J. Biol. Macromol., **4**(1) 29-32 (2004)

Article

Carbonyl reductase activity of a pluripotent enzyme, 3α-hydroxysteroid dehydrogenase from *Pseudomonas* sp. B-0831

Shigeru Ueda[1], Masayuki Oda[2, 3], Shigeyuki Imamura[1], and Masatake Ohnishi[2]

[1]Dept. Diagnostics Research & Development, Div. Fine Chemical & Diagnostics, Asahi Kasei Pharma Corporation, 632-1, Mifuku, Ohito-cho, Shizuoka 410-2321, Japan,
[2]Dept. Cellular Macromolecule Chemistry, Graduate School of Agriculture, The Prefecture University of Kyoto, 1-5, Shimogamo Nakaragi-cho, Sakyo-ku, Kyoto 606-8522, Japan.

Received December 20, 2003; Accepted January 14, 2004

We found that 3α-hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas* sp. B-0831 can catalyze the carbonyl reduction of non-steroid substrates, such as metyrapone and *p*-nitrobenzaldehyde, in addition to the activity of 3α-hydroxysteroid dehydrogenase. The results of an inhibition study in metyrapone reduction by androsterone and cholic acid indicated that metyrapone bound to the same catalytic site as the steroids. The K_m values for the carbonyl reduction were relatively higher than those for the oxidoreduction at position 3 of the steroid nucleus. It should be noted that the k_{cat} value of metyrapone using NADPH as a cofactor was less than 1% of that with NADH, indicating that the cofactor binding modulates the catalytic activity. The present result clearly showed that 3α-HSD from *P.* sp. B-0831 has pluripotent substrate specificity and can be named 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR).

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

Introduction

3α-Hydroxysteroid dehydrogenase (3α-HSD) is an $NAD(P)^+$-dependent enzyme which catalyzes the reversible inter-conversion of hydroxy and oxo groups at position 3 of the steroid nucleus and has been found in many mammalian cells and microorganisms [1-3]. Besides oxidation of 3α-hydroxysteroids and the reduction of corresponding 3-oxostertoids, 3α-HSD from *Comamonas* (*Pseudomonas*) *testosteroni*, which has been studied extensively among bacterial 3α-HSDs, is capable of catalyzing the carbonyl reduction of non-steroidal xenobiotic ketones and aldehydes, such as the potent cytochrome P-450 inhibitor, metyrapone, and *p*-nitrobenzaldehyde (Fig. 1). Due to this pluripotent substrate specificity, the enzyme was named 3α-hydroxysteroid dehydrogenase/ carbonyl reductase (3α-HSD/CR) [4].

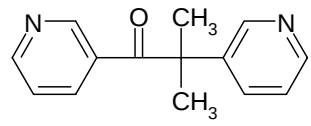
The carbonyl reductase activity has been found in several HSDs such as 11β-HSD type I from mouse liver [5-7]. Carbonyl reduction is significant in the detoxification process of potentially toxic ketone or aldehyde compounds in humans and animals [8]. In contrast, the physiological role of prokaryotic carbonyl reductase remains obscure. The structural similarity of *C. testosteroni* 3α-HSD/CR to the mammalian enzymes shown in an antibody cross-reactivity study and a crystal structural analysis [6, 9] evokes the idea that both prokaryotic and mammalian 3α-HSD/CRs have evolved from the same ancestral protein for the catabolism of a variety of carbonyl compounds [10].

In the present study, we found the carbonyl re-

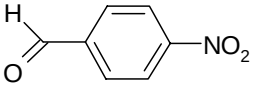
[3]To whom all correspondence should be addressed.
Tel & Fax: +81-75-703-5673,
E-mail: oda@kpu.ac.jp

The abbreviations used are: 3α-HSD, 3α-hydroxysteroid dehydrogenase; 3α-HSD/CR, 3α-hydroxysteroid dehydrogenase/carbonyl reductase; NTB, nitro tetrazolium blue; K_i, inhibitor constant; AKR, aldo-keto reductase; SDR, short-chain dehydrogenase/reductase.

