Mechanistic Roles of Ser-114, Tyr-155, and Lys-159 in 3α-Hydroxysteroid Dehydrogenase/Carbonyl Reductase from Comamonas testosteroni*  

Chi-Ching Hwang†, Yi-Hsun Chang, Chao-Nan Hsu, Hsien-Hua Hsu‡, Chen-Wei Li, and Hwa-I Pon  
From the Department of Biochemistry, Kaohsiung Medical University, Kaohsiung 807, Taiwan.

3α-Hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) from Comamonas testosteroni, a short chain dehydrogenase/reductase, catalyzes the oxidation of androsterone with NAD⁺ to form androstenedione and NADH. A catalytic triad of Ser-114, Tyr-155, and Lys-159 in 3α-HSD/CR has been proposed based on structural analysis and sequence alignment of the short chain dehydrogenase/reductase family. The 3α-HSD/CR-catalyzed reaction has not been kinetically analyzed in detail, however. In this study, we combined steady-state kinetics, site-directed mutagenesis, and pH profile to explore the function of Ser-114, Tyr-155, and Lys-159 in 3α-HSD/CR-catalyzed reaction. The catalytic efficiency of wild-type and mutants S114A, Y155F, K159A, and Y155F/K159A is 4.3 × 10⁷, 7.3 × 10⁴, 1.7 × 10³, 2.4 × 10⁵, and 71 M⁻¹ s⁻¹, respectively. The values of pK₅ on kcat/Km for the wild-type, S114A, Y155F, K159A, and Y155F/K159A are 7.2, 7.4, 8.4, 9.1, and 10.2, respectively. Mutant S114A/Y155F exhibits a pH-independent profile with 10⁻⁸ times of wild-type activity at pH 10.5. The activity decreases as the pH lowers, which indicates that a functional group with an apparent pK₅ of 7.2 is involved in the general base catalysis for wild-type 3α-HSD/CR. The pK₅ shift to 9.1 for mutant K159A suggests the role of Lys-159 is to lower the pK₅ of the residues involved in the general base catalysis. Because pH dependence is observed for both S114A and Y155F mutants and pH independence is observed in S114A/Y155F, Tyr-155 may be important as a general base catalysis in the wild-type, whereas Ser-114 may act as a general base on mutant Y155F to catalyze the reaction.

3α-Hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) from Comamonas testosteroni catalyzes the stereospecific hydrogen transfer of the 3β-proton of androsterone to NAD⁺ and forms androstenedione and B-side NADH in the for-ward reaction (Fig. 1). 3α-HSD/CR from C. testosteroni is inducible when testosterone and progesterone are present (1, 2), the steroid preventing repressor repA from binding to the regulatory regions (3, 4). The induced enzyme is then involved in the initial stage of the steroid catabolic pathway by oxidating the hydroxyl group at the C3 position, thereby forming a ketone group. This oxidation is followed by isomerization, dehydrogenation, and hydroxylation of the oxidized steroid, which leads to the opening of the B-ring. The complete degradation of the steroid molecule yields CO₂ and H₂O and provides carbon and energy (3).

