Engineering Steroid 5β-Reductase Activity into Rat Liver 3α-Hydroxysteroid Dehydrogenase†

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ABSTRACT: Δ^4-3-Ketosteroid-5β-reductase (5β-reductase) precedes 3α-hydroxysteroid dehydrogenase (3α-HSD) in steroid hormone metabolism. Both enzymes are members of the aldo−keto reductase (AKR) superfamily and possess catalytic tetrads differing by a single amino acid. In 3α-HSD, the tetrad consists of Tyr55, Lys84, Asp50, and His117, but a glutamic acid replaces His117 in 5β-reductase. By introducing the H117E point mutation into 3α-HSD, we engineered 5β-reductase activity into the dehydrogenase. Homogeneous H117E 3α-HSD reduced the double bond in testosterone to form 5β-dihydrotestosterone with k_{cat} = 0.25 min^{-1} and K_m = 19.0 μM and reduced the double bond in progesterone to generate 5β-dihydroprogesterone with k_{cat} = 0.97 min^{-1} and K_m = 33.0 μM. These kinetic parameters were similar to those reported for homogeneous rat liver 5β-reductase [Okuda, A., and Okuda, R. (1984) *J. Biol. Chem. 259*, 7519−7524]. The H117E mutant also reduced 5β-dihydrosteroids to 5β,3α-tetrahydrosteroids with a 600−1000-fold decrease in k_{cat}/K_m versus wild-type 3α-HSD. The ratio of 5β-reductase:3α-HSD activity in the H117E mutant was approximately 1:1. Although the H117A mutant reduced Δ^4-3-ketosteroids, the 3α-HSD activity predominated because the 5β-dihydrosteroids were rapidly converted to the 5β,3α-tetrahydrosteroids. The pH−rate profiles for carbon−carbon double-bond and ketone reduction catalyzed by the H117E mutant were superimposable, suggesting a common titratable group (pK_b = 6.3) for both reactions. In wild-type 3α-HSD, the titratable group responsible for 3-ketosteroid reduction has a pK_b = 6.9 and is assignable to Tyr55. The pH−rate profiles for 3-ketosteroid reduction by the H117A mutant were pH-independent. Our data indicate that Tyr55 functions as a general acid for both 3α-HSD and 5β-reductase activities. We suggest that a protonated Glu117 increases the acidity of Tyr55 to promote acid-catalyzed enolization of the Δ^4-3-ketosteroid substrate. Further, the identity of amino acid 117 determines whether an AKR can function as a 5β-reductase by reorienting the substrate relative to the nicotinamide cofactor. This study provides functional evidence that utilization of modified catalytic residues on an identical protein scaffold is important for evolution of enzymatic activities within the same metabolic pathway.

The aldo−keto reductase (AKR)[1] superfamily contains two classes of steroid transforming enzymes: the hydroxysteroid dehydrogenases (HSDs) and the Δ^4-3-ketosteroid-5β-reductases (5β-reductases) (*1*−*7*). The HSDs catalyze the position-specific and stereospecific interconversion of steroid alcohols and ketones. In peripheral tissues the HSDs inactivate steroid hormones, but in steroid target tissues they modulate steroid hormone action by converting active steroid hormones into their cognate inactive metabolites (*8*). The 5β-reductases reduce the carbon−carbon double bond in Δ^4-3-ketosteroids, a functionality present in nearly all steroid hormones. Metabolically, 5β-reductase precedes 3α-HSD in steroid hormone metabolism (*9*) and bile acid biosynthesis (*10*) (Figure 1). The high homology between the HSDs and 5β-reductases of the AKR superfamily suggests that these distinct activities arose through divergent evolution from a common ancestral protein (*7*).

Of the steroid-metabolizing AKRs, rat liver 3α-HSD (AKR1C9)[2] is the best studied (*12*). Cloning of the cDNA (*1*) allowed overexpression, purification, and kinetic characterization of the recombinant protein from *Escherichia coli* (*13*). Site-directed mutagenesis (*13*−*16*) and crystallographic

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[1] Abbreviations: ACN, acetonitrile; AKR, aldo−keto reductase; 3α-HSD, 3α-hydroxysteroid dehydrogenase (EC 1.1.1.213; A-face specific also designated AKR1C9); 5β-reductase, Δ^4-3-ketosteroid-5β-reductase (EC 1.3.99.6); testosterone, 4-androsten-17β-ol-3-one; 5α- or 5β-DHT, 5α- or 5β-dihydrotestosterone, i.e., 5α- or 5β-androstan-17β-ol-3-one; 5β,3α-THT, 5β,3α-tetrahydrotestosterone, i.e., 5β-androstane-3α,17β-diol; progesterone, 4-pregnene-3,20-dione; 5β-DHP, 5β-dihydroprogesterone, i.e., 5β-pregnane-3,20-dione; 5β,3α-THP, 5β,3α-tetrahydroprogesterone, i.e., 5β-pregnan-3α-ol-20-one.

[2] The nomenclature of the AKR superfamily was recommended by the 8th International Symposium on Enzymology and Molecular Biology of Carbonyl Metabolism (*11*).

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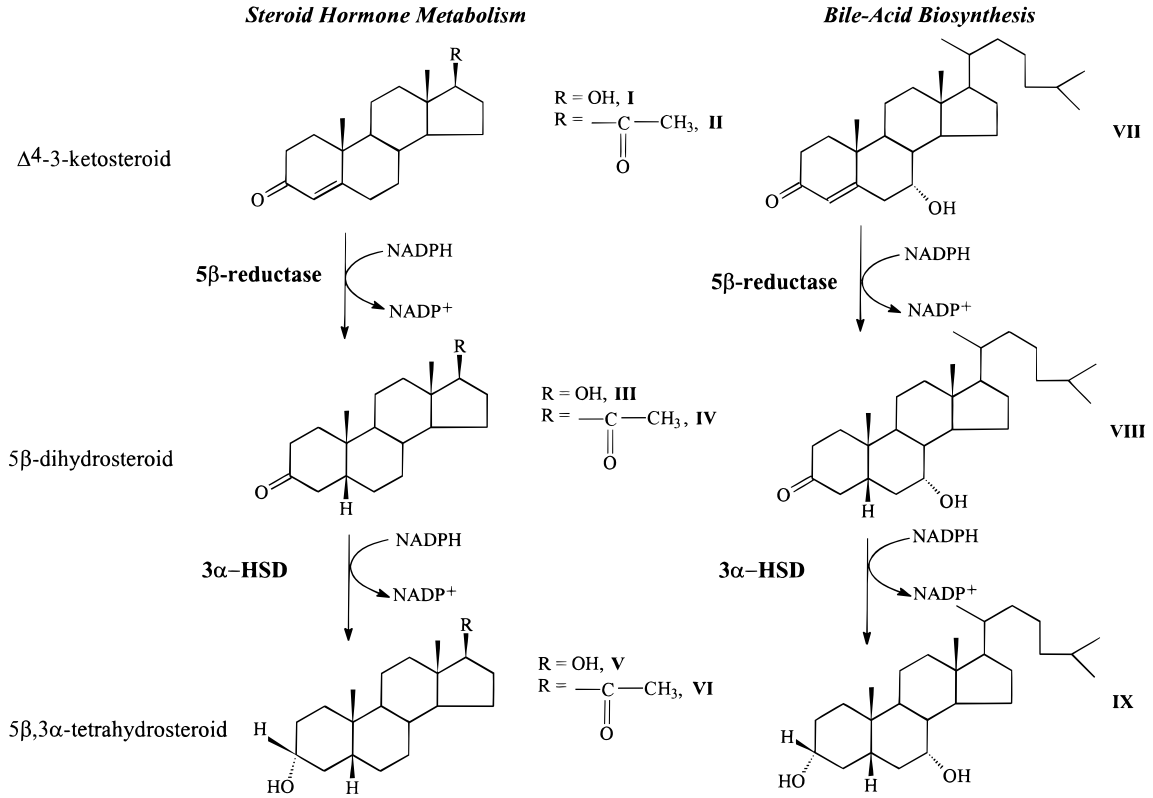

