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ESTROPHILIC 3α,3β,17β,20α-HYDROXYSTEROID DEHYDROGENASE FROM RABBIT LIVER—II. MECHANISMS OF ENZYME–STEROID INTERACTION

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Summary—Binding of [^{3}H]estradiol, [^{3}H]testosterone and [^{3}H]progesterone to purified NADP-dependent estrophilic 3α,3β,17β,20α-hydroxysteroid dehydrogenase (EHSD) from rabbit liver cytosol has been examined. The three steroids bind to the enzyme with mode-rate affinity ($K_a \simeq 10^{-17}$ M^{-1} at 4°C) and equal binding capacity. High-rates were shown for both association and dissociation processes. The steroids competitively inhibited the binding of each other to EHSD. At the same time, their relative binding affinities (RBA) were dependent on the nature of [^{3}H]ligand. The results of RBA determinations for 72 steroids and their analogues by inhibition of [^{3}H]progesterone binding to EHSD suggest that androgens and gestagens bind preferentially to the same site on EHSD molecule, while estrogens (at least by their D-ring) bind to another site. The assumption that EHSD molecule has more than one binding site for steroids is corroborated by (i) substrate inhibition revealed for a number of steroids; (ii) the estrogen ability to potentiate 20α-reduction of progesterone; (iii) stimulatory effect of 5α(β)-androstane-3α(β), 17β-diols on [^{3}H]testosterone and progesterone binding; and (iv) reciprocal effect of NADP on [^{3}H]estradiol and [^{3}H]testosterone binding to EHSD. Significant differences in sensitivity to pH and changes in NaCl concentration upon metabolism and binding of various steroids have been found. At concentrations of 16 mM dithiothreitol potentiated catalytic conversion of some steroids and had no effect on metabolism of others. Both the affinity for steroids and binding capacity of EHSD are found to be cofactor-dependent.

It is speculated that EHSD has a complex active center including at least two mutually influencing steroid-binding sites tightly related with cofactor-binding site. The polyfunctionality of EHSD may be due to both the excess of functional protein groups that form individual constellations upon binding of any steroid and also to conformational lability of EHSD molecule implying alternative orientations of steroids at the binding site.

INTRODUCTION

Many of microbial and mammalian hydroxysteroid dehydrogenases (HSD) are polyfunctional, i.e. they catalyze oxidoreduction of more than one oxygenic group in steroid molecules [1–10]. When these groups are at a distance from one another, a steroid may be anchored to the active center in alternative orientations [9–13]. Similar situations may occur with other steroid-binding proteins, for example, hydroxysteroid sulphotransferase that catalyzes the formation of steroid sulphates at positions 3 and 17 [14], 3-keto-steroid-Δ^4-Δ^5-isomerase [15] and *P*-450-dependent hydroxylases [16]. This is confirmed by the fact that the D-rings of estrogens and androgens interact with different loci in *Pseudomonas testosteroni* 3-keto-steroid-Δ^4-Δ^5-isomerase or the unusual estrogen-binding protein (UEBP) from male rat liver [17, 18]. Numerous steroid-binding proteins are capable of binding steroids of different groups and even compounds whose structure markedly differs from that of steroids. For example, 3α-HSDs from mouse and rat brain or liver cytosol in addition to androgens bind and metabolize estrogens, gestagens, prostaglandins, anti-inflammatory steroid and nonsteroid drugs, polycyclic aromatic carbohydrates and exhibits prostaglandin dehydrogenase, carbonyl reductase and dihydrodiol dehydrogenase activities [19–22]. Mouse and guinea-pig liver testosterone 17β-dehydrogenases also show high dihydrodiol dehydrogenase activities [23, 24]. The absence of significant steroid-, regio- and stereospecificity is characteristic of numerous cytochrome *P*-450-dependent steroid hydroxylases [25–27]. A question arises: what are the mechanisms responsible for the polycompetency of steroid-binding sites in such enzymes? In the present study we describe a possible approach to the resolution of this question on the example of estrophilic 3α,3β,17β,20α-hydroxysteroid dehydrogenase isolated from rabbit liver cytosol by affinity chromatography on estradiol–Sepharose [28]. This NADP(H)-dependent enzyme catalyzes 4 types of oxydoreductase reactions on androgens, gestagens and, to a lesser extent, estrogens, i.e. exhibits polyfunctionality. Its activity is inhibited

by submicromolar concentrations of stilbene estrogen hexestrol, thus confirming a relatively high affinity of the protein for estrogens. In addition, EHSD can be easily detected by its binding of [^{3}H]estradiol, [^{3}H]testosterone, and [^{3}H]progesterone [28], which provides a way for direct measurements of the EHSD–steroid interactions. From these considerations, EHSD may be regarded as a more convenient object compared with other enzymes of the steroid metabolism, whose interactions with ligands were examined indirectly: by inhibition of catalytic activities.

EXPERIMENTAL

Materials

The following [^{3}H]steroids from Amersham, England were used: [2,4,6,7,16,17-^{3}H]estradiol (197 Ci/mmol); [1,2,6,7-^{3}H]progesterone (131 Ci/mmol); 5α[1,2,4,5,6,7-^{3}H]dihydrotestosterone (138 Ci/mmol); [1,2,6,7-^{3}H]androst-4-ene-3,17-dione (100 Ci/mmol); [2,4,6,7-^{3}H]estrone (80 Ci/mmol). Most unlabelled steroids were from Sigma, U.S.A. Compounds 6, 62, 65, 71 and 72 were kindly supplied by Professor D. N. Kirk (MRC Steroid Reference Collection, Chemistry Dept, Westfield College, University of London), compounds 10 and 69—by Dr S. N. Ananchenko (Institute of Bioorganic Chemistry, Academy of Sciences, Moscow), compounds 26 and 30—by Professor A. G. Reznikov (Institute of Endocrinology, Kiev). Co-factors were from Reanal, Hungary. EHSD was isolated from rabbit liver cytosol as described in the preceding paper [28]. The protein preparation obtained at an affinity chromatography step was concentrated and stored at 4°C for up to 2 weeks.

Characterization of EHSD

The EHSD enzyme activity was assayed fluorimetrically or by chromatographic separation of [^{3}H]products and substrates [28]. Hormone-binding activity was determined by solid phase adsorption of unbound steroid on dextran-coated charcoal [28]. The standard conditions included the incubation of about 10^3 Bq of [^{3}H]steroid with or without excess of the unlabeled counterpart in 10 mM Tris–HCl, 10 mM KCl, 1 mM EDTAS (pH 7.5) buffer at 0–4°C for 5 min with 1.5–2.5 μg of EHSD in a final volume of 130 μl in the presence of 1 mg/ml of ovalbumin, unless indicated otherwise. Dissociation kinetics for the [^{3}H]steroid–enzyme complexes was examined by the dynamics of bound [^{3}H]steroid after the addition of a 500-fold excess of the same unlabeled steroid. When EHSD hormonal specificity was examined, the samples contained [^{3}H]steroid and 1–1000 ng of its unlabeled counterpart and/or unlabeled compound being tested. The relative binding affinity (RBA) was calculated as the ratio of the concentrations of two ligands (the reference steroid and the compound under examination) that produce 50% inhibition of specific binding of the [^{3}H]steroid [29]. The character of inhibition was assessed by double reciprocal plots [30, 31]. The affinity and binding capacity of EHSD were determined by the method of equilibrium dialysis [28] using the Scatchard plots [32].

To adjust pH to the desired value 50 mM solutions of the following buffers were used: sodium citrate (pH 3.7–6.5), Tris–HCl (pH 6.9–8.6), sodium carbonate (pH 8.9–11.4), pH was measured at 20°C. The protein tested was added in a solution containing (in mM) Tris–HCl 10, KCl 10, EDTA 1 (pH 7.5). The ratio between the volume of the protein solution and the 50 mM buffer solution was varied from 1:9 to 1:200. When the pH effect on the EHSD steroid-binding activity was examined, the protein preparation was incubated with the buffer for 1 h at 0–4°C. In all other experiments EHSD, steroid and the buffer tested were set in contact simultaneously. Effect of NaCl, dithiothreitol and co-factors on EHSD were examined in a similar manner.

