Characterization of Two Isoforms of Mouse 3(17) α -Hydroxysteroid Dehydrogenases of the Aldo-Keto Reductase Family

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Mouse kidney contains two 3(17) α -hydroxysteroid dehydrogenases (HSDs) that show essentially the same properties except for their isoelectric points. However, the structural differences and physiological roles of the two enzymes remain unknown. In this study, we have isolated cDNAs for the two 3(17) α -HSDs from a total RNA sample of mouse kidney by reverse transcription-PCR. The identity of the cDNAs was confirmed by characterization of the recombinant enzymes that showed the same molecular weights, pI values, pH optima, substrate specificity and inhibitor sensitivity as those of the enzymes from mouse kidney. We also found that the recombinant enzymes reduce precursors of neuroactive progesterone derivatives, 5 α -dihydrotestosterone, deoxycorticosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate and estrone at low K_m values of 0.3–2 μ M. The two enzymes belonged to the aldo-keto reductase (AKR) family, and their 323-amino acid sequences differed only by five amino acids. The sequences of the two isoforms are identical to those of proteins that are predicted to be encoded in a gene for AKR1C21 in the database of the mouse genome. However, the mRNAs for the two isoforms were expressed in mouse kidney and other tissues, in which their expression levels were different. The results indicate an important role of 3(17) α -HSD in controlling the concentrations of various steroid hormones in the mouse tissues, and suggest the existence of two genes for the two isoforms of the enzyme.

Key words 3(17) α -hydroxysteroid dehydrogenase; aldo-keto reductase family; neurosteroid; androgen; dehydroepiandrosterone

Hydroxysteroid dehydrogenases (HSDs) play pivotal roles in the biosynthesis and inactivation of steroid hormones and bile acids.¹⁾ Many mammalian HSDs have been structurally characterized and divided into two protein families, the aldo-keto reductase (AKR) family²⁾ and short-chain dehydrogenase/reductase (SDR) family.³⁾ Cytosolic 3 α -HSD, 17 β -HSD type 5 and 20 α -HSD¹⁾ are members of the AKR family, whereas microsomal 3 α -HSD,⁴⁾ the other types of 17 β -HSD,⁵⁾ 3 β -HSD, 11 β -HSD¹⁾ and 20 β -HSD⁶⁾ belong to the SDR family. Thus, there is no connection between the primary structures of HSDs and the regio- and stereo-specificity of the reactions catalyzed by the enzymes.

3(17) α -HSDs have been purified from rabbit⁷⁾ and mouse⁸⁾ kidney. The enzyme is different from the above structurally known HSDs in catalyzing the oxidation of 17 α -hydroxysteroids, but its primary structure has yet to be determined. The mouse enzyme exists as two isoforms that show almost the same properties, the exception being their respective isoelectric points. The two isoforms of the enzyme were also called DD1 and DD3, because they were purified as multiple forms of dihydrodiol dehydrogenase (DD) that catalyzes the oxidation of *trans*- and *cis*-dihydrodiols of aromatic hydrocarbons into their catechol derivatives.⁸⁾ Yet no effort has been reported concerning the structural difference of the two isoforms and their roles in steroid metabolism.

In this report, we isolated cDNAs for the two isoforms of mouse 3(17) α -HSD, and examined the substrate specificity with the recombinant enzymes. The two isoforms differed by five amino acids, and are identical to proteins deduced from cDNAs that are deposited as transcripts of a gene for AKR1C21 in the databases of the NCBI and Mouse Genome Informatics (MGI, <http://www.informatics.jax.org/>). Therefore, we also examined the distribution of the two isoforms

and their expression levels in mouse tissues. These studies suggest novel roles of mouse 3(17) α -HSD in the metabolism of neuroactive steroids, androgens and dehydroepiandrosterone (DHEA), and include evidence that the two isoforms are encoded in the different genes.

