J. Biochem. 2015;1–10 doi:10.1093/jb/mvv057 Journal of Biochemistry Advance Access published June 17, 2015 THE JOURNAL OF BIOCHEMISTRY

Characterization of hamster NAD$^+$-dependent 3(17)β-hydroxysteroid dehydrogenase belonging to the aldo-keto reductase 1C subfamily

Received February 19, 2015; accepted April 28, 2015; published online May 22, 2015

Satoshi Endo[1], Misato Noda[1], Akira Ikari[1], Kenjiro Tatematsu[2], Ossama El-Kabbani[3], Akira Hara[4], Yukio Kitade[4] and Toshiyuki Matsunaga[1,*]

[1]Laboratory of Biochemistry and [2]Laboratory of Radiochemistry, Gifu Pharmaceutical University, Gifu 501-1196, Japan; [3]Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan; and [4]Faculty of Engineering, Gifu University, Gifu 501-1193, Japan

[*]Toshiyuki Matsunaga, Laboratory of Biochemistry, Gifu Pharmaceutical University, Gifu 501-1196, Japan. Tel: +81-58-230-8100, Fax: +81-58-230-8105, email: matsunagat@gifu-pu.ac.jp

The cDNAs for morphine 6-dehydrogenase (AKR1C34) and its homologous aldo-keto reductase (AKR1C35) were cloned from golden hamster liver, and their enzymatic properties and tissue distribution were compared. AKR1C34 and AKR1C35 similarly oxidized various xenobiotic alicyclic alcohols using NAD$^+$, but differed in their substrate specificity for hydroxysteroids and inhibitor sensitivity. While AKR1C34 showed 3α/17β/20α-hydroxysteroid dehydrogenase activities, AKR1C35 efficiently oxidized various 3β- and 17β-hydroxysteroids, including biologically active 3β-hydroxy-5α/β-dihydro-C_{19}/C_{21}-steroids, dehydroepiandrosterone and 17β-estradiol. AKR1C35 also differed from AKR1C34 in its high sensitivity to flavonoids, which inhibited competitively with respect to 17β-estradiol (K_i 0.11–0.69 μM). The mRNA for AKR1C35 was expressed liver-specific in male hamsters and ubiquitously in female hamsters, whereas the expression of the mRNA for AKR1C34 displayed opposite sexual dimorphism. Because AKR1C35 is the first 3(17)β-hydroxysteroid dehydrogenase in the AKR superfamily, we also investigated the molecular determinants for the 3β-hydroxysteroid dehydrogenase activity by replacement of Val54 and Cys310 in AKR1C35 with the corresponding residues in AKR1C34, Ala and Phe, respectively. The mutation of Val54Ala, but not Cys310Phe, significantly impaired this activity, suggesting that Val54 plays a critical role in recognition of the steroidal substrate.

Keywords: aldo-keto reductase/flavonoid/3(17)β-hydroxysteroid dehydrogenase/morphine 6-dehydrogenase/sexually dimorphic expression.

Abbreviations: AKR, aldo-keto reductase; DHT, dihydrotestosterone; 5α,3α-DIOL, 5α-androstane-3α,17β-diol; 5α,3β-DIOL, 5α-androstane-3β,17β-diol; HSD, hydroxysteroid dehydrogenase; Δ^5-3β-HSDI, Δ^5-3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase; LC/MS, liquid chromatography/mass spectrometry; M6DH, morphine 6-dehydrogenase; RT, reverse transcription; SDR, short-chain dehydrogenase/reductase; TLC, thin-layer chromotography.

Hydroxysteroid dehydrogenases (HSDs) catalyze the oxidoreduction between hydroxysteroids and ketosteroids using NAD$^+$ or NADPH as the coenzyme, and play important roles in the activation/inactivation of all classes of steroid hormones (*1*). Mammalian HSDs have been structurally divided into two protein superfamilies, the short-chain dehydrogenase/reductases (SDR) (*2*) and the aldo-keto reductases (AKR) (*3*). The SDR superfamily members are microsomal 3α-HSD, Δ^5-3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (Δ^5-3β-HSDI), 11β-HSD and most isozymes of 17β-HSD that are distributed in various subcellular fractions (*4*). In addition, hamster mitochondrial P26h protein (*5*), human peroxisomal dehydrogenase/reductase (SDR family) member 4 (*6*) and pig cytosolic carbonyl reductase (*7*), belonging to this superfamily, exhibit NAD$^+$-dependent 3α-HSD, NADPH-dependent reductive 3β-HSD, and 3α/3β/20β-HSD activities, respectively. The AKR superfamily includes cytosolic NADPH-dependent 3α-HSD, 17α-HSD, 17β-HSD and 20α-HSD, most of which exist in multiple forms and show overlapped steroid specificities (*3*). For example, human 20α-HSD (AKR1C1), 3α-HSD type-3 (AKR1C2), 17β-HSD type-5 (AKR1C3) and 3α-HSD type-1 (AKR1C4) exhibit reductive 3α-, 17β- and 20α-HSD activities (*8*), and also non-stereospecifically reduce 5α-dihydrotestosterone (DHT) into its 3α- and 3β-hydroxy metabolites (*9*). In addition to the NADPH-dependent HSDs, NAD$^+$-dependent enzymes that oxidize 3α-, 17β- and/or 20α-hydroxysteroids are identified in rats (AKR1C16, AKR1C17 and AKR1C24) and mice (AKR1C12 and AKR1C13) (*10–13*). Recently, rabbit aldose reductase-like protein (AKR1B19) that reduces various aldehydes has been reported to exhibit NADPH-dependent reductive 3β-HSD activity towards 3-keto-C_{19}/C_{21}-steroids (*14*). However, no NAD$^+$-dependent AKR with 3β- or 17α-HSD activity has yet been identified.

We previously characterized three rabbit NAD$^+$-dependent HSDs (AKRs: 1C26, 1C27 and 1C28) that belong to the AKR superfamily (*15*). The rabbit HSDs show broad substrate specificity for 3α-, 17β- and/or 20α-hydroxysteroids and xenobiotic alcohols, and

AKR1C26 is identified as morphine 6-dehydrogenase (M6DH). M6DH with 17β-HSD activity belonging to the AKR superfamily was purified from hamster liver (*16*), but isolation of the corresponding complete cDNA has not been reported previously. In this study, we have isolated cDNAs for two AKRs that share >86% amino acid sequence identity with the partial sequence of hamster liver M6DH (*16*). The proteins encoded in the genes have been assigned as AKR1C34 and AKR1C35 in the AKR superfamily (http://www.med.upenn.edu/akr/). Although AKR1C34 was identical to hamster liver M6DH, AKR1C35 was found to be 3(17)β-HSD, which is the first NAD^+-dependent member with 3β-HSD activity in the AKR superfamily. We describe the enzymatic properties and tissue distribution of the two hamster AKRs. In addition, the molecular determinant for 3(17)β-HSD activity was investigated by site-directed mutagenesis studies of AKR1C35.

