Archives of Biochemistry and Biophysics 527 (2012) 23–30

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Characterization of rabbit aldose reductase-like protein with 3β-hydroxysteroid dehydrogenase activity

Satoshi Endo [a,*], Toshiyuki Matsunaga [a], Sho Kumada [a], Airi Fujimoto [a], Satoshi Ohno [b], Ossama El-Kabbani [c], Dawei Hu [d], Naoki Toyooka [d], Jun'ichi Mano [e], Kazuo Tajima [f], Akira Hara [a]

[a] Laboratory of Biochemistry, Gifu Pharmaceutical University, Gifu 501-1196, Japan
[b] Faculty of Engineering, Gifu University, Gifu 501-1193, Japan
[c] Monash Institute of Pharmaceutical Sciences, Monash University, Victoria 3052, Australia
[d] Graduate School of Science and Technology for Research, Graduate School of Innovative Life Science, University of Toyama, Toyama 930-8555, Japan
[e] Science Research Center, Yamaguchi University, Yamaguchi 753-8515, Japan
[f] Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa 920-1181, Japan

ARTICLE INFO

Article history:
Received 15 June 2012
and in revised form 9 July 2012
Available online 1 August 2012

Keywords:
AKR1B10
Aldo–keto reductase
Aldose reductase
3β-Hydroxysteroid dehydrogenase
4-Oxo-2-nonenal

ABSTRACT

In this study, we isolated the cDNA for a rabbit aldose reductase-like protein that shared an 86% sequence identity to human aldo–keto reductase (AKR)[1] 1B10 and has been assigned as AKR1B19 in the AKR superfamily. The purified recombinant AKR1B19 was similar to AKR1B10 and rabbit aldose reductase (AKR1B2) in the substrate specificity for various aldehydes and α-dicarbonyl compounds. In contrast to AKR1B10 and AKR1B2, AKR1B19 efficiently reduced 3-keto-5α/β-dihydro-C19/C21/C24-steroids into the corresponding 3β-hydroxysteroids, showing K_m of 1.3–9.1 μM and k_{cat} of 1.1–7.6 min^{-1}. The stereospecific reduction was also observed in the metabolism of 5α- and 5β-dihydrotestosterones in AKR1B19-overexpressing cells. The mRNA for AKR1B19 was ubiquitously expressed in rabbit tissues, and the enzyme was co-purified with 3β-hydroxysteroid dehydrogenase activity from the lung. Thus, AKR1B19 may function as a 3-ketoreductase, as well as a defense system against cytotoxic carbonyl compounds in rabbit tissues. The molecular determinants for the unique 3-ketoreductase activity were investigated by replacement of Phe303 and Met304 in AKR1B19 with Gln and Ser, respectively, in AKR1B10. Single and double mutations (F303Q, M304S and F303Q/M304S) significantly impaired this activity, suggesting the two residues play critical roles in recognition of the steroidal substrate.

Introduction

3β-Hydroxysteroid dehydrogenase (HSD[1])/Δ^5–Δ^4 isomerase is a NAD^+-dependent membrane-bound enzyme that is responsible for the oxidation and isomerization of Δ^5-3β-hydroxysteroid precursors into Δ^4-3-ketosteroids (Fig. 1), thus catalyzing an essential and irreversible step in the biosynthesis of all classes of active steroid hormones and bile acids [1]. In humans, three 3β-HSD/Δ^5–Δ^4 isomerase isoenzymes, type-I, type-II and type-VII, are identified, and oxidize 3β-hydroxy-Δ^5-C_{19}/C_{21}-steroids and 3β-hydroxy-Δ^5-C_{27}-steroids. In addition, several human NADPH-dependent enzymes, belonging to the aldo–keto reductase (AKR) superfamily (http://www.med.upenn.edu/akr/) [2] and the short-chain dehydrogenase/reductase (SDR) superfamily [3], have been reported to reduce 3-keto-5α/β-dihydrosteroids into the corresponding 3β-hydroxysteroids (Fig. 1). Cytosolic 20α-HSD (AKR1C1), 3α-HSD type-3 (AKR1C2), 17β-HSD type-5 (AKR1C3), and 3α-HSD type-1 (AKR1C4) non-stereospecifically reduce 5α-dihydrotestosterone into its 3α- and 3β-ol forms, and of the four enzymes AKR1C1 exhibits the most efficient 3β-HSD activity [4]. The stereospecific reduction of 3-keto-C_{19}/C_{21}-steroids into 3β-hydroxysteroids is catalyzed by membrane-associated 17β-HSD type-7 [5] and peroxisomal dehydrogenase/reductase (SDR family) member 4 (DHRS4) [6] that belong to the SDR superfamily. These NADPH-dependent enzymes are thought to be involved in the synthesis of the following biologically active 3β-hydroxy-5α/β-dihydro-C_{19}/C_{21}-steroids.

* Corresponding author. Fax: +81 58 230 8105.
E-mail address: sendo@gifu-pu.ac.jp (S. Endo).

[1] *Abbreviations used:* HSD, hydroxysteroid dehydrogenase; AKR, aldo–keto reductase; SDR, short-chain dehydrogenase/reductase; DHRS4, dehydrogenase/reductase (SDR family) member 4; AR, aldose reductase; SI, sequence identity; ONE, 4-oxo-2-nonenal; AKR1B19, rabbit aldose reductase-like protein; PHPC, (*Z*)-2-(phenylimino)-7-hydroxy-*N*-(pyridin-2-yl)-2*H*-chromene-3-carboxamide; HAHE, 3-(4-hydroxy-2-methoxyphenyl)acrylic acid 3-(3-hydroxyphenyl)propyl ester; TBE, 6-*tert*-butyl-2,3-epoxy-5-cyclohexene-1,4-dione; GS-ONE, glutathione adduct of ONE; RT, reverse transcription; bp, base pair; U, unit; LC/MS, liquid chromatography/mass spectrometry; BAEC, bovine aortic endothelial cell; FBS, fetal bovine serum.

0003-9861/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.abb.2012.07.012

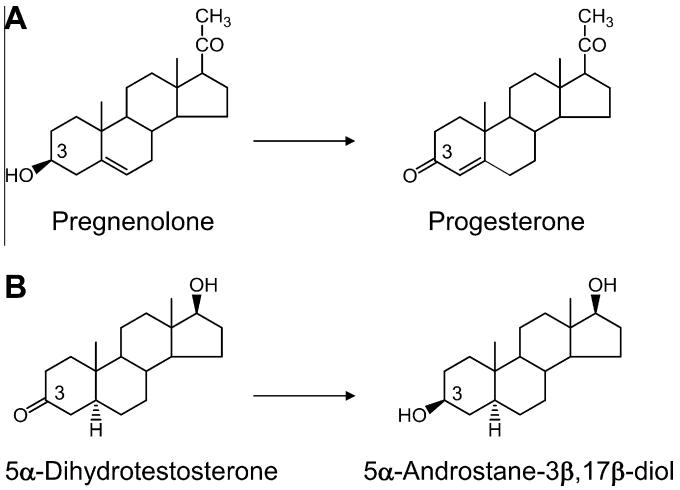

Fig. 1. Conversion catalyzed by NAD^+-dependent 3β-HSD/Δ^5–Δ^4 isomerase (A) and NADPH-dependent enzymes with reductive 3β-HSD activity (B).

5α-Androstane-3β,17β-diol strongly binds to the estrogen receptor β, regulates brain function, and modulates the growth of prostate and its cancer [7–10]. 3β-Hydroxypregnanes are pregnenolone sulfate-like antagonists of γ-aminobutyric acid type-A receptor [11], and 5β-pregnan-3β-ol-20-one shows stimulatory action on preoptic neurons [12].

