Carbonyl Reductase Activity Exhibited by Pig Testicular 20β-Hydroxysteroid Dehydrogenase

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The carbonyl reductase activity exhibited by pig testicular 20β-hydroxysteroid dehydrogenase (20β-HSD) was examined using a recombinant enzyme. Kinetic parameters were obtained for 48 carbonyl group-containing substrates, including aromatic aldehydes, aromatic ketones, cycloketones, quinones, aliphatic aldehydes and aliphatic ketones. 20β-HSD showed a high affinity towards quinones, such as 9,10-phenanthrenequinone, α-naphthoquinone and menadione (K_m values of 4, 2 and 5 μM, respectively), and the substrate utilization efficiency (V_{max}/K_m) of the enzyme against these quinones was very high. Cyclohexanone and 2-methylcyclohexanone were also reduced with a high V_{max}/K_m value, but not cyclopentanone or 2-methylcyclopentanone. Various aromatic aldehydes and ketones including benzaldehyde- and acetophenone-derivatives were reduced by 20β-HSD. Especially, 4-nitrobenzaldehyde and 4-nitroacetophenone were reduced with high V_{max}/K_m values in the related compounds. The enzyme also reduced the pyridine-derivatives, 2-, 3-, and 4-benzoylpyridine, with the V_{max}/K_m value for 2-benzoylpyridine being the highest. 20β-HSD reduced aliphatic aldehydes and aliphatic ketones, but was more effective on the former. The correlation between the structure of carbonyl compounds and their substrate V_{max}/K_m is discussed.

Key words 20β-hydroxysteroid dehydrogenase; testis; pig; carbonyl reductase; kinetics; recombinant enzyme

20β-Hydroxysteroid dehydrogenase (20β-HSD) was first reported to be found in the prokaryote *Streptomyces hydrogenans* in which it reduces the 20-carbonyl group of C_{21}-steroids.[1] We previously reported that a high level of 20β-HSD activity, which catalyzes the conversion of 17α-hydroxyprogesterone to 17α,20β-dihydroxy-4-pregnen-3-one with NADPH, was present in pig testes during the neonatal stage,[2,3] and subsequently purified and characterized the enzyme.[4–7] The properties of the testicular 20β-HSD were different from those of prokaryotic 20β-HSD. The cDNA encoding testicular 20β-HSD was also isolated, sequenced and expressed.[8,9] Interestingly, pig testicular 20β-HSD was found to have high amino acid homology to human[10,11] and rat[12] carbonyl reductase except that the pig enzyme had an extra 13 amino acid residues at the C-terminus, and exhibited carbonyl reductase-like activity. Here, we report in detail that pig testicular 20β-HSD expressed in *Escherichia coli* exhibits carbonyl reductase activity, that is, it utilizes carbonyl compounds, such as quinones, cycloketone, aromatic aldehydes, aromatic ketones, cycloketones, aliphatic aldehydes and aliphatic ketones as substrates. Kinetic parameters for the enzyme using these substrates were also determined.

MATERIALS AND METHODS

Chemicals 1-Decanal, 4-nitrobenzaldehyde, 4-benzoylpyridine and 9,10-phenanthrenequinone were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). 2-Decanone, 2-methylglyoxal, 4-ethylbenzaldehyde, butyrophenone, 2-methylcyclopentanone and 2-decanone were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Other carbonyl compounds (of the highest grade commercially available) were obtained from Wako Chemicals (Tokyo, Japan). NADPH and DE-52 were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan) and Whatman International Ltd. (Maidstone, UK), respectively. Other materials and chemicals were obtained from sources previously reported[8,9]

Preparation of Recombinant Pig Testicular 20β-HSD Expression and purification of the recombinant 20β-HSD was performed according to the method of Nakajin *et al.*[9] The purified enzyme was concentrated with an ultrafiltration apparatus using a PM-10 Diaflo-membrane (Amicon, Inc. Beverly, MA) and stored at −20 °C until used.

Enzyme Assay Carbonyl reductase activity was determined spectrophotometrically by measuring the oxidation rate of NADPH with an enzyme rate calculator apparatus (Hitachi 200-0045) attached to a spectrophotometer (Hitachi 200-20) at 37 °C. The standard assay mixture consisted of 60 mM potassium phosphate buffer (pH 6.5), 80 mM NADPH, substrate at various concentrations (0.3 μM—10 mM) and enzyme in a total volume of 1.0 ml. The reaction was initiated by addition of NADPH to the assay mixture. Blanks without substrate or enzyme were routinely included. Water-insoluble substrates were dissolved in ethanol or dimethyl sulfoxide. The final concentration of the solvent in the assay mixture did not exceed 1%. The kinetic parameters were calculated from Woolf plots ([S]/v against [S] plots) using a personal computer, and the correlation coefficients (r) obtained from each plot were 0.904—0.999.

