A KINETIC ANALYSIS OF THE 5α-REDUCTASES FROM HUMAN PROSTATE AND LIVER

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ABSTRACT

A kinetic analysis of the 5α-reductases from human liver and prostate is presented. Human prostatic 5α-reductase follows an ordered sequential mechanism in which NADPH binds first followed by testosterone. The order of release of products is DHT followed by $NADP^+$. The apparent K_m of prostatic 5α-reductase for testosterone is 0.0339 + 0.006μM, while the apparent K_m for NADPH is 2.52 + 0.65μM. Human liver 5α-reductase also follows a sequential mechanism. The apparent K_m of the liver enzyme is 0.110 + 0.08μM; the apparent K_m for NADPH is 6.2 + 0.6μM. The fact that both the liver and prostatic 5α-reductases have a sequential kinetic mechanism rules out the possibility that the reduction of testosterone to dihydrotestosterone involves an electron transport system as previously proposed.

INTRODUCTION

A number of organs such as prostate, epididymis, hypothalamus, and pituitary contain significant amounts of the enzyme 5α-reductase. In androgen-dependent target tissues this enzyme catalyzes the formation of dihydrotestosterone (DHT) from testosterone. This is an essential step in the promotion of androgen action in these organs, since, in individuals who are deficient in 5α-reductase, organs such as the prostate do not develop. In addition, an increase in 5α-reductase activity has been implicated in several clinical conditions such as benign prostatic hyperplasia and female hirsutism (1-6). The liver also has high levels of 5α-reductase activity, although the role of the enzyme in this gland is not clear.

The 5α-reductase in several tissues has been characterized extensively with regard to substrate and inhibitor specificity, co-factor requirement, tissue and subcellular distribution, and

detergent solubilization (7-18).

However, it is not clear whether the 5α-reductase in liver is distinct from that in other tissues. Golf and Graef have suggested that in the liver the transfer of a proton from NADPH to testosterone is not direct but involves an FAD-linked cytochrome C oxidoreductase and Co-enzyme Q (CoQ) (19,20). This conclusion was based on data which show that electron transport inhibitors reduce hepatic 5α-reductase activity and that CoQ can replace NADPH. Little is known regarding the mechanism of 5α-reductase in target tissues, although Cooke and Robaire (21) have suggested that an electron transport system is not involved in the epididymal 5α-reductase. This suggests that the 5α-reductases in liver and epididymis are quite distinct. In this study we have compared the kinetic mechanisms of the 5α-reductases in human prostate and liver and have elucidated the order of binding of substrates to and release of products from the prostatic enzyme.

MATERIALS AND METHODS

Chemicals

Glucose-6-phosphate, $NADP^+$, NADPH, glucose-6-phosphate dehydrogenase, and unlabeled steroids were obtained from Sigma Chemical Co. Ltd., Poole, UK. 5α-Dihydro-[4-^{14}C]testosterone (100-150mCi/mmol) and [1,2,6,7-^{3}H]testosterone (85-100Ci/mmol) were purchased from Amersham International, Amersham, UK. [^{3}H]Testosterone was adjusted to a specific radioactivity of 4.54μCi/nmol and purified as described in (15). The sources of all other materials have been described previously (15,16).

Tissue procurement

Human prostatic tissue was obtained from patients undergoing open prostatectomy for benign prostatic hyperplasia. Human liver was obtained post mortem from male organ donors. Both liver and prostatic tissues were transported to the laboratory within 60 min, snap frozen in liquid nitrogen, and stored at -70°C until use. Benign prostatic

hyperplasia was confirmed by histological examination.

5α-Reductase: Preparation and assay

Prostatic and hepatic microsomes were used as a source of 5α-reductase activity and were prepared as described in (15) using homogenization buffer (b). 5α-Reductase was assayed by the radiometric method of Houston et al (15) at the concentrations of NADPH and testosterone indicated in the figure legends.

