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Mechanism-Based Inhibition of Human Steroid 5 α -Reductase by Finasteride: Enzyme-Catalyzed Formation of NADP–Dihydrofinasteride, a Potent Bisubstrate Analog Inhibitor

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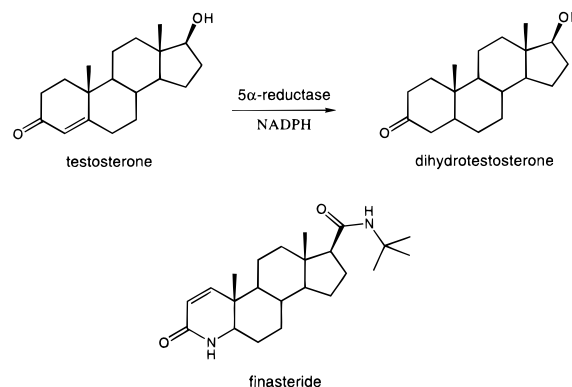
Received September 6, 1995[⊗]

Abstract: Finasteride is employed in treatment of benign prostatic hyperplasia in man, where its target enzyme is steroid 5 α -reductase. It is a novel, potent mechanism-based inhibitor of the human prostate (type 2) isozyme. Although it is accepted as an alternate substrate and is ultimately reduced to dihydrofinasteride, this proceeds through an enzyme-bound NADP–dihydrofinasteride adduct. Finasteride is processed with a second-order rate constant, $k_i/K_i = 1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, that approaches k_{cat}/K_m for reduction of testosterone, $3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, and essentially every catalytic event is lethal (partition ratio ≤ 1.07). The membrane-bound enzyme–inhibitor complex formed from [³H]finasteride appears to release [³H]dihydrofinasteride with a half-life of 1 month at 37 °C ($k = (2.57 \pm 0.03) \times 10^{-7} \text{ s}^{-1}$), as identified by mass spectroscopy. The intermediate NADP–dihydrofinasteride adduct can be recovered intact by denaturation of the enzyme–inhibitor complex and has been purified. Free in solution, it likewise decomposes to dihydrofinasteride (half-life = 11 days). An extremely potent bisubstrate analog inhibitor, this NADP–dihydrofinasteride adduct binds to the free enzyme with a second-order rate constant equal to k_{cat}/K_m for turnover of testosterone and has a dissociation constant $K_i \leq 1 \times 10^{-13} \text{ M}$. Finasteride is also a mechanism-based inhibitor of the human skin (type 1) isozyme, but it is processed with a much smaller second-order rate constant, $k_i/K_i = 3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, which attenuates its activity against this isozyme in vivo. The mechanism explains the exceptional potency and specificity of finasteride in treatment of benign prostatic hyperplasia, and the concept may have application to other pyridine nucleotide-linked enzymes.

Introduction

The enzyme steroid 5 α -reductase (NADPH– Δ^4 -3-oxosteroid-5 α -oxidoreductase (EC 1.3.99.5)) is an NADPH-dependent enzyme that catalyzes the reduction of testosterone to the more potent androgen dihydrotestosterone, as shown below. Finasteride (17 β -(*N*-*tert*-butylcarbamoyl)-4-aza-5 α -androst-1-en-3-one (MK-906)) is a potent inhibitor of the human prostate enzyme and, under the trademark Proscar, is employed in treatment of benign prostatic hyperplasia,¹ a condition afflicting to some degree the majority of men over age 55.

The medical rationale for development of 5 α -reductase inhibitors derives principally from identification of a genetic deficiency in this enzyme in a group of male pseudohermaphrodites in the Dominican Republic.² Afflicted males are born with ambiguous external genitalia and are commonly raised as girls until, at puberty, increased testosterone levels cause development of the typical male phenotype. After puberty, these men lead normal lives and display three characteristic traits: they do not develop enlarged prostates, do not have acne, and do not acquire male pattern baldness. This finding secured early work of others implicating dihydrotestosterone rather than testosterone in these endocrine disorders and advanced the



proposal that 5 α -reductase was an attractive target for medical intervention.³

More recently, it has been established that this genetic deficiency is attributable to loss of only one isozyme (designated type 2), which predominates in the prostate; the other isozyme (designated type 1) contributes one-third of circulating levels of dihydrotestosterone and, paradoxically, is the predominate isozyme in facial skin, where its role in androgen disorders remains obscure.^{4–6}

In clinical trials, the potency of finasteride in man^{1,7} far exceeded expectations based on its perceived affinity for the

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human prostate isozyme, for which it was first thought to be a simple, rapidly-reversible inhibitor with $K_i = 26$ nM.⁸ Closer evaluation led to appreciation that finasteride and certain analogs are slow-binding, extremely high affinity inhibitors of the human enzymes.⁶ From a detailed analysis of its time-dependent inhibition, one group concluded that it is a functionally irreversible inhibitor of the native human prostate (type 2) isozyme with $K_i \ll 1$ nM.⁹ Another group reached the same conclusion for the human skin (type 1) isozyme¹⁰ and proposed that finasteride binds covalently to the enzyme as a Michael acceptor to explain the apparent irreversible inhibition. Recently, they have presented large secondary kinetic tritium isotope effects at the double bond in finasteride as evidence for this mechanism,¹¹ despite the lack of adequate chemical evidence.

We would like to propose a mechanism more intimately related to catalysis, in which finasteride is a novel mechanism-based inhibitor. Although it is accepted as an alternate substrate and is ultimately reduced to dihydrofinasteride, this proceeds through an enzyme-bound NADP–dihydrofinasteride adduct. This adduct, a bisubstrate analog inhibitor, ranks among the most potent noncovalently-bound enzyme inhibitors known for any enzyme.