3α-HSD/CR from C. testosteroni is a member of the short chain dehydrogenase/reductase (SDR) family (5). SDR constitutes a large protein family with a majority of oxidoreductases with NAD(P)⁺ as cofactor (6, 7). Structurally, SDR displays an α/β folding pattern highly similar to a Rossmann fold, which consists of a central β-sheet flanked by α-helices. Although there is little sequence identity within 15–30% between different SDR enzymes, the conserved sequences include an N-terminal Gly-X₃-Gly-X-Gly cofactor binding motif and a triad of catalytically important Ser, Tyr, and Lys residues, of which Tyr is the most conserved in the SDR family. Mechanistically, the catalytic roles of Tyr, Lys, and Ser in the active site in SDR family have been proposed through extensive studies by chemical modification, site-directed mutagenesis, sequence alignment, and structural comparison (6). Mutation of tyrosine to phenylalanine has resulted in a complete loss of the enzyme activity in 7α-HSD (8), human 3β-HSD (9), 11β-HSD (10), 3β/17β-HSD (11, 12), Drosophila alcohol dehydrogenase (13, 14), and 15-hydroxyprostaglandin (15, 16). Mutants of K183R from 11β-HSD and K156I from Drosophila alcohol dehydrogenase have been found to have no enzyme activity, whereas the mutants K163I and K163R from 7α-HSD and K156R from alcohol dehydrogenase have been found to have enzyme activities decreased to 5.3, 64, and 2.2%, respectively. S146A and S146H mutants from 7α-HSD have enzyme activities decreased to 20.3 and 35.6% of kcat of the wild-type. The mutant S138A of 3β/17β-HSD results in an almost complete loss of enzyme activity, whereas substitution of Thr restores the full enzymatic activity with S138T (11), indicating the ability of the side chain of Ser to form a hydrogen bond, an essential factor in the catalysis. The conserved Tyr residue acts as a general base to extract the proton of the hydroxyl group of steroid and facilitate the hydride transfer to NAD⁺. This function of Tyr is assisted by the adjacent amino group of Lys through electrostatic interaction to lower the pK₅ of Tyr. The formation of the carbonyl group in the product is then stabilized by the hydro- gen bond with the hydroxyl group of Ser during the catalysis. In addition to the triad of Tyr, Ser, and Lys, a residue of Asn was found to be important in the catalysis as it is involved in the proton relay system (6, 12).
The structures of the binary complex with NAD\(^+\) cofactor and apoenzyme of 3α-HSD/CR have been solved (3, 17). In the binary complex, the NAD\(^+\) cofactor is bound at the C-terminal ends of the β-strands in the 3α-HSD/CR from C. testosteronae. The orientation of the nicotinamide ring is bound in a syn conformation to facilitate the stereo-specific hydrogen transfer of the 3β-proton of androsterone to nicotinamide adenine dinucleotide (NAD\(^+\)) and forms androstanediol and B-side-reduced nicotinamide adenine dinucleotide (NADH) in the forward reaction (Fig. 1). Kinetically, the 3α-HSD/CR-catalyzed reaction shows an ordered bi bi mechanism with the pyrimidine base and the product to be released (18). The chemical mechanism for the 3α-HSD/CR-catalyzed reaction is based on sequence comparisons and structural analyses in the SDR family (17). A triad of Ser-114, Tyr-155, and Lys-159 in 3α-HSD/CR is conserved within the SDR family. It has been suggested that this triad acts as a catalytic group involved in enzyme catalysis. The functional role of Ser-114, Tyr-155, and Lys-159 in the 3α-HSD/CR-catalyzed reaction, however, has not been analyzed in detail kinetically. To shed more light on the conserved catalytic residues in 3α-HSD/CR and the SDR family, using mutagenesis and kinetic studies we investigated the roles of the triad of Ser-114, Tyr-155, and Lys-159 in the catalytic mechanism of 3α-HSD/CR. Mutants S114A, Y155F, K159A, and double mutants S114A/Y155F and Y155F/K159A were constructed, expressed, and purified. The enzyme activities were measured and compared with the mutants to probe the catalytic role. The acid-base chemistry was studied through pH profiling to evaluate the residues involved in the general base to catalysis.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Mutagenetic replacements were performed using a QuickChange site-directed mutagenesis kit (Stratagene) using *Pfu* polymerase and pet15b-3α-HSD/CR plasmid obtained from Dr. E. Maser (5) as template. The primers used to create the cDNA encoding of the S114A, Y155F, K159A, S114A/Y155F, and Y155F/K159A mutants are shown in Table I. The plasmid S114A/Y155F mutant was constructed with plasmid S114A and Y155F primer. The parental DNA template was digested by DpnI endonuclease, which is specific for methylated and hemimethylated DNA and targets the sequence of 5′-Gm′A/ATC-3′. The resulting nicked vector DNA incorporating the desired mutations was then transformed into DH5α supercomplement cells. The selected colonies were then chosen, and plasmid from DH5α cells was purified. The mutant vectors were used to transform into competent *Escherichia coli* BL21(DE3) cells. All mutants of 3α-HSD/CR gene or mutants were transferred to E. coli BL21(DE3) cells. Recombinant proteins were then overexpressed in BL21(DE3) cells grown in 1 liter of Luria Bertani medium containing 50 mg of ampicillin at 37 °C by adding 0.5 mM isopropyl-1-thio-β-D-galactopyranoside when the culture had reached an optical density of 0.6–1 at 600 nm. After 4 h, the cells were harvested and lysed by sonication. After ultracentrifugation at 8000 rpm for 20 min, the resulting supernatant was loaded into a His-bind nickel-nitrilotriacetic acid resin column. The protein was eluted with a gradient from 0 to 500 mM imidazole. The purity of the protein was confirmed by separation on 15% SDS-PAGE. The protein concentrations were determined by Bradford assay with bovine serum albumin as a standard (19). The dimeric properties of 3α-HSD/CR and the mutants were confirmed through gel filtration chromatography (20). A Sephadex 75 HR 10/30 column was equilibrated with 0.15 M NaCl, 50 mM phosphate buffer, pH 7.0. The sample consisting of 20 μl of protein solution with a concentration of 2 mg/ml was loaded into the column. Elution was carried out at a flow rate of 0.3 ml/min, and the eluted protein was detected by absorbance at 280 nm. The molecular weight of mutants was calculated by a plot of log MW versus elution volume.

