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CHARACTERIZATION OF THE PURIFIED PITUITARY CYTOSOLIC NADPH:5α-DIHYDROPROGESTERONE 3α-HYDROXYSTEROID OXIDOREDUCTASE

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Summary—The purified cytosolic 3α-hydroxysteroid oxidoreductase (3α-HSOR) from female rat pituitary which catalyzes the reversible conversion of 5α-dihydroprogesterone (5α-DHP) to 3α,5α-tetrahydroprogesterone (3α,5α-THP) has been characterized in terms of its steroid substrate specificity, dihydrodiol dehydrogenase activity and inhibition by drugs such as medroxyprogesterone and indomethacin. The purified enzyme has a strong preference for the C_{21} progestin steroid substrates, 5α-DHP and 3α,5α-THP, over the corresponding C_{19} androgenic steroid substrates, 5α-dihydrotestosterone (5α-DHT) and 3α,5α-tetrahydrotestosterone (3α,5α-THT). The apparent K_m for 5α-DHP (80 nM) is about 250 times lower than the K_m for the androgenic steroid, 5α-DHT (21 μM). In the oxidative direction, the apparent K_m for 3α,5α-TP (1.4 μM) is about 3-fold lower than the K_m for the androgenic steroid, 3α,5α-THT (4.2 μM). A number of other naturally occurring 3-keto- and 3α(β)-hydroxy-steroids were assessed for their ability to act as inhibitors (alternate substrates) of the 3α-reduction of 5α-DHP catalyzed by the purified 3α-HSOR. None of the 3β- or 5β-isomers had any effect. Of the other 3-keto and 3α- steroids tested, only deoxycorticosterone and the ovarian progestins showed any significant inhibition. These may be acting as inhibitors since there was little, if any, direct 3α-reduction of progesterone to 3α-hydroxy-4-pregnen-20-one. Unlike the liver cytosolic 3α-HSOR, the pituitary enzyme has no associated dihydrodiol (quinone) dehydrogenase activity. This enzyme is similar to other cytosolic 3α-HSORs from liver and brain in that it is potentially inhibited by indomethacin and by medroxyprogesterone.

INTRODUCTION

The conversion of progesterone to 3α- and 5α-reduced metabolites appears to be an important component in bringing about progesterone's neuroendocrine effects [1, 2]. In the anterior pituitary of the female rat, progesterone is rapidly metabolized to 5α-pregnane-3,20-dione (5α-dihydroprogesterone: 5α-DHP) and 3α-hydroxy-5α-pregnan-20-one (3α,5α-tetrahydroprogesterone: 3α,5α-THP) [1, 2]. Both of these metabolites have significant effects on progesterone-sensitive neuroendocrine events such as gonadotropin regulation [1–5]. 3α,5α-THP has been shown to be more potent than progesterone in its effects on anesthesia, and on the modulation of the GABA receptor complex [6–8]. Recently, 3α,5α-THP has also been demonstrated to have potent anti-epileptic properties at physiological levels [9].

This laboratory has previously demonstrated that the formation of 3α,5α-THP from 5α-DHP in the pituitary is catalyzed by two distinct 3α-hydroxysteroid oxidoreductases (3α-HSOR): one that is a cytosolic 3α-HSOR, which prefers NADPH as a cofactor, and the second that is a particulate 3α-HSOR which prefers NADH as a cofactor [10–12]. Recently, we reported on the

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Trivial names and abbreviations: 5α-dihydroprogesterone (5α-DHP), 5α-pregnane-3,20-dione; 3α,5α-tetrahydroprogesterone (3α,5α-THP), 3α-hydroxy-5α-pregnan-20-one; 5α-dihydrotestosterone (5α-DHT), 17β-hydroxy-5α-androstan-3-one; 3α,5α-tetrahydrotestosterone (3α,5α-THT), 5α-androstane-3α,17β-diol; 3α-dihydroprogesterone (3α-DHP), 3α-hydroxy-4-pregnen-20-one; 20α-dihydroprogesterone (20α-DHP), 20α-hydroxy-4-pregnen-3-one; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; 3β,5α-tetrahydroprogesterone, 3β-hydroxy-5α-pregnan-20-one; 3α,5β-tetrahydroprogesterone, 3α-hydroxy-5β-pregnan-20-one; 3β,5β-tetrahydroprogesterone, 3β-hydroxy-5β-pregnan-20-one; 5β-dihydroprogesterone (5β-DHP), 5β-pregnane-3,20-dione; medroxyprogesterone, 17-hydroxy-6-methyl-4-pregnene-3,20-dione; medroxyprogesterone acetate, 17α-acetoxy-6-methyl-4-pregnene-3,20-dione; indomethacin, 1-(*p*-chlorobenzyl)-5-methoxy-2-methylindol-3-yl acetic acid.

purification and some of the kinetic properties of the rat pituitary cytosolic NADPH: 3α-HSOR [13]. In this article, we describe the steroid substrate specificity of this purified enzyme, its sulfhydryl sensitivity and its inhibition by various drugs such as medroxyprogesterone and indomethacin. To further assess its similarity to other 3α-HSORs from rat brain and liver we also tested for associated dihydrodiol dehydrogenase activity.