Results

Enzyme Source. The native isozymes are membrane-bound, are intractable to purification,¹² and are rare, constituting less than 0.001% the total protein in human prostate or skin tissue. Consequently, membrane-bound preparations of the recombinant human isozymes obtained from a baculovirus-expression system,¹³ which enriched the enzyme to 0.1% the total protein, were employed in this research. The K_m and K_i values of the recombinant enzymes for substrates and inhibitors appear to be essentially identical to those of the native isozymes.

The formal kinetic mechanism of 5 α -reductase is ordered bi bi,¹⁴ the cofactor adding first and leaving last, as is common among pyridine nucleotide-dependent enzymes. For the recombinant type 2 isozyme (at standard conditions of pH 7.20 and 37 °C), the catalytic constants are $K_m = 24.6 \pm 0.7$ nM for testosterone and $K_m = \sim 1$ μ M for NADPH and the turnover number $k_{cat} = 0.075 \pm 0.006$ s⁻¹. From these kinetic constants, the second-order rate constant for reduction of testosterone is $k_{cat}/K_m = (3.0 \pm 0.3) \times 10^6$ M⁻¹ s⁻¹. Enzyme concentrations were determined by titration of enzyme activity with finasteride or, equivalently, from the amount of [¹⁴C]finasteride that remained protein-bound after dialysis.

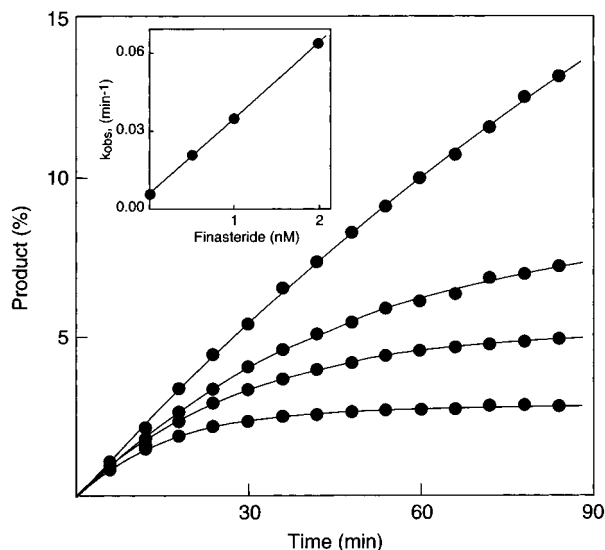
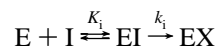


Figure 1. Slow-binding inhibition by finasteride. Plots show time courses for inhibition of recombinant human prostate isozyme by finasteride. Reactions contained 20 pM membrane-bound enzyme, $1 \times K_m$ of [7-³H]testosterone, and $100 \times K_m$ of NADPH in 0.1 M MOPS, 0.1% BSA, and 1 mM EDTA at pH 7.20 and 37 °C. Finasteride concentrations were 0, 0.5, 1, and 2 nM, in order of increasing inhibition. The solid lines are theoretical and were obtained by nonlinear regression to eqs 1 and 2. As shown in the inset, the first-order rate constants for these time courses are nearly a linear function of inhibitor concentration and correspond to a second-order rate constant $k_i/K_i = (0.957 \pm 0.008) \times 10^6$ M⁻¹ s⁻¹ and initial binding constant $K_i > 100$ nM.

Rate Constant for Association. The slow-binding inhibition data fit the standard model for mechanism-based inhibition:¹⁵



Progress curves defining slow-binding inhibition of the recombinant human prostate (type 2) isozyme are shown in Figure 1. The potency of finasteride is evident in the nearly complete suppression of the activity seen at even the lowest practical inhibitor concentrations at infinite time. The solid lines are theoretical and correspond to a second-order rate constant $k_{on} = k_i/K_i = 1 \times 10^6$ M⁻¹ s⁻¹ (pH 7.20 and 37 °C). There is no evidence of saturation over this range of inhibitor concentrations, which implies that the initial Michaelis complex has $K_i > 100$ nM. These conclusions concur with those reported earlier for the native enzyme from human prostate tissue.⁹

Despite the apparent potency, enzyme denaturation experiments indicated that finasteride was noncovalently bound to the enzyme. For instance, solution of the membrane-bound enzyme–[³H]finasteride complex in 7 M guanidine hydrochloride resulted in release of >98% of the label, as identified by ultrafiltration. Consequently, the rate constant for spontaneous dissociation was determined in order to establish K_i for this complex.

Rate Constant for Dissociation. The rate constant for dissociation was obtained from the rate of isotope exchange out of the membrane-bound enzyme–[³H]finasteride complex. The time course for appearance of free radioactivity over a period of several weeks is shown in Figure 2. The solid line is theoretical for a first-order rate constant $k_{off} = (2.57 \pm 0.03) \times 10^{-7}$ s⁻¹ (pH 7.20 and 37 °C), which is equivalent to a half-life of 31 days for the enzyme–inhibitor complex. In contrast to these long lifetimes, free enzyme in these crude membrane preparations at 37 °C is quite labile, having a lifetime of minutes

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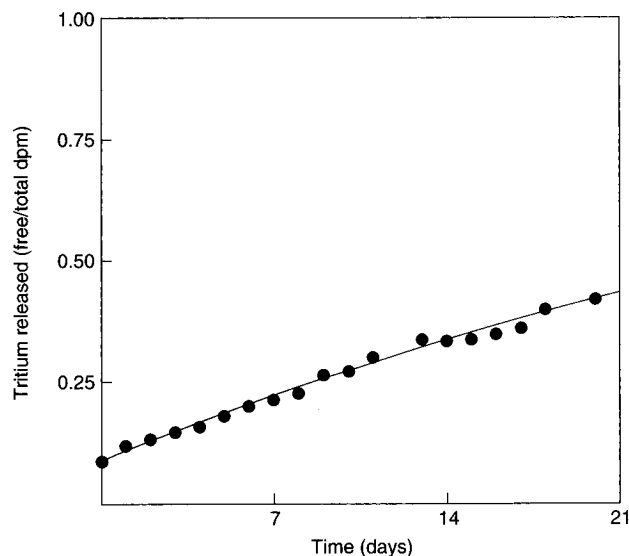