In rats and mice, in addition to the NAD^+-dependent 3β-HSD/Δ^5–Δ^4 isomerase, its NADPH-dependent isoenzymes (type-III, type-IV and type-V) are expressed and function as 3-ketosteroid reductases which produce 3β-hydroxy-C_{19}/C_{21}-steroids [1]. Such a NADPH-dependent isoenzyme has not been identified in human tissues [1,13], where human AKRs (1C1–1C4), 17β-HSD type-7 and DHRS4 exhibit the reductive 3β-HSD activity as described above. In contrast to the human 17β-HSD type-7 and DHRS4, mouse 17β-HSD type-7 does not reduce 3-keto-C_{19}-steroids [5], and rat and rabbit DHRS4s exhibit 3α-HSD activity [14]. Rabbit 20α-HSD (AKR1C5), which corresponds to human AKR1C1, exhibits 3α-HSD activity towards 3-ketosteroids [15]. Thus, there is a clear species difference in function of enzymes that act as reductive 3β-HSDs.

Recently, mouse aldose reductase (AR)-like protein (AKR1B7) has been reported to reduce 3-keto bile acids into the corresponding 3β-hydroxy bile acids, in addition to their inherent roles in the metabolism of aldehydes [16]. In contrast, 3-ketosteroids including bile acids are not reduced by human AR (AKR1B1) and AR-like protein (AKR1B10) [17] and rat AR-like proteins (AKR1B13 [18], AKR1B14 [19] and AKR1B18 [20]), and rather inhibit AKR1B1 and AKR1B10 [17]. The human and rodent AR-like proteins also differ in their kinetic constants for common carbonyl substrates [16–20] and reactivity to prostaglandins [21,22]. Current rabbit genomic analysis has predicted only one gene encoding an AR-like protein that shares more than 80% amino acid sequence identity (SI) with the human and rodent AR-like proteins. In this paper, we isolated the cDNA for the rabbit AR-like protein that has been assigned as AKR1B19 in the AKR superfamily, and examined the enzymatic properties of the recombinant AKR1B19 and tissue distribution. The data indicated that AKR1B19 acts not only as a reductase for reactive carbonyl compounds derived from lipid peroxidation like AR-like proteins of other species, but also as a superior reductive 3β-HSD for 3-keto-5α/β-dihydro-C_{19}/C_{21}/C_{24}-steroids. Therefore, the molecular determinant for 3β-HSD activity was also investigated by site-directed mutagenesis of residues of this enzyme.

Materials and methods

Materials

Steroids were obtained from Steraloids (Newport, RI); 4-oxo-2-nonenal (ONE), 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and prostaglandins were from Cayman Chemical (Ann Arbor, MI); resins for column chromatography were from Amersham Biosciences (Piscataway, NJ); and (*Z*)-2-(4-methoxyphenylimino)-7-hydroxy-*N*-(pyridin-2-yl)-2*H*-chromene-3-carboxamide (PHPC) was from Asinex (Moscow, Russia). *Pfu* DNA polymerase was purchased from Stratagene; a pCold I expression vector and *Taq* DNA polymerase were from Takara (Kusatsu, Japan); and pCR2.1 plasmid, restriction enzymes, RACE kits, Lipofectamine 2000 reagent and *Escherichia coli* BL21 (DE3) pLysS were from Invitrogen (Carlsbad, CA). AR inhibitors were kindly donated by Dr. P.F. Kador (University of Nebraska Medical Center).

Synthesis of inhibitor and substrates

3-(4-Hydroxy-2-methoxyphenyl)acrylic acid 3-(3-hydroxyphenyl)propyl ester (HAHE, a human AKR1B10 inhibitor) [23], 6-*tert*-butyl-2,3-epoxy-5-cyclohexene-1,4-dione (TBE) [24], isocaproaldehyde [25] and geranylgeranial [26] were synthesized as described previously. 4-Oxo-2-nonenol, 4-hydroxy-2-nonenol and a glutathione adduct of ONE (GS-ONE) were prepared by the methods of Doorn et al. [27]. 4-Oxo-2-hexenal was synthesized according to the method of Grée et al [28]. Briefly, to a stirred solution of 2-ethylfuran (1 g) and *N*-bromosuccinimide (2.72 g) in tetrahydrofuran–acetone–water (10:8:2; v/v, 20 mL) at −20 °C was added dropwise 1.62 g of pyridine and kept stirred for 3 h. The temperature was then allowed to 25 °C, and the mixture was stirred overnight. A 0.5 M HCl aqueous solution (20 mL) was then added. The product was extracted into ether (20 mL) three times. The solvent was dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was chromatographed on a silica gel column with pentane–ether (85:15; v/v) as the eluent to yield pure 4-oxo-2-hexenal (300 mg). Identity and purity of 4-oxo-2-hexenal were confirmed with NMR [29].

CDNA isolation and site-directed mutagenesis

The cDNAs for AKR1B19 and rabbit AR (AKR1B2) [30] were isolated from the total RNA preparation of lungs of male Japanese white rabbits by reverse transcription (RT)-PCR. The preparation of total RNA, RT, and DNA techniques followed the standard procedures described by Sambrook et al. [31]. PCR was performed with *Pfu* DNA polymerase and a pair of sense and antisense primers, which contain *Nde*I and *Eco*RI sites. The primer sequences and PCR conditions are summarized in Table S1 (Supplementary Data). 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 by using a Beckman CEQ8000XL DNA sequencer, and was confirmed to encode the 316-amino acid sequences of AKR1B19 and AKR1B2 fused to the N-terminal 6-His tag. The 3′- and 5′-untranslated regions of the AKR1B19 mRNA were generated by using 3′- and 5′-RACE kits and the gene-specific primers. The fragments were subcloned into the pCR2.1 plasmids and sequenced as described above. The 1324-base pair (bp) sequence of the cDNA including the poly(A) sequence was deposited in DDBJ database with the accession number AB724112.

Mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) and the pCold I expression plasmid harboring the cDNA for AKR1B19 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 L116F, F303Q, M304S and F303Q/M304S mutant AKR1B19s (Table S1, Supplementary Data). The coding regions of the cDNAs in the expression plasmids were sequenced in order to confirm the presence of the desired mutation and ensure that no other mutation had occurred.

Purification of enzymes

The recombinant AKR1B19, its mutant enzymes, AKR1B2 and human AKR1B10 were expressed in *E. coli* BL21 (DE3) pLysS cells transformed with the expression plasmids harboring their cDNAs as described previously [17]. The enzymes were purified from the cell extracts using a nickel-charged Sepharose 6FF resin 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 1 mM dithiothreitol, 0.5 mM EDTA and 20% (v/v) glycerol. Purity was confirmed by SDS–PAGE, and protein concentration was determined by Bradford's method using bovine serum albumin as the standard [31]. The identity of the purified recombinant protein with AKR1B19 encoded in the cDNA was verified by the mass spectrometry analysis of its 15 lysylendopeptidase-digested peptides using a Bruker–Franzen Mass Spectrometry System Ultraflex TOF/TOF [32].

AKR1B19 was also partially purified at 4 °C from rabbit lung. The rabbit lung (4 g) was homogenized with four volumes of 50 mM Tris–HCl, pH 7.4, containing 5 mM EDTA and 0.15 M KCl. The homogenate was centrifuged at 105,000*g* for 1 h, and the supernatant fraction was subjected to $(NH_4)_2SO_4$ fractionation (35–75% saturation). The protein fraction was dissolved in 10 mM Tris–HCl, pH 8.0, containing 5 mM EDTA (Buffer A) plus 0.15 M KCl, and then applied to a Sephadex G-100 column (3×70 cm) equilibrated with the buffer. The fraction with both pyridine-3-aldehyde reductase and 5β-androstan-3β-ol-17-one dehydrogenase activities was applied to a Q-Sepharose column (2×15 cm) that had been equilibrated with Buffer A. After the column was washed with the buffer, the adsorbed proteins were eluted with a linear gradient of 0–0.1 M NaCl in the buffer.

