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ESTROPHILIC 3α,3β,17β,20α-HYDROXYSTEROID DEHYDROGENASE FROM RABBIT LIVER—I. ISOLATION AND PURIFICATION*

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Summary—A procedure for isolation of a highly-purified estrophilic hydroxysteroid dehydrogenase (EHSD) from rabbit liver, including ammonium sulphate fractionation, gel filtration, ion-exchange and affinity chromatography on estradiol–Sepharose, has been developed. The enzyme possesses NADP-dependent 3α,3β,17β,20α-HSD activities with a wide spectrum of androgenic, progestagenic, and estrogenic substrates. EHSD is a monomeric protein whose molecular mass determined by different methods is 35,000–39,000. The protein exhibits microheterogeneity due to the differences in molecular surface charge. The catalytic and hormone-binding properties and molecular sizes of the two protein fractions obtained by chromatography on DEAE–Toyopearl are close or identical. The enzymatic activity of EHSD is minor as compared to other HSDs from rabbit liver. However, the low values of K_m, the high affinity for steroid ligands, and high tissue levels of EHSD suggest the protein to play a role in the biodynamics of sex hormones.

INTRODUCTION

In addition to the presently-known mechanisms of hormonal activity regulation a local dynamic control of hormone accessibility for receptors and steroid metabolizing enzymes can be provided, in our opinion, by way of steroid complexation with specific intracellular steroid-binding nonreceptor proteins, which we call stereomodulins [1]. To this group of proteins one may relate the unusual estrogen-binding protein (UEBP) of male rat liver [2, 3], sex steroid-binding protein of chicken oviduct [6, 7], estrogen-binding protein of immature rat ovaries [8, 9], UEBP-like protein of guinea-pig liver [10], transcortin-like protein of rat hypophysis [11, 12], and some others [13–15]. Proteins of this group share several common properties, such as their high content in the cells, moderate affinity for ligands, high rates of steroid association and dissociation, etc. Many of those can bind hormonal compounds of more than one group. By virtue of the above capabilities these proteins may perform the buffer-reserving function for their ligands and provide for mutual effects of various steroids on their biodynamics. In this paper we describe the isolation and purification of estrophilic 3α, 3β, 17β, 20α-hydroxysteroid dehydrogenase (EHSD) from rabbit liver, which, perhaps, controls the biodynamics of sex hormones simultaneously in two ways: by enzymatical and steromodulin mechanisms.

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EXPERIMENTAL

Materials

[2,4,6,7,16,17-^{3}H]Estradiol, [1,2,6,7-^{3}H]testosterone, [1,2,6,7-^{3}H]progesterone, 5α[1,2,4,5,6,7-^{3}H]dihydrotestosterone with specific activities of 197, 139, 131, and 138 Ci/mmol, respectively, were obtained from Amersham, England. Nonlabeled steroids were purchased from Sigma, U.S.A.: Ultrogel AcA 44 was from Industrie Biologique Francaise; DEAE–Sephadex A-50, Sepharose 6B, AH–Sepharose were from Pharmacia, Sweden; DEAE–Toyopearl 650M was from Toyo Soda, Japan; Silica gel-coated aluminium plates were from Kavalier, Czechoslovakia; Viscking dialyzing tubes type 27/32 and 8/32 were purchased from Serva, F.R.G. Estradiol–Sepharose with immobilized steroid concentration 1 μmol per 1 g of wet gel was synthesized as described earlier [16].

Hydroxysteroid dehydrogense activity assay

During fluorimetric determinations of the EHSD activity we measured the fluorescence of NADPH accumulated or utilized for the oxidation or reduction of steroid substrates. The 3α-HSD activity was measured from the rate of oxidation of the 3α-hydroxy group of androsterone; the 3β-HSD activity—from the oxidation rate of the 3β-hydroxy group of epiandrosterone; the 17β-HSD activity—from the oxidation rate of the 17β-hydroxy group of 5α-dihydrotestosterone; 20α-HSD activity—from the rate of reduction of 20-keto group of progesterone. The standard test systems contained, respectively, 18, 18,

9 and 4 μM of steroid substrate and 25, 25, 25 μM of NADP or 10 μM of NADPH in a volume of 2 ml of TKE-buffer. The reaction was initiated by adding of 10–100 μl of the protein solution. All measurements were performed at 37°C continuously during 4–10 min using Hitachi MPF-4 spectrofluorometer (λ_{excit} = 340 nm, λ_{emis} = 460 nm, slit width 10 nm).

