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PURIFICATION OF THE NADPH:5α-DIHYDROPROGESTERONE 3α-HYDROXYSTEROID OXIDOREDUCTASE FROM FEMALE RAT PITUITARY CYTOSOL

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Summary—The NADPH:5α-dihydroprogesterone 3α-hydroxysteroid oxidoreductase (3α-HSOR) [EC 1.1.1.50] which catalyzes the reversible conversion of 5α-pregnane-3,20-dione (5α-dihydroprogesterone; 5α-DHP) to 3α-hydroxy-5α-pregnan-20-one (3α-,5α-tetrahydroprogesterone; 3α,5α-THP) was purified to apparent homogeneity from female rat anterior pituitary cytosol by a three step micro-purification procedure. Specific activity of purified 3α-HSOR was enriched 438-fold from that in pituitary cytosol using successive ion exchange, chromatofocusing and affinity column chromatography purification steps. 3α-HSOR appears to be a monomer with an approximate molecular weight of 36 kDa and an isoelectric point of about 5.75. The purified enzyme appears as a single protein staining band (36 kDa) when examined by polyacrylamide gel electrophoresis and with both silver or Coomassie blue staining. Under non-dissociating electrophoretic conditions, all of the 3α-HSOR activity co-migrated with the 36 kDa protein staining band. The purified enzyme in the presence of the preferred cofactor, NADPH, has an apparent K_m for 5α-DHP of 82 nM and a V_{max} of 1.2 μmol of 3α,5α-THP formed per mg protein/30 min. The K_m for NADPH was 0.71 μM. In the oxidative direction, the enzyme in the presence of $NADP^+$ has a K_m for 3α,5α-THP of 1.4 μM and a V_{max} of 9.7 μmol of 5α-DHP formed per mg protein/30 min. The K_m for $NADP^+$ was 1.6 μM.

INTRODUCTION

The biochemical mechanisms by which progesterone influences progesterone-sensitive events such as gonadotropin regulation in the anterior pituitary are not well understood. In the anterior pituitary, progesterone is rapidly converted 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 5α-DHP and 3α,5α-THP have significant progesterone-like neuroendocrine effects on gonadotropin regulation and ovulation [1–6]. Recently 3α,5α-THP has been shown to be more potent than either progesterone or 5α-DHP in augmenting GABA-induced inhibition of synaptic transmission [7–9]. These progesterone-like effects of 3α,5α-THP suggest that *in situ* conversion of progesterone to 3α,5α-THP may be important in progesterone's neuroendocrine actions.

Previous work has demonstrated that the conversion of 5α-DHP to 3α,5α-THP is catalyzed by two distinct 3α-hydroxysteroid oxidoreductases (3α-HSOR): either a cytosolic 3α-HSOR, which prefers NADPH as cofactor, or a particulate 3α-HSOR, which prefers NADH as cofactor [10, 11]. These studies also partially characterized their subcellular location, pH and temperature optima, and kinetic parameters [10–12]. In this paper, we report the purification of the NADPH:5α-DHP 3α-HSOR from female rat anterior pituitary cytosol and some of its kinetic properties.

EXPERIMENTAL

Materials

[1,2-^{3}H]5α-DHP (sp. act.: 57.5 Ci/mmol) was purchased from the New England Nuclear Corp. [^{3}H]3α,5α-THP was synthesized enzymatically [10]. DEAE–Sephacel, Polybuffer Exchanger 94, Polybuffer 74 and Blue Sepharose CL-6B were purchased from Pharmacia PL Biochemicals (Milwaukee, Wis.). The sources and purity of the steroids and reagents used in

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TLC, GLC and enzymatic assays have also been described previously [10, 13]. The molecular weight markers for electrophoresis and all other reagents were purchased from Sigma Chemical Co. (St Louis, Mo.) and were of the highest purity available.

