

# Characterization of the proton relay system in the 3 $\alpha$ -hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni*: the function of Lys 159 and Asn 86 in proton transfer

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3 $\alpha$ -Hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni*, a short chain dehydrogenase/reductase, catalyzes the oxidation of androsterone with NAD<sup>+</sup> to form androstenedione and NADH with a concomitant releasing of protons to bulk solvent. A proton relay system is proposed in the catalysis of short chain dehydrogenase/reductase, which is involved in the Tyr, 2'-OH of the nicotinamide ribose, Lys, Asn and waters at the active site. The role of Lys159 and Asn86 in the proton relay system of 3 $\alpha$ -hydroxysteroid dehydrogenase/carbonyl reductase is studied through mutagenesis, chemical rescue, and solvent kinetic isotope effect to probe the proton transfer in catalysis.

The mutant enzymes of K159A, K159M, N86A, and N86L were generated to block the pathway of proton transfer. The activity is decreased in the mutant enzymes, but only K159A and K159M mutant enzymes can be rescued by the primary amine. N86A and N86L mutant enzymes show a difference in the circular dichroism spectrum, suggesting a change in the secondary structure and implying the decrease in activity. The Brønsted relationship between the  $\log(k_{\text{cat}}/K_{\text{d-base}})$  of the external amine (corrected for molecular size effects) and  $\text{p}K_{\text{a}}$  is linear for the K159A and K159M mutant enzymes-catalyzed reaction at pH10.4 with  $\beta = 0.85 \pm 0.09$  and  $1.2 \pm 0.2$ , respectively. The kinetic isotope effects of  $^{\text{D}}V$  and  $^{\text{D}2\text{O}}V$  for wild-type enzyme are 1 and 2.1 at pL 10.4, respectively, and suggest a rate-limiting step in the intramolecular proton transfer. Substitution of alanine for Lys159 changes the rate-limiting step to the hydride transfer, evidenced by an equal deuterium isotope effect of 1.8 on  $V_{\text{max}}$  and  $V/K_{\text{androsterone}}$ , and no solvent kinetic isotope effect at saturating Caps. However a value of 4.4 on  $V_{\text{max}}$  is observed at 10 mM Caps at pL 10.4, indicating a rate-limiting proton transfer. Furthermore, a bowl-shaped proton inventory for the K159A enzyme-catalyzed reaction was observed at 10 mM Caps, pH10.4. These results suggest that (1) the external amine binds at the active site of the K159A mutant enzyme and occupies the vacancy created by the substitution of alanine for lysine, replacing the function of lysine in the proton transfer, (2) the rescued efficiency for K159M mutant enzyme is more sensitive to the size of the external amine than that of K159A mutant enzyme as a result of the smaller space at the active site in the K159M mutant enzyme (Volume coefficient of -0.020 vs -0.011 for K159M mutant and K159A mutant enzymes, respectively), (3) the proton transfer to the external base with a late transition state occurred in a rate-limiting step, and (4) Asn86 residue may be important in maintaining the active site configuration and participating in the proton relay during the catalysis.