Inhibition of $3\alpha/\beta,20\beta$ -Hydroxysteroid Dehydrogenase by Dexamethasone, Glycyrrhetic Acid and Spironolactone Is Attenuated by Deletion of 12 Carboxyl-Terminal Residues

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We constructed a pig $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase ($3\alpha/\beta,20\beta$ -HSD) mutant, which lacks 12 carboxyl-terminal amino acids residues. Enzyme activity studies indicated that the deleted amino acids have a role in steroid metabolism and may assist in substrate binding in wild-type $3\alpha/\beta,20\beta$ -HSD. Furthermore, substrate binding likely induces a conformational change allowing the 12 carboxyl-terminal amino acids interact with the steroid substrate [Nakajin S. *et al.*, *Biochim. Biophys. Acta*, 1550, 175–182 (2001)]. In this paper, we clarified that although pig $3\alpha/\beta,20\beta$ -HSD is potently inhibited by dexamethasone, glycyrrhetic acid and spironolactone, this inhibition is remarkably attenuated by deleting the 12 carboxyl-terminal residues. The inhibition constant (K_i) of pig $3\alpha/\beta,20\beta$ -HSD for dexamethasone increased 115-fold. These observations also indicate that these amino acid residues interact with steroid substrates or steroid inhibitors and have an important role in substrate or inhibitor binding to the active site.

Key words inhibition; hydroxysteroid dehydrogenase; short-chain dehydrogenase/reductase

Pig $3\alpha/\beta,20\beta$ -hydroxysteroid dehydrogenase ($3\alpha/\beta,20\beta$ -HSD) metabolizes androgens and progestins, and is one of the short chain dehydrogenase/reductase (SDR). This enzyme was first isolated from neonatal pig testes, suggesting that $3\alpha/\beta,20\beta$ -HSD may regulate steroid hormone concentrations in the testes during development.^{1–3} Later studies revealed that pig $3\alpha/\beta,20\beta$ -HSD belongs to the superfamily of SDRs and is expressed in many other tissues including the kidney, liver, brain, lung, thymus, heart, spleen and adrenal gland. The wide distribution of pig $3\alpha/\beta,20\beta$ -HSD suggested a broader role for this enzyme in steroid hormone metabolism.^{4–6} Interestingly, pig $3\alpha/\beta,20\beta$ -HSD shares a high degree of amino acid homology with human, rat, mouse and rabbit carbonyl reductases.^{7–10} However, pig $3\alpha/\beta,20\beta$ -HSD contains 12 amino acids at its carboxyl-terminus that is absent in these other mammalian carbonyl reductases. We constructed a pig $3\alpha/\beta,20\beta$ -HSD mutant (G278opal) which lacks these amino acids and found that G278opal has a 10-fold lower catalytic efficiency for testosterone and progesterone than wild-type $3\alpha/\beta,20\beta$ -HSD.¹¹ G278opal also has decreased 3α - and 20β -reductase activities and increased 3α -reductase activity, but shares similar NADPH binding characteristics with wild-type $3\alpha/\beta,20\beta$ -HSD. The recently determined three-dimensional structure of substrate-free $3\alpha/\beta,20\beta$ -HSD shows the 12 carboxyl-terminal amino acids in a random configuration.¹² Our data indicates that these terminal amino acids have a role in steroid metabolism and that substrate binding induces a conformational change, resulting in terminal amino acid interaction with the steroid substrate.¹¹

In the present study, we found that dexamethasone, glycyrrhetic acid and spironolactone inhibited pig $3\alpha/\beta,20\beta$ -HSD, but this inhibition was remarkably attenuated by the deletion of 12 carboxyl-terminal residues. The present study indicates that the 12 carboxyl-terminal amino acids residues play an important role in binding substrate or inhibitor to the active site.

MATERIALS AND METHODS

Chemicals Dexamethasone and glycyrrhetic acid were obtained from Wako Chemicals (Tokyo, Japan). Spironolactone was obtained from Sigma-Aldrich Japan (Tokyo, Japan). NADPH and DE-52 were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan) and Whatman International Ltd. (Maidstone, U.K.), respectively. Other materials were obtained as reported in previous papers.^{6,11}