The molecular weight of recombinant enzymes was determined by nanoelectrospray ionization/mass spectrometry analysis. Mass spectrometric analysis was carried out on a Quadrupole Time-Of-Flight (Q-Tof) Ultima™ Global (Micromass MS Technologies). For all spectra, a myoglobin solution (10 pmol/μl) was used to calibrate the time of flight. Intact proteins were diluted with 1:1 aconitrile/water containing 0.1% formic acid. The sample was introduced into the mass spectrometer by infusion through a Z-spray nanoelectrospray ion source with a flow rate of 1 μl/min. Data were obtained over an m/z range of 500–2500. The raw combined mass spectra were background subtracted, peak centered, and subjected to Transform Algorithm (MassLynx Software 4.0) to produce reconstructed zero-charge spectra.

The conformations of wild-type and mutant were assessed by circular dichroism spectra by recording the ellipticity as a function of wavelength between 240 and 200 nm using a Jasco J-810 spectropolarimeter. Spectra were obtained by the average of multiple measurements with 2.8 μm enzymes in 10 mM phosphate buffer, pH 7.4. The rate of scan was 10 nm/min between 240 and 200 nm at room temperature.

**Kinetic Studies**—The 3α-HSD/CR-catalyzed oxidation of androstenedione was monitored by the formation of NADH spectrophotometrically at 340 nm. Initial rates were measured at varied concentrations of

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**TABLE I**

| Mutation         | Direction | Nucleotide sequence of primer\(^a\) |
|------------------|-----------|-----------------------------------|
| S114A            | Forward   | 5′-gcgcttctgcgctggctctggctccctggcc-3′ |
| Y155F            | Forward   | 5′-ggcggagaaacccggaggaagcaggagaagc-3′ |
| K159A            | Reverse   | 5′-cattttctgtcagccagcacaagttctggcc-3′ |
| S114A/Y155F\(^b\) | Forward   | 5′-gcgcttctgcgctggctctggctccctggcc-3′ |
| Y155F/K159A      | Forward   | 5′-ggcggagaaacccggaggaagcaggagaagc-3′ |

\(^a\) The boldface codons indicate the mutation on the amino acid residues by the replacement of the underlined codons.

