Archives of Biochemistry and Biophysics 529 (2013) 131–139

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

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

Characterization of rabbit morphine 6-dehydrogenase and two NAD$^+$-dependent 3α(17β)-hydroxysteroid dehydrogenases

Satoshi Endo [a,*], Toshiyuki Matsunaga [a], Airi Fujimoto [a], Sho Kumada [a], Yuki Arai [a], Yoko Miura [b], Hiroshige Mikamo [b], Ossama El-Kabbani [c], Shigeru Yamano [d], Munekazu Iinuma [a], Akira Hara [a]

[a] Gifu Pharmaceutical University, Gifu 501-1196, Japan
[b] Aichi Medical University Graduate School of Medicine, Aichi 480-1195, Japan
[c] Monash Institute of Pharmaceutical Sciences, Victoria 3052, Australia
[d] Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka 814-0180, Japan

ARTICLE INFO

Article history:
Received 9 October 2012
and in revised form 27 November 2012
Available online 7 December 2012

Keywords:
Aldo–keto reductase
Morphine 6-dehydrogenase
3α/17β-hydroxysteroid dehydrogenase
Flavonoids
Substrate recognition

ABSTRACT

Mammalian morphine 6-dehydrogenase (M6DH)[1] converts morphine into a reactive electrophile, morphinone. M6DH belongs to the aldo–keto reductase (AKR) superfamily, but its endogenous substrates and entire amino acid sequence remain unknown. A recent rabbit genomic sequencing predicts three genes for novel AKRs (1C26, 1C27 and 1C28) that share >87% amino acid sequence identity and are similar to the partial sequence of rabbit liver M6DH. We isolated cDNAs for the three AKRs, and compared the properties of their recombinant enzymes. Like M6DH, only AKR1C26 that shares the highest sequence identity with hepatic M6DH oxidized morphine. The three AKRs showed NAD$^+$-dependent dehydrogenase activity towards other non-steroidal alicyclic alcohols and 3α/17β-hydroxy-C_{18}/C_{19}/C_{21}-steroids, and their mRNAs were ubiquitously expressed in rabbit tissues. The kinetic constants for the substrates suggest that at least AKR1C26 and AKR1C28 act as NAD$^+$-dependent 3α/17β-hydroxysteroid dehydrogenases. AKR1C27 differed from AKR1C28 in its high K_m values for the substrates and low sensitivity towards competitive inhibitors (ikarisoside A, hinokitiol, hexestrol and zearalenone), despite their 95% sequence identity. The site-directed mutagenesis of Tyr118 and Phe310 in AKR1C27 to the corresponding residues (Phe and Ile, respectively) in AKR1C28 produced an enzyme that was similar to AKR1C28, suggesting their key roles in ligand binding.

Introduction

Morphine 6-dehydrogenase (M6DH[1], EC1.1.1.218) catalyzes the oxidation of the 6-hydroxy group of morphine into its 6-carbonyl metabolite, morphinone, using either NAD$^+$ or NADP$^+$ as the coenzyme. The structure of morphine is shown in Fig. S1 (Supplementary Data). M6DH was first purified from the guinea pig liver cytosol as an enzyme responsible for a metabolic pathway of morphine to morphinone [1], which is nine-fold more toxic than morphine [2] and potently antagonizes the morphine analgesia [3] in mice. Subsequently, the pathway catalyzed by M6DH is demonstrated to be one of the major routes in morphine metabolism in guinea pigs and rats [4,5]. The metabolite, morphinone, is a reactive electrophile that nonenzymatically reacts with glutathione and tissue macromolecules through their sulfhydryl groups [2,4-7], and is cytotoxic to rat hepatocytes [7,8] and human cultured cells [9]. These findings suggest that M6DH is involved in the toxic action of morphine including the development of tolerance to this drug. The enzyme activity has been found in the livers of rats, rabbits, hamsters, mice, cows [5] and humans [10]. M6DHs were purified from rabbit and hamster livers [11,12], and shown to be members of the aldo–keto reductase (AKR) 1C subfamily in the AKR superfamily (http://www.med.upenn.edu/akr/) by their partial amino acid sequence analyses. The rabbit and hamster enzymes show the coenzyme preference for NAD$^+$ over NADP$^+$, which differs from the guinea pig enzyme that utilizes the two coenzymes with equal efficiency [1]. The three animal M6DHs oxidize morphine congeners, but differ from one another in their substrate specificity for xenobiotic alicyclic alcohols and in particular, for hydroxysteroids. Only the hamster enzyme efficiently oxidizes several 17β-hydroxy-C_{19}-steroids and has been suggested to act as a NAD$^+$-dependent 17β-hydroxysteroid dehydrogenase (HSD) [12]. Rabbit M6DH exhibits low dehydrogenase activity towards some 17β-hydroxy-C_{19}-steroids and is competitively inhibited by 3α- and 17β-hydroxysteroids including lithocholic acid and 17β-estradiol [11]. 17β-Hydroxysteroids and alicyclic

