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Molecular determinants for the stereospecific reduction of 3-ketosteroids and reactivity towards all-*trans*-retinal of a short-chain dehydrogenase/reductase (DHRS4)

Satoshi Endo [a,*], Satoshi Maeda [a], Toshiyuki Matsunaga [a], Urmi Dhagat [b], Ossama El-Kabbani [b], Nobutada Tanaka [c], Kazuo T. Nakamura [c], Kazuo Tajima [d], Akira Hara [a]

[a] *Laboratory of Biochemistry, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan*
[b] *Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash University, Vic. 3052, Australia*
[c] *School of Pharmaceutical Sciences, Showa University, Tokyo 142-8555, Japan*
[d] *Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa 920-1181, Japan*

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ABSTRACT

DHRS4, a member of the short-chain dehydrogenase/reductase superfamily, reduces all-*trans*-retinal and xenobiotic carbonyl compounds. Human DHRS4 differs from other animal enzymes in kinetic constants for the substrates, particularly in its low reactivity to retinoids. We have found that pig, rabbit and dog DHRS4s reduce benzil and 3-ketosteroids into *S*-benzoin and 3α-hydroxysteroids, respectively, in contrast to the stereoselectivity of human DHRS4 which produces *R*-benzoin and 3β-hydroxysteroids. Among substrate-binding residues predicted from the crystal structure of pig DHRS4, F158 and L161 in the animal DHRS4 are serine and phenylalanine, respectively, in the human enzyme. Double mutation (F158S/L161F) of pig DHRS4 led to an effective switch of its substrate affinity and stereochemistry into those similar to human DHRS4. The roles of the two residues in determining the stereospecificity in 3-ketosteroid reduction were confirmed by reverse mutation (S158F/F161L) in the human enzyme. The stereochemical control was evaluated by comparison of the 3D models of pig wild-type and mutant DHRS4s with the modeled substrates. Additional mutation of T177N into the human S158F/F161L mutant resulted in almost complete kinetic conversion into a pig DHRS4-type form, suggesting a role of N177 in forming the substrate-binding cavity through an intersubunit interaction in pig and other animal DHRS4s, and explaining why the human enzyme shows low reactivity towards retinoids.

The short-chain dehydrogenase/reductases (SDRs)[1] are a large superfamily [1–3], which includes over 15,000 primary structures annotated in various sequence databases, and over 100 crystal structures exist in the Protein Data Bank. The majority of the functionally characterized members in this superfamily are NAD(P)(H)-dependent enzymes that play physiological roles in the metabolism of steroid hormones, prostaglandins, carbohydrates and retinoids, as well as in the metabolism of xenobiotics, drugs and carcinogens. The SDRs have low sequence identity, but share common sequence motifs that define the N-terminal coenzyme binding-site residues (TGxxxGxG) and the catalytic residues (N-S-Y-K) [4]. The SDR 3D structures also share a common α/β-folding pattern characterized by a central β-sheet typical of a Rossmann-fold with helices present on either side [5,6]. The coenzyme binds with the nicotinamide ring situated at the base of the active-site cavity, where the carbonyl or hydroxyl group of the substrate or product interacts with the catalytically important residues, so called catalytic triad (S-Y-K) [7]. On the other hand, there is no common feature for substrate recognition, particularly because of the large superfamily and low sequence identity among various SDRs with diverse functions.

Peroxisomal tetrameric carbonyl reductase is a member of the SDR superfamily, and the human enzyme is currently annotated as dehydrogenase/reductase (SDR family) member 4 (DHRS4) in the HUGO Gene Nomenclature Database. We previously characterized human, pig, rabbit, dog and rat DHRS4s [8–11]. These enzymes share a broad specificity for substrates including aromatic ketones, α-dicarbonyl compounds and retinals, but the human enzyme has features different from the other animal enzymes [8]. Human DHRS4 shows low reactivity towards retinoids that is believed to be physiological substrates of other animal DHRS4s including the mouse enzyme [12], which efficiently catalyze the reduction of

* Corresponding author. Fax: +81 58 237 5979.
E-mail address: sendo@gifu-pu.ac.jp (S. Endo).

