	2402
5-Androstene-3 β ,17 β -diol $\rightarrow$ dehydroepiandrosterone	22.0	3852	2075
Testosterone $\rightarrow$ androstenedione	18.8	206	747
5 α -Androstane-3 α ,17 β -diol $\rightarrow$ androsterone	16.0	208	384
5 α -Dihydrotestosterone $\rightarrow$ 5 α -androstanedione	12.8	432	683
20 α -Dihydroprogesterone $\rightarrow$ progesterone	2.0	0	0
Dehydroepiandrosterone $\rightarrow$ androstenedione	0	0	0
Androsterone $\rightarrow$ 5 α -androstanedione	0	0	0

membrane fraction from which EDH_M and VHF had been isolated (Table 2). All substrates for dehydrogenation were analysed at pH 7.8 with NAD⁺ as cosubstrate. The specific activities for reduction of the corresponding 17-ketones (pH 6.6; NADPH) are not listed. They amounted to less than 0.4 % of the dehydrogenase activity for EDH_M and VHF, down from 4 % in the crude membranes.

The specific activity of EDH_M for the dehydrogenation of oestradiol is similar to that for 5-androstene-3 β ,17 β -diol. The oxidations of the 17 β -hydroxy groups of the androgens, testosterone (5 %), 5 α -dihydrotestosterone (10 %) and its 3 α -OH-reduction product (5 %) are considerably less pronounced. VHF with roughly half the specific activities for the oxidation of oestradiol and reduced dehydroepiandrosterone contains markedly higher activities for the dehydrogenation of 17 β -OH androgens, in proportions closer to those measured in crude membranes.

Neither EDH_M nor VHF contain any activity for the oxidation of 20 α -hydroxyprogesterone and the oxidation/isomerization of dehydroepiandrosterone, which were marginally detectable in crude membranes. The membranes were devoid of any 3 α -hydroxydehydrogenase activity.

DISCUSSION

Homogenates of porcine uteri contain a host of proteolytic activities [22]. Our protocol for the purification of oestradiol 17 β -dehydrogenase therefore aimed at a speedy operation rather than at high yields. We also knew that the organelles suspected to contain the enzyme are quite vulnerable and demand cautious homogenization, as provided by the constant-tolerance shearing device [10]. The device peels endometrium off the muscle layer of the organ mince and ruptures endometrial cells gently leaving a sizeable fraction of them intact. This explains the modest enzyme activity (approx. 16 %) recovered in the 11000 g particles. Only a small fraction of their activity could be extracted by Brij 35 and remained stable during the subsequent steps. The related membrane-bound enzymes from horse placenta [24] and from

porcine testes [25,26] tolerate more aggressive detergents in higher concentrations.

The decreased specific activity in the Brij 35 extract indicates a proportionally higher solubilization of inert proteins from the Mg²⁺-packed sediment. Some of them might arise from co-sedimented microsomal membranes containing oestrone reductase [6,27], which is eluted at higher ionic strength from Blue-Sepharose than EDH. This observation corroborates the presumption that in the endometrium tissue, dehydrogenase and reductase activities reside in distinct enzymes [6]. The subsequent hydrophobic interaction chromatography of the Blue-Sepharose pool on butyl-Sepharose separated components left unresolved by the Brij treatment.

The Stokes radius of EDH_M and its sedimentation coefficient in sucrose gradients [28] correspond to a homodimer of the 32 kDa protein observed on SDS/PAGE. The dissociation of the approx. 70 kDa dimer during SDS/PAGE can be prevented by cross-linking with dimethyl pimelidate [28]. The masses of monomer and dimer resemble those of the soluble oxidoreductase from human placenta [1,2], the microsomal 17 β - and 17 α -dehydrogenases from horse placenta [23] and the microsomal oxidoreductase from porcine testes [25,26]. A close relationship to the soluble enzyme can be excluded by the presently known partial (70 %) sequence of EDH_M [29].

The 17 β -dehydrogenase activity of EDH_M is equally high with oestradiol and with 5-androstene-3,17 β -diol. Other 17 β -ol androgens are poor substrates. Oxidations of 20 α - and of 3 α /3 β hydroxy groups are not catalysed. The inherent 17-oxoreductase activity of the enzyme amounts to 0.4 % of the forward reaction. This indicates an involvement of the enzyme in the termination of oestradiol action, as can be assumed for the 17 β -dehydrogenases in human endometrium [4] and breast [30,31].

VHF differs from EDH_M in having lower specific activities for the prime substrates and higher oxidation rates with the aforementioned androgens. The heterogeneous behaviour of VHF in chromatography, gel filtration and density-gradient centrifugation [28] is contrasted by the ever recurrent SDS/PAGE pattern of silver(ink)-stained bands at 80, 45 and 32 kDa.

The cross-reactivities of antibodies raised against EDH_M (polyclonal G436) and VHF (monoclonal F1) with 32 (27) kDa EDH_M and with the 32 kDa and the 80 kDa bands of VHF suggest that the 32 (27) kDa peptide of EDH_M is an integral part of the 80 kDa peptide in VHF. We have no explanation for the inability of mAb W1, raised against EDH_M, to react with the 80 kDa protein in Western blots other than the obvious absence of a common epitope. The relationships between 32 and 80 kDa proteins of VHF and the 32 kDa protein of EDH_M remain to be clarified. The 45 kDa protein of VHF could be an admixed impurity or an integral membrane constituent.

The antibodies now available will be helpful in exploring the subcellular localization of EDH.

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