Data analysis

Enzyme kinetic data were analyzed on a BBC microcomputer using an enzyme kinetics package provided by Drs. D.A. Fell and H.M. Sauro, Oxford Polytechnic, Oxford, England. The initial velocity data were fitted to the two-substrate rate equations 1-3 (sequential, double-displacement, and equilibrium ordered respectively):

$$v = VAB/(K_{ia} K_b + K_a B + K_b A + AB) \quad (1)$$

$$v = VAB/(K_a B + K_b A + AB) \quad (2)$$

$$v = VAB/(K_{ia} B + K_b A + AB) \quad (3)$$

Product inhibition data were fitted to equations 4-6 (competitive, uncompetitive, and non-competitive inhibition respectively):

$$v = VA/(K(I/K_{is}) + A) \quad (4)$$

$$v = VA/(K + A\,(1 + I/K_{ii})) \quad (5)$$

$$v = VA/(K(1 + I/K_{is}) + A\,(1 + I/K_{ii})) \quad (6)$$

The definitions and symbols of Cleland (22,23) are used: v is the reaction velocity and V is the maximum velocity; K_a and K_b are Michaelis constants for substrates A and B; K_{ia} is a dissociation constant for substrate A; K_{is} and K_{ii} are respectively the slope and intercept inhibition constants; I is the inhibitor concentration. The initial parameter estimates required by the fitting program were devised from slope and intercept replots of Lineweaver-Burk plots of the data (Figures 1-8). Thereafter the program used an iterative non-linear regression analysis to establish the final parameter values. The best fit of the kinetic data to a given model was established on the basis of the least residual mean square value and a minimum % error value.

Miscellaneous procedures

Protein concentrations were determined by the method of Bradford (24) using bovine serum albumin as standard. Testosterone concentrations in stock solutions were measured by radioimmunoassay (25). NADPH was measured spectrophotometrically at 340nm using an absorption coefficient of $6.22 \times 10^3 L \times mol^{-1} \times cm^{-1}$. $NADP^+$ was assayed

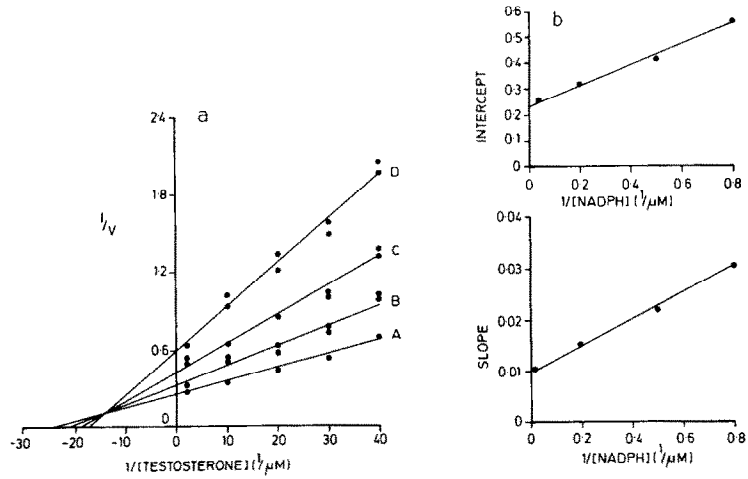


Figure 1. Initial velocity analysis of prostatic 5α-reductase with testosterone as the varied substrate. (a) Double-reciprocal plots at the following NADPH concentrations: (A) 50μM; (B) 5μM; (C) 2μM; (D) 1.25μM. Each substrate combination was assayed in duplicate, and initial velocity is in units of pmol/min/mg protein. (b) Slope and intercept replots of the data in (a).

