Finasteride: A Slow-Binding 5α-Reductase Inhibitor

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ABSTRACT: A microsomal preparation of human prostatic tissue was used to study the kinetics of interaction of steroid 5α-reductase with finasteride, a known 5α-reductase inhibitor. This molecule has been reported to reversibly bind 5α-reductase in a competitive manner to testosterone with a K_i value in the 10 nM range. The results presented in this paper show that enzyme–inhibitor complex formation does not take place instantaneously as assumed in previous studies. At neutral pH and 37 °C, the association of enzyme with inhibitor is governed by a rate constant, k_{on}, of 2.7×10^5 M^{-1} s^{-1}. This low k_{on}value, in combination with the high energy of activation of the association reaction (150 kJ mol^{-1}), indicates that the association process is not diffusion controlled and may proceed through intermediate steps. However, such an intermediate was not detected kinetically under the inhibitor concentrations investigated. We therefore conclude that the equilibrium dissociation constant, K_i*, for the initial binding of the enzyme to the inhibitor is higher than 1.5×10^{-7} M. Even at inhibitor concentrations as low as 1 nM, the reaction was completely displaced to the EI complex and no residual activity detected once the equilibrium was reached. Hence, the interaction between finasteride and 5α-reductase can also be characterized by a very low overall equilibrium dissociation constant ($K_i \ll 10^{-9}$ M), at least 1 order of magnitude lower than previously reported values. pH profiles of k, the pseudo-first-order rate constant for the association of enzyme with inhibitor, and V_m/K_m were similar, indicating that the same ionizable groups are involved in the interaction of the enzyme with both testosterone and finasteride. Our results show that finasteride can efficiently compete with testosterone for the binding to 5α-reductase since we demonstrate that $K_i \ll K_m$. The data presented in this paper support what is observed *in vivo*, predicting that pseudoirreversible inhibition of steroid 5α-reductase will take place in the presence of testosterone.

Testosterone (T)[1] is the main circulating androgen in man responsible for increase in muscle mass, deepening of the voice, spermatogenesis, and libido. However, from numerous studies the concept has evolved that in some tissues, such as the skin or the prostate, T functions as a prehormone which is converted to the more potent androgen dihydrotestosterone (DHT) (McGinley et al., 1974; Walsh et al., 1974; McGinley et al., 1979). The NADPH-dependent reduction of T to DHT is catalyzed by a membrane-bound enzyme named steroid 5α-reductase (5-AR) (Moore et al., 1972). A decrease in the DHT levels by inhibition of 5-AR may be a therapeutic approach to reducing prostate size and thus alleviating symptoms associated with certain disease states, such as benign prostatic hyperplasia (BPH). About ten years ago, a series of 4-aza steroids were synthesized as inhibitors of 5-AR (Liang et al., 1983b). One of these compounds, 17β-[*N*-(1,1-dimethylethyl)carbamoyl]-4-aza-5α-androst-1-en-3-one (finasteride, also named MK-906) was found to have a high specificity for 5-AR and very little affinity for the androgen receptor (Liang et al., 1984). Several reports of clinical trials have confirmed the *in vivo* efficacy of this molecule in decreasing DHT levels in man (Geller, 1990; McConnell et al., 1992). Finasteride is reported to be a competitive, reversible inhibitor of human 5-AR (Liang et al., 1985). Published K_i values for finasteride vary from 3 to 26 nM (Jenkins et al., 1992; Andersson et al., 1991; Liang et al., 1985). Human 5-AR can be characterized as having a high affinity for T with K_m values from 10 to 30 nM commonly reported in the literature (Bruchosvky et al., 1988; Houston et al., 1987). From these data it appears that the K_m/K_i ratio is close to 1, and therefore it is likely that a local concentration of T in the prostatic tissue will efficiently compete with the inhibitor for the target enzyme. Comparison of these kinetic data with what is observed from the clinical trials (90% decrease in the plasma DHT level, consistent with a pseudoirreversible inhibition of the enzyme) indicates that there is a discrepancy between the *in vitro* kinetic data (K_m/K_i close to 1) and the *in vivo* efficacy of the inhibitor. For this reason, we have further investigated the kinetics of interaction between 5-AR and finasteride. From these studies we were able to demonstrate that the binding of finasteride with 5-AR is not instantaneous but occurs at a relatively slow rate which, if not taken into account, leads to an overestimation of the real K_i value.

