ISOLATION OF ESTROPHILIC FRACTIONS OF RABBIT LIVER HYDROXYSTEROID DEHYDROGENASE

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One of the mechanisms of regional regulation of steroid hormone reception and metabolism may be reversible interaction of these hormones with specific intracellular nonreceptor proteins, or steromodulins [3, 5, 7]. This group of substances includes proteins similar or identical to blood transport proteins, and specialized, tissue-specific proteins of the special estrogen-binding protein (SEBP) type of rat liver [1, 12, 13]. The basic for undertaking the present investigation was provided by results of preliminary experiments showing the presence of a nonreceptor protein, intensively binding ^{3}H-estradiol, in the soluble fraction of rabbit liver. It was found in the course of the work that this protein possesses oxidoreductase activity relative to androgens and gestagens.

EXPERIMENTAL METHOD

Estrophilic fractions of hydroxysteroid dehydrogense (HSDH) were isolated from liver cytosol of male rabbits by the scheme used to purify rat liver SEBP [2, 4], with following modifications: a) the buffer for tissue homogenization also contained 10 mM Na_2MoO_4 and 0.5 mM phenylmethylsulfonyl fluoride; b) the concentration range of ammonium sulfate used to precipitate the cytosol proteins was widened to 50-75% saturation; c) dithiothreitol was excluded from the composition of the buffer used for the chromatographic procedures; d) protein adsorbed on estradiol-sepharose was eluted by a solution containing 15 μg/ml of progesterone and 15 μg/ml of estradiol.

HSDH activity was detected by two methods. Method 1 was based on chromatographic separation of the tritium-labeled reaction product and substrate on Silufol plates. Method 2 consisted on recording changes in fluorescence of NADPH formed or utilized during oxidation or reduction of steroid substrates. The test system in this case (pH 7.5) contained 17 μM androsterone, 8.5 μM 5α-dihydrotestosterone or 4 μM progesterone, and 25 μM NADP or 5 μM NADPH respectively. The reaction was initiated by the addition of 50-100 μliters of the test protein fraction. Fluorescence was recorded continuously for 4-10 min at 37°C on an MPR-4 spectrofluorometer ("Hitachi," Japan). The wavelength of exciting and emitted light was 340 and 460 nm, corresponding to the maxima of absorption and emission of NADPH. The transmission band width was 10 nm. Calibration was carried out with NADPH solutions of known concentration. Tritiated products of the HSDH reaction were identified by comparing the chromatographic mobility of the ^{3}H-compound and of standard samples of steroids, by recrystallization from assumed homonymous unlabeled compounds, and also by chemical modification of the ^{3}H-products.

Hormone-binding activity of the protein fractions was determined by adsorption of the unbound hormone by activated charcoal after incubation of the protein samples with ^{3}H-steroid [1]. Electrophoresis in 10% polyacrylamide gel with dodecylsulfate was carried out by Laemmli's method [8]. The protein concentration was measured by Coomassie staining [6].

Reagents. The (1,2,4,5,6,7-^{3}H)-5α-dihydrotestosterone, (2,4,6,7,16,17-^{3}H)-estradiol, (1,2,6,7-^{3}H)-testosterone, and (1,2,3,6,7-^{3}H)-estradiol, (1,2,6,7-^{3}H)-testosterone, and (1,2,3,6,7-^{3}H)-progesterone were obtained from "Amersham International" (England) and had specific radioactivity of 5.10, 5.17, 3.65, and $3.44 \cdot 10^{12}$ Bq/mmole, respectively. Unlabeled steroids were obtained from "Sigma" (USA) and coenzymes from "Reanal" (Hungary).