Assay of enzyme activity

Reductase and dehydrogenase activities of AKR1B19, AKR1B2 and AKR1B10 were assayed by measuring the rate of change in NAD(P)H absorbance (at 340 nm) and its fluorescence (at 455 nm with an excitation wavelength of 340 nm), respectively. The standard reaction mixture for the reductase activity consisted of 0.1 M potassium phosphate, pH 7.4, 0.1 mM NADPH, 0.2 mM pyridine-3-aldehyde and enzyme, in a total volume of 2.0 mL. In the assay for the dehydrogenase activity, 0.25 mM $NADP^+$ was used as the coenzyme. The reductase and dehydrogenase activities of the lung extract and fractions during the enzyme purification were assayed with 0.2 mM pyridine-3-aldehyde and 50 μM 5β-androstan-3β-ol-17-one, respectively, as the substrates. 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 1.25%. The concentration of methanol did not affect the activities of the enzyme. The reductase activity for all-*trans*-retinal was measured by the method of Parés and Julià [33], except that 0.1 M potassium phosphate, pH 7.4, containing 0.01% Tween 80 was added as the buffer. One unit (U) of enzyme activity was defined as the enzyme amount that catalyzes the reduction of 1 μmol of NADPH or retinal per min at 25 °C.

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 NADPH and $NADP^+$ were determined in the presence of 0.2 mM pyridine-3-aldehyde and 40 μM 5β-androstan-3β-ol-17-one, respectively, as the substrates. After the IC_{50} (inhibitor concentrations required for 50% inhibition) values were determined with pyridine-3-aldehyde as the substrate, the kinetic studies in the presence of three concentrations of an inhibitor ($0.2–1 \times IC_{50}$) were carried out in a similar manner in both pyridine-3-aldehyde reduction and 5β-androstan-3β-ol-17-one oxidation. The inhibitor constants, K_{is} and K_{ii}, were calculated from the replots of the slopes and intercepts, respectively, of the double reciprocal plot versus the inhibitor concentration. The kinetic constants and IC_{50} are expressed as the means of two determinations or the means ± SD of three determinations.

Product identification

To identify reaction products, reduction was conducted in a 2.0-mL system containing 0.2 mM NADPH, substrate (0.05–1 mM), enzyme (1–20 μg) and 0.1 M potassium phosphate, pH 7.4. The substrate and products were extracted into 4 mL ethyl acetate 5–20 min after the reaction was started at 37 °C. The steroidal products were identified by TLC and liquid chromatography/mass spectrometry (LC/MS) using a Chiralcel OJ-H 5 μm column [6]. The TLC chromatogram was developed in chloroform–acetone (9:1, v/v) twice, and 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. The reduced products of ONE were also analyzed by the TLC method [27].

Tissue distribution analysis

The total RNA samples were prepared from the tissues of male rabbits [31]. The total RNA samples were subjected to RT-PCR using *Taq* DNA polymerase and primers to amplify cDNAs for AKR1B19, AKR1B2 and rabbit Δ^4-3-ketosteroid 5β-reductase (AKR1D3, accession no. AB023951). The cDNA for rabbit β-actin was also amplified as an internal control with the specific primers. The primer sequences and PCR conditions are described in Table S1 (Supplementary Data). The PCR products were separated by agarose gel electrophoresis, and revealed with ethidium bromide.

Cell culture experiments

Bovine aortic endothelial cells (BAECs) were generous gift from Taisho Pharmaceutical Co. (Saitama, Japan), and cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a humidified incubator containing 5% CO_2. The construction and transfection of the pGW1 plasmids harboring the cDNA for AKR1B19 into BAECs were performed as described previously [34]. Briefly, the cDNA was amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase and a pair of the forward and reverse primers (Table S1, Supplementary Data), which contain underlined *Eco*RI and *Sal*I sites, respectively. The PCR products were ligated into the pGW1 vectors that had been digested with the two restriction enzymes. The expression constructs were transiently transfected with the expression plasmids using the Lipofectamine 2000 reagent. The transfected cells were maintained in the medium containing 2% FBS for 24 h, and then used for experiments. The cell extract was subjected to the assay of the pyridine-3-aldehyde reductase activity. In the analysis of cellular 3-ketosteroid metabolism, the cells were plated into a 6-cm dish and cultured in the medium containing 50 μM 5α-dihydrotestosterone or 5β-dihydrotestosterone for 6 h. The steroid and its metabolites in the medium were extracted into 8 mL ethyl acetate, and the organic layer was taken and

evaporated. The 3-hydroxy metabolites of dihydrotestosterone were analyzed by the above LC/MS method, in which the eluted metabolites were monitored using the negative ion mode of m/z 293.5.

Results

Cloning, expression and purification of AKR1B19

The full-length cDNA (1324-bp) for AKR1B19 was isolated by RT-PCR from the total RNA sample of rabbit lung. The nucleotide sequence of the coding region was identical to that of the mRNA for *Oryctolagus cuniculus* AKR1B10 (Accession No. XM_002711986) predicted from the rabbit genomic analysis, with exception of three nucleotide replacements (^{135}T → C, ^{264}G → T, ^{459}A → G) that do not affect the amino acid sequence of AKR1B19. The protein encoded in the cDNA was expressed in *E. coli* cells, and detected as a 37-kDa protein on SDS–PAGE analysis of the cell extract. The recombinant AKR1B19 was purified to homogeneity by detecting the 37-kDa protein on the SDS–PAGE analysis. The sequences (composed of a total 238 residues) of 15 lysylendopeptidase-digested peptides derived from the purified enzyme were identical to those deduced from the cDNA for AKR1B19 (Fig. S1, Supplementary Data).

Substrate specificity of AKR1B19

The purified AKR1B19 exhibited NADPH-linked reductase activity (1.1 U/mg) towards 0.2 mM pyridine-3-aldehyde at pH 7.4, which was higher than the specific activity (0.25 U/mg) of recombinant rabbit AR (AKR1B2) determined under the same conditions. The optimum pH for the activity of AKR1B19 was observed between pH 6.2 and 6.4, while AKR1B2 showed a broad pH optimum from pH 6.2 to 6.8. AKR1B19 showed high coenzyme preference to NADPH. The K_m and k_{cat} values for NADPH were 1.0 μM and 44 min^{-1}, respectively, whereas the NADH (100 μM)-linked reductase activity was only 18% of the NADPH-linked activity. Therefore, the substrate specificity of AKR1B19 was examined using the preferable coenzyme NADPH at a physiological pH of 7.4, and compared with those of AKR1B2.

AKR1B19 reduced endogenous aliphatic aldehydes with various carbon chain lengths from acrolein to geranylgeranial (Table 1). Among them, ONE was the best substrate showing the highest catalytic efficiency (k_{cat}/K_m value). In the product analysis of the reduction of ONE by TLC, its alcohol, 4-oxo-2-nonenol, was detected as only one product. Although no reductase activity for 4-oxo-2-nonenol by the enzyme was detected in the spectrophotometric activity assay, GS-ONE was reduced with a sixfold higher K_m value than that for ONE. The enzyme also reduced endogenous α-dicarbonyl compounds, and xenobiotic aromatic aldehydes, *p*-quinones (TBE and 2-*tert*-butyl-1,4-benzoquinone) and ketones (3-nitroacetophenone and α-tetralone), of which the two *p*-quinones were excellent substrates, showing high k_{cat}/K_m values. AKR1B19 showed low activity towards D- and L-glyceraldehydes and D-threose, but no reductase activity was observed for D-glucose and D-xylose that were reduced by AKR1B2. The comparison of the kinetic constants for the above substrates between AKR1B19 and AKR1B2 is summarized in Table S2 (Supplementary Data). Only 4-hydroxy-2-hexenal, 4-oxo-2-hexenal, methylglyoxal and monosaccharides were efficiently reduced by AKR1B2, whereas the k_{cat}/K_m values for other substrates of AKR1B19 were higher than those of AKR1B2.

Since AKR1B19 reduced the xenobiotic ketones, its substrate specificity for endogenous ketones, prostaglandins and ketosteroids, was examined. The enzyme reduced 3-keto-5α/β-dihydro-C_{19}/C_{21}-steroids and 3-keto bile acids, showing K_m values of 1.3–9.1 μM and k_{cat} values of 1.1–7.6 min^{-1} (Table 2). No reductase activity was observed for 17- and 20-ketosteroids, Δ^4-3-ketosteroids (testosterone, 4-androstene-3,17-dione and progesterone),

Table 1
Substrate specificity for carbonyl compounds of AKR1B19.