[1] *Abbreviations used:* AKR, aldo–keto reductase; BAEC, bovine aortic endothelial cell; DHRS4, dehydrogenase/reductase (SDR family) member 4; DHT, dihydrotestosterone; HSD, hydroxysteroid dehydrogenase; hSF, human S158F mutant DHRS4; hSFFL, human S158F/F161L mutant DHRS4; hSFFL-TN, human S158F/F161L-T177N mutant DHRS4; hWT, human wild-type DHRS4; LC/MS, liquid chromatography/mass spectrometry; PDO, pregnane-3,20-dione; pFSLF, pig F158S/L161F mutant DHRS4; pWT, pig wild-type DHRS4; SDR, short-chain dehydrogenase/reductase.

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retinals and thus called NADPH-dependent retinal reductase [9,10,12]. Human DHRS4 efficiently reduces α-dicarbonyl compounds with aromatic rings such as benzil, while it shows lower activity for α-dicarbonyl compounds with short aliphatic chains compared to the rabbit, pig and dog enzymes. The outstanding feature of the human enzyme is its ability to reduce 3-ketosteroids into 3β-hydroxysteroids, suggesting its novel role in the metabolism of 5β-dihydro-3-ketosteroids into 3β-hydroxysteroids, some of which exert biological actions [13,14].

In this study, we re-examined the reactivity of pig, rabbit and dog DHRS4s towards steroids and benzil, and found that the three animal enzymes exhibit low 3α-hydroxysteroid dehydrogenase (HSD) activity and *S*-enantio-preference in the reduction of benzil, which are in contrast to the 3β-HSD activity of human DHRS4 and its *R*-enantio-selective reduction of benzil (Fig. 1). The human enzyme shares >84% amino acid sequence identity with the pig, rabbit and dog enzymes, and differs by several residues in the putative substrate-binding region, which is predicted from the crystal structure of the pig enzyme [15]. Among such residues, S158 and F161 in human DHRS4 are phenylalanine and leucine, respectively, in the pig, rabbit, dog and mouse enzymes (Fig. 2). The crystal structure of pig DHRS4 [15] also suggests that N177 of one subunit is involved in the proper orientation of the putative substrate-binding residues by forming an H-bond with N165 of the other subunit. The residue 177 is threonine in the human enzyme, which is unable to form the H-bond [8]. Therefore, we also prepared mutant enzymes by exchanging the three residues between the human and pig enzymes, and analyzed the alterations in the kinetic constants for the substrates including all-*trans*-retinal and the stereochemistry in the reduction of 3-ketosteroids and benzil by both wild-type and mutant enzymes. Our analyses showed that double mutation (F158S/L161F) of pig DHRS4 leads to an effective switch of its substrate affinity and stereochemistry into those of the human enzyme. The roles of the two residues in determining the stereospecific reduction of 3-ketosteroids were confirmed by reverse double mutation in the human enzyme, but additional mutation of T177N was required to convert its kinetic constants for substrates into those of the pig enzyme. Furthermore, we performed molecular modeling studies by docking 3-ketosteroid and all-*trans*-retinal in the structures of the pig wild-type and mutant DHRS4–NADPH binary complexes, in order to understand the mechanisms underlying the differences in the stereochemical control and reactivity towards retinoids between human and the other animal DHRS4s.

