Retention of NADPH-Linked Quinone Reductase Activity in an Aldo-Keto Reductase Following Mutation of the Catalytic Tyrosine†

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ABSTRACT: Aldo-keto reductases (AKR) are monomeric oxidoreductases that retain a conserved catalytic tetrad (Tyr, Lys, Asp, and His) at their active sites in which the Tyr acts as a general acid−base catalyst. In rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD, AKR1C9), a well-characterized AKR, the catalytic tyrosine is Tyr 55. This enzyme displays a high catalytic efficiency for a common AKR substrate 9,10-phenanthrenequinone (9,10-PQ). Surprisingly, Y55F and Y55S mutants of 3α-HSD reduced 9,10-PQ with high k_{cat} values. This is the first report whereby the invariant catalytic tyrosine of an AKR has been mutated with retention of k_{cat} values similar to wild-type enzyme. The Y55F and Y55S mutants displayed narrow substrate specificity and reduced select aromatic quinones and α-dicarbonyls. k_{cat} versus pH profiles for steroid oxidoreduction catalyzed by wild-type 3α-HSD exhibited a single ionizable group with a pK = 7.0−7.5, which has been assigned to Tyr 55. This group was not evident in the k_{cat} versus pH profiles for 9,10-PQ reduction catalyzed by either wild-type or the Tyr 55 mutant enzymes, indicating that the protonation state of Tyr 55 is unimportant for 9,10-PQ turnover. Instead, wild-type and the active-site mutants Y55F, Y55S, H117A, D50N, K84R, and K84M showed the presence of a new titratable group with a pK_b = 8.3−9.9. Thus, the group being titrated is not part of the tetrad. All the mutants decreased k_{cat}/K_m considerably more than they decreased k_{cat}. Thus, the K84R mutant demonstrated a 30-fold decrease in the pH-independent value of k_{cat} but 2200-fold decrease in the pH-independent value of k_{cat}/K_m. This suggests that all the tetrad residues influence quinone binding and that Lys 84 plays a dominant role in maintaining proper substrate orientation. Using wild-type enzyme, the energy of activation (E_a) for 9,10-PQ reduction was ∼11 kcal/mol less than steroid oxidoreduction. The E_a for 9,10-PQ reduction was unchanged in the Tyr 55 mutants, suggesting that the reaction proceeds through the same low-energy barrier in the wild-type enzyme and these mutants. The retention of quinone reductase activity in this AKR in the absence of Tyr 55 with k_{cat} versus pH rate profiles and activation energies identical to wild-type enzyme suggests that quinone reduction occurs via a mechanism that differs from 3-ketosteroid reduction. In this mechanism, the electron donor (NADPH) and acceptor (*o*-quinone) are bound in close proximity, which permits hydride transfer without formal protonation of the acceptor carbonyl by Tyr 55. This represents a rare example where one enzyme can catalyze the same chemical reaction (carbonyl reduction) by either acid catalysis or by a propinquity effect and where these two mechanisms can be discriminated by site-directed mutagenesis.

Aldo-keto reductases (AKRs)[1] are monomeric NAD(P)(H)-linked oxidoreductases that catalyze the transformation of carbonyl groups to alcohols on diverse substrates including steroid hormones, prostaglandins, aldehydes derived from biogenic amines, and monosaccharides (*1*, *2*).[2] These reactions can result in a dramatic change in the potency of a steroid hormone for its receptor, a change in the biological properties of a prostaglandin, and play a central role in monoamine neurotransmitter and aldose sugar metabolism. Many of these enzymes turnover nonphysiological substrates including aromatic aldehydes and ketones as well as *o*-quinones (*3*−*6*). For example, 9,10-phenanthrenequinone (9,10-PQ), a common substrate for many AKRs, is often turned over with the highest catalytic efficiency (k_{cat}/K_m) (*3*, *6*).

A number of structures have been elucidated for members of the AKR superfamily, including human placental and pig

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[1] Abbreviations: 3α-HSD, 3α-hydroxysteroid dehydrogenase (EC 1.1.1.213, A-face specific and now designated as AKR1C9); AKR, aldo-keto reductase; ADH, alcohol dehydrogenase; ADR, aldose reductase; 9,10-PQ, 9,10-phenanthrenequinone.

[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 (*1*).

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lens aldose reductase (AKR1B subfamily) (*7*, *8*), porcine kidney aldehyde reductase (AKR1A subfamily) (*9*), and rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD, AKR1C9) (*10*, *11*). The known structures adopt an $(\alpha/\beta)_8$-barrel motif suggesting that this topology will be shared by the entire superfamily. In these structures, a tetrad of amino acids is situated at the base of a hydrophobic substrate-binding pocket and is in close proximity to the C4-position of the nicotinamide ring that is involved in hydride transfer. In 3α-HSD, this tetrad is Tyr 55, Lys 84, His 117, and Asp 50. Site-directed mutagenesis on these tetrad residues coupled with extensive pH-rate profiles support the role of Tyr 55 as the general acid involved in 3-ketosteroid reduction (*12*, *13*). Similar mutagenesis studies have assigned an identical function to Tyr 48 in aldose reductase (*5*, *14*, *15*) and Tyr 49 in aldehyde reductase (*16*). We have also shown that Tyr 55 acts as a general base in the oxidation of 3α-hydroxysteroids catalyzed by 3α-HSD. The ability of Tyr 55 to act as both a general acid and base relies on the effects of the adjacent His 117 and Lys 84. In the reduction direction, His 117 facilitates proton donation, and in the oxidation direction, Lys 84 facilitates proton removal by Tyr 55 in a "push−pull" mechanism involving proton transfer (*13*). On the basis of the high degree of conservation at the active site in the AKRs the tyrosine of the tetrad is likely to a play this central role in all AKR catalysis.

