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Article

Steady-state kinetic properties of 3α-hydroxysteroid dehydrogenase from *Pseudomonas* sp. B-0831: Steroid substrate specificity and nucleotide cofactor dependency

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We investigated the steroid substrate specificity of 3α-hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas* sp. B-0831. Similar to 3α-hydroxysteroid dehydrogenase/carbonyl reducetase (3α-HSD/CR) from *Comamonas testosteroni,* the recombinant 3α-HSD from *P.* sp. B-0831 catalyzed the oxidation of the antibiotic fusidic acid with the lowest K_m value among 3α-hydroxysteroid substrates examined in this study, while the k_{cat} value was 17% of that for one of the other substrates, androsterone. The enzyme showed the utility of cofactor analogues, such as thio-NAD^+, 3-acetylpyridine adenine dinucleotide ($APAD^+$), and nicotinamide hypoxanthine dinucleotide (deamino-NAD^+). Comparing the effects of cofactors, NAD^+ and thio-NAD^+, on the k_{cat} values, NAD^+ was preferred for cholic acid, while thio-NAD^+ was preferred for fusidic acid. These results suggest that the enzyme is adaptable to various substrates and cofactors, thus generating the broad substrate specificity with each different reaction mode.

Keywords: 3α-hydroxysteroid dehydrogenase, nucleotide cofactor, *Pseudomonas* sp. B-0831, steady-state kinetics, substrate specificity

Introduction

3α-Hydroxysteroid dehydrogenase (3α-HSD) catalyzes the reversible inter-conversion of hydroxy and oxo groups at position 3 of the steroid nucleus. Prokaryotic 3α-HSDs have been described in several microorganisms and belong to the short-chain dehydrogenase/reductase (SDR) superfamily (EC 1.1.1.50) [1-5]. Prokaryotic 3α-HSDs are supposed to participate in steroid degradation by their steroid utilization as a sole carbon source [6]. 3α-HSD from *Comamonas* (*Pseudomonas*) *testosteroni*, which has been studied extensively among bacterial 3α-HSDs, is an NAD^+ dependent enzyme and shows broad substrate specificity to various steroids, C_{19} to C_{27} [6, 7]. In addition, since it can catalyze the carbonyl reduction of a variety of non-steroidal aldehydes and ketones, it was named as 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) [8]. *C. testosteroni* was able to grow on steroids as a sole carbon source and the enzyme, 3α-HSD/CR, was induced by these steroids, such as testosterone and progesterone [6, 9].

We previously cloned 3α-HSD from *Pseudomonas* sp. strain B-0831 and expressed it in *E. coli* [10]. The amino acid sequence shares about 50% identity with 3α-HSD/CR from *C. testosteroni* ATCC11996 [7]. Unlike NAD^+-dependent 3α-HSD/CR from *C. testosteroni*, 3α-HSD from B-0831 has the ability to use not only NAD^+ but also

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The abbreviations used are: 3α-HSD, 3α-hydroxysteroid dehydrogenase; SDR, short-chain dehydrogenase/reductase; 3α-HSD/CR, 3α-hydroxysteroid dehydrogenase/carbonyl reductase; TBA, total bile acids; $APAD^+$, 3-acetylpyridine adenine dinucleotide; NTB, nitro tetrazolium blue; $NAAD^+$, nicotinic acid adenine dinucleotide; deamino-NAD^+, nicotinamide hypoxanthine dinucleotide.

$NADP^+$. In clinical diagnostics, the enzyme has been used for the measurement of total bile acids (TBA) in serum [11]. Furthermore, a highly sensitive and unique enzyme cycling method for TBA assay has been developed using the advantage of the higher molar extinction coefficient of an NAD^+ analogue, thio-NAD^+, and the assay system has been used for commercially available reagents [12]. Since other NAD^+ analogues also have unique properties, their use in the application of the assay system is considered practical. For instance, 3-acetylpyridine adenine dinucleotide ($APAD^+$) can alter the equilibrium of NAD^+ related enzyme reactions in favor of substrate oxidation [13].

In the present study, we analyzed the dehydrogenase activity of 3α-HSD from *P.* sp. B-0831 toward various steroid substrates (Fig. 1) and compared the results with those of 3α-HSD/CR from *C. testosteroni*. Using the results of this study together with the previous reports, we were able to elucidate the biological roles of steroids and steroid decomposition in prokaryotes. In addition, we evaluated the effects of the cofactors, NAD^+ and its analogues, on the catalytic activity.