Enzyme assay

Cytosolic NADPH:5α-DHP 3α-HSOR activity was measured using radiolabeled steroid substrates and reverse isotopic dilution analysis [10, 13]. Unless otherwise indicated, NADPH-linked enzymatic activity was assayed in the reductive direction under saturating conditions in a 5.0 ml reaction mixture containing 200 μM NADPH, 1 μM [^{3}H]5α-DHP in a 100 mM potassium phosphate buffer, pH 7.2 containing 1 mM dithiothreitol (DTT) and 1 mM EDTA and any indicated additions. Bovine gamma immunoglobulin (10 μg/ml) was added as a stabilizing carrier protein in assays with purified enzyme. The reactions were initiated after a 10 min pre-incubation period at 37°C by the addition of the radiolabeled steroid substrates in 50 μl ethanol [10, 13]. Control incubations contained all assay components except enzyme. Incubations were carried out for 30 min in a shaking water bath at 37°C. Termination of the enzyme reaction and quantitation of product steroids were performed as described previously [10, 13]. NADPH:5α-DHP 3α-HSOR activity is expressed as nmol of product (3α,5α-THP) formed per mg protein per 30 min. Assays for NADH-linked 3α-HSOR activity employed the same conditions as above except that NADH was present at 200 μM instead of NADPH.

Purification

Preparation of cytosol. Anterior pituitaries were obtained from 60-day-old female Holtzman rats at random stages of the estrous cycle [10]. All procedures were performed at 0–4°C. Generally, 90–100 anterior pituitaries (0.9–1.0 g) were homogenized in 6 ml 20 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 5 μM NADPH, 1 mM phenylmethylsulfonyl fluoride, 0.7 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 0.32 M sucrose (homogenizing buffer). The homogenization was performed with 10 complete up and down strokes in a Thomas homogenizer [10]. The homogenate was centrifuged at 1000 *g* for 10 min. The pellet was resuspended with vortexing in 2.5 ml homogenizing buffer and the resuspended homogenate was centrifuged at 1000 *g* for 10 min [10]. The two 1000 *g* supernatants were combined and centrifuged at 105,000 *g* for 60 min. Unless otherwise indicated, the resulting cytosol (supernatant fluid) was used as the source of the cytosolic NADPH:3α-HSOR enzyme in subsequent studies.

DEAE–Sephacel chromatography. Prior to application, the cytosol was diluted with water (containing 1 mM DTT and 5 μM NADPH) to a conductivity of 0.5 mmho and its pH adjusted to 8.3 with 1 M Tris–OH. This preparation (usually 80–100 ml) was then applied to a DEAE–Sephacel column (13.5 × 0.8 cm) pre-equilibrated with "Tris equilibrating buffer" (10 mM Tris–HCl buffer, pH 8.3, containing 0.1 mM EDTA and 1 mM DTT). The column was eluted with a 60 ml linear salt gradient of 0–250 mM NaCl in "Tris equilibrating buffer". Fractions of 60 drops were collected and assayed for conductivity, protein and enzymatic activity. The NADPH:3α-HSOR activity eluted in fractions with salt concentrations of 75–100 mM NaCl. After measuring conductivity, each fraction received 35 μl 0.5 M imidazole–HCl buffer, pH 7.4, containing 10% glycerol to stabilize the enzymatic activity.

Chromatofocusing chromatography. The pooled active fractions from the DEAE–Sephacel step (usually 8–10 ml) were applied to an Amicon ultrafiltration apparatus with a YM10 membrane in order to reduce the volume and to exchange the buffer with "imidazole equilibrating buffer" used for the chromatofocusing column (25 mM imidazole–HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM DTT and 20% glycerol). When the volume in the ultrafiltration apparatus was reduced to 25%, sufficient "imidazole equilibrating buffer" was added to restore the starting volume. Ultrafiltration was continued until the volume was again reduced to 25% (2–3 ml). To this concentrate, sufficient "imidazole equilibrating buffer" was added to adjust the pH to 7.4 and reduce the conductivity to 1 mmho. This preparation (usually 5–7 ml) was applied to a column packed with Polybuffer Exchanger 94 (19 × 0.7 cm) topped with a layer (0.5–1 cm) of Sephadex-G25 Coarse and pre-equilibrated with the "imidazole equilibrating buffer" (Pharmacia Chromatofocusing manual). After the sample was loaded, 2–3 ml of the imidazole equilibrating buffer was applied to provide a protective buffer zone between sample proteins and the extremes of pH

with the Polybuffer 74 (Pharmacia Chromatofocusing manual). NADPH:3α-HSOR activity was eluted with an internally generated linear pH gradient using Polybuffer 74-HCl, pH 4.5 containing 1 mM EDTA, 1 mM DTT and 20% glycerol. Fractions of 72 drops were collected and assayed for pH, protein and enzymatic activity. NADPH:3α-HSOR activity eluted in fractions with pH values in the range of 5.9–5.6. After measuring the pH, but before assaying for protein and enzyme activity, all fractions received 80 μl 1 M Tris–HCl (pH 8.5) to stabilize the enzyme activity. This was usually sufficient to adjust the pH to 7.4.