Like many other AKRs, 9,10-PQ is reduced by 3α-HSD with a high catalytic efficiency. We now show that 3α-HSD mutants in which Tyr 55 has been altered to phenylalanine or serine retain robust quinone reductase activity using 9,10-PQ as a substrate. A survey of various classes of substrates for 3α-HSD revealed that the substrate specificity of the Tyr 55 mutants was now limited to select *o*-quinones and α-dicarbonyls. We also show that retention of quinone reductase activity in this AKR in the absence of Tyr 55 is accompanied by retention of pH-rate profiles and activation energies characteristic of wild-type enzyme. This suggests that quinone reduction occurs via a mechanism that differs from 3-ketosteroid reduction. This mechanism relies on proximity effects and does not require the formal participation of a catalytic tyrosine. This represents a rare example where the same enzyme catalyzes the same reaction (carbonyl reduction) by either acid catalysis or by propinquity effects and where these mechanisms can be discriminated by site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials and Chemicals

Materials. $NADP^+$ and NADPH were obtained from Boehringer-Mannheim. Androsterone and androstanedione were obtained from Steraloids. 1-Acenaphthenol, acenaphthenequinone, and camphorquinone were purchased from Aldrich. Naphthalene-1,4-dione, menadione, and 9,10-phenanthrenequinone were purchased from Aldrich and recrystallized before use. All other compounds were of ACS grade or better and obtained from Sigma or Aldrich. Naphthalene-1,2-dione was synthesized as previously described (*17*).

Synthesis of 1-Acenaphthenone. 1-Acenaphthenol (200 mg) was dissolved in 75 mL of ether. Jones reagent (CrO_3 in H_2SO_4) was added in a 2-fold molar excess and the reaction was stirred vigorously overnight at room temperature. The course of the reaction was monitored by TLC using benzene:ethyl acetate (1:1 v/v) and visualized by spraying with a 1:1 methanol/H_2SO_4 solution and heating. Once the reaction went to completion, the organic phase was washed three times with H_2O, neutralized with 2 N Na_2CO_3, and washed with saturated NaCl. The sample was dried and recrystallized in nanograde acetone. The identity of the final product was confirmed by high-field proton [^{1}H]NMR. NMR ([^{2}H]-chloroform): 3.82 ppm (2H s CH_2), loss of 5.73 ppm (1H d C<u>H</u>OH), 7.45−8.06 ppm (6H multiplets, aromatic) assigned as 7.45 ppm (1H d C<u>H</u> 6 aromatic), 7.58 ppm (1H m C<u>H</u> 5 aromatic), 7.70 ppm (1H m C<u>H</u> 8 aromatic), 7.81 ppm (1H d C<u>H</u> 4 aromatic), 7.95 ppm (1H d C<u>H</u> 7 aromatic), and 8.75 ppm (1H d C<u>H</u> 9 aromatic).

Enzyme Measurements

Enzymes. The purification of recombinant 3α-HSD (wild-type enzyme) and the mutants Y55F, Y55S, K84M, K84R, D50N, D50E, and H117A to homogeneity from an *Escherichia coli* expression system has been previously described (*13*, *18*).

Steady-State Enzyme Kinetics. All assays were performed on either a Gilford 260 or Beckman DU640 recording spectrophotometer by monitoring the change in absorbance of pyridine nucleotide cofactor at 340 nm (NAD(P)H ϵ = 6270 M^{-1} cm^{-1}) at 25 °C. Kinetic measurements were made using a triple buffer system containing 50 mM sodium phosphate buffer, 50 mM sodium pyrophosphate, and 50 mM (3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid) (AMPSO). This buffer system permits assays to be performed over a wide range of pH values using the same ions and eliminates the concern that changes in buffer composition may have differential effects on enzyme activity. As a result, plots of kinetic constants versus pH are continuous. Kinetic constants for androsterone (3.0−75 μM) oxidation were measured in 1.0 mL systems containing saturating concentrations of $NADP^+$ (2.3 mM) at pH 9.0, and kinetic constants for androstanedione (3.0−30 μM) reduction were determined in 1.0 mL systems containing saturating concentrations of NADPH (180 μM) at pH 6.0. Measurement of k_{cat} and K_m values for 9,10-PQ (1.0−60 μM) reduction were made using 180 μM NADPH. The conditions for measuring the reduction of other substrates are described in the appropriate tables. All reactions were initiated by 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 (*19*) to fit untransformed data with a hyperbolic function (*20*) to yield estimates of the kinetic constants and their associated standard errors.

HPLC Assay for 1-Acenaphthenol and 1-Acenaphthenone Turnonver. Measurement of 1-acenaphthenol oxidation or 1-acenaphthenone reduction by monitoring the absorbance change of the cofactor at 340 nm can be complicated by contributing absorbances from the substrate and product. To circumvent this problem, we developed an HPLC-based assay using the unique UV absorbance spectrum and retention time for each compound. 1-Acenaphthenol displays a λ_{max} at 226 nm, and 1-acenaphthenone exhibits a λ_{max} at 253 nm. Each 1 mL reaction contained 50 mM triple buffer pH 9.0, cofactor

(2.3 mM $NADP^+$ or 180 μM NADPH) and substrate (400 μM 1-acenaphthenol or 1-acenaphthenone) and was initiated by the addition of 100−200 μg of enzyme. Aliquots (150 μL) were removed at various time intervals and the reaction quenched in ether. The samples were vortexed 30 s and extracted 3 times with 2 mL of ether, washed with anhydrous Na_2SO_4, and dried under N_2. Samples were reconstituted in 100 μL of 30% acetonitrile. The samples were separated by reversed-phase HPLC on a Spherisorb ODS2 column (5 micron; 4.6 × 250 mm; PhaseSep) using a 30:70 (acetonitrile: H_2O) mobile phase under isocratic conditions on a Beckman System Gold 125 Programmable Solvent Module connected to a 166 Programmable Detection Module. The samples were eluted with a retention time of 12.8 and 23.1 min for 1-acenaphthenol and 1-acenaphthenone, respectively, with a sensitivity of detection at 50 pmol. Peak areas were calculated and total amounts of substrate and product calculated using a standard curve. Initial velocities remained linear with time up to 4 h.

pH-Rate Profiles. k_{cat} and K_m values were calculated from initial velocity studies over a wide range of pH values using the triple-buffer system. The reactions were initiated by the addition of a small aliquot of enzyme stored in 100 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM β-mercaptoethanol, and 20% glycerol. No significant change in the pH of the reaction mixture was evident before or after the addition of enzyme. Data which showed a decrease in the measured parameter at high pH were fit to HBBELL (eq 1), and data which demonstrated a decrease in the measured parameter at low pH were fit to HABELL (eq 2):

$$\log Y = \log[c/(1 + K_b/H)] \quad (1)$$

$$\log Y = \log[c/(1 + H/K_a)] \quad (2)$$

Data in which the measured parameter decreased at both low and high pH were fit to BELL (eq 3):

$$\log Y = \log[c/(1 + H/K_a + K_b/H)] \quad (3)$$

In these experiments, Y represents the measured parameter, K_a and K_b are the dissociation constants for the ionizable group being titrated, and c represents the pH-independent value of Y. The best fit of the data was determined on the basis of the standard error of the fitted parameter, the lowest value of sigma, or residual least-squares, and the randomness of the residuals (*21*).