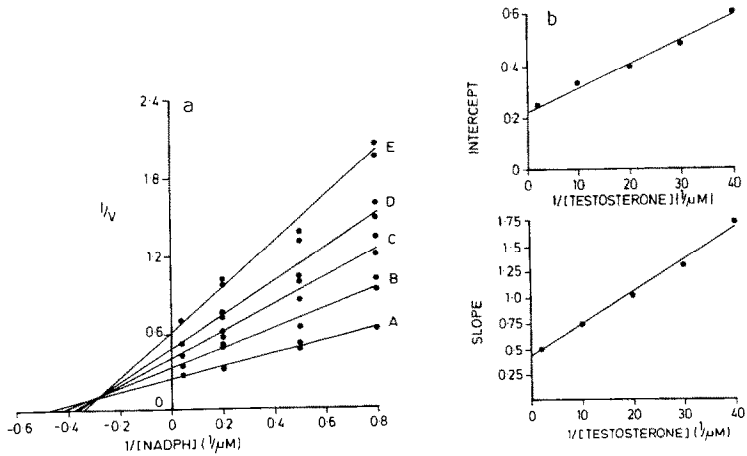


Figure 2. Initial velocity analysis of prostatic 5α-reductase with NADPH as the varied substrate. (a) Double-reciprocal plots at the following testosterone concentrations: (A) 0.5μM; (B) 0.1μM; (C) 0.05μM; (D) 0.033μM; (E) 0.025μM. Each substrate combination was assayed in duplicate. The initial velocity is in units of pmol/min/mg protein. (b) Slope and intercept replots of the data in (a).

spectrophotometrically by following its conversion to NADPH using glucose-6-phosphate and glucose-6-phosphate dehydrogenase.

RESULTS

Initial velocity studies of human prostatic 5α-reductase

The kinetic mechanism of human prostatic 5α-reductase was studied by measuring the initial rate of the reaction while systematically varying the concentrations of both substrates. The double-reciprocal plots of 1/v against 1/[testosterone] at various concentrations of NADPH form a series of lines which intersect to the left of the 1/v axis (Figure 1a). A similar pattern of intersecting lines was obtained in double-reciprocal plots of 1/v versus 1/[NADPH] at different concentrations of testosterone (Figure 2a). These intersecting patterns are diagnostic of a sequential kinetic mechanism, and the best fit to the data was provided by equation 1. The slope and intercept replots of the two primary plots were linear and in every case intersect the vertical axis at a positive value (Figures 1b, 2b). This indicates that the mechanism is not equilibrium ordered.

The apparent V_{max} determined in this experiment was 3.38 ± 0.98pmol/min/mg protein. The apparent K_m for testosterone (K_m^T) was 0.0339 ± 0.006μM while that for NADPH (K_m^{NADPH}) was 2.52 ± 0.65μM. A K_{ia} for NADPH of 2.44 ± 0.36μM was also obtained.

Product inhibition studies

The order of binding of substrates and release of products from human prostatic 5α-reductase was determined by product inhibition studies. The double-reciprocal plots of 1/v versus 1/[testosterone] at different concentrations of $NADP^+$ are shown in Figure 3a. $NADP^+$

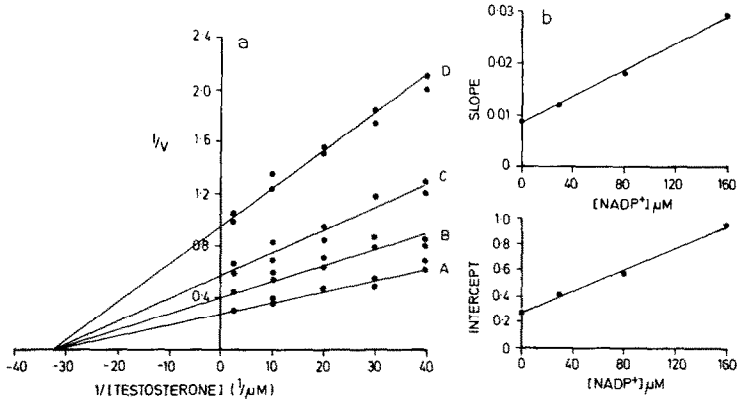


Figure 3. Inhibition of prostatic 5α-reductase by $NADP^+$ as a function of testosterone concentration. (a) Double-reciprocal plots at the following $NADP^+$ concentrations: (A) 0; (B) 30μM; (C) 80μM; (D) 160μM. Each testosterone/$NADP^+$ combination was assayed in duplicate at an NADPH concentration of 5μM. Initial velocity is in units of pmol/min/mg protein. (b) Slope and intercept replots of the data in (a).

