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Biochemistry

Identification and selective inhibition of an isozyme of steroid 5α-reductase in human scalp

(androgen metabolism/4-azasteroids/benign prostatic hyperplasia/baldness)

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ABSTRACT **Steroid 5α-reductase (EC 1.3.1.22) catalyzes the reduction of testosterone to dihydrotestosterone. The 5α-reductase found in human scalp has been compared with the enzyme found in prostate. The scalp reductase has a broad pH optimum centered at pH 7.0. This is distinctly different from the pH optimum of 5.5 observed with the prostatic form of the enzyme. These enzymes also differ in the K_m for testosterone, which is 25-fold higher for the scalp reductase. The most significant difference between the two enzymes is their affinity for inhibitors. Two 4-azasteroids and a 3-carboxyandrostadiene are potent inhibitors of the prostatic reductase but are weak inhibitors of the scalp reductase. In contrast, several *N*-4-methylazasteroids are good inhibitors of the scalp reductase. These findings support a proposal that different isozymes of 5α-reductase may exist in scalp and prostate. The scalp reductase was also compared to 5α-reductase 1, one of the two enzymes recently cloned from human prostate [Andersson, S. & Russell, D. W. (1990) *Proc. Natl. Acad. Sci. USA* 87, 3640–3644; and Andersson, S., Berman, D. M., Jenkins, E. P. & Russell, D. W. (1991) *Nature (London)* 354, 159–161]. The characteristics of the cloned reductase 1 are comparable to those of the scalp reductase.**

It is well documented that androgen-responsive tissues such as prostate, seminal vesicles, epididymis, and skin metabolize testosterone (T) to dihydrotestosterone (DHT) (1–5). This conversion is catalyzed by a steroid 5α-reductase (3-oxo-5α-steroid Δ^4-reductase, EC 1.3.1.22) in a reaction requiring NADPH.

A deficiency of 5α-reductase is known which results in a form of male pseudohermaphroditism. At birth, these individuals have ambiguous external genitalia, indicating the important role for DHT in male sexual development (6, 7). At puberty, when plasma levels of T increase, virilization of these individuals occurs. Affected males fail to develop a normal prostate and supposedly are not affected by acne or male pattern baldness; no other negative consequences are noted. This deficiency is characterized by normal to slightly elevated plasma levels of T and low, but detectable, levels of DHT. Additionally, there is a decrease in all urinary 5α-reduced metabolites of C_{19} and C_{21} steroids, resulting in an elevated 5β:5α steroid ratio. These findings led Imperato-McGinley and Gautier (8) to propose that a single gene encodes a reductase with broad substrate specificity.

Despite the suggestion from the genetic deficiency that a single 5α-reductase exists, there have been many reports regarding the presence of isozymes in both rats and humans (9–13). Martini *et al.* (10) proposed that in rat ventral prostate one isozyme may be responsible for the reduction of T, the other responsible for the reduction of androstenedione. In human prostate, Bruchovsky *et al.* (12) and Hudson (11) found different 5α-reductase activities in the stromal and epithelial fractions. Additionally, Moore and Wilson (9) described two distinct human reductases with peaks of activities at either pH 5.5 or pH 7–9. The pH 5.5 form was found only in fibroblasts from genital skin, whereas the pH 7–9 form was present in all fibroblasts assayed. To date, the enzyme has not been purified from any source. Thus, the main evidence supporting the existence of isozymes of 5α-reductase is the difference in the apparent K_m values and pH optima of enzyme activity derived from these varied preparations. However, these data are difficult to interpret, given the membrane environment of the protein.