\(^b\) Plasmid S114A/Y155F mutant was constructed with S114A plasmid and Y155F primer.

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**FIG. 1.** 3α-Hydroxy steroid dehydrogenase/carbon reductase-catalyzed reaction.
androstosterone and saturated NAD\(^+\). All reactions were initiated by the addition of enzyme. A typical assay for enzymatic reaction included 1 mM NAD\(^+\), various concentration of androstosterone, 4 \(\mu\)g/ml bovine serum albumin, and 5% methanol in 0.1 M ChEs at pH 9.0. All kinetic assays were thermostatted at 25 °C, and the pH of the assay solution was maintained with 0.1 M of the indicated buffers: Mes, pH 5.5–6.5; Hepes, pH 7.0–7.5; Taps, pH 7.9–8.5; Ches, pH 8.6–9.5; and Caps, pH 10.0–11.3. Data were fitted using Enzfitter software for appropriate rate equations to obtain kinetic parameters. Data for substrate saturation curves at a fixed concentration of the second substrate were fitted using Equation 1, \(v = V_AK_m + A\), where \(v\) and \(V\) represent the initial and maximum velocities and \(K_m\) is the Michaelis constant. The pH dependences of kinetic parameters were fitted using Equation 2, \(log\ v = log(C/H I_{1001})\) for profiles where the log of the parameter decreased at low pH with a unit slope. \(v\) is either \(k_{cat}\) or \(k_{cat}/K_m\). \(C\) is the pH-independent value of the parameter, \(H\) is the hydrogen ion concentration, and \(K_c\) is the acid dissociation constant for reactant or enzyme groups that have a given protonation state for optimum binding and/or catalysis.

**RESULTS**

**Site-directed Mutagenesis, Expression, Purification, and Characterizations of the Wild-Type and Mutant Enzymes—** Wild-type and mutant 3α-HSD/CR forms (S114A, Y155F, K159A, Y155F/K159A, and Y155F/S114A) were overexpressed in groups that have a given protonation state for optimum binding.

**Steady-state Kinetic Analysis of S114A, Y155F, K159A, Y155F/S114A, Y155F/S114A, and Wild-type 3α-HSD/CR—** 3α-HSD/CR catalyzes the reversible oxidoreductive reaction of androstosterone with cofactor NAD\(^+\). The addition of substrates is an ordered bi bi mechanism in which pyrimidine nucleotide is the first substrate to be bound and the last product to be released. The enzyme binds nucleotide NAD\(^+\), followed by androstanol, to facilitate the oxidative-reductive reaction. The steady-state kinetics of 3α-HSD/CR that catalyzed the oxidation of androstosterone was studied by varying the concentration of androstosterone at the saturated NAD\(^+\). Because the three residues, Ser-114, Tyr-155, and Lys-159, in 3α-HSD/CR are well conserved within the SDR family, they have been proposed to form a triad essential for catalysis. Site-directed mutagenesis was performed to evaluate the functional role of Ser-114, Tyr-155, and Lys-159 residues located in the active site.

| 3α-HSD/CR | Molecular mass\(^a\) (kDa) | \(k_{cat}/K_m\) 10^7 M\(^{-1}\) s\(^{-1}\) | -Fold decrease\(^a\) | \(k_{cat}\) 10^6 M\(^{-1}\) s\(^{-1}\) | -Fold decrease | \(K_m\) 10^6 M\(^{-1}\) |
|-----------|-----------------------------|--------------------------------|-----------------|-----------------------------|----------------|----------------|
| Wild-type | 28425 (28556) | (5.8 ± 0.4) | 318 ± 13 | 5.5 ± 0.6 |
| S114A     | 28412 (28540) | (1.7 ± 0.2) | 2600 | 7 ± 1 |
| Y155F     | 28410 (28540) | (2.0 ± 0.2) | 210 | 74 ± 11 |
| K159A     | 28370 (28499) | (2.9 ± 0.1) | 17 | 65 ± 5 |
| S114A/Y155F | 28389 (28524) | (6.2 ± 0.8) | 0.0028 ± 0.0001 | 1.1 ± 10^2 | 0.44 ± 0.07 |
| Y155F/K159A | 28349 (28483) | 30 ± 1 | 2 ± 10^6 | 0.0030 ± 0.0002 | 1.1 ± 10^2 | 100 ± 13 |

