Substrate Specificity and Inhibitor Sensitivity of Rabbit 20*α*-Hydroxysteroid Dehydrogenase

Satoshi Endo,*[,a] Yuki Arai,[a] Akira Hara,[b] Yukio Kitade,[b] Yasuo Bunai,[c] Ossama El-Kabbani,[d] and Toshiyuki Matsunaga[a]

[a] *Laboratory of Biochemistry, Gifu Pharmaceutical University; Gifu 502–1196, Japan:* [b] *Department of Biomolecular Science, Faculty of Engineering, Gifu University:* [c] *Department of Legal Medicine, Graduate School of Medicine, Gifu University; Gifu 501–1193, Japan: and* [d] *Department of Medicinal Chemistry, Victorian College of Pharmacy, Monash University; Parkville, Victoria 3052, Australia.*

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In this study, we examined the substrate specificity and inhibitor sensitivity of rabbit 20*α*-hydroxysteroid dehydrogenase (AKR1C5), which plays a role in the termination of pregnancy by progesterone inactivation. AKR1C5 moderately reduced the 3-keto group of only 5*α*-dihydrosteroids with 17*β*- or 20*α*/*β*-hydroxy group among 3-ketosteroids. In contrast, the enzyme reversibly and efficiently catalyzed the reduction of various 17- and 20-ketosteroids, including estrogen precursors (dehydroepiandrosterone, estrone and 5*α*-androstan-3*β*-ol-17-one) and tocolytic 5*β*-pregnane-3,20-dione. In addition to the progesterone inactivation, the formation of estrogens and metabolism of the tocolytic steroid by AKR1C5 may be related to its role in rabbit parturition. AKR1C5 also reduced various non-steroidal carbonyl compounds, including isatin, an antagonist of the C-type natriuretic peptide receptor, and 4-oxo-2-nonenal, suggesting its roles in controlling the bioactive isatin and detoxification of cytotoxic aldehydes. AKR1C5 was potently and competitively inhibited by flavonoids such as kaempferol and quercetin, suggesting that its activity is affected by ingested flavonoids.

Key words aldo-keto reductase 1C5; 20*α*-hydroxysteroid dehydrogenase; 4-oxo-2-nonenal; 17*β*-hydroxysteroid dehydrogenase; dehydroepiandrosterone; isatin

20*α*-Hydroxysteroid dehydrogenase (20*α*-HSD, EC 1.1.1.49) catalyzes the conversion of an active progestin, progesterone, into 20*α*-hydroxyprogesterone, and plays a major role in the termination of pregnancy.[1)] The cDNAs for 20*α*-HSDs have been cloned from human[2)] and other mammalian tissues.[3–6)] The mammalian enzymes are composed of 323-amino acids, and belong to the aldo-keto reductase (AKR) superfamily (http://www.med.upenn.edu/akr/).[1)] Among them, human 20*α*-HSD (AKR1C1) is the most characterized enzyme. AKR1C1 reduces various 20-ketosteroids, and exhibits moderate 3*α*- and 3*β*-HSD activities for 5*β*-dihydro-3-ketosteroids and 5*α*-dihydro-3-ketosteroids, respectively, although its 17*β*-HSD activity is negligibly low.[7–9)] It also shows broad substrate specificity for endogenous and xenobiotic non-steroidal carbonyl compounds, which include lipid peroxidation products (4-hydroxy-2-nonenal[10)] and 4-oxo-2-nonenal[11)]), prostaglandin (PG) D_2,[9)] *p*-quinones and drug ketones.[3,12,13)] In the reverse reaction, AKR1C1 exhibits dihydrodiol dehydrogenase activity, which oxidizes *trans*-dihydrodiol metabolites of aromatic hydrocarbons.[14)] Due to possible involvement of AKR1C1 in inactivation of active steroids and cancer development, its various inhibitors have been reported.[11,15)]