Energy of Activation (E_a). k_{cat} values were calculated for androsterone oxidation and for androstanedione and 9,10-PQ reduction from initial velocity studies over a range of temperatures from 10 to 40 °C. Reactions were initiated by the addition of enzyme. The E_a was calculated from the slope of a plot of log k_{cat} versus $1/T$ (K) using the relationship slope $= -E_a/2.3R$, derived from the Arrhenius equation:

$$\log k_2/k_1 = E_a/2.3R(T_2 - T_1/T_2T_1) \quad (4)$$

where k_1 and k_2 are the rate constants measured at T_1 and T_2, respectively, and R is the gas constant (1.98 cal mol^{-1} deg^{-1}). Q_{10} values were calculated from the equation

$$E_a = 2.3RT_1T_2 \log Q_{10}/10 \quad (5)$$

where Q_{10} is the change in activity over a 10 °C range.

Table 1: Kinetic Constants for 9,10-Phenanthrenequinone 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	171 ± 6	2.0 ± 0.2	86
Y55F	87 ± 8	14 ± 4	6.2
Y55S	87 ± 5	26 ±3	3.3
K84M	ND[b]	ND	ND
K84R	ND	ND	ND
D50N	0.7 ± 0.2	56 ± 31	0.01
D50E	0.9 ± 0.1	12 ± 3	0.08
H117A	2.3 ± 0.3	44 ± 10	0.05

[a] Reactions performed at pH 6.0. [b] Not detectable using 100 μg/mL of enzyme.

The $\Delta G^{\#}$, $\Delta H^{\#}$, and $\Delta S^{\#}$ for the reactions were calculated as follows:

$$\ln k_{cat} = \ln A - E_a/RT \quad (6)$$

where A is the y-intercept of the Arrhenius plot

$$\Delta H^{\#} = E_a - RT \quad (7)$$

$$\Delta S^{\#} = R \ln\frac{(AN_a h)}{RT} - R \quad (8)$$

where N_a = Avogadro's number

$$\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#} \quad (9)$$

RESULTS

Tyr 55 Mutants Retain the Ability to Reduce 9,10-Phenanthrenequinone. We have previously reported detailed analysis of the following tetrad mutants (Y55F, Y55S, K84M, K84R, D50N, D50E, and H117A) with respect to steroid oxidoreduction and their ability to form binary (E·NADPH) and ternary complexes (E·NADH·testosterone) (*13*). These studies supported the assignment of Tyr 55 acting as the general acid−base catalyst. We subsequently chose to conduct kinetic studies on wild-type enzyme and tetrad mutants using 9,10-PQ as a nonsteroidal substrate since it exhibits a low K_m value for 3α-HSD (2 μM) and concentrations of 20−40 times K_m are easily obtained. Such concentrations are unattainable with steroid substrates. 9,10-PQ also has the highest turnover number of all substrates for 3α-HSD, thereby providing increased sensitivity for detecting activity in these mutants. When 9,10-PQ was examined as a substrate, H117A, D50N, and D50E mutants were shown to be severely impaired with respect to quinone reduction (Table 1), while the K84M and K84R mutants were almost inactive. These data were generally consistent with the limited ability of these mutants to reduce 3-ketosteroids (*13*). Remarkably, the Tyr 55 mutant enzymes proved to be efficient catalysts for quinone reduction. Both Y55F and Y55S mutants exhibited only a modest 2-fold decrease in k_{cat}, and a 10-fold increase in K_m relative to wild-type. This observation is specific for the mutation of Tyr 55, and suggests that either Tyr 55 is not the general acid as originally proposed or that quinone reduction occurs by a different mechanism to steroid oxidoreduction which does not require participation of Tyr 55.

Substrate Specificity of Tyr 55 Mutants of 3α-HSD. To determine whether the Y55F and Y55S mutants could

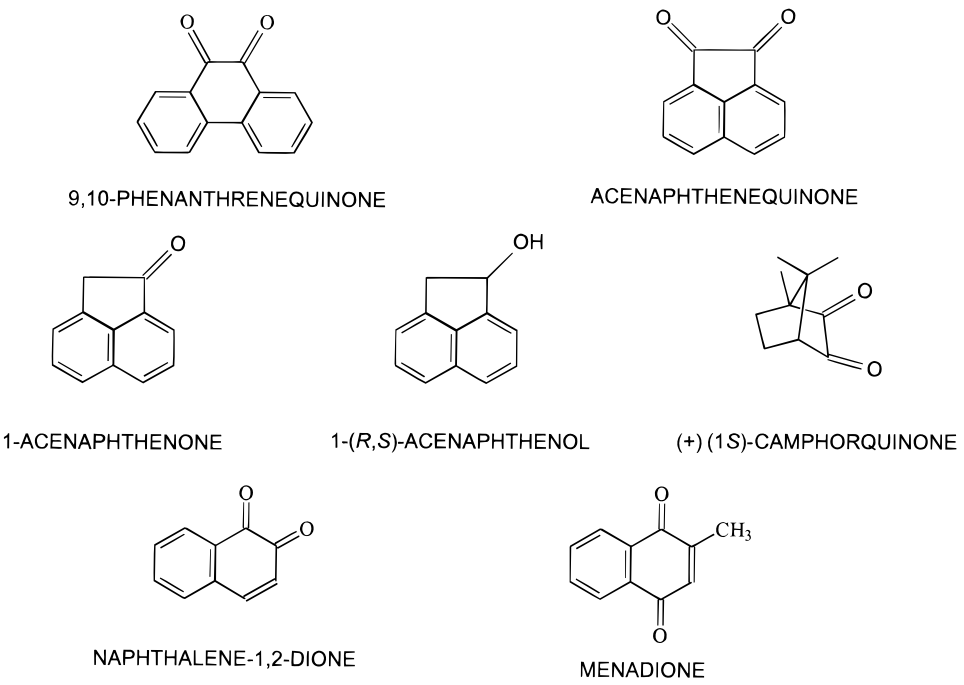

FIGURE 1: Structures of substrates for wild-type 3α-HSD and the Tyr 55 mutants.