MATERIALS AND METHODS

Materials Pyridine nucleotide coenzymes and pI markers were obtained from Oriental Yeast (Tokyo, Japan), and steroids were from Sigma Chemicals and Steraloids (Newport, RI). *Pfu* DNA polymerase and a QuickChange site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA, U.S.A.), and endonucleases and a pCR T7/CT-TOPO TA Expression kit were from Invitrogen (Carlsbad, CA, U.S.A.). *Taq* DNA polymerase and pkk223-3 expression plasmids were obtained from Takara (Kusatsu, Japan) and Amersham Biosciences (Piscataway, NJ, U.S.A.), respectively. All other chemicals were of the highest grade that could be obtained commercially.

cDNA Isolation The extraction of total RNA from a ICR mouse kidney and reverse transcription (RT)-PCR were carried out as described previously.⁹⁾ The cDNA of the mouse 3(17) α -HSD coding region was amplified with the sense primer 5'-atgaactcctcaaatgcttg and the antisense primer 5'-ttagtattcatccaaaatggcc. PCR using *Pfu* DNA polymerase was performed for 5 min at 94 °C and 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, followed by a final incubation at 72 °C for 5 min. The PCR products of 972 base pairs (bp) were cloned into pCR T7/CT-TOPO vectors. When the DNA inserts were sequenced using a CEQ2000XL DNA sequencer (Beckman Coulter), two cDNAs that encoded DD1 and DD3 were found.

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Expression and Purification of Recombinant Proteins

For the expression of the recombinant proteins using pkk223-3 expression plasmids, the two *EcoRI* sites in the cDNAs for DD1 and DD3 (at bp 690–695 and bp 853–858) were changed to inactive restriction sites using the QuickChange site-directed mutagenesis kit and following primers. The sequences of the sense primers were 5'-ggagatggtagac-caAaaCtcccctgttct and 5'-gagaacatgcagggttttgaGtTcagct-gagttcagag (capital letters represent the mutated nucleotides), which do not affect the deduced amino acid sequences of the two isolated cDNAs. The mutated cDNAs were amplified by PCR using *Pfu* DNA polymerase and primers, mddEco1 and mddHin3. mddEco1 primer (5'-ttgaattcatgaactccaaatgtcattg) corresponds to bp 1–20 of the cDNAs and includes an underlined *EcoRI* site. mddHin3 primer (5'-ccaagcttagtattcat-caaaatggc) is complimentary to bp 951–972 of the cDNAs and includes an underlined *HindIII* site. The PCR products were digested by *EcoRI* and *HindIII*, and ligated into the restriction sites of the pkk223-3 vectors. *Escherichia coli* JM109 was transfected with the expression plasmids as described previously.⁹⁾ The integrity of the construct was verified by DNA sequencing.

Expression of the recombinant enzymes in the *E. coli* cells and preparation of the cell extract were carried out as described previously.⁹⁾ The recombinant enzymes were purified according to the procedures for purification of DD1 and DD3.⁸⁾ The protein concentration was determined by the Bradford method.¹⁰⁾ The molecular weights and pI values of the purified enzymes were determined by SDS-PAGE¹¹⁾ on 12.5% slab gels and isoelectric focusing¹²⁾ on 7.5% polyacrylamide disc gels, respectively.

Assay of Enzyme Activity Dehydrogenase and reductase activities of the recombinant enzymes were assayed by measuring the rate of change in NADPH fluorescence (at 455 nm with an excitation wavelength of 340 nm) and its absorbance (at 340 nm), respectively. The standard reaction mixture for the dehydrogenase activity consisted of 0.1 M potassium phosphate buffer, pH 7.0, 0.25 mM NADP⁺, substrate and enzyme, in a total volume of 2.0 ml. The activities in the cell extracts and the enzyme preparations during the purification were assayed with 0.1 M glycine–NaOH buffer, pH 10.0, instead of the phosphate buffer, in order to detect low enzyme activity. R-indan-1-ol (1 mM) was used as the substrate, unless otherwise noted. The reductase activity was determined in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 mM NADPH and an appropriate amount of carbonyl substrate. The steroids and other water-insoluble compounds were dissolved in methanol, and added into the reaction mixture, in which the final concentration of methanol was less than 2.5%. The concentration of methanol did not affect the activities of the enzymes. One unit (U) of enzyme activity was defined as the amount that catalyzes reduction or formation of 1 μ mol NADPH per minute at 25 °C.

The apparent K_m and V_{max} values for substrates were determined by fitting the initial velocities to the Michaelis–Menten equation. The kinetic constants and IC_{50} values (inhibitor concentrations required for 50% inhibition) are expressed as the means of at least three determinations. The standard deviations of the determinations were less than 15%, unless otherwise noted.

