Mutagenesis of 3α-Hydroxysteroid Dehydrogenase Reveals a "Push−Pull" Mechanism for Proton Transfer in Aldo−Keto Reductases†

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ABSTRACT: Rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD, E.C. 1.1.1.213, AKR1C9) is a member of the aldo−keto reductase (AKR) superfamily which inactivates circulating steroid hormones. We have proposed a catalytic mechanism in which Tyr 55 acts as a general acid with its p*K* value being lowered by a hydrogen bond with Lys 84 which is salt-linked to Asp 50. To test this mechanism, residues at the active site were mutated and the mutant enzymes (Y55F, Y55S, K84M, K84R, D50N, D50E, and H117A) were purified to homogeneity from an *Escherichia coli* expression system. Spectrophotometric assays showed that mutations of Tyr 55 and Lys 84 gave enzymes that were apparently inactive for steroid oxidation and reduction. All mutants appeared inactive for steroid reduction. The catalytic efficiencies for steroid oxidation were reduced 4−10-fold for the Asp 50 mutants and 300-fold for the H117A mutant. Fluorescence titration with NADPH demonstrated that each mutant bound cofactor unimpeded. Equilibrium dialysis indicated that the competitive inhibitor testosterone formed E·NADH·testosterone complexes only with the Y55F, Y55S, and D50N mutants with K_d values 10-fold greater than those for wild-type. Therefore the loss of steroid oxidoreductase activity observed for the Tyr 55 mutants cannot be attributed simply to an inability to bind steroid. Using a highly sensitive radiometric assay in which the conversion of [^{14}C]-5α-dihydrotestosterone (DHT) to [^{14}C]-3α-androstanediol (3α-Diol) was measured, the rate enhancement (k_{cat}/k_{noncat}) for the reaction was estimated to be 2.6×10^9. Using this assay, all mutants formed steroid product with decreases in an overall rate enhancement of 10^1-10^4. It was found that Tyr 55 made the single largest contribution to rate enhancement. This is the first instance where point mutations in the conserved catalytic tetrad of an AKR yielded enzymes which were still catalytically active. This enabled the construction of pH vs k_{cat} profiles for the reduction of [^{14}C]-5α-DHT catalyzed by the tetrad mutants. These profiles revealed that the titratable group assigned to the general acid (p$K = 6.50 \pm 0.42$) was eliminated in the Y55F and H117A mutants. The pH-independent value of k_{cat} was decreased in the H117A and Y55F mutants, by 2 and 4 log units, respectively. pH vs k_{cat}(app) profiles for the oxidation of [^{3}H]-3α-Diol showed that the same titratable group (p$K = 7.50 \pm 0.30$) was eliminated in both the Y55F and K84M mutants but was retained in the H117A mutant. Since only the Y55F mutant eliminated the titratable group in both the reduction and oxidation directions it is assigned as the catalytic general acid/base. The differential effects of His 117 and Lys 84 on the ionization of Tyr 55 are explained by a "push−pull" mechanism in which His 117 facilitates proton donation and Lys 84 facilitates proton removal by Tyr 55.

Mammalian hydroxysteroid dehydrogenases (HSDs)[1] play pivotal roles in the biosynthesis and inactivation of steroid hormones. In steroid target tissues, HSDs act as molecular switches by converting potent hormones into their cognate inactive metabolites and regulate the occupancy and activation of steroid hormone receptors. Mammalian HSDs belong to two protein superfamilies: the short-chain dehydrogenase/reductases (SDR) (*1*) and the aldo−keto reductases (AKR) (*2, 3*).

Rat and human liver 3α-HSDs and human prostatic 3α-HSDs are members of the AKR superfamily (*4*−*8*). Hepatic 3α-HSDs inactivate circulating steroid hormones (*9*) and are

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[1] Abbreviations: 3α-HSD, 3α-hydroxysteroid dehydrogenase (E.C. 1.1.1.213); AKR, aldo−keto reductase; SDR, short-chain dehydrogenase/reductase; androsterone, 5α-androstan-3α-ol-17-one; androstanedione, 5α-androstane-3,17-dione; 5α-dihydrotestosterone (5α-DHT), 5α-androstane-17β-diol-3-one; 3α-androstanediol (3α-Diol), 5α-androstane-3α,17β-diol; NADH, nicotinamide adenine dinucleotide (reduced form); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form).

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involved in bile acid biosynthesis (*10, 11*). In contrast, prostatic 3α-HSD regulates occupancy of the androgen receptor by converting 5α-dihydrotestosterone (5α-DHT, a potent androgen) to 3α-androstanediol (3α-Diol, a weak androgen) (*12*). Recently, we have shown that human type 2 3α-HSD can perform this function in prostate (*8*). These 3α-HSDs share high sequence identity and structure−function studies performed on one are pertinent to the rest.

Rat liver 3α-HSD (AKR1C9,[2] E.C. 1.1.1.213) was the first HSD identified as a member of the AKR superfamily, and it is the most thoroughly characterized. This enzyme exhibits a bi−bi ordered kinetic mechanism in which NAD(P)(H) binds first and leaves last (*13*). Direct hydride transfer occurs from the C4 position of the nicotinamide ring to the acceptor carbonyl at C3 of the steroid substrate. The reaction is stereospecific, in which the 4-pro-*R* hydrogen is transferred from the A-face of the cofactor to the β-face of the steroid to form a 3α- or axial alcohol (*13*). Hydride transfer is facilitated by polarization of the acceptor carbonyl and because HSDs are nonmetalloenzymes (*14, 15*), this occurs by either an amino acid or water molecule acting as a general acid. The reaction is reversible, so the same group may act as a general base in steroid oxidation.

X-ray crystal structures of several AKRs provide insight into the identity of the general acid. Human and porcine aldose reductase were the first AKR structures determined and revealed an $(\alpha/\beta)_8$-barrel fold (*16, 17*). These structures identified four ionizable residues (Tyr 48, Lys 77, His 110, and Asp 43) in close proximity to the nicotinamide ring of bound $NADP^+$. These residues are positionally conserved in porcine aldehyde reductase (*18*) and a fibroblast growth factor induced AKR (*19*). Rat liver 3α-HSD shares 58% sequence similarity with human aldose reductase, which allowed the crystallographic structure determination of the apo 3α-HSD, the 3α-HSD·$NADP^+$ binary complex and the 3α-HSD·$NADP^+$·testosterone ternary complex by molecular replacement (*20*−*22*). These are the only structures determined for an HSD member of the AKR superfamily. The positional conservation of the tetrad residues Tyr 55, Lys 84, His 117, and Asp 50 indicated a catalytic mechanism in common with other AKRs. The binary complex revealed a water molecule in the proposed anionic binding site, indicating the site potentially occupied by the carbonyl group of a 3-ketosteroid substrate. This water molecule was displaced by the C3-ketone of testosterone in the ternary complex structure, effectively ruling out water as the immediate proton donor.

The optimal position of the conserved Tyr in each of the AKRs suggests that this residue functions as the general acid/base in catalysis. However, the observed pH-dependence for carbonyl reduction reveals an ionizable group with a pK value of ∼7.5−8.5 for most substrates (*23*−*25*). We and others proposed a mechanism for catalysis in which the pK_a of 10.5 for tyrosine was lowered by forming a hydrogen bond with Lys 84 which in turn is salt-linked to Asp 50 (*16, 20, 26*), Figure 1. Site-directed mutagenesis has been performed to test the role of the active site residues in the catalytic

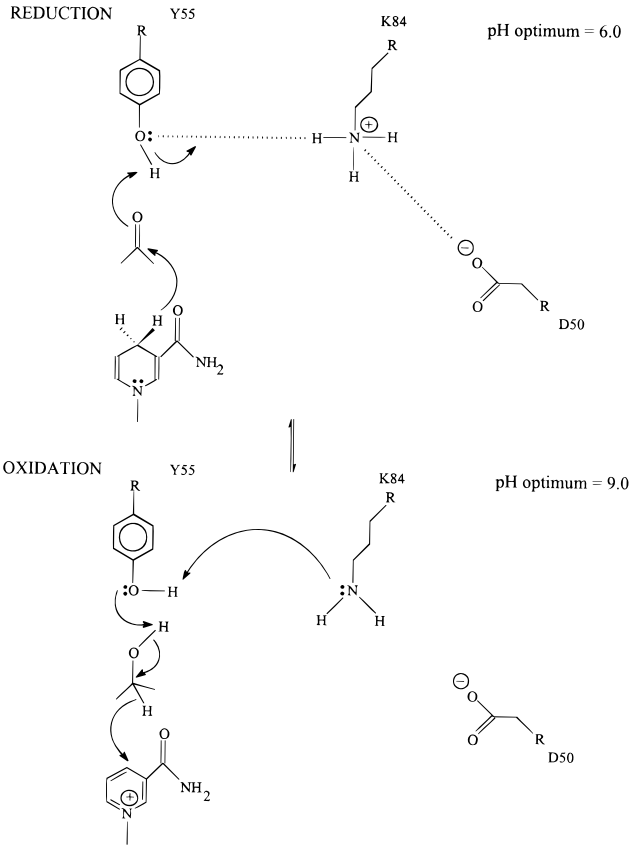

FIGURE 1: Proposed catalytic mechanism for 3α-HSD catalysis.

mechanism of aldose reductase (*24, 27, 28*) and aldehyde reductase (*25*). Mutation of the conserved tyrosine and lysine produced inactive enzymes. However, the ability of these active site mutants to form ternary complexes was not examined. Therefore, it is unclear whether the loss of activity was due to the removal of a catalytic residue or an inability to bind substrate.