FIGURE 1: 5*β*-Reductase and 3α-HSD activities catalyzed by the AKR superfamily. Testosterone (**I**), progesterone (**II**), 5*β*-DHT (**III**), 5*β*-DHP (**IV**), 5*β*,3α-THT (**V**), 5*β*,3α-THP (**VI**), 7α-hydroxycholest-4-en-3-one (**VII**), 5*β*-cholestane-7α-ol-3-one (**VIII**), and 5*β*-cholestane-3α,7α-diol (**IX**) are shown.

(*17*−*19*) studies have elucidated the catalytic mechanism of 3α-HSD and how steroids bind to this protein. These findings may be applicable to other HSDs in the AKR superfamily. In contrast, biochemical characterization of the 5*β*-reductases is less complete. Purification of rat liver 5*β*-reductase allowed kinetic studies of the enzyme (*20*, *21*), and recent cloning of cDNAs for 5*β*-reductase from human (AKR1D1) (*6*) and rat (AKR1D2) (*5*) showed that these proteins are 52.1% and 54.3% identical in amino acid sequence to rat liver 3α-HSD, respectively. Testosterone, a substrate for 5*β*-reductase, is a competitive inhibitor for 3α-HSD (*22*), indicating that both proteins can bind the same steroid at their respective active sites.

The three-dimensional structures of the AKRs display an $(\alpha/\beta)_8$-barrel motif that binds NAD(P)(H) cofactor in an extended conformation so that hydride transfer occurs from the 4-*pro*-(*R*) hydrogen of the cofactor to the substrate carbonyl (*17*, *23*−*26*). This implies that both 3α-HSD and 5*β*-reductase exhibit the same stereochemistry of hydride transfer, and this is observed (*27*, *28*). Oxidoreductases of the AKR superfamily catalyze a common reaction mechanism by using a tetrad of amino acids, Tyr55, Lys84, Asp50, and His117 (numbered according to 3α-HSD), at the active site (*16*, *29*−*31*). In the proposed mechanism, Tyr55 functions as the general acid/base. Recent site-directed mutagenesis of rat liver 3α-HSD revealed a "push−pull" mechanism involving other residues of the tetrad (*16*). The interaction between Tyr55 and Lys84 is essential for oxidation of the 3α-alcohol. This interaction lowers the pK_a of the tyrosine, allowing it to function as a general base so that it can deprotonate the substrate alcohol. A salt bridge between Lys84 and Asp50 facilitates this interaction. In contrast, the interaction between Tyr55 and His117 is essential for the reduction of 3-ketosteroids. This interaction alters the pK_b of the tyrosine, allowing it to function as a general acid so that it can protonate the substrate ketone. Comparison of the tetrad residues in oxidoreductases of the AKR superfamily with those of the rat and human 5*β*-reductases shows conservation of the tetrad except that His117 is a glutamic acid in both 5*β*-reductases (*7*). This comparison suggested that 5*β*-reductase activity in the AKR superfamily may result from a single amino acid substitution in the catalytic tetrad.

We sought to test this hypothesis by engineering steroid double-bond reductase activity into rat liver 3α-HSD. Mutation of His117 into either a glutamate or an alanine introduced 5*β*-reductase activity into 3α-HSD with varied effects. The H117E mutant functioned as both a 5*β*-reductase and a 3α-HSD. The 5*β*-reductase activity of the mutant was comparable with that of homogeneous rat liver 5*β*-reductase (*20*, *21*). Surprisingly, the H117A mutant possessed a detectable 5*β*-reductase activity but remained predominantly a 3α-HSD. We propose that Tyr55 functions as the general acid for both 3α-HSD and 5*β*-reductase activities and that the identity of the amino acid at position 117 plays a role in determining whether an AKR can function as a 5*β*-reductase. This study supports the concept that new enzyme activities can evolve in a metabolic pathway by modification of the catalytic residues within a conserved structural motif.

MATERIALS AND METHODS

Materials. Primers for PCR-based site-directed mutagenesis were purchased from Gibco. All steroids were obtained

from Steraloids; the nicotinamide cofactors were from Boehringer-Mannheim. Radiolabeled [4-^{14}C]testosterone (57.3 mCi/mmol), [4-^{14}C]progesterone (55.4 mCi/mmol), and [4-^{14}C]-5α-dihydrotestosterone (DHT) (58.3 mCi/mmol) were purchased from NEN/DuPont. Whatman LK6D TLC plates were bought from Fisher. All other reagents were ACS-grade or better and were obtained from Sigma.

Mutagenesis, Expression, and Purification of Recombinant Wild-Type and Mutant 3α-HSDs. The pKK-3α-HSD expression vector and details of the PCR-based site-directed mutagenesis protocols have been described elsewhere (*13*). Generation of the H117E mutant used the following oligonucleotide primers in the forward and reverse directions, respectively: 5′-dTTTATATTATTGAATTCCCAATGGC-3′ and 5′-dGCCATTGGGAATTCAATAATATAAA-3′ (underline denotes mutated codon). Generation of the Y55F and H117A mutants has been previously described (*13*, *16*). Ligation of the *Hin*dIII fragment (+251 to +1059 bp) of the H117E PCR product into pKK-3α-HSD(Y55F) lacking the corresponding fragment generated pKK-3α-HSD(Y55F/H117E). Dideoxy sequencing ensured fidelity of the mutant constructs. Recombinant wild-type and mutant 3α-HSDs were purified from *E. coli* sonicates by DE-52 cellulose anion-exchange and Blue Sepharose affinity column chromatographies with protein quantification, SDS−PAGE, and Western blot analysis performed as previously described (*15*).