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
Aldehydes			
ONE	1.0	30	30
GS-ONE	5.8	28	4.6
trans-2-Nonenal	8.0	22	2.8
Geranylgeranial	1.1	2.2	2.0
Farnesal	1.1	2.0	1.8
Acrolein	28	22	0.77
4-Hydroxy-2-nonenal	15	7.1	0.47
Isocaproaldehyde	9.0	3.7	0.41
Crotonaldehyde	52	20	0.38
all-*trans*-Retinal	4.0	1.4	0.35
4-Hydroxy-2-hexenal	13	2.5	0.19
trans-2-Hexenal	87	12	0.13
4-Oxo-2-hexenal	37	3.9	0.11
α-Dicarbonyls			
Isatin	8.6	3.3	0.38
3-Deoxyglucosone	74	11	0.15
Methylglyoxal	222	18	0.080
Diacetyl	455	30	0.066
Monosaccharides			
D-Glyceraldehyde	1220	13	0.011
L-Glyceraldehyde	2100	15	0.007
D-Threose	1100	5.3	0.005
Xenobiotics			
TBE	1.6	35	22
TBQ [a]	7.0	77	11
Pyridine-3-aldehyde	11	43	3.9
Benzaldehyde	15	11	0.73
3-Nitroacetophenone	346	12	0.035
α-Tetralone	141	2.2	0.016

[a] TBQ, 2-*tert*-butyl-1,4-benzoquinone.

Table 2
Substrate specificity for steroids.

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
Reduction			
5β-Pregnane-21-ol-3,20-dione	2.4	5.5	2.3
Dehydrolithocholic acid	1.3	3.1	2.3
5β-Dihydrotestosterone	3.5	6.8	1.9
5β-Androstane-3,17-dione	4.1	7.6	1.9
5β-Pregnan-20β-ol-3-one	3.0	4.0	1.3
5β-Pregnane-3,20-dione	5.5	6.4	1.2
5α-Dihydrotestosterone	2.2	2.4	1.1
5α-Pregnane-21-ol-3,20-dione	9.1	5.2	0.57
5α-Pregnane-3,20-dione	8.8	5.0	0.57
5β-Dihydrocorticosterone	7.3	2.7	0.37
5β-Dihydrocortisone	6.4	2.2	0.35
5α-Androstane-3,17-dione	7.6	2.6	0.34
5β-Cholanic acid-3,7-dione	4.9	1.1	0.22
Oxidation			
Isolithocholic acid	1.6	2.2	1.4
5β-Androstane-3β,17β-diol	9.4	5.2	0.55
5β-Pregnan-3β,21-diol-20-one	7.8	3.2	0.41
5β-Androstan-3β-ol-17-one	7.3	2.7	0.36
5β-Pregnan-3β-ol-20-one	22	4.9	0.22
5β-Pregnane-3β,20β-diol	13	2.7	0.21
5β-Pregnane-3β,20α-diol	22	2.6	0.11
5α-Cholanic acid-3β-ol	3.1	0.23	0.079
Geranylgeraniol	1.0	0.060	0.060
Farnesol	9.6	0.24	0.025

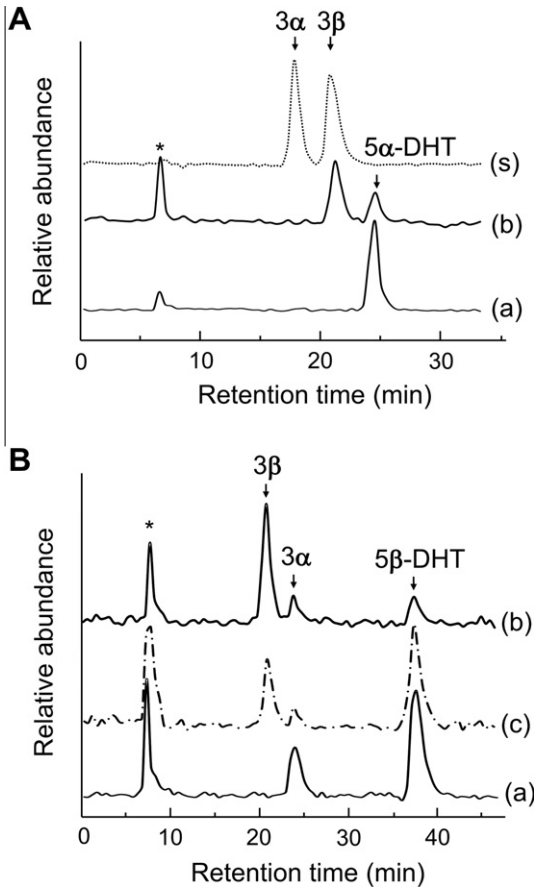

Fig. 2. LC/MS analysis of reduced products of 5α- and 5β-dihydrotestosterones in AKR1B19-overexpressed BEACs. The cells were cultured with 50 μM substrate for 6 h, and the products and remaining substrate in the medium were extracted and subjected to LC/MS. (A) Reduction of 5α-dihydrotestosterone (5α-DHT). Chromatograms: (s) authentic 5α-androstane-3α,17β-diol (3α) and 5α-androstane-3β,17β-diol (3β), (a) the control cells, and (b) the overexpressed cells. (B) Reduction of 5β-dihydrotestosterone (5β-DHT). Chromatograms: (a) the control cells, (b) the overexpressed cells, and (c) the overexpressed cells cultured with 2 μM HAHE. The control cells produced 5β-androstane-3α,17β-diol (3α), whereas the overexpressed cells showed the formation of large amounts of 5β-androstane-3β,17β-diol (3β), which was significantly decreased by the addition of the AKR1B19 inhibitor, HAHE. The peaks other than the above reduced products and substrates are due to unknown substances (*).

and prostaglandins (D_2, E_2, and A_1). In the reverse reaction, the enzyme oxidized 3β-hydroxy-5α/β-dihydrosteroids, but show no significant dehydrogenase activity for Δ^5-3β-hydroxysteroids (dehydroepiandrosterone, pregnenolone, and 5-pregnene-3β,20α-diol) and 3α-hydroxysteroids (5α/β-androstan-3α-ol-17-ones, 5α/β-androstane-3α,17β-diols, 5α/β-pregnan-3α-ol-20-ones and lithocholic acid). The k_{cat}/K_m values for the 3β-hydroxysteroids were higher than those for geranylgeraniol and farnesol, which were most efficiently oxidized by AKR1B19 among non-steroidal substrates including 4-oxo-2-nonenol, 4-hydroxy-2-nonenol, and benzyl alcohol (Table S3, Supplementary Data). $NADP^+$ was a preferable coenzyme (K_m = 1.3 μM), and the dehydrogenase activity for 40 μM 5β-androstan-3β-ol-17-one with 1 mM NAD^+ was only 8.7% of the $NADP^+$-linked activity. It should be noted that no activities of 3-ketosteroid reductase and 3β-HSD were observed for AKR1B2 and human AKR1B10.

As AKR1B19 specifically oxidized 3β-hydroxysteroids, the reduced products of 5β-pregnane-21-ol-3,20-dione, dehydrolithocholic acid, 5β-dihydrotestosterone (androstan-17β-ol-3-one), 5β-androstane-3,17-dione and 5β-pregnane-3,20-dione were identified as 5β-pregnane-21,3β-diol-20-one, isolithocholic acid, 5β-androstane-3β,17β-diol, 5β-androstan-3β-ol-17-one and 5β-pregnan-3β-ol-20-one, respectively, by TLC. To further confirm the stereo-specific reduction of 3-ketosteroids by AKR1B19, we examined the metabolism of 5α-dihydrotestosterone and 5β-dihydrotestosterone in the BAECs transfected with the expression plasmid harboring the cDNA for the enzyme. The overexpression of AKR1B19 was validated by detecting fivefold higher pyridine-3-aldehyde reductase activity in the extract of the transfected cells compared to the activity in the control cells transfected with the vector alone. In the reduction of 5α-dihydrotestosterone, only one product, 5α-androstane-3β,17β-diol, was formed, as shown in the LC/MS chromatogram (Fig. 2A). The incubation with 5β-dihydrotestosterone in the transfected cells produced both 5β-androstane-3β,17β-diol and 5β-androstane-3α,17β-diol as the major and minor products, respectively, in contrast to the formation of only 5β-androstane-3α,17β-diol in the control cells due to their intrinsic reductive 3α-HSD activity for 5β-dihydrotestosterone (Fig. 2B). The addition of the AKR1B19 inhibitor, HAHE (Table 3), into the medium of the transfected cells significantly decreased the amount of 5β-androstane-3β,17β-diol, but did not have any effect on that of 5β-androstane-3α,17β-diol, being indicative of the stereo-selective reduction of the 3-ketosteroids by AKR1B19.