Figure 2. Rate constant for dissociation of enzyme-inhibitor complex. Plot shows the slow release of radioactivity from the enzyme- ^3H -finasteride complex at pH 7.20 and 37 °C. The enzyme-inhibitor complex was formed from ~ 20 nM membrane-bound enzyme and 10 nM ^3H -finasteride and $500 \times K_m$ of NADPH, and after several hours a 100-fold excess of unlabeled finasteride was introduced to initiate isotope exchange. The solution also contained 0.1 M MOPS and 0.3 M sucrose, and 10 mM EDTA was included to block bacterial growth. Free radioactivity was determined by ultrafiltration and has been corrected for 16% nonspecific binding. Assuming the process is first-order and proceeds to completion (since $>96\%$ release is observed on heat denaturation), the solid line is theoretical for a rate constant $k_{\text{off}} = (2.57 \pm 0.03) \times 10^{-7} \text{ s}^{-1}$, equivalent to a half-life of 31.2 ± 0.4 days.

in the absence of cofactor to hours in its presence. Thus, the true rate constant for dissociation from catalytically competent enzyme is almost certainly even smaller than indicated by these measurements.

These rate constants for formation and dissociation of the enzyme-inhibitor complex imply that the (steady-state) dissociation constant of finasteride is $K_i^* = k_{\text{off}}/k_{\text{on}} \leq 3 \times 10^{-13}$ M.

Conversion to Dihydrofinasteride. The first clue to the mechanism came when it was found that heat denaturation of enzyme-inhibitor complex formed from ^3H -finasteride appeared to release the reduction product ^3H -1,2-dihydrofinasteride. This assignment was tentatively made by co-chromatography with authentic material on a reverse phase column, as shown in Figure 3.

Identification was confirmed by mass spectroscopy. The radioactive component released by heat denaturation of the enzyme-inhibitor complex was purified on a nanomole scale in relatively low yield (37% recovery). The positive ion mass spectrum indicated a parent ion mass of 375 Da, consistent with protonated dihydrofinasteride. As shown in Figure 4, comparison of its daughter ion fragmentation pattern to that of authentic dihydrofinasteride provided incontrovertible evidence for the assignment.

Returning to the isotope exchange experiment above, the free radiolabel was likewise found to be ^3H -dihydrofinasteride rather than ^3H -finasteride. The absence of ^3H -finasteride in these solutions, despite the potential for back-exchange, indicates that formation of the enzyme-inhibitor complex is irreversible. Although this means the determination of k_{off} lacked the intended driving force (isotope exchange), this probably did not influence its outcome. In support of this contention, continuous dialysis

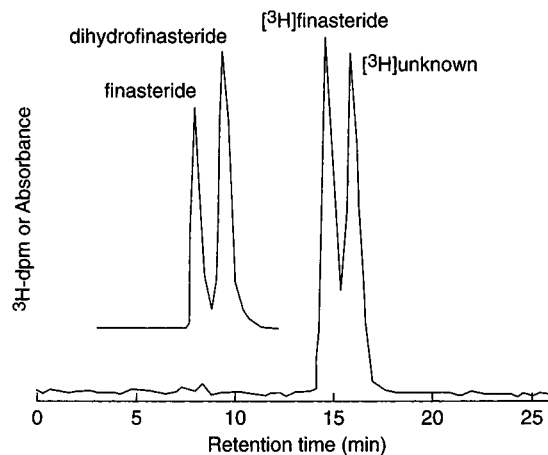


Figure 3. Chromatographic identification of dihydrofinasteride. Enzyme- ^3H -finasteride complex (1 pmol, 25 000 dpm) was denatured in a boiling water bath at neutral pH for 20 min, combined with 0.5 equiv of fresh ^3H -finasteride and chromatographed on a reverse-phase column (Vydac C-18, 4.6×250 mm, 300 Å, 5 μm , flow rate 1 mL/min) in 60% aqueous methanol containing 0.1% trifluoroacetic acid. This indicated that the ^3H -labeled compound released from the enzyme-inhibitor complex was distinct from ^3H -finasteride. Comparison to retention times for a mixture of unlabeled finasteride (3 nmol) and authentic dihydrofinasteride (10 nmol), as monitored at A_{210} and shown offset -7 min, tentatively identified the unknown as dihydrofinasteride.

of the enzyme-inhibitor complex against infinite dilution gave comparable results, indicating a half-life of ~ 1 year for the enzyme-inhibitor complex at ambient temperature.

Covalent Intermediate. At this point it was certain that finasteride was being processed as a substrate, but this did not offer a compelling explanation for the potent inhibition. Among the inconsistencies, the product dihydrofinasteride was found to be a simple, reversible inhibitor with $K_i = \sim 1$ nM and gave no evidence of slow-binding inhibition or of potentiation by the second product, NADP^+ . Moreover, recoveries of ^3H -dihydrofinasteride were routinely low (30%-45%) from heat-denatured enzyme-inhibitor complex, even though ultrafiltration indicated that all of the radiolabel (96%) was released from the enzyme. Most disconcerting, although denaturation of the enzyme- ^3H -finasteride complex in guanidine hydrochloride released $>98\%$ of the radiolabel to ultrafiltration (as mentioned), it was found that $<5\%$ would partition into chloroform, a polarity completely inconsistent with ^3H -dihydrofinasteride. These considerations implied the existence of a more potent, noncovalently-bound inhibitor that decomposes readily to dihydrofinasteride.