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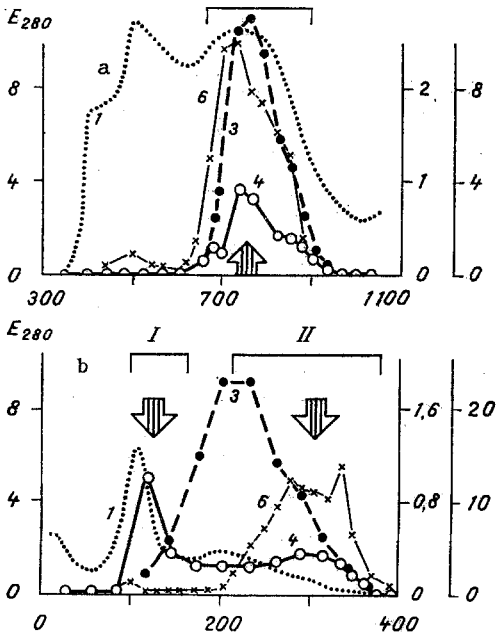

Fig. 1

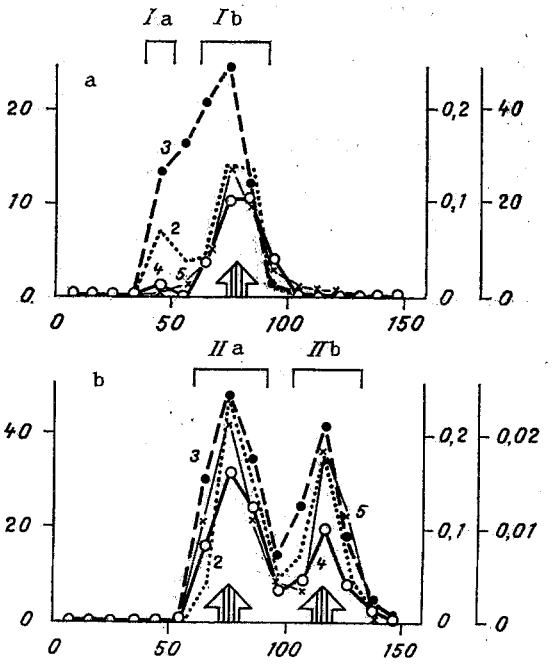

Fig. 2

Fig. 1. Chromatographic stages of isolation of estrophilic forms of hydroxysteroid dehydrogenase from rabbit liver. a) Gel-filtration on column (4.5 × 75 cm) of Ultragel AcA44 of protein fraction precipitated from cytosol from ammonium sulfate (50-75% saturation); b) ion-exchange chromatography on column (2.2 × 15 cm) of DEAE-Sephadex A-50 of material marked by bracket in Fig. 1a, after application of NaCl concentration gradient (0-0.3 M). Here and in Fig. 2: 1) extinction at 280 nm (in conventional units); 3) 17β-HSDH activity of 5α-dihydrotestosterone (scale 1 on the right); 4) 3α-HSDH activity on androsterone (scale 1 on the right); 6) radioactivity of protein-bound ^{3}H-progesterone (scale 2 on the right). Abscissa, elution volume (in ml); arrows indicate maxima of estradiol-binding activity. Ordinate: on left extinction, on right activity of HSDH.

Fig. 2. Additional fractionation of purified preparations of hydroxysteroid dehydrogenase from rabbit liver. a, b) Ion-exchange chromatography on DEAE-Toyopearl 650 M columns (0.8 × 25 cm) of material isolated from fractions I and II (Fig. 1b) by affinity chromatography. Elution by NaCl concentration gradient (0-0.15 M). 2) Protein concentration (scale on left); 5) 20α-HSDH activity on progesterone (scale 2 on right), measured relative to percent of metabolized ^{3}H steroid (a) or fluorometrically (b). Arrows indicate maxima of estradiol-, testosterone-, and progesterone-binding activity. Remainder of legend as to Fig. 1. Abscissa, elution volume, in ml. Ordinate, protein concentration (in μg/mg); on right — HSDH activity.