In this study, we have performed site-directed mutagenesis on the residues of the catalytic tetrad of 3α-HSD to assess for the first time the individual contribution of each residue to k_{cat} and steroid binding in an AKR family member. Our results indicate that Tyr 55 is essential for steroid oxidation and reduction. Interestingly, all the mutant enzymes were shown to be catalytically active using a sensitive radiometric assay in which [^{14}C]-5α-DHT is reduced to [^{14}C]-3α-Diol. Estimates of rate enhancement (k_{cat}/k_{noncat}) for each of the mutants showed that Tyr 55 is the major contributor to rate enhancement. pH vs k_{cat} profiles support the assignment of Tyr 55 as the general acid/base but in order to achieve this bifunctional role a novel "push−pull" mechanism is proposed. In this mechanism His 117 facilitates proton donation and Lys 84 facilitates proton removal by Tyr 55 by altering the pK_b and the pK_a of this residue, respectively. This mechanism may be applicable to all AKR family members.

EXPERIMENTAL PROCEDURES

Materials. The DNA Synthesis Service in the Department of Chemistry at the University of Pennsylvania synthesized the primers used for PCR-based site-directed mutagenesis. $NADP^+$, NADH, and NADPH were from Boehringer-Mannheim. All steroids were obtained from Steraloids.

[2] The nomenclature for the aldo−keto reductase superfamily was recommended by the 8th International Symposium on Enzymology & Molecular Biology of Carbonyl Metabolism, Deadwood, SD, June 29−July 3, 1996 (*2*).

Radiolabeled [9,11^{3}H(N)]-testosterone (92.5 Ci/mmol), [4-^{14}C]-5α-DHT (58.3 mCi/mmol), and [9,11^{3}H(N)]-3α-Diol (40.0 Ci/mmol) were purchased from NEN DuPont. All other compounds were ACS Grade or better and 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 were previously described, as well as the construction of the Y55F mutant (*26*). Site-directed mutagenesis to produce the Y55S, K84M, K84R, D50N, D50E, and H117A mutant enzymes used the following oligonucleotide primer pairs, respectively: 5′-dGCTTATTTGTCCGAAGTAGAA-3′ and 5′-dTTCTACTTCGGACAAATAAGC-3′; 5′-dTATACTTCAATGCTTTGGAGC-3′ and 5′-dGCTCCAAAGCATTGAAGTATA-3′; 5′-dTATACTTCAAGGCTTTGGAGC-3′ and 5′-dGCTCCAAAGCATCCTTGAAGTATA-3′; 5′-dCGCCATTTTAACTCTGCTTAT-3′ and 5′-dATAAGCAGAGTTAAAATGGCG-3′; 5′-dCGCCATTTTGAGTCTGCTTAT-3′ and 5′-dATAAGCAGACTCAAAATGGCG-3′; and 5′-dTATATTATT*GCT*TTCCCAATGGCT-3′and 5′-dAGCCATTGGGAAAGCAATAATATA-3′; underlined codons indicate the site of the mutation. Dideoxy sequencing ensured fidelity of the mutant constructs. The mutant expression vectors were used to transform competent *Escherichia coli* DH5α cells, and the overexpressed proteins were purified as previously described for wild-type recombinant 3α-HSD (r3α-HSD) (*29*). SDS−PAGE was used to analyze protein purity (*30*), and protein concentrations were determined by the method of Lowry (*31*). Immunoblots on the purified proteins were performed as previously described (*26*).

Steady-State Enzyme Kinetics. Initial velocities were measured on either a Gilford 260 spectrophotometer or a Beckman DU-640 spectrophotometer by observing the rate of change in absorbance of pyridine nucleotide at 340 nm ($\epsilon = 6270$ M^{-1} cm^{-1}) in 1 mL systems at 25 °C using a 1 cm path length. Kinetic measurements were made using a triple 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) at pH 6.0 for steroid reduction and at pH 9.0 for steroid oxidation. Measurements of the K_m and k_{cat} values for androsterone oxidation were made at 2.3 mM NADP$^+$ with varied steroid concentrations (3.0−75.0 μM) in 4% AcN. Kinetic constants for androstanedione reduction were measured at 180 μM NADPH with varied steroid concentration (3.0−30 μM) in 4% AcN. All reactions were initiated by the addition of enzyme and were corrected for nonenzymatic rates. Calculation of all k_{cat} and K_m values used the ENZFITTER nonlinear regression analysis program (*32*) to fit untransformed data to a hyperbolic function (*33*) yielding estimates of the kinetic constants and their associated standard errors.

Determination of Binding Constants for NADPH by Fluorescence Titration. Measurements of the binding constants for NADPH to wild-type r3α-HSD, and the Y55F, Y55S, K84M, K84R, D50N, D50E, and H117A mutants were made by titrating the protein fluorescence on a Perkin-Elmer model 650-10M fluorometer following the incremental addition of NADPH (0−2.5 μM) (*29*). Untransformed fluorescence data were plotted as percent change in fluorescence (%ΔF) at emission λ_{max} (340 nm) vs NADPH concentration. These data were fit to a saturation absorption isotherm by ENZFITTER which provided an estimate of the K_d and the associated standard error. Transformation of these data used the Lineweaver−Burk equation to generate a linear plot of 1/%ΔF vs 1/[NADPH].

Measurement of Binding Constants for Testosterone to the E·NADH Complex. Binding of [^{3}H]-testosterone to the E·NADH binary complex was measured by equilibrium dialysis (*29*) using microdialysis chambers constructed according to Orr et al., 1995 (*34*). Since steroid binding does not occur in the absence of NADH, corrections for nonspecific binding were not necessary. Determination of the K_d values for the binding of [^{3}H]-testosterone to wild-type r3α-HSD and mutant 3α-HSDs were determined by Scatchard analysis using the ENZFITTER program.

Fluorometric Measurement of Androsterone Oxidation. Each 1-mL reaction mixture contained 50 mM triple buffer pH 9.0, 2.3 mM NADP$^+$, and 75 μM androsterone with 4% AcN as cosolvent, and the reaction was initiated by the addition of enzyme. The formation of NADPH was measured by monitoring the fluorescence emission of NADPH at 450 nm (slit-width 5 mm) with excitation at 340 nm (slit-width 5 mm) on a Perkin-Elmer model 650-10M fluorometer at 25 °C. A standard curve was constructed by monitoring the ΔF at 450 nm with incremental additions of NADPH, which permitted rates of fluorescence change to be calculated as nmoles of NADPH formed per min (*35*).