\(^a\) The molecular masses of recombinant enzymes determined by nanoelectrospray ionization/mass spectrometry analysis on a Q-TOF (quadrupole time of flight) Ultima\(^\text{TM}\) Global. The calculated masses shown in the parentheses are based on the cDNA for the His tag fusion proteins of monomer.

\(^b\) The -fold of the decrease is the ratio of the kinetic parameters for wild-type versus mutants.
catalysis, decreasing it by 10^5-fold in k_cat. The drop in k_cat/K_m values was more pronounced for Y155F/K159A with 10^5-fold decrease. Mutant S114A had a similar K_m value to wild-type enzyme, whereas the mutants Y155F, K159A, and Y155F/K159A increased 10–20-fold. The K_m value for S114A/Y155F was 0.1 times that for wild-type. The low activities observed confirm that the residues of S114, Y155F, and K159A are important groups involved in the 3α-HSD/CR-catalyzed reactions.

**pH Dependence of the Mutants and Wild-type 3α-HSD/CR**—To ascertain the important functional groups involved in binding/catalysis on the enzyme-catalyzed reactions, the pH profile on the oxidation of androsterone with NAD^+ was studied. The stability of the 3α-HSD/CR at extreme pH was studied by incubating at fixed pH for a period of time. An aliquot of enzyme was taken to assay the relative enzyme activity at pH 9. Our data indicated the enzyme was stable for at least 2 h at pH 5.5 and 10.5 (data not shown).

The kinetic parameters of k_cat and k_cat/K_m were obtained by varying the concentration of androsterone at saturated concentrations of NAD^+ at pH 5.5–11. Plots of log k_cat and log k_cat/K_m versus pH for the wild-type 3α-HSD/CR and mutants are shown in Fig. 3. The pK_a and the pH-independent constants (C) of the kinetic parameter of wild-type and mutants obtained by fitting to Equation 2 are shown in Table III. The reactions catalyzed by 3α-hydroxysteroid dehydrogenase showed a pH dependence of the kinetic parameter k_cat/K_m with pK_a = 7.2 ± 0.1. The kinetic parameter of k_cat was slightly increased with the higher pH values, but no break in the pH rate profile could be assigned. Substitution of amino acids caused a shift of the pK_a of mutants in the kinetic parameter k_cat/K_m and k_cat to the basic side. The mutant Y155F showed a pH-dependent profile, whereas the double mutant Y155F/S114A exhibited a pH-independent one, which indicated the group(s) involved in the general base had disappeared. A comparison of pH-independent constants of the kinetic parameter k_cat/K_m between wild-type and mutants is shown in Table III. Our results gave decreases in kinetic parameter k_cat/K_m of mutants ranging from 180- to 6 × 10^5-fold.

**DISCUSSION**

The catalytic groups in the short chain dehydrogenase family studied include the most conserved residues of serine, tyrosine, and lysine and form the catalytic triad. Studies of sequence alignment, structure analysis, site-directed mutagenesis, and kinetic characterization elucidate the role of the catalytic triad. The most conserved residue of tyrosine acts as a general base in the extraction of the proton from the hydroxyl group of steroid and facilitates the hydride transfer to NAD^+ (Fig. 4). Lysine lowers the pK_a of tyrosine, whereas serine is involved in the hydrogen bonding with substrate. In addition to the catalytic groups Ser, Tyr, and Lys, the residue Asn is also important in maintaining the active site configuration (12). Asn is involved in proton relay during catalysis. The proton relay system involves proton transfer from the hydroxyl group of Tyr, 2′OH of the ribose, the Lys side chain, and a bound water connected to the carbonyl group of Asn. Mutation of Asn to Leu renders a loss of activity in 3β/17β hydroxysteroid dehydrogenase. Hence, it has been proposed that the catalytic tetrad consists of Asn, Ser, Tyr, and Lys and is involved in the catalysis of the SDR family (6, 12).

