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Article

Molecular and enzymatic properties of 7α-hydroxysteroid dehydrogenase from *Pseudomonas* sp. B-0831

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A novel 7α-hydroxysteroid dehydrogenase (7α-HSD) was purified from *Pseudomonas* sp. B-0831. The molecular weight of the purified enzyme was 25 k on SDS-PAGE and 108 k on gel filtration analysis, suggesting that the enzyme exists as a tetramer of an identical subunit, similar to those of 7α-HSD from *E. coli* HB101 and *Eubacterium* sp. VPI 12708. 7α-HSD from *P.* sp. B-0831 showed high NAD^+-dependence and was able to catalyze the oxido-reduction of 7α-hydroxy bile acids including glycine and taurine conjugates. The K_m value for cholic acid was determined to be 0.25 mM, which is similar to that for chenodeoxycholic acid and is about four times smaller than those for the conjugates of cholic acid. The k_{cat} values for the conjugates were determined to be about 60-70% of that for free cholic acid. In addition to NAD^+, 7α-HSD from *P.* sp. B-0831 can utilize thio-NAD^+ to the same extent.

Keywords: 7α-hydroxysteroid dehydrogenase, nucleotide cofactor, *Pseudomonas* sp. B-0831, purification, steady-state kinetics, substrate specificity

Introduction

Many members of the human intestinal microflora can modify the glycine and taurine conjugates of the primary bile acids, such as cholic acid and chenodeoxycholic acid. Microbial biotransformations of bile acids include hydrolysis of the conjugate amide bond, 7-dehydroxylation, and the dehydrogenation of the hydroxyl groups at positions 3, 7, and 12 of the steroid skeleton. The major reaction of physiological significance is the 7α-dehydroxylation of cholic acid and chenodeoxycholic acid, producing deoxycholic acid and lithocholic acid, respectively [1]. 7α-Hydroxysteroid dehydrogenase (7α-HSD) (EC 1.1.1.159), catalyzing the dehydrogenation of a hydroxyl group at position 7 of the steroid skeleton of bile acids, was found in numerous bacteria and mammalian liver [2-10]. The enzymes elicited by these bacteria have alkaline pH optima for bile acid oxidation. The two 7α-HSDs, the NAD^+-dependent enzyme from *E. coli* HB101 and the $NADP^+$-dependent enzyme from *Eubacterium* sp. VPI 12708 were purified to homogeneity and extensively characterized [10-13].

We previously reported that a bacterium, *Pseudomonas* sp. B-0831, grown on cholic acid produces dual nucleotide cofactor-specific 3α-hydroxysteroid dehydrogenase (3α-HSD) [14]. The substrate specificity and the nucleotide cofactor dependency of the 3α-HSD have been analyzed [15, 16]. Besides, the bacterium was found to produce another enzyme with an amount smaller than the 3α-HSD. In the present study, we purified this enzyme to electrophoretic homogeneity and characterized its molecular and enzymatic properties. This enzyme was revealed to be 7α-HSD and has unique properties in comparison with other 7α-HSDs.

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The abbreviations used are: 7α-HSD, 7α-hydroxysteroid dehydrogenase; 3α-HSD, 3α-hydroxysteroid dehydrogenase; $APAD^+$, 3-acetylpyridine adenine dinucleotide; PCMB, *p*-chloromercuribenzoate; SDR, short-chain dehydrogenase/reductase.

Materials and Methods

Materials

All steroids were obtained from Sigma. NAD^+, $NADP^+$, and thio-NAD^+ were purchased from Oriental Yeast Co., Ltd., in Japan, and 3-acetyl-pyridine adenine dinucleotide ($APAD^+$) was purchased from Sigma. All other chemicals were of the highest commercial quality available.

Microorganism and cultivation

Pseudomonas sp. B-0831, isolated from soil in Nerima Ward of Tokyo and classified in the genus *Pseudomonas*, was grown in a 20-liter medium of 1% peptone, 1% yeast extract, 1% meat extract, 0.1% K_2HPO_4, and 0.05% cholic acid adjusted to pH 7.5 at 37°C for 20 h under aerobic conditions using a 30-liter jar fermentor.

