Kinetic Analysis of Rat Steroid 5α-Reductase Activity in Prostate and Epididymis Homogenates at Neutral pH: Evidence for Type I Activity in Epididymis

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Immunocytochemical studies and mRNA measurements have shown that the rat epididymis—like the rat prostate—expresses both rat steroid 5α-reductase isozymes, i.e. type I and II. So far, enzyme activity measurements in rat epididymis homogenates, however, do not support the presence of type I 5α-reductase activity. Incubating homogenates of both tissues with a wide range of substrate concentrations, we were able to detect activity of both isozymes in rat prostate and epididymis tissues at neutral pH. In rat prostate the amount of type I activity, as measured by the $V_{\text{max}}$ at pH 7.0, exceeds that of type II 5α-reductase 50-fold. The efficiency ratio, $V_{\text{max}}/K_m$, of the type I isozyme accounts for 25% of the total in vivo potential activity. A possible anabolic role for the type I isozyme in rat prostate was thus surmised. In rat epididymis the $V_{\text{max}}$ of type I and type II 5α-reductase at pH 7.0 were similar. Comparison of the efficiency ratio $V_{\text{max}}/K_m$ of either isozyme in the rat epididymis, however, suggested that the type II isozyme would play the major role in the 5α-reduction of testosterone at physiological concentrations and at neutral pH. The specific localization of the isozymes should be considered to allow for correct quantification of their in vivo contribution to dihydrotestosterone formation. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

Dihydrotestosterone (DHT) is the major androgen formed by 5α-reduction of testosterone (T) in both androgen target- and non-target tissues [1]. The enzyme responsible for this conversion, 5α-reductase (5α-RED, EC 1.3.99.5), is membrane-bound, NADPH dependent, and capable of 5α-reducing a number of other steroids with a 4,5 double bond and a 3-oxo-group [1].

To date, two subtypes of steroid 5α-RED with specific pH-optima and inhibitor sensitivities, designated type I and type II, have been described in both human and rat [2–7]. Their existence is well documented, but has found little rationality. The tissue specific expression and affinity constants of the isozymes has led investigators to propose an anabolic role for the type II isozyme, which is expressed in the prostate and other classical androgen target tissues, and a catabolic role for the type I isozyme, as it is amply detectable in liver tissue [7]. Both the human and rat type I isozymes have a broad pH-optimum of 6.0–8.0, whereas the type II isozymes have a pH-optimum of 5.0–5.5 [5–7]. The rationale for the acidic pH-optimum of the type II isozyme has fascinated many researchers over the years. Recently, applying the efficiency ratio $V_{\text{max}}/K_m$ to the question of pH-optimum for human 5α-RED isozymes expressed in a Chinese hamster ovary cell line, it has been shown that the type II isozyme is capable of metabolizing physiological T concentrations most efficiently at pH 7.0. The acidic pH-optimum of the type II isozyme would be an artifact [8]. Both 5α-RED subtypes are now thus considered to operate at neutral pH.

We studied the 5α-reduction of T in rat prostate and epididymis homogenates at pH 7.0 to gain insight into the contribution of both isozymes to DHT formation. In rat prostate both subtypes are present and their activities have been reported [7]. In rat epididymis
both subtypes have also been described. The type II isozyme is unequivocally present and its activity has been described. Type I 5α-RED has been detected immunocytochemically [1, 9] and by mRNA measurements [7, 10, 11]. Enzyme activity measurements, however, do not support type I activity in the rat epididymis [7, 9, 11]. Because of this discrepancy between 5α-RED protein and activity we investigated whether type I activity could be found in rat epididymis homogenates at pH 7.0 using an in-depth kinetic analysis of 5α-RED activity in this tissue, with the rat prostate as a control.

**MATERIALS AND METHODS**

**Materials**

[1,2,6,7-H]T (3.74 TBq/mmol) and [1α,2α(n-3H)]17β-hydroxy-5α-androstan-3-one (DHT) (2.00-TBq/mmol) were purchased from Amersham (Amersham, U.K.). [9,11-3H]5α-androstan-3α,17β-diol (1.48 TBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). All radiolabeled steroids were purified by high performance liquid chromatography (HPLC, see below) before use. T was purchased from Steraloids (Wilton, NH). Diethylether NaOH. T metabolism was not allowed to exceed 15% between 5α-RED activity, with steroid in Pyrex culture tubes (borosilicate glass, Wilms). Metabolites were dissolved in 100 µl hexane for HPLC.