EXPERIMENTAL PROCEDURES

Materials

[1,2,6,7-^{3}H]Testosterone (92.1 Ci/mmol) was purchased from New England Nuclear (NEN). Cold testosterone and dihydrotestosterone were from Sigma, and NADPH was from Fluka. Finasteride, a known inhibitor of 5-AR, was synthesized by Dr. M. Biollaz (Ciba-Geigy Ltd., Basel). Silica-impregnated glass fiber sheets were from Gelman Sciences Inc. Irgascint A300 was from Ciba-Geigy Chemicals. Protein concentrations were estimated by the BCA method (Pierce Chemical Co.) using bovine serum albumin as a standard. 3,5,7,2′,4′-Pentahydroxyflavone was from BDH Chemicals. Radioactivity was measured using an LKB Wallach 125-Rackbeta scintillation counter calibrated to disintegrations per minute with LKB standards. Nonlinear regression

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[1] Abbreviations: T, testosterone; DHT, 5α-dihydrotestosterone; 5-AR, 3-oxo-5α-steroid Δ^4-reductase; finasteride, MK-906, 17β-[N-(1,1-dimethylethyl)carbamoyl]-4-aza-5α-androst-1-en-3-one; 4-MA, 17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one.

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analyses were performed using Enzfitter software (Elsevier-Biosoft).

Methods

Preparation of Microsomes from Human Prostate. Microsomes containing 5-AR active enzyme were prepared as previously described (Sargent & Habib, 1991) except that the solubilization with octyl β-D-glycopyranoside, the addition of brain lipids, and the dialysis step were omitted in our procedure. The preparations were stable for at least one-half year at −70 °C.

5-AR Activity Assay. All experiments (except the pH-dependence study) were performed in 100 mM Tris–citrate, 100 mM KCl, 5mM DTT, 1mM EDTA, and 20% glycerol, pH 7.0. Stock solutions of testosterone and finasteride were prepared in ethanol. The final concentration of ethanol in the reaction medium was 1% (v/v). The activity of the enzyme was monitored by following the conversion of [^{3}H]testosterone into DHT. No significant 5α-androstane-3α(3β)-17β-diols were detected under our experimental conditions. Microsomes were introduced into a medium containing NADPH and [^{3}H]testosterone, which was preincubated at 37 °C for 5 minutes. After various times of incubation, 500-μl aliquots were transferred to fresh tubes, and reactions were stopped by the addition of 2 mL of diethyl ether. The organic phase was then transferred into a new tube and evaporated to dryness in a water bath at 40 °C for 30 min. Samples were resuspended by addition of 50 μL of a solution of ethanol containing cold T and DHT (2 mg/mL each) as markers and applied to a silica-impregnated glass fiber sheet. Metabolites were separated by chromatography with dichloromethane/diethyl ether (9:1) as the running solvent. Steroids were located by spraying the dried sheets with a solution of pentahydroxyflavone (250 mg/500 mL of MeOH) and visualized under illumination at 366 nm. Spots were then cut out and transferred into polyethylene vials containing 6 mL of Irgascint A300 for scintillation counting.

Determination of K_m for Testosterone at pH 7.0 and 37 °C. Enzyme activity was measured in the presence of varying testosterone concentrations (5-120 nM). Stock solutions of testosterone were prepared in ethanol, whose concentration was maintained constant (1% v/v) throughout all the assays. Reactions were initiated by the addition of microsomes (25 μg/mL final concentration) in a buffered solution containing 0.4 mM NADPH and [^{3}H]testosterone. Five-hundred-microliter aliquots were removed after 5, 15, 25, and 35 min of incubation at 37 °C, and the [^{3}H]DHT formed was measured as described above. Velocities were plotted against testosterone concentrations, and K_m was calculated using a nonlinear regression procedure (Enzfitter) based on the Michaelis–Menten equation.

Measurement of Association Rate Constants. The rate of association of 5-AR with finasteride was measured using the progress curve method. The reaction was initiated by adding the enzyme to a mixture of substrate (T + NADPH) and inhibitor. The association reaction between enzyme and inhibitor was monitored by measuring the amount of DHT formed with time. Final concentrations for NADPH and testosterone were 0.4 mM and 10 nM, respectively. The enzyme concentration was adjusted in each assay so that no more than 10% of the substrate was turned over during the time of the reaction.

Dialysis Experiments. One hundred microliters of human prostatic microsomes (15 mg/mL) was incubated at 37 °C for 5 h with 1 mM NADPH in the presence or absence (control experiment) of 15 nM finasteride. The volume was then increased to 1 mL by the addition of 900 μL of Tris–citrate buffer, and the sample was packed in a dialysis membrane with a cutoff of 12-14 kDa. Each sample was dialyzed overnight at 4 °C against 500 mL of 100 mM Tris–citrate, 100 mM KCl, 1 mM EDTA, 5 mM DTT, 20% glycerol, 0.1 mM NADPH, and 10 nM testosterone. Each sample was then analyzed for 5-AR activity.