EXPERIMENTAL

Materials

[1,2-^{3}H]5α-DHP (sp. act. 57.5 Ci/mmol), [1,2-^{3}H]progesterone (sp. act. 57.5 Ci/mmol), [1,2-^{3}H]17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone: 5α-DHT) (sp. act. 60 Ci/mmol), and [1,2-^{3}H]5α-androstane-3α,17β-diol (3α,5α-tetrahydrotestosterone: 3α,5α-THT) (sp. act. 17 Ci/mmol) were purchased from the New England Nuclear Corp. [1,2-^{3}H]3α,5α-THP was synthesized enzymatically [10]. The radioactive steroid substrates were purified by TLC [10] using the solvent systems: benzene:methanol (19:1, v/v) for the C_{21} steroids and benzene:acetone (4:1, v/v) for the C_{19} steroids [14, 15]. Medroxyprogesterone was purchased from Steraloids (Wilton, N.H.) and 9,10-phenanthrenequinone was purchased from Aldrich Chemical Company (Milwaukee, Wis.). Unless otherwise indicated, we used purified pituitary cytosolic 3α-HSOR obtained from female rat cytosol [13]. The sources and purity of the steroids, reagents for TLC the enzyme assay and GLC have been previously described [10, 13]. All other reagents were purchased from Sigma Chemical Company (St Louis, Mo.).

3α-HSOR assay

Cytosolic NADPH: 5α-DHP 3α-HSOR activity was measured using radiolabeled steroid substrate, and the product steroids were quantitated by reverse isotopic dilution analysis [10, 13]. Unless otherwise indicated, enzyme activity was assayed in the reductive direction using 5.0 ml of reaction buffer containing 1 mM dithiothreitol, 1 mM EDTA, 200 μM NADPH, 1 μM [^{3}H]5α-DHP, 10 μg/ml of bovine gamma immunogobulin, 100 mM potassium phosphate buffer, pH 7.2, and any indicated additions. For assays in the oxidative direction, [^{3}H]3α,5α-THP was used as the substrate. Control reactions contained all assay components except the enzyme. The reactions were initiated after a 10 min pre-incubation period by the addition of the appropriate radiolabeled steroid substrates in 50 μl of ethanol as previously described [10]. Incubations were carried out for 30 min in a shaking water bath at 37°C. Termination of the enzyme assays and quantitation of product steroids were carried out as detailed earlier [10, 11].

Steroid substrate specificity

To assess the C_{19} vs C_{21} steroid substrate specificity of the purified enzyme, we examined the corresponding C_{19} androgenic steroid substrates, 5α-DHT and 3α,5α-THT, in the reductive and oxidative directions using radiolabeled steroid substrates and reverse isotopic dilution analysis to measure product steroids. The cofactors, NADPH and $NADP^+$, were present at saturating concentrations of 500 μM and 1 mM, respectively. [^{3}H]5α-DHT was present at 0.75, 1, 10 and 50 μM and [^{3}H]3α,5α-THT was present at 0.75, 1, 10 and 50 μM. The assays for the C_{19} steroid substrates were identical to those described previously for the C_{21} steroid substrates [13] except for the TLC separation system. For both the reductive and oxidative assays, the radiolabeled product steroids, 3α,5α-THT or 5α-DHT, were separated with a one-dimensional solvent system of benzene:acetone (4:1, v/v) [14, 15]. The values for the various kinetic parameters for the C_{19} steroids were determined using a non-linear regression routine [16, 17].

A number of naturally occurring 3-keto-and 3α(β)-hydroxysteroids were examined for their ability to act as competing inhibitors (possible alternate substrates) of the 3α-reduction of 5α-DHP catalyzed by the purified pituitary cytosolic 3α-HSOR [18, 19]. The assays with the test steroids were identical to the 3α-HSOR assays described above for the reductive reaction except that the concentration of 5α-DHP was reduced to 100 nM. All of these steroids were tested at a concentration of 100 nM. The test steroid was present in the assay mixture before the initiation of the reaction by [^{3}H]5α-DHP. Control reactions, incubation times, termination of the reaction and quantitation of product steroids were carried out as described above.

