Mechanism of Proton Transfer in the 3α-Hydroxysteroid Dehydrogenase/Carbonyl Reductase from Comamonas testosteroni

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3α-Hydroxysteroid dehydrogenase/carbonyl reductase from Comamonas testosteroni catalyzes the oxidation of androsterone with NAD\textsuperscript{+} to form androstane-3-one and NADH with a concomitant releasing of protons to bulk solvent. To probe the proton transfer during the enzyme reaction, we used mutagenesis, chemical rescue, and kinetic isotope effects to investigate the release of protons. The kinetic isotope effects of \textsuperscript{1}H and \textsuperscript{2}H\textsubscript{O} for wild-type enzyme are 1 and 2.1 at pH 10.4 (where L represents H, \textsuperscript{2}H), respectively, and suggest a rate-limiting step in the intramolecular proton transfer. Substitution of alanine for Lys\textsuperscript{159} changes the rate-limiting step to the hydride transfer, evidenced by an equal deuterium isotope effect of 1.8 on \textit{V}_\text{max} and \textit{V}/\textit{K}_{\text{androsterone}} and no solvent kinetic isotope effect at saturating 3-(cyclohexylamino)propanesulfonic acid (CAPS). However, a value of 4.4 on \textit{V}_\text{max} is observed at 10 mM CAPS at pH 10.4, indicating a rate-limiting proton transfer. The rate of the proton transfer is blocked in the K159A and K159M mutants but can be rescued using exogenous proton acceptors, such as buffers, small primary amines, and azide. The Bronsted relationship between the log(\textit{V}/\textit{K}_{\text{d,hydride}})\textsubscript{Et} of the external amine (corrected for molecular size effects) and pK\textsubscript{a} is linear for the K159A mutant-catalyzed reaction at pH 10.4 (\textit{β} = 0.85 ± 0.09) at 5 mM CAPS. These results show that proton transfer to the external base with a late transition state occurred in a rate-limiting step. Furthermore, a proton inventory on \textit{V}/\textit{Et} is bowl-shaped for both the wild-type and K159A mutant enzymes and indicates a two-proton transfer in the transition state from Tyr\textsuperscript{155} to Lys\textsuperscript{159} via 2'-OH of ribose.

β-sheet flanked by α-helices (2, 3). In the binary complex, the NAD\textsuperscript{+} cofactor is bound at the carboxyl-terminal ends of the β-strands in the 3α-HSD/CR from C. testosteroni. 3α-HSD/CR reversibly catalyzes the oxidation of the steroid alcohol using NAD\textsuperscript{+} as the oxidant. The reaction catalyzed by 3α-HSD/CR shows an ordered Bi Bi kinetic mechanism with oxidized dinucleotide added first and the reduced dinucleotide released last (4). Studies of the pH profile and structural determinations elucidated the chemical mechanism of the 3α-HSD/CR-catalyzed reaction (2, 3, 5). The triad of Ser\textsuperscript{114}, Tyr\textsuperscript{155}, and Lys\textsuperscript{159} in the active site participates in the enzyme catalysis. The role of Lys\textsuperscript{159} is to lower the pK\textsubscript{d} of Tyr\textsuperscript{155} through electrostatic interaction between the protonated ε-amino of Lys\textsuperscript{159} and the hydroxy group of Tyr\textsuperscript{155}, whereas the unprotonated form of Tyr\textsuperscript{155} with an apparent pK\textsubscript{d} of 7.2 acts as a catalytic base that abstracts a proton from the hydroxyl group of the substrate. Ser\textsuperscript{114} is in hydrogen bonding with the substrate during the reaction catalyzed by the WT enzyme and alternatively acts as the general base to rescue catalysis of proton transfer in the Y155F mutant enzyme. NAD\textsuperscript{+} cofactor bound in the \textit{syn} conformation accepts a hydride from the 3β-position of androsterone through the “\textit{si} face” of the nicotinamide ring to form androstane-3-one and NADH with the concomitant release of protons in the forward reaction direction.

The overall oxidoreductive reaction catalyzed by 3α-HSD/CR is composed of the deprotonation of tyrosine, proton abstraction by the tyrosinate anion, and hydride transfer from the hydroxysteroid to NAD\textsuperscript{+}, followed by the release of a proton from the hydroxyl group of tyrosine to the solution (Fig. 1). A proton relay system is proposed in the SDR family through which protons are shuttled to bulk solvent. Analysis of the structures of 3α/17β-hydroxysteroid dehydrogenase and R-specific alcohol dehydrogenase suggests a proton transfer from the hydroxyl group of Tyr, via the 2'-OH of the nicotinamide ribose, the Lys side chain, and a water molecule hydrogen-bonded to the backbone carbonyl of Asn (6, 7). The theoretical calculations of the ionization properties of the hydroxyl group of Tyr\textsuperscript{155}, the O2' ribose hydroxyl, and the Lys\textsuperscript{159} ε-amino group in the active site of Drosophila alcohol dehydrogenase are consistent with the proton relay occurring in the SDR family (8). SDR is a large protein family with highly diverse functions in pro- and eukaryotes and is composed of a majority of oxidoreductases with NAD(P)\textsuperscript{+} as cofactor (9, 10). Although sequence alignment between different SDR enzymes typically shows 15–30% identity, the conserved sequences include an
N-terminal Gly-X3-Gly-X-Gly cofactor binding motif and a tetrad of catalytically important Ser, Tyr, Lys, and Asn residues, of which Tyr is the most conserved in the SDR family. The mechanistic roles of a catalytic tetrad have been elucidated through chemical modification, site-directed mutagenesis, sequence alignment, and structural comparison (5, 6, 9). Mutation of tyrosine, serine, and lysine residue results in the loss of most activity in the SDR family. Mutation of Asn111 to Leu in 3β/17β hydroxysteroid dehydrogenase results in inactivation of the enzyme. The Asn residue is also important in maintaining the active site configuration and is involved in the proton relay during the catalysis. Hence, the catalytic tetrad of Asn, Ser, Tyr, and Lys is involved in catalysis in the SDR family.