Product Identification To identify reaction products,

ketosteroid reduction was conducted in a 2.0 ml system containing 0.1 mM NADPH, substrate (10 μ M), enzyme (3 μ g), and 0.1 M potassium phosphate, pH 6.5. The substrate and products were extracted into 6 ml ethyl acetate 30 min after the reaction was started at 37 °C, and the resulting extract was evaporated to dryness. The residue was dissolved into 0.1 ml of propan-2-ol, and analyzed in a HP 1100 Series liquid chromatography/mass spectrometry (LC/MS) diode array detector in a LC/MSD system (Hewlett-Packard, Palo Alto, CA, U.S.A.) using a CHIRALCEL OJ-H 5 μ m column (4.6 $\times$ 250 mm, Daicel Chemical Industries, Osaka, Japan). The column was eluted with *n*-hexane/propan-2-ol (90 : 10) at 0.5 ml/min and the column temperature of 40 °C. Steroids were detected by total ion monitoring mode or single ion monitoring of their expected molecular ion (positive ion mode). The rest of the residue was then analyzed by TLC¹³⁾ using chloroform/acetone (5 : 1) as the developing solvent. The reaction products were identified by comparing their R_f values, retention times and mass spectra with those of authentic steroids.

Tissue Distribution Tissues were excised from 12–14 week-old male and female ICR mice. For analysis of expression of mRNAs for 3(17) α -HSDs, the total RNA samples were prepared from the tissues, and then RT-PCR was carried out using *Taq* DNA polymerase. To discriminate the cDNAs for AKR1C21* (DD1) and AKR1C21 (DD3), the following primers were employed. The sense primers correspond to bp 61–79 of the cDNAs, and differ in the 3'-ends, which are g and c for the cDNAs for AKR1C21* (DD1) and AKR1C21 (DD3), respectively. The antisense primers are complimentary to bp 299–317 of the cDNAs, and differ in the 3'-ends, which are a and t for the cDNAs for AKR1C21* (DD1) and AKR1C21 (DD3), respectively. The amplification of the fragment (256 bp) of the AKR1C21* (DD1) cDNA was performed for 5 min at 94 °C and 40 cycles of 94 °C for 30 s, 63 °C for 30 s and 72 °C for 30 s, followed by a final incubation at 72 °C for 5 min. The same conditions were used for the amplification of the fragments of the AKR1C21 (DD3) cDNA, except that the reactions reduced to 30 cycles. Specificity of the primers was verified by establishing that each primer pair only amplified a product of the desired size and sequence from the correct cDNA template under the above conditions.

For analysis of the enzyme activities in the tissues, the cytosolic fractions of the tissue homogenates were prepared as described previously.¹⁴⁾ The fractions were analyzed for 17 α -HSD activity with 5 μ M epitestosterone and 0.1 M glycine–NaOH, pH 10.0, as the substrate and buffer, respectively.

RESULTS AND DISCUSSION

Isolation of cDNAs for Two Isoforms of 3(17) α -HSD

Several HSDs in the AKR family exhibit DD activity.^{1,15–17)} Mouse 3(17) α -HSDs were expected to be members of this family, because they also show high DD activity.⁸⁾ The databases of the NCBI and MGI include several cDNAs for mouse proteins, AKR1C14, AKR1C19, AKR1C20 and AKR1C21, which are composed of 323 amino acids and predicted to be HSDs.¹⁸⁾ The sequence of cDNA for AKR1C14 is identical to a partial sequence of cDNA for mouse 3 α -HSD,¹⁹⁾ and the amino acid sequence of AKR1C20 is similar

to that of mouse 17 β -HSD type 5, which exists in three multiple forms in the liver.¹⁷⁾ The sequence of AKR1C19 is highly similar to those of rodent morphine dehydrogenases,^{20,21)} which exhibit low HSD activity for 17 β -hydroxysteroids and utilize NAD(H) as the preferable coenzymes, in contrast to the NADP(H)-preferring mouse 3(17) α -HSDs.⁸⁾ Therefore, we amplified the cDNA for AKR1C21 as a candidate gene for either of two mouse 3(17) α -HSDs from the total mouse kidney RNA by RT-PCR. The amplified products contained two distinct cDNA species. The amino acid sequence deduced from one cDNA is the same as that of AKR1C21, and the other cDNA encodes a protein that differs from AKR1C21 by five amino acids at positions 27, 60, 91, 100 and 170 (Fig. 1). The sequence of the latter protein is identical to that predicted from a cDNA [accession no. BC013531], which is reported to be another transcript of the *Akr1c21* gene.¹⁸⁾ Therefore, the protein encoded in the latter cDNA is tentatively named as AKR1C21*, and the properties of recombinant AKR1C21 and AKR1C21* were examined.