RESULTS

Hormone-binding properties of EHSD

According to the results of Scatchard analysis of radioligand binding to EHSD, the enzyme has the highest affinity for progesterone ($K = 3.65 \pm 0.37 \times 10^7\ M^{-1}$, $n = 4$), followed by testosterone and estradiol, whose K_a-values are $0.90 \pm 0.06 \times 10^7\ M^{-1}$ ($n = 4$) and $0.56 \pm 10 \times 10^7\ M^{-1}$ ($n = 4$), respectively. From the nearly linear plots it can be concluded that the binding sites are homogeneous. The concentrations of the binding sites for estradiol, testosterone and progesterone are similar: according to the data of 4 experiments, they are related as $(1.04 \pm 0.17):(0.98 \pm 0.13):1$. The stoichiometric ratio between the concentration of the hormone-binding sites to the protein concentration is 0.38 ± 0.044 ($n = 9$). Additional ion-exchange fractionation of the preparation [28] did not increase the stoichiometric ratio of the progesterone binding (0.4, $n = 2$). The ratio value obtained (< 1) may be due to conformational changes in EHSD molecular that hamper the binding of steroid ligands. The data confirming this assumption are presented below.

The association of the three steroids with the enzyme and dissociation of the protein–steroid complexes are accomplished essentially high rate. The equilibrium of the system in both directions is achieved within 3–5 min (data not shown). The kinetic analysis of dissociation of the protein–hormone complexes showed that this process is biphasic for the three steroids. The component with the higher association rate constant (k_{-1}) contributes up to 50–80% of the steroid binding; its k_{-1} is equal to $(3–7) \times 10^{-2}\ s^{-1}$; however, this value may be underestimated. The second component is characterized by $k_{-1} = (1.1–1.6) \times 10^{-2}\ s^{-1}$. The heterogeneity of the bound ligand pools may be ascribed to conformational heterogeneity of EHSD.

To find out whether estradiol, testosterone and progesterone interact with the same or different enzyme molecules, we have examined the effect of each of these steroids on the binding of two others to EHSD. It can be seen from Fig. 1A that estradiol and testosterone competitively inhibit the binding of [^{3}H]progesterone. The analysis of these data using secondary plots (the slope – the inhibitor concentration) yields the following ratio between $1/K_d(\equiv K_a)$ for progesterone and I/K_i for estradiol and testosterone 1:0.11:0.29 which is close to directly determining the K_a ratio for these steroids (1:0.15:0.25) (see above). Figure 1B illustrates the inhibition of [^{3}H]estradiol binding by progesterone. This inhibition is also of a competitive character. The ratio between I/K_i for progesterone and $1/K_d(\equiv K_a)$ for estradiol was 1:0.21, which is somewhat different from the data obtained previously.

Another protocol was also used to examine the mutual effects of the steroids on their binding to EHSD: we have compared the steroid concentrations that induce equal (50%) inhibition of specific binding of [^{3}H]steroid. The results of a typical experiment are shown in Fig. 2A–C. The ratio between the RBA for estradiol, testosterone and progesterone determined by displacement of [^{3}H]progesterone from its complex with the enzyme was 0.1:0.2:1. A similar ratio was obtained with [^{3}H]testosterone as a ligand (0.1:0.21:1). Within the limits of error this ratio agrees with the ratio of steroid K_a determined by equilibrium dialysis and their I/K_i-values (see above). With [^{3}H]estradiol as a ligand, the situation is entirely different. The RBA ratio for the three steroids was 0.66:1.02:1. Thus, the relative efficacies of estradiol and testosterone in displacement of [^{3}H]estradiol from the enzyme complexes are considerably higher as compared with their relative efficacies in displacement of [^{3}H]progesterone or [^{3}H]testosterone. This discrepancy suggests the presence of more than one binding site in the enzyme molecule. This suggestion is confirmed

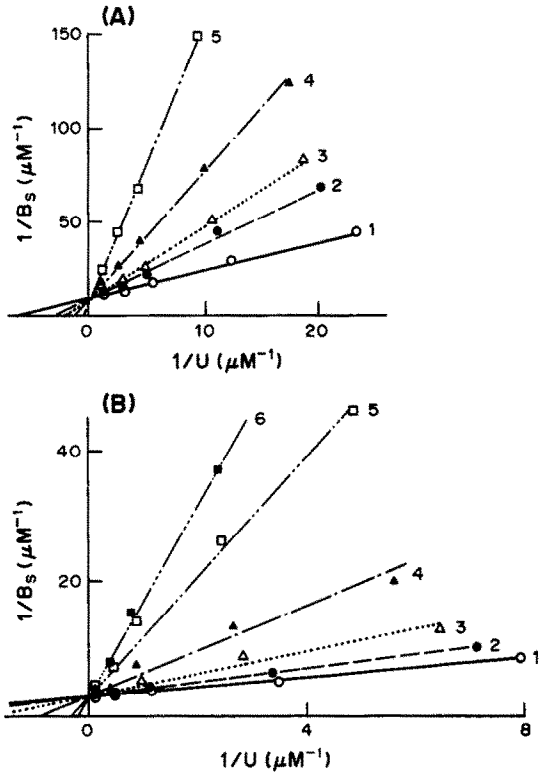

Fig. 1. Competitive inhibition of sex steroid binding to rabbit liver EHSD (pH 7.5). (A) Inhibition of [^{3}H]progesterone binding by estradiol [(2) 1.4 μM; (4) 7.1 μM; $K_i = 1.65$ μM] and testosterone [(3) 1.3 μM; (5) 6.7 μM; $K_i = 0.65$ μM]. (1) Progesterone binding without inhibitors ($K_d = 0.19$ μM). (B) Inhibition of [^{3}H]estradiol binding by progesterone [(2) 0.15 μM; (3) 0.3 μM; (4) 0.61 μM; (5) 1.22 μM; (6) 2.45 μM; $K_i = 0.75$ μM]. (1) Estradiol binding without inhibitor ($K_d = 0.35$ μM). B_s, specifically bound [^{3}H]ligand, U, unbound ligand. Each point is the mean of two determinations. Results of adsorption on activated charcoal.

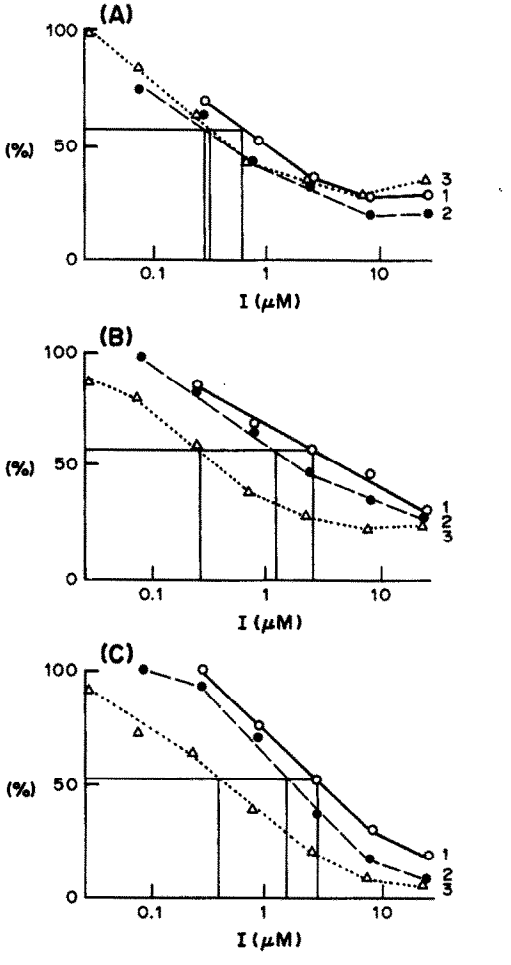

Fig. 2. Efficiency of mutual competition of sex steroids upon binding to rabbit liver EHSD determined by 50% inhibition of [^{3}H]ligand binding (pH 7.5). (A) [^{3}H]Estradiol; (B) [^{3}H]testosterone; (C) [^{3}H]progesterone. Unlabeled steroids: (1) estradiol; (2) testosterone; (3) progesterone. Binding without inhibitors (I) was assumed to 100% (I). Each point is the mean of two determinations. Results of adsorption on activated charcoal.