Blue-Sepharose CL-6B affinity chromatography. Active fractions from the chromatofocusing step were pooled (usually 20–35 ml) and, when necessary, the pH was adjusted to 7.4 with 1 M Tris buffer. To this pool and to all subsequent enzyme preparations, 10 μg/ml leupeptin was added to lessen non-specific binding losses and to stabilize the enzyme [14]. The pooled preparation was applied to a column packed with Blue Sepharose CL-6B (2.8 × 0.5 cm) pre-equilibrated with "KP_i eluting buffer" (20 mM KP_i buffer, pH 7.4, containing 1 mM EDTA, 1 mM DTT, 20% glycerol and 10 μg/ml leupeptin). The column was then washed twice; first with 10 ml "KP_i eluting buffer" and then 10 ml "KP_i eluting buffer" at pH 8.3. The column was eluted with 12 ml 8 mM $NADP^+$ in "KP_i eluting buffer" at pH 8.3. Fractions of 22 drops were collected and were assayed for protein and enzymatic activity. Peak fractions were pooled and Triton X-100 was added to a final concentration of 0.05% to lessen non-specific binding of the enzyme to plastic or glass. This pooled enzyme preparation (4.5–7 ml) was concentrated with an Amicon Centricon 10 microconcentrator which was prerinsed with 20 mM "KP_i eluting buffer" containing 0.05% Triton X-100. Unless otherwise indicated, the resulting concentrated enzyme preparation was used as the source of the purified cytosolic NADPH:5α-DHP 3α-HSOR in subsequent studies (purified or "affinity-purified" enzyme).

Protein determination. Protein concentrations in the cytosol and various column chromatography fractions were determined by the micro-Bradford procedure [15] using crystalline bovine serum albumin as the standard. However, when protein concentrations in these samples fell below 1 μg; protein concentrations were estimated with a colloidal gold protein detection procedure [16] as modified by Bio-Rad for their colloidal gold protein detection kit (see Bio-Rad instruction manual).

Electrophoretic analyses for purity

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE electrophoresis of the various enzyme preparations was performed [17] using 12.5% discontinuous slab mini-gels (5.9 × 10.2 × 0.75 mm for the resolving gel and 1.2 × 10.2 × 0.75 mm for the stacking gel). Gels were stained for proteins using the Coomassie brilliant blue [18] and silver staining [19] procedures.

Non-dissociating PAGE. A modified version of the "high pH" non-dissociating PAGE procedure [18] was employed to assess the purity of the non-dissociated enzyme and coincidence of the 3α-HSOR activity with the non-dissociated purified enzyme. To facilitate solution of the gel matrix for subsequent measurement of 3α-HSOR activity in the gels, *N,N'*-bisacrylylcystamine (BAC) was used as crosslinker [20] instead of bisacrylamide. The 30% stock acrylamide solution was made with 28.8 g of acrylamide and 1.2 g of BAC crosslinker. The resolving gel (5.9 × 10.2 × 0.5 mm) contained 10% acrylamide/BAC in the Tris–HCl resolving gel buffer, pH 8.8 [18]. The resolving gel was pre-electrophoresed for 2 h at 10 mA in Tris–glycine reservoir buffer, pH 8.3, containing 1 μM 5α-DHP, 10 μM NADPH and 1 mM EDTA to equilibrate the gel and to remove ammonium persulfate and unreacted reagents. The stacking gel [19] measured 1.2 × 10.2 × 0.5 mm.