Expression and Isolation of Recombinant Enzyme Expression and purification of recombinant $3\alpha/\beta,20\beta$ -HSD were as previously described.¹³ The cDNA of the $3\alpha/\beta,20\beta$ -HSD 12 carboxyl-terminal deletion mutant (G278opal) was constructed by the mutation of glycine-278 to the opal stop codon (TGA). Site-directed mutagenesis was performed using the Mutant-Express Km kit and protocol (Takara Biomedicals, Tokyo, Japan) following the oligonucleotide-directed dual amber method.¹⁴ The following oligonucleotide was used: 5'-pCTCAGGGGGAAGTACCATTCTACAAC-3'. The mutant sequence was confirmed and subcloned into an expression plasmid, and functional protein was expressed as described below. Rat carbonyl reductase cDNA was cloned by reverse transcription-polymerase chain reaction (RT-PCR). Messenger RNA was prepared from Wistar rat testis using the FastTrack 2.0 kit (Invitrogen, Carlsbad, CA, U.S.A.). The reverse transcription reaction was carried out at 42 °C, for 20 min using rat carbonyl reductase-reverse primer (5'-GGAAGCTTGGATTCCACCATGGTTCAA-3') to facilitate DNA extension beyond the carboxyl terminal site and the inclusion of a Hind III restriction site. After terminating the reverse transcription reaction by heating at 99 °C for 5 min, PCR was performed for 35 cycles (each cycle: 94 °C, 30 s; 62 °C, 30 s; 72 °C, 90 s) using rat CR-forward primer (5'-GGGAATTCATGTCTTCCGACAGACCCGT-3') to facilitate DNA extension beyond the amino terminal site, as well as the inclusion of an EcoRI restriction site and a rat carbonyl reductase reverse primer. PCR products including the coding region of rat carbonyl reductase were subcloned into

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Fig. 1. Effect of Deletion of 12 Carboxyl-terminal Residues from $3\alpha/\beta,20\beta$ -HSD on Enzyme Activity

Enzyme activity was determined spectrophotometrically by measuring oxidation rate of NADPH at 37°C. Details are described in materials and methods. Left and right panels show enzyme activity when 5α -DHT and 4-NBA, respectively, were used as substrate. ●: $3\alpha/\beta,20\beta$ -HSD, ■: G278opal, ▲: Rat CR.

an expression plasmid. The nucleotide sequence of rat carbonyl reductase cDNA was confirmed to be identical to that reported by Wermuth *et al.*⁸⁾ The pKK223-3 plasmid was used for expression of G278opal and rat carbonyl reductase. *E. coli* JM 105 cells were transformed with these plasmids and grown in LB media containing 50 μ g/ml of ampicillin. Expression and purification of G278opal and rat carbonyl reductase were carried out as previously described.¹³⁾ G278opal and rat carbonyl reductase were identified in column fractions by enzyme assay and Western blot using a specific antibody to carbonyl reductase.⁵⁾ Purity was verified with sodium dodecyl sulfate gel electrophoresis using the method of Laemmli (data not shown).¹⁵⁾ The purified enzyme was concentrated with ultrafiltration apparatus using a PM-10 Diaflo membrane and stored at -30°C until use.

Enzyme Assay Enzyme activity was determined spectrophotometrically by measuring the oxidation rate of NADPH at 37°C. The standard assay mixture consisted of 60 mM potassium phosphate buffer (pH 6.5), 80 mM NADPH, substrate at various concentrations and enzyme in a total volume of 1.0 ml. The reaction was initiated by addition of NADPH to the assay mixture. Blanks without substrate or enzyme were routinely included. Water-insoluble substrates and inhibitors were dissolved in ethanol. The final concentration of solvent in the assay mixture did not exceed 1%. For determination of kinetic constants for 5α -dihydrotestosterone (5α -DHT) and 4-nitrobenzaldehyde (4-NBA), the substrate concentration was changed from 5 to 120 μ M and 50 to 1500 μ M, respectively, and the NADPH concentration was fixed at 80 mM. The K_m and V_{max} values were calculated from Lineweaver-Burk plots, K_i values were calculated from Dixon plots using a personal computer and the correlation coefficients were (r) 0.876 to 0.999.

RESULTS AND DISCUSSION

Pig $3\alpha/\beta,20\beta$ -HSD utilizes numerous steroids as substrates, particularly androgens and progestins.²⁾ Furthermore, xenobiotics such as quinone, aromatic aldehydes and ketones, as well as aliphatic aldehydes and ketones also act as substrates for this enzyme. One such xenobiotic substrate, 4-NBA, has been used a model substrate for the study of reac-