Proton transfer plays many roles in the enzyme-catalyzed reaction. It participates in the stabilization of the transition state to enhance the rate through the acid-base catalysis and the formation of a low barrier hydrogen bond (11–13) and is important in bioenergetics (14, 15). The electron transfer is coupled with the movement of protons in many respiratory oxidases and results in a proton gradient across the membrane to drive ATP synthesis. The movement of protons through a protein scaffold is often essential for enzyme-catalyzed reactions. Protons may move inside proteins along pathways provided by a network of hydrogen-bonded amino acid side chains and water molecules (16, 17). The intramolecular proton transfer involved in the enzyme reaction is generally characterized by the Brønsted acid-base analysis (12, 18), solvent deuterium isotope effect (19, 20), and chemical model study (21). Here, we studied proton transfer in the 3α-HSD/CR-catalyzed reaction, examined the number of proton transfers in the transition state in the rate-limiting step, and evaluated the roles of the Lys159 residue in the active site. We studied deuterium and solvent kinetic isotope effects to determine the rate-limiting step in the overall reaction. We then substituted alanine or methionine for Lys159 to block the pathway for proton transfer, used small molecules to restore the proton shuttle, and carried out a proton inventory to investigate intramolecular proton transfer. Buffers and exogenous proton acceptors are capable of participating in catalysis when proton transfer is blocked in the K159A and K159M mutant enzymes. Furthermore, the bowl-shaped proton inventory gives two protons participating in proton transfer in the rate-limiting step. In addition to lowering the pKa of the general base Tyr155, the results further demonstrated the role of Lys159 in shutting the protons to bulk solvent in the 3α-HSD/CR-catalyzed reaction.

**EXPERIMENTAL PROCEDURES**

We used the QuikChange site-directed mutagenesis kit from Stratagene. Androsterone and androstane-3α,17β-dione were purchased from Steraloids, Inc., and NAD+ was from Roche Applied Science. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and CAPS were from Sigma. Ammonia was obtained from Showa (Japan). Methylamine, ethanalamine, 2,2,2-trifluoroethylamine, and azide were purchased from Lancaster. Ethylamine and propylamine were from Acros, and ethylenediamine was from Tedia (Japan). Deuterated chemicals 1-deutero-glucose (95% 2H), perdeuterated ethanol, H2O (99.9% 2H), and NaO2H (30% (w/w), 99.5% 2H) came from Cambridge Isotope Laboratories. All chemicals were of the highest purity available.

[3α-2H]Androsterone was prepared by enzymatic synthesis from [4S-2H]NADH with 5α-androstan-3,17-dione catalyzed by 3α-HSD/CR. B-side [4S-2H] NADH was prepared according to the method of Viola et al. (22). Glucose-6-phosphate dehydrogenase from *L. mesenteroides* reversibly catalyzes the stereospecific reduction of NAD+ from 1-deutero-glucose to form B-side-labeled NADH and gluconic acid-γ-lactone. The reaction essentially goes to completion due to the unstable gluconic acid-γ-lactone at pH 8.0, which is hydrolyzed to give gluconate. [4S-2H]NADH was separated from NAD+ by gradient elution of ammonium bicarbonate (10–500 mM) at pH 9.3 through DEAE-cellulose ionic exchange column. Fractions of [4S-2H]NADH were pooled and lyophilized. The labeled 3α-androstan-3,17-dione was further prepared by stereospecific oxidation of the resulting [4S-2H] NADH from 5α-androstan-3,17-dione as reversibly catalyzed by 3α-HSD/CR. The progress for reverse reduction of androstane-3α,17β-dione with [4S-2H]NADH catalyzed by 3α-HSD/CR was followed by the disappearance of [4S-2H]NADH at 340 nm spectrophotometrically at pH 7.0.
Proton Transfer in 3α-HSD/CR

The reaction was quenched by the addition of CHCl₃, to denature the enzyme after it reached equilibrium. Deuterated androsterone was extracted by ethyl acetate and further purified by reverse phase HPLC.

Expression and Purification of Wild-type and Mutant Enzymes—Both wild-type and mutant genes were expressed in the Escherichia coli strain BL21(DE3). Mutagenic replacements were performed using the Quickchange site-directed mutagenesis kit and Pfu polymerase with pET-15b-3α-HSD/CR plasmid as template. The synthetic oligonucleotide primers used to create the cDNA for K159M were 5’-CTGGCCTATGCGGCA-GCTGGAATGCTTTTGACGGTGCGC-3’ (sense) and 5’-GCC-AAGGCCTAAGGATCCATGCTGGCCCGATAGGCGCA-3’ (antisense). The boldface codons indicate the mutation on the amino acid residue by the replacement of the underlined codon.

The mutant vector thus obtained was transformed into competent E. coli BL21(DE3) cells. The gene was sequenced for K159M mutation and compared with that of the wild-type 3α-HSD/CR using BLAST. The K159A mutant enzyme was prepared as described previously (5). In brief, recombinant proteins were overexpressed in BL21(DE3) cells and grown at 37 °C to an optical density of 0.6–1 at 600 nm in LB medium containing 50 μg/ml ampicillin. Isopropyl β-D-thiogalactopyranoside (0.5 mM) was added to the culture to induce protein expression. Growth was continued for an additional 4 h at 37 °C. The cells were then harvested and lysed by sonication. The overexpressed proteins were purified via an Ni²⁺-nitrilotriacetic acid affinity column making use of the enzyme’s His tag. SDS-PAGE was used to analyze protein purity. The protein concentrations were determined by a Bradford assay with bovine serum albumin as a standard (23).

Kinetic Studies—The oxidation of androsterone catalyzed by 3α-HSD/CR was monitored by the formation of NADH spectrophotometrically at 340 nm. A typical assay for the enzymatic reaction included 1 mM NAD⁺ and varied concentrations of androsterone in 0.1 M CAPS at pH 10.4 at 25 °C. All reactions were initiated by the addition of enzyme. Initial velocities for wild type and mutants were measured at varying concentrations of androsterone and fixed concentrations of NAD⁺.