Aliquots of purified enzyme and marker proteins were applied to the stacking gel in pH 6.8 stacking gel buffer [18] containing 500 μM NADPH, 0.1% 2-mercaptoethanol and 10% glycerol. In some studies, pre-stained molecular weight standards were used to aid estimation of the location of the 36 kDa 3α-HSOR. The gels were electrophoresed for about 5 h at 10 mA. After electrophoresis, half of the gel containing one lane of purified enzyme and one lane of reference proteins were stained with Coomassie blue [18]. The other half was assayed for NADPH:5α-DHP 3α-HSOR activity. The lane containing the purified enzyme was cut into successive 1 cm sections. To facilitate the assay of 3α-HSOR activity, the gel sections were treated with 2-mercaptoethanol to dissolve the gel by reducing the disulfide bonds in the BAC crosslinker. Gel sections were placed in enzyme

assay flasks (25 ml Erlenmeyer flasks) containing 200 μl 2-mercaptoethanol and 100 μl 100 mM KP_i buffer, pH 7.2, containing 1 mM EDTA, 1 mM DTT and 2 mM NADPH. After 10 min, the mixture was appropriately diluted with the enzyme assay components. For each flask, the final reaction mixture contained 100 mM KP_i, 1 mM EDTA, 1 mM DTT, 240 μM NADPH, 1 μM [^{3}H]5α-DHP and 4% 2-mercaptoethanol. Reactions were initiated with 1 μM [^{3}H]5α-DHP. With this procedure, there was a loss of approx. 25–30% of the NADPH:3α-HSOR activity which is similar to that reported previously [20]; this may be due to incomplete dissolution of gel sections or denaturation of the enzyme.

Kinetic analysis of purified NADPH:5α-DHP 3α-HSOR

To determine the kinetic parameters for the steroid substrates, the reductive and oxidative cofactors (NADPH and $NADP^+$) were present at constant saturating concentrations, while the steroid substrate concentrations were varied. For the reductive direction, the cofactor and the steroid substrate were present at the following concentrations: [^{3}H]5α-DHP = 50, 100, 250, and 1000 nM, NADPH = 400 μM. For the oxidative direction, the cofactor and the steroid substrate were present at the following concentrations: [^{3}H]3α,5α-THP = 0.75, 1, 10, 50 μM, $NADP^+$ = 1 mM. To determine the kinetic parameters for the cofactors, [^{3}H]5α-DHP and [^{3}H]3α,5α-THP were present at saturating amounts (1 and 50 μM, respectively), while NADPH and $NADP^+$ were varied at the following concentrations: NADPH = 1, 5, 10, 250 μM; $NADP^+$ = 1, 5, 10, 500 μM.

Kinetic analyses

Enzyme kinetic data were analyzed using an IBM personal computer and fitted to the equation [21]:

$$v = V_{max} A/[K_m + A].$$

Initial parameter estimates (K_m, V_{max}) were obtained by a linear regression analysis using the reciprocal form of this equation [21, 22]. A non-linear regression method was used to establish the final parameter estimates [22].

RESULTS

Purification of NADPH:5α-DHP 3α-HSOR

The three step micro-purification scheme employing DEAE–Sephacel ion exchange, chromatofocusing, and Blue-Sepharose-affinity chromatography (Figs 1–3) provides a successful, rapid method for purifying NADPH:5α-DHP 3α-HSOR from pituitary cytosol. DEAE–Sephacel chromatography of the pituitary

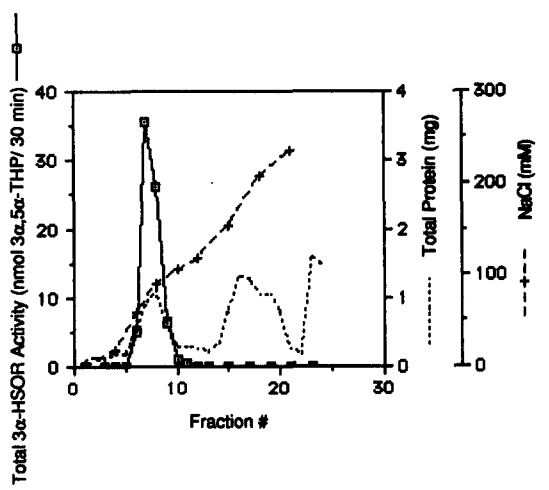

Fig. 1. DEAE–Sephacel chromatography. Pituitary cytosol (90 ml) containing 70 nmol NADPH:3α-HSOR activity was applied to a DEAE–Sephacel column (13.5 × 0.8 cm). The column was eluted with a linear NaCl gradient (60 ml) from 0–250 mM NaCl in "Tris equilibrating buffer" (10 mM Tris–HCl buffer, pH 8.3, containing 0.1 mM EDTA and 1 mM DTT). Fractions of 60 drops (3.2 ml) were collected. Aliquots of 50 μl were assayed for enzymatic activity as described in Experimental.