**Buffers**

Homogenization buffer consisted of 20 mM phosphate (Merck), 1 mM monothioglycerol and 0.25 M sucrose (Merck), pH 7.0. The incubation buffer consisted of 200 mM Tris (2-amino-2-hydroxymethylpropane-1,3-diol, Merck) and citric acid monohydrate (Merck), pH 4.0–8.0 or pH 7.0, and 2 mM NADPH tetrasodium salt (Merck).

**Tissue preparation**

Wistar rats of 7–13 weeks old (150–250 g) were killed by decapitation and whole prostates and epididymides were removed, freed of adhering fat and placed into liquid nitrogen for transport. Tissues were kept at −80°C or processed immediately. All subsequent procedures were performed on ice. Pooled tissues were thawed and minced with razor blades into small pieces. Minced tissue was homogenized in ice-cold homogenization buffer with a 7 ml Dounce tissue grinder (Kontes Glass Co., Vineland, NJ, U.S.A.) with a loose and a tight fitting pestle. The homogenate was filtered twice through nylon netting of 50 and 140 mesh, respectively, to remove cell debris. By this procedure—without centrifugation—a full homogenate with nuclei and cytosol is obtained. The pooled prostates homogenate was diluted with homogenization buffer to 29.1 mg protein/ml, while rat epididymis homogenate was diluted to 1.1 mg protein/ml.

Protein levels were determined by a modification of the method of Lowry et al. [12] against a standard of bovine serum albumin (OHRD 20/21, Hoechst-Behring, Marburg, Germany). The assay was modified for microtiter-plates and had a sensitivity of 25 µg per well.

**5α-reductase assay**

Radiolabeled T in ethanol was brought to final concentration by isotopic dilution with non-labeled steroid in Pyrex culture tubes (borosilicate glass, 12 × 75 mm, Corning Inc., Corning, NY). Ethanol was evaporated under a mild nitrogen stream at room temperature. Incubation buffer (800 µl) was added and the tubes were put into a shaking water bath at 37°C at least 10 min before the start of the incubation to ensure the substrate was dissolved (97%). A tube with the homogenate (10–50 µl) and the appropriate amount of cofactor (NADPH, 2 mM final concentration) was diluted to 200 µl with 150–190 µl incubation buffer and kept on ice. The incubation was started by adding 200 µl of the homogenate and cofactor mixture to the pre-heated tubes with substrate [13]. After 10–30 min the incubation was terminated by adding 100 µl of 3 M NaOH. T metabolism was not allowed to exceed 15% by varying enzyme amount and incubation time. This 5α-reductase assay protocol was validated and optimized as reported in an earlier paper [13]. To extract metabolites, 4 ml of ice-cold diethylether was added, and the tubes were capped and shaken. The water phase was frozen in an alcohol bath with dry-ice, the organic phase decanted and evaporated under nitrogen. Metabolites were dissolved in 100 µl hexane for HPLC.

**HPLC**

Metabolized steroids were separated on a Hiber LiChrosorb Diol-column (length 250 mm, 5 µm, Merck), equipped with a guard column (Resolve Silica, Waters Corp., Milford, MA). The HPLC-system included a Waters 610 Fluid Unit, a Waters 600E System Controller and a Waters U6K injector. The isocratic flow of the mobile phase (hexane–propanol, 96:4 v/v) was 1.5 ml/min at a pressure of 680 psi. Radioactivity was monitored with a FloOne Beta Radiomatic A500 radio-chromatography detector (Packard–Canberra Benelux, Tilburg, The Netherlands) with a 500 µl cell and a liquid scintillation flow of 1.5 ml/min (Aqua–Luma, Lumac–LSC, Olen, Belgium). The counting efficiency for tritium was 47%. The percentage formation of DHT and 5α-androstan-3α,17β-diol was used to estimate 5α-RED activity. Overall experimental recoveries for T, DHT and 5α-androstan-3α,17β-diol were 90–93%.