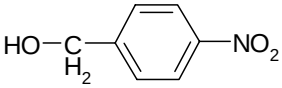
ductase activity of 3α-HSD from *Pseudomonas* sp. strain B-0831 to reduce non-steroid compounds such as metyrapone and *p*-nitrobenzaldehyde, and the substrate binding site to be similar to that for 3α-hydroxysteroid dehydrogenation. We analyzed the steady-state kinetics of this activity, together with the effects of the cofactor type on the activity.



Metyrapone



p-Nitrobenzaldehyde



p-Nitrobenzyl alcohol

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

Materials and Methods

Materials

The substrates, metyrapone, *p*-nitrobenzaldehyde, and *p*-nitrobenzyl alcohol (Fig. 1), were obtained from Sigma. The nucleotides, NAD^+, NADH, and NADPH, were purchased from Oriental Yeast Co., Ltd., in Japan. All other chemicals were of the highest commercial quality available. Recombinant 3α-HSD from *Pseudomonas* sp. strain B-0831 was prepared as described previously [3, 11].

Steady-state kinetic study

Assays were carried out at 37°C in 40 mM Tris-HCl, pH 8.5, in the presence of 0.3 mM NAD(P)H for reduction reaction, and in the presence of 1 mM NAD^+, 0.025% nitro tetrazolium blue (NTB), 0.4% Triton X-100, and 2.5 units/ml diaphorase (Asahi Kasei) for oxidation reaction. The reaction pH was chosen due to the stabilities of the enzyme and the chemicals, NTB and NAD^+. The carbonyl reductase activity was determined by measuring the decrease of NAD(P)H at 340 nm using a molar extinction coefficient of 6,220 mol^{-1} l cm^{-1}. The substrates were dissolved in methanol and added in the assay mixture within 5% (v/v). In the assay for substrate specificity, 5.0 mM substrate was used for the determination of k_{cat}, and various substrate concentrations were used for the determination of K_m. In the inhibition study, 0.4 mM to 4.0 mM substrate, metyrapone, was used in the absence or presence of 1.0 mM inhibitor, cholic acid and androsterone. The K_m and K_i values were calculated by the nonlinear least squares method using Taylor expansion [12].

Results and Discussion

The steady-state kinetic analysis revealed that 3α-HSD from *Pseudomonas* sp. strain B-0831 is able to catalyze the carbonyl reduction of non-steroid substrates, metyrapone and *p*-nitrobenzaldehyde (Table 1). No reverse oxidation of *p*-nitrobenzyl alcohol, the hydroxy product of *p*-nitrobenzaldehyde, could be observed, nor could 3α-HSD/CR from *C. testosteroni*. The K_m values for the carbonyl reduction are relatively higher

Table 1. Steady-state kinetic parameters for 3α-HSD from *Pseudomonas* sp. B-0831 with non-steroid and steroid substrates

Substrate	Cofactor	K_m (μmol/l)	k_{cat} (1/sec)	k_{cat}/K_m (l/μmol·sec)
Metyrapone	NADH	3370	13.3	3.94×10^{-3}
Metyrapone	NADPH	n.d.[a]	0.058	–
p-Nitrobenzaldehyde	NADH	4910	1.12	2.28×10^{-4}
p-Nitrobenzyl alcohol	NAD^+	No activity detectable		
Androsterone[b]	NAD^+	210	134	0.64
Cholic acid[b]	NAD^+	31	75	2.41
Fusidic acid[b]	NAD^+	6.2	23	3.75

[a] Not determined due to the small k_{cat} value.
[b] Data were taken from Ueda *et al*. [3].

than those for the oxidoreduction at position 3 of the steroid nucleus and are on the same order of those of 3α-HSD/CR from *C. testosteroni* [13]. It should be noted that the k_{cat} value for metyrapone using NADPH as a cofactor was less than 1% of that with NADH. Considering that the binding affinity of 3α-HSD to NADH is higher than that of NADPH (Ueda *et al*., manuscript in preparation), the cofactor binding can modulate the different catalytic activity. A similar correlation between the cofactor preference and the catalytic activity was also observed in the NADPH preference enzyme, mouse liver 11β-HSD, and the NADH-dependent enzyme, *C. testosteroni* 3α-HSD/CR [7, 13].