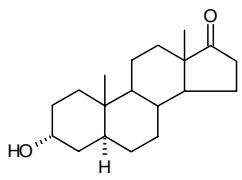
Materials and Methods

Materials

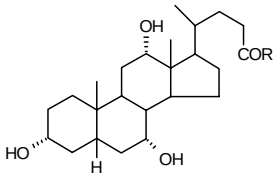
NAD^+, $NADP^+$, and nicotinic acid adenine dinucleotide ($NAAD^+$) were purchased from Oriental Yeast Co., Ltd., in Japan. Nicotinamide hypoxanthine dinucleotide (deamino-NAD^+), $APAD^+$, thio-NAD^+, and all steroids used in this study (Fig. 1) were purchased from Sigma. All other chemicals were of the highest commercial quality available. Recombinant 3α-HSD from *Pseudomonas* sp. strain B-0831 was expressed in *E. coli* and purified as described previously [10]. The protein purity was determined to be over 95% by SDS-PAGE analysis, and the protein concentration was determined by the method of Lowry [14].

Steady-state kinetic study

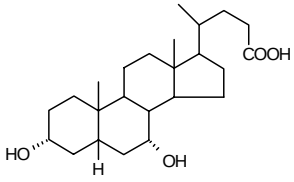
The oxidataion reactions were carried out at 37°C in 1 ml solution containing 40 mM Tris-HCl, pH 8.5, 0.025% nitro tetrazolium blue (NTB), 0.4% Triton X-100, 2.5 units/ml diaphorase, and 1.0 mM cofactor, NAD^+ or thio-NAD^+, in the presence of either 1.0 mM substrate for the determination of k_{cat} or various concentrations of substrate for the determination of K_m [10]. The lower solubility steroids, such as androsterone, lithocholic acid, and testosterone, were dissolved in methanol and were added to the assay mixture in which the final solvent concentration did not exceed 2% (v/v). Other steroids were dissolved in distilled water and adjusted to neutral pH. The reaction was started by adding the enzyme and



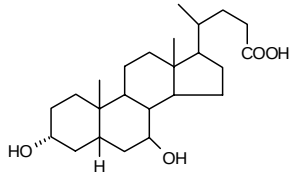
Androsterone



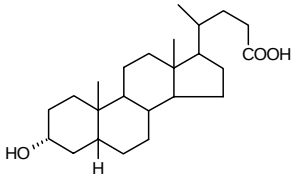
Cholic acid (R: OH)
Glycocholic acid (R: $NHCH_2COOH$)
Taurocholic acid (R: $NH(CH_2)_2SO_3H$)



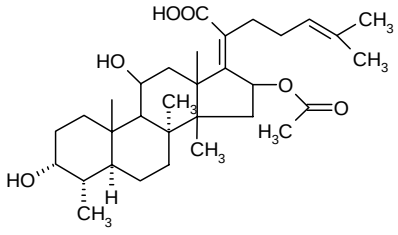
Chenodeoxycholic acid



Ursodeoxycholic acid



Lithocholic acid



Fusidic acid

Figure 1. Structures of steroid substrates used in this study.

stopped according to necessity by adding 2 ml of 0.5% SDS after incubation for 5 min. The formation of formazan dye at 550 nm was measured using a Shimadzu UV-2200 spectrophotometer and calculated using a molar extinction coefficient of 16,700 mol^{-1} l cm^{-1}. The K_m and K_i values were calculated by the nonlinear least squares method using Taylor expansion [15].

Quantitative analysis of cholic acid

Assays were carried out at 37°C in 1 ml solution of 3α-HSD containing 40 mM Tris-HCl, pH 8.0, 0.025% NTB, and 0.4% Triton X-100 with either 1.0 mM NAD^+, 2.5 units/ml diaphorase (EC 1.6.99.2) (Asahi Kasei Pharma), or 1.0 mM $NADP^+$, 2.5 units/ml NADPH diaphorase (EC 1.6.99.1) (Asahi Kasei Pharma). The reaction was started by adding 0.05 ml of various concentrations of cholic acid in distilled water and stopped by adding 2 ml of 0.5% SDS after incubation for 15 min. The amount of cholic acid was determined by measuring the formation of formazan dye at 550 nm.

Relative catalytic activity for various cofactors

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. The k_{cat} values for the oxidation reaction were determined by measuring the reduction of a cofactor at 340 nm for NAD^+, deamino-NAD^+, and $NAAD^+$; at 400 nm for thio-NAD^+; and at 365 nm for $APAD^+$.