Experimental Procedures

Materials

Steroids were obtained from Sigma Chemicals (Perth, WA) and Steraloids (Newport, RI). A pCold I expression vector was purchased from Takara (Kusatsu, Japan); *Pfu* DNA polymerase was from Stratagene; KOD FX DNA polymerase was from Toyobo (Osaka, Japan); and pCR2.1 plasmid, restriction enzymes, RACE kit and *Escherichia coli* BL21 (DE3) pLysS were from Invitrogen (Carlsbad, CA). *trans*-Benzene dihydrodiol was synthesized as described (*17*). α- and β-3-Hydroxyhexobarbitals were kindly denoted by Dr R. Takenoshita (Fukuoka University, Japan). All other chemicals were of the highest grade that could be obtained commercially.

cDNA isolation and site-directed mutagenesis

The cDNAs for AKR1C34 and AKR1C35 were isolated from the total RNA preparation of a liver of male golden hamster (Japan SLC Inc., Shizuoka, Japan) by reverse transcription (RT)-PCR. The preparation of total RNA, RT and DNA techniques followed the standard procedures described by Sambrook *et al.* (*18*). PCR was performed with *Pfu* DNA polymerase and a pair of sense and antisense primers, which contain NdeI and SalI sites. The primer sequences and PCR conditions are summarized in Supplementary Table S1. The PCR products were purified, digested with the two restriction enzymes and ligated into the pCold I vectors that had been digested with the two restriction enzymes. The insert of the cloned cDNA was sequenced using a Beckman CEQ8000XL DNA sequencer, and was confirmed to encode the 324-amino acid sequences of AKR1C34 and AKR1C35 fused to the N-terminal 6-His tag. The 3′-untranslated regions of the mRNAs for the two enzymes were generated using the RACE kit and the gene-specific primers. The fragments were subcloned into the pCR2.1 plasmids and sequenced as described earlier. The sequences of the cDNAs for AKR1C34 (1,192 bp) and AKR1C35 (1,203 bp) were deposited in the DDBJ database with the accession numbers LC006027 and LC006028, respectively.

Mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) and the pCold I expression plasmid harbouring the cDNA for AKR1C35 as the template according to the protocol described by the manufacturer. The primer pair used for the mutagenesis was composed of sense and antisense oligonucleotides to give V54A and C310F mutant enzymes (Supplementary Table S1). The cDNA for the double mutant of V54A/C310F was prepared using the expression plasmid harbouring the cDNA for V54A mutant as the template. The coding regions of the cDNAs in the expression plasmids were sequenced to confirm the presence of the desired mutation and ensure that no other mutation had occurred.

Purification of enzymes

The recombinant AKR1C35, its mutant enzymes, and AKR1C34 were expressed in *E. coli* BL21 (DE3) pLysS cells transformed with the expression plasmids harbouring their cDNAs as described previously (*19*). The enzymes were purified from the cell extracts using a nickel-charged Sepharose 6FF resin (GE Healthcare Ltd, Amersham, UK) according to the manufacturer's manual. The enzyme fraction was concentrated by ultrafiltration and dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 5 mM 2-mercaptethanol, 0.5 mM EDTA and 20% (v/v) glycerol. Purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentration was determined by Bradford's method using bovine serum albumin as the standard (*20*).

Assay of enzyme activity

The dehydrogenase activities for the enzymes were assayed by measuring the rate of change in fluorescence (at 455 nm with an excitation wavelength of 340 nm) or absorbance (at 340 nm) of NAD(P)H. The standard reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4, 1.0 mM NAD^+, 1.0 mM *S*-(+)-1,2,3,4-tetrahydro-1-naphthol (*S*-tetralol) and enzyme, in a total volume of 2.0 ml. The reductase activities were determined by measuring the rate of change in NAD(P)H absorbance in the phosphate buffer, pH 7.4, containing 0.1 mM NADH or NADPH and an appropriate amount of carbonyl substrate. The steroids and other substrates, which are hardly soluble in water, were dissolved in methanol or 50% methanol, and added into the reaction mixture, in which the final concentration of methanol was <2.5%. The concentration of methanol did not affect the activity of the enzymes. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation or oxidation of 1 μmol NAD(P)H per min at 25°C.

The pH dependency of the enzyme activity was estimated with 0.1 M potassium phosphate (pH 5.8–8.0), Tris-HCl (pH 7.5–9.0) and glycine-NaOH (pH 8.5–11) buffers. The apparent K_m and k_{cat} values were determined over a range of five substrate concentrations at a saturating concentration of coenzyme by fitting the initial velocities to the Michaelis–Menten equation. The kinetic constants for $NAD(P)^+$ and NADH were determined in the presence of saturated concentrations of *S*-tetralol and *S*-camphorquinone, respectively, as substrates. The IC_{50} (inhibitor concentrations required for 50% inhibition) values were determined with 20 μM *S*-tetralol (for AKR1C34) or 5 μM 17β-estradiol (for AKR1C35) as the substrate in the standard reaction mixture. The inhibitor constant, K_i, was estimated from the Dixon plot and/or Cornish–Bowden plot of the velocities obtained in the 17β-estradiol range ($1–30 \times K_m$) with three concentrations of the inhibitor. The kinetic constants and IC_{50} are expressed as the means ± SD of three determinations.

Product identification

To identify products, reaction was conducted in a 2.0-ml system containing 1.0 mM NAD^+ or 0.2 mM NADH, substrate (20–50 μM), enzyme (30–200 μg) and 0.1 M potassium phosphate, pH 7.4. The substrate and products were extracted into 4 ml ethyl acetate 20 min after the reaction started at 37°C. The steroidal products were identified by thin-layer chromatography (TLC) and liquid chromatography/mass spectrometry (LC/MS) using a Chiralcel OJ-H 5 μm column (*21*). The TLC chromatogram was developed in chloroform–ethyl ether (9:1, v/v), chloroform–ethyl ether–acetic acid (90:10:1, v/v) and chloroform–acetone–aqueous ammonia (70:30:1, v/v). The products and co-chromatographed authentic steroids were visualized by spraying with ethanol/H_2SO_4 (1:1, v/v) solution and heating at 110°C for 1 h. In LC/MS analysis, 3β-hydroxyprogesterone and progesterone were separated at a flow rate of 0.5 ml/min using hexane/isopropanol (90:10) as the mobile phase. The flow rate and mobile phase were 1.0 ml/min and hexane/isopropanol (85:15), respectively, for the separation of 17β-estradiol and estrone, and were 0.4 ml/min and hexane/isopropanol (95:5), respectively for the separation of 5α-pregnan-20α-ol-3-one and 5α-pregnane-3,20-dione. The steroids were detected by total ion monitoring mode or single ion monitoring of their expected molecular or fragment ion (positive ion mode). The products were also identified by comparing their retention times with those of their authentic steroids.

Tissue distribution analysis

The total RNA samples were prepared from the tissues of 6-week-old male and female hamsters (*18*). The total RNA samples were subjected to RT-PCR using KOD FX DNA polymerase and the primers were employed for the cloning of the cDNAs for AKR1C34 and AKR1C35. The cDNA for hamster β-actin was also amplified as an internal control with the specific primers (*22*). The PCR products were separated by agarose gel electrophoresis and revealed with ethidium bromide.

Results

cDNA cloning and sequence comparison

The cDNAs for AKR1C34 and AKR1C35 were isolated by RT-PCR from the total RNA sample of hamster liver with primers that were designed based on the sequences of mRNAs for *Mesocricetus auratus* AKR family 1 member C13-like transcripts (accession no. XM_005071750 and XM_005071747, respectively) predicted from the genomic analysis in the NCBI database. The nucleotide sequences of the coding regions of the isolated cDNAs for AKR1C34 and AKR1C35 (each 972 bp) were identical to those of the transcripts predicted from the genomic analysis, with the exception of a nucleotide at position 679 in AKR1C35 cDNA. Although this is deposited as an unknown nucleotide in XM_005071747, it was found to be T so that the encoded amino acid was Cys227 (Fig. 1). The amino acid sequence identity of AKR1C34 and AKR1C35 was 88%. The sequence of AKR1C34 was identical to those (composed a total of 303 residues) of peptides derived from the purified hamster liver M6DH (*16*), with the exception of four replacements (Ser34→Ala, Leu35→Met, His170→Met and Val244→Gly). The two proteins exhibited high sequence identity (83–87%) with NAD^+-dependent HSDs of rats (AKRs: 1C16, 1C17 and 1C24) and mice (AKRs: 1C12 and 1C13), followed by rabbit NAD^+-dependent HSDs (AKRs: 1C26, 1C27 and 1C28; 70–72%), but shared lower sequence identity (<67%) with NADP(H)-dependent human HSDs (AKRs: 1C1–1C4) and hamster 3-hydroxyhexobarbital dehydrogenase/3α(17β)-HSD (*23*).