Table 1. Relative binding affinities (RBA) of hormonal compounds and their analogs for rabbit liver EHSD at 50% inhibition of [^{3}H]progesterone binding (pH 7.5)

N	Compound	RBA [% ± SEM(n)]
The androstane derivatives		
(1)	1,4-Androstadiene-3,17-dione	230 ± 42 (3)
(2)	17β-Hydroxy-1,4-androstadien-3-one (Δ^1-testosterone)	196 ± 38 (3)
(3)	17α-Methyl-17β-hydroxy-4-androsten-3-one (methyltestosterone)	65.6 ± 20.3 (3)
(4)	17α-Ethynyl-17β-hydroxy-4-estren-3-one (norethyndrone)	42.5 ± 14.9 (4)
(5)	17β-Hydroxy-4-androsten-3-one (testosterone)	39.6 ± 4.8 (4)
(6)	17α-Hydroxy-4-androsten-3-one (epitestosterone)	32.5 ± 5.1 (3)
(7)	17β-Hydroxy-4-androsten-3-one 17 propionate (testosterone propionate)	27.1 ± 15.6 (3)
(8)	4-Androstene-3,17-dione	20.8 ± 4.9 (3)
(9)	17α-Ethynyl-17β-hydroxy-5(10)-estren-3-one (norethynodrel)	20.0 ± 7.1 (3)
(10)	17β-Hydroxy-4-estren-3-one (19-nortestosterone)	19.3 ± 1.9 (3)
(11)	17α-Ethynyl-17β-hydroxy-4-androsten-3-one (ethynyltestosterone)	11.7 ± 1.7 (3)
(12)	5β-Androstane-3,17-dione	9.68 ± 0.91 (3)
(13)	17α-Methyl-17β-hydroxy-4,9,11-estratrien-3-one (methyltrienolone, R1881)	5.57 ± 1.78 (3)
(14)	17β-Hydroxy-5β-androstan-3-one (5β-dihydrotestosterone)	4.45 ± 0.99 (3)
(15)	5α-Androstane-3,17-dione	2.18 ± 0.42 (3)
(16)	17β-Hydroxy-5α-androstan-3-one (5α-dihydrotstosterone)	1.07 ± 0.27 (3)
(17)	3β-Hydroxy-5β-androstan-17-one	0.95 ± 0.31 (3)
(18)	3β-Hydroxy-5α-androstan-17-one (epiandrosterone)	0.75 ± 0.19 (3)
(19)	2α-Methyl-17β-hydroxy-5α-androstan-3-one	0.60 (1)
(20)	3β-Hydroxy-5-androsten-17-one (dehydroepiandrosterone)	0.58 ± 0.15 (3)
(21)	5α-Androstan-3-one	0.47 ± 0.01 (3)
(22)	11β,17β-Dihydroxy-4-androsten-3-one (11β-hydroxytestosterone)	0.34 (3)
(23)	5α-Androstan-3β-ol	0.31 ± 0.09 (3)
(24)	3α-Hydroxy-5β-androstan-17-one (ethioholanolone)	0.26 ± 0.07 (3)
(25)	3α-Hydroxy-5α-androstan-17-one (androsterone)	0.25 (1)
(26)	Hydroxyflutamide	<0.26 (3)
(27)	5α-Androstan-17-one	<0.26 (3)
(28)	5α-Androstan-17β-ol	<0.16 (3)
(29)	5α-Androstane	<0.16 (3)
(30)	Flutamide	<0.14 (3)
(31)	4-Androstene-3,11,17-trione	<0.14 (3)
(32)	5α-Androstane-3β,17β-diol	<0.07 (3)
(33)	5β-Androstane-3β,17β-diol	<0.05 (3)
(34)	5β-Androstane-3α,17β-diol	<0.05 (3)
(35)	5α-Androstane-3α,17β-diol	<0.04 (3)
(36)	17α-Methyl-5-androstene-3β,17β-diol	<0.02 (1)
(37)	5-Androstene-3β,17β-diol	<0.02 (1)
(38)	3α-Hydroxy-5β-androstane-11,17-dione	<0.01 (1)
The pregnane derivatives		
(39)	17α-Hydroxy-4-pregnene-3,20-dione (17α-hydroxyprogesterone)	122 ± 9 (3)
(40)	17α,21-Dihydroxy-4-pregnene-3,20-dione (11-deoxycortisol)	104 (1)
(41)	4-Pregnene-3,20-dione (progesterone)	100
(42)	17α,21-Dimethyl-19-nor-4,9(10)-pregnadiene-3,20-dione (promegestone, R5020)	50.4 ± 12.9 (3)
(43)	20β-Hydroxy-4-pregnen-3-one (20β-dihydroprogesterone)	36.5 ± 7.0 (3)
(44)	5β-Pregnane-3,20-dione (5β-dihydroprogesterone)	19.3 ± 4.4 (3)
(45)	6-Methyl-17α-hydroxy-4,6-pregnadiene-3,20-dione 17-acetate (megestrol acetate)	3.59 ± 1.15 (3)
(46)	9α-Fluro-11β,16α,17α,21-tetrahydroxy-1,4-pregnadiene-3,20-dione 16,17 acetonide (triamcinolone acetonide)	2.70 ± 1.22 (4)
(47)	1,2-Methylene-6-chlor-4,6-pregnadiene-17α-hydroxy-3,20-dione 17 acetate (cyproterone acetate)	2.09 ± 0.44 (3)
(48)	16α-Methyl-9α-fluoro-1,4-pregnadiene-11β,17α,20-trihydroxy-3,20-dione (dexamethasone)	0.86 ± 0.17 (3)
(49)	5α-Pregnane-3,20-dione (5α-dihydroprogesterone)	0.84 ± 0.26 (4)
(50)	11β,17α,21-Trihydroxy-4-pregnene-3,20-dione (cortisol)	0.64 ± 0.25 (4)
(51)	17α,21-Dihydroxy-4-pregnene-3,11,20-trione (cortisone)	0.62 (1)
(52)	21-Hydroxy-4-pregnene-3,11,20-trione (11-dehydrocorticosterone)	0.41 ± 0.14 (4)
(53)	11β,21-Dihydroxy-4-pregnene-3,20-dione (corticosterone)	0.18 ± 0.13 (3)
(54)	5α-Pregnane-3β,20β-diol	<0.18 (3)
(55)	3β-Hydroxy-5-pregnen-20-one 3 acetate (pregnenolone acetate)	<0.18 (3)
(56)	11β,21-Dihydroxy-3,20-dioxo-4-pregnen-18-al (aldosterone)	<0.14 (3)
(57)	3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione (tetrahydrocortisone)	<0.1 (1)
The estrane derivatives		
(58)	Hexestrol	49.0 ± 14.6 (4)
(59)	Diethylstilbestrol	33.6 ± 11.3 (3)
(60)	1,3,5(10)-Estratriene-3,17β-diol(estradiol)	14.9 ± 4.6 (4)
(61)	3-Hydroxy-1,3,5(10)-estratriene-6,17-dione (6-ketoestrone)	9.3 ± 2.4 (4)
(62)	1,3,5(10)-Estratriene-3,16β,17β-triol (16-epiestriol)	5.1 ± 1.0 (4)
(63)	1,3,5(10)Estriatriene-3,16α,17β-triol (estriol)	5.0 ± 1.8 (4)
(64)	3-Hydroxy-1,3,5(10)-estratrien-17-one (estrone)	4.8 ± 1.6 (4)
(65)	3,16α-Dihydroxy-1,3,5(10)Estratrien-17-one (16α-hydroxyesterone)	3.9 ± 1.6 (4)
(66)	17α-Ethynyl-1,3,5(10)-estratriene-3,17β-diol (ethynylestradiol)	1.7 ± 0.7 (4)
(67)	Clomiphene cytrate	<0.56 (3)
(68)	Tamoxyphene cytrate	<0.41 (3)
(69)	1,3,5(10)-Estratrien-3-ol (17-deoxyestradiol)	<0.41 (3)
(70)	2-Methoxy-3-hydroxy-1,3,5(10)-estratrien-17-one (2-methoxyestrone)	<0.32 (3)
(71)	1,3,5(10)-Estratriene-3,16α,17α-triol (17-epiestriol)	0.30 ± 0.20 (4)
(72)	1,3,5(10)-Estratriene-3,17α-diol (epiestradiol)	<0.20 (4)
(73)	3-Methoxy-17α-ethynyl-1,3,5(10)-estratrien-17β-ol (mestranol)	<0.16 (3)

by the examination of hormonal specificity of the EHSD affinity.