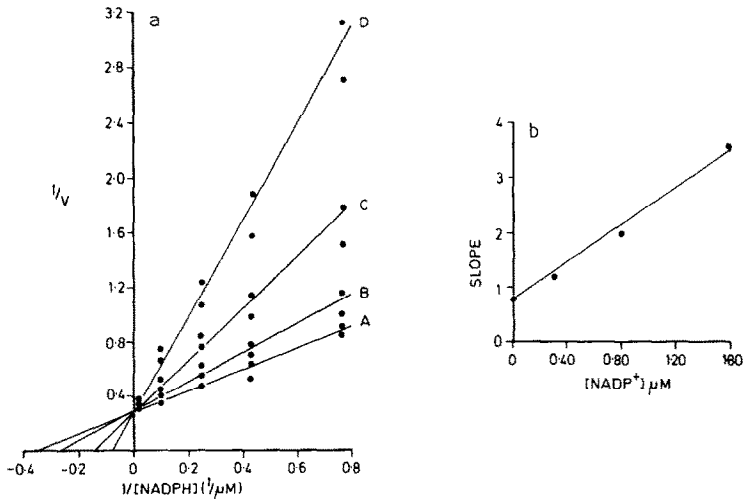


Figure 4. Inhibition of prostatic 5α-reductase by $NADP^+$ as a function of NADPH concentration. (a) Double-reciprocal plots at the following $NADP^+$ concentrations: (A) 0; (B) 30μM; (C) 80μM; (D) 160μM. Each NADPH/$NADP^+$ combination was assayed in duplicate at a testosterone concentration of 0.125μM. Initial velocity is in units of pmol/min/mg protein. (b) Slope and intercept replots of the data in (a).

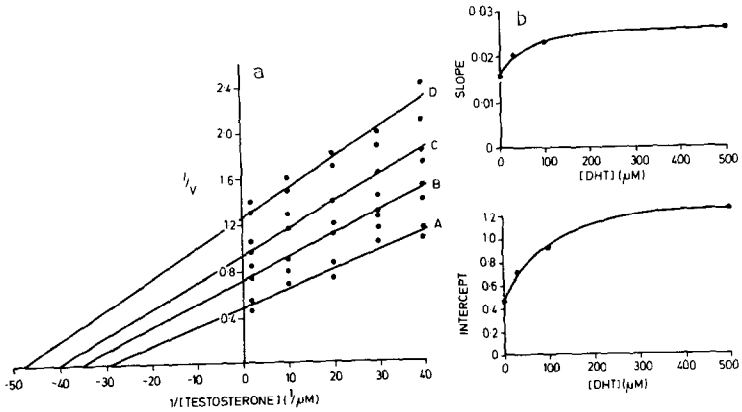


Figure 5. Inhibition of prostatic 5α-reductase by DHT as a function of testosterone concentration. (a) Double-reciprocal plots at the following DHT concentrations: (A) 0; (B) 30μM; (C) 100μM; (D) 500μM. Each combination of testosterone and DHT was assayed in duplicate at an NADPH concentration of 5μM. Initial velocity is in units of pmol/min/mg protein. (b) Slope and intercept replots of the data in (a).

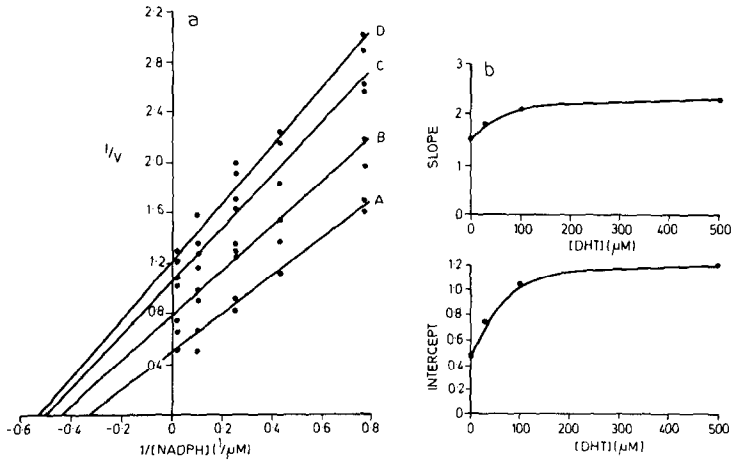


Figure 6. Inhibition of prostatic 5α-reductase by DHT as a function of NADPH concentration. (a) Double-reciprocal plots at the following concentrations of DHT: (A) 0; (B) 30μM; (C) 100μM; (D) 500μM. Each combination of NADPH and DHT was assayed in duplicate at a testosterone concentration of 0.03μM. Initial velocity is in units of pmol/min/mg protein. (b) Slope and intercept replots of data in (a).