Identification of HSD reaction products and measurements of reaction rates were performed by using a chromatographic analysis of [^{3}H]steroids. The incubation system contained [^{3}H]substrate, 25–50 μM cofactor and 10 μl of protein in a final volume 200 μl of TKE-buffer. The incubation was performed for 5–30 min at 37°C. The reaction was stopped by cooling and subsequent extraction of steroids with ethylacetate (0.5 ml × 2). The extract was dried with anhydrous Na_2SO_4, evaporated, and chromatographed on Silufol plates (9 cm long) in a chloroform/ethyl ether/methanol system (8:2:0.35). When 5α[^{3}H]-dihydrotestosterone was used as a substrate in the reduction reaction the extract was chromatographed twice in the same direction on 15–20 cm long plates using toluene/acetone system (3:2). Prior to chromatography 2–4 μg of the unlabeled steroid substrate and product were added as an internal standard. The final yield of radioactivity was 60–80%. The HSD activity was expressed as a percentage ratio of the [^{3}H]product to the net [^{3}H]product and [^{3}H]substrate. The [^{3}H]products were identified on the basis of their chromatographic mobility relative to nonlabeled standards (as visualized by chromogenes in u.v., staining in I_2 vapours or by concentrated H_2SO_4) in 2–3 chromatographic systems. Besides, the products of 5α[^{3}H]dihydrotestosterone 5α[^{3}H]androstane-3α, 17β-diol and 5α[^{3}H]androstane-3β,17β-diol isolated by TLC were identified by recrystallization from methanol with corresponding unlabeled steroids. The specific radioactivity of crystals did not differ from that of the initial solution. Similarly, the identity of the [^{3}H]testosterone oxidation product to 4-androsterone-3,17-dione was demonstrated. Additional evidence of this product formation was obtained by chemical modification of the [^{3}H]product in a reaction with dicarbethoxyhydrasine with a subsequent chromatographic analysis and recrystallization. The mobility of the [^{3}H]product of [^{3}H]progesterone reduction in different chromatographic systems was close (but not identical) to that of 20β-hydroxyprogesterone. The presence of a hydroxy group in the [^{3}H]product was evidenced from its ability to react with acetic anhydride. Incubation of the [^{3}H]product and 20β-hydroxyprogesterone with this reagent caused an increase of their chromatographic mobilities (which for acetylated forms is higher for 20α-hydroxyprogesterone). The progestagens, which have no hydroxy group (progesterone, 5α-dihydroprogesterone) did not change their mobility after such incubation.

It is noteworthy that the degree of the observed metabolic conversions of steroids was strongly proportional to the concentration of the isolated protein. Substitution of NADP(H) for NAD(H) led to a practically complete disappearance of the HAD activity. In kinetic studies protein concentrations and time of exposure were chosen in such a way that metabolic conversions involved not more than 15–20% of the steroid substrate.

Measurement and characterization of hormone-binding activity of EHSD

To determine the hormone-binding activity of EHSD during its isolation, 100 μl of the tested material was incubated for 5 min at 0–4°C with ~10 Bq of [^{3}H]steroid in a final volume of 110–130 μl. When highly-purified protein preparations were used, ovalbumin was added to the samples in a final concentration of 1 mg/ml to prevent charcoal adsorption of EHSD. The bound and unbound hormones were separated by dextran-coated charcoal adsorption for 1 min. The final concentration of charcoal (Norit A) was 0.5–0.66%, that of dextran-80 was 0.1–0.13%. After 5-min centrifugation at 3000 *g* aliquots of the supernatant were assayed for radioactivity. Specific binding of [^{3}H]ligands was measured from the difference in the radioactivities of samples which contained (nonspecific binding) or did not contain (full binding) an excess (1000 ng) of the same unlabeled ligand, or as the difference in bound radioactivity, measured in the samples with and without EHSD. All measurements were performed in duplication. The affinity and binding capacity of EHSD for [^{3}H]steroids were measured by equilibrium dialysis. To this purpose 1.5–2.0 μg of purified protein and 200 μg of ovalbumin (total volume 200 μl) were dialyzed upon periodic stirring for 24–36 h at 4°C against 5 ml of a TKE-buffer containing ~10 Bq [^{3}H]steroid and 4–2000 ng of the same unlabeled ligand. Radioactivity was measured in aliquots (150 μl) of solution inside and outside of the dialysis sacs. The values of apparent equilibrium association constant (K_a) and the concentration of binding sites (b_{max}) were determined by the Scatchard analysis [17].