Recently, Andersson *et al.* (14) isolated a cDNA which encodes a rat liver 5α-reductase. They found a single mRNA encodes both the liver and prostatic reductases in rats. This rat gene was later used to identify a human prostatic cDNA encoding a 5α-reductase (15). Analysis of this DNA sequence predicts the human protein to be highly hydrophobic with a molecular mass of 29 kDa. The protein sequence of the cloned human enzyme (5α-reductase 1) showed 60% identity with the rat sequence, which is surprisingly low for a rat–human comparison of identical genes. Expression studies in COS cells revealed that the cloned human reductase has a markedly different pH optimum compared with the enzyme present in crude human prostatic homogenates. In addition, the cloned human reductase was 50- to 100-fold less sensitive to inhibition by MK906 [17β-(*N*-*t*-butyl)carbamoyl-4-aza-5α-andros-1-en-3-one] than was the reductase activity present in human prostatic preparations.

Recently, the apparent discrepancy was resolved by the cloning of a second, more abundant, reductase (5α-reductase 2) from human prostate with properties identical to the form found in crude human prostatic extracts (16). These data support the existence of at least two genes for 5α-reductase in humans. Andersson *et al.* (16) reported that a deletion in 5α-reductase 2 is present in patients with a 5α-reductase deficiency. In these same patients the 5α-reductase 1 gene is normal.

Although two genes encoding 5α-reductase are now known, no tissue distribution of the enzyme encoded by 5α-reductase 1 gene has been reported. Herein, we describe a pharmacological characterization of an isozyme of steroid 5α-reductase present in human scalp. We propose that there are two distinct isozymes of 5α-reductase in humans: one form is found in prostate and the other is found in scalp. These enzymes can be distinguished by their pH/rate profile, their K_m for T, and their sensitivity to two classes of reductase inhibitors. Unexpectedly, the properties of the 5α-reductase 1 cloned by Andersson and Russell (15) from human prostate match those of the predominant 5α-reductase in human scalp rather than those of the human prostatic

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Abbreviations: T, testosterone; DHT, dihydrotestosterone.

enzyme. The availability of selective inhibitors will facilitate the elucidation of the functions of these isozymes.

MATERIALS AND METHODS

Materials. [4-^{14}C]T was purchased from New England Nuclear. All other chemicals were obtained from Sigma. Protein concentrations were estimated by using the method developed by Lowry *et al.* (17). Compounds **1a–e** (see Table 2) were prepared in these laboratories as previously described (18, 19). Compound 2 (SK&F105657) was prepared by Thomas Walsh as described by Holt *et al.* (20).

Human Prostatic and Scalp 5α-Reductase. Prostatic tissue was obtained from men undergoing transurethral resection of the prostate for benign prostatic hyperplasia. Scalp samples were obtained from men at the time of hair transplant. Tissues were quickly frozen and stored at −80°C until use. Samples of human tissue were pulverized in a freezer mill and homogenized in 40 mM potassium phosphate, pH 6.5/5 mM magnesium sulfate/25 mM potassium chloride/1 mM phenylmethylsulfonyl fluoride/1 mM dithiothreitol containing 0.25 M sucrose in a Potter–Elvehjem homogenizer. A crude nuclear pellet was prepared by centrifugation of the homogenate at 1500 × *g* for 15 min. The crude nuclear pellet was washed two times and resuspended in 2 vol of buffer. Glycerol was added to the resuspended pellet to a final concentration of 20% (vol/vol). The enzyme suspension was frozen in aliquots at −80°C. The prostatic and scalp reductases were stable for at least 4 months when stored under these conditions.

Expression of 5α-Reductase 1 in COS Cells. A pCMV4 expression vector containing human prostatic steroid 5α-reductase 1 cDNA was kindly provided by David Russell. Transient transfections of COS-7 cells were carried out by using Lipofectin (Bethesda Research Laboratories). The cells were harvested 48 hr after transfection and were broken by three cycles of freezing and thawing in 10 mM potassium phosphate, pH 7.0/150 mM KCl/1 mM EDTA. The homogenate was centrifuged at 100,000 × *g* and the pellet was resuspended in buffer containing 20% glycerol. Enzyme activity was stable for at least 4 months when stored at −80°C.