Table 2: Specific Activities for the Oxidation and Reduction of Select Substrates Catalyzed by Wild-type and Tyr 55 Mutant Forms of 3α-HSD

substrate	reaction conditions[a]	WT (μmol/min/mg)	Y55F (μmol/min/mg)	Y55S (μmol/min/mg)
9,10-phenanthrenequinone	40 μM, pH 6.0	4.36	1.78 (40.8)[b]	1.55 (35.6)
acenaphthenequinone	100 μM, pH 6.0	3.79	0.61 (16.1)	0.59 (15.6)
(*R*,*S*)-camphorquinone	800 μM, pH 6.0	2.54	0.003 (0.1)	0.02 (0.8)
naphthalene-1,2-dione	40 μM, pH 6.0	0.263	ND[c]	ND
menadione	40 μM, pH 6.0	0.024	ND	ND
naphthalene-1,4-dione	40 μM, pH 6.0	0.047	ND	ND
1-acenaphthenone	400 μM, pH 6.0	0.06	ND	ND
1-acenaphthenol	400 μM, pH 9.0	0.15	0.002 (1.2)	ND

[a] Reactions performed using substrate concentrations indicated and saturating concentrations of NADPH or $NADP^+$. [b] Numbers in parentheses indicate percent of wild-type value. [c] Not detectable.

catalyze the reduction of substrates other than 9,10-PQ, a selection of nonsteroidal substrates were surveyed (Figure 1). This survey failed to identify substrates other than *o*-quinones or α-dicarbonyls that could be turned over by the Tyr 55 mutants in a robust fashion. For example, 4-nitroacetophenone, 4-nitrobenzaldehyde, and dehydroascorbic acid, all of which are nonsteroidal substrates for the wild-type enzyme (*3*, *22*), were not reduced by the Y55F mutant (data not shown). However, substrates similar in structure to 9,10-PQ were turned over to different extents (Table 2). Y55F and Y55S mutants displayed 16.1 and 15.6% of wild-type activity using acenaphthenequinone as a substrate, respectively. This compound is similar to 9,10-PQ in that it has an α-dicarbonyl in conjugation with an aromatic ring system. 1-Acenaphthenone, a compound similar to acenaphthenequinone, except that it contains a single carbonyl, was not a substrate for these mutants. The corresponding alcohol, 1-acenaphthenol, was oxidized at an activity of <0.1% of wild-type when product formation was followed by HPLC. This is consistent with the low activity observed for the oxidation of [^{3}H]androstanediol catalyzed by these mutants (*13*). (*R*,*S*)-Camphorquinone, an α-dicarbonyl which lacks aromatic rings, is turned over efficiently by the wild-type enzyme (*23*) but was not reduced by the Tyr 55 mutants to any substantial degree. Naphthalene-1,2-dione and *p*-quinones such as menadione and naphthalene-1,4-dione which are reduced by native enzyme were not substrates for the Tyr 55 mutants. This narrow substrate specificity illustrates the need for a α-dicarbonyl functionality in conjugation with other aromatic rings. However, if the α-dicarbonyl is on a terminal benzo-ring, these compounds are not substrates, e.g., naphthalene-1,2-dione. This unique substrate specificity for the Y55F mutant suggests that the enzyme has been mutated to retain a NADPH-linked quinone reductase activity for select quinones.

pH Dependence of 9,10-PQ Reduction Catalyzed by Wild-Type and Mutant Forms of 3α-HSD. Our observation that mutation of Tyr 55, the putative proton donor, results in a loss of 3-ketosteroid reductase activity but retention of 9,10-PQ reductase activity, led us to reexamine the effect of pH on catalysis. In an ordered bi−bi mechanism, k_{cat} reflects all the steps in the reaction and is governed by the rate-determining step whereas k_{cat}/K_m can reflect steps involved in binding the second ligand and the chemical step. In previous work, k_{cat} versus pH profiles obtained with tetrad mutants revealed that only the Y55F mutants eliminated the titratable group ($pK_b = 7.0$ and $pK_a = 7.5$) for steroid reduction and oxidation. The loss of the titratable group was accompanied by a 10000-fold decrease in the pH-independent value of k_{cat}. In the reduction direction, the titratable group

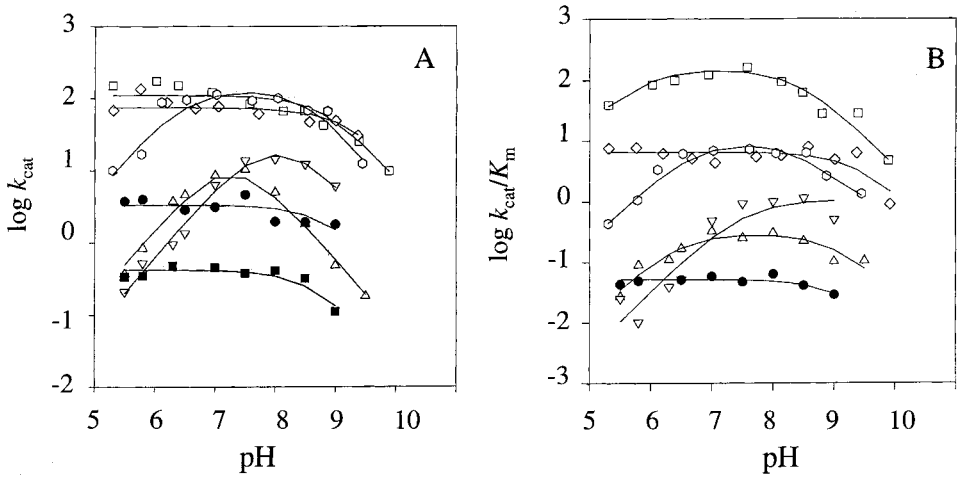

FIGURE 2: pH dependence of 9,10-PQ reduction catalyzed by wild-type 3α-HSD. Lines indicate best fit to eqs 1, 2, or 3. (A) log k_{cat} versus pH and (B) log k_{cat}/K_m versus pH plots of 9,10-PQ reduction catalyzed by wild-type enzyme (□) and Y55F (◇), Y55S (⬡) D50N (▽), K84R (●), K84M (■), H117A (△) mutants of 3α-HSD.

was also eliminated in the H117A mutant, and in the oxidation direction, the titratable group was also lost in the K84M mutant. It was proposed that His 117 facilitated proton donation and Lys 84 facilitated proton removal by Tyr 55 in a "push−pull" mechanism (*13*). These data were interpreted in terms of the chemical step since none of the tetrad mutants adversely affected the K_d for NADPH, and the changes in steroid affinity were between 10 and 30-fold. We estimated that Tyr 55 was the most important contributor to rate enhancement providing a k_{cat}/k_{noncat} of 10^4. At least a factor of 10 was due to its role in steroid binding and a factor of 10^2 was attributed to its role as an acid catalyst (*13*).