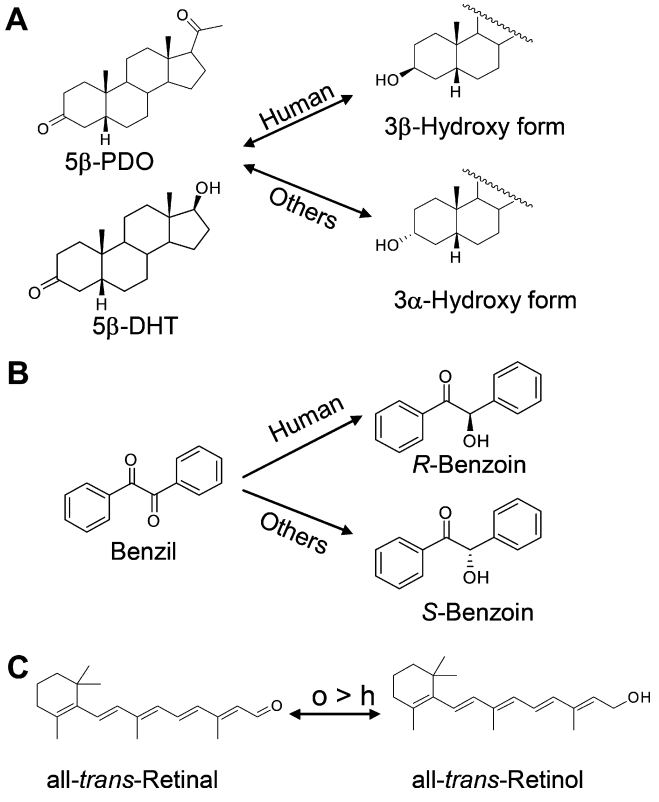

Fig. 1. Differences in the stereochemistry in the reduction of 3-ketosteroids and benzil and reactivity towards retinoids between human and other animal DHRS4s. (A) Reduction of 5β-pregnane-3,20-dione (PDO) and 5β-dihydrotestosterone (DHT). The human enzyme produces the 3β-hydroxy forms of steroids, whereas the pig, rabbit and dog enzymes (others) yield the 3α-hydroxy forms. (B) Irreversible reduction of benzil. The human and other animal enzymes preferentially produce *S*- and *R*-benzoins, respectively. (C) Reduction of all-*trans*-retinal. The other enzymes (o) catalyze the reversible reaction more efficiently than the human enzyme (h).

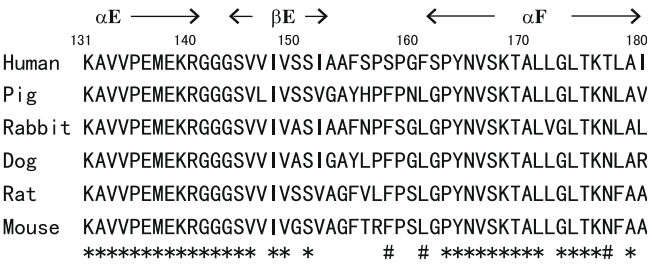

Fig. 2. Alignments of amino acids around the substrate-binding loop (between βE and αF) of mammalian DHRS4s. Secondary structure elements of the pig enzyme are indicated above the alignment. Residues conserved in all enzymes are shown with asterisks, and the residues mutated in this study are highlighted by # below the alignment.

Materials and methods

Materials

Steroids and retinoids were obtained from Steraloids (Newport, RI) and Sigma–Aldrich. A pCR T7/CT-TOPO expression kit, Lipofectamine 2000 reagent and *Escherichia coli* BL21 (DE3) pLysS were purchased from Invitrogen (Carlsbad, CA), and a QuickChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Bovine aortic endothelial cells (BAECs) were generously gifted from Taisho Pharmaceutical Co. (Saitama, Japan). All other chemicals were of the highest grade that could be obtained commercially.

Mutagenesis and purification of recombinant enzymes

Mutagenesis was performed using the site-directed mutagenesis kit and the pCR T7/CT-TOPO expression plasmids harboring the cDNAs for human and pig DHRS4s [8,9] as the templates according to the protocol described by the manufacturer (Stratagene). The single and double mutations were carried out using each set of forward and reverse mutagenic oligonucleotides (Supplementary data, Table S1). The cDNA for human S158F/F161L-T177N mutant DHRS4 (hSFFL-TN) was prepared using the primers for the T177N mutation and the expression plasmid harboring the cDNA for human S158F/F161L mutant DHRS4 (hSFFL), as the template. The complete coding region of the cDNAs in the expression plasmids were sequenced by using a Beckman CEQ2000XL DNA sequencer to confirm the presence of the desired mutation and to insure that no other mutation had occurred.

The expression constructs were transfected into *E. coli* BL21 (DE3) pLysS. The *E. coli* cells were cultured in a LB medium (1 L)