Molecular properties of EHSD

The purity of EHSD preparations and the lengths of its polypeptide chains were estimated by vertical disc electrophoresis on 10% polyacrylamide gel slabs (1 mm thick) with sodium dodecyl-sulphate [18]. Visualization of polypeptide bands was performed after fixation in 10% trichloroacetic acid for 30 min using 0.25% Coomassie R-250 in 10% acetic acid–40% isopropanol for 2 h. The dye excess was removed using 10% acetic acid. Electrophoresis without denaturating agents was performed in 7.5% PAG, pH 8.9. One of the sample tracks was stained with Coomassie, the other was cut into fractions (2.3 mm thick) and homogenized; aliquots of homogenate were incubated with [^{3}H]steroids in the presence of the corresponding cofactor. The resulting products were separated from the substrates by TLC and measured for radioactivity. Hydrodynamic properties

of EHSD were studied by centrifugation in sucrose density gradients (5–15%) and by gel filtration on Sepharose 6B columns (1.6 × 30 cm) [2]. The values of sedimentation coefficient and Stokes radius for EHSD were determined from maximal activities of bound [^{3}H]progesterone and 3α- or 17β-HSD. The molecular mass and values of friction coefficient ratios were computed according to [19], taking the partial volume of the protein to be equal to 0.74 cm^3/g.

Radioactivity was measured on a Rackbeta 1217 (LKB) liquid scintillation counter in a dioxane-based scintillator fluid [20]. Effectiveness of counting was 340–35%. Protein concentration was determined by Coomassie staining [21].

RESULTS

During gel filtration on Sepharose 6B columns of male and female rabbit liver cytosol, preincubated with [^{3}H]estradiol, the bound radioactivity is eluted in three peaks with $V_e/V_0 \sim 1.5$, 2.1, and 2.6 (Fig. 1). The first peak is presumably an estrogen receptor; its bound radioactivity can be fully displaced by a 500-fold excess of unlabelled estradiol. The radioactivity in the second peak is displaced only partly under these conditions, while that in third peak is hardly displaced whatsoever. This finding points to a lower affinity and/or to a very high capacity for estradiol of the second and third components as compared to the first component. The observed phenomenon remarkably resembles a situation which takes place in liver cytosols of male rats and of male and female guinea-pigs, from which the UEBP-like proteins were isolated [2, 10, 22]. This led us to apply the UEBP purification procedure for the isolation of the second estradiol-binding component from rabbit lever cytosol. After the capability of the protein to interact with

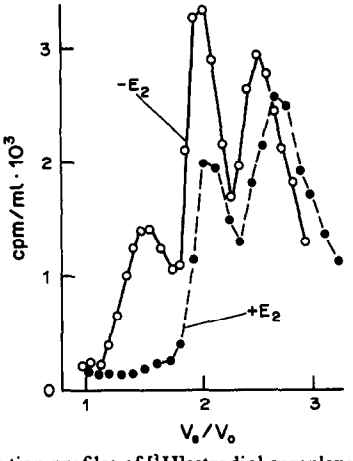

Fig. 1. Elution profiles of [^{3}H]estradiol complexes with male rabbit liver cytosol proteins obtained by Sepharose 6B column chromatography. 1 ml (20 mg of protein) of cytosol was incubated for 45 min at 0–4°C with [^{3}H]estradiol in the absence (−E_2) or presence (+E_2) of a 500-fold excess of unlabeled estradiol and applied to a column (1.6 × 30 cm) of Sepharose 6B, V_0—void volume, V_e—elution volume.

testosterone and progesterone with a greater intensity than with estradiol had been demonstrated, the course of routine fractionation was followed by measuring the progesterone-binding activity.

Estrophilic hydroxysteroid dehydrogenase was isolated by the method used for purification of UEBP [16, 23] with the following modifications: (i) 10 mM Na_2MoO_4 and 0.5 mM phenylmethyl-sulphonyl fluoride were additionally introduced to the buffer used for homogenization; (ii) the concentration range of ammonium sulphate was broadened from 55–75% up to 70–75% of saturation; (iii) dithiothreitol was excluded from the buffer used for chromatographic procedures; and (iv) the protein retained on estradiol–Sepharose was eluted by a solution containing 15 μg/ml of progesterone with or without 15 μg/ml of estradiol instead of estriol.