Inhibitor sensitivity

The reductase activity of AKR1B19 was inhibited by inhibitors of human AKR1B10 [23,35–37] and AR (AKR1B1), and the inhibitory effects were compared with those of rabbit AR, AKR1B2 (Table 3). PHPC is a potent and nonselective inhibitor of the two human enzymes [36], and most potently inhibited both AKR1B19 and AKR1B2. HAHE, a selective inhibitor of human AKR1B10 [23], showed the highest selectivity ratio of AKR1B19 to AKR1B2 among the inhibitors tested. Minalrestat was a potent inhibitor of AKR1B19, but all AR inhibitors including minalrestat inhibited AKR1B2 more potently than AKR1B19. AKR1B19 and AKR1B2 were also inhibited by sulindac, quercetin and diethylstilbestrol. Although its inhibitory potency was low, diphenic acid, an aldehyde reductase inhibitor [38], inhibited only AKR1B19. It should be noted that another aldehyde reductase inhibitor, valproate, did not affect the activities of both AKR1B19 and AKR1B2.

Table 3
Comparison of inhibitor sensitivity between AKR1B19 and AKR1B2.

Inhibitor[a]	IC_{50} (μM)		Ratio 1B2/1B19
	AKR1B19	AKR1B2	
AKR1B10 inhibitors			
PHPC	0.008	0.011	1.3
HAHE	0.060	5.9	98
Oleanolic acid	0.40	15	38
Bisdemethoxycurcumin	0.60	0.51	0.9
AR inhibitors			
Minalrestat	0.043	0.023	0.5
Tolrestat	0.16	0.016	0.1
Zopolrestat	1.2	0.27	0.2
Epalrestat	1.5	0.68	0.5
AL1567	1.7	1.8	1
Sorbinil	5.3	2.1	0.4
Other inhibitors			
Sulindac	0.86	31	36
Quercetin	1.5	0.17	0.1
Diethylstilbestrol	2.8	2.4	0.9
Diphenic acid	260	>2000[b]	>8

[a] Abbreviations: PHPC, (*Z*)-2-(4-methoxyphenylimino)-7-hydroxy-*N*-(pyridin-2-yl)-2*H*-chromene-3-carboxamide; HAHE, 3-(4-hydroxy-2-methoxyphenyl)acrylic acid 3-(3-hydroxyphenyl)propyl ester; and AL1567, DL-spiro(2-fluoro-9*H*-fluoren-9,4′-imidazolidine)-2′,5′-dione.
[b] No significant inhibition at 2 mM.

Table 4
Inhibition patterns and constants of AKR1B19 inhibitors.

Inhibitor [a]	Inhibition pattern [b]	Inhibition constant (nM)	
		K_{is}	K_{ii}
Reduction reaction			
PHPC	Noncompetitive	6.7 ± 0.4	7.8 ± 0.9
HAHE	Uncompetitive		40 ± 4
Oxidation reaction			
PHPC	Competitive	2.7 ± 0.4	
HAHE	Competitive	26 ± 5	
Oleanolic acid	Competitive	97 ± 5	
Minalrestat	Competitive	16 ± 2	
Tolrestat	Competitive	78 ± 5	
Diphenic acid	Competitive	6600 ± 900	

[a] Abbreviations of PHPC and HAHE are described in Table 3.
[b] The varied substrates are pyridine-3-aldehyde and 5β-androstan-3β-ol-17-one in the reduction and oxidation, respectively.

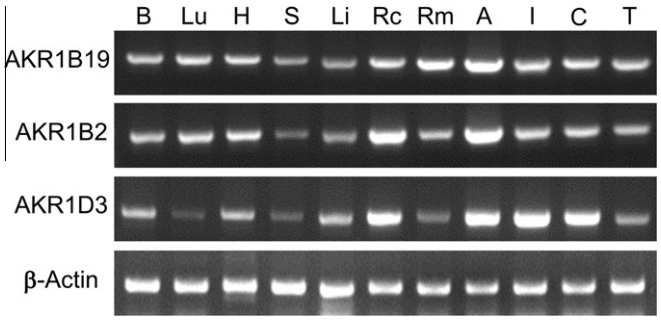

Fig. 3. RT-PCR analysis for expression of mRNAs for AKR1B19, AKR1B2 and AKR1D3 in rabbit tissues. Tissues: brain (B), lung (Lu), heart (H), stomach (S), liver (Li), renal cortex (Rc), renal medulla (Rm), adrenal gland (A), small intestine (I), colon (C), and testis (T). The expression of mRNA for β-actin is shown as the control.

In the reduction of pyridine-3-aldehyde by AKR1B19, the inhibition patterns of PHPC and HAHE were noncompetitive and uncompetitive, respectively, with respect to the substrate (Table 4). In the reverse reaction of 5β-androstan-3β-ol-17-one oxidation, the inhibition patterns of PHPC and HAHE were competitive with respect to the substrate. Other inhibitors, minalrestat, tolrestat, oleanolic acid and diphenic acid, were all competitive inhibitors with respect to the alcohol substrate.

Tissue distribution

The tissue distribution of AKR1B19 was accessed by RT-PCR, and compared with that of AKR1B2 (Fig. 3). The mRNAs for the two enzymes were detected in many rabbit tissues, of which the adrenal gland showed high expression of the two mRNAs. Since 5β-dihydro-C_{19}/C_{21}-steroids that were better substrates for AKR1B19 than their 5α-isomers (Table 1) are metabolically formed by Δ^4-3-ketosteroid 5β-reductase, the expression of the mRNA for rabbit Δ^4-3-ketosteroid 5β-reductase (AKR1D3) in rabbit tissues was also examined by RT-PCR. This mRNA was detected in all rabbit tissues, although its expression levels in the renal cortex, adrenal gland, small intestine and colon were high.

To examine the expression of AKR1B19 protein and its identity with cytosolic 3β-HSD in rabbit tissues, the $NADP^+$-linked 5β-androstan-3β-ol-17-one dehydrogenase and NADPH-linked pyridine-3-aldehyde reductase activities were partially purified from the lung, from which the cDNA for the enzyme was isolated. The two enzyme activities were eluted at the same low-molecular weight (around 35-kDa) fraction on the Sephadex G-100 filtration, but separated on the Q-Sepharose chromatography (Fig. 4). The

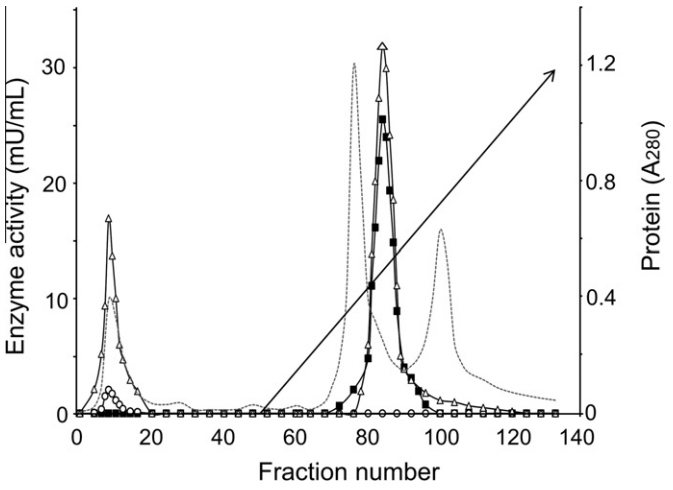

Fig. 4. Elution patterns of the activities of lung $NADP^+$-linked 3β-HSD and NADPH-linked aldehyde reductases on Q-Sepharose chromatography. After the enzyme fraction purified by the Sephadex G-100 filtration of male rabbit lung cytosolic proteins was applied, the adsorbed proteins (···) were eluted with a linear gradient of 0–0.1 M NaCl (→). Activities: 3β-HSD towards 40 μM 5β-androstan-3β-ol-17-one (○), pyridine-3-aldehyde reductase (Δ), and D-glucuronate reductase (■).