Data were fitted using SigmaPlot software for appropriate rate equations. Data for substrate saturation curves at a fixed concentration of the second substrate were fitted using Equation 1. In Equation 1, ν and V represent the initial and maximum velocity, respectively, and Kₘ is the Michaelis constant. Data for a sequential and a rapid equilibrium order kinetic mechanism were fitted to Equation 2 and 3, respectively, where A and B are the varied substrates, Kₐ is the inhibition constant for A, and Kₐ and K₉ are the Michaelis constants for substrate A and B, respectively.

\[ ν = \frac{V}{K_m + A} \quad \text{(Eq. 1)} \]

\[ ν = \frac{VAB}{(K_{A\theta}K_B + K_A + K_B)} \quad \text{(Eq. 2)} \]

\[ ν = \frac{VAB}{(K_{A\theta} + K_A + AB)} \quad \text{(Eq. 3)} \]

Chemical Rescue of the K159A and K159M Mutant Enzymes—The effects on the K159A and K159M mutant enzyme-catalyzed reaction of exogenous small molecular proton acceptors were determined by measuring the initial velocity of the oxidation of androsterone at 25 °C as a function of the added amine. Primary amines included ammonia, methylamine, ethylamine, propylamine, ethanolamine, and 2,2,2-trifluoro-ethanolamine. The assay mixtures contained 1 mM NAD⁺, 46.6 μM androsterone, and various concentrations of the rescue agents in 5 mM CAPS, pH 10.4, at 25 °C. The reactions were initiated by the addition of the mutant enzyme (0.19 μg for K159A mutant and 0.37 μg for K159M mutant). The initial rate was measured in the presence of different concentrations of the rescue agents ([amine]). Data were then fitted to Equation 4 to obtain the chemical rescue parameters, \( V/E_P, V/K_{d-base}E_t \), and \( K_{d-base} \) of chemical rescue reagents.

\[ ν_{amine} = \frac{V(amine)}{(K_{d-base} + [amine])} \quad \text{(Eq. 4)} \]

In Equation 4, \( V/K_{d-base}E_t \) is the second order rate constant for the chemical rescue. \( K_{d-base} \) is the concentration of the rescue agent for which half of \( V \) was observed. \( ν_{amine} \) is the initial rate obtained by subtracting the rate in the absence of the external amine from the rate in the presence of amine. The concentration of conjugate base of the amine, [amine], was calculated based on Equation 5.

\[ [amine] = [amine]_{total}/(1 + ([H^+]/K_d)) \quad \text{(Eq. 5)} \]

where \( K_d \) is the acid dissociation constant of the amine, and [amine]_{total} is the total concentration of the added amine. Contributions from the electronic properties (pKₐ) and molecular volume of the primary amine to the efficiency of chemical rescue (\( V/K_{d-base}E_t \)) were analyzed by fitting data to Equation 6.

\[ \log V/K_{d-base}E_t = \beta pK_a + V(\text{molecular volume}) + C \quad \text{(Eq. 6)} \]

where \( \beta \) is the Brønsted coefficient, V is the molecular volume correction coefficient, and C is the constant term for the specific reaction.

Isotope Effects and Proton Inventory—The primary kinetic isotope effects, V and \( \nu(V/K_{androsterone}) \), were determined by a direct comparison of the kinetic parameters \( V_{max} \) and \( U/V/K_{androsterone} \) with unlabeled and deuterated androsterone. \( \nu_{max} \) and \( V/K_{androsterone} \) were obtained by measuring the initial rate as a function of either the deuterated or unlabeled androsterone concentration at 1 mM NAD⁺. The concentrations of deuterated and unlabeled androsterone were determined in triplicate by end point assay. The solvent kinetic isotope effects (SKIE) were measured according to the method of Quinn and Sutton (24). In the case of reactions measured in \( ^2H_2O \), all reactants were prepared and lyophilized twice in \( ^2H_2O \). Buffers were titrated to the desired pH (where pH is equal to the pH meter reading plus 0.4) using NaO₂H. Initial rates (\( ν \)) were measured at varied concentrations of androsterone and 1 mM NAD⁺ in the presence of \( H_2O \) or \( ^2H_2O \). Data from the experiments were fitted to Equations 7–10, when an isotope effect was observed on both \( V \) and \( V/K \), an equal isotope effect on \( V \) and \( V/K \) only, and \( V/K \) only, respectively.

\[ ν = VA/(K_m + A) \quad \text{(Eq. 7)} \]

\[ ν = VA/(K_m + A) \quad \text{(Eq. 8)} \]


**TABLE 1**
Comparison of the kinetic constants for wild-type and mutant 3α-HSD/CR in the presence of methylamine

| Enzyme    | CAPS   | Methylamine | $K_{\text{andr}}$ | $V/KEt$ | $V/K_{\text{andr}}Et$ |
|-----------|--------|-------------|-------------------|---------|----------------------|
| WT        | 5 mM   | 0           | 5.1 ± 0.6         | 118 ± 6 | (22 ± 1) × 10⁻⁶      |
| WT        | 5 mM   | 400         | 8 ± 2             | 138 ± 15 (0.9) | (18 ± 3) × 10⁻⁶ (1.2) |
| WT        | 100    | 0           | 7 ± 1             | 133 ± 11 (0.9) | (19 ± 2) × 10⁻⁶ (1.2) |
| K159A     | 5 mM   | 0           | 33 ± 5            | 63 ± 0.5 (19) | (0.19 ± 0.03) × 10⁻⁶ (116) |
| K159A     | 5 mM   | 400         | 31 ± 4            | 35 ± 2 (3)  | (1.15 ± 0.08) × 10⁻⁶ (19) |
| K159A     | 100    | 0           | 38 ± 4            | 137 ± 0.7 (9) | (0.36 ± 0.04) × 10⁻⁶ (61) |
| K159A     | 200    | 400         | 42 ± 6            | 42 ± 3 (3)  | (1.00 ± 0.07) × 10⁻⁶ (22) |

$^{a}$ The fold decrease shown in parenthesis is the ratio of the kinetic parameters for wild type at 5 mM CAPS versus enzyme in different concentrations of CAPS and methylamine.

\[
v = VA/(K_m + A(1 + FE_v)) \quad \text{(Eq. 9)}
\]
\[
v = VA/(K_m(1 + FE_v/K) + A) \quad \text{(Eq. 10)}
\]

In Equations 7–10, $F_i$ is the fraction of the deuterium label in the substrate, $E_{V/V/K}$ is the isotope effect minus 1 for an equal isotope effect on $V$ and $V/K$, and $E_V$ and $E_{V/K}$ are the isotope effect minus 1 on $V$ and $V/K$, respectively (25).