In the present study, it was found that the pH dependency of 9,10-PQ reduction catalyzed by the wild-type enzyme was different to that observed for 3-ketosteroid reduction. The k_{cat} value for 9,10-PQ reduction was found to be pH dependent with the maximal rate decreasing with increasing pH but revealed an ionizable group with a pK_b of 8.90 ± 0.10 that must be protonated for maximal activity (Figure 2A). The k_{cat}/K_m value displayed a bell-shaped curve that was pH-dependent with the catalytic efficiency decreasing at both low and high pH, yielding a pK_a value of 5.80 ± 0.50 and a pK_b value of 8.40 ± 0.30, respectively (Figure 2B).

The pH dependency of k_{cat} for 9,10-PQ reduction catalyzed by the Y55F mutant was identical to wild-type. An ionizable group with a pK_b value of 9.90 ± 0.10, that must be protonated for maximal activity, was observed (Figure 2A). Thus, the ionizable group responsible for 9,10-PQ reduction cannot be Tyr 55. The Y55F mutant showed a similar shaped curve for both the k_{cat} and the k_{cat}/K_m versus pH plots, indicating that K_m had become pH independent (Figure 2). The Y55S mutant displayed a k_{cat} versus pH plot that was unique. The k_{cat} value exhibited a bell-shaped curve, with a decrease in activity at both low and high pH, yielding both a pK_a and pK_b (Figure 2A). A nearly identical-shaped curve was obtained for the plot of k_{cat}/K_m versus pH (Figure 2B). The ionizable group with a pK_b = 8.60 ± 0.20, that must be protonated for maximal 9,10-PQ reduction, appears to be the same group found in wild-type enzyme, the Y55F and the Y55S mutants and is not Tyr 55.

The failure to identify an ionizable group for 9,10-PQ reduction that had the same pK_b value as that required for steroid reduction suggests that either the same amino acid is not involved in both reactions or that the same amino acid is involved but it has undergone a shift in its pK_b of 2 log units, possibly because of an increase in substrate "stickiness" (*24*). Further, although the pH dependency of k_{cat} is similar for wild-type and the Y55F mutant, the entire k_{cat}/K_m curve for 9,10-PQ reduction catalyzed by the Tyr 55 mutants is decreased by 1 order of magnitude. Therefore, mutation of Tyr 55 results in an increase in the K_m value for 9,10-PQ reduction. The magnitude of this effect resembles the contribution of Tyr 55 to the binding of steroid substrates (*13*).

In an effort to assign the titratable group necessary for 9,10-PQ reduction, the H117A, D50N, K84M, and K84R mutants were also used to determine k_{cat} versus pH and k_{cat}/K_m versus pH plots. In each instance, a titratable group with a pK_b value of 8.3−9.9 was observed that must be protonated for maximal 9,10-PQ reduction. Further, since this titratable group can be detected in each tetrad mutant, the residue being titrated is not part of the catalytic tetrad.

A summary of pK values and pH-independent values of the measured parameter is presented in Table 3. None of the tetrad mutants affect K_d for NADPH binding; therefore, effects on k_{cat} and k_{cat}/K_m are expected to be similar unless the K_m is significantly altered. Inspection of the data shows that while each of the tetrad mutants decreased k_{cat}, their effects on k_{cat}/K_m are considerably greater. Thus, the K84R mutant demonstrated a 30-fold decrease in the pH-independent value of k_{cat} but a 2200-fold decrease in the pH-independent value of k_{cat}/K_m. Likewise, the K84M mutant showed a 200-fold decrease in the pH-independent value of k_{cat}, but k_{cat}/K_m could not be determined over the dynamic pH range because its value was too low. These data suggest that all of the tetrad mutants affect *o*-quinone binding and that Lys 84 plays a dominant role in determining catalytic efficiency because of its large effect on K_m.

Rate Enhancement for 9,10-PQ Reduction. The unique substrate specificity of the Tyr 55 mutants for aromatic quinones coupled with a difference in the pH dependency of the reaction point to a different mechanism for quinone

Table 3: p*K* Values for the Kinetic Constants Obtained from Substrate Oxidoreduction Catalyzed by Wild-type and Mutant 3α-HSDs

enzyme	substrate	parameter	equation	pK_a	pK_b	C[a]
wild-type	androsterone[b]	k_{cat}	HABELL	8.1 ± 0.20	NA[c]	86.0 ± 19.0
		k_{cat}/K_m		ND[d]	ND	ND
wild-type	androstanedione[b]	k_{cat}	HBBELL	NA	7.0 ± 0.10	49.0 ± 6.4
		k_{cat}/K_m		ND	ND	ND
wild-type	9,10-PQ	k_{cat}	HBBELL	NA	8.9 ± 0.10	110.0 ± 14.0
		k_{cat}/K_m	BELL	5.8 ± 0.50	8.4 ± 0.30	114 ± 19.0
Y55F	9,10-PQ	k_{cat}	HBBELL	NA	9. 9 ± 0.10	77 ± 1.0
		k_{cat}/K_m	HBBELL	NA	9.3 ± 0.30	7.3 ± 2.0
Y55S	9,10-PQ	k_{cat}	BELL	6.5 ± 0.20	8.6 ± 0.40	130.0 ± 1.0
		k_{cat}/K_m	BELL	6.6 ± 0.10	8.6 ± 0.20	10.0 ± 2.0
H117A	9,10-PQ	k_{cat}	BELL	7.0 ± 0.20	7.7 ± 0.10	13.0 ± 3.0
		k_{cat}/K_m	BELL	6.4 ± 0.20	9.0 ± 0.20	0.30 ± 0.05
D50N	9,10-PQ	k_{cat}	BELL	7.7 ± 0.20	8.3 ± 0.30	33.0 ± 14
		k_{cat}/K_m		7.5 ± 0.30		1.1 ± 0.50
K84R	9,10-PQ	k_{cat}	HBBELL	NA	8.9 ± 0.20	3.30 ± 0.40
		k_{cat}/K_m	HBBELL	NA	9.1 ± 0.20	0.05 ± 0.003
K84M	9,10-PQ	k_{cat}	HBBELL	NA	8.7 ± 0.10	0.4 ± 0.04
		k_{cat}/K_m		ND[d]	ND	ND