Enzyme assay

The assays were carried out as described previously [15]. The substrate concentrations for the determination of the relative catalytic activity and the steady-state kinetic constants were 1.0 mM and 2.5 mM, respectively. One unit of the enzyme activity was defined as the amount catalyzing the consumption of 1 μmol of cholic acid per min.

Purification

The cells were harvested by centrifugation at 5,000 × *g* for 30 min and washed with 20 mM Tris-HCl buffer, pH 8.0. The washed cells were suspended in 2 liter of the same buffer containing 0.025% (w/v) lysozyme and 0.1 mM EDTA, and the suspension was incubated for 30 min at 37°C. After the mixture was centrifuged to remove cell debris and to obtain the cell-free extract, the resultant supernatant was fractionated with ammonium sulfate. The precipitation occurring at 45% saturation was collected by centrifugation at 8,000 × *g* for 30 min and dialyzed against 10 mM Tris-HCl buffer, pH 8.0. The dialyzed enzyme solution was loaded onto a DEAE-Sepharose (Amersham Biosciences) column (2.5 x 10 cm) and eluted with a 300 ml linear KCl gradient from 0 to 0.5 M at a flow rate of 1 ml/min. After the enzyme fractions were dialyzed against 10 mM KH_2PO_4-NaOH buffer, pH 7.5, the enzyme solution was loaded onto a Red-Sepharose (Amersham Biosciences) column (1.2 x 10 cm) and eluted with a 100 ml linear KCl gradient from 0 to 0.5 M at a flow rate of 0.5 ml/min. The eluted 7α-HSD fractions were concentrated with a membrane filter apparatus equipped with an XM-50 (Amicon) and dialyzed against 10 mM KH_2PO_4-NaOH buffer, pH 7.5.

Analytical methods

SDS-PAGE was performed by PhastSystem (Amersham Biosciences) using PhastGel Gradient 8-25. Proteins in gels were stained by Coomassie brilliant blue R-250. The isoelectric point (pI) of the enzyme was estimated by the isoelectric focusing method with a pH 3-10 carrier ampholyte [17]. The protein concentration was determined by the method of Lowry with bovine serum albumin as the standard [18]. Gel filtration was performed on a Sephadex G-150 column (1.5 x 85 cm) equilibrated with a buffer containing 50 mM Tris-HCl, pH 8.0, and 0.2 M NaCl. The following proteins were used as standards to calibrate the column; cytochrome C (13 k), chymotrypsin (25 k), ovalbumin (45 k), bovine serum albumin (68 k), and catalase (240 k).

Results

Purification

7α-HSD was produced in the culture medium of *P.* sp. B-0831 together with 3α-HSD. Partial separation of 7α-HSD from 3α-HSD was achieved by ammonium sulfate fractionation, and subsequent DEAE-Sepharose column chromatography enabled each enzyme to be separated completely,

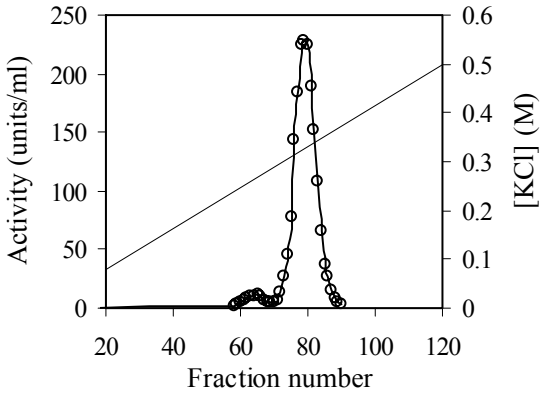

Figure 1. Elution profile on DEAE-Sepharose column chromatography. Each 2 ml fraction was collected. The enzymatic activities to catalyze cholic acid of respective fractions are indicated by open circles, and the KCl concentration to elute the sample is indicated by linear line.