The liver(s) of 1–2 adult male rabbit(s) was (were) perfused with cold 0.9% NaCl, minced and homogenized in a Teflon–glass homogenizer for 1 min, 10–15 strokes of pestle in 10 mM Tris–HCl, 10 mM KCl, 1 mM EDTA, pH 7.5 (TKE-buffer) containing 10 mM Na_2MoO_4, 6 mM dithiothreitol and 0.5 mM phenylmethylsulphonyl fluoride at 2:1 (v/w). This and subsequent procedures were performed at 0–4°C. The homogenate was centrifuged for 1 h at 40,000 *g*; the supernatant was used to isolate a protein fraction precipitated at 50–75% of $(NH_4)_2SO_4$ saturation. (In this fraction almost the whole progesterone-binding activity of liver cytosol was found. The distribution of other steroid-binding activities as well as of HSD activities in the 0–50% and 75–100% fractions was not investigated.) The material was dissolved in a homogenization buffer (6–10 ml), applied to a column (4.5 × 75 cm) of Ultrogel AcA 44 and eluted with a TKE-buffer at a rate of about 2–3 ml/min. The resulting fractions were assayed for hormone-binding and enzymatic activities (Fig. 2A and B). It can be seen that with exception of the 20α-HSD activity and, partly, of the estradiol-binding activity, the enzymatic and hormone-binding activities are eluted within one compact region. The fractions containing the hormone-binding activity, were combined, applied to a column (2 × 12 cm, 1.5 g) of DEAE–Sephadex A-50 equilibrated with a TKE-buffer, and eluted at a rate of 1.5 ml/min. The column was washed with 50 ml of TKE-buffer. The proteins were eluted in 400 ml of a linear gradient of NaCl (0–0.3 M) in TKE-buffer. At this stage of purification (Fig. 2C and D) the hormone-binding activity was separated from the bulk of 3α, 20α- and, partly, 17β-HSD activities (see also Table 3). It should be noted that the observed difference in the relative binding of progesterone and estradiol in the peaks in Fig. 2A, C and E is a result of some variations in the concentrations of [^{3}H]steroids used in separate experiments. The ratio of the number of binding sites for the three [^{3}H]steroids does not change during chromatographic procedures as determined by the Scatchard analysis (data not shown). The fractions containing the

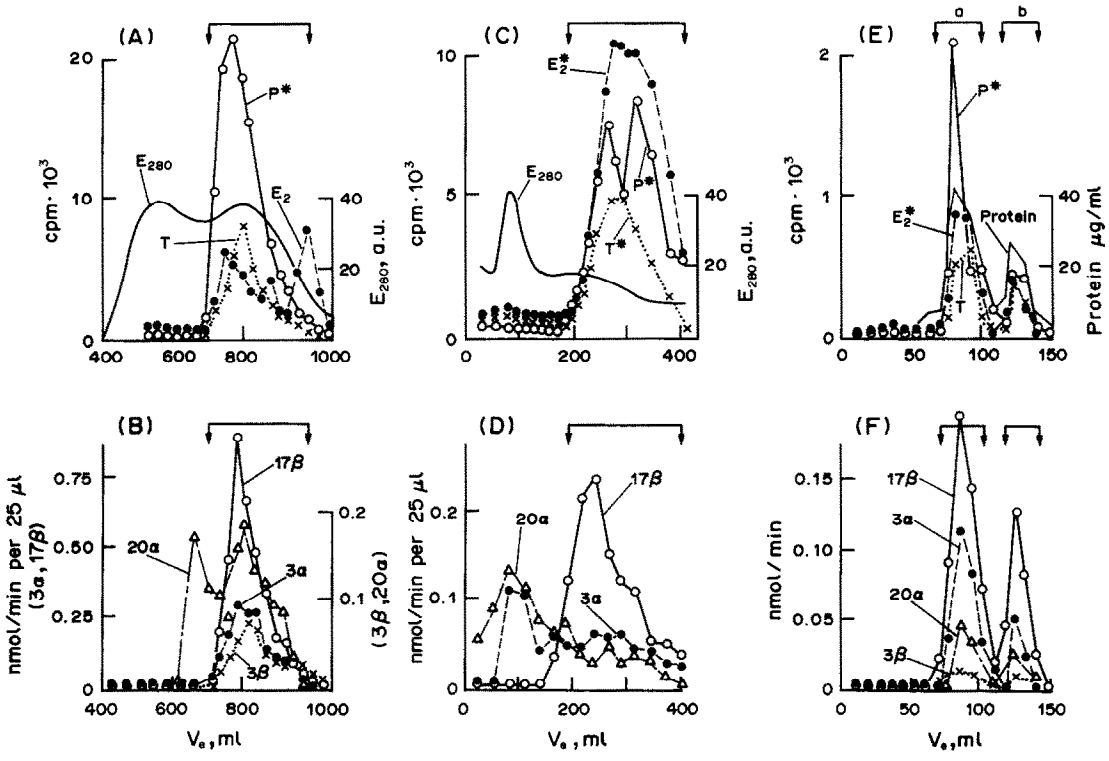

Fig. 2. Purification of rabbit liver EHSD. Ammonium sulphate (50–75% of saturation) precipitated cytosolic protein fraction has been used as a starting material. (A, B) Gel-filtration on a Ultrogel AcA 44 column. (C, D) Ion-exchange chromatography on a DEAE–Sephadex A-50 column. (E, F) Additional fractionation on a DEAE–Toyopearl 650M column after affinity chromatography on estradiol–Sepharose. (A, C, E) Steroid-binding activity and protein. (B, D, F) Hydroxysteroid dehydrogenase activity. V_e—elution volume (C, D, E, F: O-application of NaCl gradient of concentration). The fractions used in the following purification steps (A–D) or experiments (E, F) are marked by brackets. P*, T*, E_2^*—bound [^{3}H]progesterone, [^{3}H]testosterone, and [^{3}H]estradiol in 100 μl; 3α, 3β, 17β and 20α-corresponding dehydrogenase activities. (F) 3α- and 17β-HSD activities are expressed per 25 μl, 3β- and 20α-HAD activities—per 50 μl.