The extracts of the *E. coli* cells transfected with the expression plasmids harboring the cDNAs for AKR1C21 and AKR1C21* exhibited NADP⁺-linked dehydrogenase activities towards epitestosterone (0.03 and 0.05 U/mg), *R*-indan-1-ol (0.10 and 0.13 U/mg) and *cis*-benzene dihydrodiol (0.12 and 0.19 U/mg), which are representative substrates for mouse kidney DD1 and DD3.⁸⁾ No significant activities towards the substrates were detected in the extract of the cells transfected with the vector alone. During purification, the recombinant enzymes were eluted at the identical position around apparent molecular mass of 35 kDa on the Sephadex G-100 gel-filtration, but showed different elution patterns on the anion-exchange chromatography on a DEAE-Sephacel column. While the recombinant AKR1C21* was not adsorbed on the resin, the recombinant AKR1C21 was eluted by a linear gradient of 0–0.12 M NaCl. The elution patterns of the recombinant AKR1C21* and AKR1C21 are similar to those of mouse kidney DD1 and DD3, respectively. The two enzymes purified by the subsequent chromatography on a Red A matrix column showed 36-kDa single bands on SDS-PAGE (Fig. 2). The specific activities and yields (amounts) from each liter of the cultured cells were 1.0 U/mg and 13% (2.6 mg) for AKR1C21*, and 1.2 U/mg and 17% (2.2 mg) for AKR1C21, respectively. The isoelectric points of AKR1C21* and AKR1C21 were 8.3 and 7.4, respectively, which are also identical to the values of mouse kidney DD1 (pI 8.4) and DD3 (pI 7.4).⁸⁾

The purified recombinant AKR1C21* and AKR1C21 oxidized 3 α -hydroxysteroids, epitestosterone, *cis*-benzene dihydrodiol and *R*-indan-1-ol at pH 7.0 (Table 1). The substrate specificity and K_m values were similar to those determined at pH 10.0 with DD1 and DD3 of mouse kidney,⁸⁾ and substrate inhibition, which is a characteristic of the mouse kidney enzymes, was observed at high concentrations (more than 10 folds of the K_m values) of the steroid substrates. The recombinant enzymes are also similar to the kidney enzymes in the pH optima and inhibitor sensitivity. The pH dependency of the NADP⁺-linked dehydrogenase activities of the two recombinant enzymes were the same, and the maximal rates were observed at pH 10.0. The activities of the two enzymes were inhibited by synthetic estrogens and 17 β -estradiol:

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DD3 MNSKCHCVILNDGNFIPVLGFGTALPLECPKSKAKELTKIAIDAGFHFDSSASVYNTEDH 60
DD1 -----V-----R-----

DD3 VGEAIRSKIDAGTVRRREDIFYTSKVWCTSLHPELVASRLERSLOKLQFDYDYLILHYPM 120
DD1 -----R-----V-----

DD3 ALKPGEENFPVDEHGKLI FDRVDLCATWEAMEKCKDAGLTKSIGVSNFNRYQLEMI LNKP 180
DD1 -----S-----

DD3 GLKYKPVGNQVECHPYLNNQMKLLDFCKSKDILVAVGVLTORYGGWVDQNSPVLLDEPV 240
DD1 -----

DD3 LGSMKKYNRTPALIALRYQLORGIVVLNTSLKEERIKENMQVFEFQLSSEDMKVL DGLN 300
DD1 -----

DD3 RNMRYIPAAIFKGHPNWPFLDEY 323
DD1 -----

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Fig. 1. Amino Acid Sequences Deduced from cDNAs for AKR1C21 (DD3) and AKR1C21* (DD1)

The residues identical to those of AKR1C21 (DD3) are indicated with hyphens in the sequence of DD1. The sequence of DD3 is the same as that of the polypeptide (accession no. Q9CX32) encoded in the *Akr1c21* gene and those deduced from cDNAs (accession no. NM_029901, BC061057 and AK020439). The residue at position 148 is unidentified in the sequences of Q9CX32, NM_029901 and AK020439 and the residue at position 294 is replaced with a Glu in the sequence of BC061057. The sequence of AKR1C21* (DD1) is identical to that of another polypeptide (accession no. Q91WR5) encoded in the *Akr1c21* gene and that deduced from a cDNA (accession no. BC013531).

Fig. 2. SDS-PAGE of Purified AKR1C21 (DD3) and AKR1C21* (DD1)

Lanes: a, AKR1C21 (DD3) (5 μ g); b, AKR1C21* (DD1) (2.5 μ g); and c, molecular mass markers. The gel was stained with 0.2% Coomassie brilliant blue. Arrows indicate the positions of the markers (in kDa).