Radiochemical Measurement of 5α-DHT Reduction and 3α-Diol Oxidation. In the reduction direction, the assay system contained 200 μM NADPH and 35 μM [^{14}C]-5α-DHT (40 000 cpm) in a final volume of 100 μL of 50 mM triple-buffer at pH 6.0. In the oxidation direction, the assay system contained 2.3 mM NADP$^+$ and 35 μM [^{3}H]-3α-Diol (100 000 cpm) in a final volume of 100 μL of 50 mM triple-buffer at pH 9.0. These reactions were initiated by the addition of enzyme. All reactions were incubated at 37 °C, and aliquots were removed at various time points. The reaction rate was linear over the time course. Reactions were quenched by the addition of 400 μL of ethyl acetate, and the resulting extracts were evaporated to dryness and redissolved in 40 μL of methanol and applied to LK6D Silica TLC plates. Chromatograms were developed in chloroform: ethyl acetate (4:1 v/v). The positions of 5α-DHT (R_f value = 0.44) and 3α-Diol (R_f value = 0.25) were identified by reference to standards and were visualized by spraying with a 1:1 methanol/H_2SO_4 solution and heating. The amounts of substrate and product were quantitated by scraping the corresponding sections of the TLC plate into a toluene-based scintillation fluid, measuring [^{14}C]- or [^{3}H]-radioactivity with a scintillation counter, and converting the corrected cpm into nmoles of product using the final specific radioactivity of the isotope used in the assay. Extraction efficiency was consistently between 85 and 90% (*36*).

pH-Rate Profiles. k_{cat} and K_m values for either androstanedione reduction or androsterone oxidation were calculated for wild-type r3α-HSD from initial velocities performed over a wide range of pH values using the triple buffer system. This buffer system was used so that the same ions were employed to maintain pH over the entire range studied. This negated concerns that different ions may have differential effects on enzyme activity and ensured that the resultant plots

of log k_{cat} vs pH were continuous. pH rate profiles for [^{14}C]-5α-DHT reduction and [^{3}H]-3α-Diol oxidation catalyzed by wild-type r3α-HSD and tetrad mutants were conducted at a single concentration of substrate (35 μM) using the triple buffer system. This substrate concentration is 10 times K_m for 5α-DHT and 8 times K_m for 3α-Diol observed with wild-type and represents the limit of steroid solubility. Thus the initial velocities measured are the maximal velocities that can be determined and reflect V_{max}(app). The resultant values of V_{max}(app) were normalized and plotted as k_{cat}(app) vs pH. In analyzing these data, estimates of pK_a and pK_b were obtained along with the pH-independent value (*C*) of the measured parameter *Y* (k_{cat}(app)). The best fit of these data to the Cleland programs HABELL, HBBELL, BELL, and WAVL was determined on the basis of the standard error of the fitted parameter, the lowest value of σ, or residual least-squares, and the randomness of the residuals (*37*).

RESULTS

Expression and Purification of Wild-Type and Mutant 3α-HSDs. To test the proposed enzymatic mechanism (Figure 1), we performed site-directed mutagenesis on the four conserved residues implicated in catalysis by the X-ray crystal structures of rat liver 3α-HSD and its complexes. Wild-type and mutant 3α-HSD cDNA constructs were used to transform DH5α *E. coli*. The resultant mutant enzymes were overexpressed and purified to homogeneity to yield 20−40 mg of each mutant. One exception was the Lys 84 mutants which were obtained in quantities of only 3−4 mg. SDS−PAGE analysis confirmed the purity of each enzyme. Immunoblot analysis using rabbit anti-rat 3α-HSD anti-serum (*38*) demonstrated that each mutant was purified to a single immunoreactive species (data not shown). The immunoblots indicated that the correct protein had been purified even when the mutant enzyme had no detectable activity in a spectrophotometric assay.

Kinetic Comparisons for Steroid Oxidoreduction. To assess the effect of each mutation upon reduction and oxidation, the k_{cat} and K_m values for the substrate pair androstanedione and androsterone were determined using NADP(H) as cofactor in a spectrophotometric assay. Each mutant was unable to reduce androstanedione. Only the Tyr 55 and Lys 84 mutant enzymes were devoid of activity in both directions (Table 1). This observation is consistent with the crucial role of these two residues in the proposed mechanism. Comparison of the kinetic constants for androsterone oxidation catalyzed by wild-type r3α-HSD and the D50N mutant showed that the k_{cat} value for the mutant was decreased 10-fold, resulting in an overall 12-fold decrease in the catalytic efficiency. The D50E mutant displayed modest increases in the k_{cat} and K_m values for androsterone oxidation resulting in a 4-fold decrease in the catalytic efficiency. In contrast, the H117A mutant demonstrated a 30-fold decrease in the k_{cat} value and a 10-fold increase in the K_m value resulting in a 300-fold decrease in the catalytic efficiency for androsterone oxidation. Of these two residues His 117 appears to be more important than Asp 50 for steroid oxidation, but neither are essential. The kinetic constants for NADP$^+$ reduction catalyzed by these mutants were also determined, Table 2. The k_{cat} values are consistent with those presented in Table 1. The K_{mNADP^+} values for both D50N and H117A mutants were similar to that for wild-type r3α-HSD. Surprisingly, the K_{mNADP^+} value for the D50E mutant was increased 10-fold. Since we were unable to reach saturation of the D50E mutant with NADP$^+$, the kinetic constants for the cofactor have a larger associated standard error.

Table 1: Kinetic Comparisons between Wild-Type and Mutant Forms of 3α-HSD

	androsterone[a]		
enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
wild type[b]	75 ± 3	6.6 ± 1.1	11.4
D50N	8.3 ± 0.3	8.7 ± 1.0	1.0
D50E	112 ± 6	40 ± 5	2.8
H117A	2.2 ± 0.1	59 ± 5	0.04
	androstanedione[c]		
enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
wild type	32 ± 2	<3[d]	ND
all mutants	ND[e]	ND[e]	ND[e]

[a] Reactions performed at pH 9.0. See "Experimental Procedures" for buffer composition. [b] No detectable rates were measured for androsterone oxidation using up to 100 μg/mL of Y55F, Y55S, K84M, or K84R mutants. [c] Reactions performed at pH 6.0. [d] Accurate determination of the K_m value is not possible since the absorbance changes at substrate concentrations (0.2−3)K_m are too small to measure accurately. [e] Not detectable at 100 μg/mL of enzyme.

Table 2: Kinetic Constants for NADP$^+$ Reduction Catalyzed by Wild-Type and Mutant Forms of 3α-HSD[a]

enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
wild type	69.1 ± 4.5	104 ± 30	0.66
D50N	8.2 ± 0.2	164 ± 13	0.05
D50E	90 ± 20[b]	1140 ± 560[b]	0.08
H117A	1.3 ± 0.1	88.2 ± 18.8	0.02

[a] Reactions performed using 75 μM androsterone at pH 9.0 under the same conditions as described for Table 1. No detectable rates were measured using up to 100 μg/mL of Y55F, Y55S, K84M, or K84R. [b] Did not reach saturation.

Formation of Binary and Ternary Complexes in Mutant 3α-HSDs. We have previously shown that native rat liver 3α-HSD and wild-type r3α-HSD can form E·NADPH and E·NADH·testosterone complexes, but not an E·testosterone complex (*26*). This is consistent with the ordered bi−bi kinetic mechanism in which pyridine nucleotide binds first and steroid binds second. To measure the effect of each mutation on pyridine nucleotide binding the protein fluorescence of each mutant was titrated with NADPH. Each enzyme exhibited a K_d value for NADPH similar to wild-type r3α-HSD (Table 3). This result demonstrated that the catalytic tetrad is not critical for binding NADPH. The X-ray crystal structure of the E·NADP$^+$ binary complex predicts that a total of 12 residues running from the base of the steroid binding pocket to the periphery of the α/β barrel are involved in cofactor binding. The ability of each mutant to bind NADPH unimpeded with nanomolar affinity indicates that the amino acid substitutions made have not resulted in an alteration of this extended pocket and suggests that the global structure of the protein has not been affected by these mutations.

Table 3: Cofactor and Inhibitor Binding Constants for Wild-Type and Mutant Forms of 3α-HSD

enzyme	NADPH[a] K_d (nM)	testosterone[b] K_d (μM)	testosterone[b] relative B_{max}
wild type	190 ± 9	3.2 ± 1.0	1.0
Y55F	120 ± 10	32 ± 18.0	0.38[c]
Y55S	400 ± 13	33 ± 15.0	0.51
K84M	400 ± 57	>50[d]	NE[e]
K84R	180 ± 22	>50	NE
D50N	210 ± 16	30.0 ± 5.5	0.85
D50E	290 ± 13	>50	NE
H117A	180 ± 4	>50	NE

[a] E·NADPH complex measured by fluorescence titration. [b] E·NADH·testosterone complex measured by equilibrium dialysis using [^{3}H]-testosterone. See "Experimental Procedures" for details of both methods. [c] The low B_{max} values observed are due to an inability to reach saturation. [d] No binding detectable at the limit of testosterone solubility. [e] Not estimated.