The inhibitor was proposed to be a covalent adduct formed by attack of dihydrofinasteride enolate (the immediate product of hydride transfer) on NADP^+ and to have the structure shown in Figure 5. Chemical precedent for this proposal comes from analogous high-affinity, abortive complexes that are formed spontaneously (in the reverse direction) by several pyridine nucleotide-linked dehydrogenases, notably the NAD-pyruvate adduct formed by lactate dehydrogenase.¹⁶ Adopting conditions more commensurate with NADPH chemistry, as described in detail in the Experimental Section, this intermediate was subsequently isolated by denaturation of the enzyme- ^{14}C -finasteride complex in ethanol and purified by ion exchange chromatography in 62% overall recovery.

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A. Dihydrofinasteride

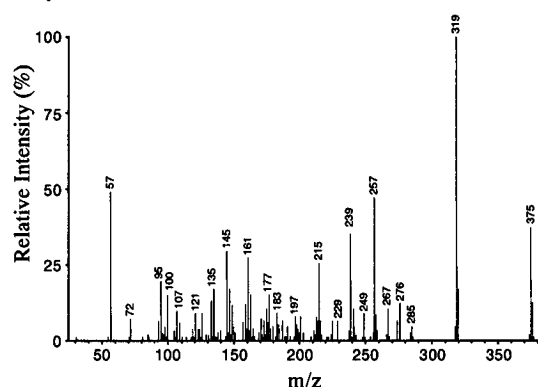
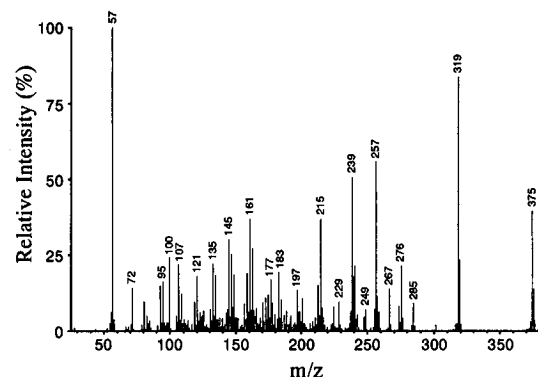
B. ^3H -Inhibitor

Figure 4. Mass spectral identification of dihydrofinasteride. Dialyzed membrane-bound enzyme— ^3H finasteride complex (containing 16 nmol of inhibitor in 140 mg of protein) was heat-denatured in a boiling water bath (20 min), and the released radioactive component was purified by centrifugation (90% recovery), ultrafiltration (84% recovery), and reverse-phase high-pressure liquid chromatography (37% recovery). The positive-ion mass spectrum of the radioactive component indicated a dominant parent ion at 375 Da, as predicted for protonated dihydrofinasteride. This ion gave a fragmentation pattern (B) that was identical to that of authentic dihydrofinasteride (A), securing the assignment.

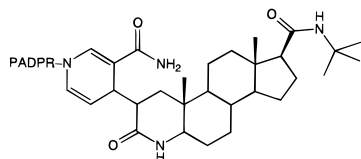


Figure 5. Tentative structure of the NADP-dihydrofinasteride adduct. PADPR = phosphoadenosine diphosphoribose.

Several lines of evidence point to the structure proposed in Figure 5: (1) The radioactive product is anionic and cochromatographs with NADPH absorbance at 340 nm. (2) As shown in Figure 6, the product has an absorbance spectrum consistent with known NAD adducts. The λ_{max} and extinction coefficient at 326 nm are virtually the same as described for the NAD-cyanide adduct¹⁷ and are similar to those for NAD-pyruvate and related adducts, which have $\lambda_{\text{max}} = 340\text{--}347\text{ nm}$.¹⁶ The fluorescence spectrum (not shown) also resembles that of NADPH and known NAD-adducts, with an emission maximum at 441 nm. (The reason that λ_{max} coincides with that for the more polar CN^- adduct is unclear; taken alone it could indicate attack of nitrogen or oxygen rather than carbon, but this is inconsistent with the other evidence at hand.) (3) The compound is unstable in solution and, among other reactions, decomposes to [^{14}C]dihydrofinasteride in 75% yield with a half-

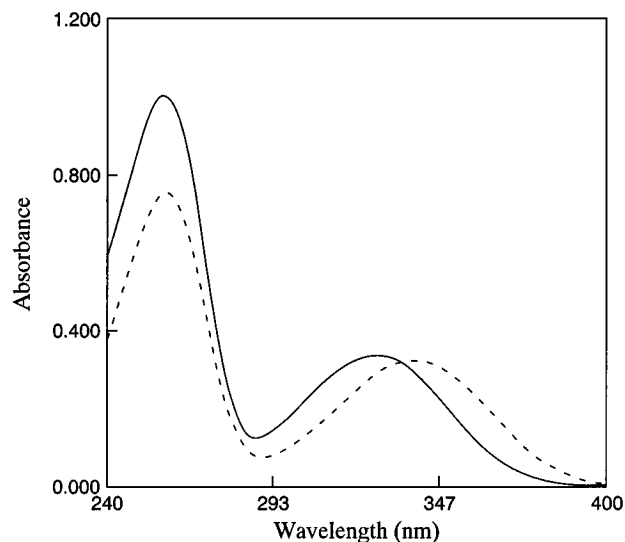


Figure 6. Comparison of absorbance spectra of the NADP-dihydrofinasteride adduct (solid line, 58 μM) and NADPH (broken line, 50 μM). The adduct had been purified as described in the Experimental Section and was subsequently chromatographed on a polymethacrylate reverse phase column (Waters, DE-613) to remove contaminating absorbance at 260 nm (84% recovery). Spectra were recorded in 25% methanol/75% 0.1 M ammonium bicarbonate at pH 9. The extinction coefficient of the adduct is $5.83 \times 10^3\text{ M}^{-1}\text{ cm}^{-1}$ at $\lambda_{\text{max}} = 326\text{ nm}$ based on ^{14}C content. Residual impurities may still contribute to A_{260} .

life of 11 days under the standard conditions at 37 $^{\circ}\text{C}$. This nonenzymatic decomposition explains the recovery of [^3H]dihydrofinasteride in the earlier experiments, and appears to be consistent with chemical properties described for acetyl-NAD-pyruvate¹⁸ (an analog that also is not susceptible to further rapid cyclization and oxidation). (4) As anticipated, decomposition to [^{14}C]dihydrofinasteride was abolished by treatment with phenazine methosulfate, a selective oxidant of the dihydropyridine ring.¹⁶ And (5), securing the identification, the most prominent parent ion peak in its negative-ion mass spectrum (identified as the dianion at 558 Da) had the predicted mass/charge ratio.