The IC₅₀ values for hexestrol, dienestrol, diethylstilbestrol and 17 β -estradiol were 0.3 $\pm$ 0.05, 6.8 $\pm$ 0.7, 6.0 $\pm$ 0.9 and 3.5 $\pm$ 0.1 μ M, respectively, for AKR1C21*, and the respective values were 0.2 $\pm$ 0.06, 5.7 $\pm$ 0.5, 4.0 $\pm$ 0.3 and 4.5 $\pm$ 0.2 μ M for AKR1C21. Judging from their isoelectric points and elution patterns on the anion-exchange chromatography, AKR1C21* and AKR1C21 were concluded to be DD1 and DD3, respectively, of mouse 3(17) α -HSD.

Substrate Specificity for Ketosteroids The two recombinant 3(17) α -HSDs displayed dual coenzyme specificity for NAD(H) and NADP(H), of which NADP(H) were the preferable coenzymes. The K_m and k_{cat}/K_m values of recombinant AKR1C21 (DD3) for NADP⁺ were 1.8 μ M and 10 min⁻¹ μ M⁻¹ respectively (with 5 μ M epitestosterone as the substrate), and the respective values for NADPH were 1.0 μ M and 11 min⁻¹ μ M⁻¹ (with 5 μ M 5 α -pregnane-3,20-dione as the substrate). The enzyme showed high K_m and low k_{cat}/K_m values for NAD⁺ (1580 μ M and 0.01 min⁻¹ μ M⁻¹) and NADH (280 μ M and 0.05 min⁻¹ μ M⁻¹). Similar kinetic constants for the coenzymes were obtained with recombinant AKR1C21* (DD1). It is believed that a NADP(H)-preferring enzyme

Table 1. Kinetic Constants for Hydroxysteroids and Non-steroidal Alcohols

Substrate	AKR1C21* (DD1)			AKR1C21 (DD3)		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\mu\text{M}^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\mu\text{M}^{-1}$)
3α-Hydroxysteroids						
5 β -Pregnane-3 α ,20 α -diol	0.4	8	20	0.6	13	22
Lithocholic acid ^{a)}	0.1	1.9	19	0.1	2.8	28
3 α -Hydroxy-5 β -pregnan-20-one	0.4	7.5	19	0.7	12	17
3 α -Hydroxy-5 β -androstan-17-one	0.5	6.7	13	0.4	7.3	18
3 α ,21-Dihydroxy-5 β -pregnan-20-one	1.1	8.8	8	1	13	13
5 α -Pregnane-3 α ,20 α -diol	1	4.6	5	0.5	6.3	13
3 α -Hydroxy-5 α -pregnan-20-one	1.4	3.8	3	2.6	7	3
3 α ,21-Dihydroxy-5 α -pregnan-20-one	1.9	3.5	2	3	7.9	3
17α-Hydroxysteroids						
Epitestosterone	0.6	13	22	0.6	18	30
17 α -Hydroxy-5 α -androstan-3-one ^{a)}	0.4	8.4	21	0.4	10	25
17 α -Hydroxy-5 β -androstan-3-one ^{a)}	0.4	2.5	6	0.4	8.4	21
17 α -Hydroxyestr-4-en-3-one	3.3	5.3	2	2.5	7.6	3
Androst-5-ene-3 β ,17 α -diol	2.1	3.7	2	2.6	5.1	2
17 α -Estradiol	2.5	1.6	0.6	1.6	2	1
Non-steroidal alcohol						
cis-Benzene dihydrodiol	1910	38	0.02	1240	62	0.05
R-Indan-1-ol	370	19	0.05	370	27	0.07

a) The standard deviations of the K_m values for the substrates were less than 25%, and those for the other substrates were less than 15%.