To investigate the spectrum of the compounds capable of binding to EHSD and to determine the major steroid determinants responsible for their affinities for EHSD. RBA was evaluated for 72 compounds, using [^{3}H]progesterone as a radioligand (Table 1). It can be seen that the EHSD preparation binds a broad spectrum of steroid and nonsteroid (stilbene) ligands. With the exception of 11-desoxycortisol (a weakly-secreted corticoid) and triamcinolone acetonide (a potent synthetic glucocorticoid), all most efficient competitors (RBA > 1%) are the agents with androgenic, gestagenic, estrogenic or antihormonal activity. It should be emphasized that there is no direct correlation between the affinity for EHSD and biological activity of the compounds tested. However, with the exception of 17α-hydroxyprogesterone and 6-ketoestrone, for major secreted sex hormones (testosterone, progesterone, and estradiol) there is correlation between RBA value and the position of the steroid in the metabolic chains (taking into account reversability of reactions under physiological conditions).

The analysis of the significance of each substitutor in the structure of steroid of each group was performed by comparing RBA values for pairs of compounds differing in one substitutor (for details see Ref. [33]). The major determinants of steroid ligands of pregnane, androstane and estrane derivatives are shown in Fig. 3. It is noteworthy that there is a certain similarity between the effects of structure modifications in androstane and pregnane derivatives on their affinities for EHSD. In both cases a decrease in RBA has been observed upon the reduction of the Δ^4 bond and 3-keto groups. In both cases 5β-reduced metabolites are more active than 5α-derivatives, in both cases the contribution of substitutor in the D-ring to the steroid–enzyme interaction is relatively low, and oxygen at position 11 in keto- or β-hydroxyl form produces an appreciable inhibitory effect. Taken together with competitive mutual inhibition of progesterone and testosterone binding, these results suggest that androgens and gestagens bind to the same site on EHSD molecule. With estrane derivatives, however, the substitutors in both A- and D-rings are of significance. Bearing in mind that D-rings in estrogen and androgen molecules have similar structures, one can suggest that estrogens (at least by D-ring) bind to the site other than the androgens (and gestagens) do. This suggestion is consistent with the dependence of RBA-values on the nature of the [^{3}H]ligand. On the basis of dissimilarity of the A-ring structure in estranes, on the one hand, and androstanes and pregnanes, on the other, one can propose that the A-rings of these compounds also bind to different loci of the protein. Some experimental findings (see below) support the assumption on the full noncoincidence of binding sites for these steroids. It should be mentioned that referring to androgens includes only potent competitors of progesterone and testosterone. There is evidence to suggest that at least a part of androgens with low RBA (androstanediols) may interact with other enzyme locus which probably coincides with the estrogen locus (see below).

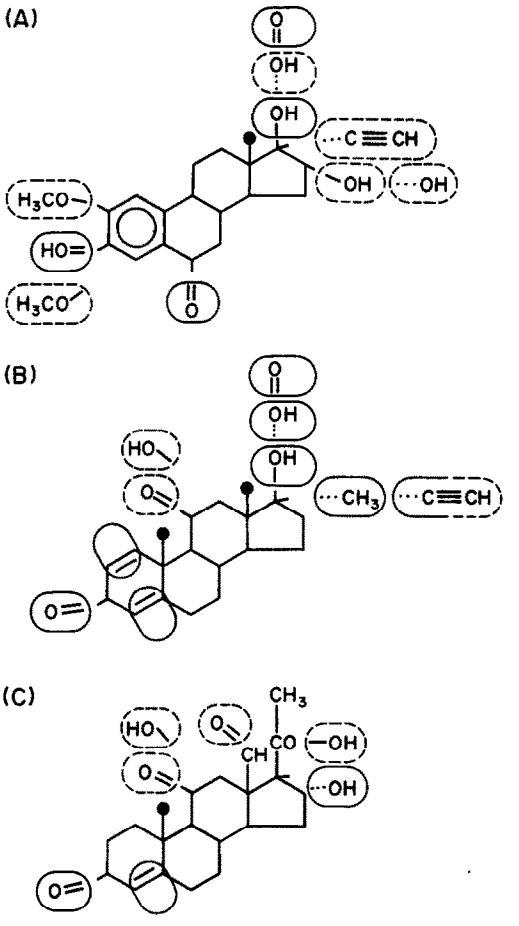

Fig. 3. Major structural determinants of steroid ligands responsible for their affinity for rabbit liver EHSD determined by inhibition of [^{3}H]progesterone binding (pH 7.5). (A) Estrogens; (B) androgens; (C) gestagens. Solid frame indicates the binding-potentiating substitutors, broken frame indicates binding-reducing substitutors.

Substrate inhibition of EHSD and modulation by estrogens

Substrate inhibition has been demonstrated for a number of ligands with high and moderate affinity for

Table 2. I/K_s-values for substrate inhibition of rabbit liver EHSD (pH 7.5)

Substrate	I/K_s (μM^{-1})[a]
5β-Dihydroprogesterone	0.84
Progesterone	0.67
5β-Dihydrotestosterone	0.46
5β-Androstane-3,17-dione	0.12
5α-Dihydrotestosterone	0.091
Testosterone	0.002

[a] K_s values were obtained in "1/V vs S" plots by extrapolation of the line for high substrate concentrations to the abscissa axis.

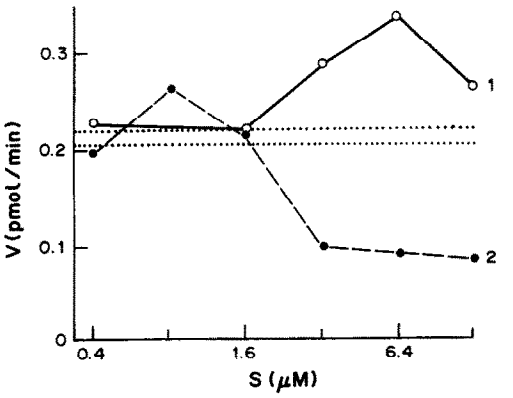

Fig. 4. Potentiating and inhibitory effects of estradiol (1) and hexestrol (2) on the intensity of 20α-reduction of [^{3}H]progesterone in the presence of rabbit liver EHSD and NADPH (pH 7.5). The area indicated with point line is the reaction rate in the absence of modulators (S).

EHSD (Table 2). Besides, moderate concentrations of estradiol and hexestrol are able to potentiate the 20α-reduction of progesterone (Fig. 4). These findings may be regarded as an additional evidence in favour of the presence of more than one site for steroid binding on EHSD molecule.

The effect of pH on hormone-binding and enzyme activities of EHSD

The polyfunctionality of EHSD at a broad ligand spectrum suggests essentially labile organization of the steroid-binding sites. This includes an excess of functional enzyme groups that participate in anchoring of any substrate and/or steroid-like regulator. This suggestion may be checked by examination of the effect of ionization of amino acid residues of EHSD on its steroid-binding and metabolizing activities. Figure 5 illustrates the effect of pH on oxidating by EHSD 17β-, 3α- and 3β-hydroxygroups of a number of steroids. In all cases, the curves are of a bell-like shape with the maximum in the alkaline region. Comparing the curves for different substrates, we have found that its form and optimal pH-value vary considerably depending on the steroid structure. It should be mentioned that these differences manifest themselves not only when substrates with different localizations of oxidated hydroxyl groups are used (Fig. 5B,J), but also within the same group of steroids (Fig. 5B,G).

Different pH sensitivity of EHSD with various substrates has also been observed when reverse reactions were examined, i.e. the reduction of 3-, 17-, 20-keto groups (Fig. 6). The width of the zone in the alkaline region in which the EHSD activity is realized considerably differs for various substrates. This phenomenon is not associated with the localization of reduced keto-group, but may be related to the steroid affinity for the enzyme.