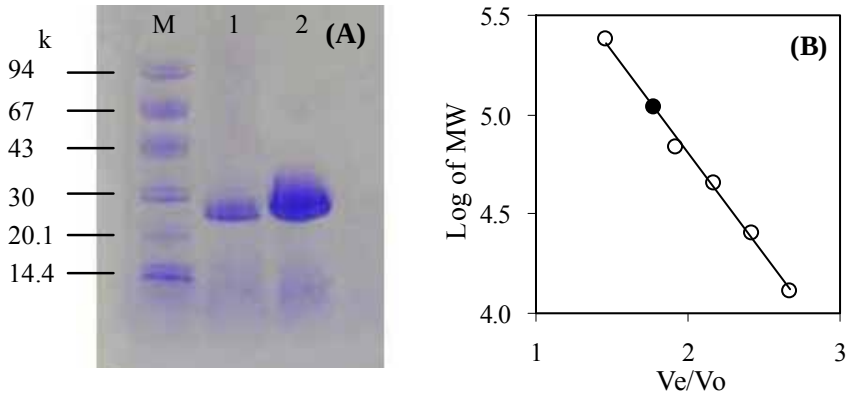

Figure 2. Molecular weight determination of the purified 7α-HSD. (A) Coomassie-stained polyacrylamide gel after SDS-PAGE under reducing conditions. Lane M, markers; Lane 1, 7α-HSD from *P.* sp. B-0831 (0.5 μg); Lane 2, recombinant 3α-HSD from *P.* sp. B-0831 (1.8 μg). (B) A standard curve obtained by plotting the log of molecular weights vs. relative elution volume (Ve/Vo) of standard proteins (○) on gel filtration analysis. The Ve/Vo of 7α-HSD (●) was plotted on the standard curve to determine the native molecular weight.

Table 1. Purification of 7α-HSD from *P.* sp. B-0831

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Cell-free extract	28600	4000	0.14	1	100
Ammonium sulfate	296	3200	10.8	77	80
DEAE-Sepharose	60	2090	35	250	52.3
Red-Sepharose	0.52	493	941	5881	12.3

with the minor and major peaks containing 3α-HSD and 7α-HSD, respectively (Fig. 1). The procedure of affinity chromatography using a Red-Sepharose column gave a 27-fold purification factor, resulting in nearly a single band at about 25 k on Coomassie stained gel after SDS-PAGE (Fig. 2A). The purification procedures of 7α-HSD are summarized in Table 1. The pI of the enzyme was determined to be 4.6. The gel filtration analysis of the purified 7α-HSD gave a calculated molecular weight of about 108 k (Fig. 2B). The results of SDS-PAGE and gel filtration indicated that the enzyme exists in a tetrameric form.

Nucleotide cofactor dependency

To analyze the effects of nucleotide cofactors on enzyme activity, the k_{cat} values for oxidation of 1.0 mM cholic acid were determined in the presence of various cofactors. The relative activities, in which the k_{cat} values were normalized by setting the value with NAD^+ to 100%, are summarized in Table 2. The results clearly show that 7α-HSD from *P.* sp. B-0831 is NAD^+-dependent, which is in contrast to the dual nucleotide cofactor-specific 3α-HSD produced by the same bacterium. In addition, 7α-HSD can utilize an NAD^+ analogue, thio-NAD^+, with the same extent of activity.

Substrate specificity

The enzyme was active toward the substrates, each of which has a hydroxy group at position 7 of the steroid skeleton in the α orientation, and inactive toward the 7α-hydroxy-lacking substrates, deoxycholic acid, glycodeoxycholic acid, ursodeoxycholic acid, lithocholic acid, and androsterone. The steady-state kinetic rate parameters of 7α-

Table 2. Effect of cofactors on the 7α-HSD-catalyzed reaction for cholic acid

Cofactor	Relative activity (%)
NAD^+	100
thio-NAD^+	93
$APAD^+$	4.9
$NADP^+$	0.7

Assays were carried out at 37°C in 40 mM Tris-HCl, pH 9.5, 1.0 mM cholic acid, 0.05 units/ml 3α-HSD, and 1.0 mM cofactor.

hydroxysteroid dehydrogenation, K_m and k_{cat}, were determined (Table 3). The K_m value for cholic acid was similar to that for chenodeoxycholic acid and about four times smaller than those for the conjugates of cholic acid. The k_{cat} values for the conjugates were about 60-70% of that for cholic acid.