tion mechanism.¹⁶⁾

The effects of the 12-residue deletion from the carboxyl-terminus of $3\alpha/\beta,20\beta$ -HSD on enzyme activity are shown in Fig. 1. When the typical steroid substrate 5α -DHT was used, wild type $3\alpha/\beta,20\beta$ -HSD had a K_m of 0.029 mM, a V_{max} of 217 nmol/min/mg and a substrate utilizing efficiency (V_{max}/K_m) of 7.4×10^3 , whereas G278opal had a K_m of 0.187 mM, a V_{max} of 962 nmol/min/mg and a V_{max}/K_m of 5.1×10^3 . When 4-NBA was used as a substrate, wild type $3\alpha/\beta,20\beta$ -HSD had a K_m of 0.036 mM, a V_{max} of 182 nmol/min/mg and a V_{max}/K_m of 5.1×10^3 , whereas G278opal had a K_m of 0.177 mM, a V_{max} of 512 nmol/min/mg and a V_{max}/K_m of 2.9×10^3 . The apparent K_m and V_{max} increased 4.9 to 6.5-fold and 2.8 to 4.4-fold, respectively. Both substrates were similarly catalyzed by G278opal, although V_{max}/K_m decreased 0.57 to 0.69-fold. In other words, substrate utilizing efficiency decreased by 57 to 69% as a result of deleting the 12 carboxyl-terminal residues. This suggests that the 12 carboxyl-terminal residues have a role in substrate affinity and/or substrate binding. Conversely, 4-NBA is a good substrate for rat CR ($K_m = 1.56$ mM, $V_{max} = 1407$ nmol/min/mg and $V_{max}/K_m = 0.90(103)$), while 5α -DHT is not as good a substrate ($K_m = 0.216$ mM, $V_{max} = 55$ nmol/min/mg and $V_{max}/K_m = 0.26 \times 10^3$).

Figure 2 shows inhibition of wild type $3\alpha/\beta,20\beta$ -HSD enzyme activity by dexamethasone, glycyrrhetic acid and spironolactone. We previously found that these steroids and steroid analogs inhibit $3\alpha/\beta,20\beta$ -HSD.^{17,18)} These chemicals possess bulky substituents on the D-ring of the steroid skeleton or at a corresponding position in the steroid analogs. Inhibition of enzyme activity occurred at concentrations of 1 to 100 μ M. G278opal was only slightly inhibited by these chemicals, whereas rat CR was not inhibited at all. Dexamethasone, glycyrrhetic acid and spironolactone were not utilized as substrates for $3\alpha/\beta,20\beta$ -HSD.

Lineweaver-Burk plots for inhibition of wild type $3\alpha/\beta,20\beta$ -HSD and G278opal by dexamethasone are shown in Fig. 3. For $3\alpha/\beta,20\beta$ -HSD, 1 and 5 μ M dexamethasone was added and for G278opal, 50 to 300 μ M dexamethasone was added. These results demonstrate that dexamethasone induces mixed type inhibition for pig $3\alpha/\beta,20\beta$ -HSD. Using Dixon plots, the inhibition constants (K_i) were found to be

Fig. 2. Inhibitory Effect of Dexamethasone, Glycyrrhetic Acid and Spironolactone on Enzyme Activity of Wild Type $3\alpha/\beta,20\beta$ -HSD, G278opal and Rat Carbonyl Reductase

Enzyme activity was determined spectrophotometrically by measuring the oxidation rate of NADPH at 37°C and was expressed as % of control. 4-NBA (0.5 mM) was used as substrate. Details are described in materials and methods. Left panel: dexamethasone, center panel: glycyrrhetic acid, right panel: spironolactone, ●: $3\alpha/\beta,20\beta$ -HSD, ■: G278opal, ▲: Rat CR.

Fig. 3. Lineweaver-Burk Plots of Inhibition of Wild Type $3\alpha/\beta,20\beta$ -HSD and G278opal by Dexamethasone

Enzyme activity was determined spectrophotometrically by measuring the oxidation rate of NADPH at 37°C. Details are described in materials and methods. Left panel: wild type, right panel: G278opal.

2.4 μM for wild type $3\alpha/\beta,20\beta$ -HSD and 275 μM for G278opal. Deletion of the 12 residues at the carboxyl terminus resulted in a 115-fold increase in K_i for dexamethasone.

The three dimensional structure of $3\alpha/\beta,20\beta$ -HSD was recently resolved by X-ray crystallography.¹² It became clear that the substrate-entry loop is almost non-existent in this enzyme and the entrance to the active site is more open than in any other HSD. However, only residues Glu281 to Ala288 from the carboxyl-terminal tail are in the vicinity of the active site. Based on these observations, these carboxyl-terminal amino acid residues are very important for substrate interaction and binding to the active site.

In the present study, we demonstrated that although pig $3\alpha/\beta,20\beta$ -HSD is potentially inhibited by steroids, such as dexamethasone, glycyrrhetic acid and spironolactone, this inhibition is remarkably attenuated by deletion of 12 carboxyl-terminal residues. These observations also indicate that the 12 carboxyl-terminal residues interact with steroid substrates or steroid inhibitors and have an important role in substrate or inhibitor binding to the active site.

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