[a] pH independent values for the indicated kinetic constants. k_{cat} values are expressed in units of min^{-1} and k_{cat}/K_m values are expressed in units of inverse minutes micromolarity. [b] Values taken from ref *13*. [c] Not applicable. [d] K_m values could not be accurately determined over the pH range used.

reduction to that observed with steroid-oxidoreduction. We have observed a small but significant nonenzymatic rate for 9,10-PQ reduction by NADPH. The k_{noncat} for the reduction of 20 μM 9,10-PQ by 180 μM NADPH was found to be 0.02 min^{-1}. This concentration of quinone is sufficient to saturate wild-type enzyme (K_m = 2.0 μM), permitting the calculation of the rate enhancement k_{cat}/k_{noncat} (171 min^{-1}/0.02 min^{-1}) which was found to be 8×10^3. This value should be compared with the rate enhancement observed for the reduction of 3-ketosteroids which is estimated to be 3×10^9 (*13*). These values clearly indicate that the enzymatic reduction of 9,10-PQ is preferred. This preference may be related to a more favorable redox potential. The difference in redox potentials between the $NADP^+$/NADPH couple and the 9,10-PQ/catechol couple is 0.791 V and that between the $NADP^+$/NADPH and the androstanedione/androsterone couple is 0.042 V. Thus, the driving force for 9,10-PQ reduction by NADPH is very much greater than that for steroid reduction by NADPH, and the former reaction is energetically favored (*25*).

Energy of Activation for 9,10-PQ Reduction. To establish whether steroid oxidoreduction or 9,10-PQ reduction had the lowest activation energies (E_a), we constructed Arrhenius plots for these reactions catalyzed by wild-type 3α-HSD and the Tyr 55 mutants (Figure 3). We measured E_a from plots of k_{cat} versus inverse temperature by calculating the slopes of the curves (Figure 3A). According to elementary collision theory, E_a represents the threshold energy a molecule must obtain to participate in a productive reaction. As would be expected for a substrate pair in a reversible reaction, the E_a values for androsterone oxidation and androstanedione reduction were virtually identical (Table 4). The E_a value for 9,10-PQ reduction by wild-type 3α-HSD is 11.2 kcal/mol less than androstanedione reduction. Thus, under identical reaction conditions, the energy barrier that has to be climbed to reach the transition state for 9,10-PQ reduction is less than that for 3-ketosteroid reduction. The temperature dependency exhibited by the Tyr 55 mutants from 10 to 25 °C is nearly identical to wild-type, indicating that in the absence of Tyr 55, quinone reduction proceeds through the same low-energy barrier intermediate observed in wild-type

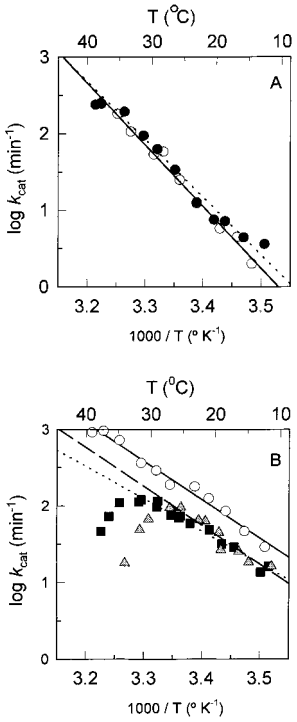

FIGURE 3: Arrhenius plots for substrate oxidoreduction catalyzed by wild-type and Tyr 55 mutants of 3α-HSD. Lines are drawn from a linear regression analysis of the data. (A) log k_{cat} versus 1/*T* plot for androsterone oxidation (open circles, solid line) and androstanedione reduction (filled circles, dotted line) catalyzed by wild-type 3α-HSD. (B) log k_{cat} versus 1/*T* plot of 9,10-PQ reduction catalyzed by wild-type (open circles, solid line), Y55F (filled squares, dotted lines), or Y55S (gray triangles, dashed line) 3α-HSD.

enzyme. The Y55F and Y55S mutants displayed a decrease in activity as the temperature was increased above 30 and 25 °C (Figure 3B), respectively, so these values were not used in calculating E_a. Preincubation of enzyme at 40 °C did not result in a loss of activity at 25 °C, indicating that the temperature-dependent loss of activity was a reversible process (data not shown).

The large difference in E_a between steroid oxidoreduction and 9,10-PQ reduction catalyzed by both wild-type and Tyr

Table 4: Energy of Activation and Thermodynamic Parameters for Oxidoreduction Catalyzed by Wild-Type and Tyr 55 Mutant 3α-HSDs

enzyme	E_a (kcal/mol)	substrate[a]	Q_{10}	$\Delta G^{\#}$ [b] (kcal/mol)	$\Delta H^{\#}$ [b] (kcal/mol)	$\Delta S^{\#}$ [b] (cal/mol deg)
wild-type	33.1	androsterone	6.6	17.5	32.6	50.7
wild-type	34.1	androstanedione	7.1	17.7	33.5	53.0
wild-type	22.9	9,10-PQ	3.7	16.7	22.3	18.9
Y55F	19.9	9,10-PQ	3.2	17.2	18.7	5.03
Y55S	24.2	9,10-PQ	4.2	17.1	23.6	21.8

[a] Reactions performed using 50 mM triple buffer at pH 7.0 using 2.3 mM $NADP^+$ or 180 μM NADPH. [b] $\Delta G^{\#}$, $\Delta H^{\#}$, and $\Delta S^{\#}$ values were calculated at 25 °C.

55 mutants cannot be explained by differences in binding energy for the substrates. Mutation of Tyr 55 produces a 10-fold decrease in binding affinity for testosterone (*12*, *13*). If it is assumed that this mutation would have a similar effect on the K_d for 9,10-PQ, this would account for an increase of 1.3 kcal/mol in $\Delta\Delta G^{\#}$, when in fact a decrease in $\Delta\Delta G^{\#}$ is observed. These data suggest that a different transition state and, hence, a different reaction mechanism must account for the decrease in E_a measured.

We have also used the E_a to calculate the thermodynamic parameters associated with 3-ketosteroid and 9,10-PQ reduction. These calculations indicate that the $\Delta S^{\#}$ for 3-ketosteroid reduction is considerably greater than that observed for 9,10-PQ reduction and that the Tyr mutants do not affect $\Delta G^{\#}$, $\Delta H^{\#}$, or $\Delta S^{\#}$ for quinone reduction. They also indicate that the $\Delta G^{\#}$ for quinone reduction is dependent upon large changes in enthalpy but little change in entropy suggesting that the reaction trajectory is highly ordered.