Standard Radiometric Assay. Assays contained 100 mM potassium phosphate buffer (pH 7.0), 2.3 mM NADPH, 4% acetonitrile (ACN), and 30 000 cpm of radiolabeled steroid (the final concentration was 50 μM testosterone (**I**), 50 μM progesterone (**II**), or 35 μM 5α-DHT in a 100 μL reaction volume at 25 °C. Reactions were quenched with ethyl acetate and extracted. The extracts were evaporated to dryness, redissolved in methanol, and applied to Whatman LK6D TLC plates. Chromatograms from reactions with testosterone were developed twice in chloroform/ethyl acetate (4:1, v/v), while those from reactions with 5α-DHT were developed once in the same solvent system. The chromatograms from reactions with progesterone were developed once in toluene/ethyl acetate (3:1 v/v). Visualization and quantitation were as previously described (*16*).

Measurement of Steady-State Kinetic Parameters for 5β-Reductase and 3α-HSD Activity. The k_{cat} and K_m values for testosterone and progesterone reduction catalyzed by the H117E mutant were determined with varied steroid concentrations (2.5−50.0 μM) in the standard radiometric assay. Initial rates were calculated from aliquots of reactions in which only the 5β-dihydrosteroid product was detected over the time course employed. Estimates of the kinetic constants were obtained from ENZFITTER (*32*). For comparison, measurement of initial velocities for reduction of testosterone and progesterone with a Beckman DU-640 spectrophotometer were performed in parallel by observing the rate of change in absorbance of pyridine nucleotide at 340 nm ($\epsilon = 6270$ M^{-1} cm^{-1}) in 1 mL systems. Reaction components (100 mM potassium phosphate, pH 7.0, and 4% ACN) were identical to those used in the standard radiometric assays except that 200 μM NADPH and no radiolabeled steroid was used. For these measurements, calculation of initial velocities used only the first 2 min of data. Independent measurements with the radiometric assay confirmed that when 200 μM NADPH was substituted, only 5β-dihydrosteroids were observed in the first 5 min. Determination of the k_{cat} and K_m values for 4-androstene-3,17-dione (2.0−40.0 μM) and 4-pregnen-20α-ol-3-one (2.5−50.0 μM) reduction used the spectrophotometric assay system described above. Kinetic constants for reduction of 5β-androstan-17β-ol-3-one [5β-dihydrotestosterone, 5β-DHT (**III**)], 5α-androstan-17β-ol-3-one (5α-dihydrotestosterone, 5α-DHT), 5β-pregnane-3,20-dione [5β-dihydroprogesterone, 5β-DHP (**IV**)], and 5α-pregnane-3,20-dione were obtained spectrophotometrically under the same assay conditions by varying substrate concentration (2.5−50.0 μM) at a constant NADPH concentration (200 μM).

pH−Rate Profiles. To determine k_{cat} and K_m values over a range of pH values, a buffer system containing 50 mM sodium phosphate, 50 mM sodium pyrophosphate, and 50 mM (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid) (AMPSO) was used in place of the potassium phosphate buffer. The triple buffer system maintains constant ionic strength over the pH range used and negates the concern that different ions could have differential effects on enzyme activity. Initially, the pH−rate profiles for testosterone and progesterone reduction catalyzed by the H117E mutant were determined radiochemically to ensure that only 5β-dihydrosteroids were formed over the first 5 min of the reaction at the chosen pH. The log k_{cat} and log (k_{cat}/K_m) data were then generated spectrophotometrically with initial rates calculated from the first 2 min of the reaction. The pH−rate profiles were fitted to HBBELL, BELL, or WAVL as described by Cleland (*33*).

RESULTS

Identification of 5β-Reductase Activity in Rat Liver 3α-HSD Mutants. To test whether a point mutation could introduce 5β-reductase activity into 3α-HSD, we generated the H117E mutant and purified the expressed protein from *E. coli*. As controls, the previously characterized recombinant wild-type 3α-HSD and the H117A mutant were overexpressed and purified. Each protein appeared as a single homogeneous band of the same molecular weight by SDS−PAGE; and each was immunoreactive with rabbit anti-rat 3α-HSD antiserum upon Western blot analysis (not shown).

[^{14}C]Testosterone was used as a substrate to survey the purified wild-type 3α-HSD and the H117E and H117A mutants for 5β-reductase activity (Figure 2). As expected, wild-type 3α-HSD exhibited no 5β-reductase activity. The H117E mutant generated a product that comigrated in TLC analysis with 5β-DHT. However, this mutant also reduced 5β-DHT to 5β-androstane-3α,17β-diol [5β,3α-tetrahydrotestosterone; 5β,3α-THT (**V**)] during the assay. The TLC system used could resolve other stereochemical products that may arise from testosterone reduction (i.e., 5α-DHT and 5α-androstane-3α,17β-diol). These products did not appear in any of the reactions. Thus, mutation of His117 into a glutamic acid introduced a stereospecific 5β-reductase activity, but the H117E mutant retained 3α-HSD activity. Surprisingly, the H117A mutant also reduced testosterone, but the primary product observed in this reaction was 5β,3α-THT. This indicated that 3α-HSD and 5β-reductase activities were also present in the H117A mutant.

Kinetic Characterization of the H117E and H117A Mutants. Time courses were conducted with [^{14}C]testosterone

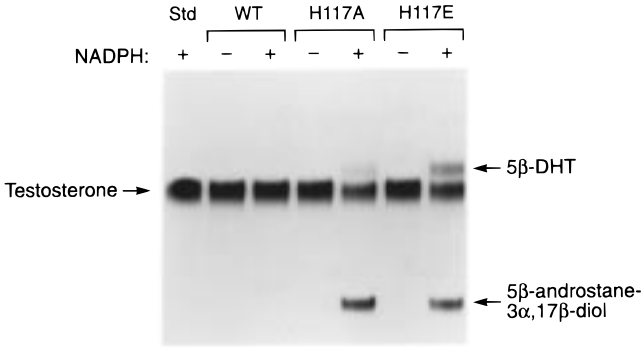

FIGURE 2: Detection of 5β-reductase activity in the H117E and H117A mutants. Purified recombinant wild type 3α-HSD and the His117 mutants (10−15 μg each) were incubated with 50 μM [^{14}C]-testosterone in the absence (−) and presence (+) of 2.3 mM NADPH for 30 min in the standard radiometric assay as described under Materials and Methods. Positions of authentic standards for substrate and products are indicated by the arrows.