hormone-binding activity were combined and applied to successively connected columns of AH–Sepharose (2 × 3 cm) and estradiol–Sepharose (2 × 6 cm) equilibrated with a TKE-buffer containing 0.5 M NaCl. The elution rate was 1.5 ml/min. The affinity column was then washed for 16–20 h with 1.5–21 of the same solution. EHSD was eluted with a solution of progesterone (15 μg/ml) and estradiol (15 μg/ml). The fractions containing the Coomassie-stained material were combined, dialyzed for 20 h against 1.51 × 2 of a TKE-buffer, and concentrated on a mini-column (0.6 × 2 cm) of DEAE–Sephadex A-50. The protein eluted form this column with 0.5 M NaCl in a TKE-buffer (0.6–1.0 mg/ml, 3–4 ml), was dialyzed against 0.51 × 2 of a TKE-buffer for 20 h and stored at 4°C. Upon SDS–PAGE the resulting protein preparation gives one Coomassie-stained polypeptide band with $M_r \sim 39{,}000$. This value markedly exceeds that for UEBP, isolated by the same method [23]. In some experiments this preparation was additionally fractionated on a column (0.8 × 30 cm) of DEAE–Toyo-pearl 650 M equilibrated with a TKE-buffer. Elution was performed with 150 ml of a linear gradient of NaCl (0–0.15 M) in a TKE-buffer at a rate of 1 ml/min. This additional fractionation step (Fig. 2E and F) gives two protein fractions designated as *a* and *b*, which possess similar or identical properties. Both fractions bind estradiol, testosterone and progesterone and express 3α-, 3β-, 17β-, 20α-HSD activities. The ratios of all activities in the two fractions reflect the distribution pattern of the protein. Additional evidence of similarity of the both protein with regard to their affinity and binding capacity for progesterone. Michaelis constants, maximal rates, inhibitory constants of hexestrol for some EHSD-catalyzed reactions is given in Table 1. Moreover, the two fractions have similar molecular sizes, as measured by SDS–PAGE (Fig. 3). and by gel filtration and sucrose density centrifugation (Table 2). An electrophoretic analysis without denaturing agents revealed that each fraction is represented by a number of subfractions (Fig. 4A). It is important that the majority of these subfractions are common for fractions *a* and *b*, although their ratios are different. These data together with the observed homogeneity of protein molecules in respect of their molecular sizes testify to the heterogeneity of the isolated protein in terms of its molecular charge which may, at least partly, be due to conformation changes of the protein. The isolated subfractions seem to possess similar enzymatic properties. Figure 4B–E

Table 1. A comparison of properties of rabbit liver EHSD fractions *a* and *b* (pH 7.5). The number of determinations is given in parenthesis

Parameter	Fraction *a*	Fraction *b*
Apparent equilibrium association constant with progesterone ($\times 10^7\ M^{-1}$)	2.25 (2)	1.80 (2)
Number of binding sites for progesterone ($\times 10^{-8}$ mol per mg of protein)	1.14 (2)	0.88 (2)
Oxidation of 5α-dihydrotestosterone 17β-hydroxy group, K_m (μM)	1.68 (2)	1.24 (2)
V_{max} (μmol/min per mg of protein)	0.37 (2)	0.26 (2)
K_i for hexestrol (μM)	0.040 (2)	0.054 (2)
Oxidation of androsterone 3α-hydroxy group K_m (μM)	6.40 (2)	5.83 (2)
V_{max} (μmol/min per mg of protein)	0.11 (2)	0.15 (2)
K_i for hexestrol (μM)	0.042 (2)	0.036 (2)
α-Reduction of progesterone 20-keto group, K_m (μM)	0.31 (3)	0.33 (3)
V_{max} (nmol/min per mg of protein)	3.5 (3)	3.0 (3)
K_s for substrate inhibition (μM)	1.9	1.1
K_i for hexestrol (μM)	2.4	1.2

shows that all subfractions express all the four types of the HSD activity: 3α, 3β, 17β, 20α. For a more detailed analysis these subfractions must be isolated in preparative quantaties. By reason of the apparent