Test for dihydrodiol dehydrogenase activity

To determine if the purified pituitary 3α-HSOR also had dihydrodiol dehydrogenase activity, as reported for the male rat liver enzyme [20], we examined the commonly used quinone test substrate, 9,10-phenanthrene-

quinone, for its ability to inhibit the 3α-HSOR catalyzed 5α-DHP reduction. The assays were identical to those described above for the steroid specificity studies. 9,10-Phenanthrenequinone was tested at concentrations of 0.5 and 1 μM.

Testing for the conversion of progesterone to 3α-DHP or 20α-DHP

Because several of the Δ^4-3-ketosteroids, especially the ovarian progestins, progesterone, 17α-hydroxyprogesterone and 20α-dihydroprogesterone, were able to inhibit the 3α-HSOR catalyzed 3α-reduction of 5α-DHP, there was the possibility that these steroids were alternate substrates [18, 19]. To test for the possible 3α-reduction of progesterone to 3α-hydroxy-4-pregnen-20-one (3α-dihydroprogesterone: 3α-DHP), we substituted 1 μM [^{3}H]progesterone in place of 1 μM [^{3}H]5α-DHP in the 3α-HSOR assay described above for the reductive direction. The incubations were performed in triplicate. For these studies only, we used a preparation of female rat pituitary cytosolic 3α-HSOR derived from the DEAE–Sephacel purification step from our previously published purification procedure [13]. The NADPH:5α-DHP 3α-HSOR preparation had a total activity of 35.9 nmol of 3α,5α-THP/mg protein/30 min and had no progesterone 5α-reductase activity [17]. With these same incubations, we also tested for the possibility that the enzyme was catalyzing the 20α-hydroxysteroid oxidoreduction of [^{3}H]progesterone to [^{3}H]20α-hydroxy-4-pregnen-3-one (20α-dihydroprogesterone: 20α-DHP). When the reaction was terminated, we added 100 μg of the following non-radioactive carrier steroids to each incubation flask: 3α-DHP, 20α-DHP, 5α-DHP and 3α,5α-THP. After extraction with ether, the steroids were separated with a series of either two or three 2-dimensional TLC separation systems. We found no conversion of progesterone to 5α-DHP or 3α,5α-THP. The other test product steroids, 3α-DHP and 20α-DHP were quantitated by reverse isotopic dilution analysis after each of two TLC separation steps for 20α-DHP and each of three TLC separations for 3α-DHP. The first TLC system employed benzene:methanol (19:1) in the first dimension and n-butyl acetate:cyclohexane (2:1) in the second dimension. The second system employed chloroform:ether (10:3) twice in the first dimension and hexane:ethyl acetate (5:20) twice in the second dimension [21]. The third system employed benzene:methanol (9:1) twice in the first dimension and benzene:ether:hexane (6:4:1) twice in the second dimension. The three TLC separation systems were used only for 3α-DHP. After the first two separation systems, the radioactivity associated with non-radioactive carrier 20α-DHP was too low for further analysis.

Kinetic analyses

Enzyme kinetic data were analyzed using an IBM XT personal computer and the inhibition data were fitted to equations (1–3) (corresponding to competitive, uncompetitive and non-competitive inhibition, respectively):

$$v = VA/[K(1 + I/K_{i_{slope}}) + A]. \quad (1)$$

$$v = VA/[K + A(1 + I/K_{i_{intercept}})]. \quad (2)$$

$$v = VA/[K(1 + I/K_{i_{slope}}) + A(1 + I/K_{i_{intercept}})] \quad (3)$$

Initial parameter estimates were obtained by a weighted multiple linear regression analysis using the reciprocal form of the appropriate equation [16, 22]. Because the experimental velocities exhibited a constant proportional error [22], the data were generally weighted by the square of the reciprocal of the calculated velocity. Final parameter estimates were established using a nonlinear regression routine [16]. The best fit of the experimental data to a given model was assumed on the basis of the least residual mean square value and on estimates of the kinetic parameters not encompassing zero.

RESULTS

Steroid substrate specificity

To assess the C_{19} vs C_{21} steroid substrate specificity of the purified cytosolic NADPH:5α-DHP 3α-HSOR, we tested the corresponding C_{19} androgenic substrates, 5α-DHT and 3α,5α-THT, in the reductive and oxidative directions, respectively. Table 1 compares the apparent K_m

Table 1. Kinetic constants for purified cytosolic 3α-HSOR[a]

Substrate	Apparent K_m (μm)	Apparent V_{max} (μmole product/mg/30 min)	V_{max}/K_m
5α-DHT	21 ± 5	45 ± 9	2.1
3α,5α-THT	4.2 ± 0.2	13.0 ± 0.3	3.1
5α-DHP[b]	0.08 ± 0.01	1.2 ± 0.1	1.5
3α,5α-THP	1.4 ± 0.4	9.7 ± 1.3	6.9

[a]The 3α-HSOR activities were determined using 85 ng of purified enzyme as described in the Kinetic analysis section of Experimental. Enzyme activity at each substrate concentration was the average of duplicate determinations. The derived kinetics constants (±SE) were calculated as detailed under Experimental.