5α-Reductase Assay. The reaction mixture contained in a final volume of 100 μl: 40 mM buffer (human scalp or COS cell-expressed reductase, potassium phosphate, pH 6.5; human prostatic 5α-reductase, sodium citrate, pH 5.5), 0.3–10 μM [^{14}C]T, 1 mM dithiothreitol, and 500 μM NADPH. Typically, the assay was initiated by the addition of 50–100 μg of prostatic homogenate, 75–200 μg of scalp homogenate, or 5–10 μg of COS cell-expressed reductase 1 and incubated at 37°C. After 10–50 min the reaction was quenched by extraction with 250 μl of a mixture of 70% (vol/vol) cyclohexane/30% ethyl acetate containing DHT and T at 10 μg each. The aqueous and organic layers were separated by centrifugation at 14,000 rpm in an Eppendorf microcentrifuge. The organic layer was subjected to normal-phase HPLC (10-cm Whatman Partisil 5 silica column equilibrated in 70% cyclohexane/30% ethyl acetate; at a flow rate of 1 ml/min retention times were DHT, 6.8–7.2 min; androstanediol, 7.6–8.0; T, 9.1–9.7 min). The HPLC system consisted of a Waters model 680 gradient system equipped with a Hitachi model 655A autosampler, Applied Biosystems model 757 variable UV detector, and a Radiomatic model A120 radioactivity analyzer. The conversion of [^{14}C]T to [^{14}C]DHT was monitored, using the radioactivity flow detector, by mixing the HPLC effluent with one volume of Flo Scint 1 (Radiomatic Instruments and Chemical, Tampa, FL). Under the conditions described, the production of DHT was linear for at least 25 min. The only steroids observed with the human prostate and scalp preparations were T, DHT, and androstanediol.

For the assay of the 5α-reductase expressed in COS cells, the HPLC separation was modified for the COS cell enzyme to include tandem 10-cm Partisil columns equilibrated in the cyclohexane/ethyl acetate mixture described above. The retention times for the steroid products were as follows: DHT, 8.3 min; androstenedione, 10.1 min; androstanediol, 12.2 min; and T, 14.2 min.

Inhibition Experiments. The relative potency for inhibitors is the concentration of compound required to produce 50% inhibition during the assay period at constant concentrations of T corresponding to K_m (Table 1) and saturating NADPH (500 μM). In these experiments the reaction time was 10 min. Some inhibitors appeared to have a slow time-dependent component to their mechanism of inhibition (G.H. and H. Bull, unpublished observations) but their effects were not taken into account in the pharmacological comparison of the 5α-reductase subtypes.

RESULTS

Identification of Steroid 5α-Reductase in Human Scalp. The subcellular localization of 5α-reductase in scalp tissue was determined by differential centrifugation. The crude nuclear fraction of the scalp contained 85% of the enzyme activity. The remainder of the activity was equally divided between