In other mammals, 20*α*-HSDs of monkeys (AKR1C25),[3,9)] rats (AKR1C8)[12,16,17)] and mice (AKR1C18)[9)] show dual 3*α*- and 3*β*-HSD activities and broad substrate specificity for 20-ketosteroids and xenobiotic compounds. AKR1C25 shares 94% amino acid sequence identity with AKR1C1 exhibiting almost identical properties including PGD_2 reductase activity and inhibitor sensitivity, whereas AKR1C8 and AKR1C18 share lower amino acid sequence identity with AKR1C1 (72 and 69%, respectively), do not reduce PGD_2 and show an inhibitor sensitivity different from AKR1C1. In rabbits, 20*α*-HSD (AKR1C5) that shares 80% sequence identity with AKR1C1 was molecularly cloned from the ovary,[6)] but its substrate specificity for steroids and PGs differs from that of the human enzyme. AKR1C5 displays comparable activity for the 3*α*-, 17*β*- and 20*α*-reduction of 5*α*-dihydrotestosterone, 4-androstene-3,17-dione and progesterone, respectively,[18)] and is identical to ovarian PGE_2 9-ketoreductase.[19)] However, it remains unknown whether AKR1C5 exhibits such a broad specificity for other steroids and nonsteroidal compounds reported for the human and rodent 20*α*-HSDs. In this study, we examined the substrate specificity of AKR1C5 for various steroids and nonsteroidal compounds, in order to elucidate its role other than the progesterone inactivation. Additionally, we compared the inhibitor sensitivity of AKR1C5 with that of AKR1C1.

MATERIALS AND METHODS

Materials Nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and their reduced forms (NADH and NADPH) were obtained from Oriental Yeast Co. (Tokyo, Japan); steroids were from Steraloids (Newport, RI, U.S.A.); 4-oxo-2-nonenal, 4-hydroxy-2-nonenal and PGD_2 were from Cayman Chemical (Ann Arbor, MI, U.S.A.). *trans*-Benzene dihydrodiol,[20)] 6-*tert*-butyl-2,3-epoxy-5-cyclohexen-1,4-dione (TBE),[21)] isocaproaldehyde,[22)] 4-oxo-2-nonenal[23)] and 4-oxo-2-hexanal[24)] were synthesized as described previously. 3-Deoxyglucosone and befunolol were gifts from Nippon Zoki Pharmaceutical Co. (Osaka, Japan) and Kaken Pharmaceutical Co. (Tokyo, Japan), respectively.

Preparation of Recombinant Enzymes A cDNA for AKR1C5 was isolated from a total RNA sample of ovaries of a Japanese white rabbit by reverse transcription (RT)-poly-

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* To whom correspondence should be addressed. e-mail: sendo@gifu-pu.ac.jp

merase chain reaction (PCR). The preparation of total RNA, RT, and DNA techniques followed the standard procedures described by Sambrook *et al.*[25] The PCR was performed using *Pfu* DNA polymerase (Stratagene) and a pair of sense and antisense primers, 5′-ttttcatatgatggatcccaaatttcagcg-3′ and 5′-ttttgtcgacttaatattcatcagaaaatgggt-3,′ which contain underlined *Nde*I and *Sal*I sites, respectively. The PCR products were purified, digested with the two restriction enzymes (Invitrogen), and ligated into the pCold I vectors (TaKaRa) that had been digested with the two restriction enzymes. The sequence of the coding region of the cDNA was confirmed to encode the 323-amino acid sequences of AKR1C5[7] fused to the N-terminal 6-His tag by DNA sequencing. The expression construct was transfected into *Escherichia coli* BL21 (DE3) pLysS, and the recombinant AKR1C5 was expressed and purified to homogeneity from the cell extracts using the nickel-charged Sepharose 6FF resin (GE Healthcare) as described previously.[24] Recombinant AKR1C1 was expressed from the cDNA and purified to homogeneity as described previously.[26]

Assay of Enzyme Activity The dehydrogenase and reductase activities of the recombinant proteins were assayed at 25°C by measuring the rate of change in NADPH fluorescence and its absorbance, respectively, in 0.1 M potassium phosphate buffer, pH 7.4, containing enzyme, substrate and coenzyme (0.25 mM $NADP^+$ and 0.1 mM NADPH, respectively).[24] The reductase activity for all-*trans*-retinal was measured by the method of Parés and Julià,[27] except that 0.1 M potassium phosphate, pH 7.4, containing 0.01% Tween 80 was added as the buffer.