Inhibition by Free Intermediate. The kinetic pattern for inhibition by the purified intermediate also supports its assignment as NADP-dihydrofinasteride. Like finasteride, this compound is also a slow-binding inhibitor and produces progress curves identical in nature to those shown for finasteride (Figure 1), but the rate of binding is now competitive with the first substrate (NADPH) and noncompetitive with the second (testosterone). This pattern is consistent with binding of a bisubstrate analog inhibitor in an ordered bi bi mechanism. By extrapolation to zero pyridine-nucleotide cofactor concentration, as $k_{\text{on}} = k_{\text{obsd}}(1 + [\text{NADPH}/K_m])$, the rate constant for binding of the adduct to free enzyme is $\sim 1 \times 10^6\text{ M}^{-1}\text{ s}^{-1}$ at 37 $^{\circ}\text{C}$. Confirming this model, direct determination in the absence of both substrates gave $k_{\text{on}} = (3 \pm 1) \times 10^6\text{ M}^{-1}\text{ s}^{-1}$. The ratio of this rate constant to that for spontaneous dissociation of the enzyme-inhibitor complex (ultimately to dihydrofinasteride) indicates the dissociation constant of the adduct is $K_i \leq 1 \times 10^{-13}\text{ M}$.

Partition Ratio. A characteristic of mechanism-based inhibitors¹⁹ is that, once activated by an enzyme, they may partition between two fates: dissociation away to free solution and lethal inactivation of the enzyme. This partition ratio (total

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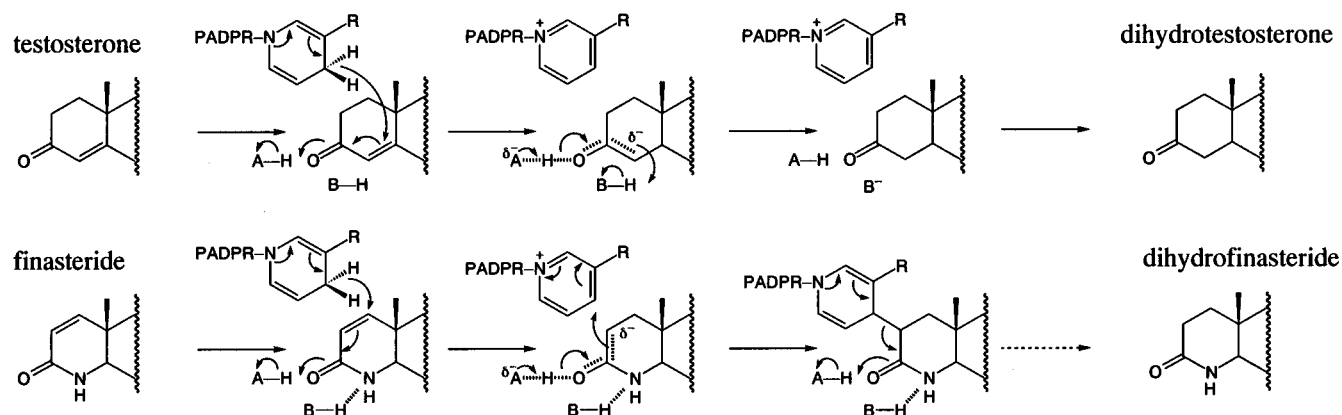


Figure 7. Proposed mechanism of inhibition. The scheme illustrates how reduction of testosterone and mechanism-based inhibition by finasteride proceed through closely related enol(ate) intermediates, but then diverge because finasteride reroutes the carbanion center to a position where it escapes the proton transfer that normally completes the reduction. As indicated by the broken arrow, the decomposition of the adduct to produce dihydrofinasteride could be either enzyme-catalyzed or nonenzymatic. PADPR = phosphoadenosine diphosphoribose.

turnover/lethal events) for finasteride is ≤ 1.07 against the prostate isozyme, as seen in the small percentage of radiolabel that appears to be free after formation of enzyme–inhibitor complex has reached completion in the presence of excess enzyme (y intercept in Figure 2). Finasteride is an unusually efficient mechanism-based inhibitor by this criterion.

Inhibition of the Type 1 Isozyme. Parallel experiments with the recombinant human type 1 isozyme, found in scalp and facial skin, indicate that finasteride is also a mechanism-based inhibitor of this form of 5 α -reductase. The only significant difference is that the adduct is formed with a smaller rate constant, $k_i/K_i = 3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, which is $<1\%$ the value for the type 2 isozyme. This can be attributed to slower processing of finasteride, rather than inefficient capture of the enolate intermediate, since more than half the catalytic events remain lethal (partition ratio = 1.55 ± 0.05).

This rate constant contributes to making finasteride a more effective inhibitor of the type 2 than the type 1 isozyme *in vivo*, since it dictates the concentration of inhibitor required to influence steady-state pools of each isozyme. This expectation agrees qualitatively with clinical results in treatment of benign prostatic hyperplasia, where plasma concentrations of dihydrotestosterone tend to plateau ($\sim 70\%$ decrease) at levels that mimic genetic deficiency in the type 2 isozyme.¹

After its formation, the properties of the enzyme–inhibitor complex are virtually identical to those described for the type 2 isozyme. This includes a comparable rate constant for release of radiolabel, equivalent to a half-life of 14 ± 2 days at 37°C , the concomitant production of free [^3H]dihydrofinasteride in solutions of this complex, and the quantitative release of the radiolabel as the NADP-[^3H]dihydrofinasteride adduct upon denaturation of the enzyme in guanidine hydrochloride.