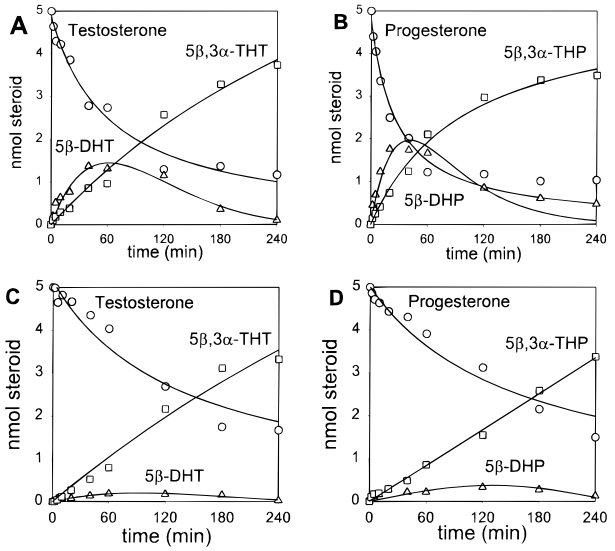

FIGURE 3: Time course for the reduction of testosterone and progesterone catalyzed by the purified H117E and H117A mutants. Incubation of purified H117E (12.5 μg) with [^{14}C]testosterone (A) or [^{14}C]progesterone (B) or purified H117A (13.2 μg) with [^{14}C]-testosterone (C) or [^{14}C]progesterone (D) was monitored with aliquots taken at intervals from 0 to 240 min and developed by TLC as described under Materials and Methods. The Δ^4-3-ketosteroid substrates (○) and the 5β-dihydrosteroid (△) and 5β,3α-tetrahydrosteroid products (□) are indicated on the graphs. Values shown are the mean for $n = 3$ with a standard deviation of less than 10% for each time point.

and [^{14}C]progesterone as substrates to monitor the progress of the 5β-reductase and 3α-HSD activities in the histidine mutants. The H117E mutant reduced testosterone to 5β-DHT (Figure 3A). However, as the concentration of 5β-DHT increased, the 3α-HSD activity of the mutant reduced 5β-DHT to 5β,3α-THT. Likewise, a time course of the H117E mutant with [^{14}C]progesterone as substrate showed a similar product pattern in which 5β-DHP appeared first and was subsequently reduced to 5β-pregnan-3α-ol-20-one [5β,3α-tetrahydroprogesterone, 5β,3α-THP (**VI**)] (Figure 3B). Based on the initial rates of Δ^4-3-ketosteroid disappearance and appearance of 5β-dihydrosteroid products (Figure 3A,B), the specific activities of the H117E mutant were 4.1 nmol of testosterone reduced min^{-1} (mg protein)$^{-1}$ and 9.8 nmol of progesterone reduced min^{-1} (mg of protein)$^{-1}$.

Table 1: Kinetic Parameters of the 3α-HSD H117E Mutant for 5β-Reductase Activity[a]

substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1}/mM^{-1})
testosterone	0.25 ± 0.02	19 ± 4	13.0
4-androstene-3,17-dione	0.34 ± 0.07	22 ± 10	15.0
progesterone	0.97 ± 0.12	33 ± 8	29.0
4-pregnen-20α-ol-3-one	0.28 ± 0.01	20 ± 3	15.0

[a] All reactions were performed spectrophotometrically in potassium phosphate buffer (pH 7.0) and 4% ACN as described under Materials and Methods. All k_{cat} and K_m values were determined with the ENZFITTER program (*32*) and are expressed as a mean ± SE for $n = 4$.

The H117A mutant reduced [^{14}C]testosterone and [^{14}C]-progesterone but the 5β-dihydrosteroids did not accumulate to the same degree as observed with the H117E mutant; instead the 5β,3α-tetrahydrosteroids were the major products (Figure 3C,D). In the H117A mutant, the rates of Δ^4-3-ketosteroid disappearance corresponded to the rates observed for tetrahydrosteroid formation (Figure 3C,D). Despite a large excess of Δ^4-3-ketosteroids, the 5β-dihydrosteroids were barely detectable and were rapidly converted to the 5β,3α-tetrahydrosteroids by the 3α-HSD activity of the H117A mutant. The inability to measure the formation of 5β-dihydrosteroids reliably with the H117A mutant prohibited accurate determination of the kinetic constants for its 5β-reductase activity. However, on the basis of the rate of disappearance of the Δ^4-3-ketosteroid substrate, the H117A mutant had estimated specific activities of 0.6 nmol of testosterone reduced min^{-1} (mg of protein)$^{-1}$ and 1.7 nmol of progesterone reduced min^{-1} (mg of protein)$^{-1}$. These values were approximately 90-fold lower than the specific activities of the H117A mutant for the reduction of 5β-DHT and 5β-DHP, indicating that the 3α-HSD activity predominated.

The kinetic constants for the reduction of the carbon−carbon double bonds in testosterone, progesterone, 4-androstene-3,17-dione, and 4-pregnen-20α-ol-3-one catalyzed by the H117E mutant were determined by either radiometric or spectrophotometric assays (Table 1). Both assay methods gave similar k_{cat} and K_m values for testosterone and progesterone reduction. As reduction of the last two steroids could only be followed spectrophotometrically, the values reported for all Δ^4-3-ketosteroids are those determined by this method. It was found that the H117E mutant exhibited similar k_{cat} values to those reported for native 5β-reductase purified to homogeneity from rat liver and demonstrated only 10-fold higher K_m values (*20*). For example, the H117E mutant reduced testosterone to 5β-DHT with $k_{cat} = 0.25$ min^{-1} and $K_m = 19.0$ μM, while native 5β-reductase has $k_{cat} = 1.30$ min^{-1} and $K_m = 2.30$ μM for testosterone. Similarly, reduction of progesterone to 5β-DHP by the H117E mutant occurred with $k_{cat} = 0.97$ min^{-1} and $K_m = 33.4$ μM, while native 5β-reductase exhibits $k_{cat} = 1.80$ min^{-1} and $K_m = 2.20$ μM for progesterone. Purified native 5β-reductase has no reported 3α-HSD activity (*20*, *21*).