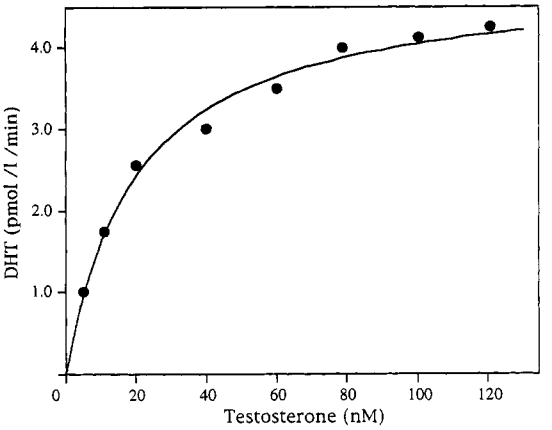

FIGURE 1: Determination of the K_m value of human prostatic 5-AR for testosterone. Reactions were initiated by the addition of enzyme (25 μg/mL final concentration) to a buffered solution containing 0.4 mM NADPH and [^{3}H]testosterone (5–120 nM). The solid line represents the best fit to the Michaelis–Menten equation. A K_m value of 20 ± 3 nM is calculated from nonlinear regression analysis of the experimental data.

pH Dependence of the Kinetic Parameters. Enzyme activity and the association rate constants for the binding of finasteride to 5-AR were determined at 37 °C and constant ionic strength (μ = 0.1 M). In order to maintain constant ionic strength over the pH range investigated (5–9), we used a buffer solution made of a mixture of succinic acid, imidazole, and diethanolamine (Ellis & Morrison, 1982). The buffer contained 33 mM succinic acid, 44 mM imidazole, 44 mM diethanolamine, 5 mM DTT, 1 mM EDTA, and 20% glycerol and was adjusted to the desired pH value. The effect of pH on the enzyme activity was evaluated by adding 5-AR to a buffered mixture containing 0.8 mM NADPH and 5 or 10 nM [^{3}H]testosterone. Aliquots were removed after various periods of incubation, and the amount of DHT was quantified as described above. The activity was determined from the slope of the DHT *vs* time curve. All the activities were normalized to the concentration of protein present in the assay. Determination of k_{on}, the association rate constant, was performed using the progress curve method. Reactant concentrations were the same as described for the enzyme activity determination, except 15 nM inhibitor was added to the reaction medium.

RESULTS

K_m of Human Prostatic 5-AR for Testosterone. Figure 1 shows the progressive saturation of the enzyme with increasing testosterone concentrations. The K_m value for testosterone calculated from these data is 20 ± 3 nM. This value, rather than those from the literature, was used throughout this work.

Time Dependence of the Inhibition. Figure 2 depicts the inhibition of microsomes (100 μg/mL) containing 5-AR by 10 nM finasteride. One can see that under these conditions the inhibition of 5-AR by finasteride does not take place immediately, as hypothesized in previous inhibition studies (Liang et al., 1985; Jenkins et al., 1992). In the presence of 10 nM inhibitor, the steady state is only reached after 30 min. The straight-line trend shown in the inset indicates that no

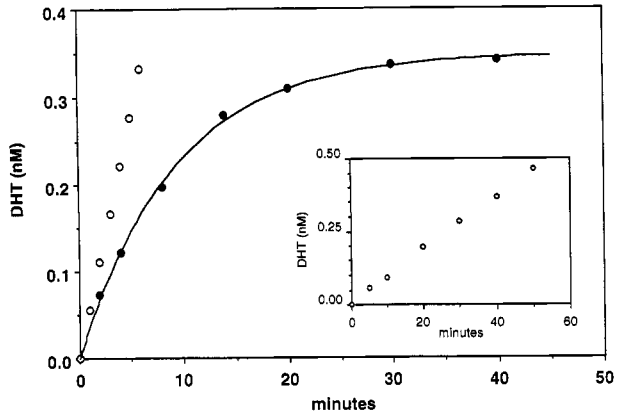

FIGURE 2: Progress curve for the inhibition of 5-AR by MK-906 in Tris–citrate, pH 7.0, at 37 °C. The enzyme was added to a mixture of substrate (NADPH + testosterone) in the presence (closed circles) or absence (open circles) of inhibitor. The amount of DHT produced was measured with time as described in the Methods section. Final concentrations were as follows: 5-AR, 100 μg/mL protein; MK-906, 10 nM; NADPH, 0.4 mM; [^{3}H]testosterone, 10 nM. The solid line represents the best exponential fit to the experimental data ($k = 1.8 \times 10^{-3}$ s^{-1}). The inset shows a control for the stability of the enzyme during the time of the experiment. As above, the enzyme was added to medium containing T (10 nM) + NADPH (0.4 mM). To prevent a decrease in the enzyme activity due to substrate consumption, the concentration of microsomes was reduced to 16 μg/mL protein.