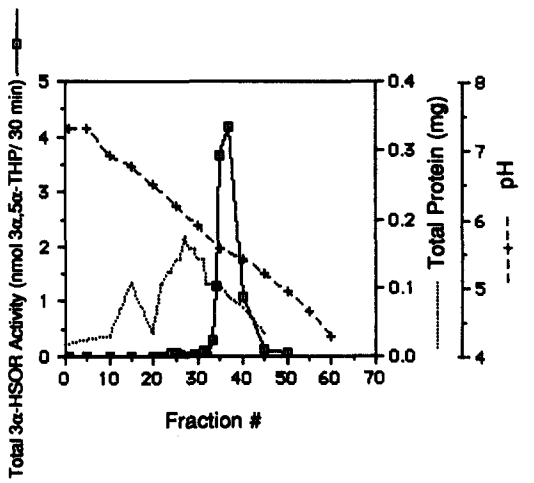

Fig. 2. Chromatofocusing chromatography. Fraction (Nos 6–8) from the DEAE–Sephacel column (Fig. 1) were pooled and concentrated as described in Experimental. The concentrated material (7 ml) was applied to a chromatofocusing column packed with Pharmacia Polybuffer Exchanger 94 (19 × 0.7 cm). The 3α-HSOR activity was eluted with an internally generated pH gradient with Polybuffer 74 (pH 4.5) containing 20% glycerol, 1 mM EDTA and 1 mM DTT. Fractions of 72 drops (3.6 ml) were collected and aliquots of 75 μl were assayed for enzymatic activity as described in Experimental.

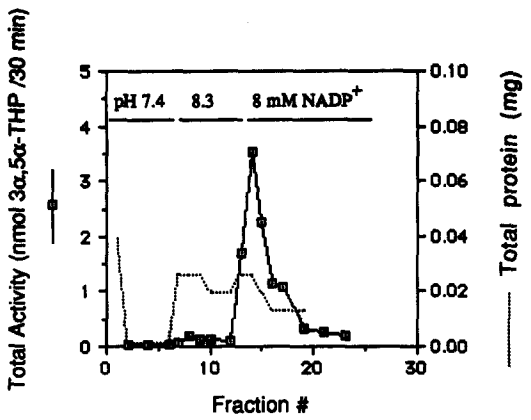

Fig. 3. Blue-Sepharose affinity chromatography of the pooled fractions from the chromatofocusing column (Fig. 2). Fractions (Nos 35–41) containing the highest 3α-HSOR specific activity were combined (23 ml) and applied to a Blue-Sepharose column (2.8 × 0.5 cm). The column was washed twice; first with "KP_i eluting buffer" (10 ml) and then "KP_i eluting buffer" at pH 8.3 (10 ml). The enzyme was eluted with 8 mM $NADP^+$ in KP_i eluting buffer at pH 8.3 (12 ml). Fractions of 22 drops (1.1 ml) were collected and aliquots of 75 μl were assayed for enzymatic activity as described in Experimental.

cytosol (Fig. 1) resulted in a 5-fold purification of 3α-HSOR with a 98% recovery. More than 99% of total 3α-HSOR activity was bound to the DEAE-column when the cytosol preparation was applied at a pH of 8.3 and low salt content (<0.5 mM NaCl). The capacity of the DEAE–Sephacel column to retain NADPH-linked enzyme was lessened when the cytosol preparations were applied with conductivity values greater than 0.5 mmho and pH values less than 8.3.

In the second step (Fig. 2), chromatofocusing gave a 3.3-fold purification with a 54% recovery. The 3α-HSOR activity eluted from the chromatofocusing column in the pH range of 5.9 to 5.6, with an estimated isoelectric point of 5.75. However, at this pH, the enzymatic activity is unstable with time. Loss of 3α-HSOR activity was reduced between steps by increasing the pH of each column fraction soon after elution by adding 80 μl of 1 M Tris–OH, pH 8.5

The final purification procedure using Blue-Sepharose affinity chromatography (Fig. 3) was the most effective purification step resulting in a 25-fold purification with a 45% recovery. All the 3α-HSOR activity was eluted with 8 mM $NADP^+$ in "KP_i eluting buffer". The flow-through sample volume contained 50–60% of the applied protein but only 3% of the applied 3α-HSOR activity. After two washes with buffer at pH 7.4 and 8.3, an additional 20% of the total loaded protein was removed.