pH optimum and coenzyme specificity

The purified preparations of the recombinant AKR1C34 and AKR1C35 showed single 37-kDa protein bands on the SDS-PAGE analysis. The enzymes exhibited NAD^+-linked dehydrogenase activity towards *S*-tetralol that is an excellent substrate for the above NAD^+-dependent HSDs of other species (*11–13*, *15*). The specific activities of AKR1C34 and AKR1C35 determined at pH 7.4 were 0.039 and 0.23 U/mg, respectively. The pH optima for the activity of AKR1C34 and AKR1C35 were observed at pH 10.0 and 9.0, respectively, where the enzyme activities were higher about 3- and 2-fold, respectively, than those assayed at pH 7.4. As Gln270 and Glu276 that are critical residues for the NAD^+ specificity (*10*) were conserved in AKR1C34 and AKR1C35 (Fig. 1), the enzymes showed high preference for NAD^+ compared with $NADP^+$. The k_{cat}/K_m values for NAD^+ in the *S*-tetralol oxidation by the two enzymes were much higher than those for $NADP^+$ (Table I). In the reverse reaction, AKR1C34 and AKR1C35 reduced *S*-camphorquinone using NADH as the coenzyme, but their reductase activities determined with 0.1 mM NADPH as the coenzyme were >17% of their NADH-linked activities. Therefore, the substrate specificity of the two enzymes was examined using the preferable coenzyme NAD^+ at a physiological pH of 7.4.

Table I. Kinetic constants for coenzymes.

Enzyme	Coenzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)
AKR1C35	$NADP^+$	2,400 ± 530	9.0 ± 2.0	0.0038
	NAD^+	23 ± 3	6.8 ± 0.3	0.30
	NADH[a]	17 ± 2	6.2 ± 0.9	0.36
AKR1C34	$NADP^+$	1,380 ± 450	5.5 ± 0.2	0.004
	NAD^+	13 ± 2	1.5 ± 0.2	0.12
	NADH[a]	13 ± 2	10 ± 1	0.76

[a]The values for NADH were determined in the reduction of *S*-camphorquinone, whose concentrations were 0.1 mM and 40 μM for AKR1C35 and AKR1C34, respectively.

Fig. 1 Alignment of amino acid sequences of AKRs (1C34 and 1C35). In the 1C34 sequence, the hyphens represent identical residues to those of AKR1C35, and the underlined residues are identical to those determined by peptide sequencing of hamster liver M6DH. The determinants for coenzyme specificity for NAD(H) are shown with asterisks. The positions of substrate-binding residues in AKRs (1C1, 1C2, 1C3, 1C5, 1C9 and 1C21) are indicated with arrowheads. The mutated residues of AKR1C35 are in boldface.

Substrate specificity

AKR1C34 and AKR1C35 oxidized non-steroidal alicyclic and long-chain aliphatic alcohols (Table II), whose structures are shown in Supplementary Fig. S1. There were some differences in the kinetic constants between the enzymes. AKR1C35 oxidized β-3-hydroxyhexobarbital more efficiently than its α-isomer, whereas AKR1C34 showed the opposite specificity for the two isomers. Among the substrates, an endogenous aliphatic alcohol, geranylgeraniol, was the most excellent substrate for AKR1C35, showing the highest k_{cat}/K_m value that was also higher than that of AKR1C34. In contrast, AKR1C34 exhibited lower K_m and higher catalytic efficiency (k_{cat}/K_m) for other alicyclic and aliphatic alcohols than AKR1C35.

The two hamster AKRs showed marked differences in the specificity for hydroxysteroids (Table III). The most striking characteristic of AKR1C35 was the oxidation of 3β-hydroxysteroids, which were not oxidized by AKR1C34. The 3β-hydroxysteroid substrates included 3β-hydroxyprogesterone, dehydroepiandrosterone and 3β-hydroxy-5α/β-dihydro-C_{19}/C_{21}-steroids, and the oxidized products of 3β-hydroxyprogesterone and 5α-androstan-3β-ol-17-one were identified as progesterone and 5α-androstane-3,17-dione, respectively, by LC/MS (Fig. 2) and TLC, respectively. The enzyme did not show significant activity for 3β-hydroxy derivatives of 5-pregnenes (pregnenolone and 17-hydroxypregnenolone), bile acids (isolithocholic acid and 5α-cholic acid-3β,7β-diol) and 5α-cholestan-3β-ol. Although 17β-hydroxysteroids were common substrates of the two AKRs, the high reactivity of AKR1C35 to 17β-estradiol was remarkable, as the k_{cat}/K_m value for 17β-estradiol was the highest among the values for all substrates. The product of 17β-estradiol oxidation was identified as estrone (Fig. 2). Among 3,17-dihydroxysteroids, 5α-androstane-3α,17β-diol (5α, 3α-DIOL), 5α-androstane-3β,17β-diol (5α,3β-DIOL), 5-androstene-3β,17β-diol and 5β-androstane-3β,17β-diol were oxidized only by AKR1C35. The product of 5α,3α-DIOL oxidation was identified as 5α-androstan-3α-ol-17-one by TLC, whereas the oxidation of 5α,3β-DIOL produced 5α-DHT and 5α-androstan-3β-ol-17-one as the major and minor products, respectively, indicating that AKR1C35 mainly oxidizes the 3β-hydroxy group of 5α,3β-DIOL.

As hamster liver M6DH exhibited 17β-HSD activity (*16*), AKR1C34 oxidized 17β-hydroxysteroids other than 17β-estradiol, 5α,3β-DIOL, 5-androstene-3β,17β-diol, 5α,3α-DIOL and 5β-androstane-3β,17β-diol (Table III). In addition, AKR1C34 exhibited dehydrogenase activity towards several 3α- and 20α-hydroxysteroids, which were not oxidized by AKR1C35. The oxidation of 5α-pregnan-20α-ol-3-one into the corresponding 20-ketosteroid was confirmed by product analysis using LC/MS (Fig. 2). It should be noted that AKR1C34 and AKR1C35 did not show significant activity towards other 3α-hydroxysteroids (5α-pregnan-3α-ol-20-one, 5α-androstan-3α-ol-17-one and lithocholic acid), 17α-hydroxysteroids (17α-estradiol and epitestosterone) and 20-hydroxysteroids (5β-pregnan-20α-ol-3-one, 20β-hydroxyprogesterone and 5α/β-pregnan-20β-ol-3-ones).

In the NADH-linked reverse reaction, AKR1C35 and AKR1C34 highly reduced non-steroidal α-dicarbonyl compounds (9,10-phenanthrenequione, isatin, 1-phenyl-1,2-propanedione, *S*-camphorquinone and methylglyoxal), and also exhibited moderate activities towards aromatic aldehydes (4-nitrobenzaldehyde and pyridine-3-aldehyde), α-tetralone and methylglyoxal (Supplementary Table S2). The two enzymes showed significant difference in the specificity for ketosteroids. AKR1C34 showed low activity towards 5β-pregnane-3,20-dione but not 3- and 17-ketosteroids (5α-androstane-3,17-dione, 5α-DHT, 5β-DHT and estrone). In contrast, AKR1C35 did not reduce 5β-pregnane-3,20-dione and accepted the 3- and

Table II. Substrate specificity for non-steroidal alcohols.