**Calculation of enzyme characteristics**

Velocities were plotted against T concentration, and $K_m$ and $V_{max}$ were calculated using a non-linear
regression procedure based on the Michaelis–Menten equation on a PC with the software-program Enzfitter. A double reciprocal plot of the obtained estimated initial velocities against substrate concentration was used. This Lineweaver–Burk plot appeared to be non-linear only at high substrate concentrations. An Eadie–Scatchard plot of velocity over substrate concentration against velocity was used for a more even weighting of points [14] and was non-linear over practically the whole substrate concentration range tested. Two 5α-RED enzyme activities with a different potential enzyme activity one has to consider that isozymes. Based on a Michaelis–Menten equation for linear regression procedure with fitting to least squares, a double reciprocal plot of the obtained estimated the type I and 21.3–10⁻⁶ Km practically the whole substrate concentration range activity in rat epididymis homogenate at a pH-range of 4.0–8.0. Enzyme activity for 5α-RED was further established at pH 7.0 with substrate concentrations ranging from as low as 2 nM to as high as 3.2 μM [Fig. 3(A)]. A double reciprocal plot of these data was non-linear at high substrate concentrations [Fig. 3(B)]. These data were also analyzed using an Eadie–Scatchard plot of V/S against V [Fig. 3(C)].

For the use of the V max/Km ratio as an index of potential enzyme activity one has to consider that endogenous T concentrations are much lower than the Km of either 5α-RED subtype [15]. Therefore applying Km ≈ [S] in the Michaelis–Menten equation gives:

\[ V \approx \frac{V_{\text{max}}}{K_m} [S]. \]

So, at physiological T concentrations, the enzyme reaction velocity is proportional to \( V_{\text{max}}/K_m \).

RESULTS

In this study we investigated the 5α-RED characteristics of rat prostate and epididymis homogenates. The validity of the 5α-RED assay was checked and reported in an earlier paper [13]. With this assay, the time course of the 5α-reduction of T in these rat tissue homogenates was linear and the enzyme stable for at least 30 min at 37°C with 2 mM NADPH. Therefore, the velocities measured can be considered valid estimates of initial velocities.

In rat prostate homogenate 5α-RED activity was determined with a single substrate concentration of 1 μM T in the pH range of 4.0–8.0 [Fig. 1(A)]. The two pH-optima suggest the presence of two isozymes in this tissue as the type II 5α-RED isozyme is primarily responsible for the 5α-RED activity at pH 5.0, whereas the type I isozyme has a broad pH-optimum from 6.0–8.0. Enzyme activity for 5α-RED was further established at pH 7.0 with substrate concentrations ranging from as low as 2 nM to as high as 3.2 μM [Fig. 2(A)]. A double reciprocal plot of these data was non-linear at high substrate concentrations [Fig. 2(B)]. For a more even weighting of points, an Eadie–Scatchard plot of V/S against V was used [Fig. 2(C)]. The affinity constants of the 5α-RED isozymes, i.e. type II 5α-reductase from rat prostate, were determined with a single substrate concentration of either 5α-RED subtype [15], Therefore applying Eadie-Scatchard plot of velocity over substrate concentration ranging from as low as 2 nM to as high as 3.2 μM [Fig. 3(A)]. As in the rat prostate, a Lineweaver–Burk plot of the data was non-linear at high substrate concentrations [Fig. 3(B)]. These data were also analyzed using an Eadie–Scatchard plot of V/S against V [Fig. 3(C)].

Figure 1(B) shows the pH dependency of 5α-RED activity in rat epididymis homogenate at a pH-range of 4.0–8.0 with 1 μM T as substrate. Highest enzyme activity was found at pH 5.0, indicative of the presence of type II 5α-RED. Activity was low at neutral pH. 5α-RED characteristics were also assessed at pH 7.0 with a substrate range of 2 nM–3.2 μM [Fig. 3(A)]. As in the rat prostate, a Lineweaver–Burk plot of the data was non-linear at high substrate concentrations [Fig. 3(B)]. These data were also analyzed using an Eadie–Scatchard plot of V/S against V [Fig. 3(C)].