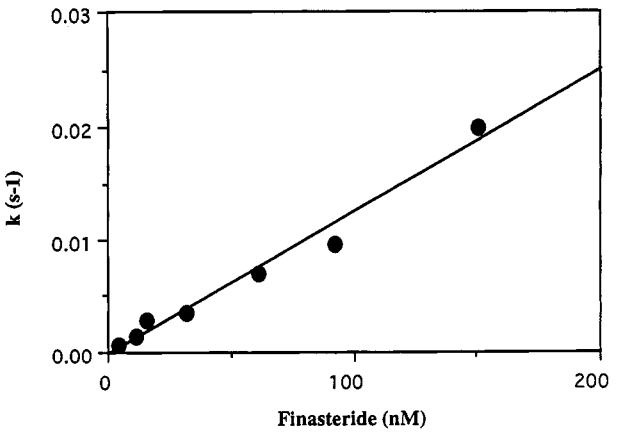

FIGURE 3: Influence of inhibitor concentration on the pseudo-first-order rate constant, k, in Tris–citrate, pH 7.0, at 37 °C. Values of k were obtained from progress curve experiments similar to that presented in Figure 1. For each inhibitor concentration, k was calculated by nonlinear regression analysis to a simple exponential (see eq 2).

significant enzyme degradation occurred during the time of the experiment and that the deviation observed when finasteride was added to the reaction medium was due to the formation of enzyme–inhibitor complexes.

Evidence That $[E_o] \ll [I_o]$ Under the Conditions of the Progress Curve Experiments. In separate experiments microsomes (10 mg/mL) were incubated for 30 min at 37 °C with 1 mM NADPH in the presence or absence of 100 nM finasteride. The residual enzyme activity was then checked as described in Methods and shows that 100 nM finasteride was able to completely inhibit the 5-AR activity contained in the 10 mg/mL microsomal preparation. This indicates that $[E_0] \ll [I_0]$ under the conditions used in the experiment shown in Figure 2 where 10 nM finasteride was reacted with a 0.1 mg/mL microsomal preparation. Therefore, pseudo-first-order conditions are obeyed and the progress curve describing the production of DHT with time is thus accounted for by the following equation (Morrisson, 1982; Morrisson & Walsh, 1988):

$$P = v_s t + \frac{(v_0 - v_s)}{k}(1 - e^{-kt}) \quad (1)$$

where P is the product concentration, v_0 is the initial velocity, v_s is the steady-state velocity, and k is the pseudo-first-order rate constant for the approach to the steady state. All inhibition curves obtained did rise to a plateau, indicating that pseudoirreversible conditions were obeyed ($[I_o] \gg K_i$). Under these conditions eq 1 simplifies to

$$P = \frac{v_0}{k}(1 - e^{-kt}) \quad (2)$$

Values of v_0 and k were determined by nonlinear regression analysis. The bimolecular association rate constant, k_{on}, can then be calculated from k using the following equation:

$$k = \frac{k_{on}[I_0]}{1 + [S_0]/K_m} \quad (3)$$

Figure 2 shows that the experimental data fit well with eq 2, which is in agreement with the postulate $[E_o] \ll [I_o]$. From this experiment, the best fit to the experimental data was obtained for $k = 1.8 \times 10^{-3}$ s^{-1} and $v_o = 7 \times 10^{-4}$ nM s^{-1}. From eq 3, using a K_m value of 20 nM, a k_{on} of 2.7×10^5 M^{-1} s^{-1} was calculated. These experiments were repeated several times using first constant, followed by increasing, inhibitor concentrations (see below). High reproducibility was obtained in determining k_{on} values (standard deviation of 5% within a set of five experiments). This can be attributed to the experimental conditions used (pseudo-first-order), where variation in the enzyme activity from one preparation to another does not affect the measurement of k_{on}.

Effect of Inhibitor Concentration on the Pseudo-First-Order Rate Constant, k. The same method as above was used to investigate the rate of association of 5-AR with finasteride over a broad range of inhibitor concentrations. Figure 3 shows that the first-order rate constant, k, increases linearly with increasing inhibitor concentrations. This linear dependence indicates that the association reaction behaves like a simple bimolecular association process up to 150 nM, the highest inhibitor concentration tested (Quast et al., 1974). The association rate constant, k_{on}, can also be deduced from the slope, m, of this straight line ($k_{on} = [m(1 + [S_0]/K_m)]$. From data presented in Figure 3, a k_{on} of 2.6×10^5 M^{-1} s^{-1} is determined, in agreement with the value determined with 10 nM inhibitor.

