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PURIFICATION OF SPECIFIC RAT LIVER ESTROGEN-BINDING PROTEIN BY AFFINITY CHROMATOGRAPHY ON ESTRADIOL-SEPHAROSE

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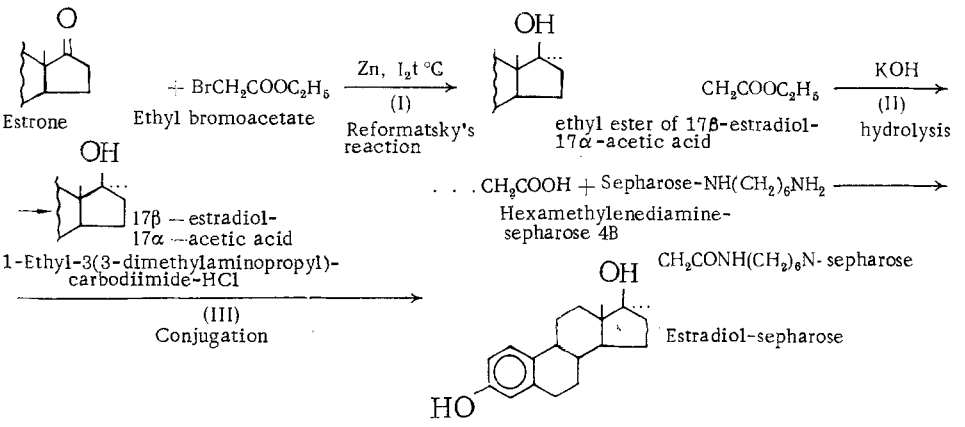
Specific estrogen-binding protein (SEBP) of rat liver differs essentially in certain properties from known receptors and transport proteins [1, 5, 7]. Besides aspects of regulation of the level of this sex-dependent protein in rats already known, new aspects of multifactorial control of determination and regulation of gonad-dependent processes have been discovered [2, 3, 8].

Purification of the SEBP, described in this paper, can be regarded, in particular, as an essential stage in the study of the as yet unknown physiological function of this protein.

EXPERIMENTAL METHOD

Experiments were carried out on sexually mature Wistar or noninbred rats weighing 150-300 g. The animals were decapitated. After perfusion with cold 0.9% NaCl solution the liver tissue from 15 to 20 animals was homogenized in 2 volumes of 10 mM Tris-HCl (pH 7.5) with 10 mM KCl and 1 mM EDTA buffer (TPE buffer), containing 6 mM dithiothreitol. The standard preparation of partially purified SEBP was obtained by precipitation of proteins with ammonium sulfate from the cytosol, gel-filtration on AcA 44 Ultrogel, and ion-exchange chromatography on DEAE-Sephadex A-50 [1]. Before further purification of the SEBP by affinity chromatography, two samples of it were pooled, one of which had previously been dialyzed against TEP-buffer and concentrated 20-30 times on a small (1 ml) column of DEAE-Sephadex A-60.

The stages of synthesis of the affinity sorbent estradiol-sepharose are illustrated in the scheme:



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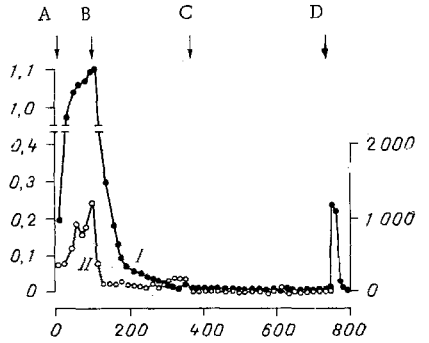

Fig. 1. Affinity chromatography of SEBP on estradiol-sepharose. A) Time of application of standard partially purified SEBP preparation, B) time of application of buffer solution, C) time of connection of second column with affinity sorbent, D) time of application of estriol solution. Abscissa, elution volume (in ml); ordinate: on left – extinction of solution of protein stained with Coomassie at 595 nm (in relative units), on right – radioactivity of protein-bound [^{3}H]estradiol (in cpm).

To a solution of 5 g of estrone and 250 μCi (0.73 μg) of [2,4,6,7-^{3}H]estrone (from Amersham Corporation) in 150 ml of a mixture of freshly distilled absolute dioxan and tetrahydrofuran (1:1) 2 g of freshly prepared zinc filings and 2 ml of ethyl bromoacetate were added during mixing and heating. The reaction was initiated with two or three crystals of metallic iodine and it continued on boiling (about 85°C) for 12 h with periodic addition of zinc (total 10 g) and ethyl bromoacetate (total 10 ml). Complexes of the product with zinc were decomposed with cold dilute HCl at pH 3.0. Steroids were extracted with ethyl ether, and then washed with water to neutral pH values. The extract was dried over $MgSO_4$ and evaporated. Estrone and the ethyl ester of estradiol-17α-acetic acid (with similar mobility in different systems of solvents on silica-gel) were separated from byproducts by column chromatography on silica gel (60-100 mesh) in a hexane–ether (2:1) system. The ester was saponified by treatment with 3.3% KOH in 30 ml of 70% methanol for 18 h at 20°C. The alkali was neutralized with 1 N HCl and the steroids were extracted with ethyl ether. The extract was washed with water, dried over $MgSO_4$, and evaporated. Estrone and estradiol-17α-acetic acid were separated by column chromatography on silica-gel in a benzene–ethyl acetate (4:1 and 1:1) system. The product was isolated from the oily solution by crystallization from hexane–ether (2:1). Yellowish crystals with mp 116-130° were obtained. Yield 25%. The structure of 17β-estradiol-17α-acetic acid was confirmed by data of the mass spectrum (m/e): 330 (M^+), 312 (M^+-H_2O), 270 (M^+-CH_3COOH), 252 ($M^+-CH_3COOH-H_2O$); and infrared spectrum (cm^{-1}): 3340 (–OH), 1690 (⟩C–O).