Discussion

Mechanism. As shown in the mechanism proposed in Figure 7, reduction of testosterone and mechanism-based inhibition by finasteride follow parallel reaction coordinates that proceed through closely related enol(ate) intermediates. The two reactions diverge in the final step, where finasteride aborts the process by translocating the enolate/carbanion center to a position where it escapes the proton transfer that normally completes the reduction.

Acceptance of finasteride as an alternate substrate is more plausible than may be evident in the drawings. The π -orbitals of the double bonds in testosterone and finasteride share a 1,3-

axial orientation in the partial chair conformation of the steroid A-ring, which places them relatively close to one another in space. Assuming the nicotinamide ring lies in a parallel plane beneath the steroid, then modest displacements in this plane would accommodate the various enzyme–reactant complexes required for all these reactions. Moreover, an equally important facet of catalysis must be stabilization of the enol(ate) intermediate, which is almost identical for testosterone and finasteride.

The proton transfer to complete the reduction presumably controls partitioning between simple turnover and lethal addition to the cofactor. Translocation of the carbanion center alone may not be sufficient to prevent this transfer. Recent research with 4-X-analogs of finasteride (not presented above) indicates a heteroatom in this position is also essential to evade proton transfer, perhaps because it ties up the proton donor by hydrogen bonding. Thus, 4-O-analogs of finasteride are also potent, time-dependent inhibitors, but 4-CH₂-analogs are not and, instead, are turned over as alternate substrates without inactivating the enzyme.

Related NAD Adducts. Inhibition by finasteride is analogous in certain respects to the formation of abortive NAD adducts by more common dehydrogenases that traffic in alcohol/ketone redox reactions.²⁰ These adducts are formed when the enzymes mistakenly bind the enol tautomer of their keto substrates to the NAD⁺ form of the enzyme.¹⁶ Lactate dehydrogenase, the preeminent example, achieves a rate enhancement of 10^{11} -fold over the nonenzymatic rate constant for formation of the NAD–pyruvate adduct,¹⁸ which it binds with $K_i = 5 \times 10^{-11} \text{ M}$. Yet such adducts are not thought to be biologically important, since their formation is attenuated by the small mole fraction of enol tautomer and an unfavorable equilibrium constant for addition to NAD⁺ in solution. In the case of 5 α -reductase, this reaction is equivalent to forming the central enolate complexes in Figure 7 by binding the (quite rare) enolates of dihydrotestosterone or dihydrofinasteride in the reverse direction and is undetectable. Finasteride circumvents these thermodynamic constraints: approaching the intermediate from the forward direction, it exploits the free energy available from reduction of the vinyl group to transiently convert every molecule to this reactive enol(ate) form in the course of the reduction.

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Concluding Remarks

In conclusion, finasteride is exquisitely selective because, as a mechanism-based inhibitor, its activation requires participation in the single catalytic reaction it was intended to inhibit. It is a nearly ideal mechanism-based inhibitor, being processed by the prostate isozyme with a rate constant, $k_i/K_i = 1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, that approaches k_{cat}/K_m for turnover of testosterone, and nearly every catalytic event aborts in capture of the cofactor (partition ratio ≤ 1.07). The resulting enzyme•NADP–dihydrofinasteride complex is extremely stable, dissociating with a half-life of 1 month at 37 °C to produce dihydrofinasteride. It is ambiguous whether the enzyme ever recovers from inhibition and, if so, whether decomposition of the adduct to dihydrofinasteride is enzyme-catalyzed or nonenzymatic. To the extent that inhibition is reversible, finasteride can be considered to be a pseudo-irreversible inhibitor, in that it is turned over to dihydrofinasteride in the course of keeping the enzyme inhibited, with a steady-state dissociation constant $K_i^* = k_{\text{off}}/k_{\text{on}} \leq 3 \times 10^{-13} \text{ M}$.

The covalent NADP–dihydrofinasteride adduct, having an apparent dissociation constant $K_i = k_{\text{off}}/k_{\text{on}} \leq 1 \times 10^{-13} \text{ M}$, ranks among the most potent noncovalently-bound inhibitors known for any enzyme.^{15,21,22} These include inhibition of adenosine deaminase by nebularine hydrate²³ ($K_i = 3 \times 10^{-13} \text{ M}$), ribulose-bisphosphate carboxylase by 2-carboxyarabinitol bisphosphate²⁴ ($K_i = 2 \times 10^{-13} \text{ M}$), and glutamine synthase by methionine sulfoximine *N*-phosphate²⁵ ($K_i \sim 10^{-20} \text{ M}$). In common with these inhibitors, which are all transition-state analogs, the NADP–dihydrofinasteride adduct is a slow-binding inhibitor ($k_{\text{on}} = 3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$). This rate constant is equal to k_{cat}/K_m for turnover of testosterone and is only slightly faster than k_i/K_i for formation of the adduct from free finasteride and cofactor-saturated enzyme. This suggests that an enzyme conformational change associated with binding the bisubstrate analog may also be rate limiting in catalytic turnover of testosterone and finasteride.

As a bisubstrate analog, the binding affinity of the NADP–dihydrofinasteride adduct can be attributed to the affinity of the enzyme for the individual components, NADPH ($K_m = 1 \times 10^{-6} \text{ M}$) and dihydrofinasteride ($K_i = 1 \times 10^{-9} \text{ M}$), and the entropy loss in joining two small molecules. This entropy loss is known to enhance the binding affinity of bisubstrate analogs by a factor of 10^{-5} M in favorable cases²⁶ and has a theoretical limit of 10^{-8} M in the complete absence of strain or distortion.²⁷ Thus, the sum of these free energy changes predicts the dissociation constant of the NADP–dihydrofinasteride adduct could approach 10^{-20} M and easily accommodates the observed $K_i \leq 1 \times 10^{-13} \text{ M}$.