In summary (Table 1), this purification of NADPH:5α-DHP 3α-HSOR from 100 anterior pituitaries resulted in a 23% overall recovery of enzymatic activity (15.9 nmol of 3α,5α-THP formed per 30 min) and yielded 11 μg protein. This suggests that the 3α-HSOR represents about 0.05% of the protein from female rat anterior pituitary cytosol. The specific activity was enhanced 438-fold over the cytosol and 1570-fold when compared to the pituitary homogenate. This purification procedure was repeated several times with similar results.

Electrophoretic analyses for purity

Aliquots of the enzyme preparation after each purification step were analyzed for purity by SDS–PAGE (Fig. 4). Coomassie blue staining of the affinity-purified enzyme lane showed a single protein band indicating that the 3α-HSOR was purified to apparent homogeneity. The molecular weight was estimated to be approx. 36 kDa by comparison to molecular weight standards. In another SDS–PAGE study (Fig. 4), the gels were analyzed with the more sensitive silver staining procedure. Again, the affinity-purified enzyme displayed one staining band (lane 5) with an approximate molecular weight of 36 kDa.

Non-dissociating electrophoretic analysis of the purified enzyme showed a single stained protein band that migrated with the 36 kDa protein marker suggesting that the native enzyme is a monomer (Fig. 5). When the gel track was analyzed for enzymatic activity, nearly all of the 5α-DHP 3α-HSOR activity (>99%) co-migrated with this single stained protein band

Table 1. Purification of NADPH:5α-DHP 3α-HSOR from female rat pituitary cytosol

Purification step	Total enzyme activity (nmol/30 min)	Total protein (mg)	Specific activity (nmol/mg/30 min)	Purification (-fold)	Overall yield (%)
Cytosol	69.2	20.9	3.3	—	100
DEAE–Sephacel	67.8	4.05	16.7	5.1	98
Chromatofocusing	35.8	0.65	55.1	16.7	52
Affinity chromatography	15.9	0.011	1445	438	23

After each purification step, NADPH:5α-DHP 3α-HSOR activity was measured as described in Experimental. To assess coincidence of NADH-linked 3α-HSOR activity with NADPH-linked activity, we also measured enzyme activity with NADH. NADH-linked activity was always coincident with NADPH-linked activity but the ratio of NADPH/NADH-linked activity ranged from 2 to 5.

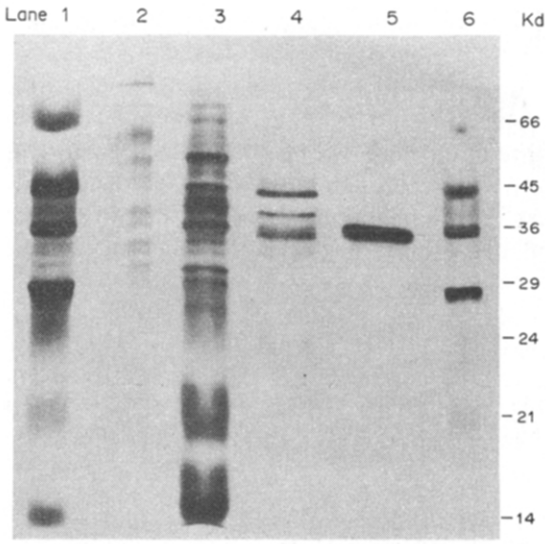

Fig. 4. SDS–Polyacrylamide gel electrophoresis. Aliquots from the pooled enzymatic preparations from each of the three chromatographic steps, as well as the cytosol, were subjected to analytical gel electrophoresis using 12.5% SDS–PAGE slab mini-gels (7.3 cm × 10.2 cm × 0.75 mm, for both resolving and stacking gel). Duplicate sets of gels were stained for proteins using the Coomassie Blue and silver staining procedures. The results shown are those with the silver staining procedure. Identical results were observed with Coomassie Blue staining. Lanes 1 and 6 contain molecular weight standards; lane 2, cytosol; lane 3, pooled and concentrated DEAE fractions; lane 4, pooled chromatofocusing fractions and lane 5, purified protein. Details are described in Experimental.