Binding of testosterone (a competitive inhibitor) to the E·NADH complex of each mutant was also measured by equilibrium dialysis and revealed that the catalytic tetrad was essential to maintain steroid binding affinity (Table 3). The binding of testosterone could not be detected in the Lys 84, His 117, and D50E mutants suggesting a $K_d \gg 50$ μM. In contrast the binding of testosterone was detectable in the D50N enzyme, the K_d value was increased 10-fold relative to wild-type r3α-HSD. Both Tyr 55 mutants bound testosterone with a 10-fold increase in the K_d value. In the Y55F, Y55S, and D50N enzymes the B_{max} values were low relative to wild-type r3α-HSD due to the inability to reach saturation, and the resultant K_d values have large associated standard errors. This 10-fold change in K_d is insufficient to explain the complete loss of enzyme activity in the Tyr 55 mutants. The Tyr 55 mutants were the only enzymes that were able to form both binary and ternary complexes that were devoid of enzyme activity in both directions in the spectrophotometric assays.

Measurement of Steroid Oxidoreduction in Mutant 3α-HSDs. The spectrophotometric assay used to measure enzyme activity has a limit of detection of 1 nmol/min. Since it was possible that the Tyr 55 and Lys 84 mutants were still catalytically active but exhibited enzymatic rates below the sensitivity of this assay, we employed more sensitive fluorometric (*35*) and radiometric assays (*36*). In the fluorometric assay, which uses androsterone and $NADP^+$ as substrates, the production of NADPH was monitored by exciting the sample at 340 nm and measuring fluorescence emission at 450 nm. This method provided a 100-fold increase in sensitivity relative to the spectrophotometric assay. Mutation of Asp 50 and His 117 gave active enzymes with low specific activities relative to wild-type r3α-HSD (Table 4), and these values are consistent with the k_{cat} values presented in Table 1. The Y55S and K84M mutants were once again inactive using this fluorometric assay. Using this assay the Y55F mutant was found to possess less than 0.04% of wild-type r3α-HSD activity.

A radiometric assay that measured the conversion of [^{14}C]-5α-DHT to [^{14}C]-3α-Diol was also employed. This assay is 2500-fold more sensitive than the spectrophotometric assay and allows direct observation of the formation of steroid product. Unlike androstanedione reduction, conversion of 5α-DHT to 3α-Diol was detectable in all the tetrad mutants

Table 4: Specific Activities for Steroid Oxidation and Reduction Catalyzed by Wild-Type and Mutant Forms of 3α-HSD and Estimated Rate Enhancement for 5α-DHT Reduction

enzyme	activity (nmol min^{-1} mg^{-1}) androsterone[a]	activity (nmol min^{-1} mg^{-1}) 5α-DHT[b]	5α-DHT reduction rate enhancement (k_{cat}/k_{noncat})
wild type	1585	766	2.6×10^9
Y55F	0.66	0.13	4.4×10^5
Y55S	ND[c]	0.05	1.7×10^5
K84M	ND	0.45	1.5×10^6
K84R	21.7	50.2	1.6×10^8
D50N	167	24.6	8.3×10^7
D50E	941	14.3	4.8×10^7
H117A	36.6	5.5	1.9×10^7

[a] Activity determined by fluorometric assay described under "Experimental Procedures". [b] Activity determined by radiochemical assay as described under "Experimental Procedures". [c] Not detectable.

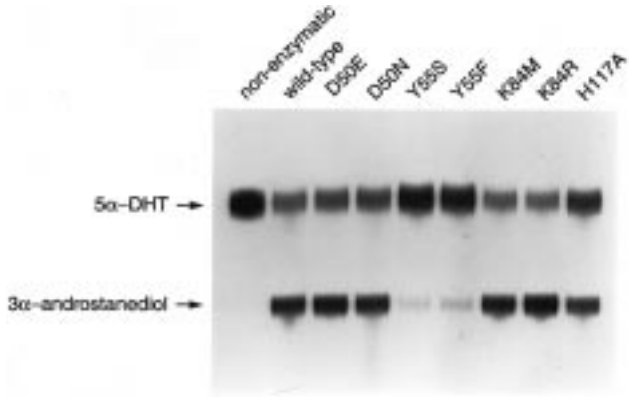

FIGURE 2: Radiochemical assay of 5α-DHT reduction catalyzed by wild-type and mutant forms of 3α-HSD. 35 μM [^{14}C]-5α-DHT, 200 μM NADPH and enzyme were incubated in 50 mM triple buffer buffer, pH 6.0, at 37 °C. Samples were extracted, resolved by TLC, and visualized by autoradiography. Key: (nonenzymatic) no enzyme, 2 h incubation; (WT) 3.0 μg, 5 min; (D50E) 7.4 μg, 10 min; (D50N) 7.8 μg, 10 min; (Y55S) 30.9 μg, 60 min; (Y55F) 26.2 μg, 60 min; (K84M) 28.4 μg, 60 min; (K84R) 19.8 μg, 60 min; (H117A) 13.0 μg, 10 min.

(Figure 2), indicating that steroid reduction had not been completely abolished. The Y55F, Y55S, and K84M mutant enzymes each possessed significantly less than 1% of wild-type activity, affirming the importance of both these residues to catalysis (Table 4). This is the first instance where product formation has been observed in an AKR after the active site tyrosine and lysine residues have been mutated.

Estimates of k_{cat} vs k_{noncat}. Since all of the mutant enzymes could still be robust catalysts relative to the noncatalyzed rate, the radiochemical assay was used to provide estimates of rate enhancement (k_{cat}/k_{noncat}). In this assay the specific activities for each enzyme were measured in the presence of 35 μM 5α-DHT. This substrate concentration is considerably greater than the K_m of 2.0 μM for wild-type r3α-HSD and represents the limit of steroid solubility so that the resultant specific activities are a reflection of the maximal attainable velocities and were used to compute k_{cat}(app). Using this assay we were unable to detect 3α-Diol formation in the absence of enzyme for up to 20 h. The enzymatic rate for the wild-type enzyme was shown to be linear up to 4 h. The limit of sensitivity for this assay at 4 h (0.4 pmol/min) can then be considered an upper limit for the nonenzymatic rate, where $v = [S]\, k_{noncat}$. Using these estimates for k_{cat} and k_{noncat}, we calculated the rate enhancement for

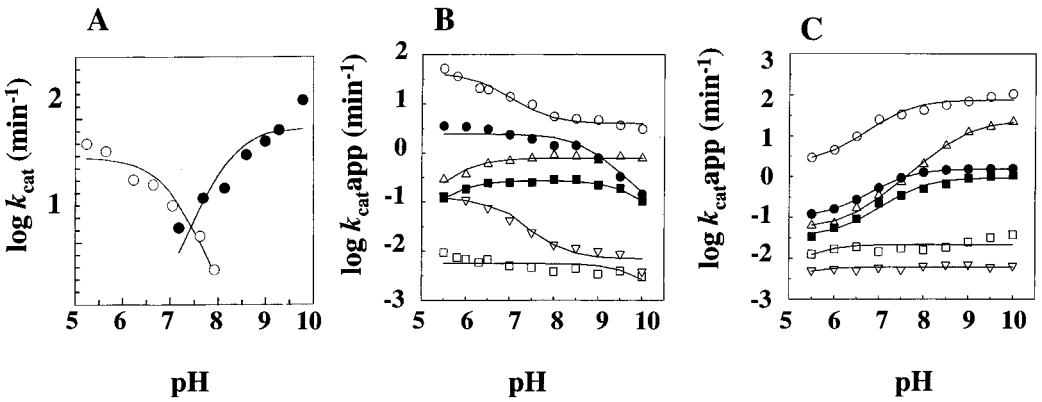

FIGURE 3: (A) Log k_{cat} vs pH for steroid oxidoreduction catalyzed by wild-type and mutant forms of 3α-HSD: androstanedione reduction (○) and androsterone oxidation (●) catalyzed by wild-type enzyme. (B) log k_{cat}(app) vs pH for [^{14}C]-5α-DHT reduction catalyzed by wild-type and tetrad mutants of 3α-HSD: wild type (○), K84R (●), D50N (△), H117A (■), K84M (▽), and Y55F (□). (C) log k_{cat}(app) vs pH for [^{3}H]-3α-Diol oxidation catalyzed by wild-type and tetrad mutants of 3α-HSD: wild-type (○), D50N (△), K84R (●), H117A (■), Y55F (□), K84M (▽). Lines indicate best fit to the equations described by Cleland (*37*).