This mechanism explains the exceptional potency and selectivity of finasteride in treatment of benign prostatic hyperplasia in man.

Experimental Section

Materials. Procedures for the synthesis of finasteride and related 4-azasteroid inhibitors have been published.²⁸ Radiolabeled compounds were [1,2-³H]finasteride (10.52 Ci/mmol) and [*tert*-butyl-¹⁴C]finasteride (11.56 Ci/mol); both were >97% pure by reverse-phase high-pressure liquid chromatography.

Enzyme Preparation. The recombinant human isozymes were produced by a baculovirus expression system.¹³ In general, the cell pellets were combined with an equal volume of buffer consisting of 0.1 M MOPS (3-(*N*-morpholino)propanesulfonic acid), 1 M sucrose, and 10 mM EDTA (ethylenediaminetetraacetic acid) at pH 7.2 and were dispersed using a high shear force homogenizer (Polytron) for several minutes in an ice bath.¹² The baculovirus expression system gave homogenates containing 1–2 μM enzyme, in which the enzyme constituted ~0.1% of the total protein. Consistent with the reported localization of the native enzymes in membranes associated with the nuclear envelope, the recombinant enzymes sedimented over a wide range of centrifugal force. In 0.5 M sucrose, the enzyme distributed as follows: 25% of the activity sedimented at $1000g \times 30 \text{ min}$, 16% sedimented at $10\,000g \times 30 \text{ min}$, 15% sedimented at $100\,000g \times 60 \text{ min}$, and 8% remained suspended at this force. Some 36% of the activity in the homogenate was unaccounted for after centrifugation. Since no fraction was substantially enriched in specific activity, those remaining suspended at the highest speeds were employed in kinetic measurements, and the remainder was devoted to preparation of radiolabeled products.

Enzyme Assay. The standard assay contained 10–100 pM membrane-bound enzyme suspended in a solution of 25 nM [7-³H]-testosterone (carrier free, 24.6 Ci/mmol, New England Nuclear) and 100 μM NADPH in a buffer consisting of 0.1 M MOPS, 1 mM EDTA, and 0.1% BSA (bovine serum albumin), at pH 7.20 and 37 °C, in a total volume of 100 μL . Ethanol was included at 1% final concentration as the vehicle for introduction of inhibitors. Reactions were quenched with an equal volume, or more, of water containing 1% trifluoroacetic acid, and the product [³H]dihydrotestosterone was isolated by direct injection on a reverse phase C-18 column (Vydac, $4.6 \times 250 \text{ mm}$, 300 Å, 5 μm , flow rate 1 mL/min), which was run in an isocratic system of 60% aqueous methanol containing 0.1% trifluoroacetic acid. When tissue extracts were the source of enzyme, the quenched reactions were clarified by centrifugation at $10\,000g$ before analysis. Retention times were 13 min for testosterone and 20 min for dihydrotestosterone. The effluent containing the [³H]dihydrotestosterone peak (6 mL) was collected in a liquid scintillation vial and counted in Aquasol 2 (14 mL) with 30% efficiency.

Determination of Bound and Free Inhibitor. Concentrations of bound and free radioactivity were determined by ultrafiltration on 10 000 Da cutoff membranes (Amicon, Centricon-10). These experiments established that finasteride does not become covalently bound to the enzyme, and the method was employed later to determine the rate constant for decomposition of the enzyme–[³H]inhibitor complex. Nonspecific binding of [³H]finasteride was assessed by including a 100-fold excess of unlabeled finasteride over enzyme concentration. These controls indicated $16 \pm 2\%$ nonspecific binding at the nanomolar concentrations of enzyme employed in the off-rate determinations, of which $12 \pm 2\%$ (the majority) was attributable to binding to the filter alone in the absence of enzyme. Heat denaturation of the enzyme–[³H]finasteride complex gave $86 \pm 2\%$ apparent free inhibitor, implying >96% release of the label after correction for nonspecific binding. Alternatively, denaturation of enzyme–inhibitor complex in 7 M guanidine hydrochloride solutions was not attended by nonspecific binding ($\pm 2\%$), and these experiments indicated >98% release of the radiolabel directly.

Mass Spectroscopy. Mass spectra were recorded by flow-injection on a PE/SCIEX API III tandem mass spectrometer. Mass spectra of dihydrofinasteride were obtained in the positive ion mode by atmospheric pressure chemical ionization, employing a heated nebulizer interface (500 °C), and daughter ion spectra were acquired using argon as the collision gas. Mass spectra of the NADP–dihydrofinasteride adduct were recorded in negative ion mode employing a pneumatic electrospray (ionspray) interface.

Purification of the NADP–[¹⁴C]Dihydrofinasteride Adduct. The starting membrane-bound enzyme–[¹⁴C]inhibitor complex was prepared from recombinant human type 2 enzyme taken from the baculovirus

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expression system, stored as frozen cell pellets. A suspension was prepared from the cell pellets to contain approximately 1000 mg of protein, 50 nmol of enzyme, 100 nmol of [^{14}C]finasteride (2.57×10^6 dpm), and 5 μmol of NADPH in a buffer of 0.05 M MOPS at pH 7.20 and a total volume of 50 mL. This suspension was homogenized at 4 $^{\circ}\text{C}$ and then stirred at room temperature for 1 h (>100 half-lives) to form the enzyme-inhibitor complex. Afterward, the excess [^{14}C]finasteride was removed by dialysis for 3 days at 4 $^{\circ}\text{C}$ against 2×4 L of 1 mM MOPS and 1 mM EDTA (pH 7.20) in Spectrapor-2 dialysis tubing, 14 000 Da cutoff. The recovered suspension (96 mL) contained 42 nmol of enzyme-[^{14}C]inhibitor complex (1.08×10^6 dpm).

