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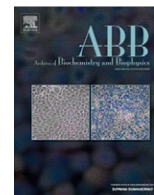

Characterization of rabbit aldose reductase-like protein with 3β-hydroxysteroid dehydrogenase activity

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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.

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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.

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[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.

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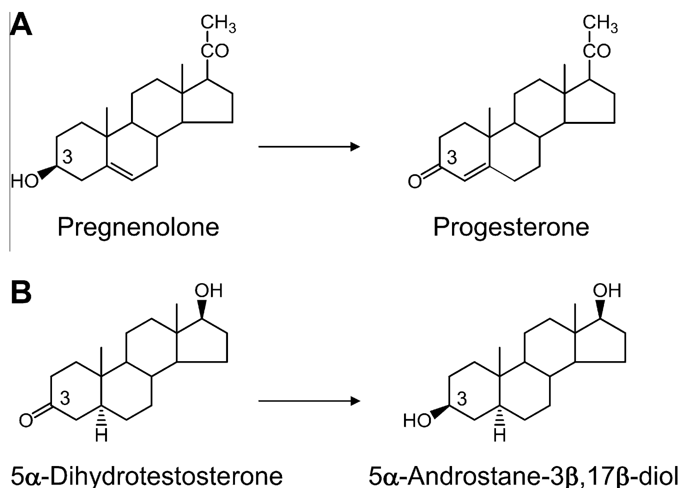

Fig. 1. Conversion catalyzed by NAD+-dependent 3β-HSD/Δ^5–Δ^4 isomerase (A) and NADPH-dependent enzymes with reductive 3β-HSD activity (B).

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

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