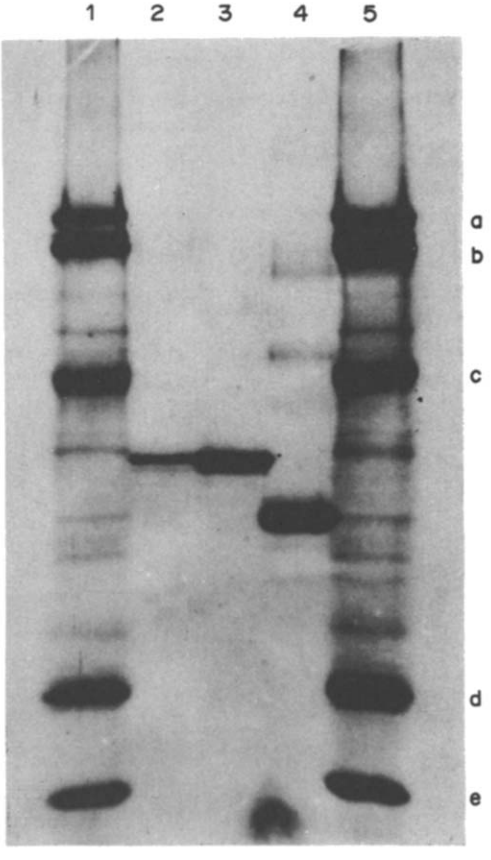

Fig. 3. SDS–PAGE of purified EHSD preparations. (1) 5-standards (a—transferrin, b—BSA, c—ovalbumin, d—myoglobin, e—cytochrome *c*). (2) Fraction *b*, 3—fraction *a*, 4—purified UEBP.

Table 2. Size characteristics of rabbit liver EHSD molecules. The number of determinations is given in parenthesis

Parameter	Fraction *a*	Fraction *b*
Sedimentation coefficient ($S_{20,w}$)	3.32 ± Q12 (3)	3.34 ± 0.03 (3)
Stokes radius (nm)	2.40 (2)	2.45 (2)
Friction coefficient ratio (f/f_0)	1.12	1.14
Molecular mass:		
sedimentation and filtration	34,700 ± 1200	35,600 ± 300
SDS–PAGE	39,500 ± 600 (5)	

functional homogeneity of the protein in further studies we avoided its additional fractionation and used the preparation, obtained at affinity chromatography step. The enzymatic activity was measured at different stages of the EHSD purification procedure (Table 3). The data are yet of a limited value because of predominance of nonEHSD forms of HSD in the initial material. If the yield of the purified protein is taken for 25% (i.e. that observed after UEBP isolation from rat liver by means of similar method [16]) the

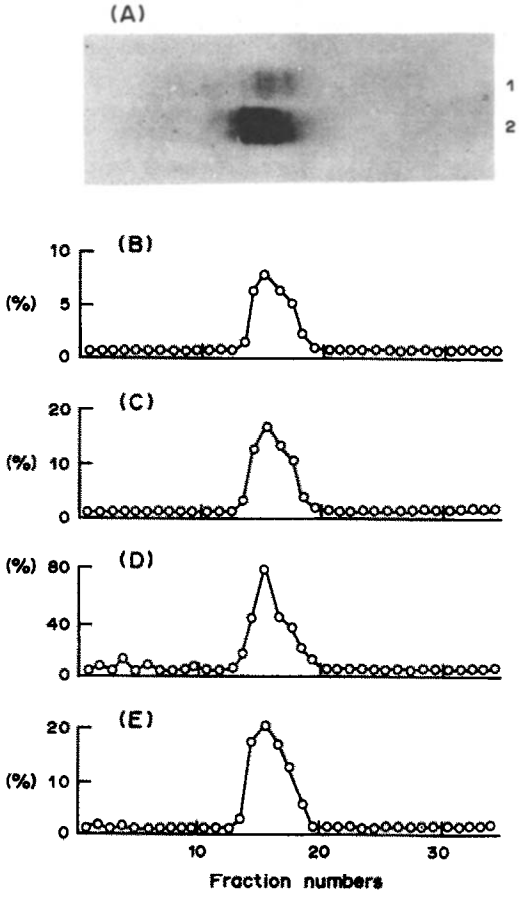

Fig. 4. Electrophoresis in 7.5% PAG of purified EHSD preparations. (A) Coomassie staining (1—fraction *b*, 2—fraction *a*). (B–E) 3α-, 3β-, 17β- and 20α-HSD activities, respectively in fraction *a*. The track was cut into pieces (2.3 mm thick), and each was homogenized in 200 μl of TKE-buffer. Aliquots of homogenate (30 μl in D or 50 μl in B, C, E) were incubated with 2000 Bq of 5α[^{3}H]dihydrotestosterone (B, C), [^{3}H]testosterone (D) or [^{3}H]progesterone (E) and 25 μM NADPH (B, C, E) or $NADP^+$ (D) for 15 min at 37°C. The results were expressed in % of the [^{3}H]product formed.