To obtain information on the number and fractionation factors for proton(s) being transferred at the transition state(s), the proton inventory method was utilized (24). The kinetic parameters $V$ and $V/K$ were obtained by measuring the initial rate at different fractional concentrations of $^2$H$_2$O in the reaction mixture by combining appropriate volumes of $^2$H$_2$O or $^3$H$_2$O. Data were then fitted to the Gross-Butler equation as follows,

\[
\eta_k = \frac{\phi_T}{Z-n}(1-n+\phi_T) \quad \text{(Eq. 11)}
\]
\[
\eta_k = \frac{\phi_T}{Z-n}(1-n+\phi_T) \quad \text{(Eq. 12)}
\]
\[
\eta_k = \frac{\phi_T}{Z-n}(1-n+\phi_T) \quad \text{(Eq. 13)}
\]

where $\eta_k$ is the ratio of the rate constants ($V$ or $V/K$) measured in different fractional concentrations of $^3$H$_2$O compared with 100% $^2$H$_2$O, $^{15}O$K is the solvent deuterium isotope effect (i.e. the ratio of the rate constants ($V$ or $V/K$) in $^2$H$_2$O and $^3$H$_2$O), $n$ is the fractional concentration of $^3$H$_2$O, $\phi_T$ is the corresponding deuterium fractionation factor for the exchangeable protonic sites relative to bulk water, and $Z$ represents a medium effect. Data for the linear and bowl-shaped proton inventory were fitted to Equation 12 and 13, respectively. In Equation 13, two exchangeable protonic sites with identical fractionation factors are involved in the transition state and a unit fractionation factor of reactant state.

**RESULTS**

Dependence of the Activity of the K159A Mutant Enzyme on Buffers—Substitution of alanine for lysine is expected to block the proton shuttle from the active site to the solvent. In an attempt to restore the proton transfer, an external proton acceptor was added to rescue the reaction catalyzed by the K159A mutant enzyme. We observed that the activity of the K159A mutant enzyme was dependent on the concentrations of CAPS. In comparison, the activity of the wild-type enzyme was not affected at either 5 or 100 mM CAPS at pH 10.4, indicating that the buffers participate in the reaction only when the Ly$^{159}$ side chain is eliminated (Table 1). The initial rates of the K159A enzyme-catalyzed reaction exhibited a hyperbolic dependence on the concentration of CAPS varying from 5 to 200 mM at 72 μM androsterone and 1 mM NAD$^+$, pH 10.4. To elucidate the role of CAPS in the kinetic mechanism, an initial velocity pattern was obtained by varying the buffer concentrations at different fixed concentrations of androsterone at 1 mM NAD$^+$, pH 10.4. The double-reciprocal plot intersected to the left of the ordinate, suggesting a sequential kinetic mechanism (see supplemental Fig. S1). Data were fitted to Equation 2; values for $V/E_t$ were 13 ± 2 s⁻¹, and $K_m$ for androsterone and CAPS in the mutant enzyme was 34 ± 11 μM and 8 ± 4 mM, respectively.

Chemical Rescue of the Activity of the K159A Mutant Enzyme by Exogenous Proton Acceptors—To further characterize the contribution of the mutated side chain on the catalytic rate enhancement provided by wild-type enzyme, noncovalent chemical rescue reagents were used to restore the activity of the mutant enzyme. The small molecules used were the primary amines, ammonia, methylamine, ethylamine, propylamine, ethanolamine, and 2,2,2-trifluoroethyamine, as well as azide. Those molecules have different $pK_a$ values and different molecular volumes (26). An increase in the initial rate of the mutant enzyme was observed in the presence of all of the chemical rescue agents with the exception of 2,2,2-trifluoroethyamine (up to 0.4 M, $pK_a = 5.7$) in the presence of 5 mM CAPS, pH 10.4. The activity of wild-type 3α-HSD/CR was unaffected by 0.4 M methylamine within the range of experimental error (Table 1). The activity of the K159A mutant enzyme in the presence of methylamine obeyed Michaelis-Menten kinetics with respect to androsterone as the varied substrate. The kinetic parameters we obtained for the K159A mutant-catalyzed reaction were compared in the absence and presence of 0.4 mM methylamine at 5 mM CAPS, pH 10.4. No significant change in affinity for androsterone was observed in response to external amine. The $V/E_t$ and $V/K_{\text{andr}}Et$ for the K159A mutant enzyme at 5 mM CAPS was increased 6-fold with 0.4 mM methylamine, which was up to 30% in $V/E_t$ and 5% in $V/K_{\text{andr}}Et$ of the wild-type level. The rescue of activity in K159A by external bases is shown in supplemental Fig. S2. The second order rate constant ($V/K_{\text{Et}}$) for methylamine increased as the pH increased, indicating that the basic form of amine is an active species (data not shown). The concentration of the conjugated base form of the chemical rescuers was then calculated based on the $pK_a$ of the bases and the pH value in the solution according to the Henderson-Hasselbach Equation 5. Data were fitted to Equation 4 to obtain the second order rate constant, $V/K_{\text{d-base}}Et$. The kinetic parameters $V/E_t$, $V/K_{\text{d-base}}Et$, and $K_{\text{d-base}}$ that we obtained are shown in Table 2.
**TABLE 2**

Chemical rescue of activity in K159A and K159 M mutant enzymes by external base at pH 10.4

The reaction included 46.6 μM androsterone, 1 mM NAD⁺, and data were fitted to Equation 4 to obtain kinetic parameters V/Er, Kd-base, and V/Kd-base, Et. NA, not available.

| External base | pKₐ | Molecular volume | Kd-base | V/Er | V/Kd-base Et | Kd-base | V/Er | V/Kd-base Et |
|---------------|-----|-----------------|---------|------|--------------|---------|------|--------------|
| Methylamine   | 10.6| 42.1            | 39 ± 4  | 41 ± 2| 1050 ± 71    | 58 ± 5  | 34 ± 2| 592 ± 28     |
| Ethylamine    | 10.6| 60.9            | 40 ± 6  | 29 ± 2| 730 ± 66     | 57 ± 2  | 21.5 ± 0.4| 382 ± 9      |
| Propylamine   | 10.5| 79.8            | 21 ± 0.4| 10.9 ± 1.0| 500 ± 8 | 15 ± 5 | 2.7 ± 0.3| 190 ± 50     |
| Ethylenediamine| 10.0| 74.6            | 103 ± 11| 11.7 ± 0.5| 114 ± 8  | 163 ± 27| 3.6 ± 0.3| 22 ± 2       |
| Ethanolamine  | 9.5 | 71.5            | 306 ± 31| 20.7 ± 0.7| 68 ± 6   | 691 ± 106| 6.4 ± 0.5| 9.1 ± 0.6    |
| Ammonia       | 9.2 | 23.2            | 145 ± 36| 19 ± 2  | 129 ± 21    | 371 ± 66| 17.6 ± 0.1| 48 ± 4       |
| Azide         | 4.72| NA              | 188 ± 50| 19 ± 3  | 101 ± 10    | 19 ± 3  | 101 ± 10|             |

* The molecular volume of the primary amine is from Ref. 26.