EXPERIMENTAL RESULTS

On gel-filtration of material obtained by precipitation of cytosol proteins with ammonium sulfate, estradiol-binding activity was eluted in one zone. Marked progesterone-binding activity and also activity of 3α- and 17α-HSDH were found in the same zone (Fig. 1a). Subsequent chromatography on DEAE-Sephadex A-50 led to separation of the estradiol-binding activity into two main fractions. Progesterone-binding activity was found mainly in fraction II. Activity of 3α-HSDH also was divided into two fractions, whereas activity of 17α-HSDH covered both these fractions (Fig. 1b). After separate chromatography of fractions I and II on estradiol-sepharose, the resulting material in each case gave one precipitation band, stained with Coomassie, on electrophoresis with dodecylsulfate (38 kD). Additional fractionation of each preparation on DEAE-Toyopearl 650 M yielded altogether four protein subfractions. The molecular weight of all the polypeptides was about 38 kD. The minor subfraction Ia virtually did not bind steroids and did not exhibit activity of 3α- and 20α-HSDH, but did possess 17β-HSDH activity. Subfractions Ib, IIa, and IIb intensively bound estradiol,

and also progesterone and testosterone. Activity of 3α-, 17β-, and 20α-HSDH was found in all three subfractions (Fig. 2a, b). Elution profiles of hormone-binding and enzymic activity coincided with the elution profile of the total protein. Besides types of activity mentioned above, activity of 3β-HSDH also was discovered in subfractions IIa and IIb.

To demonstrate 3α- and 3β-HSDH activity in purified protein preparations ^{3}H-5α-dihydrotestosterone and HADPH were used. The two tritiated reaction products were isolated by double chromatography in a toluene/acetone (3:2) system. Their mobility coincided with that of 5α-adrostane- 3α,17β-diol and 5α-androstane-β,17β-diol. On recrystallization of the tritiated products with these steroids, the specific radioactivity of the crystals obtained coincided with the initial level. The tritiated product of the 17β-HSDH reaction between ^{3}H-testosterone and HADP was identified by the same method. Proof of the identity of this product to 4-androstene-3,17-dione was obtained also by chemical modification of the steroid in the reaction with dicarbethoxyhydrazone. Essentially, unlike testosterone, estradiol which also is intensively bound by the secreted protein, is oxidized extremely slowly by the enzyme. Only after exposure of ^{3}H-estradiol for 30 min to NADP in the presence of a high protein concentration (140 μg/ml) could a small quantity (5%) of the ^{3}H-metabolite with the mobility of estrone be found. The presence of activity of 20α-HSDH protein was deduced from the formation of the ^{3}H-product in the reaction of ^{3}H-progesterone with NADPH. The ^{3}H-product exhibited stronger polarity than progesterone in different systems of solvents, close to but not identical with the polarity of 20β-hydroxyprogesterone. The presence of a hydroxy group in the ^{3}H-product was proved by its ability to undergo acetylation. Treatment of the ^{3}H-product and of 20β-hydroxyprogesterone with acetic anhydride led to an increase in their chromatographic mobility (which was a little higher for the acetylated forms in the case of 20β-hydroxyprogesterone also). Gestagens without hydroxy groups (progesterone, 5α-dihydroprogesterone) did not change their mobility after such treatment. It must be emphasized that the metabolic conversions of steroids which were observed were absolutely dependent on the protein preparations. Replacement of NADPH by NADH led to the almost complete disappearance of HSDH activity.

Highly purified preparations of several steroidophilic forms of HSDH, exhibiting activity against 3α-, 3β-, 17β-, and 20α-hydroxy groups of steroid hormones, were thus isolated in this investigation. The possibility of combining two types of activity in HSDH from different sources, including rabbit liver, is well known [9-11]. However, the existence of HSDH with four types of activity concurrently, according to our information, has been demonstrated for the first time.

The second important conclusion from the work is that very intensive interaction of steroids (estrogens) with enzyme protein is possible without any significant metabolism of the ligand. This suggests a steromodulin function of the isolated forms of HSDH.

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