200 mg of 17β-estradiol-17α-acetic acid was treated in 120 ml of 70% dioxane with 2 g of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl (from Sigma) at pH 4.0 until no further increase of pH took place (about 5 min). Next, 20 g of AH-sepharose 4B from Pharmacia, Sweden, washed with 2 liters of 0.5 M NaCl and 1 liter H_2O, was added to the mixture. The pH was adjusted to 8.0 with dilute KOH. The mixture was stirred for 2 h at 20°C. The gel was then washed with 1 liter of 50% dioxane and 0.5 liter H_2O and used for a second identical conjugation cycle. According to the content of radioactive steroid in the washed gel, the efficiency of the conjugation procedure with respect to steroid was 10%. The degree of occupation with steroid was 2 μmole/g of wet gel.

The estradiol-binding activity of the SEBP in the test fractions was determined as described previously [3].

Electrophoresis of the SEBP preparations in the presence of sodium dodecylsulfate in tubes of 10% polyacrylamide gel and staining of the gels were carried out as in [6]. Bovine serum albumin, ovalbumin, α-chymotrypsinogen, and myoglobin were used as markers.

The protein content was determined by the method in [4], using bovine serum albumin as the standard.

EXPERIMENTAL RESULTS

The standard preparation of partially purified SEBP (80-120 ml) was applied to a column (1.2 × 7 cm, 11 ml) of the affinity sorbent, equilibrated with TPE buffer containing 1 mM dithiothreitol and 0.5 M NaCl. The

TABLE 1. Stage-by-Stage Purification of SEBP

Stage of purification	Total protein content, mg	SEBP, moles/mg protein $\times 10^{-12}$	Total content of SEBP, moles $\times 10^{-9}$	Purification at stage, number of times	Yield at stage, %
Cytosol $(NH_4)_2SO_4$, 55-75% saturation	3313±61 (11)	6,1±0,9 (10)	19,7±3,0 (10)	1	100
	773±118 (9)	10,9±1,5 (9)	9,7±1,9 (11)	2,4±0,4 (8)	53,8±7,3 (10)
Ultrogel AcA 44	227±13 (11)	34,1±4,0 (11)	8,0±1,3 (11)	3,1±0,5 (8)	88,4±8,0 (11)
DEAE-Sephadex A-50	25,5±5,1 (5)	233±58 (5)	6,2±2,7 (5)	13,3±3,5 (5)	88,2±27,9 (5)
Estradiol-sepharose	0,39±0,14 (5)	13800±700 (4)	5,4	59,3	86,3
Total	—	—	—	2260	27,2

Legend. Number of determinations shown in parentheses.

rate of flow of the solution through the column was 15 ml/h. A further increase in rate led to an increase in the yield of SEBP in the unretained fraction (usually not more than 5%) and to the earlier elution of the retained fraction of SEBP (usually not before 300 ml). Proteins nonspecifically retained by the sorbent were eluted with 200 ml of the above-mentioned buffer. Excluding NaCl from the buffer sharply increased adsorption of ballast proteins, probably due to the ion-exchange properties of the unsubstituted amino groups of the AH-sepharose. After removal of the main mass of ballast proteins from the column, a second column was connected to it with a fresh portion of sorbent. The washing of the columns continued for a further 24 h at the rate of 15 ml/h, after which the SEBP was eluted with a solution of the specific ligand estriol (20 μg/ml) at the rate of 60 ml/h (Fig. 1). The resulting preparation, dialyzed for 5 days against 5 liters of 10 mM $CH_3COOH-NH_4OH$ (pH 8.0), and lyophilized gave only one band on electrophoresis under denaturing conditions, of a polypeptide with mol. wt. of 31,200 ± 400 daltons, when applied to the gel in amount up to 50 μg protein, evidence of the high degree of purity of the preparation (evidently at least 95%). Exclusion of the second column with affinity sorbent from the purification scheme did not allow a sufficiently high degree of purification to be achieved, but the use of two identical cycles of affinity chromatography on one column led to a significant decrease in yield of SEBP and did not give stable results as regards the degree of purification of the protein. The resultant purification of SEBP achieved after all stages of purification was 2260 times, with a yield of about 27%; the main losses of SEBP took place in the preliminary stages of isolation (Table 1). Specific estradiol-binding activity of the SEBP in the preparations amounted to 0.45 ± 0.03 mole of steroid per mole of protein (taking the molecular weight of SEBP to be 31.2 kilodaltons).

To conclude, the relative simplicity of synthesis of the immobilized derivative of estradiol used in the work, the integrity of the functional groups of the steroid, and the presence of commercial preparations of [^{3}H]-estrone – the original compound for synthesis – make this ligand a very promising substance for affinity chromatography not only of SEBP, but also of other estrogen-binding proteins, and also for induction of antibodies to estradiol and for the study of interaction between estrogens and cell membranes.

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