To evaluate the steric effect caused by the external amine, the kinetic parameters V/Kd-base Et for methyamine, ethyamine, and propylamine with similar pKₐ values were compared. A plot of log V/Kd-base Et versus molecular volume showed a linear relationship with a slope of −0.0085 and intercept of 3.38. The decrease in log V/Kd-base Et as the side chain increased in length indicates a steric discrimination in the active site of the K159A mutant enzyme. The result clearly indicated that the size of the external amine affected the catalytic efficiency in proton transfer for the K159A mutant enzyme. In contrast, the plot comparing log V/Kd-base Et and pKₐ did not show a clear correlation. Therefore, data for rescue efficiency were fitted to Equation 6, resulting in a Brønsted coefficient β of 0.85 ± 0.09, a volume coefficient V of −0.011 ± 0.003, and a constant value C of −5.5 ± 0.9. Corrected by the steric effect, the plot comparing rescue efficiencies (log V/Kd-base Et − V × molecular volume) and pKₐ of the primary amines showed a linear relationship during the reaction catalyzed by K159A mutant enzyme (Fig. 2). Similarly, the external amines were capable of restoring the activity of the K159M mutant enzyme (Table 2 and supplemen-

tal Fig. S3). Data for rescue efficiency were fitted to Equation 6, resulting in a Brønsted coefficient β of 1.2 ± 0.2, a volume coefficient V of −0.020 ± 0.004, and a constant value C of −9 ± 2 (Fig. 2).

Since CAPS also participates in binding and catalysis, the steric discrimination in the active site of the K159A mutant enzyme may exclude CAPS from binding at the active site. To distinguish between the binding sites of CAPS and the small amines, the initial rate pattern was studied by varying the concentrations of CAPS at fixed concentrations of methylamine in the presence of 46.6 μM androsterone and 1 mM NAD⁺ at pH 10.4. The activity of K159A mutant enzyme is dependent on the concentrations of methylamine at saturating CAPS. The concentrations of methylamine are 0 mM (○), 50 mM (□), 100 mM (▲), and 200 mM (▼). The lines represent a fit of data to Equation 1.

**FIGURE 2.** Bronsted relationship of the activity of K159A and K159M mutant enzymes with the pKₐ of the external amine. The second rate constant V/Kd-base Et of K159A mutant (●) and K159M mutant (◎) is from Table 2. By correcting the volume effect of the external amine, the log (V/Kd-base Et) is linearly dependent on the pKₐ of the external amine. The lines represent a fit of data to Equation 6, and give the Bronsted coefficient β = 0.85 ± 0.09, volume coefficient V = −0.011 ± 0.003, and C = −5.5 ± 0.9 for the K159A mutant enzyme (solid line) and the Bronsted coefficient β = 1.2 ± 0.2, volume coefficient V = −0.020 ± 0.004, and C = −9 ± 2 for the K159M mutant enzyme (dashed line), respectively.

**FIGURE 3.** Dependence of the activity of K159A mutant enzyme on both CAPS and methylamine. The initial rate pattern was carried out by varying the concentrations of CAPS at fixed concentrations of methylamine in the presence of 46.6 μM androsterone and 1 mM NAD⁺ at pH 10.4. The activity of K159A mutant enzyme is dependent on the concentrations of methylamine at saturating CAPS. The concentrations of methylamine are 0 mM (○), 50 mM (□), 100 mM (▲), and 200 mM (▼). The lines represent a fit of data to Equation 1.

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the reduction of androstanedione with B-side [4S-2H]NADH catalyzed by 3α-HSD/CR. [4S-2H] NADH was prepared by the oxidation of 1-deutero-glucose with NAD\(^+\) catalyzed by glucose 6-phosphate dehydrogenase. The primary kinetic isotope effect was obtained by direct comparison of the kinetic parameters of \(V_{\text{max}}\) and \(V/K_{\text{androst}}\) for labeled and unlabeled androsterone. The initial velocity patterns obtained by varying the concentrations of androsterone at several fixed concentrations of NAD\(^+\) indicated a sequential order and a rapid equilibrium order kinetic mechanism for the wild-type and K159A mutant enzymes, respectively (data not shown). Therefore, data obtained from the isotope effect study were fitted to Equation 10 for the wild-type enzyme and Equation 8 for the K159A mutant enzyme, which gives an equal isotope effect on \(V_{\text{max}}\) and \(V/K_{\text{androst}}\). The primary deuterium KIE for wild-type and K159A mutant enzymes is shown in Table 3. We measured the solvent deuteron kinetic isotope effect to further explore the intramolecular proton transfer. Previously, the pK\(_d\) of wild-type and K159A mutant enzymes were obtained as 7.2 and 9.1, respectively (5). Hence, the kinetic parameters \(V_{\text{max}}\) and \(V/K_{\text{androst}}\) for the wild-type and mutant enzymes were measured in H\(_2\)O and D\(_2\)O at pL 10.4 (where L represents H, \(^2\)H), where enzyme activity is in the range of pH independence. This reduced the thermodynamic effect caused by the differential deprotonation of functional groups in H\(_2\)O and D\(_2\)O (27). A SKIE of D\(_2\)O \(\delta^{2}V = 2.1 \pm 0.2\) was observed, whereas \(\delta^{2}O(V/K_{\text{androst}})\) is unity for the wild-type enzyme when the concentration of androsterone is varied at a fixed concentration of NAD\(^+\) (1 mM). In the K159A mutant enzyme, the SKIE is dependent on the concentration of CAPS. No SKIE was observed on either \(V_{\text{max}}\) or \(V/K_{\text{androst}}\) for the K159A mutant enzyme in the presence of 200 mM CAPS, but a large \(\delta^{2}O(V/K_{\text{androst}})\) of 4.4 ± 0.3 was observed in the presence of 10 mM CAPS (Table 3).