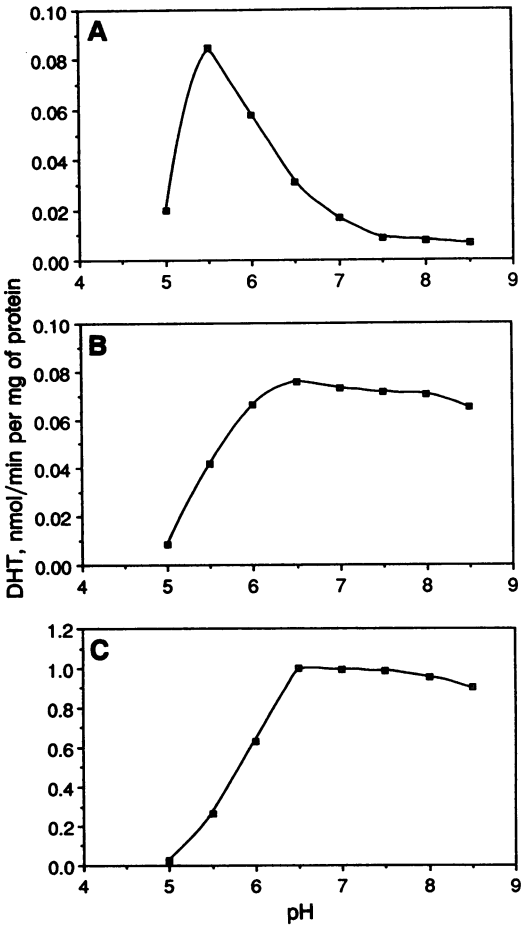


FIG. 1. Steroid 5α-reductase activity as a function of pH. All enzymes were assayed for 15 min at 37°C with 500 μM NADPH in constant ionic strength buffers consisting of succinic acid, imidazole, and diethanolamine as described by Levy *et al.* (21). (*A*) pH dependence of the prostatic reductase. (*B*) pH dependence of the scalp reductase. (*C*) pH dependence of the cloned human reductase 1 expressed in COS cells.

Table 1. Kinetic constants for 5α-reductases

Enzyme	Conditions*	Apparent K_m, μM	
		T	NADPH
Prostatic	pH 5.5	0.3 ± 0.06	3.9 ± 0.7
Scalp	pH 6.5	7.7 ± 0.4	26 ± 2.0
Cloned	pH 6.5	11.2 ± 0.9	29 ± 1.9

*Assay conditions described in text.

the 10,000 × *g* and 100,000 × *g* membrane fractions. A similar distribution was found with the prostatic 5α-reductase, with 87% of the activity located in the nuclear fraction. The product of the reaction was confirmed as 5α-DHT by thin-layer chromatography and reverse-phase HPLC (data not shown). No metabolites of T or DHT, other than androstanediol, were observed under these reaction conditions.

Kinetic Properties. The pH dependence for the human prostatic and scalp 5α-reductase activities is shown in Fig. 1. As originally reported by Liang *et al.* (22), the prostatic reductase has a sharp pH optimum of 5.5 and a low level of activity between pH 6.5 and 8.0 (Fig. 1*A*). In contrast, the scalp reductase has a broad pH optimum between 6.0 and 8.0 (Fig. 1*B*). Thus, it appears that the tissues have similar levels of activity, but the pH dependence of the prostatic reductase is significantly different from that of the scalp reductase.

To further characterize the two reductases, the kinetic constants for T and NADPH were determined. Comparison of the apparent K_m values for T indicates that there are substantial differences in the affinity of this substrate for the two enzymes at their respective pH optima (Table 1). The K_m for T for the prostatic reductase (0.3 μM) is 1/25th that of the scalp reductase (7.7 μM) (see Fig. 2). Similarly, there is a 7-fold difference in the apparent K_m values for NADPH observed for the prostatic and scalp enzymes (4 and 26 μM, respectively; Table 1).

Inhibitors of 5α-Reductase. The effect of reductase inhibitors on the human 5α-reductase subtypes is summarized in Table 2. Some compounds display slow time-dependent inhibition which will be addressed in a future article. For the present, it should be recognized that for certain inhibitors the potencies determined in the single time-point assay may reflect rate constants for binding to the enzyme rather than the overall equilibrium constant. This ambiguity does not compromise the goal at hand: distinguishing the two reductase subtypes by their sensitivities to different inhibitors. Compounds **1a**, **1b**, **1c**, and **2**, while belonging to different

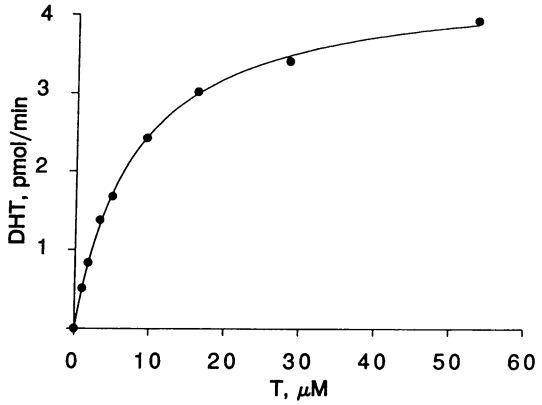
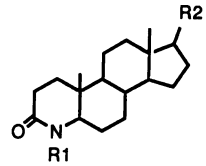