* Corresponding author. Address: Laboratory of Biochemistry, Gifu Pharmaceutical University, Gifu 501-1196, Japan. Fax: +81 58 230 8105.
E-mail address: sendo@gifu-pu.ac.jp (S. Endo).

[1] *Abbreviations used:* M6DH, morphine 6-dehydrogenase; AKR, aldo-keto reductase; HSD, hydroxysteroid dehydrogenase; TBE, 6-*tert*-butyl-2,3-epoxy-4-hydroxy-5-cyclohexen-1-one; RT, reverse transcription; *S*-tetralol, *S*-(+)-1,2,3,4-tetrahydro-1-naphthol; BAEC, bovine aortic endothelial cell; LC/MS, liquid chromatography/mass spectrometry.

0003-9861/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.abb.2012.11.013

alcohols are not substrates for guinea pig M6DH [1]. Thus, in addition to their full-length amino acid sequences, endogenous substrates of rabbit and guinea pig M6DHs remain unknown.

17β-HSDs play a crucial role in the activation/inactivation of C_{19}-androgens and C_{18}-estrogens in both steroidogenic and peripheral target tissues, and to date fourteen different mammalian 17β-HSDs have been annotated and characterized [13]. While most 17β-HSDs belong to the short-chain dehydrogenase/reductase superfamily (http://sdr-enzymes.org/), only type 5 17β-HSD belongs to the AKR superfamily, in which the human and mouse enzymes are named AKR1C3 and AKR1C6, respectively. Type 5 17β-HSD is NADP(H)-dependent, and therefore is distinct from NAD^+-dependent M6DH that belongs to the AKR1C subfamily and acts as a 17β-HSD in hamster liver [12]. However, each of these two members of the AKR1C subfamily (AKR1C12 and AKR1C13 in mice, and AKR1C16 and AKR1C24 in rats) have recently been characterized as NAD^+-dependent 17β-HSDs associated with 3α- and/or 20α-HSD activities [14–16]. In addition, the mouse and rat enzymes exhibited dehydrogenase activities towards alicyclic alcohols, which are substrates of hamster and rabbit M6DHs. These findings have suggested the possibility that hamster and rabbit M6DHs may show broad specificity for 3α- and/or 20α-hydroxysteroids as well as 17β-hydroxysteroids.

Current rabbit genomic analysis has predicted three genes encoding the AKR1C subfamily proteins that show high sequence identity with the partial sequence of rabbit M6DH. In order to identify the gene for rabbit M6DH and elucidate the physiological roles of M6DH and the proteins encoded in the above genes, we isolated the cDNAs for the proteins that have been assigned as AKR1C26, AKR1C27 and AKR1C28 in the AKR superfamily, and examined the properties of the recombinant enzymes and their tissue distribution. The data indicated that the three enzymes are NAD^+-dependent 3α/17β-HSDs with broad substrate specificity for alicyclic alcohols and long-chain aliphatic alcohols, although only AKR1C26 exhibited M6DH activity. Among the proteins, AKR1C27 and AKR1C28 structurally differ by only 15 amino acids, but their K_m values for the substrates and sensitivity to several competitive inhibitors were markedly different. Therefore, the molecular determinants responsible for their affinities towards the substrates and inhibitors were also investigated by mutating two important residues in AKR1C27 to the corresponding residues in AKR1C28.