In order to determine the substrate binding site for the carbonyl reduction, in comparison with that for the oxidoreduction of the steroid nucleus, we conducted the inhibition study by using androsterone and cholic acid as the steroid inhibitor. As shown in Fig. 2, the carbonyl reduction was dramatically inhibited by these substrates following the competitive nature, suggesting that both steroids and metyrapone bind to the catalytically active site on the 3α-HSD. The inhibitor constants (K_i) of metyrapone reduction by androsterone and cholic acid were determined to be 0.174 mM and 0.118 mM, respectively. These values are similar to the K_i value of metyrapone reduction by corticosterone for mouse liver 11β- HSD, which is approximately 0.1 mM [7].

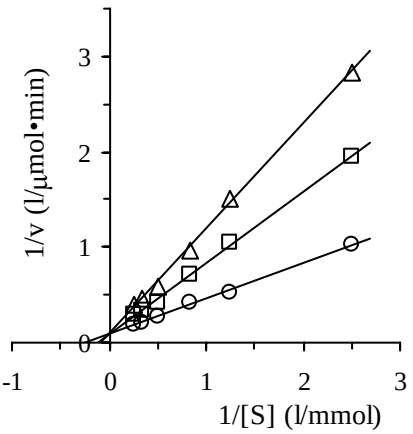


Figure 2. Lineweaver-Burk plot for the competitive inhibition of metyrapone reduction by androsterone and cholic acid. The carbonyl reductase activity of 3α-HSD was assayed for the metyrapone concentration range from 0.4 mM to 4.0 mM in the absence (circle) or presence of 0.1 mM inhibitor, androsterone (square) and cholic acid (triangle).

Carbonyl-reducing enzymes have been found in several HSDs and classified into two protein superfamilies: the aldo-keto reductase (AKR) superfamily and the short-chain dehydrogenase/ reductase (SDR) superfamily. In SDRs, much of the carbonyl reductase activity has been found in mammalian enzymes, such as 11β-HSD, 3α,20β-HSD, and 17β-HSD [10]. Particularly in 11β-HSD, carbonyl reductase activity has been estimated to be involved with detoxification of biologically active carbonyl compounds [8]. On the other hand, the physiological function of the prokaryotic enzymes, such as 3α-HSD/CR from *C. testosteroni* and 3α,20β-HSD/CR from *Streptomyces hydrogenans*, which also have the function of carbonyl reduction, is still obscure. Recently, putative 3α-HSD/CR genes were found in plant pathogen, such as *Xantomonas axonopodis*, *Xantomonas campestris*, and *Ralstonia solanacearum* [14, 15]. We found that 3α-HSD/CR from *P*. sp. B-0831 can also catalyze antibiotic fusidic acid with lower K_m value (Table 1), similar to 3α-HSD/CR from *C. testosteroni* [13]. These findings support the notion that the steroid detoxification function is one of the resources for those prokaryotes to establish the infectious or symbiotic relationship with their corresponding hosts.

In conclusion, this study has shown that 3α-HSD from *P*. sp. B-0831 can catalyze the reduction of xenobiotic carbonyl compounds with higher K_m values than those for the steroids. It has pluripotent substrate specificity, similar to 3α-HSD/CR from *C. testosteroni*, and can be named 3α-HSD/CR.

References

1. Cheng, K. C., White, P. C., and Qin, K. N. (1991) Molecular cloning and expression of rat liver 3α-hydroxysteroid dehydrogenase. *Mol. Endocrinol.* **5**, 823-828.
2. Jez, J. M., Flynn, T. G., and Penning, T. M. (1997) A new nomenclature for the aldo-keto reductase superfamily. *Biochem. Pharmacol.* **54**, 639-647.
3. Ueda, S., Oda, M., Imamura, S., and Ohnishi, M. (2004) Steady-state kinetic properties of 3α-hydroxysteroid dehydrogenase from *Pseudomonas* sp. B-0831: Steroid substrate

specificity and nucleotide cofactor dependency. *J. Biol. Macromol.* **4**, 23-28.