The analysis of the enzymatic kinetics at different pH (Fig. 7) suggests that the changes in the H^+

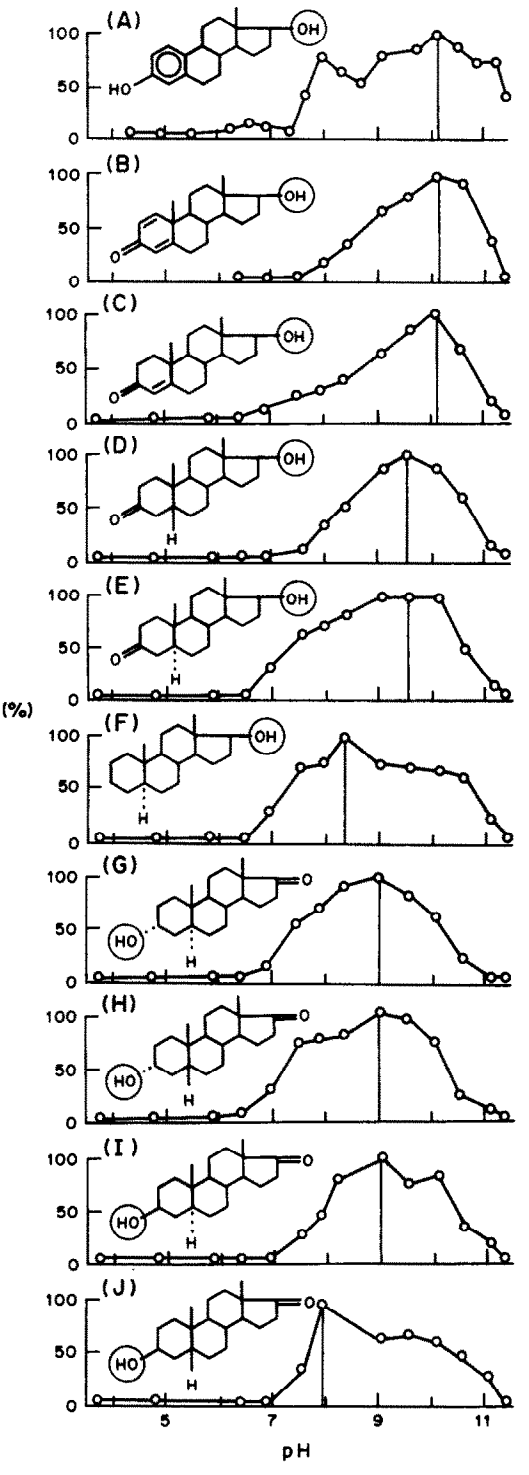

Fig. 5. Effect of pH on the initial rate of oxidation of steroid hydroxy groups in the presence of rabbit liver EHSD and $NADP^+$. The data are presented as % of the maximal rate for the conversion of each substrate which was (in nmol/min per 1 mg protein): (A) estradiol, 10.7; (B) Δ^1-testosterone, 90; (C) testosterone, 415; (D) 5β-dihydrotestosterone, 432; (E) 5α-dihydrotestosterone, 660; (F) 5α-androstane-17β-ol, 36; (G) androsterone, 516; (H) etiholanolone, 818; (I) epiandrosterone, 50; (J) 3β-hydroxy-5β-androstan-17-one, 17.5. Results of chromatographic separation of [^{3}H]product and substrate (A) and fluorimetry by NADP formation (B–J).

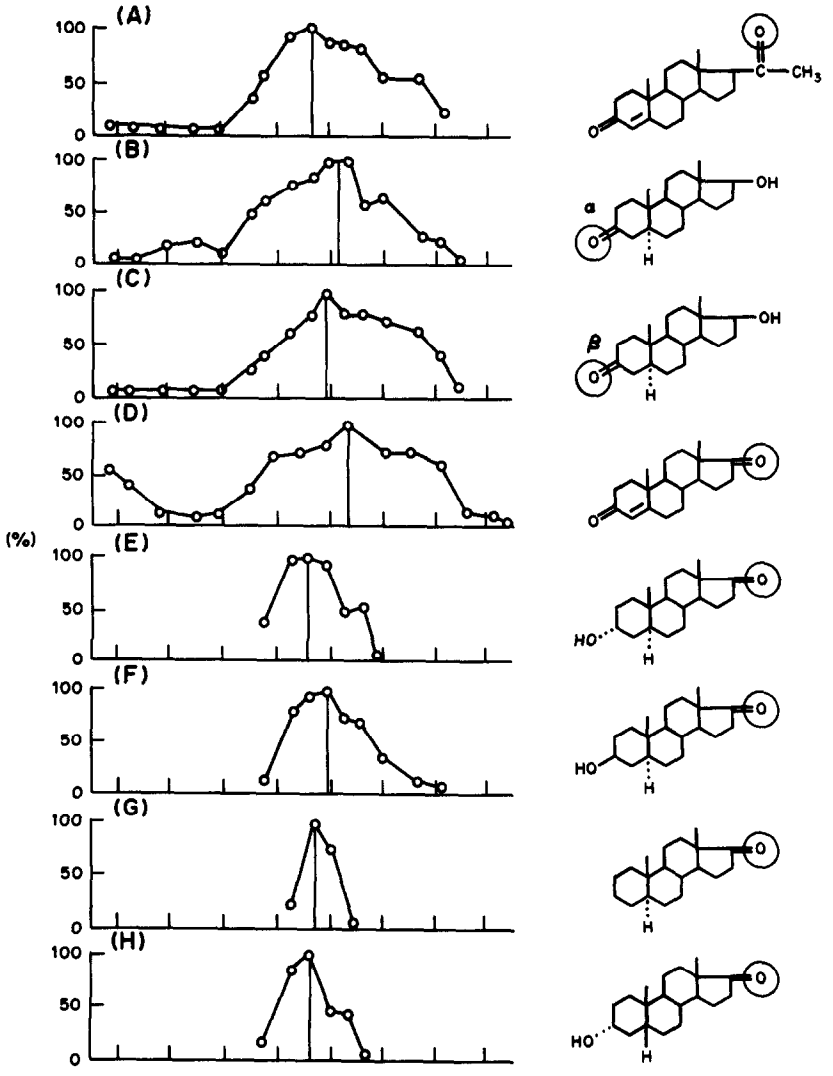

Fig. 6. Effect of pH on the initial rate of reduction of steroid keto-groups in the presence of rabbit liver EHSD and NADPH. The data are presented as % of the maximal rate for the conversion of each substrate which was (in nmol/min per 1 mg protein): (A) progesterone, 7.2; (B) 5α-hihydrotestosterone (3α), 23; (C) 5α-dihydrotestosterone (3β), 53; (D) 4-androstene-3,17-dione, 10.4; (E) androsterone, 123; (F) epiandrosterone, 117; (G) 5α-androstane-17-one, 131; (H) etioholanolone, 75. The results of chromatographic separation of [^{3}H]product and substrate (A–D) and fluorimetry by NADPH expense (E–H).

concentration induce complex rearrangements in the enzyme structure which may express in the efficiency of the catalytic process, the EHSD–steroid interaction, and probably the degree of mutual effects of steroid and cofactor on their interaction with the enzyme.

Direct determination of [^{3}H]steroid binding to EHSD at different pH confirms this suggestion. This binding is pH-dependent (Fig. 8). There is considerable variability in pH-dependence of binding of different steroids. For example, at pH 6.9 the hormone-binding activity of EHSD towards progesterone and testosterone almost reached the maximum, while binding of estradiol or estrone at this pH is insignificant. At pH 10.6 the situation is entirely opposite. The binding of 4-androstene-3,17-dione and 5α-dihydrotestosterone to the enzyme in the pH range tested does not reach maxima. The Scatchard plots of the equilibrium dialysis data (Fig. 9) show that the pH changes influence both the affinity and the concentration of available binding sites of the protein for steroids.