each mutant (Table 4). The Tyr 55 mutant enzymes exhibited a decrease in rate enhancement of 4 orders of magnitude relative to wild-type r3α-HSD. In contrast, Lys 84 mutants showed a decrease of 3 orders of magnitude in rate enhancement, and the Asp 50 and His 117 mutants showed a decrease of only 2 orders of magnitude in rate enhancement relative to wild-type enzyme. This comparison emphasizes the primary importance of Tyr 55 in the chemical mechanism and suggests that the remaining residues have a facilitatory role.

pH-Rate Profiles for Wild-Type r3α-HSD. Our observation that Tyr 55 is the single largest contributor to rate-enhancement suggested that it may function as the proton donor/acceptor and led us to examine the effect of pH on catalysis more closely. The pH-dependency of steroid oxidoreduction catalyzed by wild-type r3α-HSD was examined using the substrate pair androstanedione and androsterone. The k_{cat} value for androstanedione reduction decreased with increasing pH, revealing an ionizable group with a pK_b value of 7.0 ± 0.11 that must be protonated for maximal activity (Figure 3A). The k_{cat} value for androsterone oxidation increased with increasing pH. Thus androsterone oxidation requires that an ionizable group with a pK_a value of 8.10 ± 0.20 that must be deprotonated for maximal activity (Figure 3A). Although, the pK values for androstanedione reduction and androsterone oxidation differ by almost a full pH unit, the pH vs k_{cat}(app) plots are mirror images of one another and probably reflect the influence of different amino acid residues on the same titratable group. Therefore, an average pK value of about 7.5 was attributed to this group. A similar residue with a pK value of 7.0 for DL-glyceraldehyde reduction has been tentatively identified as Tyr 48 in aldose reductase (*24, 39*), which is analogous to Tyr 55 in 3α-HSD. The K_m value for steroid oxidoreduction catalyzed by wild-type 3α-HSD could not be determined accurately in the triple buffer system over the pH range utilized. This would require using substrate concentrations so low that the resultant absorbance changes would be difficult to detect, and hence k_{cat}/K_m vs pH plots were not constructed.

pH-Rate Profiles on Tetrad Mutants. With the availability of the sensitive radiochemical assay, pH rate profiles were also determined for the reduction of [^{14}C]-5α-DHT and the oxidation of [^{3}H]-3α-Diol catalyzed by the tetrad mutants. It was thought that the mutation which eliminates the titratable group with a significant concomitant decrease in the pH-independent value of k_{cat} would identify the mutated residue as the general acid or base. Furthermore, these studies could provide information on the significance of hydrogen bonds and salt bridges that may exist between tetrad residues and their contribution to the catalytic mechanism. Plots of log k_{cat} vs pH in an ordered bi−bi reaction reflect the pH-dependency of all kinetic steps where as plots of log k_{cat}/K_m vs pH reflect the pH-dependency of the binding of the second substrate (steroid) and the rate of isomerization of the central complex (k_p) (*40*). Estimation of k_{cat} assumes that the initial velocity was measured at saturation. Saturation was achieved with the wild-type enzyme but for some mutants the maximal velocity was that determined at the limit of steroid solubility, i.e., V_{max}(app). Consideration was given to measuring v/S at low substrate concentrations which is effectively equivalent to V_{max}/K_m. However, initial velocities were not measured at $^1/_{10}$th K_m or 0.35 μM 5α-DHT since these could only be obtained if stoichiometric rather than catalytic quantities of mutant enzymes were used and steady-state kinetics would not apply. In the experiments that follow log V_{max}(app) was measured and normalized to log k_{cat}(app).

For [^{14}C]-5α-DHT reduction, it was found that a log k_{cat} vs pH plot for wild-type enzyme gave a titratable group with a pK_b = 6.51, which was similar to the titratable group observed for androstanedione reduction (Figure 3B and Table 5). Importantly, the Y55F, H117A, and D50N mutants eliminated the titratable group observed in wild-type enzyme. Asp 50 is not a candidate for the general acid/base since mutation of this residue had only minor effects on the catalytic efficiency for androsterone oxidation and rate enhancement. This suggests that either Tyr 55 or His 117 may function as the general acid for steroid reduction. The Y55F mutant had a much greater effect on the pH-independent value of k_{cat}(app) than the H117A mutant, suggesting that Tyr 55 is the most important contributor to the rate-limiting step, which is now presumably proton donation. Surprisingly, the K84M and K84R mutants retained the titratable group and catalyzed a pH-dependent reduction reaction. In the K84R mutant the pK_b of the group was shifted to a more basic pH.

Table 5: p*K* Values for k_{cat}(app) Obtained for Substrate Oxidoreduction Catalyzed by Wild-Type and Mutant Forms of 3α-HSD

enzyme	substrate	equation	pK_a	pK_b	C^a
wild type	androstanedione	HBBELL	NA[b]	7.0 ± 0.11	48.9 ± 6.4
wild type	androsterone	HABELL	8.1 ± 0.20	NA	86.0 ± 18.6
wild type	5α-DHT	WAVL	NA	6.5 ± 0.42	42.1 ± 3.2
K84R	5α-DHT	HBBELL	NA	8.8 ± 0.12	2.52 ± 0.30
D50N	5α-DHT	BELL	5.8 ± 0.08	pH-independent	0.78 ± 0.04
H117A	5α-DHT	BELL	5.6 ± 0.05	9.8 ± 0.05	0.27 ± 0.01
K84M	5α-DHT	WAVL	NA	6.8 ± 0.35	0.076 ± 0.03
Y55F	5α-DHT		NA	pH-independent	0.006 ± 0.0005
wild type	3α-androstanediol	WAVL	7.5 ± 0.30	NA	74.5 ± 8.5
D50N	3α-androstanediol	WAVL	9.0 ± 0.15	NA	22.9 ± 2.4
K84R	3α-androstanediol	WAVL	7.4 ± 0.18	NA	1.55 ± 0.09
H117A	3α-androstanediol	WAVL	7.8 ± 0.17	NA	0.93 ± 0.09
Y55F	3α-androstanediol		pH-independent	NA	0.022 ± 0.003
K84M	3α-androstanediol		pH-independent	NA	0.006 ± 0.002

[a] pH independent values for k_{cat}(app) are expressed in units of min^{-1} and for the WAVL fits are the highest values. [b] NA, not applicable.

For [^{3}H]-3α-Diol oxidation, log k_{cat} vs pH plots for wild-type r3α-HSD gave a titratable group with a pK_a = 7.5, similar to that observed for androsterone oxidation (Figure 3C and Table 5). This titratable group was eliminated by the Y55F and K84M mutants, which catalyzed pH-independent reactions yielding k_{cat}(app) values that were reduced by 3.5 and 4 log units, respectively. Importantly, log k_{cat}(app) vs pH plots for the H117A mutant showed the same inflection point as wild-type. Thus His 117 cannot be the general base for steroid oxidation. This observation now only permits His 117 to act as an acid in the reduction direction. As only the Y55F mutants gave log k_{cat}(app) values which were pH-independent in both the reduction and oxidation directions, this argued in favor of this residue acting as both the general acid/ base catalyst.

DISCUSSION

A plethora of kinetic, crystallographic, and mutagenesis data pertaining to the AKR superfamily has led to the proposal that a conserved tetrad of Tyr 55, Lys 84, Asp 50, and His 117 or their equivalent catalyzes a common reaction mechanism. In this mechanism, hydride transfer from the nicotinamide ring is facilitated through polarization of the substrate carbonyl by the conserved tyrosine, which acts as a general acid. It was proposed that the pK_a value of this tyrosine was lowered by a hydrogen bond with the conserved lysine which was salt-linked to an aspartate. Our report is the first in which an HSD of the AKR superfamily has been analyzed by site-directed mutagenesis to assess the role of each residue of the active site tetrad in the binding of NADPH, substrate, and catalysis. This study shows that the mechanism proposed in Figure 1 requires modification. It provides evidence that Tyr 55 acts as the general acid/base using a novel "push−pull" mechanism that utilizes different members of the tetrad to catalyze reduction and oxidation. This mechanism may be applicable to all AKRs.

Initial steady-state kinetic analysis of the tetrad mutants of 3α-HSD using androsterone and androstanedione as substrates in a spectrophotometric assay was in agreement with the proposed mechanism. For steroid oxidation, the Tyr 55 and Lys 84 mutants had no detectable activity while the Asp 50 and His 117 mutants were active. In the reduction reaction, all the mutants were catalytically inactive based on these assays. However, these data could not distinguish between residues involved in nicotinamide cofactor and steroid binding vs those involved in the chemical step.