Based on the structure determination and sequence alignment of 3α-HSD/CR with the SDR family, it could be that the conserved residues of Ser-114, Tyr-155, and Lys-159 act as a catalytic triad that facilitates the oxidation of androsterone with NAD^+ as coenzyme. However, no detailed kinetic study has been reported on how Ser-114, Tyr-155, and Lys-159 are involved in the catalysis/binding. In this study, we have characterized the roles of these residues in 3α-HSD/CR through studies using site-directed mutagenesis, steady-state kinetics, and pH profiling.

**Steady-state Kinetics of 3α-HSD/CR and Mutants**—The studies on the pH profile for wild-type 3α-HSD/CR and mutants showed a plateau above pH 10.5, which indicated an optimum enzyme-catalyzed reaction. Therefore, the activity for wild-type and mutants was further measured and compared at pH 10.5. The kinetic parameters of k_cat and k_cat/K_m on 3α-catalyzed...
oxidation of androsterone with NAD\(^+\) at pH 10.5 are shown in Table II. All mutants we studied decreased the activities in the kinetic parameters of \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) due to the loss of the functional groups involved in the catalysis. Amino acid substitutions of Ser-114 to alanine and Tyr-155 to phenylalanine removed the hydroxyl group and caused the decrease in activity. The catalytic constants \(k_{\text{cat}}\) for mutants S114A, Y155F, and K159A were decreased by 2600-, 210-, and 17-fold, respectively. Moreover, double mutants S114A/Y155F and Y155F/K159A led to extremely low activities with 10

\(k_{\text{cat}}\) slightly increases at the higher pHs. The pH dependence of the kinetic parameter \(k_{\text{cat}}/K_m\) indicates an important functional group on either the enzyme-NAD\(^+\) binary complex or substrate that is involved in the binding and/or catalysis. Because the \(pK_a\) of the 3-hydroxyl group of androsterone in solution is outside the range of pH studied, the observed \(pK_a\) of 7.2 can be attributed to the enzyme residue. Therefore, the group involved in the binding or catalysis, the steady-state kinetics and pH profile of the mutants S114A, Y155F, K159A, S114A/Y155F, and Y155F/K159A were studied. Plots of kinetic parameters of \(k_{\text{cat}}/K_m\) versus pH for mutants are shown in Fig. 3. The pH dependences of kinetic parameters \(k_{\text{cat}}/K_m\) of S114A, Y155F, and K159A showed a decrease at low pH, giving a \(pK_a\) of 7.40 ± 0.08, 8.41 ± 0.07, and 9.1 ± 0.1, respectively. The pH dependences of \(k_{\text{cat}}/K_m\) for Y155F and K159A showed a decrease at low pH, giving a \(pK_a\) of 8.7 ± 0.1 and 9.6 ± 0.2, respectively. The pH profile on the enzyme kinetics has been utilized to probe the acid-base catalysis (22). The log \(k_{\text{cat}}/K_m\) versus pH profile shows the \(pK_a\) values of groups necessary both for binding and catalysis. The \(k_{\text{cat}}\) profile allows the group to be correctly protonated for catalysis. Therefore, the group involved in the binding and catalysis will be observed in the pH dependence of kinetic parameters \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\), whereas the group participating in the binding of substrate will appear in the pH dependence of the kinetic parameter \(k_{\text{cat}}/K_m\) but not in the kinetic parameter \(k_{\text{cat}}\). Our pH profile studies clearly display the pH dependence of kinetic parameter \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) in the mutants Y155F and K159A. Therefore, the group with an apparent \(pK_a\) of 7.2 on wild-type 3α-HSD/CR is involved in the acid-base catalysis. Because the kinetic parameter \(k_{\text{cat}}\) includes all the rate constants from the enzyme-substrate complex to the release of the last product, a plot of the pH profile may be masked by a rate-limiting step outside the chemical step. The slight increase shown on kinetic parameter \(k_{\text{cat}}\) but no break at the higher pHs for the wild-type 3α-HSD/CR may be due to the slow step in the release of products. Hence, the rate-limiting step on the product release may mask the pH-dependent catalytic step and cause the slight drift observed on the pH rate profile. The release of the last product of NADH concomitant with the possible conformational change limits the overall rate. In the case of mutants Y155F and K159A, substituting tyrosine with phenylalanine or lysine with alanine slows down the chemical step and displays the pH dependences on both \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\). However, the pH profile of mutant S114A is similar to the wild-type 3α-HSD/CR with a pH dependence on \(k_{\text{cat}}/K_m\) and a linear drift in \(k_{\text{cat}}\). The activity was significantly decreased, 2600- and 3400-fold, in \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\), respectively. Replacement of Ser-114 with alanine may disrupt the hydrogen bond interaction between enzyme and substrate, reaction intermediate, and product (3, 17). This causes the decrease in rates of the hydride transfer and the release of product and displays a drift in kinetic parameter \(k_{\text{cat}}\).
on the plot of pH profile. Therefore, based on the pH profiles, the rate-limiting step(s) was determined to occur on the product release for wild-type and S114A, but this step changes to the chemical step in the mutants Y155F, K159A, and Y155F/K159A. Kinetic isotope effects on the wild-type and mutants will be studied to probe the postulation.