3β-HSD activity was eluted only in the non-adsorbed fractions, whereas the pyridine-3-aldehyde reductase activity separated into two peaks, one of which was co-eluted with the 3β-HSD activity. The enzyme in the non-adsorbed fractions showed low K_m values for pyridine-3-aldehyde, 5β-dihydrotestosterone and 5β-androstan-3β-ol-17-one (10, 6.2 and 10 μM, respectively), and sensitivity to HAHE and diphenic acid (the IC_{50} values are 0.095 and 430 μM, respectively), which are similar to those of recombinant AKR1B19 (Tables 1 and 2). On the other hand, the enzyme eluted in the NaCl gradient reduced pyridine-3-aldehyde (K_m = 0.6 mM) and D-glucuronate (K_m = 2.9 mM) at similar rates, but showed no reductase activity towards 5β-dihydrotestosterone and D-glucose. The D-glucuronate activity was inhibited by both diphenic acid and valproate (IC_{50} values are 182 and 171 μM, respectively). The properties of the adsorbed enzyme exhibiting pyridine-3-aldehyde reductase activity are similar to those of aldehyde reductase [38,39].

Alteration of reactivity for steroids by mutagenesis

The outstanding difference of AKR1B19 from human and rodent AR-like proteins was the efficient and stereo-specific reduction of 3-keto-5β-dihydrosteroids, despite of their high SI, as described above. Among the substrate-binding residues predicted from the crystal structure of AKR1B10 and site-directed mutagenesis [40], two residues at positions 303 and 304, located in a region known as the C-terminal substrate-binding loop, are different between the human and rabbit enzymes (Fig. S1, Supplementary Data). The corresponding residues are Phe and Met in AKR1B19, whereas they are Gln and Ser in AKR1B10. In addition, Leu116 that corresponds to a substrate-pocket residue of the AKR superfamily proteins [41] is replaced to Phe in AKR1B10. To examine whether the three residues are responsible for the reduction of 3-ketosteroids, we prepared three single mutant AKR1B19s (L116F, F303Q and M304S) and a double F303Q/M304S mutant enzyme, and compared the effects of the mutations on kinetic constants for 3-ketosteroids (5β-pregnane-21-ol-3,20-dione and 5β-dihydrotestosterone) and non-steroidal substrates (methylglyoxal and pyridine-3-aldehyde). The mutation of L116F did not apparently affect the kinetic constants, whereas those of F303Q and M304S resulted in significant alterations of K_m and k_{cat}/K_m values for the steroidal substrates (Table 5).

Table 5
Alteration of kinetic constants for steroidal and non-steroidal substrates by mutagenesis.

Substrate	Enzyme	K_m		k_{cat}		k_{cat}/K_m	
		(μM)	Mu/WT[a]	(min^{-1})	Mu/WT[a]	(μM^{-1} min^{-1})	Mu/WT[a]
5β-Pregnane-21-ol-3,20-dione	WT	2.4 ± 0.2	1	5.5 ± 0.5	1	2.3	1
	L116F	2.3 ± 0.1	1	3.8 ± 0.1	0.7	1.7	0.7
	F303Q	13 ± 1.0	5	3.7 ± 0.3	0.5	0.25	0.1
	M304S	23 ± 2	10	2.5 ± 0.3	0.4	0.11	0.05
	F303Q/M304S	50 ± 5	21	1.4 ± 0.1	0.2	0.028	0.01
5β-Dihydrotestosterone	WT	3.5 ± 0.2	1	6.8 ± 0.7	1	1.9	1
	L116F	7.2 ± 2.0	2	6.4 ± 2.2	1	0.88	0.5
	F303Q	17 ± 2	4	4.8 ± 0.3	0.7	0.28	0.1
	M304S	20 ± 1	6	2.8 ± 0.1	0.4	0.14	0.07
	F303Q/M304S	39 ± 3	11	1.2 ± 0.2	0.2	0.031	0.02
Methylglyoxal	WT	222 ± 25	1	18 ± 0.2	1	0.080	1
	L116F	180 ± 30	1	19 ± 3	1	0.11	1
	F303Q	253 ± 10	1	35 ± 10	2	0.13	2
	M304S	209 ± 20	1	22 ± 2	1	0.10	1
	F303Q/M304S	250 ± 14	1	24 ± 1	2	0.10	1
Pyridine-3-aldehyde	WT	11 ± 2	1	43 ± 4	1	4.1	1
	L116F	22 ± 2	2	36 ± 6	1	1.7	0.4
	F303Q	11 ± 1	1	52 ± 7	1	4.7	1
	M304S	39 ± 3	4	47 ± 6	1	1.2	0.3
	F303Q/M304S	48 ± 6	4	72 ± 10	2	1.5	0.4

[a] Ratio of mutant enzyme to WT.

The double mutation of F303Q/M304S further impaired the affinity and catalytic efficiency, although it did not affect the stereospecific reduction of the two 3-ketosteroids into the corresponding 3β-hydroxysteroids (data not shown). In contrast, the mutations did not affect the kinetic constants for the small non-steroidal substrates, except that the M304S mutation caused a small (fourfold) increase in the K_m value for pyridine-3-aldehyde.

Discussion

In the present study, for the first time we have cloned the full-length cDNA for AKR1B19, and characterized AKR1B19 as a functional NADPH-dependent AKR that reduces a variety of aldehydes and α-dicarbonyl compounds, and some xenobiotic *p*-quinones and ketones. AKR1B19 shows more than 80% amino acid SI not only with human AR-like proteins (SI) [AKR1B10 (86%) and AKR1B15 (82%)], but also with other animal AR-like proteins (SI): mouse AKRs [1B8 (85%) and 1B7 (80%)]; rat AKRs [1B13 (84%), 1B14 (81%) and 1B18 (81%)]; and Chinese hamster AKR1B9 (85%). Although the enzymatic properties of human AKR1B15 have not been characterized well [42], the broad substrate specificity for aldehydes is common among the other AR-like proteins. However, the enzymes differ in other properties. For examples, human AKR1B10 is distinct from the rodent AR-like proteins, in its great ability to reduce all-*trans*-retinal [43], isoprenyl aldehydes (farnesal and geranylgeranial) [17] and some *p*-quinones (TBE and 2-*tert*-butyl-1,4-benzoquinone) [44]. Rat AKR1B18 is distinct from the other rodent and human AR-like proteins, especially in its efficient reduction of ONE and activation by organic acids [20].

AKR1B19 mostly resembles human AKR1B10 among the AR-like proteins, in its ability to reduce all-*trans*-retinal and efficient reduction of the isoprenyl aldehydes and *p*-quinones. In addition, AKR1B19 was inhibited by the human AKR1B10 inhibitors, of which HAHE and oleanolic acid showed high selectivity to AKR1B19 over AKR1B2, which is similar to that of human AKR1B10 over AKR1B1 [23,35]. Compared to human AKR1B10 [43,45], the reactivity towards all-*trans*-retinal of AKR1B19 is low, but, instead, that towards ONE is significantly high. The catalytic efficiency for ONE of AKR1B19 is the highest among those reported with other AR-like proteins (AKR1B7 [46], AKR1B14 [19] and AKR1B18 [20]), and ARs (AKR1B1 [27] and AKR1B2). AKR1B19 also reduced GS-ONE that is formed by nonenzymatic reaction and glutathione-S-transferase [27,47], and its k_{cat}/K_m values are about an order magnitude higher than those of AKR1B1 [27], AKR1B14 [19], and AKR1B18 [20]. In addition, this enzyme reduced other α,β-unsaturated aldehydes derived from lipid-peroxidation [29,48,49]. The substrate specificity, together with the ubiquitous tissue distribution, suggests that AKR1B19 is implicated in detoxification of the reactive aldehydes, especially the most highly reactive lipid-derived aldehyde, ONE.