	AKR1C35			AKR1C34		
Substrate	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]
Alicyclic alcohols						
β-3-Hydroxyhexobarbital	13	5.8	0.45	40	1.5	0.038
α-3-Hydroxyhexobarbital	30	2.4	0.13	14	1.1	0.078
1-Acenaphthenol	148	17	0.11	23	1.3	0.057
S-Tetralol	61	6.3	0.10	1.5	1.5	1.0
S-1-Indanol	181	6.0	0.033	1.2	1.7	1.4
R-Tetralol	95	0.45	0.005	28	1.3	0.047
trans-Benzene dihydrodiol	1,000	2.7*	0.003*	11	2.0	0.18
cis-Benzene dihydrodiol	630	1.9	0.003*	9.7	1.5	0.15
2-Cyclohexen-1-ol	569	1.7	0.003*	26	2.4	0.093
Cyclohexanol		ns		74	0.54*	0.007*
Aliphatic alcohols						
Geranylgeraniol	1.2	2.3	2.0	1.0	0.46	0.46*
β-Ionol	9.7	3.2	0.32	1.0	1.3	1.3
Farnesol	11	0.27	0.025	1.2	1.1	0.85
Geraniol	76	0.92	0.012*	4.1	1.4	0.34
Nerol	162*	0.69*	0.004*	11	1.4	0.13

[a] K_m (μM), k_{cat} (min^{-1}) and k_{cat}/K_m ($min^{-1}\mu M^{-1}$) were determined with 1.0 mM NAD^+ as the coenzyme. The experimental errors were less than 15%, except that those indicated by asterisks were 15–20%. ns, no significant activity (>0.05 min^{-1}).

17-ketosteroids as substrates. The product of the 5α-androstane-3,17-dione reduction was identified as 5α-androstan-3β-ol-17-one by TLC.

Inhibitor sensitivity

AKR1C35 and AKR1C34 also differed in inhibitor sensitivity (Table IV). The dehydrogenase activity of AKR1C35 was inhibited by flavonoids, lithocholic acid and zearalenone, which did not show potent inhibitory effects on AKR1C34. AKR1C34 was selectively inhibited by phenolphthalein and hinokitiol, which have been reported to be inhibitors of rabbit M6DH (AKR1C26) (*15*). The inhibition patterns of AKR1C35 by these inhibitors were all competitive inhibitors with respect to the alcohol substrate, 17β-estradiol. The inhibitory flavonoids are 7-hydroxyflavones, with the exception of genistein that is an isoflavone. Flavone without hydroxyl group and daidzein, a des-5-hydroxy derivative of genistein, did not inhibit at a high concentration of 20 μM, suggesting that the 7-hydroxy group of flavones and 5-hydroxy group of isoflavones are structural requisites for binding to the enzyme.

Tissue distribution

The tissue distribution of AKR1C35 in male and female hamsters was assessed by RT-PCR, and compared with that of AKR1C34 (Fig. 3). The expression of the mRNA for AKR1C35 was markedly sex-different: In the male hamster, its expression was almost liver-specific, and was much lower in the brain, kidney, adrenal gland and small intestine. In the female hamster, the mRNA for AKR1C35 was expressed in many tissues except for the colon. In contrast, opposite sexual dimorphism was observed in the expression of the mRNA for AKR1C34. In the male hamster, the mRNA for AKR1C34 was expressed in many tissues, of which the liver showed high expression. In the female hamster, it was predominantly detected in the liver.

Effect of mutagenesis on steroid specificity of AKR1C35

The most significant difference between AKR1C35 and AKR1C34 was the oxidation of 3β-hydroxysteroids and 17β-estradiol, despite their high sequence identity, as described earlier. Previous crystallographic studies of several members of the AKR1C subfamily (*24–29*) show 16 substrate-binding residues, of which five residues at positions 54, 118, 227, 308 and 310 are different between AKR1C35 and AKR1C34 (Fig. 1). Among them, the residues 54 and 310 are situated inside (adjacent to the catalytic residue, Tyr55) and outside, respectively, of the active sites of known AKR crystal structures. In addition, Val54 and Cys310 in AKR1C35 are Ala and Phe, respectively, in AKR1C34 and other AKRs (1C12, 1C13, 1C16, 1C17, 1C24, 1C26, 1C27 and 1C28) with NAD^+-dependent 3α- and/or 3α/17β-HSD activity. To examine whether the two residues are responsible for the unique steroid specificity of AKR1C35, we prepared two single mutant AKR1C35s (V54A and C310F) and a

Table III. Substrate specificity for hydroxysteroids.

	AKR1C35			AKR1C34		
Substrate	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]	K_m[a]	k_{cat}[a]	k_{cat}/K_m[a]
3α-Hydroxysteroids						
3α-Hydroxyprogesterone		ns		0.5	0.35	0.70
5β-Pregnan-3α-ol-20-one		na		1.0	0.12	0.12
5β-Androstan-3α-ol-17-one		ns		0.5	0.058*	0.12*
3β-Hydroxysteroids						
3β-Hydroxyprogesterone	0.9	3.4*	3.8*		ns	
5α-Androstan-3β-ol-17-one	2.5	1.6	0.64		na	
5α-Pregnan-3β-ol-20-one	3.9	0.78	0.20		na	
Dehydroepiandrosterone	4.3	0.60	0.14		na	
5β-Androstan-3β-ol-17-one	46	1.3*	0.028*		na	
5β-Pregnan-3β-ol-20-one	6.8	0.17*	0.025*		na	
5β-Pregnane-3β,21-diol-20-one	15	0.26	0.017*		na	
17β-Hydroxysteroids						
17β-Estradiol	0.4	2.8	7.0		ns	
Testosterone	8.1	1.7	0.21	3.4	0.53	0.16
5α-DHT	7.6	1.3	0.17	0.6	0.61	1.0
5β-DHT	13	0.47	0.036*	1.2	0.37	0.31
20α-Hydroxysteroids						
20α-Hydroxyprogesterone		na		0.5	0.37	0.74
5α-Pregnan-20α-ol-3-one		ns		1.4	0.78	0.56
3,17-Dihydroxysteroids						
5α,3β-DIOL	4.5	3.4	0.76		ns	
5-Androstene-3β,17β-diol	3.3	1.9	0.58		ns	
5α,3α-DIOL	7.2	2.6	0.36		ns	
4-Androstene-3α,17β-diol	8.1	2.6	0.33	2.0	0.22*	0.11*
5β-Androstane-3α,17β-diol	3.9	0.72	0.18	0.7	0.73	1.0
5β-Androstane-3β,17β-diol	15	2.2	0.15		ns	

[a] K_m (μM), k_{cat} (min^{-1}) and k_{cat}/K_m ($min^{-1}\mu M^{-1}$) were determined with 1.0 mM NAD^+ as the coenzyme. The experimental errors were <15%, except that those indicated by asterisks were 15–20%. ns, no significant activity (>0.05 min^{-1}). na, no activity.