The apparent K_m and k_{cat} values for coenzymes and substrates were determined by fitting the initial velocities to the Michaelis–Menten equation. The inhibitor constant, K_i, was estimated from the Dixon plot of the velocities obtained in the substrate β-ionol range (20–200 μM) with three concentrations of the inhibitor. The kinetic constants and IC_{50} values 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 The reaction was conducted at 37°C for 30 min in a 2.0-mL reaction mixture, containing 0.2 mM NADPH, substrate (0.05–0.1 mM), enzyme (0.1 mg), and 0.1 M potassium phosphate, pH 7.4. The products were extracted into 4 mL ethyl acetate, and identified by TLC and liquid chromatography/mass spectrometry.[9,24] The reduced products of ONE were also analyzed by TLC.[23]

RESULTS AND DISCUSSION

Specificity for Steroids The 3-, 17- and 20-ketosteroids reduced by the purified recombinant AKR1C5 in the presence of NADPH are listed in Table 1. Among 3-ketosteroids, 5α-dihydrotestosterone (5α-androstan-17β-ol-3-one) and 5α-pregnan-20α-ol-3-one were reduced into their 3α-hydroxy metabolites, but their 5β-isomers and Δ^4-unsaturated-3-ketosteroids were not the substrates. In contrast, the enzyme efficiently reduced 17-keto groups of 5α/β-androstanes, dehydroepiandrosterone (DHEA, 5-androsten-3β-ol-17-one) and its 7α-hydroxy and 3-sulfate derivatives, as well as 4-androstene-3,17-dione and estrone. The reduced products of 4-androstene-3,17-dione, 5β-androstan-3α-ol-17-one, 5α-androstan-3β-ol-17-one, DHEA and estrone were identified as their 17β-hydroxy metabolites. Similarly, AKR1C5 reacted towards various 20-ketosteroids as excellent substrates. The reduced products of 5α/β-pregnane-3,20-diones and progesterone were identified as their 20α-hydroxy metabolites, indicating that the enzyme preferentially reduces the 20-keto group of the 3,20-diketosteroids. Thus, the binding of the 17- and 20-ketosteroids to the enzyme may not be significantly affected with their differences in the fusion types between A- and B-rings, the unsaturated bond on the rings and the configuration of 3-hydroxy group on A-ring.

In the reverse reaction, AKR1C5 oxidized various 17β- and 20α-hydroxysteroids (Table 2), and the oxidized products of 5α/β-androstane-3α,17β-diols were identified as their 17-keto metabolites. The results suggest that the oxidoreduction of the 17β- and 20α-hydroxy groups on the steroid substrates by the enzyme is reversible. In contrast, no significant activities were observed with 5α/β-androstan-3α/β-ol-17-ones, 5α/β-pregnan-3α/β-ol-20-ones, 5α/β-pregnane-3α,21-diol-20-ones, 4-pregnen-3α-ol-20-one and lithocholic acid as substrates, indicating that the reduction of the 5α-dihydro-3-ketosteroids is apparently irreversible.

The stereospecific reduction of only 5α-dihydro-3-ketosteroids by AKR1C5 is clearly different from those by AKR1C1 and rodent 20α-HSDs (AKR1C8 and AKR1C18) that act as reversible 3α- and 3β-HSDs towards 5α- and 5β-dihydrosteroids, respectively.[8,9,12] In addition, the high 17β-HSD activity is a unique property of AKR1C5, because the 17-keto- and 17β-hydroxy-steroids listed in Tables 1 and 2 were not significantly reduced or oxidized by AKR1C1 (data not shown), AKR1C18[9] and AKR1C8.[12,16] A crystallographic study of AKR1C5-$NADP^+$-testosterone ternary complex has shown that the substrate specificity of the enzyme is medi-