AKR1B19 also reduced isocaproaldehyde, which is the product of side-chain cleavage of cholesterol in the steroidogenesis of endocrine glands. Previously, three forms of 36-kDa NADPH-dependent isocaproaldehyde reductase were isolated from rabbit adrenal gland [25]. Although the two of the three isocaproaldehyde reductases are identified as ARs, the nature of the third enzyme form remains unknown. This third enzyme form shows an acidic pH optimum of 6.3, inability to reduce D-glucose, low sensitivity to tolrestat, AL1567 and sorbinil, and moderate inhibition by diphenic acid. The properties are similar to those of AKR1B19, whose mRNA was highly expressed in the adrenal gland. Therefore, AKR1B19 may be identical to the third form of adrenal isocaproaldehyde reductase previously isolated.

The most striking difference of AKR1B19 from human and rodent AR and AR-like proteins is its ability to efficiently reduce 3-ketosteroids into 3β-hydroxysteroids. Among mammalian ARs and AR-like proteins, bovine AR (AKR1B5) [50] and Chinese-hamster AKR1B9 [51] reduce the 20-keto group of 17α-hydroxyprogesterone, and human AKR1B10 exhibits low 20α-HSD activity [17]. Although AKR1B7 exhibits low reductase activity for 3-keto bile acids [16], 3-keto-5α/β-dihydro-C_{19}/C_{21}-steroids are not reduced by human AKR1B10 and AKR1B1 [17], rat AKRs (1B13, 1B14 and 1B18) [18–20], mouse AKR1B7 [46, unpublished data] and rabbit AKR1B2 [this study]. Bile acids and 3-ketosteroids are inhibitors for human AKR1B10 and AKR1B1 [17], and bile acids, other than dehydrocholic acid, are activators for AKR1B14 [46]. Thus, AKR1B19 is the first AKR1B subfamily enzyme that exhibits significant reductive 3β-HSD activity towards 3-keto-5α/β-dihydrosteroids.

The k_{cat}/K_m values for the 3-ketosteroid substrates of AKR1B19 are comparable or superior to those of human AKR1C1 [4], 17β-HSD type-7 [5] and DHRS4 [6]. In addition, AKR1B19 reduces a large number of 3-ketosteroids compared to these human enzymes. The metabolism of 5α- and 5β-dihydrotestosterones in the AKR1B19-overexpressed cells supports that the enzyme catalyzes the strict 3β-stereoselective reduction *in vivo*. Currently, there are no reports on rabbit enzymes with the reductive 3β-HSD activity, as described in the Introduction section. AKR1B19 mRNA was expressed in many rabbit tissues, and the identity of AKR1B19 with cytosolic 3β-HSD was evidenced by the co-purification of the two enzyme activities from rabbit lung. AKR1B19 may play a role in the intracellular pre-receptor formation of the active 3β-hydroxyandrostanes and 3β-hydroxypregnanes [8–13], as well as in the inactivation of the potent androgen 5α-dihydrotestosterone. The enzyme did not reduce Δ^4-unsaturated steroids, but reduced various 5α- and 5β-dihydrosteroids that are formed from testosterone, progesterone and corticoids by steroid reductases. Considering the ubiquitous expression of the mRNA for Δ^4-steroid 5β-reductase (AKR1D3) in rabbit tissues, AKR1B19 in concert with AKR1D3 also contributes to catabolic pathways of these steroid hormones. Thus, the enzyme is important for the metabolism of

endogenous steroids and cytotoxic aldehydes rather than that of xenobiotic carbonyl compounds.

The results of the mutagenesis of F303Q/M304S have revealed that both Phe303 and Met304 play critical roles in recognition of 3-ketosteroid substrates by AKR1B19, although other residues are probably involved in the steroid binding because of lack of complete abolishment of reductive 3β-HSD activity by the mutagenesis. The two residues are present in a so-called C-terminal loop of the tertiary structures of enzymes of the AKR superfamily, in which amino acid difference in this and other two loop regions between different enzymes has been suggested to determine the substrate specificity of the enzymes [2,41]. In the crystal structures of HSDs belonging to the AKR1C subfamily, apolar residues (at positions 306, 308 and 310) in the C-terminal loop interact the steroidal substrates or inhibitors [15,41]. The C-terminal loop of AKR1B1 is critical for catalytic efficiency and substrate specificity [52], and a role of Cys304, a C-terminal loop residue of AKR1B10, in binding of the cyclohexene ring of all-*trans*-retinal has been shown by crystallographic and site-directed mutagenesis studies [40,43]. Based on the present mutations and the crystal structure of AKR1B10, the binding mode of the 3-ketosteroids in AKR1B19 can be speculated as follows. The 3-keto group on the A ring of the steroid must be close to the catalytic residues of Tyr48 and His111, so that the opposite side of this steroid molecule may orientate towards hydrophobic residues, Phe303 and Met304 to form hydrophobic interactions. The formation of this hydrophobic interaction is supported by no significant effects of the F303Q/M304S mutation on the kinetic constants for the smaller and more hydrophilic substrates, methylglyoxal and pyridine-3-aldehyde. Structural studies using molecular modeling techniques and crystallization of AKR1B19 complexed with the coenzyme and steroid are now in progress in order to clarify the steroid recognition of the rabbit enzyme leading to efficient 3β-HSD activity.

Acknowledgments

This work was partly founded by Grant-in-aid for Young Scientists (B) and Scientific Research (C) from the Japan Society for the Promotion of Science, and by a Sasakawa Scientific Research Grant from Japan Science Society.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2012.07.012.