**Proton Inventory Study**—To obtain information on the number of protons involved in the proton shuttle and their fractionation factors in the transition state, a proton inventory of the wild-type and mutant enzymes was carried out. A dependence of \(^{2}V_{\text{max}}\) versus \(n\), the mole fraction of solvent deuteron, was observed for both the wild-type enzyme at 100 mM CAPS and K159A mutant enzyme at 10 mM CAPS when the concentration of androsterone was varied at a fixed concentration of NAD\(^+\) (1 mM) (Fig. 4). Data obtained from the wild-type and K159A mutant enzymes were fitted to Equation 12 or Equation 13 for one- and two-transition state protons that contributed to the isotope effect, respectively. The latter regressed to less variance for both wild-type (\(\sigma^2 = 0.0106\) and 0.0068, respectively) and K159A mutant enzymes (\(\sigma^2 = 0.0662\) and 0.0154, respectively). Hence, the results gave a better fit for two protons with \(\delta^{2}O(V = 2.19\pm 0.06\) and \(\phi = 0.64\pm 0.02\) for wild-type enzyme, and

**DISCUSSION**

**Chemical Rescue in the K159A Mutant Enzyme by Proton Acceptors**—In a previous study, the pH profile of the K159A mutant enzyme showed similar pH dependence compared with wild-type enzyme with a shift of the pK\(_{\alpha}\) in V/K\(_{\text{androst}}\) Et from 7.2 to 9.1, suggesting that Lys\(^{159}\) lowers the pK\(_{\alpha}\) of the Tyr\(^{155}\), which acted as a general base. Although the phenol group of Tyr\(^{155}\) was deprotonated for an optimal reaction in 100 mM CAPS at pH 10.4, the activity of K159A mutant enzyme was 50-fold less than that of the wild-type in V/K\(_{\text{androst}}\) Et and 10-fold less in V/Et (Table 1). In the present study, a series of small amines and buffers were added to investigate the proton transfer performed by the amino group. The activity of wild-type enzyme was not affected by the presence of either CAPS or methylamine at pH 10.4, but that of the K159A mutant enzyme was found to depend on the concentration level of CAPS and the external amine. The initial velocities exhibit a saturated kinetics with respect to the base rescuers, implying that CAPS and the external amine participate in the binding and catalysis of this enzyme (Table 1 and supplemental Fig. S1). Buffer has been proposed to act as a proton acceptor assisting in proton transfer. A proton relay system for human liver alcohol dehydrogenase is composed of the proton transfer from the Zn\(^{2+}\)-bound alcohol substrate, via the hydroxyl group of Ser\(^{48}\), the \(2',3'\)-OH of the nicotinamide ribose, and the His\(^{51}\) side chain to the solvent. Replacement of His\(^{51}\) with Gln decreases the V/K\(_{\text{alcohol}}\) 6-fold at pH 7, but it can be restored by buffers (28). Furthermore, carbonic anhydrase II has evolved a proton shuttle to allow buffer components to participate in the reaction.

### Table 3

| CAPS   | \(\delta^{2}V\) | \(\delta^{2}O(V/K_{\text{androst}})\) | \(\delta^{2}V\) | \(\delta^{2}O(V/K_{\text{androst}})\) |
|--------|----------------|---------------------------------|----------------|---------------------------------|
| WT     | 200 0.97 ± 0.07 | 1.0 ± 0.1                      | 1.82 ± 0.05    | 1.82 ± 0.05                     |
| K159A  | 10 4.4 ± 0.3    | 1.0 ± 0.1                      | 1.82 ± 0.05    | 1.82 ± 0.05                     |

**FIGURE 4.** Proton inventory on \(V_{\text{max}}\) of wild-type and K159A mutant 3α-HSD/CR-catalyzed reactions. Kinetic parameters of \(V_{\text{max}}\) were obtained from initial rate measurements in mixed isotopic solvents of H\(_2\)O and D\(_2\)O containing a varying atom fraction of deuterium (n). The reactions were carried out by varying the concentrations of androsterone (1 mM NAD\(^+\)) at pL 10.4 with 100 and 10 mM CAPS for wild-type and K159A mutant enzyme, respectively. The proton inventory of \(V_{\text{max}}\) on wild-type (○) and K159A mutant (□) enzymes is bowl-shaped. The lines represent a fit of the data to Equation 13, giving \(\delta^{2}O(V = 2.19 \pm 0.06\), \(\phi = 0.64 \pm 0.02\) for the wild-type enzyme (solid line) and \(\delta^{2}O(V = 4.4 \pm 0.1\), \(\phi = 0.44 \pm 0.02\) for the K159A mutant enzyme (dashed line).
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from solution. The rate of proton abstraction from Zn$^{2+}$-bound H$_2$O is dependent on the external buffer, imidazole, which exhibits saturated kinetics (29–33). We argue that buffer is capable of binding with K159A mutant enzyme to facilitate a proton release into solvent. This phenomenon is similar to that for human liver alcohol dehydrogenase (28). The initial rate pattern indicates a sequential kinetic mechanism for the 3α-HSD/CR-catalyzed reaction (i.e. all reactants of NAD$^+$, androstosterone, and CAPS must combine with the enzyme before reaction can take place and any product can be released). This kinetic mechanism is different from the ping-pong mechanism of the carbonic anhydrase-catalyzed reaction in which imidazole is bound with the intermediate of Zn$^{2+}$-bound H$_2$O and facilitates proton transfer to solution in the second half of the reaction (29).