Effect of NaCl on the hormone-binding activity of EHSD

At pH 7.5, NaCl inhibited binding of the three steroid tested, [^{3}H]progesterone binding being the most and [^{3}H]estradiol the least sensitive (Fig. 10). These results are consistent with the data on the pH-sensitivity of binding of these steroids to EHSD. In fact, the relative contribution of ionized groups of the protein to estradiol binding at pH 7.5 is considerably lower compared with their contribution to progesterone binding. Therefore masking of these

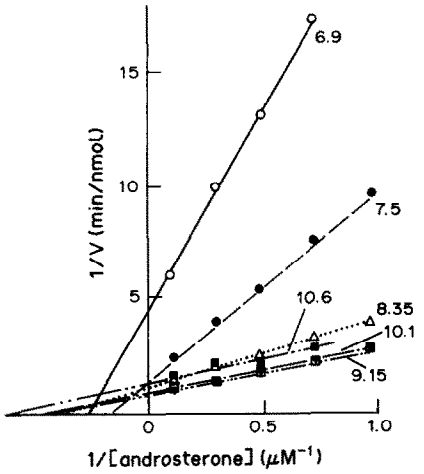

Fig. 7. Kinetic analysis of the pH effect on oxidation of 3α-hydroxy group of androsterone in the presence of rabbit liver EHSD (2.5 μg/ml) and $NADP^+$ (50 μM). Results of fluorimetry. The numbers at the lines indicate pH. The K_m-values (μM) and V_{max} (nmol/min per 1 mg protein) are at pH 6.9–3.7 and 90; at pH 7.5–5.2 and 260; at pH 8.35–2.9 and 390; at pH 9.15–2.18 and 560; at pH 10.1–2.5 and 490; at pH 10.6–1.7 and 320, respectively.

groups by Na^+ has lower effect on estradiol binding compared with progesterone binding.

Effect of dithiothreitol on enzymatic and hormone-binding activities of EHSD

The degree of the involvement of SH-groups in EHSD catalytic activity towards different substrates varies considerably (Fig. 11A–E). In the presence of dithiothretiol (≥ 16 mM), 17β-, 3β-, and 20α-reduction of 4-androstene-3,17-dione, 5α-dihydrotestosterone and progesterone, respectively, markedly increased. The same was observed with 17β-oxidation of 5α-dihydrotestosterone (pH 7.9). At the same time the intensity of this reaction is dithiothreitol-independent at optimal pH. The reaction with testosterone is virtually independent of dithiothreitol concentration at pH 7.65 and 9.7. It can be suggested that free SH-groups somehow participate in catalytic conversion of some, but not all substrates, and this involvement may be dependent on the degree of ionization of alkaline amino acids in the enzyme molecule. The binding of the three examined [^{3}H]ligands to EHSD lowered as dithiothreitol concentration increased (Fig. 11E). The inhibitory activity of dithiothreitol towards different steroids varied from the minimal for progesterone and maximal for estradiol. This may confirm the assumption that the combination of functional enzyme groups involved in ligand binding is individual for each steroid.

Effects of cofactors on the steroid–EHSD binding

The results of kinetic analysis of EHSD reactions with varied steroid and cofactor concentrations are summarized in Table 3. It can be seen from the table that on oxidation of 3α-hydroxy group of androsterone cofactor and steroid do not produce any effect on their affinities for EHSD. Upon 17β-reduction of this substrate steroid and cofactor mutually decreased their affinities for the enzyme, while on oxidation of 17β-hydroxy group of 5α-dihydrotestosterone substrate and cofactor mutually potentiated their binding to EHSD.

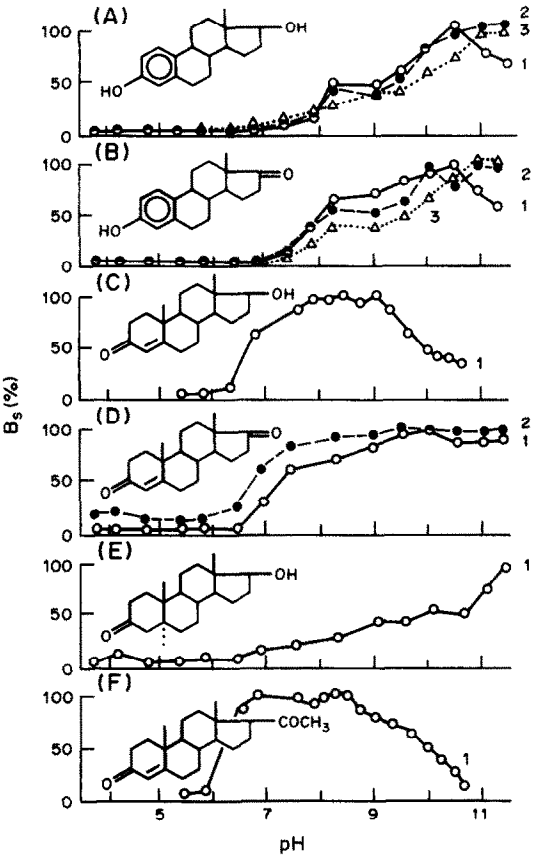

Fig. 8. Effect of pH and the presence of cofactor (50 μM) on [^{3}H]steroid binding to rabbit liver EHSD. The data are expressed as % of maximal binding for each [^{3}H]ligand. The ratio between the maximal binding with and without $NADP^+$ is: (A) 2.5; (B) 2.5; (C) 1.6. The correspondent values for NADPH are: (A) 0.83; (B) 0.62. (A) Estradiol; (B) estrone; (C) 5α-dihydrotestosterone; (D) 4-androstene-3,17-dione; (E) testosterone; (F) progesterone; (1) without cofactor; (2) in the presence of $NADP^+$; in the presence of NADPH. Results of adsorption on activated charcoal. Each point is the mean of two determinations.

To confirm the cofactor effect on steroid affinity for the enzyme in a direct experiment, the method of equilibrium dialysis with [^{3}H]steroids has been employed. The ligand metabolism during incubation was prevented by the use of the cofactor only in a form which is inefficient in enzymatic reaction with the steroid. The results given in Fig. 12 and Table 4 indicate that the affinity of steroid ligands for EHSD is influenced by cofactors: in the case of 4-androstene-3,17-dione and progesterone a positive effect was observed with $NADP^+$, whereas in the case of testosterone the same effect is produced by NADPH. The effects of oxidated and reduced cofactor forms on

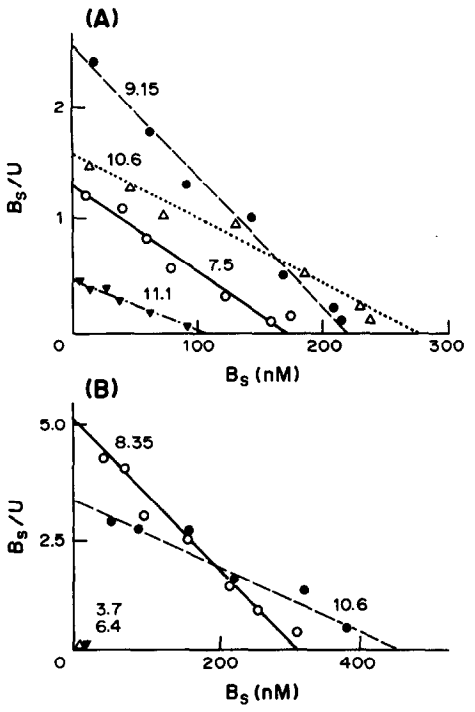

Fig. 9. Scatchard analysis of interaction between [^{3}H]estradiol (A) or [^{3}H]progesterone (B) and rabbit liver EHSD at varied pH. Protein concentration was 17.5 (A) and 12.5 μg/ml (B). The numbers at the lines indicate pH, b_s, specifically bound steroid; U, unbound steroid. The K_a (μM^{-1}) and b_{max} (nmol/mg protein) were (A) at pH 7.5–7.8 and 9.7; at pH 9.15–11.5 and 12.4; at pH 10.6–5.8 and 15.6; at pH 11.1–4.5 and 5.9; (B) at pH 8.35–16.4 and 24.8; at pH 10.6–7.6 and 35.7, respectively. Results of equilibrium dialysis.

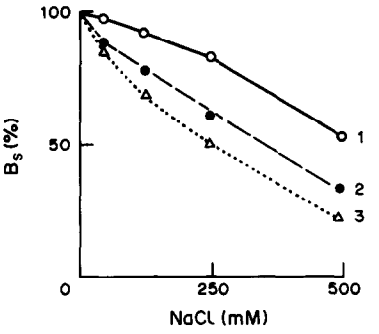

Fig. 10. Effect of NaCl on [^{3}H]steroid binding to rabbit liver EHSD (pH 7.5). The data are expressed as % of the values obtained without NaCl. (1) Estradiol; (2) testosterone; (3) progesterone, b_s specifically bound [^{3}H]ligand. Results of adsorption on activated charcoal.