Further study of the pH-dependent profile of Y155F is needed to find out which groups are involved in the general base. Based on the sequence alignment of the SDR family, Tyr-155 is the most conserved residue. It has been suggested that Tyr-155 acts as a general base to extract the proton from Tyr-155 and hydroxyl group of the general base and causes a shift of the nearby Lys-159 in the wild-type 3α-HSD/CR. Alternately, Ser-114 may have acted as the general base to catalyze the reaction when residue Tyr-155 mutated to phenylalanine.

Catalytic Role of Lysine 159—The catalytic constant \(k_{cat}\) of the mutant K159A was slightly decreased, by 17-fold, at pH 10.5, but it dropped significantly for double mutant Y155F/K159A to 10^{-5} times of that for wild-type 3α-HSD/CR. Similarly, the catalysis efficiencies in \(k_{cat}/K_m\) at pH-independent value decreased by 200- and 2 \times 10^{6}-fold for K159A and Y155F/K159A, respectively. The enzyme activity decreased significantly on mutant Y155F/K159A and decreased slightly in K159A. Both mutant forms displayed a pH-dependent profile with \(pK_a\) of 9.1 for K159A and 10 for Y155F/K159A, i.e., a similar change in the \(pK_a\) value of Tyr-159 of 1.9 units on the mutant K159A and that of Ser-114 of 1.8 units on the mutant Y155F/K159A. This suggests that residue Lys-159 has a similar electrosstatic interaction with residue Tyr-155 of the wild-type and residue Ser-114 of mutant Y155F. Replacement of lysine with Alanine breaks down the electrostatic interaction between the positive charge of the e-aminium ion of Lys-159 and nicotinamide N1 of NAD^+.