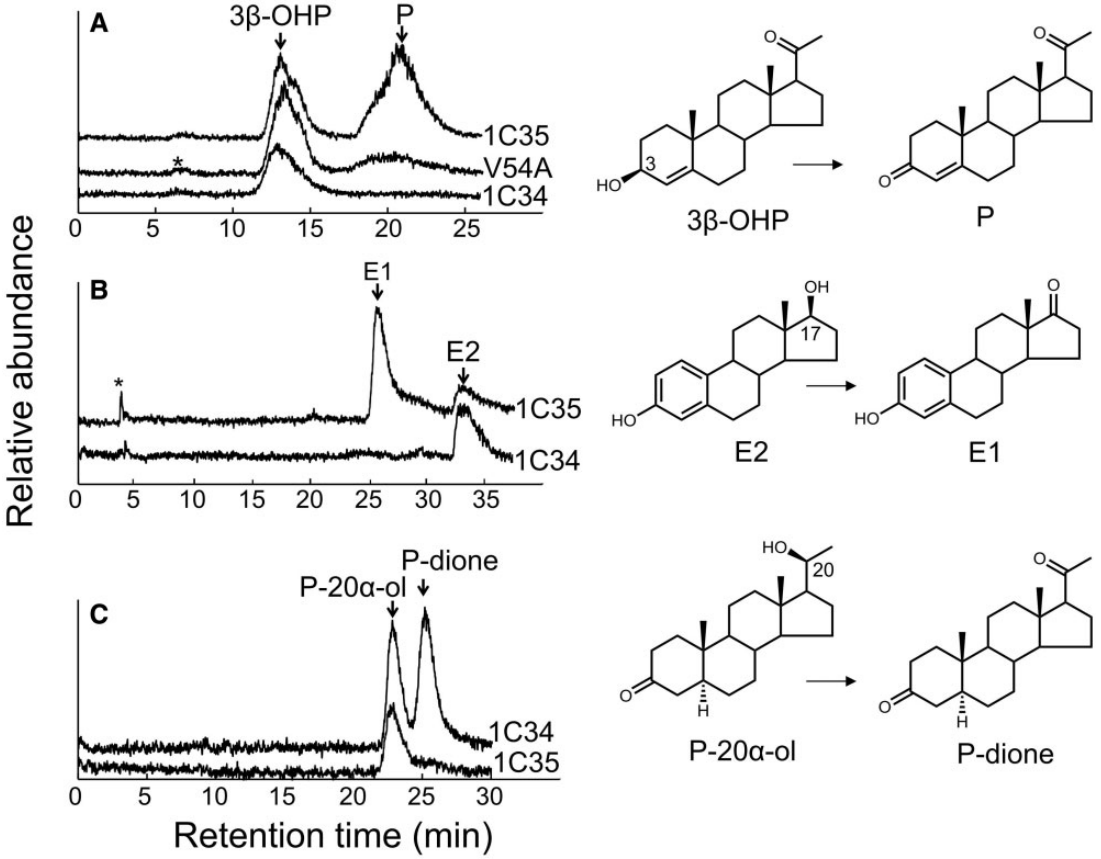

Fig. 2 LC/MS analysis of oxidized products of 3β-hydroxyprogesterone (3β-OHP), 17β-estradiol (E2) and 5α-pregnan-20α-ol-3-one (P-20α-ol). (**A**) Oxidation of 3β-OHP by AKR1C34 (1C34), AKR1C35 (1C35) and its V54A mutant. 3β-OHP and progesterone (P) were monitored at their total ions of *m*/*z* 317.4 and 299.4, respectively. (**B**) Oxidation of E2 by 1C34 and 1C35. E2 and estrone (E1) were monitored at their total ions of m/z 271.1 and 255.1, respectively. (**C**) Oxidation of P-20α-ol by 1C34 and 1C35. P-20α-ol and 5α-pregnane-3,20-dione (P-dione) were monitored at their fragment ions of *m*/*z* 319.2 and 317.2, respectively. The peaks other than the above reduced products and substrates are due to unknown substances (*).

Table IV. Inhibitor sensitivity of AKR1C34 and AKR1C35.

Inhibitor	AKR1C35		AKR1C34
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM) [a]
Flavonoids			
7-OH flavone	0.92 ± 0.02	0.11 ± 0.02	>20[a]
Chrysin	1.0 ± 0.2	0.11 ± 0.02	>20[a]
Quercetin	2.6 ± 0.2	0.46 ± 0.03	>20[a]
Genistein	4.2 ± 0.2	0.55 ± 0.02	>20[a]
Galangin	5.0 ± 0.3	0.69 ± 0.05	>20[a]
Others			
Lithocholic acid	4.4 ± 0.2	0.39 ± 0.1	>20[a]
Hexestrol	6.5 ± 0.5	0.54 ± 0.06	1.3 ± 0.2
Zearalenone	15 ± 1	3.3 ± 0.6	>50[a]
Phenolphthalein	>50[a]	nd[b]	0.65 ± 0.02
Hinokitiol	>50[a]	nd[b]	*3.2* ± 0.2

[a] The inhibition by the indicated concentration of inhibitor was <36% inhibition.
[b] nd, not determined.

double V54A/C310F mutant enzyme, and compared the effects of mutations on kinetic constants for 3β-hydroxysteroids (3β-hydroxyprogesterone, 5α-androstan-3β-ol-17-one and 5α,3β-DIOL), 17β-estradiol and *S*-tetralol (Table V). The mutation of V54A resulted in >20-fold decreases in k_{cat} and k_{cat}/K_m values for steroidal substrates. The large impairment of the 3β-HSD activity was also evident from the insignificant formation of progesterone in the product analysis of the 3β-hydroxyprogesterone oxidation by this mutant enzyme (Fig. 2). Compared with the V54A mutation, the C310F mutation showed smaller effects on the kinetic constants, with the exception of its larger effect on the K_m value for 5α,3β-DIOL. Like wild-type AKR1C35, the C310F mutant enzyme formed 5α-DHT and 5α-androstan-3β-ol-17-one as the major and minor products of the oxidation of 5α,3β-DIOL. The double mutation of V54A/C310F impaired the k_{cat} and k_{cat}/K_m values for all steroidal substrates more greatly than the single V54A mutation. No significant dehydrogenase activities for 3α- and 20α-hydroxysteroids were detected for the V54A and double mutant enzymes, whereas the C310F mutant enzyme showed 3α- and 20α-HSD activities towards 3α-hydroxyprogesterone (K_m = 0.8 ± 0.1 μM, k_{cat} = 1.2 ± 0.1 min^{-1}), 5β-pregnan-3α-ol-20-one (K_m = 0.8 ± 0.1 μM, k_{cat} = 0.48 ± 0.08 min^{-1}), 5α-pregnan-20α-ol-3-one (K_m = 0.2 ± 0.03 μM, k_{cat} = 0.13 ± 0.02 min^{-1}), and 20α-hydroxyprogesterone (K_m = 1.7 ± 0.2 μM, k_{cat} = 0.09 ± 0.01 min^{-1}). In contrast to the alteration in the steroid specificity,

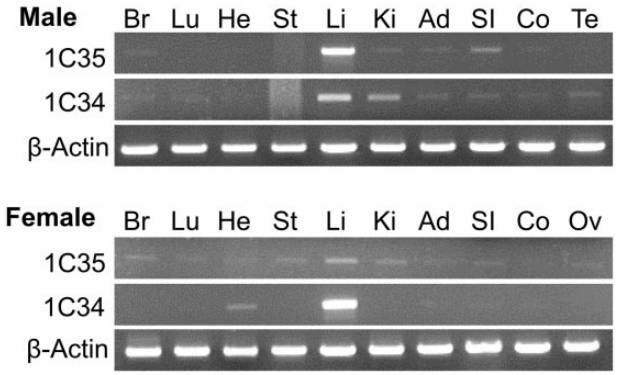

Fig. 3 RT-PCR analysis for expression of mRNAs for AKR1C35 and AKR1C34 in male and female hamster tissues. Tissues: brain (Br), lung (Lu), heart (He), stomach (St), liver (Li), kidney (Ki), adrenal gland (Ad), small intestine (SI), colon (Co), testis (Te) and ovary (Ov). The expression of mRNA for β-actin is shown as the control.

the three mutations did not significantly alter the kinetic constants for the small and non-steroidal substrate, *S*-tetralol, except that the V54A and double mutations caused 5-fold decreases in the k_{cat} value.