FIG. 2. Substrate dependence of DHT formation for the scalp reductase. The data were fit by NLIN, an in-house nonlinear regression kinetics program, to give K_m = 7.7 ± 0.4 μM for T.

structural classes, are all potent inhibitors of the human prostatic reductase, with IC_{50} values of less than 15 nM. The *N*-4-methylazasteroids **1d** and **1e** were found to be much less potent (IC_{50} > 100 nM). Interestingly, the observed rank order of inhibitor potency is very different for the human scalp reductase: the *N*-methylazasteroids are better inhibitors than the *N*—H azasteroids. Compounds **1a**, **1b**, and **2** show poor affinity for the scalp enzyme but potent inhibition of the prostatic reductase. In contrast, the *N*-methylazasteroids **1d** and **1e** are potent inhibitors of the scalp reductase but weak inhibitors of the prostatic enzyme. Compound **1c** is very active against both reductases. From these data it is clear that inhibitors can be classified as prostate selective, dual active, and scalp selective.

Properties of the Cloned Human Reductase Compared with Those of the Native Prostatic and Scalp Reductases. The poor inhibition of the scalp reductase by **1a** was reminiscent of that found with the prostatic 5α-reductase 1 expressed in COS cells (15). This observation prompted a closer examination of the properties of this cloned enzyme compared with the prostatic and scalp reductases. As shown in Fig. 1*C*, the 5α-reductase 1 expressed in COS cells has a broad pH optimum centered at pH 7.0 which closely resembles that of the scalp reductase. In addition, the reductase 1 has a K_m of 11.2 μM for T, which is similar to the K_m of the scalp enzyme (Table 1). To definitively establish that the expressed 5α-reductase 1 represents a scalp-like enzyme, we studied its

Table 2. Inhibition of human 5α-reductase subtypes



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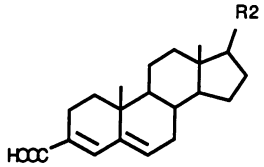

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Compound	R1	R2	A-ring modification	Apparent potency,* nM		
				Prostatic homogenate	Scalp homogenate	Cloned reductase
1a	H	$CONHC(CH_3)_3$	Δ^1	4.2	500	1200
1b	H	$COCH_2CH(CH_3)_2$	Δ^1	12.5	240	400
1c	CH_3	$CON(CH_2CH_3)_2$		0.9	11	19
1d	CH_3	CO_2CH_3	Δ^1	960	34	15
1e	CH_3			140	5	ND
2		$CONHC(CH_3)_3$		0.7	350	830

*The apparent potency is the concentration of inhibitor required to produce 50% inhibition. ND, not determined.

sensitivity to inhibitors. As summarized in Table 2, the cloned reductase 1 is most strongly inhibited by the *N*-methyl-4-azasteroids **1c** and **1d**. The *N*—H azasteroids (**1a** and **1b**) and the 3-carboxyandrostadiene (**2**) are poor inhibitors of the cloned prostatic enzyme. Given the similar pharmacological properties of the human scalp and cloned human reductase 1, we propose that the cloned enzyme represents a scalp-type isozyme in prostate. However, at most, this isozyme would contribute less than 10% of the activity in prostate and is therefore below the level of detection.

DISCUSSION

The 5α-reductase present in prostate has been relatively well characterized in regard to its substrate and inhibitor specificity (11, 18, 19, 21, 22). In contrast, little has been done to characterize the enzyme in scalp. In this study we compared the properties of the scalp and prostatic 5α-reductases. We find a 25-fold difference in the K_m for T for the scalp and prostatic reductases. In addition, these enzymes can be differentiated on the basis of their sensitivity to inhibitors. Compound **2** is 500-fold more effective against the prostatic reductase, whereas compound **1e** is 28-fold more effective against the scalp reductase (Table 2). We have also found that inhibitors can be very potent against both enzymes (compound **1c**; Table 2). Similarly, Mellin *et al.* (23) found that the 5α-reductase in the hair follicle and prostate differ significantly in their sensitivity to 4-azasteroids. These data support the existence in humans of at least two isoforms of 5α-reductase which differ in their sensitivity to inhibitors.