appears to inhibit the enzyme non-competitively with respect to testosterone with an apparent K_{is} = 70 ± 8μM and an apparent K_{ii} = 56 ± 11μM. The slope and intercept replots are both linear, which suggests that only one molecule of $NADP^+$ is binding to the enzyme (23). The double-reciprocal plots of 1/v against 1/[NADPH] at various levels of $NADP^+$ give a set of lives which intersect on the 1/v axis (Figure 4) and the data were best fitted to equation 4. Thus $NADP^+$ is a linear competitive inhibitor of 5α-reductase with a K_{is} = 48 ± 3μM.

Double-reciprocal plots in which 1/v versus 1/[testosterone] or 1/v versus 1/[NADPH] is plotted at different concentrations of DHT are shown in Figures 5a and 6a. In both cases a non-competitive pattern is observed. However the slope and intercept replots are all hyperbolic, indicating partial inhibition. We believe that the hyperbolic patterns observed in this case indicate that the DHT is not completely soluble at the highest concentration used (500μM). The values of the kinetic parameters and the best fit to equations 4-6 were therefore determined using only the data for 0, 30, and 100μM DHT. In both cases the data were best fitted to equation 6 and the values for the inhibition constants were: (Testosterone versus DHT) K_{is} = 240 ± 12μM, K_{ii} = 110 ± 24μM; (NADPH versus DHT) K_{is} = 260 ± 17μM, K_{ii} = 100 ± 8μM.

Initial velocity studies of human liver 5α-reductase

The kinetic mechanism of human liver 5α-reductase was studied by the method used for the prostatic enzyme. A series of intersecting double-reciprocal plots was obtained when 1/v against 1/[testosterone] was plotted at different concentrations of NADPH (Figure 7a) or when

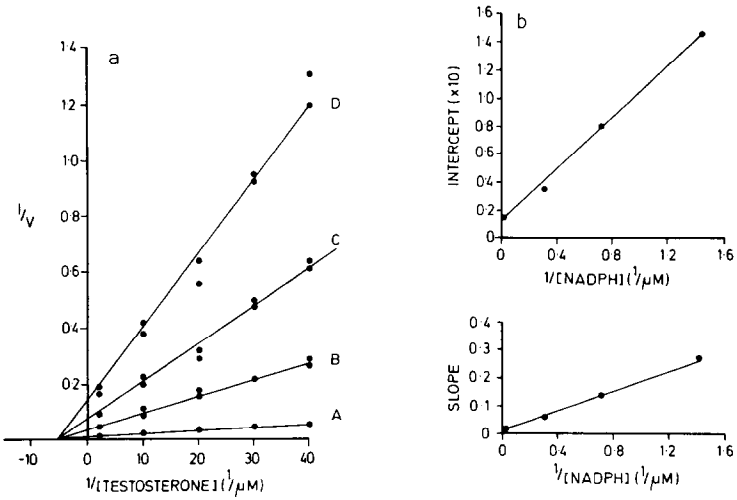


Figure 7. Initial velocity analysis of hepatic 5α-reductase with testosterone as the varied substrate. (a) Double-reciprocal plots of 1/v against 1/[testosterone] at the following concentrations of NADPH: (A) 50μM; (B) 3.3μM; (C) 1.4μM; (D) 0.7μM. Each substrate combination was assayed in duplicate and initial velocity is in units of pmol/min/mg protein. (b) Slope and intercept replots of the data in (a).