Discussion

The present cDNA cloning and characterization of the encoded proteins, AKR1C34 and AKR1C35, reveal that AKR1C34 is NAD^+-dependent M6DH that was previously purified from hamster liver (*16*). The identity of AKR1C34 and M6DH is evidenced by the match between their amino acid sequences and similarity of their enzymatic properties including substrate specificity for xenobiotic alcohols and 17β-hydroxysteroids and low inhibitor sensitivity to quercetin and lithocholic acid. In addition, AKR1C34 resembles rabbit M6DH (AKR1C26) (*15*) in their substrate specificity for endogenous isoprenyl alcohols (geranylgeraniol and farnesol) and 3α/20α-hydroxysteroids. However, the tissue expression of hamster AKR1C34 is different from ubiquitously expressed rabbit M6DH (*15* and S. Endo, unpublished results) in its sexual dimorphism (ubiquitous in the males versus apparently liver-specific in the females). Because AKR1C34 showed high catalytic efficiency for 5α-DHT, it may function in metabolism of the active androgen especially in male hamster tissues. AKR1C34 also showed lower K_m and higher k_{cat}/K_m values for most xenobiotic alcohols compared with AKR1C35, suggesting that the enzyme is involved in the hepatic metabolism of xenobiotic alcohols, as well as biologically active steroids.

AKR1C35, that was newly identified in this study, is the first NAD^+-dependent dehydrogenase with 3(17)β-HSD activity in the AKR superfamily. Among the substrates of AKR1C35, 17β-estradiol is the best substrate showing the highest k_{cat}/K_m value, which is also higher than those of NAD^+-dependent 3α/17β/20α-HSDs of rabbits (AKR1C27 and AKR1C28) (*15*), rats (AKR1C16 and AKR1C24) (*11*, *12*) and mice (AKR1C12 and AKR1C13) (*12*, *13*). This ability most likely contributes to controlling the intracellular concentration of the active estrogen in female hamster tissues, in which AKR1C35 was ubiquitously distributed. Among other 17β-hydroxysteroids and 3α,17β-dihydroxysteroids, the neurosteroid 5α,3α-DIOL (*30*) was most efficiently oxidized into inactive 5α-androstan-3α-ol-17-one by AKR1C35. In the brain of female hamsters, the enzyme might be involved in inactivation of this neurosteroid.

The most striking difference of AKR1C35 from AKR1C34 and the aforementioned rodent and rabbit NAD^+-dependent AKRs is its ability to oxidize various 3β-hydroxy-5α/β-dihydrosteroids and 3β-hydroxy-Δ^5-androstenes (Table III). In this respect, the enzyme is also distinct from NAD^+-preferring 3α(17β)-HSD that was purified from hamster liver cytosol (*31*). It has been known that NAD^+-dependent isozymes of rat and mouse Δ^5-3β-HSDIs exhibit 3β-HSD activity towards 3β-hydroxy-5α-androstanes (*32*), and alcohol dehydrogenase isozymes such as human ADH1C(γ) and horse ADH1S oxidize 3β-hydroxy-5β-dihydro-C_{19}/C_{21}-steroids (*33*). The steroid specificity of AKR1C35 is broader than those of the isozymes of Δ^5-3β-HSDI and alcohol dehydrogenase. In hamsters, such a 3β-HSD activity has not been examined for NAD^+-dependent isozymes (types I and II) of Δ^5-3β-HSDI that are expressed in the adrenal gland and gonads (*34*), but alcohol dehydrogenase BB-isoenzyme that is mainly distributed in the liver oxidizes only 5β-androstan-3β-ol-17-one of the 3β-hydroxysteroids (*35*). Although AKR1C35 did not efficiently oxidize 5β-androstan-3β-ol-17-one, it may act as a major 3β-HSD for other 3β-hydroxy-5α/β-dihydrosteroids in this animal. The 3β-hydroxysteroid substrates of AKR1C35 include ligands of estrogen receptor β (5α,3β-DIOL and 5-androstene-3β,17β-diol) (*36*, *37*) and antagonists of γ-aminobutyric acid type-A receptor (5α/β-pregnan-3β-ol-20-ones and 5β-pregnane-3β,21-diol-20-one) (*38*), of which 5β-pregnan-3β-ol-20-one also induces erythropoiesis (*39*). AKR1C35 may participate in the metabolism of the active 3β-hydroxysteroids, particularly, in female hamster tissues including the brain.

As described earlier, the ubiquitous expression of AKR1C34 in male hamster tissues and AKR1C35 in the female tissues is probably related to their roles in controlling the intracellular concentrations of 5α-DHT (for AKR1C34), 17β-estradiol and biologically active 3β-hydroxysteroids (for AKR1C35). In contrast, AKR1C34 and AKR1C35 were expressed almost specifically and highly in the livers of female and male hamsters, respectively. Although the relevance of the female liver-specific expression of AKR1C34 to steroid metabolism is unclear, the hepatic enzyme would contribute to generation of progesterone from its metabolites 3α/20α-hydroxyprogesterones. The male-liver specific expression of AKR1C35 is similar to that of hamster Δ^5-3β-HSDI type III isozyme that catalyzes the NADPH-dependent conversion of 5α-DHT into 5α,3β-DIOL (*34*). In this respect, AKR1C35 with the opposite activity (converting 5α,3β-DIOL back to 5α-DHT) may play a role in controlling the concentrations of the two active steroids in male hamster liver in concert with Δ^5-3β-HSDI type III isozyme. Alternatively, AKR1C34 and AKR1C35 may act as

Table V. Alteration of kinetic constants for steroidal and non-steroidal substrates by mutagenesis.

		K_m		k_{cat}		k_{cat}/K_m	
Substrate	**Enzyme**	**(μM)**	**Mu/WT[a]**	**(min^{-1})**	**Mu/WT[a]**	**($\mu M^{-1} min^{-1}$)**	**Mu/WT[a]**
3β-Hydroxyprogesterone	WT [b]	0.9	1	3.4	1	3.8	1
	V54A	6.3 ± 0.2	7	0.18 ± 0.02	0.05	0.028	0.007
	C310F	0.7 ± 0.1	0.8	1.2 ± 0.3	0.4	1.7	0.5
	V54A/C310F	5.5 ± 0.6	6	0.078 ± 0.010	0.02	0.014	0.004
5α-Androstan-3β-ol-17-one	WT [b]	2.5	1	1.6	1	0.64	1
	V54A	6.2 ± 0.7	4	0.074 ± 0.009	0.05	0.012	0.02
	C310F	2.3 ± 0.4	1	2.4 ± 0.6	2	1.0	2
	V54A/C310F	3.7 ± 0.5	2	0.053 ± 0.007	0.03	0.014	0.02
5α,3β-DIOL	WT [b]	4.5	1	3.4	1	0.76	1
	V54A	2.0 ± 0.2	0.4	0.069 ± 0.009	0.02	0.035	0.05
	C310F	0.8 ± 0.1	0.2	2.7 ± 0.4	0.8	3.4	4
	V54A/C310F	5.1 ± 0.3	1	0.018 ± 0.003	0.005	0.0035	0.005
17β-Estradiol	WT [b]	0.4	1	2.8	1	7.0	1
	V54A	1.6 ± 0.2	5	0.20 ± 0.03	0.09	0.13	0.02
	C310F	0.5 ± 0.1	1	0.31 ± 0.06	0.5	0.62	0.09
	V54A/C310F	2.3 ± 0.2	6	0.088 ± 0.009	0.03	0.038	0.005
S-Tetralol	WT [b]	61	1	6.3	1	0.10	1
	V54A	68 ± 3	1	1.4 ± 0.3	0.2	0.021	0.2
	C310F	60 ± 5	1	5.6 ± 0.7	0.9	0.093	0.9
	V54A/C310F	31 ± 2	0.5	1.1 ± 0.2	0.2	0.035	0.4

[a] Ratio of mutant enzyme to wild-type (WT).
[b] The values of WT are taken from Tables II and III.

major dehydrogenases for xenobiotic alcohols in the livers of the females and males, respectively, because the xenobiotic substrates include long-chain aliphatic alcohols, constituents of plants ingested by animals. The regulation mechanism of the expression of the AKR1C35 and Δ^5-3β-HSDI isozyme genes in relation to the sexual dimorphism needs to be established in further metabolomic and molecular genetic studies.