References

[1] J. Simard, M.-L. Ricketts, S. Gingras, P. Soucy, F.A. Feltus, M.H. Melner, Endocr. Rev. 26 (2005) 525–582.
[2] O.A. Barski, S.M. Tipparaju, A. Bhatnagar, Drug Metab. Rev. 40 (2008) 553–624.
[3] U. Oppermann, C. Filling, M. Hult, N. Shafqat, X. Wu, M. Lindh, J. Shafqat, E. Nordling, Y. Kallberg, B. Persson, H. Jörnvall, Chem. Biol. Interact. 143–144 (2003) 247–253.
[4] S. Steckelbroeck, Y. Jin, S. Gopishetty, B. Oyesanmi, T.M. Penning, J. Biol. Chem. 279 (2004) 10784–10795.
[5] S. Törn, P. Nokelainen, R. Kurkela, A. Pulkka, M. Menjivar, S. Ghosh, M. Coca-Prados, H. Peltoketo, V. Isomaa, P. Vihko, Biochem. Biophys. Res. Commun. 305 (2003) 37–45.
[6] T. Matsunaga, S. Endo, S. Maeda, S. Ishikura, K. Tajima, N. Tanaka, K.T. Nakamura, Y. Imamura, A. Hara, Arch. Biochem. Biophys. 477 (2008) 339–347.
[7] Z. Weihua, S. Makela, L.C. Andersson, S. Salmi, S. Saji, J.I. Webster, E.V. Jensen, S. Nilsson, M. Warner, J.A. Gustafsson, Proc. Natl. Acad. Sci. USA 98 (2001) 6330–6335.
[8] R.J. Handa, T.R. Pak, A.E. Kudwa, T.D. Lund, L. Hinds, Horm. Behav. 53 (2008) 741–752.
[9] N. Sugiyama, P.R. Barros, M. Warner, J.A. Gustafsson, Trends Endocrinol. Metab. 21 (2010) 545–552.
[10] D. Dondi, M. Piccolella, A. Biserni, S. Della Torre, B. Ramachandran, A. Locatelli, P. Rusmini, D. Sau, D. Caruso, A. Maggi, P. Ciana, A. Poletti, Endocr. Relat. Cancer 17 (2010) 731–742.
[11] M. Wang, Y. He, L.N. Eisenman, C. Fields, C.M. Zeng, J. Mathews, A. Benz, T. Fu, E. Zorumski, J.H. Steinbach, D.F. Covey, C.F. Zorumski, S. Mennerick, J. Neurosci. 22 (2002) 3366–3375.
[12] M. Matsunaga, K. Okuhara, K. Ukena, K. Tsutsui, Brain Res. 1007 (2004) 160–166.
[13] M.W. McBride, A.J. McVie, S.M. Burridge, B. Brintnell, N. Craig, A.M. Wallace, R.H. Wilson, J. Varley, R.G. Sutcliffe, Genomics 61 (1999) 277–284.
[14] S. Endo, S. Maeda, T. Matsunaga, U. Dhagat, O. El-Kabbani, N. Tanaka, K.T. Nakamura, K. Tajima, A. Hara, Arch. Biochem. Biophys. 481 (2009) 183–190.
[15] J.F. Couture, P. Legrand, L. Cantin, L. Labrie, V. Luu-The, R. Breton, J. Mol. Biol. 339 (2004) 89–102.
[16] D.R. Schmidt, S. Schmidt, S.R. Holmstrom, M. Makishima, R.T. Yu, C.L. Cummins, D.J. Mangelsdorf, S.A. Kliewer, J. Biol. Chem. 286 (2011) 2425–2432.
[17] S. Endo, T. Matsunaga, H. Mamiya, C. Ohta, M. Soda, Y. Kitade, K. Tajima, H.T. Zhao, O. El-Kabbani, A. Hara, Arch. Biochem. Biophys. 487 (2009) 1–9.
[18] S. Endo, T. Matsunaga, H. Mamiya, A. Hara, Y. Kitade, K. Tajima, O. El-Kabbani, Chem. Biol. Interact. 178 (2009) 151–157.
[19] S. Endo, T. Matsunaga, A. Fujita, K. Tajima, O. El-Kabbani, A. Hara, Biol. Pharm. Bull. 33 (2010) 1886–1890.
[20] S. Endo, T. Matsunaga, T. Kuragano, S. Ohno, Y. Kitade, K. Tajima, O. El-Kabbani, A. Hara, Arch. Biochem. Biophys. 503 (2010) 230–237.
[21] Z. Kabututu, M. Manin, J.C. Pointud, T. Maruyama, N. Nagata, S. Lambert, A.M. Lefrançois-Martinez, A. Martinez, Y. Urade, J. Biochem. 145 (2009) 161–168.
[22] S. Lambert-Langlais, J.C. Pointud, A.M. Lefrançois-Martinez, F. Volat, M. Manin, F. Coudoré, P. Val, I. Sahut-Barnola, B. Ragazzon, E. Louiset, C. Delarue, H. Lefebvre, Y. Urade, A. Martinez, PLoS One 4 (2009) e7309.
[23] M. Soda, D. Hu, S. Endo, M. Takemura, J. Li, R. Wada, S. Ifuku, H.T. Zhao, O. El-Kabbani, S. Ohta, K. Yamamura, N. Toyooka, A. Hara, T. Matsunaga, Eur. J. Med. Chem. 48 (2012) 321–329.
[24] K. Tajima, M. Hashizaki, K. Yamamoto, T. Mizutani, Drug Metab. Dispos. 20 (1992) 816–820.
[25] K. Matsuura, Y. Deyashiki, Y. Bunai, I. Ohya, A. Hara, Arch. Biochem. Biophys. 328 (1996) 265–271.
[26] M.P. Doyle, M. Yan, ARKIVOC Part viii (2002) 180–185.
[27] J.A. Doorn, S.K. Srivastava, D.R. Petersen, Chem. Res. Toxicol. 16 (2003) 1418–1423.
[28] R. Grée, H. Toubah, R. Carrié, Tetrahedron Lett. 27 (1986) (1986) 4983–4986.
[29] D. Rindgen, M. Nakajima, S. Wehrli, K. Xu, I.A. Blair, Chem. Res. Toxicol. 12 (1999) 1195–1204.
[30] A. Garcia-Perez, B. Martin, H.R. Murphy, S. Uchida, H. Murer, B.D. Cowley Jr, J.S. Handler, M.B. Burg, J. Biol. Chem. 264 (1989) 16815–16821.
[31] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
[32] S. Endo, T. Matsunaga, K. Kitade, S. Ohno, K. Tajima, O. El-Kabbani, A. Hara, Biochem. Biophys. Res. Commun. 377 (2008) 1326–1330.
[33] X. Parés, P. Julià, Methods Enzymol. 189 (1990) 436–441.
[34] N. Tanaka, K. Aoki, S. Ishikura, M. Nagano, Y. Imamura, A. Hara, K.T. Nakamura, Structure 16 (2008) 388–397.
[35] M. Takemura, S. Endo, T. Matsunaga, M. Soda, H.-T. Zhao, O. El-Kabbani, K. Tajima, M. Iinuma, A. Hara, J. Nat. Prod. 74 (2011) 1201–1206.
[36] S. Endo, T. Matsunaga, K. Kuwata, H.-T. Zhao, O. El-Kabbani, Y. Kitade, A. Hara, Bioorg. Med. Chem. 18 (2010) 2485–2490.
[37] T. Matsunaga, S. Endo, M. Soda, H.-T. Zhao, O. El-Kabbani, K. Tajima, A. Hara, Biochem. Biophys. Res. Commun. 389 (2009) 128–132.
[38] G. Branlant, Eur. J. Biochem. 129 (1982) 99–104.
[39] B. Wermuth, Prog. Clin. Biol. Res. 174 (1985) 209–230.
[40] O. Gallego, F.X. Ruiz, A. Ardèvol, M. Domínguez, R. Alvarez, A.R. de Lera, C. Rovira, J. Farrés, I. Fita, X. Parès, Proc. Natl. Acad. Sci. USA 104 (2007) 20764–20769.
[41] J.M. Jez, M.J. Bennett, B.P. Schlegel, M. Lewis, T.M. Penning, Biochem. J. 326 (1997) 625–636.
[42] J.K. Salabei, X.P. Li, J.M. Petrash, A. Bhatnagar, O.A. Barski, Chem. Biol. Interact. 191 (2011) 177–184.
[43] F.X. Ruiz, S. Porté, X. Parés, J. Farrés, Front. Pharmacol. 3 (2012) 58.
[44] T. Matsunaga, S. Endo, M. Takemura, M. Soda, K. Yamamura, K. Tajima, O. El-Kabbani, A. Hara, Drug Metab. Pharmacokinet. (in press).
[45] H.J. Martin, E. Maser, Chem. Biol. Interact. 178 (2009) 145–150.
[46] S. Endo, T. Matsunaga, A. Fujita, T. Kuragano, M. Soda, J. Sundaram, U. Dhagat, K. Tajima, O. El-Kabbani, A. Hara, Biochimie 93 (2011) 1476–1486.
[47] K. Kamada, S. Goto, T. Okunaga, Y. Ihara, K. Tsuji, Y. Kawai, K. Uchida, T. Osawa, T. Matsuo, I. Nagata, T. Kondo, Free Radic. Biol. Med. 37 (2004) 1875–1884.
[48] H. Esterbauer, R.J. Schaur, H. Zollner, Free Radic. Biol. Med. 11 (1991) 81–128.
[49] E.K. Long, M.J. Picklo Sr, Free Radic. Biol. Med. 49 (2010) 1–8.
[50] J.C. Warren, G.L. Murdock, Y. Ma, S.R. Goodman, W.E. Zimmer, Biochemistry 32 (1993) 1401–1406.
[51] D.J. Hyndman, R. Takenoshita, N.L. Vera, S.C. Pang, T.G. Flynn, J. Biol. Chem. 272 (1997) 13286–13291.
[52] K.M. Bohren, C.E. Grimshaw, K.H. Gabbay, J. Biol. Chem. 267 (1992) 20965–20970.