Table 1. Kinetic Constants for Ketosteroids

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)
3-Ketosteroids [a]			
5α-Pregnan-20α-ol-3-one	5.9	4.6	0.78
5α-Dihydrotestosterone	3.4	1.5	0.44
17-Ketosteroids			
4-Androstene-3,17-dione	1.0	2.6	2.6
5β-Androstan-3α-ol-17-one	1.7	4.0	2.4
DHEA	6.0	9.3	1.6
5α-Androstan-3α-ol-17-one	4.1	5.1	1.2
DHEA-3-sulfate	4.2	4.8	1.1
5β-Androstan-3β-ol-17-one	2.8	2.7	0.96
Estrone	1.1	1.0	0.91
7α-Hydroxy-DHEA	2.9	2.6	0.90
Estrone-3-sulfate	6.7	5.9	0.88
5α-Androstan-3β-ol-17-one	6.0	5.0	0.83
20-Ketosteroids			
Progesterone	0.6	1.6	2.7
5α-Pregnane-3,20-dione	0.5	1.1	2.2
4-Pregnen-3α-ol-20-one	1.0	1.9	1.9
5β-Pregnan-3α-ol-20-one	1.0	1.8	1.8
5β-Pregnane-3,20-dione	1.0	1.7	1.7
5α-Pregnan-3α-ol-20-one	1.3	1.9	1.5
5β-Pregnane-3α,21-diol-20-one	2.6	1.0	0.38
5α-Pregnane-3α,21-diol-20-one	6.2	1.1	0.18

a) 5β-Androstan-17β-ol-3-one, 5β-pregnan-20α-ol-3-one, testosterone and 20α-hydroxyprogesterone were not reduced.

ated by three structural elements; the relaxation of loop B (residues 223–230), the nature of the residue at position 54 and the residues in C-terminal tail.[18] In particular, the relaxation movement of loop B induced upon the binding of the substrate is proposed to be mostly related to the 17β-HSD activity of AKR1C5.

Specificity for Non-steroidal Compounds AKR1C5 reduced various endogenous and xenobiotic carbonyl compounds (Table 3). Among the endogenous substrates, isatin was the most excellent substrate, showing the highest k_{cat}/K_m value. Most of the endogenous aldehydes were reduced at low K_m values, which were comparable to those for the steroidal substrates. These substrates include cytotoxic aldehydes (4-oxo-2-nonenal, 4-hydroxy-2-nonenal and 4-oxo-2-hexenal)[23,28,29] and all-*trans*-retinal, and the reduced product of 4-oxo-2-nonenal was identified as its alcohol metabolite, 4-oxo-2-nonenol. The enzyme also showed high activities for xenobiotic pyridine-3-aldehyde, *p*-quinones (2-*tert*-butyl-1,4-benzoquinone, TBE and menadione) and ketones (4-nitroacetophenone, befunolol, α-tetralone and ketotifen). While AKR1C5 is known to reduce PGE_2,[19] no reductase activity was detected with 0.1 mM PGD_2 as the substrate. In addition to the inability to reduce PGD_2, the high k_{cat}/K_m values for isatin and the *p*-quinones of AKR1C5 is distinct from the low values of AKR1C1.[13,30] In contrast, AKR1C5 is similar to AKR1C18 in the high activity for isatin,[9] but the two enzymes clearly differ in the reactivity to befunolol, which is not reduced by AKR1C18.

In the reverse reaction, AKR1C5 oxidized xenobiotic alicyclic and aliphatic alcohols, including *trans*-benzene dihydrodiol that is a representative substrate of dihydrodiol dehydrogenase[12,14] (Table 2). Among the xenobiotic substrates, β-ionol was most efficiently oxidized. The substrate specificity is similar to those of AKR1C1[3,12,13] and AKR1C18,[9] but the kinetic constants for the substrates are different among the three enzymes. In fact, AKR1C1 oxidized β-ionol at lower K_m (1.2 μM) and k_{cat} values (3.2 min^{-1}) than the values of AKR1C5.