7-Hydroxyflavones and an isoflavone, genistein, were AKR1C35-specific inhibitors, suggesting that its activity is affected by ingested flavonoids. NAD^+-dependent 3α/17β/20α-HSDs of rats (AKR1C24) (*11*) and rabbits (AKR1C27 and AKR1C28) (*15*), and rabbit ovarian NADPH-dependent 20α-HSD (AKR1C5) (*40*) were also sensitive to flavonoids, but these enzymes are insensitive to isoflavones and therefore differ from genestin-sensitive AKR1C35. Genistein also inhibits NAD^+-dependent Δ^5-3β-HSDI isozymes (*41*, *42*) that are expressed in steroidogenic glands (*32*). When the isoflavone is ingested in the hamster, it may decrease not only the synthesis of steroid hormones in the endocrine glands by inhibiting the Δ^5-3β-HSDI isozymes, but also the metabolism of 5α-DHT, 17β-estradiol and active 3β-hydroxysteroids by inhibiting AKR1C35. Therefore, the isoflavone might not exert large effects on concentrations of these active steroids in this animal.

The results of the mutagenesis have revealed that Val54 plays more critical role in recognition of 3β- and 17β-hydroxysteroid substrates by AKR1C35 than Cys310. In the previous studies on 3β-HSD activity of human NADPH-dependent AKRs (1C1 and 1C2), the residue at position 54 is suggested to play an important role in determining the stereospecific reduction of 3-keto group of 5α-DHT into 3α- or 3β-hydroxy group (*9*). The conversion of the steroid specificity from AKR1C1 into AKR1C2 and vice versa is achieved by replacement of the residue 54 between Val in AKR1C1 and Leu in AKR1C2 (*43*, *44*). In the light of these studies, the present mutation of V54A can be interpreted to alter the orientation of 3β- and 17β-hydroxy groups of the steroidal substrates in the active site of AKR1C35. However, the mutations of the residues in AKR1C35 to the corresponding residues in AKR1C34 did not convert it to a 3α/17β/20α-HSD that is similar to AKR1C34. As the double mutation further impaired the 3β- and 17β-HSD activities in contrast to the appearance of 3α- and 20α-HSD activities by the single C310F mutation, the steroid specificity of AKR1C35 may be dictated by a combination of Val54, Cys310 and other substrate-binding residues, yet to be identified.

Supplementary Data

Supplementary Data are available at *JB* Online.

Funding
This work was partly funded by grant-in-aid for Scientific Research (C) from the Japan Society for the Promotion of Science (26460149).

Conflict of Interest
None declared.

References

1. Penning, T.M. (1997) Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocr. Rev.* **18**, 281–305
2. Kavanagh, K.L., Jörnvall, H., Persson, B., and Oppermann, U. (2008) The SDR superfamily: functional

and structural diversity within a family of metabolic and regulatory enzymes. *Cell. Mol. Life Sci.* **65**, 3895–3906

3. Barski, O.A., Tipparaju, S.M., and Bhatnagar, A. (2008) The aldo-keto reductase superfamily and its role in drug metabolism and detoxification. *Drug Metab. Rev.* **40**, 553–624
4. Wu, X., Lukacik, P., Kavanagh, K.L., and Oppermann, U. (2007) SDR-type human hydroxysteroid dehydrogenases involved in steroid hormone activation. *Mol. Cell. Endocrinol.* **265-266**, 71–76
5. Ishikura, S., Usami, N., Kitahara, K., Isaji, T., Oda, K., Nakagawa, J., and Hara, A. (2001) Enzymatic characteristics and subcellular distribution of a short-chain dehydrogenase/reductase family protein, P26h, in hamster testis and epididymis. *Biochemistry* **40**, 214–224
6. Matsunaga, T., Endo, S., Maeda, S., Ishikura, S., Tajima, K., Tanaka, N., Nakamura, KT., Imamura, Y., and Hara, A. (2008) Characterization of human DHRS4: an inducible short-chain dehydrogenase/reductase enzyme with 3α-hydroxysteroid dehydrogenase activity. *Arch. Biochem. Biophys.* **477**, 339–347
7. Nakajin, S., Takase, N., Ohno, S., Toyoshima, S., and Baker, M.E. (1998) Mutation of tyrosine-194 and lysine-198 in the catalytic site of pig 3α/β,20β-hydroxysteroid dehydrogenase. *Biochem. J.* **334**, 553–557
8. Penning, T.M., Burczynski, M.E., Jez, J.M., Hung, C.F., Lin, H.K., Ma, H., Moore, M., Palackal, N., and Ratnam, K. (2000) Human 3α-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. *Biochem. J.* **351**, 67–77
9. Steckelbroeck, S., Jin, Y., Gopishetty, S., Oyesanmi, B., and Penning, T.M. (2004) Human cytosolic 3α-hydroxysteroid dehydrogenases of the aldo-keto reductase superfamily display significant 3β-hydroxysteroid dehydrogenase activity: implications for steroid hormone metabolism and action. *J. Biol. Chem.* **279**, 10784–10795
10. Sanai, M., Endo, S., Matsunaga, T., Ishikura, S., Tajima, K., El-Kabbani, O., and Hara, A. (2007) Rat NAD^+-dependent 3α-hydroxysteroid dehydrogenase (AKR1C17): a member of the aldo-keto reductase family highly expressed in kidney cytosol. *Arch. Biochem. Biophys.* **464**, 122–129
11. Ishikura, S., Matsumoto, K., Sanai, M., Horie, K., Matsunaga, T., Tajima, K., El-Kabbani, O., and Hara, A. (2006) Molecular cloning of a novel type of rat cytoplasmic 17β-hydroxysteroid dehydrogenase distinct from the type 5 isozyme. *J. Biochem.* **139**, 1053–1063
12. Endo, S., Sanai, M., Horie, K., Matsunaga, T., Ishikura, S., Tajima, K., El-Kabbani, O., and Hara, A. (2007) Characterization of rat and mouse NAD^+-dependent 3α/17β/20α-hydroxysteroid dehydrogenases and identification of substrate specificity determinants by site-directed mutagenesis. *Arch. Biochem. Biophys.* **467**, 76–86
13. Endo, S., Matsumoto, K., Matsunaga, T., Ishikura, S., Tajima, K., El-Kabbani, O., and Hara, A. (2006) Substrate specificity of a mouse aldo-keto reductase (AKR1C12). *Biol. Pharm. Bull.* **29**, 2488–2492
14. Endo, S., Matsunaga, T., Kumada, S., Fujimoto, A., Ohno, S., El-Kabbani, O., Hu, D., Toyooka, N., Mano, J., Tajima, K., and Hara, A. (2012) Characterization of rabbit aldose reductase-like protein with 3β-hydroxysteroid dehydrogenase activity. *Arch. Biochem. Biophys.* **527**, 23–30
15. Endo, S., Matsunaga, T., Fujimoto, A., Kumada, S., Arai, Y, Miura, Y., Mikamo, H., El-Kabbani, O., Yamano, S., Iinuma, M., and Hara, A. (2012) Characterization of a rabbit morphine 6-dehydrogenase and two NAD^+-dependent 3α(17β)-hydroxysteroid dehydrogenases. *Arch. Biochem. Biophys.* **529**, 131–139
16. Todaka, T., Yamano, S., and Toki, S. (2000) Purification and characterization of NAD-dependent morphine 6-dehydrogenase from hamster liver cytosol, a new member of the aldo-keto reductase superfamily. *Arch. Biochem. Biophys.* **374**, 189–197
17. Platt, K.L. and Oesch, F. (1977) An improved synthesis of *trans*-5,6-dihydroxy-1,3- cyclohexadiene (*trans*-1,2-dihydroxy-1,2-dihydrobenzene). *Synthesis,* **7**, 449–450
18. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor.
19. Endo, S., Matsunaga, T., Kuragano, T., Ohno, S., Kitade, Y., Tajima, K., El-Kabbani, O., and Hara, A. (2010) Properties and tissue distribution of a novel aldo-keto reductase encoding in a rat gene (Akr1b10). *Arch. Biochem. Biophys.* **503**, 230–237
20. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
21. Ishikura, S., Usami, N., Nakajima, S., Kameyama, A., Shiraishi, H., Carbone, V., El-Kabbani, O., and Hara, A. (2004) Characterization of two isoforms of mouse 3(17)α-hydroxysteroid dehydrogenases of the aldo-keto reductase family. *Biol. Pharm. Bull.* **27**, 1939–1945
22. Uehara, Y., Urata, H., Ideishi, M., Arakawa, K., and Saku, K. (2002) Chymase inhibition suppresses high-cholesterol diet-induced lipid accumulation in the hamster aorta. *Cardiovasc. Res.* **55**, 870–876
23. Takenoshita, R., Nomura, Y., and Toki, S. (2001) Cloning and expression of cDNA encoding hamster liver 3-hydroxyhexobarbital/17β(3α)-hydroxysteroid dehydrogenase 1. *Chem. Biol. Interact.* **130-132**, 863–870
24. Penning, T.M. (1999) Molecular determinants of steroid recognition and catalysis in aldo-keto reductases. Lessons from 3α-hydroxysteroid dehydrogenase. *J. Steroid Biochem. Mol. Biol.* **69**, 211–225
25. Couture, J.F., Legrand, P., Cantin, L., Labrie, F., Luu-The, V., and Breton, R. (2004) Loop relaxation, a mechanism that explains the reduced specificity of rabbit 20α-hydroxysteroid dehydrogenase, a member of the aldo-keto reductase superfamily. *J. Mol. Biol.* **339**, 89–102
26. Couture, J.F., Legrand, P., Cantin, L., Luu-The, V., Labrie, F., and Breton, R. (2003) Human 20α-hydroxysteroid dehydrogenase: crystallographic and site-directed mutagenesis studies lead to the identification of an alternative binding site for C_{21}-steroids. *J. Mol. Biol.* **331**, 593–604
27. Jin, Y., Stayrook, S.E., Albert, R.H., Palackal, N.T., Penning, T.M., and Lewis, M. (2001) Crystal structure of human type III 3α-hydroxysteroid dehydrogenase/bile-acid binding protein complexed with $NADP^+$ and ursodeoxycholate. *Biochemistry* **40**, 10161–10168
28. Qiu, W., Zhou, M., Labrie, F., and Lin, S.X. (2004) Crystal structures of the multispecific 17β-hydroxysteroid dehydrogenase type 5: critical androgen regulation in human peripheral tissues. *Mol. Endocrinol.* **18**, 1798–1807
29. Faucher, F., Cantin, L., de Jesus-Tran, K.P., Lemieux, M., Luu-The, V., Labrie, F., and Breton, R. (2007) Mouse 17α-hydroxysteroid dehydrogenase (AKR1C21)