After this point all solutions contained ammonium bicarbonate adjusted to pH 9 with ammonium hydroxide to buffer the free adduct, as employed for instance in preparation of [4- ^3H]NADH.²⁹ The dialyzed enzyme-[^{14}C]finasteride complex was denatured by mixing with an equal volume (96 mL) of 95% ethanol containing 2 mL of 1 M ammonium bicarbonate (pH 9). The suspension was stirred for 30 min at room temperature, then centrifuged at 10 000g for 45 min to remove most of the insoluble matter. The supernatant contained 93% of the radioactivity.

Preliminary purification was accomplished on a Pharmacia Fast Q-Sepharose anion exchange matrix (5 mL column volume, 150×7 mm, HCO_3^- form, equilibrated to 0.01 M ammonium bicarbonate (pH 9)). The centrifuged aqueous ethanol solution was applied to the column, which was developed with 50 mL portions of increasing concentrations of ammonium bicarbonate at pH 9. A single peak of radioactivity was recovered, eluting in the 0.20 and 0.25 M bicarbonate fractions, and accounted for 68% of the applied ^{14}C content. The flow-through contained 3% of the radiolabel, and the remainder failed to elute. The peak fractions were pooled and lyophilized in the dark overnight, and the fluffy white residue was redissolved in 2 mL of 0.01 M ammonium bicarbonate (pH 9). The recovery to this point was 63% of the ^{14}C content of the dialyzed enzyme-inhibitor complex.

Further purification was achieved by high-pressure liquid chromatography on a Pharmacia Mono-Q column. After centrifugation to remove a faint precipitate, the solution was applied to the column (HCO_3^- form, equilibrated to 0.01 M ammonium bicarbonate (pH 9) in 50% methanol). The column was developed with a 50 min linear gradient to 0.50 M ammonium bicarbonate (pH 9) at a flow rate of 1 mL/min, holding the methanol content constant at 50%. (The methanol is essential; in its absence, none of the compound elutes at any salt concentration.) The radioactivity co-chromatographed with dihydropyridine absorbance ($\lambda = 340$ nm), appearing as a sharp peak at a nominal concentration of 0.266 M bicarbonate and comprising 97% of the applied radioactivity. The peak radioactive fractions were pooled, the methanol was blown off with argon, and the solution was lyophilized in the dark to give a clear glass. The purified compound amounted to 26 nmol based on ^{14}C content (6.74×10^5 dpm), an overall recovery of 62% of the radioactivity bound in the starting enzyme-[^{14}C]inhibitor complex. It appears to be stable for several months when stored in, or lyophilized from, the ammonium bicarbonate buffer at pH 9 and -75 $^{\circ}\text{C}$, as determined by chromatography again on this column.

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Stability of Free NADP-Dihydrofinasteride. The NADP-[^{14}C]dihydrofinasteride adduct appears to be much more stable to oxidation and cyclization than described for its NAD-pyruvate counterpart.¹⁶ Its integrity was determined from its ability to decompose to produce [^{14}C]dihydrofinasteride, which was measured by reverse-phase chromatography as described in the legend to Figure 3 (but in the absence of trifluoroacetic acid). As a positive control, oxidation of the dihydropyridine ring with 1 mM phenazine methosulfate for 1 h completely abolished formation of [^{14}C]dihydrofinasteride. When the rate of decomposition under standard conditions (pH 7.20 and 37 $^{\circ}\text{C}$) was monitored over a period of 2 weeks, the data gave a good fit to a first-order rate equation with $k = (7.21 \pm 0.27) \times 10^{-7} \text{ s}^{-1}$ (half-life of 11 days) and indicated that the reaction proceeded to 75% formation of [^{14}C]dihydrofinasteride at infinite time. Alternatively, heating the compound in a boiling water bath at 100 $^{\circ}\text{C}$ decreased the half-life to ~ 20 min at pH 7, explaining the partial recovery of dihydrofinasteride upon heat denaturation of the enzyme-inhibitor complex in early experiments. Also as anticipated, the decomposition was found to be acid catalyzed, having a half-life of ~ 1 min at pH 4 and 100 $^{\circ}\text{C}$, where production of dihydrofinasteride increased to 88% conversion at infinite time.

Calculations. Data were fit to the appropriate models by nonlinear regression using a least-squares Marquardt algorithm in a computer program written by Nancy Thornberry. The program is available on request. The data for slow-binding inhibition were analyzed as reviewed by Morrison and Walsh.¹⁵ The time courses for inhibition by finasteride or the purified NADP-dihydrofinasteride adduct were fit to an integrated first-order rate equation (eq 1), where A is the product

$$A = v_s t + (v_o - v_s)(1 - e^{-kt})/k + A_o \quad (1)$$

concentration, v_o is the initial velocity, v_s is the infinite-time velocity, and k is the observed first-order rate constant for progression of the enzyme between the two states. The velocities at infinite time were essentially zero. For a mechanism-based inhibitor, or a competitive reversible inhibitor potent enough to drive the reaction to completion, the observed first-order rate constants depend on substrate concentration, S , the steady-state dissociation constant for initial binding of the inhibitor, K_i , and the first-order rate constant for conversion of the initial to the final enzyme-inhibitor complex, k_i , as described by eq 2. For

$$k = k_i(I/K_i)/(1 + (S/K_m) + (I/K_i)) \quad (2)$$

inhibition of the prostate (type 2) isozyme by finasteride or the NADP-dihydrofinasteride adduct, there was no evidence for saturation at the highest accessible inhibitor concentrations ($K_i \gg I_{\text{max}}$), so that only the second-order rate constant, k_i/K_i , is determined. This is not the case for inhibition of the skin (type 1) isozyme by finasteride, where this complex is kinetically significant and has $K_i = 360 \text{ nM}$.¹⁰

Acknowledgment. We thank Bennett M. Shapiro for first suggesting that finasteride might be a mechanism-based inhibitor.

JA953069T