EHSD binding of estradiol, a ligand weakly-metabolized by the enzyme, are reversal. The data demonstrate the cofactor effect not only on the enzyme affinity, but also on its accessibility for steroid ligands and these effects seems to be unilateral. Considerable differences in the inhibitory activity of hexestrol, a synthetic estrogen of the stilbene group, in EHSD-catalyzed reactions of steroid oxidation and reduction in, depending on the position of the modified group in the substrate molecule (Table 5), are in agreement with preceding data. Ionized residues of basic amino acids of the enzyme may be involved in the mediating of the cofactor effect on the EHSD steroid-binding activity. This is confirmed by cofactor effects not only on the maximal steroid-binding activity of EHSD at pH optimum (see legend to Fig. 8) but also on the ratio between the EHSD binding activities at suboptimal pHs (cf. Fig. 12A,B,D).

The mechanism of coupling between the binding sites for steroid and cofactor probably shares the same structures in the protein with the mechanism of conjunction of the two steroid binding sites. This assumption is based on the data on the co-factor effects on the efficiency of the potentiating action of androstanediols on the EHSD–[^{3}H]testosterone binding: in the presence of NADPH, which potentiates the protein–testosterone interaction, the stimulatory effect of androstanediols on [^{3}H]testosterone binding is reduced or abolished (Fig. 13). If the cofactor and androstanediols acted via different structures of the enzyme, summation of their effects should be expected. The absence of additivity in the effects of 5α-androstane-3α,17β-diol and cofactor on binding of various steroids has been confirmed by equilibrium dialysis (data not shown). Unlike the binding of [^{3}H]testosterone and [^{3}H]progesterone, the binding of [^{3}H]-estradiol to EHSD was not stimulated but was only inhibited by androstanediols (data not shown). This fact suggests a coincidence of the preferential binding sites for estrogen and androstanediols in the protein.

DISCUSSION

EHSD from rabbit liver binds three classes of [^{3}H]steroids: estrogens, androgens and gestagens, Some evidence has been obtained suggesting that the binding sites for these steroids overlap: concentrations of binding sites for [^{3}H]estradiol, [^{3}H]testosterone and [^{3}H]progesterone are similar and inhibition of their binding is mutually competitive (Fig. 1). At the same time, data have been obtained that seem to disagree with this suggestion. (a) The analysis of inhibition of [^{3}H]steroid binding by 50% displacement of radioligand from the enzyme–steroid complex shows considerable differences in the RBA-values with [^{3}H]estradiol, on the one hand, and [^{3}H]testosterone and [^{3}H]progesterone on the other (Fig. 2). (b) Examination of the competitive efficiency of 72 steroids and their analogues by the displacement of [^{3}H]progesterone from the complexes has shown that the same modifications in the D-rings of estrogen and androgen molecules produce different effects on RBA-values of these compounds (Table 1, Fig. 3). By contrast, similar modifications in androgen and gestagen molecules have similar effects on their RBA-values. Most probably, the observed discrepancy is due to the existence of two separate binding sites in the projection molecule with an extremely high degree of allosteric conjunction between them; one site has a

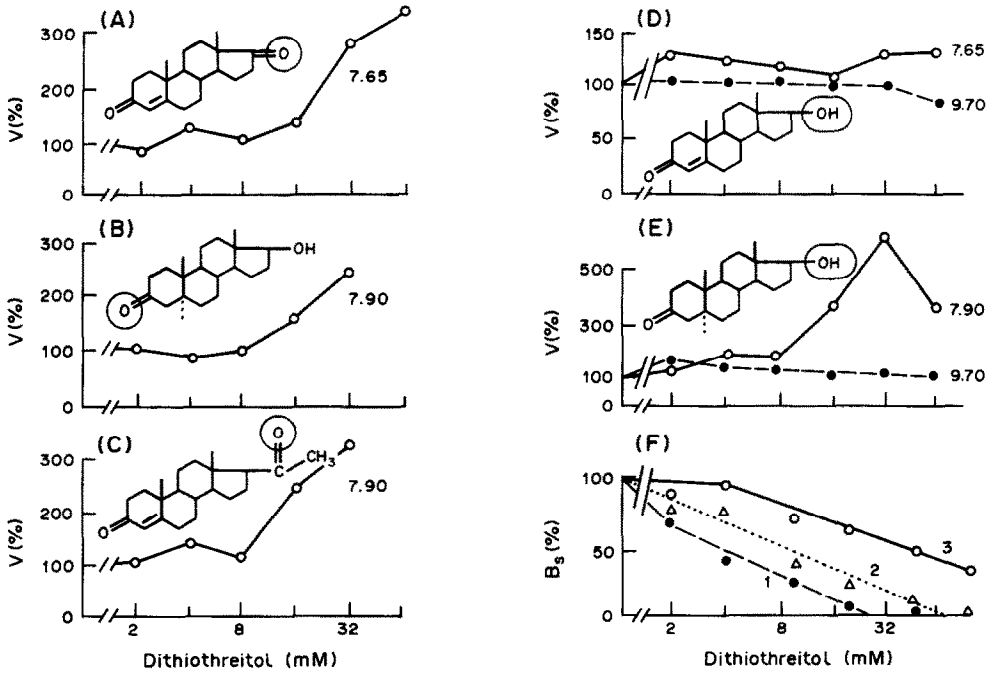

Fig. 11. Effect of dithiothreitol on the intensity of reactions catalyzed by rabbit liver EHSD (A–E) and [^{3}H]steroid binding to this enzyme (F). The data are % of the values measured without the reagent. (A) 4-Androstene-3,17-dione; (B) 5α-dihydrotestosterone (3β); (C) progesterone; (D) testosterone; (E) 5α-dihydrotestosterone (17β). The numbers at the curves indicate pH. The reaction rate was determined by chromatographic separation of [^{3}H]product and substrate. B_s specifically bound ligand. The binding was determined by adsorption on activated charcoal (pH 7.5). Each point is the mean of two determinations. (1) Estradiol; (2) testosterone; (3) progesterone.

preferential specificity for estradiol, the other one—for testosterone and progesterone. This specificity, however, seems not to be absolute. The data confirming the presence of two steroid-binding sites in the EHSD molecule have been obtained in the present study. First, substrate inhibition was observed with some high and moderate affinity ligands (Table 2). Second, at moderate concentrations estradiol and hexestrol not only inhibit, but even potentiate 20α-reduction of progesterone (Fig. 4). Third, reduced cofactor can produce opposite effects on EHSD affinity and binding capacity towards estradiol and testosterone (Fig. 12) and progesterone. Fourth, androstanediols strongly potentiate the binding of testosterone (Fig. 13) and progesterone. The fact that estradiol, though to a small degree, is metabolized by EHSD and androstanediols are good substrates for this enzyme [28] indicates that these ligands are capable of interacting with both postulated steroid-binding sites. Demonstrations of substrate inhibition [4, 20, 34], noncompetitive inhibition by steroids [17, 19, 35, 36], nonMichaelis kinetics [4] for a number of steroid-metabolizing enzymes suggest that EHSD may not be the sole protein possessing more than one steroid-binding site.

The stoichiometric ratio for the bound steroid to purified EHSD is about 0.4 and remained virtually unchanged after further fractionating of the enzyme. Taken together with the data on homogeneity of protein preparation in the respect of molecule size [28], this suggests that about a half of EHSD molecules cannot bind steroids under the conditions employed. Similar situation occurred upon purification of the another steroid-binding protein UEBP using the same method [37]. The underestimation of steroid-binding sites may not be due to denaturation of the EHSD molecules, since under certain conditions (varied pH, presence of co-factors—Figs 9, 12 and Table 4) the

Table 3. Kinetic parameters of some reactions catalyzed by rabbit liver EHSD (pH 7.5)

Reaction	Substrate	Cofactor	Parameter V_{max} (μmol/min per mg)	K_m^A (μM)	K_s^A (μM)	K_m^B (μM)	K_s^B (μM)
3α-Hydroxy	Andresterone	$NADP^+$	0.23	11.1	10.0	4.2	4.3
3-Keto,17-keto	Androsterone	NADPH	0.33	9.9	3.2	3.7	1.4
17β-Hydroxy,17β-hydroxy 17-Keto	5α-Dihydrotestosterone	$NADP^+$	0.11	2.9	5.9	1.0	2.2

A indicates cofactor, B indicates steroid.