The kinetic constants for the reduction of 5β- and 5α-dihydrosteroids catalyzed by wild-type 3α-HSD and the histidine mutants were also determined (Table 2). Previously, we reported that the reduction of androstanedione by the H117A mutant was not detectable in the triple buffer

Table 2: Kinetic Parameters of Wild-Type 3α-HSD and the H117E and H117A Mutants for 3-Ketosteroid Reduction[a]

substrate	wild type	H117E	H117A
5β-Androstan-17β-ol-3-one			
k_{cat} (min^{-1})	57 ± 4	0.29 ± 0.08	4.4 ± 0.7
K_m (μM)	6.0 ± 2.0	31 ± 16	37 ± 11
k_{cat}/K_m (min^{-1}/mM^{-1})	9500	9.4	120
5α-Androstan-17β-ol-3-one			
k_{cat} (min^{-1})	13 ± 1	*b*	0.51 ± 0.04
K_m (μM)	6.0 ± 1.5	*b*	20 ± 2
k_{cat}/K_m (min^{-1}/mM^{-1})	2200	*b*	26
5β-Pregnane-3,20-dione			
k_{cat} (min^{-1})	29 ± 1	0.27 ± 0.01	4.6 ± 1.5
K_m (μM)	3.0 ± 0.30	15 ± 2	61 ± 30
k_{cat}/K_m (min^{-1}/mM^{-1})	9700	18	75
5α-Pregnane-3,20-dione			
k_{cat} (min^{-1})	5.2 ± 1.0	*b*	0.38 ± 0.02
K_m (μM)	5.6 ± 1.0	*b*	21 ± 8
k_{cat}/K_m (min^{-1}/mM^{-1})	930	*b*	18

[a] All reactions were performed spectrophotometrically in potassium phosphate buffer (pH 7.0) and 4% ACN as described under Materials and Methods with k_{cat} and K_m values determined with the ENZFITTER program (*32*) and expressed as a mean ± SE for $n = 4$. [b] Not detectable spectrophotometrically with up to 100 μg of protein.

system by a spectrophotometric assay (*16*). However, in this study we used a potassium phosphate buffer and different steroid substrates that yielded measurable rates for 3-ketosteroid reduction with this mutant. The H117A mutant exhibited 50−140-fold decreases in the k_{cat}/K_m values for 3-ketosteroid reduction versus wild-type enzyme and affected both k_{cat} (6−25-fold decrease) and K_m (3−22-fold increase). The H117E mutant decreased the k_{cat}/K_m values for 3-ketosteroid reduction by 600−1000-fold compared to wild type, with the largest effect observed on k_{cat}. On the basis of comparison of the k_{cat}/K_m values, the H117A mutant was a superior 3α-HSD relative to the H117E mutant.

Comparison of the k_{cat}/K_m values for the reduction of testosterone and progesterone catalyzed by the H117E mutant with those for the reduction of the corresponding 5β-dihydrosteroids revealed that they were 1.5-fold higher for double-bond reduction (Tables 1 and 2). Although the H117A mutant displayed decreased catalytic efficiency for ketone reduction relative to wild-type 3α-HSD, these k_{cat}/K_m values are clearly superior to those for 5β-reductase activity, which could not be determined for this mutant.

Our results also indicated that 5β-dihydrosteroids were the preferred substrates for wild-type 3α-HSD and both histidine mutants. Wild-type 3α-HSD and the H117A mutant had 4−11-fold higher k_{cat}/K_m values for reduction of A/B *cis*-ring fused steroids than for the A/B *trans*-ring fused steroids. In the spectrophotometric assays, the H117E mutant reduced 5β-dihydrosteroids but failed to reduce 5α-dihydrosteroids.

pH Dependence of 5β-Reductase and 3α-HSD Activities. The pH dependence of k_{cat} and k_{cat}/K_m for wild-type 3α-HSD and the histidine mutants were determined over a pH range from 5.5 to 10 (Figure 4). The measured parameters obtained from fits of the experimental data are given in Table 3. The log k_{cat} versus pH profile for the reduction of 5β-DHT catalyzed by wild-type enzyme showed an inflection point with a pK_b = 6.9 and was similar to those reported for reduction of 5α-DHT and androstanedione (*16*). Previous work on 3α-HSD, aldose reductase, and aldehyde reductase

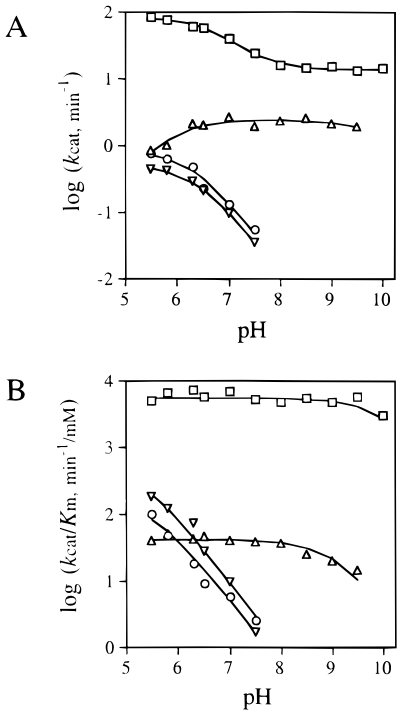

FIGURE 4: pH dependence of 5β-reductase and 3α-HSD activities catalyzed by wild-type 3α-HSD and the H117E and H117A mutants. (A) log k_{cat} versus pH and (B) log (k_{cat}/K_m) versus pH profiles for 5β-DHT reduction catalyzed by wild-type 3α-HSD (□) and the H117A (Δ) and H117E (○) mutants and for testosterone reduction catalyzed by the H117E (▽) mutant. All assays were performed spectrophotometrically with the triple buffer system as described under Materials and Methods.

has assigned this titratable group as the catalytic tyrosine, which is Tyr55 in 3α-HSD (*16*, *30*, *31*). For example, mutation of Tyr55 in 3α-HSD eliminated this inflection point with an observed 10^4-fold decrease in k_{cat} (*16*). In this study the inflection point was also eliminated in the H117A mutant with a 100-fold decrease in the pH-independent value of k_{cat} for 5β-DHT reduction. This pH profile was similar to that previously reported for the H117A mutant when 5α-DHT was used as substrate (*16*). These results implied that a protonated His117 interacts with Tyr55 through a potential proton relay to facilitate ketone reduction in 3α-HSD (*16*).