A limitation of the current work is the crude nature of the enzyme preparations. Thus far, all attempts by us and others (21, 24–26) to purify 5α-reductase have been unsuccessful. Steroid 5α-reductase in skin is found primarily in the apocrine and sebaceous glands with lower levels present in the hair follicle and dermis (27, 28). However, despite the possible heterogeneous composition of the enzyme, the close fit of the kinetic data is consistent with a single major form of the reductase in scalp. Presently, we believe that the differences between the scalp and prostatic reductases are best explained by two genes encoding two different reductases.

Steroid 5α-reductase activity has also been detected in skin slices from anatomical sites other than scalp (29–31). The highest levels are found in skin from the perineal sites. Lower levels of activity are found in regions from trunk and limbs. Whether the activity present in all regions of skin represents a single form of the enzyme such as the scalp reductase is unclear. In fact, there is evidence to suggest that the enzyme present in the dermal papilla cells of beard resembles the prostatic reductase, while the enzyme present in the occipital scalp is similar to the scalp reductase described herein (32). Additional studies are required to fully characterize the skin reductase(s).

The identification of an isozyme for 5α-reductase calls for a reexamination of the profile of the 5α-reductase-deficient individuals. These men have normal to high levels of T and low but detectable levels of DHT. The origin of the circulating DHT has as yet been unclear. Andersson *et al.* (16), using restriction fragment length polymorphism (RFLP) studies, found that the deficiency in some patients is the result of a lesion in the 5α-reductase 2 gene, whereas the 5α-reductase 1 gene is normal. In addition, Moore and Wilson (9) found that normal genital skin fibroblasts have a reductase activity with a pH optimum of 5.5 which is low or absent in mutant fibroblasts. In contrast, a 5α-reductase with a pH optimum of 7.0–9.0 is found in both normal and mutant skin fibroblasts. We suggest that the prostatic enzyme (pH 5.5 optimum form) is abnormal in the 5α-reductase-deficient patients, while the scalp-type reductase (pH 7.0 optimum) is unaffected. Thus, the circulating DHT may arise from peripheral conversion of T by a scalp-type reductase.

Additional data to support the proposal that the scalp enzyme is normal and therefore the origin of the circulating DHT in the 5α-reductase-deficient patients comes from studies of root hairs. In one report similar levels of 5α-reductase were found in scalp hair roots of normal and deficient patients (33). Thus, it can be assumed that the reductase activity associated with the root hair is unaffected in the 5α-reductase-deficient individual. Although no characterization of this enzyme was described, it is reasonable to assume that this enzyme activity represents the scalp reductase.

It is interesting to note the properties of the scalp reductase and the 5α-reductase 1 expressed in COS cells are virtually identical, especially with regard to sensitivity to inhibitors. These data imply that a scalp-type enzyme exists in prostate, since the gene for 5α-reductase 1 was originally cloned from prostate (15). However, our results would indicate that this isozyme represents a minor component of the reductase activity in prostate because we could not detect this activity in crude extracts. Interestingly, 5α-reductase 1-type activity appears to be the major reductase activity present in scalp.

The results reported in this paper may have important implications in pursuing steroid 5α-reductase as a therapeutic target. An inhibitor of 5α-reductase has been shown to be useful in the treatment of benign prostatic hyperplasia (34). Reductase inhibitors may also be useful in treating male pattern baldness, acne, and hirsutism, as these disorders also appear to be DHT dependent. However, the results of our study indicate that a single inhibitor may not be suitable, given the differences in the enzymes. We have shown that it is possible to identify scalp-selective, prostate-selective, and dual-active inhibitors.

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