[b]The kinetic constants for the C_{21} steroids were obtained from work previously done in our laboratory [13].

Table 2. The effects of various 3α/3β-hydroxysteroids on 3α-HSOR activity[a]

Test steroid	3α-HSOR activity (nmol of 3α,5α-THP/mg/30 min)
Control (5α-DHP)	1020 ± 6
3β- and 5β-isomers	
5β-DHP	950 ± 31
3α,5β-Dihydroprogesterone	940 ± 37
3β,5β-Dihydroprogesterone	950 ± 31
3β,5α-Dihydroprogesterone	980 ± 21
Ovarian progestins	
Progesterone	610 ± 14*
20α-Dihydroprogesterone	610 ± 28*
17α-Hydroxyprogesterone	710 ± 8*
Glucocorticoids and estradiol	
Deoxycorticosterone	760 ± 7*
Corticosterone	990 ± 3
Cortisol	920 ± 15
17β-Estradiol	910 ± 19

[a]NADPH:5α-DHP 3α-HSOR activity was determined as described in Experimental in the presence of 100 nM [^{3}H]5α-DHP, 200 μM NADPH and 100 nM of the competing test steroid using 28.5 ng of purified 3α-HSOR [13]. The results are the average of triplicate determinations (±SE).

*Significantly different from control at $P < 0.01\%$ using the non-parametric version of Dunnett's test [39].

and V_{max} values determined for the C_{21} progestin substrates, 5α-DHP and 3α,5α,-THP, to those determined for their C_{19} androgenic analogs. The bimolecular rate constants (V_{max}/K_m) indicate that the C_{21} steroid substrates are preferred over the corresponding C_{19} analogs. The K_m for 5α-DHP was 250-fold and 50-fold lower than K_m values determined for 5α-DHT (21 μM), and 3α,5α-THT (4.2 μM), respectively. The K_m for 3α,5α-THP was 3-fold lower than the K_m determined for 3α,5α-THT (4.2 μM). These results clearly demonstrate that the enzyme has a strong preference for the C_{21} steroid, 5α-DHP.

A number of other naturally occurring 3-keto/3-hydroxysteroids were tested as possible alternate steroid substrates by examining their ability to act as inhibitors [18, 19] of the 3α-reduction of 5α-DHP catalyzed by the purified 3α-HSOR. In these studies (Table 2), we surveyed various 3β- and 5β-isomers of the C_{21} progestin substrates as well as the major ovarian progestins, some glucocorticoids and estradiol. There was no inhibition with any of the 5β- and 3β-isomers of 5α-DHP and 3α,5α-THP. Although none of the other tested steroids strongly inhibited the 3α-HSOR catalyzed 3α-reduction of 5α-DHP, we did observe significant inhibition (25–40%) with deoxycorticosterone and the ovarian progestins, progesterone, 20α-dihydroprogesterone and 17α-hydroxyprogesterone. The lack of any strong inhibition by these steroids at the concentrations tested supports the above conclusion that 5α-DHP is the preferred substrate for the 3α-HSOR in the reductive direction.

Can the 3α-HSOR catalyze the 3α-reduction of Δ⁴-3-ketosteroids such as progesterone?

Since some of the tested Δ⁴-3-ketosteroids, especially the ovarian progestins (Table 2), were able to inhibit the 3α-reduction of 5α-DHP catalyzed by the 3α-HSOR, we considered the possibility that these steroids were alternate substrates and underwent 3α-reduction of their 3-keto groups. We further examined progesterone as a representative test steroid of this group. Among other possibilities, it was possible that the cytosolic pituitary 3α-HSOR was directly catalyzing the 3α-reduction of progesterone to the allylic alcohol, 3α-DHP, without a prior 5α-reduction to 5α-DHP. We found that the conversion of [^{3}H]progesterone to [^{3}H]3α-DHP was barely detectable. Because the radioactivity associated with non-radioactive carrier 3α-DHP after the third purification step was too low for meaningful analysis, we could not confirm the presence of [^{3}H]3α-DHP as a metabolite. If, however, we assume that the radioactivity that was still associated with carrier 3α-DHP after the third specific activity analysis was in fact [^{3}H]3α-DHP, then this conversion would represent less than 0.2% (58 pmol/mg/30 min) of that observed when the preferred substrate, 5α-DHP, was converted to 3α,5α-THP (35.9 nmol/mg/30 min).