Table 3. HSD activities in EHSD preparations at different steps of purification

Purification stage	Protein (mg)	Specific activity (nmol/min per mg) 3α	3β	17β	20α	Yield (%) 3α	3β	17β	20α
Cytosol	2880	26.8	11.4	34.0	1.44	100	100	100	100
$(NH_4)_2SO_4$	1300	5.6	0.93	17.3	1.24	9.7	3.8	24.0	40.5
Ultrogel AcA 44	423	15.2	3.08	58.0	5.0	8.25	3.96	25.1	51.0
DEAE–Sephadex A-50	29.2	85.0	8.1	367.0	26.0	3.22	0.72	10.9	18.3
Estradiol–Sepharose	3.2	78.5	4.85	156.0	10.3	0.40	0.06	0.63	0.97

Table 4. Substrate specificity of EHSD

Steroid[a]	K_m (μM)	V_{max} (μmol/min per mg)	V_{max}/K_m (l/min per mg)
3-Reduction			
17β-Hydroxy-5β-androstan-3-one (5β-dihydrotestosterone)	S.I.[b]		
17β-Hydroxy-5α-androstan-3-one (5α-dihydrotestosterone)	2.86 ± 0.43 (4)	0.26 ± 0.44 (4)	0.044
5α-Androstan-3-one	62	0.40	0.006
2α-Methyl-17β-hydroxy-5α-androstan-3-one	ND[c]		
17-Reduction			
4-Androstene-3,17-dione	2.2	0.10	0.045
1,4-Androstadiene-3,17-dione	2.1	0.07	0.033
4-Androstene-3,11,17-dione (adrenosterone)	5.0	0.80	0.160
3β-Hydroxy-5-androstene-17-one (dehydroepiandrosterone)	5.2	0.09	0.017
3β-Hydroxy-5β-androstan-17-one	3.2	0.12	0.037
3α-Hydroxy-3β-androstan-17-one (etioholanolone)	1.7	0.08	0.047
3β-Hydroxy-5α-androstan-17-one (epiandrosterone)	1.9	0.12	0.063
3α-Hydroxy-5α-androstan-17-one (androsterone)	1.80 ± 0.39 (3)	0.12 ± 0.02 (3)	0.067
3α-Hydroxy-5β-androstane-11,17-dione	7.6	0.15	0.020
3-Hydroxy-1,3,5(10)-estratrien-17-one (estrone)	1.6	0.02	0.012
2-Methoxy-3-hydroxy-1,3,5(10)-estratrien-17-one	5.5	0.10	0.018
20-Reduction			
4-pregnene-3,20-dione (progesterone)	0.40 ± 0.07 (13)	0.0046 ± 0.0011 (13)	0.011
17α-Hydroxy-4-pregnene-3,20-dione (17α-hydroxyprogesterone)	ND		
11β,21-Dihydroxy-4-pregnene-3,20-dione (corticosterone)	ND		
11β,17α,21-Trihydroxy-4-pregnen-3,20-dione (cortisol)	40	0.08	0.002
9α-Fluoro-11β,16α,17α21-tetrahydroxy-1,4-pregnadiene-3,20-dione 16,17-acetonide (triamcinolone acetonide)	ND		
17α,21-Dihydroxy-4-pregnene-3,11,20-trione (cortisone)	ND		
17α,21-Dihydroxy-4-pregnene-3,20-dione (11-deoxycortisol)	ND		
21-Hydroxy-4-pregnene-3,11,20-trione (11-dehydrocorticosterone)	4.4	0.04	0.009
3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione (3α,5β-tetrahydrocortisol)	ND		
3,17-Reduction			
5α-Androstane-3,1-dione	1.5	0.22	0.147
5β-Androstane-3,17-dione	S.I.		
3,20-Reduction			
5α-Pregnane-3,20-dione	0.23	0.07	0.305
5β-Pregnane-3,20-dione	S.I.		
3-Oxidation			
3α-Hydroxy-5α-androstan-17-one (androsterone)	5.77 ± 0.51 (13)	0.21 ± 0.05 (13)	0.036
3α-Hydroxy-5β-androstane-11,17-dione	6.7	0.33	0.049
3β-Hydroxy-5α-androstan-17-one (epiandrosterone)	100	0.10	0.001
3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione (3α,5β-tetrahydrocortisol)	15.3	0.55	0.036
17-Oxidation			
17β-Hydroxy-4-androsten-3-one (testosterone)	S.I.		
17β-Hydroxy-1,4-androstadien-3-one-(Δ^1-testosterone)	S.I.		
17β-Hydroxy-4-estren-3-one (19-nortestosterone)	1.2	0.04	0.033
17β-Hydroxy-5α-androstan-3-one (5α-dihydrotestosterone)	1.98 ± 0.30 (16)	0.36 ± 0.04 (16)	0.181
5α-Androstan-17β-ol	4.6	0.06	0.013
2α-Methyl-17β-hydroxy-5α-androstan-3-one	10	0.5	0.050
3,17-Oxidation			
5β-Androstane-3α,17β-diol	2.8	0.30	0.107
5β-Androstane-3β,17β-diol	7.2	0.67	0.093
5α-Androstane-3β,17β-diol	2.5	0.22	0.088
5α-Androstane-3α17β-diol	10.5	1.17	0.111
5-Androstane-3β,17β-diol	10.0	0.22	0.022
17α-Methyl-5-androstene-3β,17β-diol	15.3	0.09	0.006