Effects of Tetrad Mutants on Cofactor Binding. We have previously shown that the rate-limiting step in the ordered bi−bi reaction catalyzed by 3α-HSD is the binding and release of cofactor, and it was conceivable that the tetrad mutants were inactive because this step had been influenced. We discount this argument for several reasons. First, we have independently measured the effect of these mutants on the binding of NADPH at pH 7.0 and shown that there is no effect on K_d for cofactor. At this same pH, the tetrad mutants have a profound effect on k_{cat}(app), indicating that a step other than NADPH binding is being affected. Second, others have shown that in aldo−keto reductases the titratable group responsible for cofactor binding has a pK_b = 9.4 (*23*). Thus plots of $1/K_{dNADPH}$ vs pH gave an inflection point of 9.4. This group is most likely assignable to Lys 262 which is known to interact with the 2′-phosphate of 2′-AMP of the nucleotide (*19*). This titratable group is not evident in the pH vs rate profiles for any of the tetrad mutants. Third, of the tetrad residues mutated only Asp 50 is implicated by the 3α-HSD binary complex structure to be involved in cofactor binding; when this residue was mutated to Glu, there was a 10-fold increase in K_m for NADPH. This effect may be due to disruption of the hydrogen bond that exists between the carboxyl group of Asp 50 and the 2′-hydroxyl group on the ribose of the nicotinamide cofactor, in the binary and ternary complex structures (*21, 22*).

Effect of Tetrad Mutants on Testosterone Binding. Each active site residue is involved in steroid binding as measured by the formation of the E·NADH·testosterone complex by equilibrium dialysis. No significant steroid binding was detected for the Lys 84 and His 117 mutants at the limit of testosterone solubility. This is not true of the Tyr 55 mutants, which were the most catalytically impaired. They retained the ability to form ternary complexes with a 10-fold increase in K_d for testosterone. This 10-fold increase corresponds to a $\Delta\Delta G$ of 1.3 kcal/mol and could represent the loss of the contribution of the hydrogen bond that exists between the C3 ketone of testosterone and the hydroxyl group of Tyr 55 observed in the ternary complex structure (*22*). The decrease in steroid affinity in the tyrosine mutants cannot explain the complete loss of enzyme activity in the spectrophotometric assays. Based on the 3α-HSD·$NADP^+$·testosterone ternary complex (*22*), Asp 50 and Lys 84 do not make any contact

with bound testosterone, yet mutations of these residues affect steroid binding. It is possible that these mutations disrupt the anionic binding site formed by the active site residues and thereby indirectly affect steroid affinity.

There are only two other examples where inhibitor binding was measured in active site mutants of AKR proteins. Alrestatin binding to the Y48F, Y48H, and H110A mutants of aldose reductase was measured by ultrafiltration (*41*). Significant binding was detectable only for the H110A mutant and not in the Tyr 48 mutants. In another study, binding of the noncompetitive inhibitor AL1576 to the Y49H mutant of aldehyde reductase was described (*25*). Interpretation of these experiments on aldose and aldehyde reductases are clouded by several problems. Only single inhibitor concentrations were used, making calculation of K_d or B_{max} values virtually impossible. Also, alrestatin and AL1576 are not structurally analogous to substrates of these proteins and so may not reflect true effects on substrate binding caused by the active site mutations. The current study measuring testosterone binding to mutant 3α-HSDs provides a more detailed account of the contribution of each residue to steroid and hence substrate binding.

Effects of Tetrad Mutants on Rate Enhancement. To verify that the tetrad mutants of 3α-HSD were inactive, we used more sensitive fluorometric and radiometric assays. Surprisingly, in the fluorometric assay only the Y55S and K84M mutant enzymes failed to give a detectable rate, and in the radiometric assay all the active site mutants were able to convert [^{14}C]-5α-DHT into [^{14}C]-3α-Diol. This is the first instance where product formation has been directly demonstrated in point mutants of the catalytic tetrad of an AKR. Several other groups have reported an extremely low enzymatic rate when the AKR active site tyrosine was mutated to a phenylalanine, serine, or histidine (*24, 28*) or when the lysine was mutated to methionine (*27*). In many cases, mutant enzymes with low catalytic rates relative to wild-type were considered "inactive". Our ability to measure steroid reduction catalyzed by the tetrad mutants demonstrates that this is clearly not the case.

As each tetrad mutant may be capable of catalyzing significant rate enhancement over the noncatalyzed rate we compared the k_{cat}/k_{noncat} ratios for each mutant. This comparison indicated that each residue contributed to the overall rate enhancement. We estimated that Tyr 55 is the most important contributor to rate enhancement and donates a factor of 10^4. At least a factor of 10 comes from steroid binding, and a factor of 10^2 is expected from a general acid catalyst (*42*). Thus, assuming that Tyr 55 is the catalytic residue, we can account for 10^3 of the rate enhancement assigned to this residue. The rate enhancement (10^5) that remains when Tyr 55 is eliminated is still substantial and may be due to a propinquity effect brought about by the proper positioning of the nicotinamide cofactor and steroid substrate. Mutation of Asp 50, Lys 84, and His 117 all affect steroid binding, and these mutations are accompanied by decreases in rate enhancement of 10^2-10^3. It is predicted that double and triple mutants of the catalytic tetrad will be required to completely abolish enzymatic rate enhancement.

Evidence That Tyr 55 Acts as the Catalytic General Acid in a "Push−Pull" Mechanism. For wild-type 3α-HSD, similar pH-rate profiles were observed for the androstanedione/androsterone substrate pair (determined spectrophotometrically) and for the 5α-DHT/3α-Diol substrate pair (determined radiometrically). The pK_b values determined for 3α-HSD are similar to those reported for glyceraldehyde reduction catalyzed by aldose and aldehyde reductases (*24, 25*) and for *p*-chlorobenzaldehyde reduction catalyzed by aldose reductase (*23*). Likewise, the pK_a value for steroid oxidation by 3α-HSD is similar to those determined for the oxidation of chlorobenzyl and benzyl alcohols in aldose reductase (*23*). These results suggest that the conserved tetrad in 3α-HSD, aldose reductase, and aldehyde reductase catalyze a common reaction mechanism with a similar pH-dependencies. The availability of a radiometric assay which can detect activity in tetrad mutants of 3α-HSD provided us with the unique opportunity to probe the pH-dependence of enzymes which have been considered "inactive."

We assessed the pH-dependence of [^{14}C]-5α-DHT reduction and the pH-dependence of [^{3}H]-3α-Diol oxidation catalyzed by the Y55F, H117A, K84M, K84R, and D50N mutants and report the data as log k_{cat}(app) vs pH. If plots of pH vs k_{cat}(app) are a measure of the pH-dependency of all steps in the reaction, then we can rule out effects on the on and off rates for NADPH since the K_d for the cofactor is unaffected in the mutants at pH 7.0. In contrast k_{cat}(app) is significantly altered by the tetrad mutants at this pH. Furthermore, based on the binary complex structure Tyr 55, His 117, and Lys 84 are not involved in cofactor binding. This leaves only two terms that could affect the k_{cat}(app) vs pH plot, either the K_d for steroid (which was measured independently by equilibrium dialysis) must be increased or the k_p step must be decreased. Effects on steroid binding are in general ruled out. The tetrad mutants Y55F, Y55S, and D50N only resulted in a 10−30-fold increase in K_d for testosterone. Further, all mutants were able to catalyze the reduction of 35 μM 5α-DHT. To observe enzymatic rates in this concentration range suggests that significant binding of steroid still occurred even though saturation was not achieved. These effects are insufficient to account for the 2−4 log unit decreases in the pH-dependency of k_{cat}(app) observed in the Tyr 55, His 117, and Lys 84 mutants and suggests that their major effect is on k_p. With this interpretation, our data support the presence of a single titratable group acting as the general acid/base; however, this bifunctionality is dependent upon contributions from different residues of the tetrad and suggests a new mechanism (Figure 4a).

In the reduction reaction, the Y55F mutant eliminates the titratable group with an inflection point of 6.5 and the pH-dependence of the reaction. This mutant showed the largest effect on the pH-independent value of k_{cat}(app) which is decreased by 4-log units. Both of these effects are consistent with its proposed function as the proton donor in the reduction reaction. The H117A mutant also eliminates the titratable group with a concomitant decrease in the pH-independent value of k_{cat}(app) which is lowered by 2 log units suggesting that the protonation state of this residue is an important element of the reduction mechanism.