Physiological Roles Although AKR1C5 predominantly functions in the metabolism of progesterone in the ovarian and luteal tissues,[6,19] its broad substrate specificity for compounds other than progesterone and PGE_2 suggests following novel roles. 1) The enzyme efficiently reduced estrone, 4-androstene-3,17-dione and DHEA, which are precursors of 17β-estradiol synthesis. In addition to the inactivation of progesterone, the estrogen synthesis by AKR1C5 may be related to its major role in the termination of pregnancy. AKR1C5 is suggested to be implicated in luteolysis by producing $PGF_{2\alpha}$ from PGE_2,[19] but the reported K_m and k_{cat}/K_m values for PGE_2 are much higher and lower, respectively, than those for the above 17-ketosteroids. Recently, 17β-estradiol has been reported to control time of luteolysis in rats and heifers.[31,32] Thus, it is possible that the reductive 17β-HSD activity of AKR1C5 is involved in regressing post-partum rabbit corpora lutea. 2) 5-Androstene-3β,17β-diol, the reduced metabolite of DHEA by AKR1C5, binds to estrogen receptor (ER) β,[33] which is expressed in rabbit ovary and uterus.[34] Additionally, AKR1C5 reduced 5α-androstan-3β-ol-17-one into 5α-androstane-3β,17β-diol, which is another ligand of ERβ.[35] Since rabbit aldose reductase-like protein (AKR1B19) that converts 5α-androstane-3,17-dione into 5α-androstan-3β-ol-17-one is expressed in many rabbit tissues including the ovary,[24] AKR1C5 might be involved in the synthesis of this ERβ ligand in concert with AKR1B19. The production of the

Table 2. Kinetic Constants for Hydroxysteroids and Other Alcohols

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)
17β-Hydroxysteroids			
5β-Androstane-3α,17β-diol	1.4	15	11
5α-Androstane-3α,17β-diol	4.2	16	3.8
5-Androstene-3β,17β-diol	3.1	6.0	1.9
5β-Androstan-17β-ol-3-one	6.2	9.2	1.5
5α-Androstane-3β,17β-diol	2.2	1.9	0.86
5β-Androstane-3β,17β-diol	2.5	2.0	0.80
Testosterone	13	3.4	0.26
17β-Estradiol	1.3	0.67	0.52
5α-Dihydrotestosterone	18	2.8	0.16
20α-Hydroxysteroids			
5β-Pregnane-3α,20α-diol	7.8	17	2.2
5α-Pregnane-3α,20α-diol	0.8	1.5	1.8
5β-Pregnan-20α-ol-3-one	5.7	9.2	1.6
20α-Hydroxyprogesterone	2.2	2.9	1.3
5α-Pregnan-20α-ol-3-one	11	9.5	0.86
Non-steroidal alcohols			
β-Ionol	18	14	0.61
(*S*)-1-Tetralol	112	13	0.12
(*S*)-1-Indanol	131	12	0.092
4-Chromanol	152	4.1	0.027
trans-Benzene dihydrodiol	260	6.5	0.025
Geraniol	180	2.0	0.011

Table 3. Kinetic Constants for Non-steroidal Carbonyl Compounds

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)
Endogenous ketones			
Isatin	0.6	8.2	14
Diacetyl	19	9.5	0.50
Methylglyoxal	88	7.2	0.082
Acetoin	162	5.8	0.080
3-Deoxyglucosone	1400	11	0.008
Endogenous aldehydes			
4-Oxo-2-nonenal	1.8	6.7	3.7
1-Nonanal	0.9	1.9	2.1
1-Hexanal	7.6	2.6	0.34
trans-2-Nonenal	5.5	1.2	0.22
trans-2-Hexenal	7.4	1.5	0.20
Acrolein	14	2.1	0.15
4-Oxo-2-hexenal	5.9	0.69	0.11
4-Hydroxy-2-nonenal	5.3	0.52	0.098
all-*trans*-Retinal	8.9	0.51	0.057
Isocaproaldehyde	68	1.4	0.021
Xenobiotics			
TBQ[a]	1.0	11	11
4-Nitroacetophenone	1.2	6.7	5.6
TBE	2.0	10	5.0
Befunolol	34	7.0	0.79
Pyridine-3-aldehyde	61	7.9	0.13
Menadione	18	7.5	0.12
α-Tetralone	53	3.2	0.060
Ketotifen	111	1.2	0.011

a) 2-*tert*-Butyl-1,4-benzoquinone.