It has been demonstrated that catalytic activity can be restored to the inactive K258A mutant of E. coli aspartate aminotransferase by exogenous amines (26, 34) and that the observed rate constants are linearly dependent on the concentration of the free base form of the amine catalyst in the K258A mutant, suggesting that K258 acts as the general base to facilitate the reaction catalyzed by aspartate aminotransferase. This methodology has been used to study many enzyme reactions to confirm the role of lysine and arginine in the general acid-base catalysis and the reaction mechanisms. The enzymes studied included mannitol 2-dehydrogenase (35), ketopantoate reductase (36), dihydroorotate dehydrogenase (37), and inosine 5'-monophosphate dehydrogenase (38). We extended this method to investigate the proton relay system for the reaction catalyzed by 3α-HSD/CR. We aimed to further clarify the contribution of the ε-amine side chain of Lys$^{159}$ to catalysis by ionizing the group containing the K159A mutant enzyme by the exogenous proton acceptor. We added external proton acceptors with different pK$_a$ of the primary amine and azide to the reaction. The fact that by adding a series of primary amines, we could restore up to 30% of wild-type activity in K159A mutant enzyme strongly suggests that exogenous amino groups can compensate for the loss of the mutated side chain. However, the chemical rescue appears to be an incomplete restoration of activity to the K159A mutant enzyme (Fig. 3). Hence, the chemical rescue appears to be an incomplete restoration of activity to the K159A mutant enzyme, suggest-
that the proton transfer limits the overall reaction. This may include the proton transfer from Tyr\(^{155}\) via Lys\(^{159}\) to solvent. Combined with the results from the SKIE and primary KIE, we conclude that the release of NADH product catalyzed by 3\(\alpha\)-HSD/CR. Evidence for a rate-limiting dissociation of NADH was demonstrated in the reaction catalyzed by the 3\(\alpha\)-HSD from \textit{Pseudomonas} sp. The binding of NADH with the 3\(\alpha\)-HSD from \textit{Pseudomonas} sp. is a two-step mechanism with an initial loosely bound form followed by a tightly bound isomerized form in a transient phase kinetic study (40). The structures of the apo- and holo-3\(\alpha\)-HSD with NADH on the 3\(\alpha\)-HSD from \textit{Pseudomonas} sp. further demonstrated that binding with the coenzyme NADH induces a significant conformation change, resulting in a shift from the substrate-binding loop to a helix conformation (41). Hence, proton transfer could be accompanied by a conformational change during the product-releasing step and contribute to the rate-limiting step(s). The observed SKIE will be due to a decrease in the rate of conformational rearrangement of the enzyme once the protons are substituted by deuterons. This result implies that enzyme flexibility and enzyme activity are tightly coupled in 3\(\alpha\)-HSD/CR-catalyzed reactions.

The rate-limiting step in the reaction catalyzed by K159A mutant enzyme occurs upon hydride transfer at saturated CAPS, evidenced by the equal deuterium isotope effect on \(V_{\text{max}}\) and \(V/K_{\text{app}}\), no SKIE, and rapid equilibrium order kinetic mechanism. It changes to a proton transfer at limited amounts of CAPS. Hence, proton transfer to the saturated CAPS is rapid, whereas limiting the amount of CAPS probably results in a decrease in the rate of proton transfer, resulting in a larger observed SKIE of 4.

Proton Inventory Study—Proton inventory can determine the number of protons undergoing changes in a rate-limiting step and allow a breakdown of the overall SKIE into its reactant state and transition state components (20, 24). A linear relationship between the observed rate constant and the mole fraction of the deuterium fraction indicates that one proton is involved in the transition state, whereas a curve indicates either the involvement of more than one proton or a contribution effect from the medium. A bowl-shaped proton inventory indicates multiple hydrogen sites in either the reactant or transition state, and a dome-shaped proton inventory arises from offsetting normal and inverse contributions to the solvent isotope effect. Our proton inventory study of wild-type and K159A mutant enzymes showed a bowl shape between the observed rate constant and the mole fraction of the deuterium fraction indicates that one proton is involved in the transition state, whereas a curve indicates either the involvement of more than one proton or a contribution effect from the medium. A bowl-shaped proton inventory indicates multiple hydrogen sites in either the reactant or transition state, and a dome-shaped proton inventory arises from offsetting normal and inverse contributions to the solvent isotope effect. Our proton inventory study of wild-type and K159A mutant enzymes showed a bowl shape between the observed rate constant and the mole fraction of the deuterium fraction, suggesting that multiple exchangeable hydrogenic sites were involved in the proton transfer. The bowl-shaped proton inventory of \(V_{\text{max}}\) on the wild-type and K159A mutant enzyme is best fitted for two protons in flight in the transition state. The values of \(\phi_T = 0.64\) and 0.44 were obtained for the wild-type and K159A mutant enzyme, respectively, which could be assigned to the proton bridge involving the hydroxyl group of Tyr\(^{155}\), 2\(-\)OH of ribose, and the NH\(_2\) group from either Lys\(^{159}\) or an external amine. The difference in the transition state fractionation factor for the wild-type versus the K159A mutant enzyme suggests a different mechanism of proton transfer. The proton transfer for 3\(\alpha\)-HSD/CR from \textit{C. testosteroni}, a member of the SDR family, is shown in Fig. 5. The Lys\(^{159}\) residue is involved in the proton transfer. The proton transfer includes the deprotonation of Tyr\(^{155}\) to form tyrosinate anion, the abstraction of the proton from the 3-hydroxyl group of the substrate androsterone, followed by hydride transfer to NAD\(^+\), and relay of the proton to solvent through the 2\(-\)OH of the nicotinamide ribose, the amino group of Lys\(^{159}\), and to the bulk solvent. A proton transfer from the 2\(-\)OH of ribose to Lys\(^{159}\) must be a rate-limiting step and a late transition step with a transition state fractionation factor of 0.64 for the wild-type enzyme. In the case of the mutant K159A-catalyzed reaction shown in Fig. 6, the proton transfer is blocked by the replacement of Lys\(^{159}\) with alanine. The activity is restored by the external amine, and the rate of proton transfer is dependent on the strength and size of the external base. The external small amine is bound at the active site and is involved in proton transfer with a late transition state in the rate-limiting step. In addition, a proton is alternately transferred from the 2\(-\)OH of

![FIGURE 5. The proton relay system in the 3\(\alpha\)-HSD/CR-catalyzed reaction. The structure of 3\(\alpha\)-HSD/CR (Protein Data Bank accession number 1fk8) (3) shows a similar proton relay system composed of the residues Tyr\(^{155}\), 2\(-\)OH of ribose, Lys\(^{159}\), and Asn\(^{86}\) and water in a 3\(\alpha\)-HSD/CR-catalyzed reaction. The hydrogen bonding distances (in \(\AA\)) within the residues in the proton relay system are shown. The plot was generated using the program PyMOL. Water is shown as a red sphere.](image)

![FIGURE 6. The proton relay system is assisted by the external small amine in the K159A mutant-catalyzed reaction. The external small amine is bound with enzyme at the position created by the substitution of Lys\(^{159}\) with alanine to facilitate proton transfer and restore activity. The proton is transferred to the external amine in a rate-limiting step with a Brønsted coefficient of 0.85.](image)
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ribose to CAPS at the surface with a transition state fractionation factor of 0.44. The smaller fractionation factor of 0.44 in K159A mutant versus 0.64 in wild-type enzyme suggests that the proton transfer to the external CAPS in K159A mutant enzyme has a loosener transition state structure compared with Lys. In summary, the proton relay system is involved in the 3α-HSD/CR-catalyzed reaction through Tyr, 2'-OH of the nicotinamide ribose, Lys, Asn, and waters at the active site. The observed normal solvent kinetic isotope effect on $V_{\text{max}}$ indicates that proton transfer is mostly rate-limiting. The Lys residue is important because they demonstrate the role of Lys in the catalytic tetrad of SDR: 1) to lower the pK_a of the general base and 2) to serve as the proton shuttle in the enzyme catalysis, establishing the mechanistic basis for general insights into SDR catalysis proton transfer.