Fig. 1. pH-profiles of 5α-reductase activity in (A) rat prostate homogenate and (B) rat epididymis homogenate in the pH range 4.5–8.0 with 2 mM NADPH as cofactor and 1 μM T as substrate. The pH-profile in rat prostate suggested the presence of the two established 5α-reductase isozymes, i.e. type II 5α-reductase activity at pH 5.0 and type I 5α-reductase from pH 6.0 to 8.0. In the rat epididymis a pH-optimum was found at pH 5.0, indicative of type II 5α-reductase activity.

(Table 1). The efficiency ratio V max/Km in the rat prostate at pH 7.0 was 7.68·10⁻⁶ l/(min·mg protein) for the type I and 21.3·10⁻⁶ l/(min·mg protein) for the type II 5α-RED subtype (Table 1); the total V max/Km ratio for 5α-RED activity at pH 7.0 in rat prostate homogenate was 29.0·10⁻⁶ l/(min·mg protein). Almost 75% of this ratio was accounted for by the type II and 25% by the type I isozyme.
Fig. 2. (A) Estimated initial velocities (V) of 5α-reductase activity in rat prostate homogenates at pH 7.0 with 2 mM NADPH and with 2 nM to 3.2 μM T as substrate (S). Values are mean of two duplicate assays carried out on different days. (B) A double reciprocal plot of these data was non-linear at high substrate concentrations. (C) An Eadie-Scatchard plot of estimated initial velocities over substrate concentration (V/S) against velocity (V) in rat prostate homogenates at pH 7.0. This plot gives a more even weighting of points and is non-linear. It could be described by two enzyme activities, an alleged type I (---) and type II (...) 5α-reductase isoform. The abscissa intercepts give the respective Vmax, while the slope indicates −Km⁻¹.

The enzyme characteristics obtained were typical for the type I and type II 5α-RED isozymes. The mean Km values were 1397 and 3.14 nM, respectively. The Vmax values of the type I and II 5α-RED were similar: 0.98 and 0.78 pmol/(min·mg protein), respectively (Table 1). The Vmax/Km ratio was 0.70·10⁻⁶ l/(min·mg protein) for the type I and 248·10⁻⁶ l/(min·mg protein) for the type II 5α-RED subtype. Thus, in epididymis, only about 0.3% of the total Vmax/Km ratio at pH 7.0 was accounted for by the type I isoform.

In Fig. 4 the Vmax of the respective subtypes at pH 7.0 were compared with the Vmax/Km efficiency ratios at this pH of both isozymes in rat prostate and epididymis. The relative amount of isozyme activity present can be measured by the specific activity, Vmax, in rat prostate (Fig. 4(A)) a 50-fold higher Vmax for the type I isoform was found than for the type II isoform, in the epididymis (Fig. 4(B)) the specific activities for both isozymes were almost equal. Nevertheless, measuring the potential in vivo activity in the prostate as reflected by the efficiency ratio, Vmax/Km [Fig. 4(A)], it appeared that isoform type I accounts for 25% of total enzyme activity at pH 7.0. In the epididymis [Fig. 4(B)] the type II isoform was quantitatively responsible for 5α-RED activity at physiological T concentrations and at neutral pH (99.7%).

DISCUSSION

The assay employed in this paper has been validated in an earlier paper [13]. The putative hysteretic behavior of the isozyme, which would extensively hamper valid measurement of enzyme activity was only observed at the acidic pH-optimum of the type II isozone [13, 16]. With the work of Thigpen et al. in mind, we measured both isozyme activities at neutral pH, as it was reported that the acidic pH-optimum of the type II isozone was an artifact [8]. At neutral pH, we did not observe hysteresis [13]. Therefore, the enzyme activities we measured were valid estimates of initial velocity. The affinity constants established at pH 7.0 in the present study in rat prostate (752 and 5.29 nM) and epididymis (1397 and 3.14 nM) are characteristic for the type I and type II rat and human steroid 5α-RED subtypes reported in literature [7, 8, 13]. This strongly suggests that the non-linear plots found in rat prostate and epididymis homogenates can be attributed to these two isozyme activities. Moreover, as we measured isozyme activities at neutral pH and applied a citrate buffer, in which negative cooperativity as observed in an acetate buffer was not exhibited, the present findings did not represent the negative cooperativity of the human type II isozone exhibited in the hyperplastic prostate at acidic pH [16].