Miscellaneous Protein concentration was estimated by the method of Lowry *et al.*[13] with crystalline bovine serum albumin (Fraction V, Armour Pharmaceutical Co.). Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) was performed according to the method of Laemmli.[14] HPLC using a Waters 650 advanced purification system was performed by the method described previously.[7]

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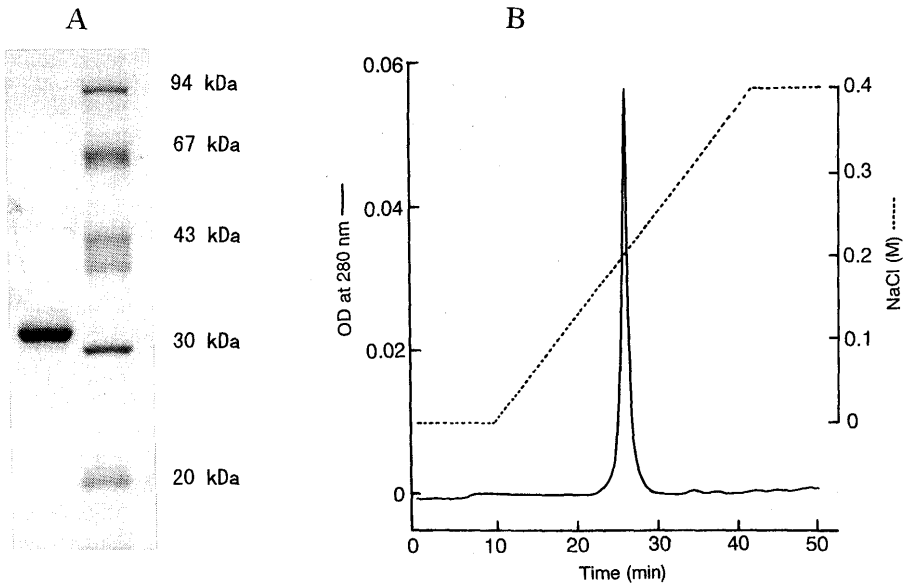

Fig. 1. SDS-Polyacrylamide Gel Electrophoresis and HPLC of Recombinant Pig Testicular 20β-HSD

Panel A: Electrophoresis was carried out with a slab of 10% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Panel B: HPLC was performed on a Protein Pack G-DEAE column (1 × 5 cm, Waters) with a concentration gradient from 50 mM potassium phosphate (pH 7.4)–0.1 mM EDTA to 0.5 M NaCl–50 mM potassium phosphate (pH 7.4)–0.1 mM EDTA.

RESULTS

The purity of the recombinant pig testicular 20β-HSD was demonstrated by SDS-PAGE and HPLC (Figs. 1A and B, respectively). The purified enzyme was a single component under both denaturing and native conditions.

Aromatic aldehydes, aromatic ketones, cycloketones, quinones, aliphatic aldehydes and aliphatic ketones, some of which were known to be typical substrates of carbonyl reductase, were used as the substrate of pig testicular 20β-HSD and the kinetic constants of the enzyme using these substrates are summarized in Table 1. 20β-HSD possessed a high affinity towards quinones, such as 9,10-phenanthrenequinone (**1**), (numbers refer to compound in Table 1) α-naphthoquinone (**2**), and menadione (**3**), which were reduced with low K_m values (4, 2 and 5 μM, respectively). The substrate utilization efficiency (V_{max}/K_m) of the enzyme against these quinones was also very high. The V_{max}/K_m values were much higher for cyclohexanone (**6**) and 2-methylcyclohexanone (**7**) than for cyclopentanone (**4**) and 2-methylcyclopentanone (**5**). That is, the enzyme was more efficient in catalyzing the reduction of the cyclohexanones than in catalyzing the reduction of the cyclopentanones, and was more efficient in catalyzing the reduction of the 2-methyl derivatives than these cycloketones. Various aromatic aldehydes and ketones including benzaldehyde- and acetophenone-derivatives were also reduced by 20β-HSD. V_{max}/K_m values of the enzyme were especially high for 4-nitrobenzaldehyde (**17**) and 4-nitroacetophenone (**19**) in the related compounds while they were relatively low for 4-methylacetophenone (**21**) propiophenone (**22**), butyrophenone (**23**) and benzophenone (**24**). Furthermore, the enzyme also reduced pyridine-derivatives, 2-, 3-, and 4-benzoylpyridine (**25**, **26** and **27**, respectively), with the V_{max}/K_m value for compound **25** being the highest. Although 20β-HSD reduced both aliphatic aldehydes and aliphatic ketones, the enzyme showed high V_{max}/K_m values against the aliphatic aldehydes. The V_{max}/K_m value of the enzyme for aliphatic aldehydes tended to increase with increasing number of carbon atoms in a straight-chain of alkyl groups reaching a maximum against 1-nonanal (**45**), and then decreased with longer chain lengths.