We also tested for the possibility that the purified 3α-HSOR was catalyzing the 20α-hydroxysteroid oxidoreduction of [^{3}H]progesterone to [^{3}H]20α-DHP. After two TLC separations, the radioactivity associated with the non-radioactive carrier 20α-DHP was again too low to draw meaningful conclusions. If present, the conversion of progesterone to 20α-DHP would have been less than 0.001%. Taken together, the results suggest that progesterone, 20α-dihydroprogesterone, 17α-hydroxyprogesterone and deoxycorticosterone may be acting as inhibitors rather than alternate substrates.

Table 3. Influence of sulfhydryl reducing or blocking reagents on purified 3α-HSOR activity[a]

Reagent added	3α-HSOR activity (μmol/mg/30 min)	% Control[b]
Control	1.2	—
β-Mercaptoethanol (1%)	1.7	142
p-Chloromercuribenzoic acid (1 mM)	1.1	92
N-ethylmaleimide (1 mM)	0.4	33

[a]Cytosolic NADPH:5α-DHP 3α-HSOR activity was assayed as described in the Experimental section using 60 ng of 3α-HSOR, 1 μM [^{3}H]5α-DHP, 200 μM NADPH and the indicated additions. The results are represented as the mean of duplicate measurements.

[b]The data are compared to control (no addition) and are expressed as percent control.

Sulfhydryl sensitivity

Because our previous report [10] with a crude enzyme preparation of cytosolic pituitary 3α-HSOR activity suggested that sulfhydryl groups may be necessary for maximal activity, we re-examined the effects of sulfhydryl-reducing and sulfhydryl-blocking reagents on the purified 3α-HSOR (Table 3). The present results again support our earlier finding that reactive sulfhydryl groups are important for maximal 3α-HSOR activity. As shown, the addition of the sulfhydryl-reducing agent, 2-mercaptoethanol, stimulated the purified activity by nearly 50%. We observed different results with the two sulfhydryl-blocking reagents. *N*-ethylmaleimide inhibited 3α-HSOR activity by 67%. *p*-Chloromercuribenzoic acid, however, had little effect in contrast to the results obtained earlier with the crude cytosolic 3α-HSOR [10]. It is possible that this sulfhydryl-blocking reagent is not as effective with the purified enzyme, as reported by others for a number of sulfhydryl-containig enzymes [23].

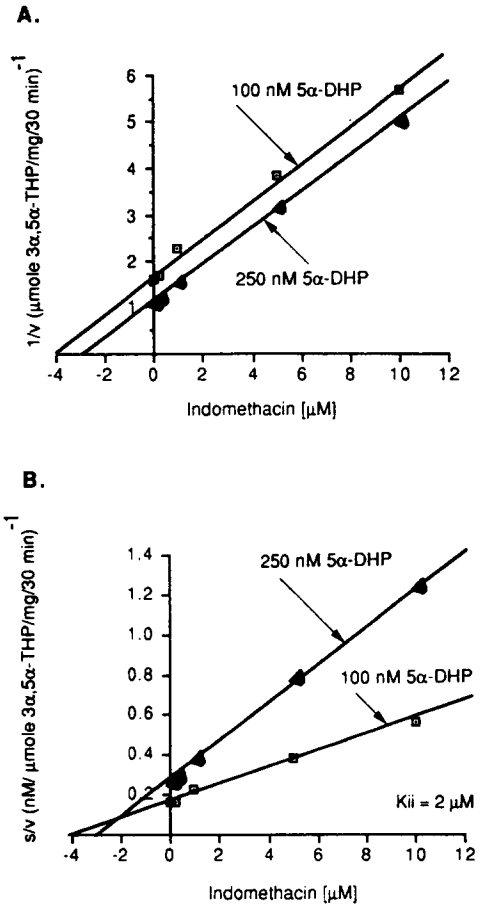

Fig. 1. Inhibition of purified NADPH:5α-DHP 3α-HSOR by indomethacin (A) Dixon Plot; (B) Cornish–Bowden Plot. All incubations contained either 100 or 250 nM [^{3}H]5α-DHP, 60 ng of purified 3α-HSOR and saturating concentrations of NADPH (200 μM). Indomethacin was present at the following concentrations: 0, 0.25, 1, 5, 10 μM. The assays were carried out as described in Experimental. Each point is the average of duplicate measurements.