The log k_{cat} versus pH profiles for reduction of testosterone and 5β-DHT catalyzed by the H117E mutant were superimposable and indicated that both reactions used the same titratable group with a pK_b = 6.3. The H117E mutant also showed a 2 log unit decrease in the pH-independent value of k_{cat} for 5β-DHT reduction versus wild-type 3α-HSD. These data suggest that the pK_b of Tyr55 was shifted to a more acidic pH in the H117E mutant and that a protonated Glu117 facilitates both the 5β-reductase and the 3α-HSD activities.

The log (k_{cat}/K_m) versus pH profile for 5β-DHT reduction catalyzed by wild-type 3α-HSD is essentially flat up to pH 9.0. The inflection point pK_b = 6.9 observed in the log k_{cat} versus pH plot is eliminated due to a systematic decrease in K_m as pH increases, resulting in an increase in catalytic efficiency. In contrast, log (k_{cat}/K_m) versus pH profiles for 5β-DHT reduction catalyzed by the H117A mutant showed a 2 log unit decrease versus wild type and gave an inflection point of pK_b = 9.0. This inflection point results from a further decrease in catalytic efficiency due to a pH-dependent

Table 3: p*K* Values for the Kinetic Constants of 5β-DHT and Testosterone Reduction Catalyzed by Wild-Type 3α-HSD and the H117E and H117A Mutants

enzyme	substrate	parameter	equation	pK_a	pK_b	C^a
wild type	5β-DHT	k_{cat}	WAVL	NA[b]	6.90 ± 0.10	86.0 ± 2
		k_{cat}/K_m	HBBELL	NA	10.00 ± 0.10	5600 ± 260
H117A	5β-DHT	k_{cat}	BELL	5.80 ± 0.10	10.00 ± 0.30	2.4 ± 0.2
		k_{cat}/K_m	HBBELL	NA	9.00 ± 0.10	40 ± 9
H117E	5β-DHT	k_{cat}	HBBELL	NA	6.40 ± 0.03	0.52 ± 0.02
		k_{cat}/K_m	HBBELL	NA	5.10 ± 0.60[c]	*d*
H117E	testosterone	k_{cat}	HBBELL	NA	6.30 ± 0.12	0.87 ± 0.15
		k_{cat}/K_m	HBBELL	NA	5.50 ± 0.40[c]	*d*

[a] pH-independent values for k_{cat} and k_{cat}/K_m are expressed in units of min^{-1} and min^{-1}/mM^{-1}, respectively, and for the WAVL and BELL fits represent the highest values. [b] NA, not applicable. [c] These values have larger associated errors because the extrapolations were greater. [d] Values are not given for *C* because the error was greater than the value since the plots did not plateau. All reactions were performed in the triple buffer system.

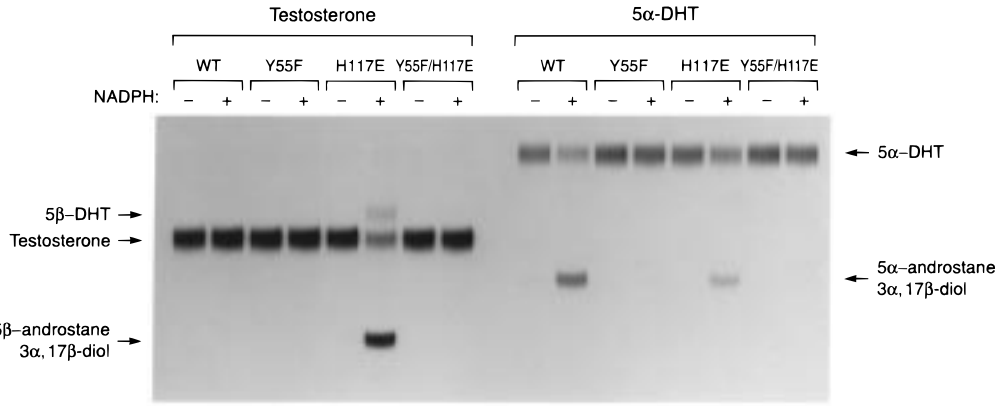

FIGURE 5: Elimination of 3α-HSD and 5β-reductase activities by the Y55F/H117E double mutant. Purified recombinant wild-type 3α-HSD (10 μg), H117E (12.5 μg), and the Y55F/H117E double mutant (15 μg) were incubated with either [^{14}C]testosterone or [^{14}C]-5α-DHT in the absence (−) and presence (+) of 2.3 mM NADPH for 30 min in the standard radiometric assay as described under Materials and Methods. Positions of authentic standards are noted.

increase in K_m. These data show that in wild type enzyme His117 is important for both catalysis and steroid binding.

The H117E mutant gave log (k_{cat}/K_m) versus pH profiles that were superimposable for the reduction of both 5β-DHT and testosterone. These plots were similar to those described for plots of log k_{cat} versus pH obtained with this mutant. Further, inspection of the changes in K_m versus pH showed these values increased with pH leading to steeper titration curves in the log (k_{cat}/K_m) profiles for both activities. Together, these results indicate that at high pH the negatively charged Glu117 adversely affects the reduction and binding of both 5β-DHT and testosterone.

Tyr55 as the General Acid for the 5β-Reductase and 3α-HSD Activities. To test the role of Tyr55 as the general acid for both 3α-HSD and 5β-reductase activities, the Y55F/H117E double mutant was overexpressed, purified, and assayed for both enzymatic activities. As expected, wild type 3α-HSD did not reduce [^{14}C]testosterone and the H117E mutant functioned as a 5β-reductase (Figure 5). The Y55F mutant was severely impaired for reduction of [^{14}C]-5α-DHT. However, the Y55F/H117E mutant did not catalyze reduction of either 3-ketosteroids or Δ^4-3-ketosteroids. These results are consistent with Tyr55 acting as the general acid for both 3α-HSD and 5β-reductase activities.

DISCUSSION

Guided by the three-dimensional structure of rat liver 3α-HSD and sequence comparisons of steroid-metabolizing AKRs, we successfully engineered 5β-reductase activity into 3α-HSD with a point mutation. Our results indicate that the AKRs use a common general acid (Tyr55) for reduction of both ketones and carbon−carbon double bonds. This work shows that the identity of the amino acid 117 in the catalytic tetrad can determine if an AKR functions as a steroid 5β-reductase. Finally, this study provides functional evidence that mutation of a common catalytic tetrad on a conserved structural motif is important for evolution of enzyme activities within the same metabolic pathway.