Table 2. Kinetic Constants for Ketosteroids and Non-steroidal Carbonyl Compounds

Substrate	AKR1C21* (DD1)			AKR1C21 (DD3)		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\mu\text{M}^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}\mu\text{M}^{-1}$)
3-Ketosteroids						
21-Hydroxy-5 β -pregnane-3,20-dione	0.4	35	88	0.3	36	120
5 β -Pregnane-3,20-dione	0.5	19	38	0.6	24	40
5 α -Pregnane-3,20-dione	0.4	8.9	22	0.4	12	30
21-Hydroxy-5 α -pregnane-3,20-dione	1	15	15	1.3	20	15
17 β -Hydroxy-5 β -androstan-3-one	1.2	21	18	1.7	37	22
Dehydrolithocholic acid	0.7	10	14	0.8	15	19
5 α -DHT	1	6.2	6	1.1	8.5	8
5 β -Dihydrocorticosterone	6.2	7	1	9.4	11	1
Testosterone ^{a)}	0.9	0.9	1	0.5	0.8	2
5 β -Dihydrocortisone	8.4	3.5	0.4	6.4	3.5	0.5
Deoxycorticosterone ^{a)}	1.5	0.5	0.3	2	1	0.5
17-Ketosteroids						
Androst-4-ene-3,17-dione ^{a)}	0.3	11	37	0.2	10	50
16-Ketosterone	0.8	30	37	0.8	35	44
5 β -Androstane-3,17-dione	1	17	17	0.7	20	29
5 α -Androstane-3,17-dione	0.6	6.3	10	1	8	8
DHEA	0.4	4.1	10	0.4	5.4	13
DHEA 3-sulfate ^{a)}	0.3	3.6	12	0.4	4.8	12
3 α -Hydroxy-5 β -androstan-17-one	0.3	2.7	9	0.3	3.2	10
3 α -Hydroxyandrost-4-en-17-one	0.5	1.2	2	0.6	1.5	3
Estrone ^{a)}	0.8	0.3	0.4	0.8	0.4	0.5
Non-steroidal compounds						
R-Camphorquinone	0.7	42	60	1.1	66	60
S-Camphorquinone	0.8	42	53	0.8	69	86
Acenaphthenequinone ^{a)}	0.5	22	44	0.7	35	50
1-Phenylisatin	2	32	16	1.9	35	18
Phenanthrenequinone ^{a)}	2.7	27	10	2.6	47	18
4-Nitrobenzaldehyde	6.1	26	4	2.3	30	13
4-Nitroacetophenone	51	5.8	0.1	43	12	0.3

a) The standard deviations of the K_m values for the substrates were less than 25%, and those for the other substrates were less than 15%.

preferentially acts as a reductase because of high cellular NADPH/NADP⁺ and NAD⁺/NADH ratios.²²⁾ Previously few ketosteroids were found as the substrates of DD1 and DD3 and the K_m values for the substrates were determined only

with DD3.⁸⁾ Therefore, we tested various ketosteroids and non-steroidal carbonyl compounds as the substrates of the two homogeneous recombinant enzymes, and determined kinetic constants for the substrates (Table 2). The two enzymes

efficiently reduced both 5β - and 5α -diastereomers of pregnane-3,20-dione and 21-hydroxypregnane-3,20-dione, that are precursors of potent and efficacious positive modulators (3α -hydroxy- $5\alpha/5\beta$ -pregnan-20-one and $3\alpha,21$ -dihydroxy- $5\alpha/5\beta$ -pregnan-20-one) of the γ -aminobutyric acid type A (GABA_A) receptors.^{23,24} The other biologically active 3-ketosteroids, such as dehydrolithocholic acid, 5α -dihydrotestosterone (5α -DHT), testosterone and deoxycorticosterone, were reduced at low K_m values. The two enzymes also showed low K_m values ($\leq 1 \mu\text{M}$) for 17-ketosteroids, of which DHEA and its sulfate are known to exert a variety of biological actions.^{25,26} 16-Ketoestrone was efficiently reduced by the enzymes. In contrast to the low catalytic efficiency (k_{cat}/K_m) for estrone. The high reactivity for 16-ketoestrone may result from the chemical properties of the α -dicarbonyl structure at positions 16 and 17 of this steroid, because the enzymes showed high k_{cat}/K_m values for non-steroidal α -dicarbonyl compounds compared to those for 4-nitrobenzaldehyde and 4-nitroacetophenone.