REFERENCES

1. Mobus, E., and Maser, E. (1998) J. Biol. Chem. 273, 30888–30896
2. Maser, E., Xiong, G., Grimm, C., Ficner, R., and Reuter, K. (2001) Chem. Biol. Interact. 130–132, 707–722
3. Grimm, C., Maser, E., Mobus, E., Klebe, G., Reuter, K., and Ficner, R. (2000) J. Biol. Chem. 275, 41333–41339
4. Skalhegg, B. A. (1975) Eur. J. Biochem. 50, 603–609
5. Hwang, C. C., Chang, Y. H., Hsu, C. N., Hsu, H. H., Li, C. W., and Pon, H. I. (2005) J. Biol. Chem. 280, 3522–3528
6. Filling, C., Berndt, K. D., Benach, J., Knapp, S., Prozorovski, T., Nordling, E., Ladenstein, R., Jornvall, H., and Oppermann, U. (2002) J. Biol. Chem. 277, 25677–25684
7. Schlieben, N. H., Niefind, K., Muller, J., Riebel, B., Hummel, W., and Schomburg, D. (2005) J. Mol. Biol. 349, 801–813
8. Koumanov, A., Benach, J., Attrian, S., Gonzalez-Duarte, R., Karshikoff, A., and Ladenstein, R. (2003) Proteins 51, 289–298
9. Oppermann, U., Filling, C., Hult, M., Shafqat, N., Wu, X., Lindh, M., Shafqat, J., Nordling, E., Kalberg, Y., Persson, B., and Jornvall, H. (2003) Chem. Biol. Interact. 143–144, 247–253
10. Jornvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J., and Ghosh, D. (1995) Biochemistry 34, 6003–6013
11. Cleland, W. W., and Kreevoy, M. M. (1994) Science 264, 1887–1890
12. Schowen, K. B., Limbach, H. H., Denisov, G. S., and Schowen, R. L. (2000) Biochim. Biophys. Acta 1458, 43–62
13. Cleland, W. W., Frey, P. A., and Gerlt, J. A. (1998) J. Biol. Chem. 273, 25529–25532
14. Brzezinski, P. (2000) Biochim. Biophys. Acta 1458, 1–5
15. Fexen, K., Gilderson, G., Adelroth, P., and Brzezinski, P. (2005) Nature 437, 286–289
16. Pankhurst, K. L., Mowat, C. G., Rothery, E. L., Hudson, J. M., Jones, A. K., Miles, C. S., Walkinshaw, M. D., Armstrong, F. A., Reid, G. A., and Chapman, S. K. (2006) J. Biol. Chem. 281, 20589–20597
17. Nagle, J. F., and Morowitz, H. J. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 298–302
18. Richard, J. P. (1998) Biochemistry 37, 4305–4309
19. Rose, I. A., Kuo, D. J., and Warsms, J. V. (1991) Biochemistry 30, 722–726
20. Schowen, K. B., and Schowen, R. L. (1982) Methods Enzymol. 87, 515–606
21. Kirby, A. J. (1997) Accounts of Chemical Research 30, 290–296
22. Viola, R. E., Cook, P. F., and Cleland, W. W. (1979) Anal. Biochem. 96, 334–340
23. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
24. Quinn, D. M., and Sutton, L. D. (1991) in Enzyme Mechanism from Isotope Effects (Cook, P. F., ed), pp. 73–126, CRC Press, Boca Raton, FL
25. Cleland, W. W. (1979) Methods Enzymol. 63, 103–138
26. Toney, M. D., and Kirsch, J. F. (1992) Protein Sci. 1, 107–119
27. Relyea, H. A., Vrtis, J. M., Woodyer, R., Rinkus, S. A., and van der Donk, W. A. (2005) Biochemistry 44, 6640–6649
28. Ehrig, T., Hurley, T. D., Edenberg, H. J., and Bosron, W. F. (1991) Biochemistry 30, 1062–1068
29. Silverman, D. N., and Lindskog, S. (1988) Acc. Chem. Res. 21, 30–36
30. Taoka, S., Tu, C., Kistler, K. A., and Silverman, D. N. (1994) J. Biol. Chem. 269, 17988–17992
31. Tu, C. K., Paranawithana, S. R., Jewell, D. A., Tanhauser, S. M., LoGrasso, P. V., Wynns, G. C., Laipis, P. J., and Silverman, D. N. (1990) Biochemistry 29, 6400–6405
32. Tu, C., Tripp, B. C., Ferry, J. G., and Silverman, D. N. (2001) J. Am. Chem. Soc. 123, 5861–5866
33. An, H., Tu, C., Ren, K., Laipis, P. J., and Silverman, D. N. (2002) Biochim. Biophys. Acta 1599, 21–27
34. Toney, M. D., and Kirsch, J. F. (1989) Science 243, 1485–1488
35. Klimacek, M., Kavanagh, K. L., Wilson, D. K., and Nidetzky, B. (2003) Biochem. J. 375, 141–149
36. Zheng, R., and Blanchard, J. S. (2000) Biochemistry 39, 16244–16251
37. Jiang, W., Locke, G., Harpel, M. R., Copeland, R. A., and Marcinkeviciene, J. (2000) Biochemistry 39, 7990–7997
38. Guillen Schlippe, Y. V., and Hedstrom, L. (2005) Biochemistry 44, 16695–16700
39. Chothia, C. (1975) Nature 254, 304–308
40. Ueda, S., Oda, M., Imamura, S., and Ohnishi, M. (2004) Eur. J. Biochem. 271, 1774–1780
41. Nakamura, S., Oda, M., Kataoka, S., Ueda, S., Uchiyama, S., Yoshida, T., Kobayashi, Y., and Ohkubo, T. (2006) J. Biol. Chem. 281, 31876–31884