EFFECT OF COENZYMES ON BINDING OF STEROID SUBSTRATES WITH RAT LIVER ESTROPHILIC HYDROXYSTEROID DEHYDROGENASE

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Estrophilic NADP(H)-dependent hydroxysteroid dehydrogenase (EHSD), which we isolated from rabbit liver cytosol in the form of a highly purified preparation, possesses 3α-, 3β-, 17β-, and 20α-hydroxysteroid (HSD) activity relative to androgens and gestagens and belongs to the class of high-affinity enzymes.

In relation to the gross metabolism of steroid hormones, the enzymic activity of this protein evidently has no definite role to play, for it amounts to something of the order of 1% of activity of the other HSD of liver cytosol. The study of the hormone-binding properties of EHSD, however, suggests that this protein has an influence on the biodynamics (reception and metabolism) steroid ligands resembling in its type of action the special estrogen-binding protein of rat liver and other proteins (stereomodulins) [4]. Preliminary experiments showed that the inhibitor efficiency of the stilbene estrogen hexestrol depends on the direction of the EHSD-catalyzed reaction of different substrates in the presence of the oxidized or reduced form of the coenzyme. The question accordingly has arisen of the possible role of coenzymes as physiological regulators of the stereomodulin and enzymic function of the isolated EHSD.

EXPERIMENTAL METHOD

EHSD was isolated from liver cytosol of male rabbits weighing 2-4 kg in accordance with the scheme used previously to purify the special estrogen-binding protein of rat liver [3], with slight modifications. Purification was carried out relative to progesterone-binding activity. The scheme included fractionation of proteins with ammonium sulfate, gel-filtration, ion-exchange chromatography, and affinity chromatography on estradiol-sepharose. The isolated protein was concentrated and kept in 10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA buffer (TEK-buffer) at 4°C. During SDS-PAG electrophoresis [5] the preparation gave one band, staining with Coomassie, with mol. wt. of about 35,000. In the absence of denaturing agents the product exhibited marked polydispersity. All protein zones found possessed 3α-, 3β-, 17β-, and 20α-HSD activity.

Enzymic activity was determined by two methods. The first included incubation of the protein preparation with ^{3}H-substrates at 37°C followed by fractionation of the protein preparation with ^{3}H-substrates at 37°C followed by fractionation of the products and substrates by thin-layer chromatography and measurement of radioactivity. With the protein concentrations used (5-20 μg/ml) accumulation of ^{3}H-products followed a linear course with time. The second method of measuring the enzyme kinetics was fluorometric determination of the accumulation or utilization of NADPH in the course of the reaction. For this purpose, during 4-10 min fluorescence of the sample was recorded continuously at 37°C, using exciting light with a wavelength of 340 nm and emitted light with a wavelength of 460 nm, on an MPF-4 "Hitachi" spectrofluorometer (Japan). The kinetics was analyzed in double reciprocal coordinates followed by plotting of secondary graphs [1]. Hormone-binding activity was measured by solid-phase adsorption of the ^{3}H-steroid on activated charcoal, covered with dextran [2]. The parameters of hormone-protein interaction were evaluated by the equilibrium dialysis method [2], using Scatchard plots [6]. Each experiment was repeated 2 or

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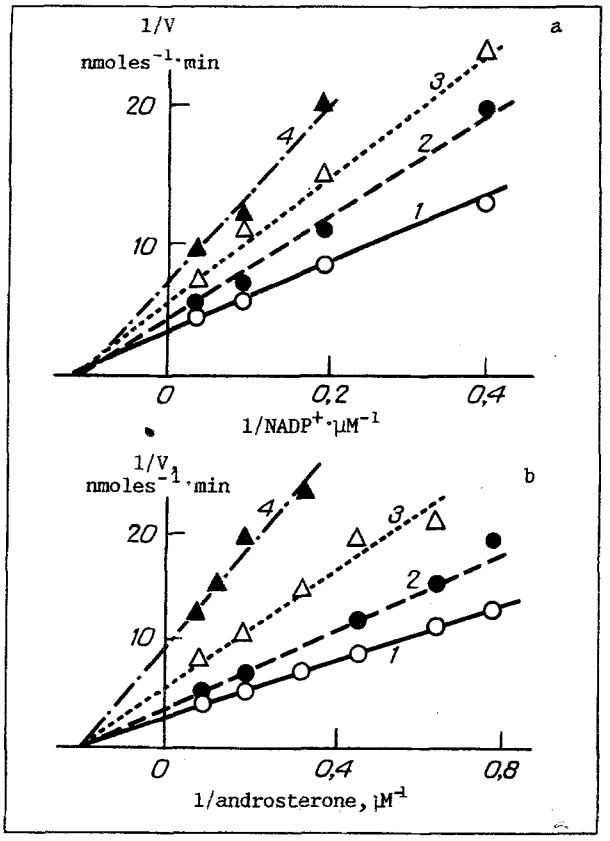