Results

Steroid substrate specificity

The recombinant 3α-HSD from *Pseudomonas* sp. B-0831 was shown to be active among a variety of steroid substrates, such as androsterone (C_{19} steroid), cholic acid (C_{24} steroid), and fusidic acid (C_{27} steroid) (Table 1), while the 3-oxosteroid reduction of testosterone was not observed. This result was similar to the substrate specificity of 3α-HSD/CR from *Comamonas testosteroni*. The steady-state kinetic constants for the 3α-hydroxysteroid oxidation of various steroid substrates using NAD^+ or thio-NAD^+ as a cofactor were determined and summarized in Table 1. The k_{cat} values for bile acids including their conjugates, such as glycocholic acid and taurocholic acid were quite similar. Comparing the activity toward the steroid substrates used in this study, the enzyme oxidized steroid antibiotic fusidic acid more effectively than androsterone and bile acids.

Quantitative analysis of cholic acid by using NAD^+ or $NADP^+$ as a cofactor

3α-HSD from *P.* sp. B-0831 was found to be a dual nucleotide cofactor-specific enzyme (Ueda *et al*., manuscript in preparation). In order to analyze the different effects of these dual cofactors, NAD^+ and $NADP^+$, on 3α-hydroxysteroid dehydrogenation, the quantitative analysis of cholic acid was

Table 1. Steady-state kinetic parameters for 3α-steroid substrates

Substrate	Cofactor	K_m (μmol/l)	k_{cat} (1/sec)	k_{cat}/K_m (l/μmol·sec)
Androsterone	NAD^+	210	134	0.64
Cholic acid	NAD^+	31	75	2.41
Cholic acid	thio-NAD^+	84	35	0.41
Chenodeoxycholic acid	NAD^+	63	76	1.21
Ursodeoxycholic acid	NAD^+	69	82	1.19
Lithocholic acid	NAD^+	68	72	1.06
Glycocholic acid	NAD^+	38	77	2.02
Taurocholic acid	NAD^+	43	74	1.72
Fusidic acid	NAD^+	6.2	23	3.75
Fusidic acid	thio-NAD^+	37	25	0.67

Experimental error was within 10%.

carried out in the presence of either NAD^+ or $NADP^+$. As shown in Fig. 2A, 0.1 units/ml enzyme was enough for NAD^+ to catalyze 2.0 mM cholic acid under the present reaction conditions, while 2.5 units/ml enzyme was needed for $NADP^+$. When either 0.5 units/ml enzyme with NAD^+ or 2.5 units/ml enzyme with $NADP^+$ was used, the same standard curves, where the absorbance change at 550 nm increased in proportion to the amount of cholic acid, were obtained (Fig. 2B).

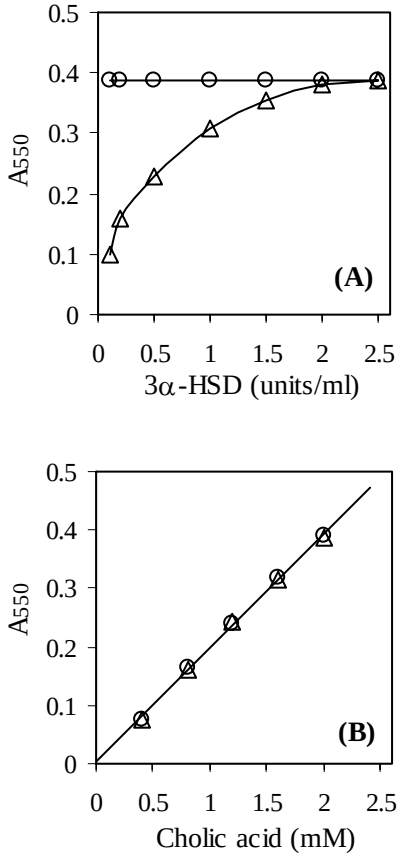


Figure 2. Effects of nucleotide cofactors, NAD^+ (circle) and $NADP^+$ (triangle), on the 3α-hydroxysteroid dehydrogenation of cholic acid by 3α-HSD from *P.* sp. B-0831. (A) Correlation between the enzyme concentration and the catalytic efficiency toward 2.0 mM cholic acid. (B) Standard curve to determine the amount of cholic acid by 0.5 units/ml enzyme with NAD^+ and 2.5 units/ml enzyme with $NADP^+$.