[a] Reductive reactions of steroids were studied with 10 μM of NADPH, oxidative reactions—with 25 μM $NADP^+$, pH 7.5.
[b] S.I.—substrate inhibition, i.e. decrease of reaction rate at high substrate concentrations.
[c] ND—not detected.

purification will be ~180-fold, e.g. EHSD makes up to about 0.5% of the net cytosolic protein. The activity of EHSD in this case will make 1.6, 0.23, 2.5, and 3.9% of the 3α-, 3β-, 17β- and 20α-HSD activities in the cytosol, respectively.

The isolated EHSD possesses a broad substrate specificity being able to use androgens, progestogens and, in a lesser degree, estrogens as substrates (Table 4).

The hormone-binding and enzymatic activity of EHSD is stable at 37°C for at least 20 min. After 5 min heating at 50°C, the hormone-binding activity is reduced by 45–75%, and at 65°C it declines to zero values. Lyophilization of the protein from 10 mM NH_4HCO_3 or from distilled water leads to the irreversible loss of its solubility. Freezing and storage at −20°C results in the loss of the hormone-binding activity which can be partly restored (up to 30–50%) after 1–3 days incubation at 4°C. Storage of the preparation at 4°C for 2–3 weeks does not evoke any noticeable changes in its hormone-binding activity; however the enzymatic activity shows a tendency to decrease presumably due to the increase in the K_m-values for some substrates.

DISCUSSION

EHSD catalyzes an unprecedentedly high number of steroid conversion reactions, although the principle of polyfunctionality of hydroxysteroid dehydrogenases is now well established. Many HSD of microorganisms and mammals are bifunctional [24–27]. One isozyme of 17β-HSD of female rabbit liver possesses a slight (but measurable) activity of the third type [25]. EHSD expresses a very weak 3β-HSD activity in a direct reaction with epiandrosterone as compared with its 3α-HSD activity with androsterone. However, the reverse reaction, e.g. the reduction of the 5α-dihydrotestosterone 3-keto group, proceeds 2 times more intensive towards the formation of 3β-derivative as compared with the 3α-derivative. Therefore one may expect that some other HSDs will show the polyfunctionality comparable with that of EHSD.

The activity of EHSD is minor relative to other HSDs from rabbit liver cytosol. This does not mean, however, that its contribution to the steroid biodynamics is equally little. The K_m-values for a number of substrates for EHSD are by about one order of magnitude lower than the K_m-values for the majority of other HSD isozymes earlier isolated from rabbit liver [25, 28]. This suggests that EHSD plays a role in steroid metabolism at physiological concentrations of substrates. Besides, the relatively high affinity for steroid ligands and the high contents of EHSD in the liver may provide the participation of this protein in steroid biodynamics due to reversible, nonenzymatic steroid–protein interactions.

The observed heterogeneity of EHSD is not a unique feature of mammalian HSDs. The protein heterogeneity established by other authors may in some cases be accounted for by the partial degradation of the enzyme during its isolation, or be due to the original heterogeneity of the protein in the other [24, 25, 28–36]. In case of EHSD, the heterogeneity may be partly due to conformational changes of the protein, which leads to the exposure on the surface of its molecules of different number of charged amino acid residues. This hypothesis is based on the coincidence of Coomassie-stained protein bands during PAGE of fractions separated by ion-exchange chromatography, as well as on the ability of some factors (cofactors, steroids, pH) to change the equilibrium between the hormone-binding and hormone-nonbinding forms of EHSD [35].

The microheterogeneity was also found in preparations of rat UEBP isolated by the same method [23]. Unlike rabbit EHSD, UEBP does not express any oxidoreductase activity with a broad spectrum of steroid substrates [33]. Nevertheless, these two proteins possess a number of common properties including the existence of at least of two separate binding subsites [2, 23, 34, 35]. Therefore these proteins may be the members of one protein family.

The convenient and rapid method for preparative isolation of highly purified fraction of HSD from rabbit liver described herein is based on a high affinity of the enzyme for immobilized estradiol. One can suppose that other immobilized steroid ligands of EHSD (nonmetabolized progestogens and androgens) will also be effective for purification of this protein.

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