DISCUSSION

We have described for the first time the retention of substantial enzymatic activity in an AKR containing a mutation of the invariant catalytic tyrosine. Y55F and Y55S mutants can reduce the nonsteroidal substrate 9,10-PQ with only a 2-fold reduction in k_{cat} relative to wild-type enzyme. This suggests that either the mechanism for AKR catalysis, which requires Tyr 55 to act as a general acid−base, is incorrect or that quinone reduction occurs via a different mechanism. Examination of the substrate specificity of the Tyr 55 mutants indicated that they are uniquely specific for select *o*-quinones or α-dicarbonyls in conjugation with aromatic rings. These findings indicate that 3α-HSD has been mutated to eliminate 3-ketosteroid reduction but retains quinone reductase activity.

Of the two choices, it seems that the retention of quinone reductase activity in 3α-HSD is due to the reliance on a different chemical mechanism. We show that wild-type 3α-HSD and the Tyr 55 mutants use the same titratable group (pK_b 8.3−9.9) for 9,10-PQ reduction and that this group cannot be Tyr 55. Once Tyr 55 has been mutated, the tetrad has been disrupted, leaving only three known catalytic amino acids (His 117, Lys 84, and Asp 50). Examination of the k_{cat} versus pH profiles shows that when each of these three residues are mutated, none eliminate the titratable group. Therefore, the titratable group does not belong to the tetrad. These titration curves most closely resemble those reported for plots of $1/K_d^{NADPH}$ versus pH reported for bovine lens aldose reductase where the pH dependency was assigned to the disruption of the electrostatic linkage that exists between the 2′-phosphate of AMP and Lys 262 (*26*). However, this is not the explanation for the k_{cat} versus pH profiles observed for 9,10-PQ reduction because the residue that forms this linkage in 3α-HSD is Arg 276, which has a $pK_a = 12.5$. It is unlikely that the pK_a of this group would be lowered by 4 pH units in the enzyme.

There is also a substantial difference in the E_a required for 3-ketosteroid versus *o*-quinone reduction. The E_a for the latter reaction is 11 kcal/mol less, indicating that the transition state for quinone reduction is more easily attained. Importantly, the Y55F and Y55S mutants yield the same E_a for 9,10-PQ reduction as wild-type enzyme, indicating that catalysis proceeds through a common low-energy barrier and/or transition state in all these enzymes. We have previously shown that Tyr 55 makes the largest contribution to rate enhancement for 3-ketosteroid reduction. This is achieved by this residue acting as both a general acid and by contributing to high-affinity steroid binding. The retention of quinone reductase activity in 3α-HSD following mutation of Tyr 55, with maintenance of pH rate profiles and activation energies characteristic of wild-type enzyme for the reaction argues that (1) quinone reduction does not require Tyr 55 to act as the general acid; (2) quinone reduction occurs via the same mechanism in the wild-type enzyme and tetrad mutants; and (3) this mechanism differs to that observed for 3-ketosteroid reduction.

Quinone reduction catalyzed by native 3α-HSD under aerobic conditions requires an obligatory two electron-transfer event from the NADPH cofactor to the α-dicarbonyl. The acceptor dicarbonyl is considerably more receptive to hydride transfer than a 3-ketosteroid substrate since quinone reduction is accompanied by a substantial nonenzymatic rate whereas 3-ketosteroid reduction is not. In fact, we show that the rate enhancement required for the enzymatic reduction of 9,10-PQ is 8×10^3 whereas that for 3-ketosteroid reduction is 3×10^9. Since hydride transfer occurs without Tyr 55 protonating the acceptor carbonyl, rate enhancement may be achieved in the mutants via a propinquity effect. Comparison of the k_{cat} versus pH and k_{cat}/K_m versus pH profiles for 9,10-PQ reduction support this assertion. With each of the tetrad mutants, the decrease in the pH-independent value of k_{cat} was less profound than the decrease in the pH-independent value of k_{cat}/K_m. This is borne out by the large decreases in the pH-independent value of k_{cat}/K_m for the Lys 84 mutants. These decreases are not because Lys 84 acts as the general acid for quinone reduction. In the structure, Lys 84 is sterically hindered by Tyr 55 and mutation of Lys 84 does not abolish the titratable group observed in quinone reduction. Thus, Lys 84 must have another role. Studies on the K84M and K84R mutants also show that they have a profound effect on the ability to bind