In AKR structures, His 117 has been modeled in one of two conformations. In the first conformation, the N_ϵ of the histidine points into the substrate binding pocket and is clearly capable of interacting with the oxygen of a potential substrate or a bound inhibitor at a distance of about 3 Å and is unlikely to form a hydrogen bond with the tyrosine (*16,*

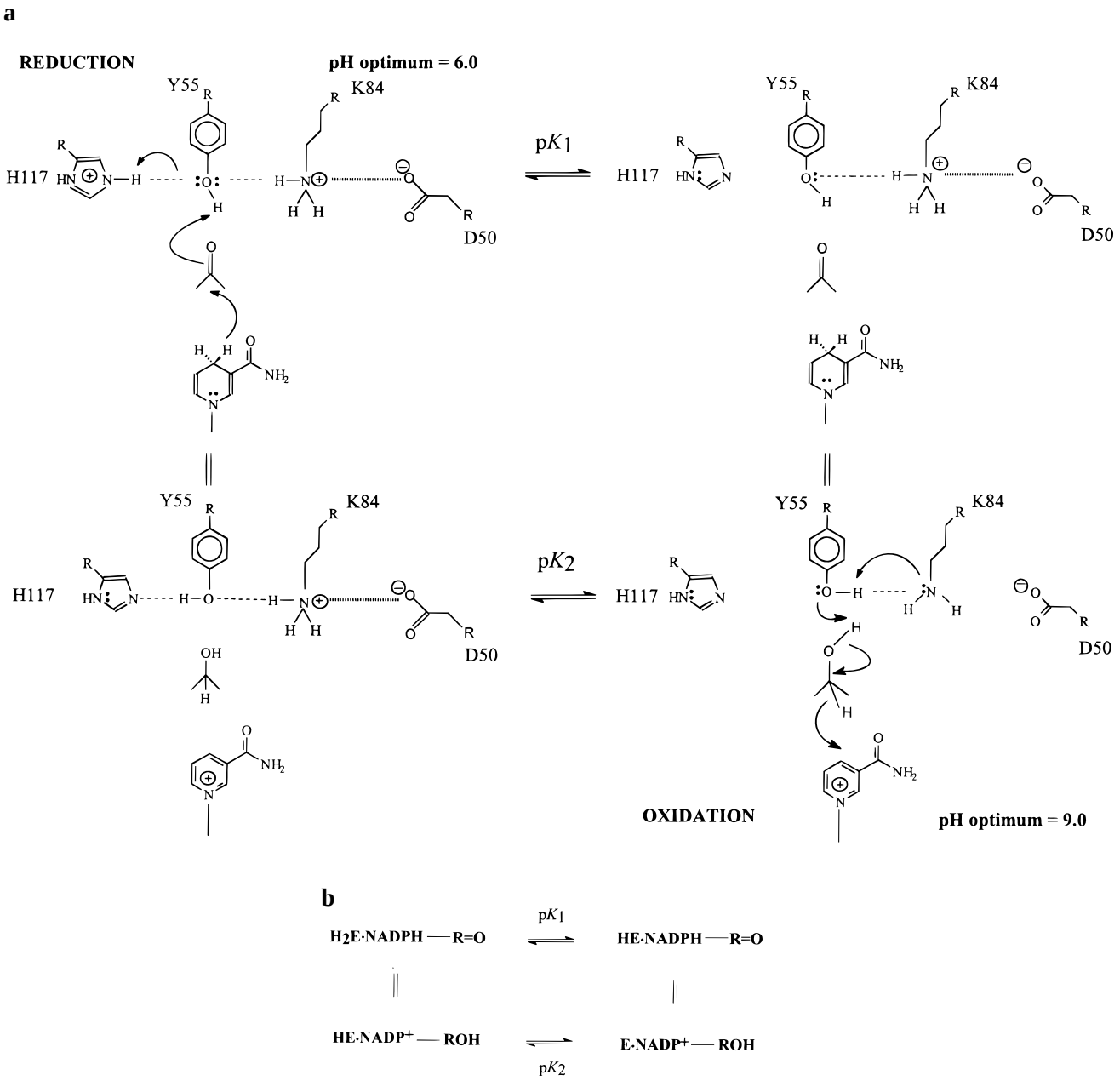

FIGURE 4: Revised catalytic mechanism for AKR catalysis. (a) Chemical steps: (reduction) Tyr 55 acting as a general acid by donating its proton to the acceptor carbonyl of the steroid, facilitated by protonation of the tyrosyl hydroxyl group by the imidazole ring of His 117 to yield a group with a pK_b; (oxidation) Tyr 55 acting as a general base by using its phenolate anion to abstract a hydrogen from the steroid alcohol, facilitated by deprotonation of the tyrosyl hydroxyl group by the ϵ-amino group of lysine, to yield a group with a pK_a. (b) Enzyme species involved in the reduction and oxidation reactions.

21). However, in the alternate orientation (*43*), the histidine N_ϵ is angled more toward the tyrosine than the oxygen of bound inhibitors (citrate, cacodylate, and glucose-6-phosphate) and could form a hydrogen bond with the hydroxyl group of Tyr 55. Thus it is unclear whether His 117 interacts with the oxygen of a bound substrate or inhibitor or the hydroxyl group of Tyr 55.

In the pH-rate profiles for the reduction reaction, the K84M and K84R mutants do not eliminate the titratable group. The overall p*K* of this group does shift to a more basic pH value with the K84R mutant, suggesting that Lys 84 can influence the p*K* of Tyr 55. Since the inflection point is retained in the K84M mutant, which is incapable of shuttling a proton, it is unlikely that a proton is shuttled between Tyr 55 and Lys 84 as originally proposed. When it is considered that the H117A mutant eliminates the titratable group and lowers the pH-independent value of k_{cat}(app) by 2 log units, the reduction reaction would appear to be dependent upon a proton shuttle between Tyr 55 and His 117, Figure 4a. The D50N mutant also eliminates the titratable group with a more modest effect on k_{cat}(app). Asp 50 in the wild-type enzyme would be deprotonated as a carboxylate over the pH-range used for the reduction reactions. In all the AKR crystal structures solved, this residue is involved in a salt bridge with Lys 84 (*17*−*20*). Our results show that mutations of His 117 and Asp 50 can eliminate the pH-dependency of k_{cat}(app), suggesting that the pK_b of Tyr 55 is affected by several tetrad residues.

In the oxidation reaction, the Y55F mutation eliminates the titratable group with a concomitant decrease in the pH-

independent value of k_{cat}(app) of 4 log units. The H117A mutant does not eliminate the titratable group in the oxidation direction indicating that the protonation state of this residue is not important in facilitating catalysis in this direction. In contrast, the K84M mutant eliminates the titratable group with a concomitant decrease in the pH-independent value of k_{cat}(app) of 4 log units. The K84R mutant retains the titratable group with only a 30-fold decrease in the pH-independent value of k_{cat}(app). Thus the pH log k_{cat}(app) profile for the K84R mutant is not too dissimilar from that for wild-type enzyme. The differential effects of the K84M and K84R mutants on the pH-rate profile clearly argue for the presence of a residue in this position for steroid oxidation that can become positively charged. These results indicate that the interaction between Tyr 55 and Lys 84 is an essential component for the oxidation reaction. Since His 117 does not participate in the oxidation reaction, a proton shuttle is proposed between Lys 84 and Tyr 55. This could occur by one of two mechanisms. First, Tyr 55 could exist as a formal phenolate ion which requires a positive charge on the lysine for stabilization. Second, a hydrogen bond between Tyr 55 and Lys 84 may result in sharing of a proton, thereby producing a partial negative character at the tyrosine oxygen which could then abstract the proton from the steroid alcohol with the proton being shuttled to Lys 84, Figure 4a. The D50N mutant showed a shift in the p*K* of the titratable group in the oxidation direction, indicating that the carboxylate character of this residue is important in balancing the positive charge that forms on Lys 84 in the reaction.

As the Y55F mutant is the only enzyme form that eliminates the titratable group in the reduction and oxidation direction, we assign this residue as the general acid/base.[3] The role of Tyr 55 as the catalytic residue is further supported by the findings that Tyr 55 is the largest contributor to rate enhancement, and that Y55F mutants still form binary and ternary complexes. For Tyr 55 to act in a bifunctional manner we invoke a "push−pull" mechanism for steroid reduction and oxidation. In this new mechanism we account for the differential effects on the H117A and K84M mutants on the pH rate profiles. The pH-independent rates catalyzed by the H117A mutants in the reduction direction and the pH-independent rates catalyzed by the K84M mutants in the oxidation direction suggest that His 117 facilitates proton donation and Lys 84 facilitates proton removal by Tyr 55. This is achieved by His 117 and Lys 84 effectively altering the pK_b and pK_a of this residue. The enzyme species involved in these reactions are reversible and this depends upon the ionization of the participating residues which in turn is governed by pH, Figure 4b.