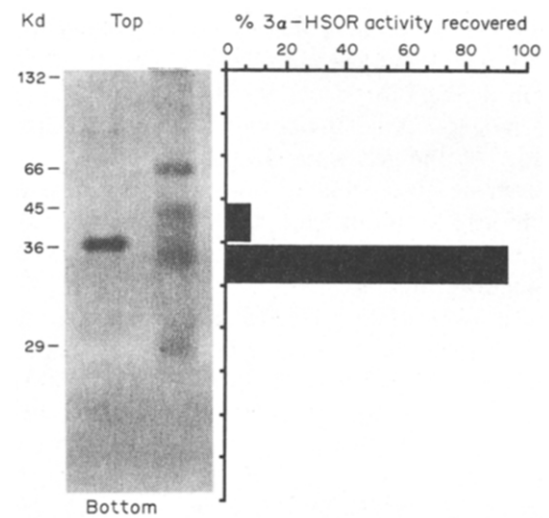

Fig. 5. Non-dissociating polyacrylamide gel electrophoresis. Aliquots of the purified enzyme were subjected to non-dissociating PAGE using a 10% slab mini-gel (7.3 cm × 10.2 cm × 0.75 mm). Lanes 1 and 3 purified enzyme, lanes 2 and 4 molecular weight standards. After electrophoresis, the gel was cut in half. One half of the gel (lanes 1 and 2) was stained with Coomassie blue. The results are in the bottom display of the figure. The top lane contains molecular weight markers and the bottom lane, purified 3α-HSOR. From the other half of the gel, the lane containing 3α-HSOR activity was cut into 1 cm sections for subsequent assay of enzymatic activity as described in Experimental. The results are shown in the bar graph in the top of the figure.

Table 2. Kinetic constants for purified cytosolic 3α-HSOR*

Substrate	K_m (μM)	V_{max} (μmol/mg/30 min)	V_{max}/K_m
5α-DHP	0.08 ± 0.01	1.2 ± 0.1	15
3α,5α-THP	1.4 ± 0.4	9.7 ± 1.3	6.9
NADPH	0.71 ± 0.09	1.2 ± 0.1	1.7
$NADP^+$	1.6 ± 0.3	5.5 ± 0.3	3.5

*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 and the kinetic constants (±SE) were calculated as detailed under Experimental.

providing further support that the (active) native enzyme is a 36 kDa monomer.

Kinetic parameters

The purified enzyme in the presence of the preferred cofactor, NADPH, had an apparent K_m for 5α-DHP of 82 nM, a V_{max} of 1.2 μmol of 3α,5α-THP/mg/30 min and a K_m for NADPH of 0.71 μM (Table 2). In the oxidative direction, the K_m for 3α,5α-THP (1.4 μM) is about 20 times greater than the K_m for 5α-DHP (80 nM), while the K_m for the oxidized cofactor, $NADP^+$ (1.6 μM), was about 2-fold greater.

The K_m for 5α-DHP (82 ± 10 nM) using purified 3α-HSOR (Fig. 6a) was comparable to the K_m determined with 3α-HSOR activity (Fig. 6b) from the pituitary cytosol (apparent $K_m = 138 \pm 13$ nM). Thus, purification did not appreciably affect the steady state affinity for this steroid since the apparent K_m-values for 5α-DHP are somewhat similar for both the purified and cytosolic 3α-HSOR.

Cofactor preference

Consistent with our earlier observations [10], all of the purified enzyme preparations from several replicates demonstrated a clear cofactor preference for NADPH over NADH. The ratios of NADPH/NADH-linked activity in the presence of equal molar concentrations of NADPH and NADH ranged from 2 to 5. These same ratios were also observed with enzymatic preparations from the previous chromatographic steps. There was no evidence that this overlap in cofactor specificity was due to separate enzymes. After each of the chromatographic steps, the elution profile of 3α-HSOR activity with NADH as cofactor was coincident with that using NADPH as cofactor.

DISCUSSION

To our knowledge, this is the first report of the purification of the cytosolic NADPH:5α-DHP 3α-HSOR from the female rat pituitary.