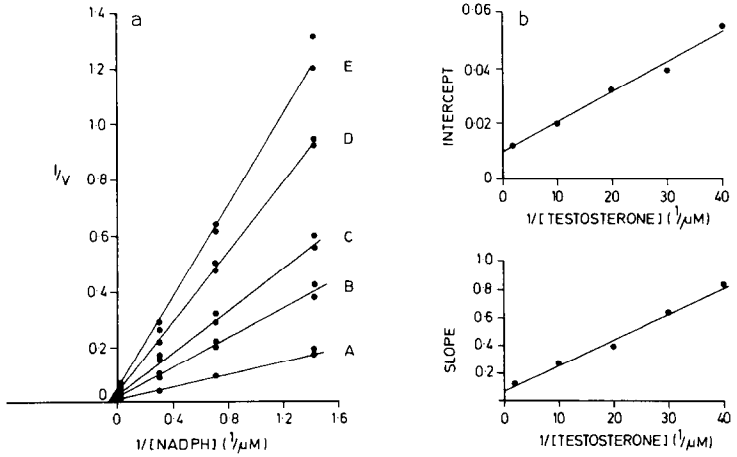


Figure 8. Initial velocity analysis of hepatic 5α-reductase with NADPH as the varied substrate. (a) Double-reciprocal plots of 1/v against 1/[NADPH] at the following concentrations of testosterone: (A) 0.5μM; (B) 0.1μM; (C) 0.05μM; (D) 0.033μM; (E) 0.025μM. Each substrate combination was assayed in duplicate. Initial velocities are in units of pmol/min/mg protein. (b) Slope and intercept replots of the data in (a).

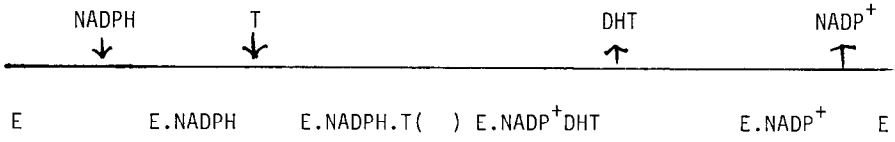
1/v versus 1/[NADPH] was plotted at different concentrations of testosterone (Figure 8a). In both cases the lines intersected to the left of the 1/v axis and the slope and intercept replots were linear. The best fit to the data was provided by equation 1 and so the kinetic mechanism appears to be sequential. The apparent V_{max} was 123 $\pm$ 24pmol/min/mg protein and the apparent K_m for testosterone and NADPH were 0.110 $\pm$ 0.08μM and 6.2 $\pm$ 0.6μM respectively. Since equation 1 is symmetrical with respect to each substrate, the data were initially analyzed on the basis that NADPH binds first and then on the basis that testosterone binds first. This yields two possible values for the K_{ia} term in equation 1: K_{ia} (testosterone) = 1.9 $\pm$ 0.8μM and K_{ia} (NADPH)=0.016 $\pm$ 0.008μM; K_{ia} (testosterone) and K_{ia} (NADPH) correspond to the dissociation constants for these substrates. The lowest residual mean square value was obtained when NADPH was the first substrate to bind, although this does not necessarily mean that NADPH binds first.

DISCUSSION

The results presented in this paper (Figures 1 and 2) indicate that human prostatic 5α-reductase follows a sequential kinetic mechanism. Subsequent to the completion of this work, Campbell *et al* (26) described the kinetic mechanism of the progesterone 5α-reductase from rat pituitary. The initial velocity data of these workers also indicated a sequential mechanism. Their data were best fitted to equation 3, that of an equilibrium ordered mechanism, with the binding of NADPH preceding that of progesterone. The initial velocity data obtained in this present study were best fitted to equation 1, which is the equation of a general sequential mechanism and the slope and

intercept replots in Figures 1b, 2b confirm this. Since equation 1 is symmetrical with respect to each substrate, it is not possible to determine the order of binding of NADPH and testosterone without further analysis.

The product inhibition patterns are described in the Results section. Three of the substrate/product pairs gave non-competitive inhibition patterns, and the data were best fitted to equation 6. In terms of the kinetic mechanism this means that, for each of the three pairs, the substrate and product bind to different forms of the enzyme (or more specifically, to different enzyme-ligand complexes). $NADP^+$ inhibits 5α-reductase competitively with respect to NADPH (Figure 4) and the data were best fitted to equation 4. This implies that $NADP^+$ and NADPH both bind to the same form of the enzyme. As the only such form to which substrate and product can both bind is the uncomplexed enzyme, this indicates that NADPH is the first substrate to bind and $NADP^+$ the last product to be released:



This order of binding is supported by the results of inhibition studies by other workers. Dead-end inhibition of prostatic 5α-reductase by 6-methylene-4-pregnene-3-ones and photo-affinity labeling of prostatic 5α-reductase by 21-diazo-4-azasteroids both require the presence of NADPH and cause competitive inhibition with respect to testosterone but uncompetitive inhibition against NADPH

(7,27). These are precisely the inhibition patterns expected, given that testosterone cannot bind to the enzyme unless NADPH is already bound.