Recently human 3α - and 20α -HSDs (AKR1C1-AKR1C4) have been reported to exhibit both 3α -HSD and 3β -HSD activities for 5α -DHT.²⁷ In the reduction of 5α -DHT, AKR1C21* (DD1) and AKR1C21 (DD3) formed only one product that was identified as 5α -androstane- $3\alpha,17\beta$ -diol by LC/MS analysis, in which the retention time (12.8 min) of the product was identical to that of the authentic 5α -androstane- $3\alpha,17\beta$ -diol and differed from that (13.8 min) of its 3β -isomer. Similarly in the reduction of 17-ketosteroids, the mouse enzymes yielded only the corresponding 17α -hydroxy metabolites, as the representative product identification in the reduction of DHEA and estrone are shown in Figs. 3a and 3b, respectively. Although the steroidal substrate is bound to the active site of mouse $3(17)\alpha$ -HSD from both its A-ring and D-ring sides, the reduction of the 3- or 17-keto group is strictly stereoselective, in contrast to the non-stereoselective human enzymes. Furthermore, mouse $3(17)\alpha$ -HSD formed only epitestosterone in the reduction of androst-4-ene- $3,17$ -dione (Fig. 3c), which is expected to be reduced into both epitestosterone and 3α -hydroxyandrost-4-en-17-one by the enzyme. This indicates that androst-4-ene- $3,17$ -dione exclusively binds to the active site of the enzyme from its D-ring side. Because the catalytic efficiency for androst-4-ene- $3,17$ -dione was much higher than that for 3α -hydroxyandrost-4-en-17-one (Table 2), the carbonyl group at C-3 in the steroid might be important for the positional selectivity in the reduction by mouse $3(17)\alpha$ -HSD.

The 17α -HSD activity of mouse $3(17)\alpha$ -HSD is unique within the members of the AKR family. Mouse $3(17)\alpha$ -HSDs share high amino acid sequence identity (69–75%) with rat 3α -HSD (AKR1C9), human $3(20)\alpha$ -HSD (AKR1C1) and human 3α -HSDs (AKR1C2, AKR1C3 and AKR1C4) and rabbit $3\alpha/17\beta/20\alpha$ -HSD (AKR1C5). Recent crystallographic and site-directed mutagenesis studies of AKR1C1,²⁸ AKR1C2,²⁹ AKR1C9³⁰ and AKR1C5³¹ have pointed out 12 substrate-binding residues, of which seven residues (Val⁵⁴, Tyr⁵⁵, Trp⁸⁶, His¹¹⁷, Phe¹²⁹, Trp²²⁷ and Ile³¹⁰) are conserved in mouse $3(17)\alpha$ -HSDs (Fig. 1). When the other substrate binding residues at positions 24, 118, 128, 306 and 308 were compared among mouse $3(17)\alpha$ -HSDs and other HSDs in the AKR family, Ala²⁴, Tyr¹¹⁸ and Asn¹²⁸ are specific for mouse $3(17)\alpha$ -HSDs, and may be responsible

Fig. 3. LC/MS Analysis of Products in the Reduction of Ketosteroids by $3(17)\alpha$ -HSD

(a) Reduction of DHEA by AKR1C21 (DD3). The retention time of the product was identical to that of authentic androst-5-ene- $3\beta,17\beta$ -diol (1, 12.5 min). The elution peaks of authentic androst-5-ene- $3\beta,17\beta$ -diol (2) and DHEA (3) are shown by arrows. (b) Reduction of estrone by DD3. Arrows show the elution peaks of authentic 17α -estradiol (4), 17β -estradiol (5) and estrone (6). (c) Reduction of androst-4-ene- $3,17$ -dione by DD3. The retention time of the product was identical with that of authentic epitestosterone (8, 26.0 min), but differed from those of 3α -hydroxyandrost-4-en-17-one (7, 14.4 min), androst-4-ene- $3\alpha,17\alpha$ -diol (9, 28.3 min), the substrate (10, 41.2 min) and testosterone (27.6 min). Similar elution patterns of the products were observed in the reduction of the substrates by AKR1C21* (DD1) (not shown).

Fig. 4. RT-PCR Analysis for Expression of mRNAs for AKR1C21* (DD1) and AKR1C21 (DD3) in Tissues of Female and Male Mice

Tissues: Cr, cerebrum; Cl, cerebellum; Lu, lung; He, heart; St, stomach; Li, liver; Ki, kidney; Sp, spleen; Si, small intestine; Mu, muscle; Ov, ovary; Te, testis; and Ut, uterus. The expression of mRNA for β -actin was analyzed as the control.

for the unique 17α -HSD activity of the mouse enzymes. Additionally, the C-terminal sequence (Ile³⁰⁶-Pro³⁰⁷-Ala³⁰⁸-Ala³⁰⁹-Ile³¹⁰) of the mouse enzymes differs from those of the other HSDs, and may also be related to the substrate specificity, because the importance of the C-terminal residues for the discrimination of the incoming steroid substrates has been suggested by crystallographic and site-directed mutagenesis studies of AKR1C5,³¹ AKR1C1^{28,32} and AKR1C4.³²