Classically, type I or II 5α-RED isozyme activities are detected by measuring their specific pH-optima. The presence of type II 5α-RED can indeed be assessed by the use of a pH-profile as presented here for the rat prostate, where the peak at pH 5.0 indicates type II activity. The acidic pH-optimum of the type II 5α-RED isozyme and the broad neutral pH-optimum of the type I isozone would indicate that the maximum velocities at pH 5.0 and pH 7.0 are a measure for,
respectively, type II and type I 5α-RED isozyme activity. This $V_{\text{max}}$ at optimal pH has been used to characterize type I and II tissue specific distribution [17]. Using this definition, according to previous results [13] in rat prostate, equal amounts of isozyme activities would be found. However, as there is now growing evidence that the type II isozyme does operate at neutral pH [8], the enzyme activity measured at acidic pH does not reflect true type II activity. In a tissue where considerable type I activity is present, like rat prostate [7], quantification of type II isozyme activity at neutral pH is usually hampered. In this paper a method is described by which both isozyme activities can be assessed simultaneously. The $V_{\text{max}}$ values at pH 7.0 of both isozymes were quite different from those measured at optimal pH: the results for 5α-RED enzyme activity in rat prostate homogenate would indicate a major role for the type I isozyme (50-fold higher $V_{\text{max}}$). The efficiency ratios, $V_{\text{max}}/K_m$, indicate a predominant role of the type II isozyme in the rat prostate at neutral pH. The type I isozyme, however, has a ratio which is still 25% of the total 5α-RED efficiency ratio at pH 7.0.

The pH-profile of 5α-RED activity in the rat epididymis reveals minimal activity at neutral pH and high activity at pH 5.0. The maximum activity found at pH 5.0 far exceeds that found at pH 7.0, indicating that the major amount of 5α-RED in this tissue must be attributed to the type II isozyme. However, the activity at neutral pH can be attributed to either subtype, depending on the affinity constants at this pH. When similar amounts of both isozyme activities are present, detection of either subtype depends on the range of substrate concentrations used. At lower, physiological, concentrations a high affinity enzyme (type II 5α-RED) will be found. At high concentrations the enzyme activity found will be largely attributable to the type I 5α-RED. In an earlier study [13] we were not able to detect type I 5α-RED activity in rat epididymis homogenates as we used T concentrations below 1 µM. However, incubating epididymis homogenates with higher T concentrations, we now did detect type I activity. The similar specific activities, $V_{\text{max}}$, of type I and type II 5α-RED at pH 7.0 indicate the presence of similar amounts of isozyme activities in this tissue. This agrees with results from immunocytochemical studies [9] and with studies in which type I and type II mRNA content is quantified in rat epididymis tissue [7, 10, 11]. However, as the efficiency ratio $V_{\text{max}}/K_m$ is a better indicator of potential 5α-RED activity [8, 15], our results indicate that at pH 7.0 only approx. 0.3% of the total $V_{\text{max}}/K_m$ ratio is accounted for by the type I isozyme. However, this type I isozyme is reportedly expressed in a small portion of the epididymis, the initial segment [9]. A highly concentrated expression in a specific segment could—notwithstanding our results presented in this paper—imply a significant contribution of this isozyme to DHT formation. Our results mark the intricacies when comparing immunocytochemical studies, 5α-RED mRNA measurements and 5α-RED activity measurements in rat epididymis, even more so as immunocytochemistry and mRNA measurements semi-quantitatively detect the presence of enzyme and not enzyme activity per se.