Equilibrium Dissociation Constant. In all the experiments where $[I_o] \geq 10$ nM, progress curves plateaued when the equilibrium was attained, indicating that the reaction was completely displaced to the enzyme–inhibitor complex formation. Thus, lower inhibitor concentrations were used to measure the dissociation equilibrium constant. Progress curves were recorded in the presence of 1 nM inhibitor and 10 nM testosterone. Under these conditions the steady state was attained only after 6 h of incubation. The DHT *vs* time curve plateaued, indicating that no significant residual enzyme activity was detected. A control experiment was performed in parallel to check the stability of the enzyme during the time of the experiment. In the presence of T and NADPH, no more than 5% loss of enzyme activity was detected. Furthermore, the progress curve obtained in the presence of the inhibitor fits well with eq 1, and a k_{on} value of 2.4×10^5 M^{-1} s^{-1} (similar to that found under higher finasteride concentration) was calculated, which is consistent with the absence of a significant decrease in the enzyme activity. However, further decrease in the inhibitor concentration was not possible

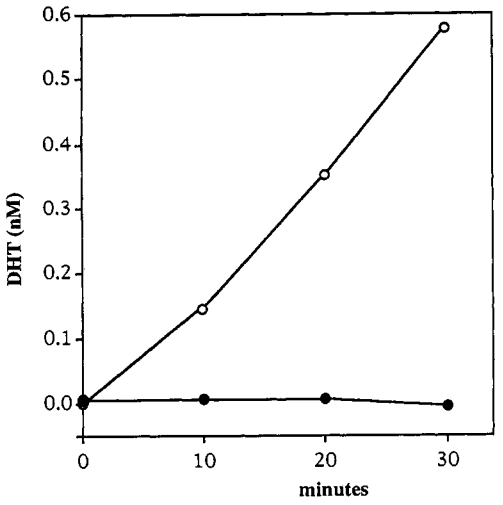

FIGURE 4: Dialysis of the finasteride–human prostatic 5-AR complex. Microsomes (15 mg/mL) ± 15 nM finasteride were incubated in the presence of 1 mM NADPH for 5 h at 37 °C. One hundred microliters of the mixture was then dialyzed against 500 mL buffer for 12 h at 4 °C, and the enzyme activity was measured in each sample. The control experiment without inhibitor is represented by the open circles; and the finasteride-treated enzyme, the closed circles.

because enzyme degradation then became significant during the time of the experiment ($t_{1/2} = 0.7/k_{on}[I_o]$). The low value of k_{on}, together with the relative lability of the enzyme, thus renders precise determination of K_i difficult. The conclusion from this experiment is that $K_i \ll 1$ nM.

A confirmation of the high affinity of finasteride for human prostatic 5-AR was obtained from a dialysis experiment. Enzyme–inhibitor (EI) complexes were preformed by mixing microsomes (15 mg/mL) containing 5-AR activity with finasteride (15 nM final concentration) in the presence of 1 mM NADPH. The mixture was incubated at 37 °C for 5 h to allow the EI complexes to be formed. One hundred microliters of this preformed EI complex mixture was then dialyzed against 500 mL of buffer to induce dissociation of the complexes. The results shown in Figure 4 indicate that no significant dissociation of the preformed EI complex had occurred (i.e., no reactivation of 5-AR was observed), thus confirming the high affinity of the interaction. In parallel, two control experiments were performed using untreated microsomes which were dialyzed under similar conditions in the absence and presence of 50 nM finasteride in the dialysis buffer in order to check (i) the stability of the enzyme and (ii) the ability of the inhibitor to penetrate the dialysis membrane. The enzyme activity was then measured after overnight dialysis. The microsomes which were dialyzed against buffer containing finasteride were totally inactivated, showing that the inhibitor has the capacity to cross the membrane, whereas microsomes dialyzed in the absence of finasteride were still active.

Temperature Dependence of the Association Reaction. As above, the progress curve method was used to follow the enzyme–inhibitor association at different temperatures, from 22 to 37 °C (295–310 K). The Arrhenius plot shown in Figure 5 yielded a straight line, from which an activation energy of 150 kJ mol^{-1} was calculated.