Inhibition by indomethacin

Other workers had reported that a number of non-steroidal anti-inflammatory drugs, including indomethacin, are potent inhibitors of similar cytosolic 3α-HSORs from male rat brain and liver [20, 24]. In those studies indomethacin was shown to be a competitive inhibitor whereas in our studies indomethacin appears to be a potent *uncompetitive* inhibitor (Fig. 1) of the pituitary cytosolic 3α-HSOR exhibiting an apparent K_{ii} of $1.9 \pm 0.2\,\mu M$ vs 5α-DHP. These results suggest the possibility that increasing levels of 5α-DHP during the estrus cycle [25] could increase the ability of indomethacin to inhibit the 3α-HSOR [26].

Inhibition by medroxyprogesterone

Medroxyprogesterone acetate, a potent synthetic progestational steroid, has been shown to inhibit cytosolic 3α-HSOR activity in rat liver and brain [20, 24]. It was of interest to determine if medroxyprogesterone acetate (17α-acetoxy-6-methyl-4-pregnene-3,20-dine) could also inhibit the pituitary enzyme. Medroxyprogesterone acetate (Fig. 2A) is a potent competitive inhibitor vs 5α-DHP with an apparent K_{is} of 4.9 ± 1.0 nM. Medroxyprogesterone, itself, is also a competitive inhibitor vs 5α-DHP (Fig. 2B) with an apparent K_{is} of 30.9 ± 3.2 nM. Although this competitive inhibition by medroxyprogesterone is quite potent, it can, nevertheless, be overcome by increasing levels of 5α-DHP [27] unlike the potent uncompetitive inhibition described above for indomethacin.

Does the 3α-HSOR also have dihydrodiol dehydrogenase activity?

The rat liver cytosolic 3α-HSOR has a broad substrate specificity beyond steroids since it can also use dihydrodiols (quinones) as substrates [20, 28], albeit less efficiently than 3α-hydroxysteroids. The male brain cytosolic 3α-HSOR, on the other hand, does not appear to have dihydrodiol dehydrogenase activity [24, 28]. To determine if the purified pituitary 3α-HSOR also has dihydrodiol dehydrogenase activity, we examined, as a representative test compound, the commonly used quinone substrate, 9,10-phenanthrenequinone, for its ability to inhibit the 3α-HSOR catalyzed 5α-DHP reduction.

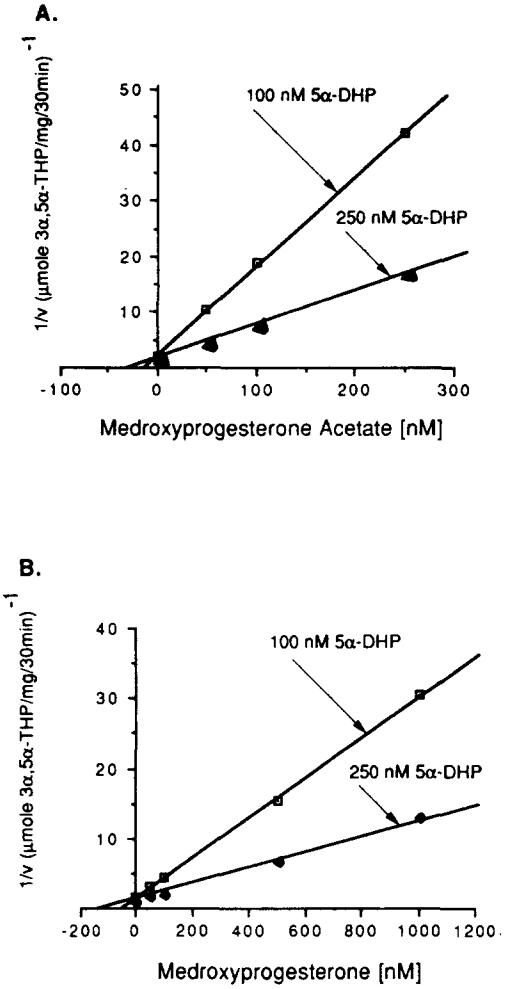

Fig. 2. Inhibition of purified NADPH:5α-DHP 3α-HSOR by medroxyprogesterone acetate (A) and medroxyprogesterone (B) (A) All incubations contained either 100 or 250 nM [^{3}H]5α-DHP, 80 ng of purified 3α-HSOR and saturating concentrations of NADPH (200 μM). Medroxyprogesterone acetate was present at the following concentrations: 0, 50, 100, 250 nM. (B) All incubations contained either 100 or 250 nM [^{3}H]5α-DHP, 60 ng of purified 3α-HSOR and saturating concentrations of NADPH (200 μM). Medroxyprogesterone was present at the following concentrations: 0, 50, 100, 500, 1000 nM. The assays for both inhibitors were carried out as described in Experimental. Each point is the average of duplicate measurements.