Insights on AKR Catalysis by Engineering 5β-Reductase Activity into 3α-HSD. Structural and kinetic studies have elucidated the reaction mechanism of the oxidoreductases in the AKR superfamily but have not addressed how the 5β-reductases of the superfamily function. 3α-HSD, aldose reductase, and aldehyde reductase share an identical catalytic tetrad (Tyr55, Lys84, Asp50, and His117) in which the tyrosine functions as the general acid/base (*16*, *29*−*31*). Our results are consistent with the mechanism proposed for ketone reduction catalyzed by rat liver 3α-HSD (*16*). The salient features of this reaction are hydride transfer from NAD(P)H to the C3-ketone of the steroid with Tyr55 acting as the general acid and His117 participating in the proton relay. This study suggests that the 5β-reductase activity uses similar features. First, the reaction is NADPH-dependent. Second, based on the pH−rate profiles of the H117E mutant and the lack of activity in the Y55F/H117E double mutant, Tyr55 also serves as the general acid for carbon−carbon double-bond reduction. Finally, the log k_{cat} versus pH profile of the H117E mutant suggests that a protonated glutamic acid facilitates double-bond reduction.

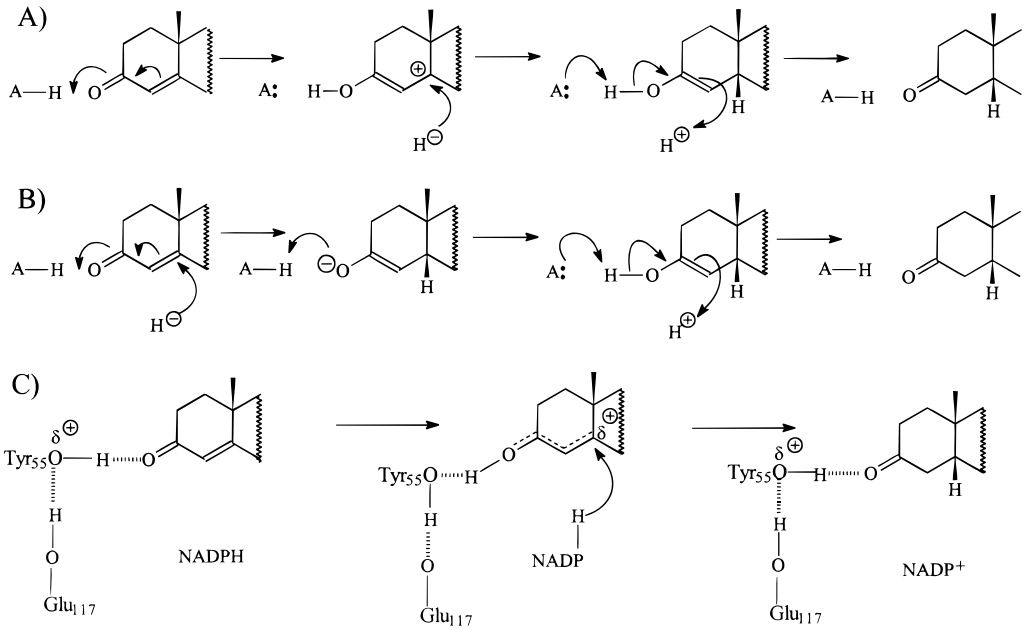

FIGURE 6: Alternate mechanisms for carbon−carbon double-bond reduction. (A) Double-bond reduction proceeding via initial protonation of the ketone at C3 and subsequent formation of a carbonium ion intermediate. (B) Double-bond reduction proceeding via initial hydride transfer to C5 and subsequent formation of an enolate anion. (C) Proposed mechanism for double-bond reduction in the 3α-HSD H117E mutant and native 5β-reductase, in which a protonated Glu117 facilitates the reaction catalyzed by Tyr55, the general acid.

It is unknown whether double-bond reduction in Δ^4-3-ketosteroids occurs by a stepwise or concerted mechanism. Akhtar et al. (*34*) proposed that steroid double-bond reduction may occur by a two-step mechanism in which either protonation or hydride transfer is the initial step. In Δ^4-3-ketosteroids, protonation at C3 would generate an enol and a partial positive charge or a carbonium ion at C5 (Figure 6A). This would permit hydride transfer to the β-face of the steroid at C5 followed by tautomerization and protonation at C4. Alternatively, hydride transfer could occur first at C5, forming an enolate anion (Figure 6B), with protonation at C4 completing the reaction. Although both the carbonium ion and enolate anion are unstable, the transition state of the reaction may resemble one of these two intermediates. Using primary and solvent kinetic isotope effects, others have shown that in aldose reductase (a related aldo−keto reductase) proton donation is slower than hydride transfer, providing evidence for an oxyanion intermediate during the catalytic mechanism (*30*). This oxyanion intermediate would be equivalent to the enolate in Figure 6B. Thus the oxyanion hole at the AKR active site (*35*) could stabilize the negatively charged enolate. However, substitution of His117 by Glu117 could compromise this situation in the H117E mutant and the native 5β-reductases. Finally, as noted for Δ^5-3-ketosteroid isomerase (*36*), the extremely apolar nature of the steroid binding site would present an unfavorable environment for generation of a formally charged species during the reaction mechanism. This would suggest a mechanism in which enolization precedes hydride transfer.

On this basis we propose the following mechanism for the steroid double-bond reductase activity of the H117E mutant and the 5β-reductases of the AKR superfamily (Figure 6C). Following binding of NADPH and testosterone, Tyr55 promotes acid-catalyzed enolization of the Δ^4-3-ketosteroid. Similar to the role of His117 in 3-ketosteroid reduction (*16*), a protonated Glu117 hydrogen-bonds with Tyr55 to lower the pK_b of the tyrosine, making it a stronger acid to facilitate the enolization step. The resultant 3,4-enol produces cationic character at C5 and allows hydride transfer from NADPH to the β-face of the steroid C5. Finally, the enol can tautomerize to generate the 5β-dihydrosteroid product. Alternatively, as proposed for 5α-reductase, the enol may dissociate from the enzyme and reprotonate nonenzymatically in solution (*37*).

A similarity exists between the reaction mechanism of 5β-reductase and that of Δ^5-3-ketosteroid isomerase. Two recent structures of *Pseudomonas* Δ^5-3-ketosteroid isomerase support assignment of Tyr14 as the general acid and suggest that both this tyrosine and Asp99 hydrogen-bond with the C3 oxygen of the bound steroid (*38*, *39*). Likewise, experimental evidence based on the binding of substituted *p*-phenols to Δ^5-3-ketosteroid isomerase in which the catalytic base (Asp38) was mutated may support this pattern of hydrogen bonds (*40*). However, Mildvan and co-workers (*41*) have demonstrated by NMR that Asp99 forms a strong low-barrier hydrogen bond with Tyr14, making it a better acid catalyst. In the AKRs with 5β-reductase activity, we propose that Glu117 hydrogen-bonds with Tyr55, making it a better acid catalyst so that acid-catalyzed enolization of the steroid enone can occur. On the basis of the three-dimensional structure of rat liver 3α-HSD, a glutamic acid at position 117 could interact with either the steroid ketone or Tyr55. However, the observed acid shift in the pK_b of Tyr55 in the H117E mutant for both 5β-reductase activity and 3-ketosteroid reduction plus the loss of pH dependence of k_{cat} in the H117A mutant suggests that Glu117 interacts with Tyr55, not the steroid ketone. It is intriguing that the mechanisms for isomerization of Δ^5-3-ketosteroids and for reducing Δ^4-3-ketosteroids resemble each other and that catalytic residues responsible for both reactions are similar.