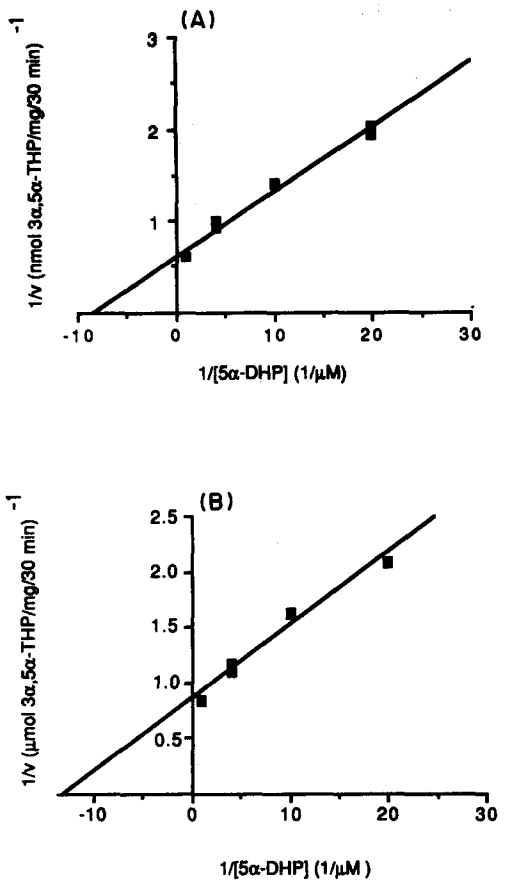

Fig. 6. K_m determination of the NADPH:5α-DHP 3α-HSOR. (A) K_m determination for 5α-DHP of the purified enzyme. 3α-HSOR activity was assayed in the reductive direction as described in Experimental with varying amounts of [^{3}H]5α-DHP (50, 100, 250 and 1000 nM) and contained constant saturating amounts of NADPH (200 μM). All incubations contained 85 ng purified 3α-HSOR. Each point was assayed in duplicate and the kinetic parameters were fitted as described in Experimental. (B) K_m for 5α-DHP for the 3α-HSOR activity in the crude cytosol. The assays were performed as described using 119 μg of cytosol protein.

The purified enzyme is a monomer with a mol. wt of 36 kDa and has an isoelectric point of about 5.75 and a cofactor preference for NADPH over NADH. The apparent K_m values determined for 5α-DHP and 3α,5α-THP are in good agreement with those previously reported for the crude cytosolic 3α-HSOR activities from female rat pituitary [10].

The kinetic studies suggest that the enzyme may preferentially operate *in situ* in the reductive direction. As noted in the Results, K_m-values for the substrates for the reductive reaction (5α-DHP and NADPH) are lower than those for the oxidative reaction (3α,5α-THP and $NADP^+$). This direction is also supported by the observation that the K_m-value for 5α-DHP (80 nM) is well within its reported blood levels [23], while the K_m-value for 3α,5α-THP (1.4 μM) is *greater* than its blood levels [23, 24]. This preference is also supported by the finding that the K_m for NADPH (710 nM) is lower than its reported cellular concentrations (10–400 μM) and the observation that the cytosolic NADPH/$NADP^+$ ratio is generally greater than unit [25, 26].

Similar cytosolic 3α-HSOR activities have been partially or fully purified from male rat liver [27], brain [28], and prostate [29, 30]. These 3α-HSORs, also known as 3α-hydroxysteroid dehydrogenases, have molecular weights in the range of 31–34 kDa and isoelectric points in the range of 5.5–5.8. They also prefer the cofactor NADPH when compared to NADH. Most of these studies have examined the oxidoreduction of C_{19} androgenic steroid substrates and not the C_{21} progestin substrates, 5α-DHP and 3α,5α-THP, used in our studies. One study with partially purified prostatic enzyme did report a K_m for 5α-DHP of 270 nM [29]. The prostatic and hepatic enzymes also have K_m-values for the C_{19} steroid substrates for the reductive reaction that are lower than those for the oxidative reaction [27, 29]. Since the female anterior pituitary and male rat 3α-HSORs have some properties in common, they may represent multiple forms of the same enzyme or isoenzymes.

The physiological importance of the pituitary cytosolic 3α-HSOR in catalyzing the reversible conversion of 5α-DHP to 3α,5α-THP and the subsequent role of these metabolites in mediating progesterone's biological effects in the pituitary is not clear at this time. 5α-DHP is more potent than 3α,5α-THP in modulating gonadotropin release [2, 6], but 3α,5α-THP is more potent than 5α-DHP in modulating the chloride ion channel associated with the $GABA_A$ receptor [7–9]. *In situ*, the 3α-HSOR enzyme may play an important role in determining the relative levels of 5α-DHP and 3α,5α-THP in the pituitary.

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