ER ligands by AKR1C5 may also stimulate roles of ERβ in the ovary and uterus.[36,37] 3) The efficient reduction of potent tocolytic 5β-pregnane-3,20-dione[38,39] by AKR1C5 may be related to the role of the enzyme in parturition. 4) Isatin, the best substrate of AKR1C5, has recently been reported to antagonize rat brain receptor for C-type natriuretic peptide (CNP),[40] which is also secreted from mouse growing follicles and stimulates preantral and antral follicle growth.[41] AKR1C5 may be involved in ovarian follicle development by metabolizing the CNP receptor antagonist, isatin. 5) The enzyme also reduced other α-dicarbonyls (diacetyl and methylglyoxal) and various aliphatic aldehydes at low K_m values, which are comparable or superior to those of rabbit aldose reductase and AKR1B19.[24] It is possible that AKR1C5 acts as a detoxification enzyme for reactive α-dicarbonyl compounds[42] and cytotoxic aldehydes[23,28,29] in the ovary, where 4-hydroxy-2-nonenal and reactive oxygen species are increased by age-related oxidative stress.[43]

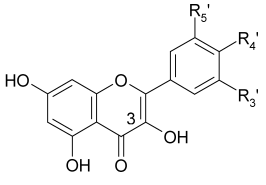
Inhibitor Sensitivity The $NADP^+$-linked β-ionol dehydrogenase activity of AKR1C5 was inhibited by known AKR1C1 inhibitors (Table 4). Among them, phenolphthalein inhibited AKR1C5 more potently than AKR1C1, whereas 3′,3″,5′,5″-tetrabromophenolphthalein (TBPP) and benzbromarone, potent competitive inhibitors of AKR1C1,[15] showed less inhibition of AKR1C5. The low sensitivity towards TBPP and benzbromarone is similar to that of AKR1C18,[9] but AKR1C5 was relatively resistant to inhibition by sulfobromophthalein, a potent AKR1C18 inhibitor showing an IC_{50} value of 20 nM.[9] Compared to AKR1C1, AKR1C5 was potently inhibited by flavonoids, suggesting that its activity is affected by ingested flavonoids. Quercetin with hydroxy groups at positions 3′ and 4′ inhibited AKR1C5 more potently than its analogs (kaempferol, naringenin galandin and myricetin), suggesting that at least the presence of two hydroxy groups at positions 3′ and 4′ on the structure of galandin is a structural rationale for the potent inhibition of AKR1C5 by flavonoids. The inhibition patterns of AKR1C5 by phenolphthalein, benzbromarone, quercetin and kaempferol were competitive with respect to β-ionol, showing K_i values of 4.7±0.5, 41±5, 13±2 and 30±6 nM, respectively. This indicates that the inhibitors bind to the substrate-binding site of the enzyme. Therefore, the difference in the inhibitor sensitivity between AKR1C5 and AKR1C1 is reflected by the difference between the substrate-binding pockets of the two enzymes.

Table 4. Inhibitor Sensitivity of AKR1C5 and AKR1C1

Inhibitor	IC_{50} (μM)[a]	
	AKR1C5	AKR1C1
AKR1C1 inhibitors		
Phenolphthalein	0.022	4.6[b]
Benzbromarone	0.12	0.048[b]
TBPP	3.0	0.033[b]
Hexestrol	6.4	8.3[b]
Mefenamic acid	10	2.2
Sulfobromophthalein	11	>20
Flufenamic acid	26	5.3[b]
Flavonoids[c]		
Quercetin	0.059	1.4
Kaempferol	0.11	7.3
Naringenin	0.37	0.31
Galangin	0.41	2.6
Myricetin	0.64	5.5

a) The values of AKR1C5 and AKR1C1 were determined in the oxidation of 0.18 mM β-ionol and 0.1 mM (*S*)-1-teralol, respectively. *b*) The values are taken from ref. 15. *c*) Structures of the flavonoids are shown below. Naringenin is a kaempferol derivative without 3-hydroxy group.

	R_3'	R_4'	R_5'
Galangin	H	H	H
Kaempferol	H	OH	H
Quercetin	OH	OH	H
Myricetin	OH	OH	OH



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