4. Möbus, M., and Maser, E. (1998) Molecular cloning, overexpression, and characterization of steroid-inducible 3α-hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni*. A novel member of the short-chain dehydrogenase/reductase superfamily. *J. Biol. Chem.* **273**, 30888-30896.
5. Maser, E., and Netter, K. J. (1989) Purification and properties of a metyrapone-reducing enzyme from mouse liver microsomes – this ketone is reduced by an aldehyde reductase. *Biochem. Pharmacol.* **38**, 3049-3054.
6. Maser, E., Oppermann, U. C., Bannenberg, G., and Netter, K. J. (1992) Functional and immunological relationships between metyrapone reductase from mouse liver microsomes and 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni*. *FEBS Lett.* **297**, 196-200.
7. Maser, E., and Bannenberg, G. (1994) 11β-hydroxysteroid dehydrogenase mediates reductive metabolism of xenobiotic carbonyl compounds. *Biochem. Pharmacol.* **47,** 1805-1812.
8. Maser, E., and Oppermann, U. C. (1997) Role of type-1 11β-hydroxysteroid dehydrogenase in detoxification processes. *Eur. J. Biochem.* **249**, 365-369.
9. Grimm, C., Maser, E., Möbus, E., Klebe, G., Reuter, K., and Ficner, R. (2000) The crystal structure of 3α-hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni* shows a novel oligomerization pattern within the short chain dehydrogenase/reductase family. *J. Biol. Chem.* **275**, 41333-41339.
10. Maser, E. (1995) Xenobiotic carbonyl reduction and physiological steroid oxidoreduction. The pluripotency of several hydroxysteroid dehydrogenases. *Biochem. Pharmacol.* **49**, 421-440.
11. Suzuki, K., Ueda, S., Sugiyama, M., and Imamura, S. (1993) Cloning and expression of a *Pseudomonas* 3α-hydroxysteroid dehydrogenase-encoding gene in *Escherichia coli*. *Gene* **130**, 137-140.
12. Sakoda, M., and Hiromi, K. (1976) Determination of the best-fit values of kinetic parameters of the Michaelis-Menten equation by the method of least squares with the Taylor expansion. *J. Biochem.* **80**, 547-555.
13. Maser, E., Möbus, E., and Xiong, G. (2000) Functional expression, purification, and characterization of 3α-hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas testosteroni*. *Biochem. Biophys. Res. Commun.* **272**, 622-628.
14. Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J. C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Lenard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thébault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J., and Boucher, C. H. (2002) Genome sequence of the plant pathogen *Ralstonia Solanacearum. Nature* **415**, 497-502.
15. da Silva, A. C. R., Ferro, J. A., Reinach, F. C., Farah, C. S., Furlan, L. R., Quaggio, R. B., Monteiro-Vitorello, C. B., Van Sluys, M. A., Almeida, N. F., Alves, L. M. C., do Amaral, A. M., Bertolini, M. C., Camargo, L. E. A., Camarotte, G., Cannavan, F., Cardozo, J., Chambergo, F., Ciapina, L. P., Cicarelli, R. M. B., Coutinho, L. L., Cursini-Santos, J. R., El-Dorry, H., Faria, J. B., Ferreira, A. J. S., Ferreira, R. C. C., Ferro, M. I. T., Formighieri, E. F., Franco, M. C., Greggio, C. C., Gruber, A., Katsuyama, A. M., Kishi, L. T., Leite, R. P., Lemos, E. G. M., Lemos, M. V. F., Locali, E. C., Machado, M. A., Madeira, A. M. B. N., Martinez-Rossi, N. M., Martins, E. C., Meidanis, J., Menck, C. F. M., Miyaki, C. Y., Moon, D. H., Moreira, L. M., Novo, M. T. M., Okura, V. K., Oliveira, M. C., Oliveira, V. R., Pereira, H. A., Rossi, A., Sena, J. A. D., Silva, C., de Souza, R. F., Spinola, L. A. F., Takita, M. A., Tamura, R. E., Teixeira, E. C., Tezza, R. I. D., Trindade dos Santos, M., Truffi, D., Tsai, S. M., White, F. F., Setubal, J. C., and Kitajima, J. P. (2002) Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* **417**, 459-463.

Communicated by Kikuko Watanabe