The role of the type I isozyme has been described as catabolic as it is amply found in liver tissue [7]. However, the presence of the type I 5α-RED isozyme in the rat androgen target tissues prostate and epididymis suggests an additional role for this subtype. On the other hand, considering the rather high affinity constant, the type I isozyme will only be active at high concentrations of T in the microenvironment of the isozyme. As all prostatic rat steroid 5α-RED activity is reportedly located in the nucleus [18-20], one can hypothesize that the type I isozyme could therefore be active when T accumulates in the nucleus, e.g. due to binding to the androgen receptor [21, 22]. As the $V_{\text{max}}/K_m$ ratio of the type I isozyme still contributes substantially to the total potential in vivo 5α-RED activity, an anabolic function for this subtype in rat prostate is plausible. In rat epididymis distinct 5α-RED activities have been detected in the nucleus and microsomes [23-25]. Immunocytochemical studies suggest a microsomal localization of the type I isozyme [9]. Accumulation of T, necessary for the type I 5α-RED to be active in the rat epididymis, should thus occur in the cytoplasm. The role of the type I isozyme in this tissue is therefore different from that in the prostate, even more so as it is reportedly confined to the initial segment of the epididymis [9].

In summary, we present here evidence for type I 5α-RED activity in rat epididymis homogenates in addition to the well established type II activity. The non-linear plots of 5α-RED activity we found in rat epididymis suggest an additional role for this subtype. The role of the type I isozyme has been described as catabolic as it is amply found in liver tissue. However, the presence of the type I 5α-RED isozyme in the rat androgen target tissues prostate and epididymis suggests an additional role for this subtype. On the other hand, considering the rather high affinity constant, the type I isozyme will only be active at high concentrations of T in the microenvironment of the isozyme. As all prostatic rat steroid 5α-RED activity is reportedly located in the nucleus, one can hypothesize that the type I isozyme could therefore be active when T accumulates in the nucleus, e.g. due to binding to the androgen receptor. As the $V_{\text{max}}/K_m$ ratio of the type I isozyme still contributes substantially to the total potential in vivo 5α-RED activity, an anabolic function for this subtype in rat prostate is plausible. In rat epididymis distinct 5α-RED activities have been detected in the nucleus and microsomes. Immunocytochemical studies suggest a microsomal localization of the type I isozyme. Accumulation of T, necessary for the type I 5α-RED to be active in the rat epididymis, should thus occur in the cytoplasm. The role of the type I isozyme in this tissue is therefore different from that in the prostate, even more so as it is reportedly confined to the initial segment of the epididymis.

### Table 1. Enzyme characteristics of type I and II 5α-RED in rat prostate and epididymis homogenates at pH 7.0

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$V_{\text{max}}$ (pmol/(min-mg protein) (SEM))</th>
<th>$K_m$ (nM (SEM))</th>
<th>$V_{\text{max}}/K_m$ $(10^{-6}$ l/(min-mg protein))</th>
<th>$V_{\text{max}}$ (pmol/(min-mg protein) (SEM))</th>
<th>$K_m$ (nM (SEM))</th>
<th>$V_{\text{max}}/K_m$ $(10^{-6}$ l/(min-mg protein))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>5.78 (0.09)</td>
<td>752 (32.5)</td>
<td>7.68</td>
<td>0.113 (0.02)</td>
<td>5.29 (1.85)</td>
<td>21.3</td>
</tr>
<tr>
<td>Epididymis</td>
<td>0.980 (0.16)</td>
<td>1397 (539)</td>
<td>0.70</td>
<td>0.780 (0.03)</td>
<td>3.14 (0.41)</td>
<td>248</td>
</tr>
</tbody>
</table>

Values are mean of two duplicate measurements with SEM ($n = 4$) carried out on two different days.
prostate and epididymis homogenates at neutral pH could be adequately explained by the presence of two isozymes with affinity constants characteristic of the type I and type II isozymes. In rat prostate the presence of both isozymes is undisputed and this tissue can thus serve as a control for the results presented here for the rat epididymis. The results obtained for the type I activity in the rat prostate indicate a possible anabolic role for this subtype. Although the $V_{\text{max}}$ in the epididymis indicates similar amounts of type I and type II 5α-RED activity at neutral pH, almost all potential in vivo 5α-RED activity, as determined by the efficiency ratio $V_{\text{max}}/K_m$, is probably attributable to the type II isozyme. The highly segmental expression of the type I isozyme in the epididymis should be considered to allow for correct quantification of its in vivo contribution to DHT formation.

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REFERENCES