Tissue Distribution and Role of $3(17)\alpha$ -HSD The

expression of the mRNAs for AKR1C21* (DD1) and AKR1C21 (DD3) in tissues of female and male mice was assessed by RT-PCR using the enzyme-specific primers. The mRNA for DD1 was not detected in male tissues, but was expressed in the kidney and spleen of the females. The result on the female mouse tissues is coinciding with the tissue-specific expression of the mRNA for AKR1C21 reported by Vergnes *et al.*¹⁸⁾ In contrast, the mRNA for AKR1C21 (DD3) was abundant in the kidney of both sexes, and was present at lower levels in female brain and in several tissues of the males. To confirm the expression of the enzymes in the brain, 17 α -HSD activity in the tissue extract was assayed using 5 μ M epitestosterone as the substrate. The activities of the brain extracts of female and male mice were 0.013 ± 0.010 and 0.018 ± 0.004 mU/mg ($n=5$), respectively, which were lower than those of the kidney extracts of female (0.97 ± 0.27 mU/mg) and male (1.4 ± 0.34 mU/mg). It should be noted that no significant 17 α -HSD activity was detected in the nuclear, mitochondrial and microsomal fractions of the kidney homogenates, indicating that the two isoforms, AKR1C21* (DD1) and AKR1C21 (DD3), of mouse 3(17) α -HSD are cytoplasmic enzymes.

The high expression of 3(17) α -HSD in mouse kidney, together with their low K_m values for various 3-ketosteroids, suggests the importance of the kidney in the metabolism of androgens (5 α -DHT and testosterone), deoxycorticosterone and metabolites of corticoids. The present study also revealed additional roles of the enzymes in the metabolism of DHEA, its sulfate and estrone. Although the reduction of estrone into 17 α -estradiol was reported with 3(17) α -HSD of rabbit kidney and liver,⁷⁾ there is no report on the enzyme that catalyzes the reduction of DHEA and its sulfate into the 17 α -hydroxymetabolites. In addition, the K_m values for DHEA of the two isoforms of mouse 3(17) α -HSD are lower by more than one order of magnitude than those of other DHEA-metabolizing enzymes, such as human 17 β -HSD type 5,³³⁾ human 17 β -HSD type 1,³⁴⁾ rat alcohol sulfotransferase,³⁵⁾ and rat and human 7 α -hydroxylase.³⁶⁾ The K_m values for DHEA sulfate of mouse 3(17) α -HSDs are also lower than that of human steroid sulfatase.³⁷⁾ Thus, the kidney is a major organ that metabolizes DHEA and its sulfate, although further study will be needed to elucidate the relationship between the metabolism and various biological actions of DHEA and its sulfate.^{25,26)} Furthermore, the expression of the two isoforms of 3(17) α -HSD in mouse brain may be important for controlling the concentrations of neuroactive steroids. DHEA sulfate, a substrate of the enzymes, is not only a positive modulator of the σ_1 receptor, but also a negative modulator of the GABA_A-receptor.^{23,24)} The high catalytic efficiency of the two enzymes for the precursors (5 α /5 β -pregnane-3,20-dione and 21-hydroxy-5 α /5 β -pregnane-3,20-dione) of the positive modulators of the GABA_A-receptor suggests the involvement of 3(17) α -HSD, as well as 3 α -HSD,²¹⁾ in the synthesis of the neurosteroids in mouse brain.

Possible Existence of Genes for DD1 and DD3 In the databases of the NCBI and MGI, the two cDNAs identical with those for AKR1C21* (DD1) and AKR1C21 (DD3) are deposited as transcripts for a gene for AKR1C21. The detection of the two mRNAs for AKR1C21* (DD1) and AKR1C21 (DD3) in a mouse tissue and the difference in

their tissue expression levels indicate that the two proteins are not caused by the strain difference, but are encoded in the distinct genes. The complete sequence identity between AKR1C21 (DD3) and AKR1C21 reveals that DD3 is the protein encoded in the gene for AKR1C21 on chromosome 13 in the NCBI database. The database also includes another gene that encodes a protein with high sequence identity (99%) with AKR1C21* (DD1), although its chromosomal localization has yet to be determined. Further genomic analyses of the genes for AKR1C21 and, probably, AKR1C21* (DD1) will be needed not only to confirm our proposal of the existence of the two genes, but also to elucidate the sex-related difference in the expression of the two enzymes that was found in this study.

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