There was no inhibition at 0.5 μM 9,10-phenanthrenequinone and only 20% at 1 μM. This suggests that the pituitary enzyme, like the brain enzyme, does not have significant dihydrodiol dehydrogenase activity.

DISCUSSION

The purified pituitary cytosolic 3α-HSOR clearly has a strong preference for the C_{21} progestin substrates, 5α-DHP and 3α,5α-THP, over the corresponding androgenic C_{19} substrates. Moreover, the results suggest that 5α-DHT is more likely to act as a weak inhibitor rather than an alternate substrate because the K_m for 5α-DHP is 250-fold lower than the K_m for 5α-DHT, while the plasma concentration of 5α-DHT in female rats is 200–1400-fold lower than its K_m [29]. The results also indicate that the reductive direction is preferred since the K_m constants for both C_{21} progestin and C_{19} androgen substrates are lower in the reductive direction than in the oxidative direction. When other 3-keto-/3-hydroxysteroids were tested as possible inhibitors and/or alternate steroid substrates, the data suggest that the enzyme may prefer 3-hydroxy-C_{21} steroids having both 3α- and 5α-reduced configurations since none of the 3β- or 5β-isomers of 3α,5α-THP nor 5β-DHP inhibited the 3α-HSOR catalyzed 3α-reduction of 5α-DHP. However, the presence of a 5α-reduced configuration may not be necessary for binding, and thus inhibition, since some of the tested Δ^4-3-ketosteroids, especially the ovarian progestins, did significantly inhibit the 3α-reduction of 5α-DHP. The influence of hydroxyl groups on inhibition was variable, i.e. some of the tested Δ^4-3-ketosteroids with C-11 hydroxyl moieties were poor inhibitors of the 3α-reduction of 5α-DHP, whereas hydroxylations at the C-17 or C-20 positions did not substantially affect inhibitory activity.

The studies examining radiolabeled progesterone as a possible substrate suggest that the ovarian progestins are not alternate substrates since the direct 3α-reduction of progesterone to the allylic alcohol, 3α-DHP, is barely detectable. If present, this conversion would have been less than 0.2% of that observed when 5α-DHP was used as the substrate. Thus, these Δ^4-3-ketosteroids may be acting as inhibitors of the 3α-reduction of 5α-DHP rather than alternate substrates. This inhibition by ovarian progestins could be important whenever their circulating levels are high enough to inhibit the 3α-reduction of 5α-DHP and/or other appropriate 3-keto/3-hydroxysteroid substrates. On the other hand, if the enzyme does catalyze the 3α-reduction of progesterone and other progestins to their corresponding 3α-reduced allylic alcohols (albeit in small amounts), then this small conversion may be an important mechanism for generating threshold levels of 3α-DHP in the anterior pituitary. Wood and Wiebe [30] have reported that 3α-DHP can have significant inhibitory effects on FSH release at very low concentrations (10^{-12}–10^{-15} M). Thus, if present, this small conversion could generate a potent antagonist

of FSH release. A similar situation obtains in the hypothalamus and other brain tissues where a small conversion of androgens by aromatase can generate potent estrogens.

Although there are some notable differences, the purified pituitary enzyme does have some properties in common with several cytosolic 3α-HSORs that have been purified and characterized from male rat liver [20, 31], brain [24] and prostate [15, 32]. These 3α-HSORs, also known as 3α-hydroxysteroid dehydrogenases [EC 1.1.1.50], also prefer NADPH and $NADP^+$ as cofactors and 3α-hyhdroxy- or 3-keto-steroids as substrates [15, 20, 24, 31]. Most of the studies to date with these 3α-HSORs [15, 24, 32, 33] have only assessed the oxidoreduction of C_{19} androgenic steroid substrates and not the C_{21} progestin substrates, 5α-DHP and 3α,5α-THP. One study, however, with partially purified prostate 3α-HSOR [32] did report a lower K_m for 5α-DHP (270 nM) than for 5α-DHT (600–820 nM), which suggests that this enzyme from this male tissue source may also prefer 5α-DHP over 5α-DHT. The K_ms for 5α-DHT and for 3α,5α-THT reported here are similar to those reported for the cytosolic 3α-HSORs from male rat brain and prostate [24, 32]. The cytosolic 3α-HSOR from female rat brain also has a similar substrate specificity profile and exhibits a K_m for 5α-DHP that is lower than that for 5α-DHT (Campbell and Karavolas, unpublished results).