Our results (Figures 7 and 8) show that human hepatic 5α-reductase also has a sequential kinetic mechanism. As we are primarily interested in prostatic 5α-reductase we have not studied the order of binding of substrates to the liver enzyme. However, it is likely that the order of binding of substrates is the same in both tissues; Liang et al (28) found that the reversible inhibition of rat liver 5α-reductase by a 4-methyl-4-aza-steroid was dependent on the presence of NADPH and competitive with respect to testosterone. These findings imply a mechanism in which NADPH binds first, followed by testosterone. The Michaelis constants determined for the hepatic enzyme were approximately 3-fold higher than those for the prostate. To our knowledge this is the only measurement of these parameters for human liver 5α-reductase, although Roy (29) has reported a K_m^{NADPH} = 200μM and a K_m^{T} = 2μM for the rat liver enzyme. Both values are significantly higher than those obtained in this study. Whether this reflects a species difference is unclear.

The finding that human prostatic and hepatic 5α-reductases follow a sequential mechanism appears to rule out the possibility that the reaction catalyzed by 5α-reductase is mediated by an electron transport system involving a cytochrome, flavins, and CoQ, as originally proposed by Golf et al (19,20). Such a system would give rise to a double-displacement kinetic mechanism and would have been distinguished by parallel double-reciprocal plots in Figures 1,2,7,8. Thus our results complement those of Cooke and Robaire (21) and

Enderle-Schmitt et al (30) who, using non-kinetic methods, concluded that rat epididymal and prostatic 5α-reductases do not utilize an electron transport system.

Values for the K_m^{NADPH} of human prostatic 5α-reductase do not appear to have been reported by earlier workers. The value determined in this study (2.52 ± 0.65μM) is somewhat lower than values reported for 5α-reductases from other species and target tissues (14,29,31). It is interesting to note that the K_m^{NADPH} of the progesterone 5α-reductase studied by Campbell et al (26) is $\simeq 0$. This is a consequence of the equilibrium ordered kinetic mechanism of that enzyme. The value for K_m^T determined in this study was 33.9 ± 6nM, which is similar to that obtained in previous studies from this laboratory (16). There is considerable disagreement in the literature regarding the value of the K_m^T for prostatic 5α-reductase. Values ranging from 21nM to 15uM have been reported (e.g., 12-14,31,32). Several different factors may be responsible for this large variation, including the stroma/epithelium ratio and the assay conditions (11,31, 33). Since the product of a reaction must interact with the enzyme it is theoretically capable of being an inhibitor. In practice however, the product is often insoluble at the concentrations necessary to demonstrate inhibition. In our hands DHT inhibited prostatic 5α-reductase non-competitively at all concentrations tested, but the slope and intercept replots were hyperbolic. This is generally interpreted as indicating that the binding of the inhibition diverts the reaction flux through an alternative, slower pathway (22). In the present case a more likely explanation is that, in the presence of all of the assay components, DHT is not completely soluble at the highest

concentration used. It should be noted that Campbell et al (26) have experienced similar insolubility problems with 5α-dihydroprogesterone.

The relationship between the 5α-reductases in target tissues is unclear. While the 5α-reductases in these tissues are in some respects similar (9,10,13,16,17,30,32), other workers have reported certain differences (12,31). This problem will be resolved only when detailed physico-chemical studies are performed on 5α-reductases purified from a number of sources.

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NOTES

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APPENDIX

List of trivial names and the abbreviations used:

Testosterone = 17β-hydroxy-4-androsten-3-one
5α-DHT = 5α-dihydrotestosterone = 17β-hydroxy-5α-androstan-3-one
Androstenedione = 4-androstene-3,17-dione
5α-Reductase = cholestenone 5α-reductase = 3-oxo-5α-steroid: $NADP^+$-4-ene-oxidoreductase;EC 1.3.1.22