K_m- and K_s-values were obtained from secondary "slope or intercept vs [steroid]$^{-1}$ or [cofactor]$^{-1}$" plots developed on the basis of double reciprocal "1/V vs 1/S" plots for a number of steroid and cofactor concentrations, assuming a kinetic mechanism of reactions to be unordered and equilibrium.

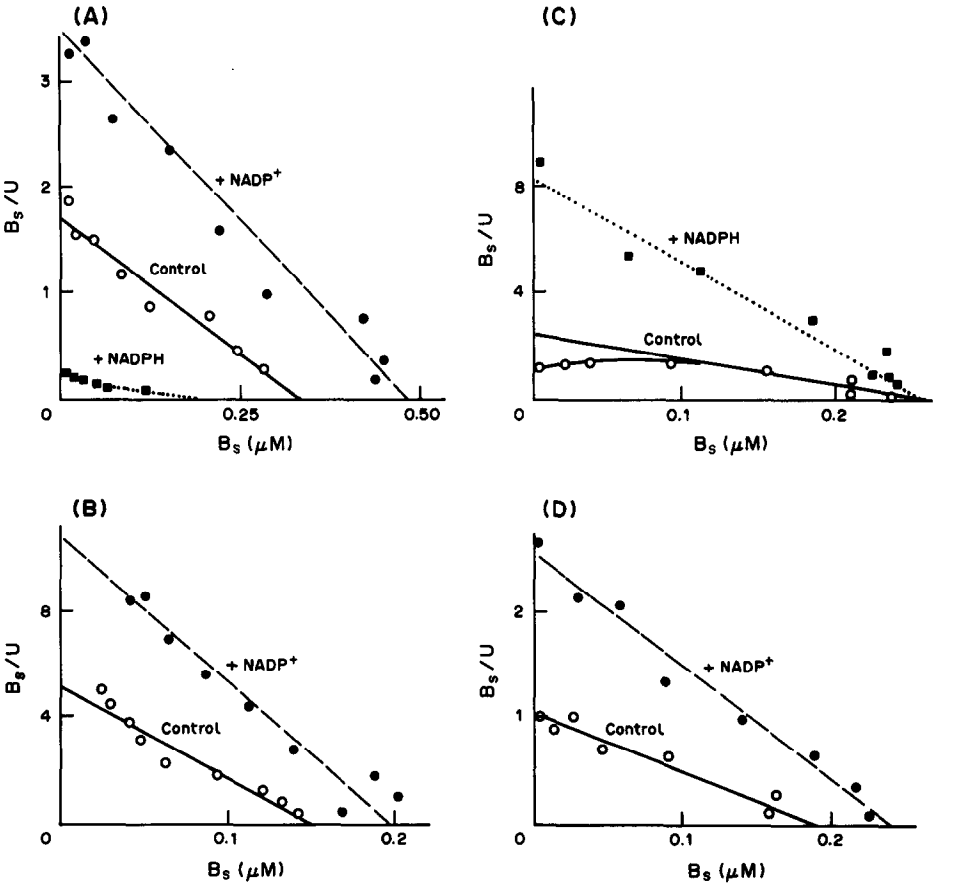

Fig. 12. Effects of cofactors on the parameters of [^{3}H]steroid interaction with rabbit liver EHSD (pH 7.5). (A) [^{3}H]estradiol; (B) [^{3}H]progesterone; (C) [^{3}H]testosterone; (D) [^{3}H]androstene-3,17-dione. b_s, specifically bound ligand; U, unbound ligand. Results of equilibrium dialysis.

Table 4. Cofactor effects on the parameters of the EHSD–steroid interaction (pH 7.5)

[^{3}H]Steroid	Cofactor (50μM)	Parameters b_{max} (nmol/mg)	K_a ($M^{-1} \times 10^7$)
4-Androstene-3,17-dione	—	7.2	0.58
	$NADP^+$	9.8	1.04
Progesterone	—	11.7	3.24
	$NADP^+$	14.9	5.44
Testosterone	—	11.0	0.91
	NADPH	10.3	3.13
Estradiol	—	9.9	0.50
	NADPH	5.2	0.10
	$NADH^+$	14.3	0.69

steroid-binding capacity can be increased considerably. In line with the homogeneity of EHSD in the respect of its molecule charge, these facts suggest conformational mobility of this enzyme.

The pH dependences for EHSD enzymatic and hormone-binding activities (Figs 5, 6 and 8) appeared to be individual for each steroid. Our findings suggest that a specific constellation of ionized amino acid residues in EHSD molecule participates in anchoring of each steroid to the binding site. Similarly, the involvement of SH-groups in the catalysis and binding of different steroids also varies (Fig. 11). Interestingly, the efficiency of the potentiating action of dithiothreitol may be either pH-dependent or pH-independent depending on the substrate structure. The participation of ionized amino acid residues and cysteine in steroid-binding and catalysis has been demonstrated for numerous HSD [3, 7, 12, 13, 38–42]. Individual combinations of functional groups of HSD in steroid binding has been suggested by Blomquist *et al.*[43] "positioning of steroids in the complexes is

Table 5. Effects of oxidized and reduced cofactor on the inhibitory activity of hexestrol in reactions catalyzed by rabbit liver EHSD

Cofactor	Substrate	Reaction	K_i (μM) (m ± SE)
NADPH	Progesterone	20-keto → 20α-hydroxy	1.80 ± 0.40 (3)
	5α-Dihydrotestosterone	3-keto → 3α-hydroxy	1.65 (2)
	5α-Dihydrotestosterone	3-keto → 3β-hydroxy	2.25 (2)
$NADP^+$	5α-Dihydrotestosterone	17β-hydroxy → 17-keto	0.047 ± 0.005 (5)
	Androsterone	3α-hydroxy → 3-keto	0.041 ± 0.005 (5)

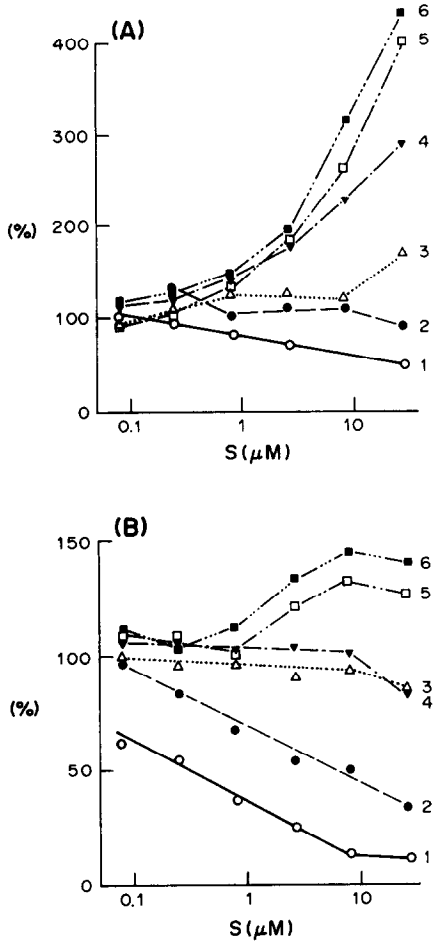

Fig. 13. Cofactor modulation of the potentiating effects of 5(α,β)-androstane-3(α,β), 17β-diols on [^{3}H]testosterone binding to rabbit liver EHSD (pH 7.5). (A) Without co-factor; (B) in the presence of 50 μM NADPH. The data are % of [^{3}H]ligand binding in the absence of unlabeled steeroids (S): (1) testosterone; (2) estradiol; (3) 5α-androstane-3β,17β-diol; (4) 5β-androstane-3α,17β-diol; (5) 5α-androstane-3α,17β-diol; (6) 5β-androstane-3β,17β-diol. Results of adsorption on activated charcoal. Each point is the mean of two determinations.

variable. Multiple amino acid residues at the steroid site might be capable of bond formation and, depending on the structure of the steroid, certain of a number of possible stabilizing interactions occurs." The present study may be regarded as experimental support for this statement.

In conclusion, EHSD from rabbit liver has a complex active center that includes at least two steroid-binding sites closely coupled to each other and to the cofactor-binding site. Ionized amino acid residues are involved in the coupling of these sites. The EHSD polyfunctionality may result from the excess of the functional groups which can be involved in steroid binding on the one hand, and from conformational lability of the enzyme molecule providing alternative orientations of different steroid substrates at the site of its anchoring.

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