Table 3. Steady-state kinetic parameters for 7α-steroid substrates

Substrate	K_m (mmol/l)	k_{cat} (1/s)
Cholic acid	0.25	423
Chenodeoxycholic acid	0.27	487
Glycocholic acid	1.20	254
Taurocholic acid	1.13	288

Assays were carried out at 37°C in 40 mM Tris-HCl, pH 8.0, 0.025% nitro tetrazolium blue, 0.4% Triton X-100, 2.5 units/ml diaphorase, 1.0 mM NAD^+, and 2.5 mM substrate.
Experimental error was within 10%.

Effects of pH, temperature, and chemicals on enzyme activity

The effect of pH on the oxidation of cholic acid was determined over a pH range of 5-11 as shown in Fig. 3A. The optimum pH range was found to be between 8 and 10.

As shown in Fig. 3B, the enzyme was stable below 50°C. After incubation at 55°C and 60°C for 10 min, the remaining activities were 55% and 10%, respectively. The incubation at 37°C for 60 min resulted in no loss of activity in the pH range of 7 to 10.

The effect of various compounds on 7α-HSD was evaluated (Table 4). The enzyme activity was strongly inhibited by $CuCl_2$ and $FeCl_3$ but was minimally affected by a sulfhydryl-blocking reagent, *p*-chloromercuribenzoate (PCMB).

Table 4. Effect of chemicals on the 7α-HSD-catalyzed reaction for cholic acid

Addition	Relative activity (%)
None (control)	100
2 mM $MgCl_2$	111
2 mM $CaCl_2$	108
2 mM $CuCl_2$	2
2 mM $ZnCl_2$	51
2 mM $FeCl_3$	8
2 mM NaF	98
1 mM EDTA	98
0.02 mM PCMB	78

The respective compounds were added into the enzyme assay solution, described in Table 2.

Discussion

7α-HSD-producing microorganisms often have produced other steroid catabolic enzymes [3, 4, 7, 19, 20]. So far, 7α-HSD from *E. coli* HB101 and *Eubacterium* sp. VPI 12708 have been sequenced and classified as members of the short-chain dehydrogenase/reductase (SDR) superfamily [11, 12]. The molecular weight analysis of 7α-HSD from *P.* sp. B-0831 shows that the subunit weight (25 k) is slightly smaller than those from *E. coli* (28 k) and *Eubacterium* sp. (32 k), while the

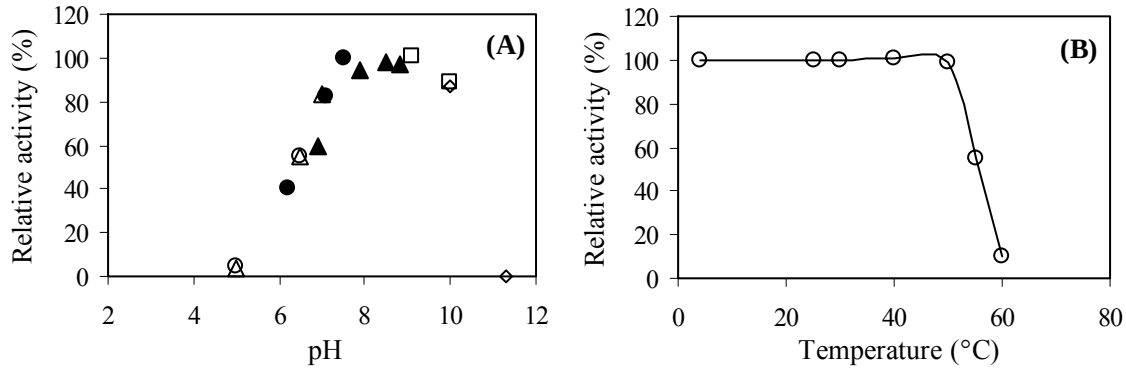

Figure 3. Effects of pH and temperature on the 7α-HSD-catalyzed reaction for cholic acid. (A) Optimization of pH. The k_{cat} values were measured over a pH range of 5-11. The following buffer was used at the respective pH: acetate-sodium acetate (○), KH_2PO_4-NaOH (●), Tris-HCl (▲), dimethylglutaric acid-NaOH (Δ), glycine-NaOH (□), and Na_2HPO_4-NaOH (◊). The relative activities were normalized by setting the k_{cat} value at pH 7.5 to 100%. (B) Thermal stability. The enzyme was incubated at various temperatures for 10 min in 10 mM Tris-HCl buffer, pH 8.0, followed by the measurement of k_{cat}. The relative activities were normalized by setting the k_{cat} value at 30°C to 100%.