Materials and methods

Materials

Morphine hydrochloride and steroids were obtained from Takeda Chemical Ind., Ltd. (Osaka, Japan) and Steraloids (Newport, RI), respectively. *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). *trans*-Benzene dihydrodiol [17], *trans*-naphthalene dihydrodiol [18] and 6-*tert*-butyl-2,3-epoxy-4-hydroxy-5-cyclohexen-1-one (TBE) [19] were synthesized as described previously. Ikarisoside A, noranhydroicaritin and icarisid II were isolated from *Epimedium* species as reported previously [20]. The flavonoids showing >98% purity were obtained by recrystallization. All other chemicals were of the highest grade that could be obtained commercially.

cDNA isolation and site-directed mutagenesis

The cDNAs for AKR1C26, AKR1C27 and AKR1C28 were isolated from the total RNA preparations of small intestine, brain and kidney, respectively, 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. [21]. PCR was performed with *Pfu* DNA polymerase and a pair of sense and antisense primers, which contain *Nde*I and *Sal*I sites. The primers for the amplification of cDNAs for AKR1C26, AKR1C27 and AKR1C28 were designed based on the mRNAs for *Oryctolagus cuniculus* prostaglandin E_2 9-reductase-like proteins (accession nos. XM_002721695, XM_002721693 and XM_002721692, respectively) predicted from the rabbit genomic analysis, and their 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 inserts of the cloned cDNAs were sequenced by using a Beckman CEQ8000XL DNA sequencer, and were confirmed to encode the 323-amino acid sequences of the three AKRs fused to the N-terminal 6-His tag. For the mRNA for AKR1C26, its 3'- and 5'-untranslated regions 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 sequences of cDNAs for AKR1C26, AKR1C27 and AKR1C28 were deposited in DDBJ database with the accession numbers AB743996, AB743997 and AB743998, respectively.

Mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) and the pCold I expression plasmid harboring the cDNA for AKR1C27 as the template according to the protocol described by the manufacturer. The sequences of the primers used for the mutagenesis of Tyr118Phe and Phe310Ile are shown in Table S1 (Supplementary Data). The cDNA for the double mutant of Tyr118Phe/Phe310Ile was prepared using the expression plasmid harboring the cDNA for Phe310Ile mutant as the template. 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.

Expression and purification of recombinant enzymes

The recombinant AKR1C26, AKR1C27, AKR1C28 and the mutant AKR1C27s were expressed in *E. coli* BL21 (DE3) pLysS cells transformed with the expression plasmids harboring their cDNAs as described previously [22]. The enzymes were purified from the cell extracts using a nickel-charged Sepharose 6FF resin (GE Healthcare) 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-mercaptoethnol, 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 [21].

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 corresponding standard reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4, 0.5 mM NAD^+, substrate and enzyme, in a total volume of 2.0 mL. *S*-(+)-1,2,3,4-Tetrahydro-1-naphthol (*S*-tetralol) was used for the substrate, and its concentrations for the activity assays of AKR1C26, AKR1C27 and AKR1C28 were 0.5 mM, 1 mM and 20 μM, respectively. The reductase activities were determined by measuring the rate of change in NAD(P)H absorbance in the phosphate buffer, pH 7.4, containing 80 μM NADH or NADPH and an appropriate amount of carbonyl substrate. The steroid and other substrates, which are hardly soluble in water, were dissolved in methanol or 50% methanol,