binds steroids differently from other aldo-keto reductases: Identification and characterization of amino acid residues critical for substrate binding. *J. Mol. Biol.* **369**, 525–540

30. Reddy, D.S. (2010) Neurosteroids: endogenous role in the human brain and therapeutic potentials. *Prog. Brain Res.* **186**, 113–137
31. Ohmura, M., Hara, A., Nakagawa, M., and Sawada, H. (1990) Demonstration of 3α(17β)-hydroxysteroid dehydrogenase distinct from 3α-hydroxysteroid dehydrogenase in hamster liver. *Biochem. J.* **266**, 583–589
32. Simard, J., Ricketts, M.L., Gingras, S., Soucy, P., Feltus, F.A., and Melner, M.H. (2005) Molecular biology of the 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase gene family. *Endocr. Rev.* **4**, 525–582
33. Plapp, B.V. and Berst, K.B. (2003) Specificity of human alcohol dehydrogenase 1C*2 (γ2γ2) for steroids and simulation of the uncompetitive inhibition of ethanol metabolism. *Chem. Biol. Interact.* **143-144**, 183–193
34. Rogerson, F.M., Courtemanche, J., Fleury, A., Head, J.R., LeHoux, J.G., and Mason, J.I. (1998) Characterization of cDNAs encoding isoforms of hamster 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase. *J. Mol. Endocrinol.* **20**, 99–110
35. Keung, W.M. (1996) Isolation and characterization of three alcohol dehydrogenase isozymes from Syrian golden hamsters. *Alcohol Clin. Exp. Res.* **20**, 213–220
36. Wang, M., He, Y., Eisenman, L.N., Fields, C., Zeng, C.M., Mathews, J., Benz, A., Fu, T., Zorumski, E., Steinbach, J.H., Covey, D.F., Zorumski, C.F., and Mennerick, S. (2002) 3β-hydroxypregnane steroids are pregnenolone sulfate-like $GABA_A$ receptor antagonists. *J. Neurosci.* **22**, 3366–3375
37. Handa, R.J., Weiser, M.J., and Zuloaga, D.G. (2009) A role for the androgen metabolite, 5α-androstane-3β,17β-diol, in modulating oestrogen receptor β-mediated regulation of hormonal stress reactivity. *J. Neuroendocrinol.* **21**, 351–358
38. Baker, M.E. (2013) What are the physiological estrogens? *Steroids* **78**, 337–340
39. Besa, E.C., Gorshein, D., Hait, W.A., and Gardner, F.H. (1973) Effective erythropoiesis induced by 5β-pregnane-3β-hydroxy-20-one in squirrel monkeys. *J. Clin. Invest.* **52**, 2278–2282
40. Endo, S., Arai Y., Hara A., Kitade Y., Bunai Y., El-Kabbani O., and Matsunaga T. (2013) Substrate specificity and inhibitor sensitivity of rabbit 20α-hydroxysteroid dehydrogenase. *Biol. Pharm. Bull.* **36**, 1514–1518
41. Ohno, S., Matsumoto, N., Watanabe, M., and Nakajin, S. (2004) Flavonoid inhibition of overexpressed human 3β-hydroxysteroid dehydrogenase type I. *J. Steroid Biochem. Mol. Biol.* **88**, 175–182
42. Hu, G.X., Zhao, B.H., Chu, Y.H., Zhou, H.Y., Akingbemi, B.T., Zheng, Z.Q., and Ge, R.S. (2010) Effects of genistein and equol on human and rat testicular 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase 3 activities. *Asian J. Androl.* **4**, 519–526
43. Matsuura, K., Deyashiki, Y., Sato, K., Ishida, N., Miwa, G., and Hara, A. (1997) Identification of amino acid residues responsible for differences in substrate specificity and inhibitor sensitivity between two human liver dihydrodiol dehydrogenase isoenzymes by site-directed mutagenesis. *Biochem. J.* **323**, 61–64
44. Zhang, B., Zhu, D.W., Hu, X.J., Zhou, M., Shang, P., and Lin, S.X. (2014) Human 3α-hydroxysteroid dehydrogenase type 3 (3α-HSD3): the V54L mutation restricting the steroid alternative binding and enhancing the 20α-HSD activity. *J. Steroid Biochem. Mol. Biol.* **141**, 135–143