Effect of pH on V_m/K_m and k_{on}. The activity of the enzyme was assessed at different pH values (from 5 to 9) by following the production of DHT with time. In all cases, straight lines were obtained, indicating that variations in the activity with [H$^+$] are not due to enzyme inactivation (data not shown). Two different testosterone concentrations were employed to

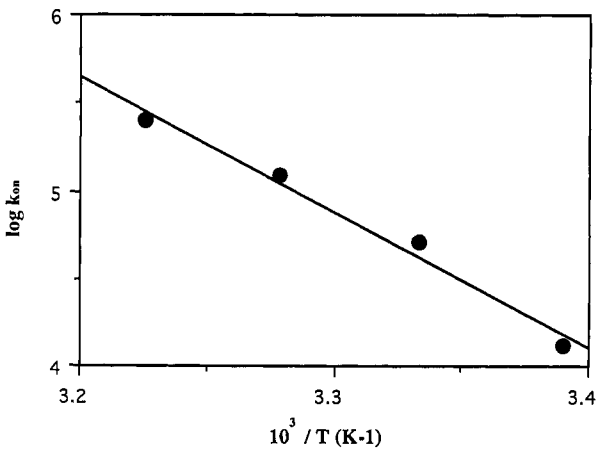

FIGURE 5: Arrhenius plot for the influence of temperature on k_{on} for the inhibition of 5-AR by MK-906 at pH 7.0 (Tris–citrate buffer). k_{on} values were determined at different temperatures, from 295 to 310 K, using the progress curve method. Final concentrations were as follows: enzyme, from 35 μg/mL protein (310 K) to 140 μg/mL protein (295 K); inhibitor, 10 nM; NADPH, 0.4 mM, and [^{3}H]testosterone, 10 nM. The activation energy obtained is 150 kJ mol^{-1}.

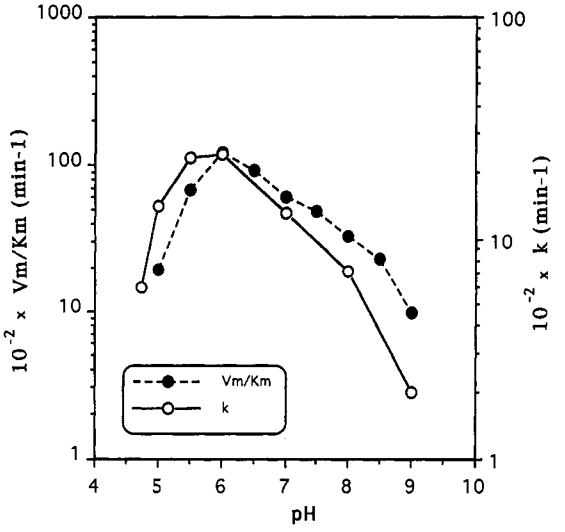

FIGURE 6: pH dependency of k (open circles) and V_m/K_m (closed circles). Both constants were measured at 37 °C at constant ionic strength (μ = 0.1 M) . The nature of the buffer and the details concerning the measurement of V_m/K_m are given in Experimental Procedures. Progress curves used to measure k were generated by adding the enzyme (100 μg/mL protein final concentration) to a mixture containing NADPH (0.8 mM), [^{3}H]testosterone (10 nM), and MK-906 (15 nM).

measure the enzyme activity (i.e., 5 and 10 nM). In all cases, the velocity measured at 5 nM was about half of that measured at 10 nM substrate. Thus, at all pH values investigated, the inequality $[S_0] < K_m$ applies and the enzyme activities measured under these conditions could therefore be attributed to V_m/K_m ratios. Inspection of the data presented in Figure 6 shows that V_m/K_m exhibits a maximum at pH 6.0. The activity decreases in both the acid and the alkaline direction. Residual activities are 16 and 8% at pH 5.0 and 9.0, respectively. The pH profile obtained is not symmetric, suggesting that more than 2 pK_a's are involved in the catalytic process.

The pH dependence of the association rate constant, k_{on}, was measured with constant inhibitor (15 nM) and testosterone (10 nM) concentrations over the pH range studied. Since the inequality $[S_o] < K_m$ applies for the whole pH range investigated, measurement of the pseudo-first-order rate constant, k, is directly linked to the variation of k_{on}(see eq 3). The k_{on}

value determined at pH 7.0 from this set of experiments was 2.4×10^5 M^{-1} s^{-1}, in good agreement with that found at pH 7.0 in the Tris–citrate buffer (2.7×10^5 M^{-1} s^{-1}).