homo tetramer form of the mature enzyme is the same as these two enzymes [10, 11]. 7α-HSD from *P.* sp. B-0831 is NAD^+-dependent and insensitive to PCMB (Tables 2 and 4). These characteristics are similar to those of 7α-HSD from *E. coli* HB101 and in contrast to those of 7α-HSD from *Eubacterium* sp. VPI 12708, which is $NADP^+$-dependent and PCMB-sensitive. The activity of 7α-HSD from *P.* sp. B-0831 was inhibited by some heavy-metal ions as well as 7α-HSDs from *E. coli* HB101 and *Eubacterium* sp. VPI 12708. The steady-state kinetic properties of 7α-HSD from *P.* sp. B-0831 were found to be unique (Table 3) in comparison with the other two 7α-HSDs [10, 11]. The K_m value of 7α-HSD from *P.* sp. B-0831 for cholic acid (0.25 mM) is larger than that of the *Eubacterium* enzyme (11 μM) and smaller than that of the *E. coli* enzyme (1.2 mM). The k_{cat} value of the *E. coli* enzyme for cholic acid is nearly eight times larger than that for chenodeoxycholic acid, while both of the corresponding values of 7α-HSD from *P.* sp. B-0831 are similar. The k_{cat} values of the *E. coli* enzyme for the conjugates of cholic acid are less than one-twentieth that of cholic acid itself, while the corresponding values of 7α-HSD from *P.* sp. B-0831 are about 60-70%. This comparatively equal k_{cat} values for free cholic acid and the conjugates of cholic acid suggest that 7α-HSD from *P.* sp. B-0831 is appropriate for the quantitative analysis of 7α-hydroxy bile acids.

In the metabolism of the primary bile acids, cholic acid and chenodeoxycholic acid are dehydroxylated by intestinal microflora to deoxycholic acid and lithocholic acid, respectively. In addition, another pathway for the 7-dehydroxylation system has been proposed in *Eubacterium* sp. VPI 12708, an intestinal anaerobic bacterium, in which 3α-HSD is related to the initial step of this pathway [21]. 3α-HSD from *Comamonas testosteroni* is known to participate in not only bile acid metabolism but also steroid decomposition, which is important for the defense strategy [22, 23]. We recently showed the close similarity in the substrate specificity between 3α-HSDs from *P.* sp. B-0831 and *C. testosteroni* [15, 16]. The characterization of different HSDs from the same bacterium can help us to understand the more precise biological roles of the respective enzymes. In comparison with 7α-HSD and 3α- HSD from *P.* sp. B-0831, the subunit molecular weights are similar, while the native oligomeric forms are different. The nucleotide cofactor specificities are different from each other: NAD^+-dependent 7α-HSD and dual nucleotide cofactor-specific 3α-HSD. The K_m values of 7α-HSD for cholic acid and its conjugates are larger than those of 3α-HSD. The slightly decreased k_{cat} values for the cholic acid conjugates relative to that for cholic acid are similar in both of the HSDs.

The present study shows that 7α-HSD from *P.* sp. B-0831 can utilize an NAD^+ analogue, thio-NAD^+ (Table 2). The activity difference of 7α-HSD with the two cofactors, NAD^+ and thio-NAD^+, is much smaller than that of 3α-HSD. Using the advantage of the higher molar extinction coefficient of thio-NAD^+, 3α-HSD with thio-NAD^+ has been used in the assay system for the measurement of total bile acids in serum as commercially available reagents [24]. From a practical standpoint, 7α-HSD with thio-NAD^+ is considered to be useful for future clinical diagnostics in the measurement of the primary bile acids.

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