It is important to consider why testosterone and progesterone are not substrates for native 3α-HSD. There are no known examples of HSDs that reduce ketones when they are present as α,β-unsaturated ketones. In examining

chemical precedents, methanolic borohydride will leave the α,β-unsaturated ketone of Δ^4-androstene-3,17-dione intact but will easily reduce the 17-ketone to yield the 17β-alcohol (*42*). The enone is resistant to reduction under these mild conditions because it is more thermodynamically stable than a saturated ketone. Since reduction of a saturated ketone is chemically preferred, testosterone and progesterone are not substrates for 3α-HSD. Under harsher conditions borohydride will selectively reduce the ketone in 2-cyclopenten-1-one (*43*). However, enzymatic formation of a 3α-alcohol containing a $\Delta^{4,5}$-ene is not observed since reduction of the enone occurs via hydride transfer to C5 and not by hydride transfer to C3. This process is facilitated in the H117E mutant by acid-catalyzed enolization of the enone.

Role of Amino Acid 117 in Orienting Steroid Substrates. Our results also suggest that amino acid 117 may serve other functions, such as orienting steroid substrates within the pocket. The 3α-HSD and 5β-reductase reactions are similar but differ in the position to which hydride is transferred. Rat liver 3α-HSD binds testosterone at the active site as a competitive inhibitor (*22*) and the ternary complex structure (*19*) provides insight on why this enzyme does not function as a 5β-reductase. The C5 of testosterone is 5 Å away from the C4 of the nicotinamide ring, a position too distant to permit hydride transfer. Since the C3 and C5 of testosterone are 2.4 Å from each other, an adjustment in the position of the steroid must place C5 nearer the 4-*pro*-(*R*)-hydrogen of the nicotinamide ring. Although the detailed structural effects of the H117E and H117A mutants on steroid binding are unknown, substitution of a glutamic acid or an alanine for His117 may reposition the C5 of testosterone closer to the C4 of the nicotinamide ring to permit hydride transfer and generation of the 5β-dihydrosteroid product. Previous work on aldose reductase, an AKR that primarily metabolizes monosaccharides, has demonstrated the importance of this conserved histidine for orienting 2-hydroxyaldehyde substrates in the active site (*30*).

Wild-type 3α-HSD and the histidine mutants preferentially reduce the C3 ketone of 5β-dihydrosteroids over 5α-dihydrosteroids even though the histidine mutants adversely affect both k_{cat} (6−30-fold decreases) and K_m (6−200-fold increases) for these substrates. This preference may have mechanistic consequences for the 5β-reductase activity of the H117E mutant. In 5α-dihydrosteroids, the C5 hydrogen is α-oriented and the A/B-ring fusion is in trans, whereas this hydrogen is β-oriented in the 5β-dihydrosteroids and the A/B-ring fusion is in cis. This subtle difference has major structural effects on the steroid. In the trans ring fusion, the A-ring is planar with the steroid nucleus, but the A-ring bends 90° relative to the steroid nucleus when the fusion is in cis. In the H117E mutant hydride transfer from the nicotinamide to C5 of the Δ^4-3-ketosteroid, in which the A-ring is roughly planar to the remainder of the steroid, produces a 5β-dihydrosteroid with a 90° bend in the A-ring. Our results suggest that the substrate pocket of wild type 3α-HSD and the histidine mutants better accommodates A/B cis ring-fused steroids and may permit binding of a bent transition state during reduction of Δ^4-3-ketosteroids to 5β-dihydrosteroids.

Evolution of Enzymatic Activity. Horowitz (*44*) originally proposed that biosynthetic pathways evolved in a stepwise manner. Identification of related enzymes catalyzing consecutive reactions for the biosynthesis of methionine in *E. coli* (*45*) and of isoleucine in *Bacillus subtilis* (*46*) suggests that gene duplication followed by divergence of function has occurred. In mammals, 5β-reductase activity immediately precedes 3α-HSD activity in steroid hormone metabolism (*9*) and bile acid biosynthesis (*10*). The sequence similarity of these proteins in both rat and human and the results reported here argue that divergent evolution from a common ancestor that potentially catalyzed both 5β-reductase and 3α-HSD activities has occurred.

Our results are consistent with a "chemistry first" view of enzyme evolution. Jensen (*47*) suggested that the evolution of enzymatic activity occurs by recruitment of a protein possessing a given catalytic machinery. This catalytic power is then harnessed to generate new activities either by modifying substrate specificity or by partitioning of reaction intermediates to form new products. Structural and sequence comparisons of protein superfamilies have formed the basis of this hypothesis. For example, all members of the enolase superfamily of proteins, including mandelate racemase and muconate lactonizing enzyme, share a common structural motif and abstract the α-proton of a carboxylic acid, generating an enolic intermediate that is subsequently partitioned into different reaction products depending on the architecture of the active site of each protein (*48*−*50*). The existence of a number of large enzyme superfamilies, including the AKRs and enolases, demonstrate that enzymes can share a common structural scaffold but catalyze different overall reactions. Assertions of the "chemistry first" idea have relied solely upon comparative methods between proteins of a given superfamily. By engineering 5β-reductase activity into 3α-HSD, we have provided functional evidence supporting this concept.

On the basis of amino acid sequence comparisons, we identified a single amino acid difference at the active site between the oxidoreductases and the 5β-reductases of the AKR superfamily and proposed that the alteration determines the enzymatic activity of an AKR protein (*7*). This hypothesis was in part true. While the H117E mutant is unlikely to be "the" ancestral AKR, this mutant represents an example whereby 3α-HSD has been de-evolved into a form that uses the same catalytic machinery for two distinct enzymatic activities. This result is consistent with the above model of enzyme evolution. Since rat liver 3α-HSD and rat liver 5β-reductase have only single enzymatic activities (*12*, *20*, *21*), complete conversion of the H117E mutant into a strict 5β-reductase may require further modification of the substrate binding pocket to optimize protein−steroid interactions.

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