DISCUSSION

The results presented in this paper demonstrate that the kinetics of interaction between 5-AR and finasteride is characterized by an association rate constant, k_{on}, several orders of magnitude lower than expected for a diffusion-controlled reaction. This finding has major consequences. Firstly, for the determination of the true equilibrium dissociation constant, K_i, the residual enzyme activity cannot be evaluated by a single time-point measurement. The affinity of the enzyme–inhibitor interaction can be significantly underestimated if the residual enzyme activity is measured before the equilibrium between E and I has been attained. The delay time (dt) for such an equilibrium to be reached is a function of the two microscopic constants k_{on} and k_{off} since $dt = 5/(k_{on}[I_0] + k_{off})$ under pseudo-first-order conditions. Secondly, this finding has consequences for evaluation of the *in vitro* potency of inhibitors since a lot of molecules designed to inhibit the conversion of T to DHT are transition-state analogs and are thus likely to act in a manner similar to finasteride (time-dependent inhibition of the enzyme). In the case of finasteride, the determination of the true K_i value (if there is one) is difficult due to the very low dissociation rate constant ($k_{off} = k_{on}K_i \ll 10^{-4}$ s^{-1}) and the lability of the enzyme. Our conclusion that k_{off} should be very low came from (i) the irreversible behavior of the progress curve recorded with 1 nM inhibitor and (ii) the absence of dissociation of the EI complex during an overnight dialysis of the complex. Human prostatic 5-AR is characterized by a low K_m for testosterone. Values from 10 to 30 nM (at pH 7.0) were reported by Bruchovsky et al. (1988), while Habib et al. (1987) measured a K_m of 33 nM at pH 7.40. In the present work we determined a K_m for testosterone of 20 nM, in agreement with the above studies. In the case of an inhibitor competitive to testosterone it is likely that a molecule can play a physiologic role within reasonable drug concentrations only if $K_m/K_i \geq 10$ (pseudoirreversible conditions). Our results indicate that the K_i for finasteride is much lower than 1 nM, a value at least 1 order of magnitude lower than the K_m for testosterone. These results are in line with the *in vivo* effect reported for finasteride in man (Geller, 1990; McConnell et al., 1992).

The pH dependence of V_m/K_m exhibits a maximum (2×10^{-4} s^{-1}) at pH 6.0 with enzyme activity decreasing in both the acidic and the alkaline direction. Inspection of Figure 6 reveals a substantial amount of enzyme activity still present at neutral pH (80% of the activity at pH 6.0). Although this conflicts with previous studies (Liang et al., 1985; Andersson et al., 1991), it confirms other reports of activity at neutral pH (Houston et al., 1985; Hudson, 1987; Sargent & Habib, 1991). In addition, residual enzyme activity at neutral pH was recently reported by Harris et al. (1992). A possible explanation for these discrepancies in the pH profiles could be related to the method used to prepare the microsomes. To summarize the literature data, 5-AR type 2 (the prostatic enzyme) is characterized by its low K_m for testosterone and a high sensitivity to finasteride as reported by Andersson et al. (1991). A K_m for testosterone of 33 nM (close to our 20 nM) measured at neutral pH was also reported in the literature (Houston et al., 1987). Our decision to perform the kinetic experiments at pH 7.0 was based on (i) the presence of a relatively high residual activity at neutral pH in our procedure and (ii) the reported inactivation of the enzyme at acidic pH (Hudson, 1987).

The pH profile shown in Figure 6 is not symmetric, suggesting that more than two ionizable residues are involved in the catalytic process which converts T to DHT. The conversion of T to DHT by 5-AR is thought to proceed through the formation of an enolate intermediate (Blohm et al., 1980; Levy et al., 1990a) whose second-order association rate constant is given by k_{cat}/K_m. It was therefore of interest to compare pH profiles of V_m/K_m and k, the pseudo-first-order rate constant for the association reaction. Figure 6 shows that the variation of these two parameters with pH is very similar, suggesting that the interaction between 5-AR and finasteride involves the same ionizable groups as with testosterone.

A time-dependent inhibition of rat liver 5-AR by 4-MA, another 3-oxo-4-aza steroid, has already been reported by Liang et al. (1983a). The interaction with 4-MA was characterized by a k_{on} of 4×10^5 M^{-1} s^{-1} and a k_{off} of 2.7×10^{-3} s^{-1}. These values are higher than the ones reported here for human prostatic 5-AR–finasteride interaction. Had the 4-MA study been performed at 37 °C instead of at 0 °C, the difference would have been even greater (see Figure 5 for temperature dependence of k_{on}). However, one has to be cautious when comparing liver 5-AR to prostatic 5-AR. Normington and Russell (1992) recently reported the presence of two 5-AR isoenzymes in the rat. From mRNA quantification, it appears that 5-AR1 is the major form present in the liver, while type 2 mRNA dominates in the prostate. Therefore, liver enzyme cannot be directly compared with the prostatic species. Isoenzymes 5-AR1 and 5-AR2 differ with regard to their K_m for testosterone and their sensitivity to finasteride. We recently found that both type 1 and type 2 are present as active enzymes in human prostatic tissue (Faller et al., 1993). This was substantiated by the detection of two successive steady states in the activity *vs* testosterone curve. The activity due to 5-AR1 becomes measurable only at testosterone concentrations higher than 1 μM at neutral pH. In the present work, a testosterone concentration of 10 nM was employed, which is far too low to activate 5-AR1. Therefore, the 5-AR activity documented in this study relates only to 5-AR2.