To examine the possibility that the Ser-114 residue can replace the function of Tyr-155 on mutant Y155F, the double mutants S114A/Y155F and Y155F/K159A were expressed and characterized. The dimer property was confirmed by gel filtration chromatography, and no global structural change was observed on the CD spectra. The activity significantly decreased by 10^{5}-fold in Y155F/K159A. The pH profile of mutant Y155F/K159A showed a dependence in both \(k_{cat}\) and \(k_{cat}/K_m\) with a \(pK_a\) of 9.9, and 10.2, respectively, whereas mutant S114A/Y155F exhibited a pH-independent profile. Therefore, either Ser-114 or Tyr-155 in the active site may be involved in the general base on the 3α-HSD/CR-catalyzed reaction. The \(pK_a\) of hydroxyl group is around 13 for serine and 10 for tyrosine in solution. Tyr-155 is an effective base in the phenolate form because of its low \(pK_a\) of 7.2 through the assistance of the nearby Lys-159 in the wild-type 3α-HSD/CR. Therefore, based on the pH profiles, Tyr-155 was found to decrease by 2800-fold in Y155F, K159A, and Y155F/K159A. Kinetic isotope effects on the wild-type and mutants will be studied to probe the postulation.

Mechanistic Implication in Short Chain Dehydrogenase/Reductase—The catalytic groups and their function of Ser-114, Y155F, and Lys-159 have been characterized in this report. The group participating in the 3α-HSD/CR-catalyzed reaction is deprotonated for the optimum binding and catalysis.
posed chemical mechanism on the 3α-HSD/CR-catalyzed reaction is shown in Fig. 5A. In the first step of the reaction, the deprotonated phenolic group of Tyr-155 forms a hydrogen bond with the hydroxyl group attached to position 3 of the steroid skeleton of the substrate to form the ternary complex. In the second step, the deprotonated tyrosine residue acts as a catalytic base that extracts a proton from the hydroxyl group of the substrate. Concomitantly, NAD⁺ transfers a hydride liberated from position 3 of the steroid skeleton through the “B-face” of the nicotinamide ring at position 4. In the case of the mutants Y155F and Y155F/K159A the acid-base catalysis is attributed to Ser-114, which is postulated to result in deprotonation of Tyr-155 in the absence of Tyr-155 (Fig. 5B). The apparent pKₐ of 8.4 for Y155F is lower than that for serine, which is around 13 in the solution. Assisted by Lys-159, the Ser-114 on mutant Y155F is deprotonated to facilitate the hydride transfer.

The mechanism by which serine alternately acts as the base catalysis has been proposed for the reactions catalyzed by Drosophila alcohol dehydrogenase (13, 24) and UDP-galactose 4-epimerase (25, 26). Both are members of the SDR family and catalyze the hydride transfer reaction through the general base catalysis in the first step. Drosophila alcohol dehydrogenase catalyzes the oxidation of alcohol with nucleotide NAD⁺ in the absence of metal ion. Substitutions of Tyr-155 to phenylalanine and Ser-138 to alanine have resulted in loss of the enzyme activities. The constructed three-dimensional ternary complex models based on the crystal structure and thermal perturbation on pKₐ suggested both Tyr-151 and Ser-138 are ionized in order to allow an alcohol molecule to bind and react (24). Therefore, a much stronger base of negatively charged serine residue would be expected to produce the alcoholate ion (24). Therefore, a much stronger base of negatively charged serine residue would be expected to produce the alcoholate ion. In the meantime, the group involved in the general base is believed to be Ser-114 to catalyze the reaction. Ser-114 is involved in the differential roles in wild-type and mutant Y155F. In the wild-type 3α-HSD/CR-catalyzed reaction, Tyr-155 is a general base, whereas Ser-114 stabilizes the substrate, reaction intermediate, and product through the hydrogen bond. Because pH-dependent kinetics is observed in the mutant Y155F that lacks phenolate oxygen, the general base is believed to be Ser-114 to catalyze the reaction. In the mean-time, the group involved in the general base is assisted by Lys-159 in lowering the pKₐ value of the hydroxyl group through electrostatic interaction. This elucidates the mechanistic roles of the residues Ser, Tyr, and Lys on the 3α-HSD/CR and may apply to the reaction catalyzed by the SDR family.

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Hwa-I Pon

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