The male rat liver cytosolic 3α-HSOR has a broader substrate specificity than the present 3α-HSOR and rat brain 3α-HSOR because it also has dihydrodiol dehydrogenase activities [28, 31]. Unlike the pituitary and brain enzymes, it also catalyzes the oxidoreduction of many dihydrodiols, quinones, prostaglandins and various proximate and ultimate carcinogens [31, 33]. Even so, the liver 3α-HSOR does have a greater preference for 3-keto/3α-hydroxy-steroids as substrates; it utilizes the C_{19} substrate, androsterone, 1000-fold more efficiently than any of the dihydrodiol substrates tested [33]. The pituitary 3α-HSOR appears to be more similar to the male rat brain 3α-HSOR [24] since neither enzyme has significant associated dihydrodiol dehydrogenase activity. Furthermore, the 3α-HSORs from different tissues also exhibit immunological differences [28]. Smithgall and coworkers [28] have reported that antibodies to male rat liver cytosolic 3α-HSOR did not cross-react with cytosol from male rat brain or prostate (pituitary was not tested).

Another notable property of these other cytosolic 3α-HSOR enzymes, especially those of the liver and brain, is that they are potently inhibited by several non-steroidal anti-inflammatory drugs, including indomethacin [20, 24, 31]. Using indomethacin as a representative test compound, our results clearly indicate that the purified pituitary 3α-HSOR also shares this property. However, indomethacin is an *uncompetitive* inhibitor vs 5α-DHP with the pituitary enzyme, in contrast to the competitive nature of its inhibition of the male rat and mouse liver 3α-HSORs [20, 34]. These differences in the type of inhibition suggest, among other things, that there may be additional tissue specific differences between these enzymes.

The uncompetitive nature of inhibition by indomethacin suggests the possibility of a regulatory site. That is, the binding of 5α-DHP to the pituitary 3α-HSOR may "create" a binding site on the enzyme for indomethacin since no inhibition was observed a low concentrations of 5α-DHP [27]. This possibility is supported by the observation that most pyridine nucleotide-linked enzymes utilize ordered sequential kinetic mechanisms [35] whereby the binding of cofactor precedes the binding of the substrate. This, taken together with the fact that indomethacin is an uncompetitive inhibitor vs 5α-DHP, suggests that indomethacin binds to the enzyme at a site other than the NADPH or steroid binding sites on the 3α-HSOR. *In situ* this uncompetitive inhibition by indomethacin may effectively antagonize the pathway [26] for the production of 3α,5α-THP in the anterior pituitary, with increasing inhibition being observed as 5α-DHP levels rise during the estrous cycle [25]. It is of interest to note that, while competitive inhibition can eventually be reversed by the increasing levels of substrate as a result of the inhibition, uncompetitive inhibition increases with higher substrate levels because more inhibitor sites are created [26]. The differential inhibitory effect of indomethacin during times of changing progestin substrate concentrations may have consequences on the *in vivo* production of 3α,5α-THP and, in turn, on 5α-DHP and 3α,5α-THP progesterone-sensitive biological end-points in the anterior pituitary.

Another property that the pituitary 3α-HSOR shares with the cytosolic enzymes from male rat liver, brain, testis and mouse liver [20, 24, 34, 36] is its potent inhibition by low concentrations of medroxyprogesterone acetate. Medroxyprogesterone acetate and medroxy-

progesterone are also competitive inhibitors of pituitary 3α-HSOR catalyzed 5α-DHP reduction. Yet, neither compound inhibits the pituitary progesterone 5α-reductase [37]. This specificity for only one of the two major progesterone metabolizing steps in the anterior pituitary suggests a means by which medroxyprogesterone may exert differential effects on pituitary progesterone metabolism *in situ*.

The physiological role of this enzyme in the female rat anterior pituitary is not clear at this time. The enzyme is clearly different in many respects from the liver cytosolic enzyme and in some respects from the brain and prostatic 3α-HSOR. Also, the purified enzyme from this important female feedback tissue strongly prefers the C_{21} progestin substrates over the C_{19} androgen analogues. The product of the reductive direction, 3α,5α-THP, is a potent modulator of the $GABA_A$ receptor complex [7, 8] which may be the underlying mechanism for its potent anesthetic and anti-epileptic effects [6, 9]. Several recent reports suggest that the $GABA_A$ receptor complex may be mediating the 3α,5α-THP stimulation of LH and FSH release [5] and prolactin release [38]. Our inhibition studies with various drugs and naturally occurring steroids suggest that the pituitary production of 3α,5α-THP during the estrous cycle may be differentially regulated. Changing substrate and ovarian steroid levels could modulate 3α,5α-THP production and consequently its ability to influence dose-dependent biological end-points in the pituitary.

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