Steroidal acrylates, another class of 5-AR inhibitors known to inhibit 5-AR in an noncompetitive way to both testosterone and NADPH, are characterized by higher k_{off} values since they are able to entirely dissociate from rat 5-AR–$NADP^+$ complex within the time of a gel filtration experiment (Levy et al., 1990b). Here again, it has to be emphasized that this higher k_{off} value was obtained with liver enzyme, presumably 5-AR1. The relatively low k_{on} value that we obtained with human prostatic 5-AR (2.7×10^5 $M^{-1}s^{-1}$) suggests that the combination of E and I to form EI cannot be described by a simple bimolecular association process, but most likely involves intermediate steps as depicted in eq 4 below. The hypothesis for the presence of such an intermediate is strengthened by the high value of the energy of activation measured for the association process (150 $kJ.mol^{-1}$), a value much higher than expected for a diffusion step (Benson, 1960). However, no accumulation of any intermediate EI* complex was detected kinetically since the variation of the pseudo-first-order constant k with $[I_0]$ remained linear up to the highest concentration tested (150 nM). This means that the association process still behaves as a single step reaction up to the above inhibitor concentration. Equation 4 summarizes how the combina-

Table I

	K_i^* (nM)	k_2 (s^{-1})	k_2/K_i^* (M^{-1} s^{-1})
MK-906[a]	>150	$>4 \times 10^{-2}$	2.7×10^5
RMI 18341[b]	35	7×10^{-4}	2×10^4
AMPD[c]	1250	4.8×10^{-3}	3.8×10^3

[a] This work. [b] RMI 18341: (5α-20-*R*)-4-diazo-21-hydroxy-20-methylpregnan-3-one. Data from Blohm et al. (1980). [c] AMPD: 17-acetoxy-6-methylenepregn-4-ene-3,20-dione. Data from Petrow et al. (1981).

tion of human prostatic 5-AR with finasteride is hypothesized to proceed:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI \qquad (4)$$

with $K_i^* = k_{-1}/k_1 > 1.5 \times 10^{-7}$ M, $k_2 > 0.04$ s^{-1}, $k_{-2} < 10^{-4}$ s^{-1}, and $k_{on} = k_2/K_i^* = 2.7 \times 10^5$ M^{-1} s^{-1}.

In the literature, at least two compounds have been described as irreversible inhibitors of rat prostatic (5-AR2) enzyme. In the first study, a time-dependent inhibition of 5-AR by RMI 18341, a compound belonging to the 3-keto-4-diazo-5-α-dihydro steroid class, was reported by Blohm et al. (1980). The second study (Petrow et al., 1981) describes the inhibition of rat prostatic 5-AR by AMPD, a 6-methylenic steroid derivative. In both cases, the presence of an EI* intermediate was detected kinetically in the association pathway. A comparison with the kinetics of interaction between 5-AR and finasteride is presented in Table I.

In this work we demonstrate that the inhibition of 5-AR by finasteride is governed by an association rate constant, k_{on}, of 2.7×10^5 M^{-1} s^{-1} and by a very low dissociation rate constant, k_{off} ($<10^{-4}$ s^{-1}). To our knowledge such a low k_{on} has never been reported in any previous kinetic investigations. However, it has to be emphasized that most of the reported K_i values for finasteride were measured at pH values close to 5. In the present work progress curves for the inhibition of 5-AR by finasteride were recorded over the pH range from 5.0 to 9.0 to measure the pH dependence of k_{on} (Figure 6). All of these curves reached a plateau, indicating that no residual 5-AR activity was present at the steady state. Therefore, it is unlikely that the pseudoirreversible behavior of 5-AR–finasteride that we further investigated at neutral pH is strongly affected by pH. Consequently, all K_i values published (3–26 nM) underestimate the affinity of finasteride for 5-AR by at least 1 order of magnitude. We were unable to precisely measure the dissociation rate constant, k_{off}, for this enzyme–inhibitor pair due to experimental restrictions or to determine whether a covalent bond is formed in the final EI complex. Compounds which belong to the 3-keto-4-diazo-5-α-dihydro steroid family (Blohm et al., 1980) and at least one of the 6-methylenic steroid derivatives (Petrow et al., 1981) are classified as putative irreversible inhibitors of prostatic 5-AR. Taking into account the data presented in this work, the question of the reversibility in the interaction of finasteride with 5-AR from human prostate thus remains open and further investigations are needed.

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