3α-HSD is the only steroid metabolizing enzyme of the AKR superfamily that has been studied in this detail. It is likely that other HSDs of the superfamily will operate this "push−pull" mechanism. Interestingly, the aldose and aldehyde reductases function predominantly as reductases yet they retain the same tetrad. Therefore it is unknown why these reductases do not behave as better bidirectional catalysts. We have also proposed that HSDs that belong to the AKR and short-chain dehydrogenase/reductase (SDRs) families have convergently evolved to retain the same catalytic mechanism, if this is true the "push−pull" mechanism may also be applicable to the SDRs. In a structural comparison of the active sites of AKRs and SDRs we have previously shown that Tyr 55, Lys 84, and His 117 of 3α-HSD are positionally conserved with respect to Tyr 152, Lys 156, and Ser 139 of bacterial 3α,20*β*-HSD (*21*, *44*). If these amino acid residues are functionally conserved then both His 117 and Ser 139 would facilitate proton donation from the catalytic tyrosine. This issue could be investigated by conducting pH-rate profiles on mutants of the conserved Ser in the SDR family.

[3] In a previous preliminary report (*44*) we showed that the Y55F and Y55S mutants catalyze 9,10-phenanthrenequinone reduction at a robust rate. Further examination of the substrate specificity of these mutants indicates that the catalytic activity retained is specific for aromatic quinones. This observation will be the focus of a separate manuscript that will deal with retention of a quinone reductase activity in aldo−keto reductase mutants.

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REFERENCES

1. Jornvall, H., Persson, M., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffrey, J., and Ghosh, D. (1995) *Biochemistry 34*, 6003−6013.
2. Jez, J. M., Flynn, T. G., and Penning, T. M. (1997) *Biochem. Pharmacol. 54*, 639−647.
3. Jez, J. M., Bennett, M. J., Schlegel, B. P., Lewis, M., and Penning, T. M. (1997) *Biochem. J. 326*, 625−636.
4. Pawlowski, J. E., Huizinga, M., and Penning, T. M. (1991) *J. Biol. Chem. 266*, 8820−8825
5. Stoltz, A., Hammond, L., Lou, H., Takikawa, H., Ronk, M., and Shively, J. E. (1993) *J. Biol. Chem. 268*, 10448−10457.
6. Deyashiki, Y., Ogasawara, A., Nakayama, T., Miyabe, Y., Sato, K., and Hara, A. (1994) *Biochem. J. 299*, 545−552.
7. Dufort, I., Soucy, P., Labrie, F., and Luu-The, V. (1996) *Biochem. Biophys. Res. Commun. 228*, 474−479.
8. Lin, H. K., Jez, J. M., Schlegel, B. P., Peehl, D. M., Pachter, J. A., and Penning, T. M. (1997) *Mol. Endocrinol. 11*, 1971−1984.
9. Tomkins, G. M. (1956) *J. Biol. Chem. 218*, 437−447.
10. Berséus, O. (1967) *Eur. J. Biochem. 2*, 493−502.
11. Stoltz, A., Takikawa, H., Sugiyama, Y., Kuhlenkamp, J., and Kaplowitz, N. (1987) *J. Clin. Invest. 79*, 427−434.
12. Jacobi, G. H., and Wilson, J. D. (1976) *Endocrinology 99*, 602−610.
13. Askonas, L. J., Ricigliano, J. W., and Penning, T. M. (1991) *Biochem. J. 278*, 835−841.
14. Murdock, G. L., Pineda, J., Nagorsky, N., Lawrence, S. S., Heritage, R., and Warren, J. C. (1986) *Biochim. Biophys. Acta 1076*, 197−202.
15. Levy, M. A., Holt, D. A., Brandt, M., and Metcalf, B. W. (1987) *Biochemistry 26*, 2270−2279.
16. Wilson D. K., Bohren, K. M., Gabbay, K. H., and Quiocho, F. A. (1992) *Science 257*, 81−84.
17. Rondeau, J.-M., Tete-Favier, F., Podjarny, A., Reymann, J.-M., Barth, P., Biellman, J.-F., and Moras, D. (1992) *Nature 355*, 469−472.
18. El-Kabbani, O., Judge, K., Ginell, S. L., Myles, D. A. A., DeLucas, L. J., and Flynn, T. G. (1995) *Nat. Struct. Biol. 2*, 687−692.
19. Wilson, D. K., Nakano, T., Petrash, J. M., and Quiocho, F. A. (1995) *Biochemistry 34*, 14323−14330
20. Hoog, S. S., Pawlowski, J. E., Alzari, P. M., Penning, T. M., and Lewis, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2517−2521.
21. Bennett, M. J., Schlegel, B. P., Jez, J. M., Penning, T. M., and Lewis, M. (1996) *Biochemistry 35*, 10702−10711.

22. Bennett, M. J., Albert, R. H., Jez, J. M., Ma, H., Penning, T. M., and Lewis, M. (1997) *Structure 5*, 799−812.
23. Liu, S.-Q., Bhatnagar, A., and Srivastava, S. K. (1993) *J. Biol. Chem. 268*, 25494−25499.
24. Bohren, K. M., Grimshaw, C. E., Lai, C.-J., Harrison, D. H., Ringe, D., Petsko, G. A., and Gabbay, K. H. (1994) *Biochemistry 33*, 2021−2032.
25. Barski, O. A., Gabbay, K. H., Grimshaw, C. E., and Bohren, K. M. (1995) *Biochemistry 34*, 11264−11275.
26. Pawlowski, J. E., and Penning, T. M. (1994) *J. Biol. Chem. 269*, 13502−13510.
27. Tarle, I., Borhani, D. W., Wilson, D. K., Quiocho, F. A., and Petrash, J. M. (1993) *J. Biol. Chem. 268*, 25687−25693.
28. Carper, D. A., Hohman, T. C., and Old, S. E. (1995) *Biochim. Biophys. Acta 1246*, 67−73.
29. Jez, J. M., Schlegel, B. P., and Penning, T. M. (1996) *J. Biol. Chem. 47*, 30190−30198.
30. Laemmli, U.K. (1970) *Nature 227*, 680−685.
31. Lowry, O. H., Rosebrough, N. J., Farr, A. J., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
32. Leatherbarrow, R. J. (1987) *ENZFITTER: A Non-Linear Regression Data Analysis Program for the IBM PC (and true compatibles)*, BioSoft, Cambridge, UK.
33. Wilkinson, G. N. (1961) *Biochem. J. 80*, 324−332.
34. Orr, A., Ivanova, V. S., and Bonner, W. M. (1995) *Biotechniques 19*, 204−206.
35. Lowry, O. H. (1972) in *A Flexible System of Enzymatic Analysis* (Passonneau, J. V., Ed.) Academic Press, New York.
36. Penning, T. M., Sharp, R. B., and Kreiger, N. R. (1985) *J. Biol. Chem. 260*, 15266−15272.
37. Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−117.
38. Smithgall, T. E., and Penning, T. M. (1988) *Biochem. J. 254*, 715−721.
39. Grimshaw, C. E., Bohren, K. M., Lai, C.-J., and Gabbay, K. H. (1995) *Biochemistry 34*, 14371−14384.
40. Northrop, D. (1982) *Methods Enzymol. 87*, 607−623.
41. Ehrig, T., Bohren, K. M., Prendergast, F. G., and Gabbay, K. H. (1994) *Biochemistry 33*, 7157−7165.
42. Jencks, W. P. (1969) Catalysis in chemistry and enzymology, in *McGraw-Hill Series in Advanced Chemistry*, Vol. xvi, McGraw-Hill, New York, NY.
43. Harrison, D. H., Bohren, K. M., Ringe, D., Petsko, G. A., and Gabbay, K. H. (1994) *Biochemistry 33*, 2011−2020.
44. Penning, T. M., Pawlowski, J. E., Schlegel, B. P., Jez, J. M., Lin, H.-K., Hoog, S. S., Bennett, M. J., and Lewis, M. (1996) *Steroids 61*, 508−523.

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