Fig. 1. Investigation of reaction kinetics of oxidation of 3α-hydroxy-group of androsterone, catalyzed by rabbit liver EHSD. 4 μg protein in 2 ml TEK-buffer incubated at 37°C with 2-13 μM androsterone and 2.5-25 μM $NADP^+$. Initial reaction velocity v determined fluorometrically. a) Concentration of androsterone: 1) 13, 2) 5.1, 3) 3.0, 4) 2.1 μM; concentration of $NADP^+$: 1) 25, 2) 10, 3) 5.4, 4) 2.5 M.

3 times. The ^{3}H-steroids used were obtained from Amersham International (England), and had specific radioactivity of 40-140 Ci/mmole.

EXPERIMENTAL RESULTS

The data on the kinetics of oxidation of the 3α-hydroxy-group of androsterone in the presence of $NADP^+$ and EHSD, shown in Fig. 1 and Table 1, suggest that this reaction proceeds by a disordered equilibrium mechanism in the absence of any significant mutual influences of the coenzyme and steroid on their affinity for protein. It is a different matter in the other reactions. During 17β-reduction of the same substrate in the presence of NADPH, the steroid and coenzyme mutually depress affinity for protein (Table 1), whereas in the reaction of oxidation of the 17β-hydroxy group of 5α-dihydrotestosterone the substrate and coenzyme mutually potentiate binding with the enzyme (Table 1). These data suggest that the coenzyme and steroid substrate can either not affect, mutual weaken, or potentiate interaction with the enzyme. The results of the kinetic experiments permit a different interpretation. It was therefore important to determine whether the coenzyme has an influence on affinity of the steroid for protein in a direct experiment. For this purpose the method of equilibrium analysis with ^{3}H-ligands was used. To prevent metabolism of the steroids during incubation, the same form of coenzyme was used as did not maintain enzymic conversions of the test steroid. The results given in Fig. 2 and Table 2 show that coenzymes have a marked influence on the affinity for steroid ligands for EHSD; in the cases of androstenedione and progesterone, $NADP^+$ has a positive action, whereas in the case of testosterone NADPH has a similar action. These results were confirmed by solid-phase adsorption on activated charcoal. Data on the opposite effects of oxidized and reduced forms of the coenzyme on binding of estradiol, a ligand virtually