Effects of NAD^+ anologues on catalytic activity

An NAD^+ analogue, thio-NAD^+, has been used for the TBA assay system, mainly due to its higher sensitivity, as described above. It should be noted that the effects of the cofactors, NAD^+ and thio-NAD^+, on the k_{cat} values for cholic acid and fusidic acid were different from each other. When thio-NAD^+ was used, the k_{cat} value for cholic acid was decreased to 47%, while that for fusidic acid was similar (Table 1), indicating that the catalytic function for each substrate can be modulated by the cofactor in a different manner. From a practical standpoint, since the lower catalytic activity with thio-NAD^+ for cholic acid can be compensated by the amount of enzyme used, the present results supported the notion that the enzymatic analysis using thio-NAD^+ is applicable and attractive.

We also analyzed the effects of other oxidized cofactor analogues on the k_{cat} values and compared the relative catalytic activity (Table 2). It was found that 3α-HSD from *P.* sp. B-0831 can utilize not only thio-NAD^+ but also $APAD^+$ and deamino-NAD^+, while it can not utilize $NAAD^+$, a precursor of NAD^+, as a cofactor.

Table 2. Effects of cofactors on the 3α-HSD-catalyzed reaction for cholic acid

Cofactor	Relative activity (%) [a]
NAD^+ [b]	100
thio-NAD^+ [b]	47
$APAD^+$	42
deamino-NAD^+	36
$NAAD^+$	0

[a] The k_{cat} values were normalized by setting the value with NAD^+ to 100%.
[b] The k_{cat} values were taken from Table 1.

Discussion

Although 3α-HSD is known to play a central role in steroid metabolism, the information regarding its molecular function is limited. The steady-state kinetics of substrate specificity in prokaryotic 3α-HSDs has been reported only for 3α-HSD/CR from *C. testosteroni* [6, 7]. This enzyme was shown to catalyze antibiotic fusidic acid with the lowest K_m value (6.1 μM), compared with those of general steroids, such as androsterone (18.4 μM) and cholic acid (31.1 μM) [7]. As well, the K_m value of 3α-HSD from *Pseudomonas* sp. strain B-0831 for fusidic acid was 6.2

μM, lower than those for androsterone and cholic acid (Table 1). The steady-state kinetic constants for each bile acid, including its conjugates, such as glycocholic acid and taurocholic acid were similar, indicating the eligibility of the enzyme for the measurement of TBA in serum.

3α-HSD from B-0831 has the ability to use not only NAD^+ but also $NADP^+$, which is different from NAD^+-dependent 3α-HSD/CR from *C. testosteroni*. The capability of $NADP^+$ utilization was confirmed by the quantitative analysis of cholic acid as shown in Fig. 2B. In clinical diagnostics, 3α-HSD has been used for the measurement of TBA in serum [11]. In this regard, the dual cofactor specificity of the enzyme could be advantageous. However, the present study revealed that, when $NADP^+$ is used as a cofactor, a larger amount of the enzyme and a longer reaction time are needed (Fig. 2A). Therefore, it could be difficult to use $NADP^+$ for practical application.

The catalytic activity of 3α-HSD from B-0831 was largely changed by the types of cofactors (Tables 1 and 2). The subtle structural differences between NAD^+ and its analogues can drastically influence the activity, in which the activity with $NAAD^+$ was completely lost. These results indicate that the type of cofactor can modulate the fine catalytic function of the enzyme. At the point of application, the activity difference using the NAD^+ analogues, such as thio-NAD^+ and $APAD^+$, was found to be small. Therefore, the advantages of these NAD^+ analogues, resulting in higher sensitivity and a larger substrate concentration range, are useful and attractive, especially for clinical diagnostics.

Prokaryotic 3α-HSDs were shown to be induced by steroids such as testosterone and progesterone [6, 9, 16]. These steroids have been considered to be potent inducers and not substrates of 3α-HSD [7]. Recently, two regulator genes for enzyme expression, *rep*A and *rep*B, which act as repressors by binding to the operator of the enzyme gene and mRNA, respectively, were identified in *C. testosteroni* [17, 18]. The presence of steroids resulted in the liberation of these bindings such that translation could proceed. The testosterone induction was reported to lead to an approximately 6-fold elevation of resistance of *C. testosteroni* ATCC11996 against fusidic acid [19]. These results are consistent with the results showing that 3α-HSD/CR from *C. testosteroni* can catalyze fusidic acid but cannot catalyze testosterone [7], similar to 3α-HSD from *P.* sp. B-0831 observed in this study. 3α-HSD-producing prokaryotes, isolated even from human sources, can utilize steroids as a sole carbon source [9]. They often have produced other steroid catabolic enzymes, such as 3β,17β-HSD from *C. testosteroni* [16] and 7α-HSD from *P.* sp. B-0831 [20]. Thus, the catabolic pathway could be related to the decomposition of the steroids, which should also be important for the defense strategy.

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