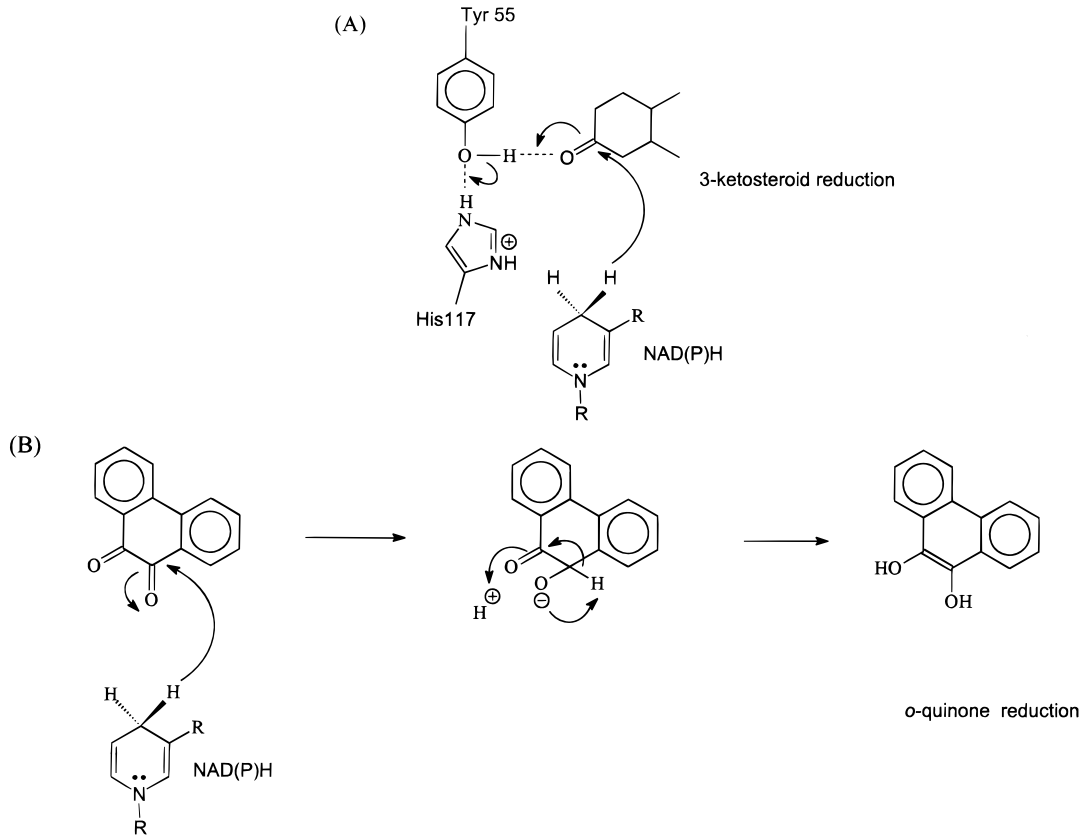

FIGURE 4: The two reaction mechanisms catalyzed by 3α-HSD. (A) 3-Ketosteroid reduction catalyzed by 3α-HSD in which there is a proton relay with Tyr 55 and His 117; (B) *o*-quinone reduction catalyzed by 3α-HSD in which adjacent carbonyls act as hydride ion and proton acceptors.

the competitive inhibitor testosterone (*13*). In each mutant, binding of the steroid ligand was not detectable. Lys 84 forms a salt link with Asp 50, and disruption of this salt link may in turn disrupt the binding site. We postulate that the presence of Lys 84 may be critical in maintaining the structure of the pocket necessary for the propinquity effect.

Three other examples exist where oxidoreductases have retained quinone reductase activity but have lost other catalytic properties. First, frog lens rho-crystallin, a member of the AKR superfamily lacks the catalytic tyrosine (*27*). This natural AKR mutant is an efficient catalyst of 9,10-PQ reduction (*4*) but does not reduce other AKR substrates. In this instance, the catalytic tyrosine is replaced by threonine which could act as a surrogate general acid. In this report, we describe the retention of quinone reductase activity when the catalytic tyrosine is replaced by phenylalanine, a residue incapable of acting as a general acid. The properties of rho-crystallin create a precedent for other structural proteins of the lens that are AKRs to redox-cycle quinones.

Second, ζ-crystallin from guinea pig lens will reduce a variety of aromatic quinones, including 9,10-PQ (*28*). ζ-Crystallin contains 27% sequence identity with the human β_1 liver alcohol dehydrogenase (*29*). Although the NAD^+-binding fold is conserved in ζ-crystallin, the zinc binding motif characteristic of mammalian alcohol dehydrogenases (ADH) is missing. In the absence of zinc, the protein will not function as an ADH but reduction of 9,10-PQ occurs at a robust rate. This substrate specificity resembles that of the 3α-HSD Tyr 55 mutant. In ADH, the only role of zinc is to polarize the acceptor carbonyl for hydride transfer. In 3α-HSD, the role of Tyr 55 is to polarize the acceptor carbonyl and act as the general acid by formally donating a proton to the ketone. Clearly, quinone or α-dicarbonyl reduction can occur in ζ-crystallin and 3α-HSD without the respective contributions of zinc or Tyr 55.

The third example is DT-diaphorase. DT-diaphorase catalyzes the obligatory two electron reduction of quinones using NAD(P)H as cofactor using a "Ping−Pong" mechanism. In this mechanism, there is initial hydride transfer from the C4 of the nicotinamide to the N^5 of FAD. In the second half-reaction, hydride transfer occurs from the N^5 of $FADH_2$ to the acceptor carbonyl on a *p*-benzoquinone, e.g., tetramethyl-1,4-benzoquinone, while the adjacent carbonyl is protonated via a proton relay involving Tyr 155 and His 161. On the basis of the crystal structure of DT-diaphorase, it has been proposed that the carbonyl which accepts a hydride ion is not the same as the carbonyl that is protonated (*30*). Mutation of Tyr 155 to either aspartate or valine results in retention of the quinone reductase activity in DT-diaphorase even though the proton relay is disrupted, suggesting that proton donation to the adjacent carbonyl does not require formal participation from Tyr 155 (*31*). This raises the possibility that α-dicarbonyl reduction catalyzed by the Y55F and Y55S mutants of 3α-HSD may occur via a similar mechanism in which adjacent carbonyls act as hydride ion and proton acceptors, respectively.

On this basis, it would seem that 3α-HSD catalyzes hydride transfer to an acceptor carbonyl by two fundamentally different mechanisms (Figure 4). In the first mechanism, characterized by 3-ketosteroid reduction, a single carbonyl accepts a hydride ion and is protonated. In this mechanism, Tyr 55 polarizes the acceptor carbonyl thereby

facilitating hydride transfer. Tyr 55 also acts as a formal general acid by donating its proton to form the alcohol product. Proton donation is facilitated by a proton relay with His 117. This mechanism is characterized by a large E_a which may be involved in stabilizing a tetrahedral oxyanion intermediate. In the second mechanism, characterized by quinone reduction, adjacent carbonyls act as hydride ion and proton acceptors. Hydride transfer to the α-dicarbonyl of an *o*-quinone occurs as a result of a propinquity effect which lowers the energy barrier as measured by a decrease in E_a. The highly favorable redox potential that exists between the adjacent reactants provides the driving force for the reaction to proceed to completion. The remaining proton may be derived from water. In the first mechanism, the enzyme has to bring the substrates together and orient them toward Tyr 55 so that it can act as a catalytic acid. In the second mechanism, the enzyme only needs to provide a scaffold to bring the cosubstrates together. Jencks has commented on how propinquity effects may contribute more to rate enhancement than the presence of a catalytic acid (*32*). Our studies represent a rare example where one enzyme can catalyze the same reaction (carbonyl reduction) by either acid catalysis or propinquity effects and where these mechanisms can be discriminated by site-directed mutagenesis.

We have also described how mutation of His 117 to Glu 117 in 3α-HSD introduces steroid-double bond reductase (5β-reductase) into this AKR (*33*). 5β-Reductase is a member of the AKR superfamily and in the native enzyme the amino acid equivalent to His 117 has been altered to Glu (*2*). The 5β-reductase activity introduced into 3α-HSD was characterized by k_{cat} values similar to those reported for rat liver 5β-reductase (AKR1D2) (*33*). In the studies reported here, the Tyr 55 mutants also displayed k_{cat} values for quinone reduction similar to those described for the wild-type enzyme. Our findings indicate that point mutations of the catalytic tetrad of AKRs can be used to tailor different catalytic activities into the same protein scaffold and that the resulting activities have significant turnover numbers for the target substrate.

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