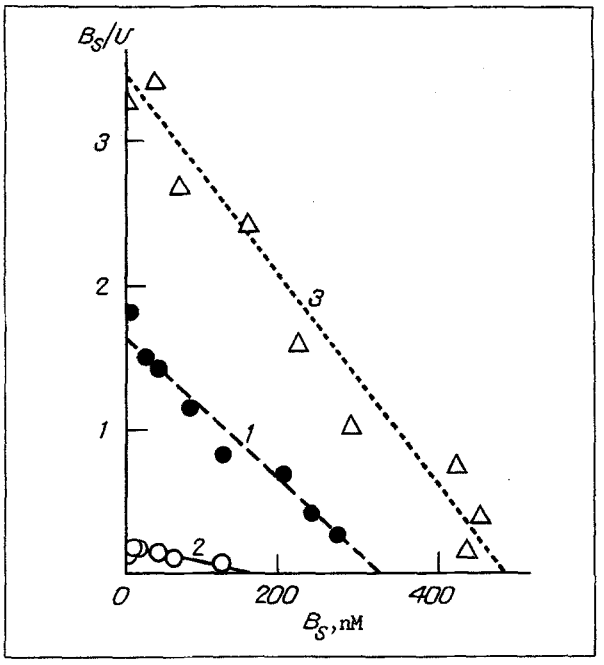

Fig. 2. Scatchard plot analysis of effect of coenzymes on interaction of ^{3}H-estradiol with rabbit liver EHSD. 6.6 μg protein in 200 μl dialyzed for 40 h at 4°C against 5 ml of TEK buffer containing $3 \cdot 10^4$ bq of ^{3}H-estradiol and various concentrations (0-7 μM) of unlabeled hormone in the absence (1) and presence of 50 μM NADPH (2) or $NADP^+$ (3). B_S) Specifically bound, V) unbound hormone.

TABLE 1. Kinetic Parameters of Some Reactions Catalyzed by Rabbit Liver EHSD

Reaction	Substrate	Coenzyme	Parameter				
			V_{max}, μmoles/min/mg	K_M^A, μM	K_S^A, μM	K_M^B, μM	K_S^B, μM
3α-Hydroxy 3-Keto	Androsterone	$NADP^+$	0,23	11,1	10,0	4,2	4,3
17-Keto 17β-hydroxy	Androsterone	NADPH	0,33	9,9	3,2	3,7	1,4
17β-hydroxy 17-Keto	5β-Dihydrotestosterone	$NADP^+$	0,11	2,9	5,9	1,0	2,2

Legend. Index A denotes coenzyme, index B denotes steroid.

TABLE 2. Effect of Coenzymes on Parameters of Interaction of Steroids with Rabbit Liver EHSD

^{3}H-Steroid	Coenzyme	Parameter	
		B_{max}, nmoles/kg	K_a, $M^{-1} \cdot 10^7$
4-Androstene-3,17-dione	—	7,2	0,58
	$NADP^+$, 50 μM	9,8	1,04
Progesterone	$NADP^+$, 50 μM	11,7	3,24
		14,9	5,44
Testosterone	—	11,0	0,91
	NADPH, 50 μM	10,3	3,13
Estradiol		9,9	0,50
	NADPH, 50 μM	5,2	0,10
	$NADP^+$, 50 μM	14,8	0,69

TABLE 3. Effect of Oxidized and Reduced Forms of Coenzyme on Inhibitory Efficiency of Hexestrol in Reactions Catalyzed by Rabbit Liver EHSD (number of determinations shown in parentheses)

Coenzyme	Substrate	Reaction	K_i, μM ($M \pm m$)
NADPH	Progesterone	20-Keto→20α-hydroxy	1,80±0,40(3)
	5α-Dihydrotestosterone	3-Keto→3α-hydroxy	1,65±0,40(2)
	» »	3-Keto→3β-hydroxy	2,25±0,75(2)
$NADP^+$	5α-Dihydrotestosterone	17β-Hydroxy→17-keto	0,047±0,005(5)
		3α-Hydroxy→3-keto	0,041±0,005(5)

not metabolized by the isolated HSD, are particularly interesting. This is evidence of the existence of not only positive, but also negative effects of coenzymes on binding of steroids by the enzyme, in agreement with data given above on the kinetics of reactions catalyzed by EHSD. The results given in Fig. 2 and Table 2 demonstrate the influence of the coenzyme not only on affinity, but also on the accessibility of the enzyme for steroid ligands; these effects, moreover, are evidently identical in direction. Data showing considerable differences in the inhibitory efficiency of hexestrol, a synthetic estrogen of the stilbene series, in reactions of oxidation and reduction of steroid substrates, catalyzed by EHSD, irrespective of the position of the modified function in the substrate molecule (Table 3).

Thus the results as a whole show conclusively that binding sites of coenzymes and steroids in molecules of rabbit liver EHSD can interact with one another. The degree and sign of this interaction are determined by the type of the bound steroid and coenzyme. In conclusion, it must be emphasized that the results provide a sound basis for the hypothetical role of the coenzyme as physiological regulator of the enzymic and stereomodulin functions of the isolated protein.

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