DISCUSSION

The preceding results show that carbonyl compounds such as quinones, followed by aromatic aldehydes and aromatic ketones, which were previously shown to be effective substrates of carbonyl reductase from human brain,[15] placenta,[16] liver[17] and testis[18] as well as rat ovary,[19] testis[20] and adrenals[21] are also substrates of the pig testicular enzyme, 20β-HSD. Pig testicular 20β-HSD was first found to be a hydroxysteroid dehydrogenase (oxidoreductase) which catalyzes the reduction of the 20-carbonyl group of C_{21}-steroids. Subsequently, we reported the sequence of this enzyme and showed that its primary structure has 85% amino acid homology to human carbonyl reductase.[8] Wermuth *et al.*[12] also reported that the primary structure of rat carbonyl reductase has 80% amino acid homology to pig testicular 20β-HSD. These preceding investigations indicated that 20β-HSD from neonatal pig testis is one of the isoforms of pig-specific carbonyl reductase.

The substitution of a functional group for the 4-hydrogen of benzylaldehyde remarkably influenced the V_{max}/K_m values: the V_{max}/K_m value increased in the order of dimethylamino (no activity), hydrogen, methyl, ethyl, chloro, methoxy and nitro group. This result shows that the V_{max}/K_m value increased in the order of intensity of the electro-drawing ability of the group, rather than in the order of the size of the functional group. The V_{max}/K_m values for these substrates were well correlated with Hammett's values (σ_p)[22] ($r=0.7870$) and Taft-Charton's

Table 1. Substrate Specificity of Testicular 20β-HSD for Various Carbonyl Compounds

No.	Compounds	Kinetic parameter		
		K_m (mM)	V_{max} (nmol/min/mg)	V_{max}/K_m
Cycloketones and quinones				
1	9,10-Phenanthrenequinone	0.004	242	60559
2	α-Naphthoquinone	0.002	73.0	36500
3	Menadione	0.005	85.6	17120
4	Cyclopentanone	4.34	101	23.3
5	2-Methylcyclopentanone	0.280	135	484
6	Cyclohexanone	0.088	162	1844
7	2-Methylcyclohexanone	0.040	246	6474
8	1-Decalone	0.106	156	1480
Aromatic aldehydes				
9	Benzaldehyde	1.05	113	108
10	4-Methylbenzaldehyde	0.584	110	188
11	4-Ethylbenzaldehyde	0.076	71.0	934
12	4-Methoxybenzaldehyde	0.04	75.7	1884
13	4-Diethylaminobenzaldehyde	n.d.	(0)	n.d.
14	4-Chlorobenzaldehyde	0.032	41.6	1301
15	2-Nitrobenzaldehyde	0.091	150	1649
16	3-Nitrobenzaldehyde	0.211	140	665
17	4-Nitrobenzaldehyde	0.036	150	4169
Aromatic ketones				
18	Acetophenone	0.37	49.5	134
19	4-Nitroacetophenone	0.015	76.5	5100
20	4-Chloroacetophenone	0.294	39.2	135
21	4-Methylacetophenone	1.37	57.9	42.3
22	Propiophenone	0.228	14.8	64.3
23	Butyrophenone	1.00	28.5	28.5
24	Benzophenone	n.d.	(12)	n.d.
25	2-Benzoylpyridine	0.133	164	1233
26	3-Benzoylpyridine	0.251	14.8	59.0
27	4-Benzoylpyridine	0.452	95.5	212
Aliphatic ketones				
28	Propanone	n.d.	n.d.	n.d.
29	2-Butanone	3.48	48.5	13.9
30	3-Pentanone	0.726	29.7	40.8
31	2-Hexanone	2.54	69.5	27.4
32	2-Heptanone	0.931	55.9	60.1
33	2-Octanone	0.159	41.0	258
34	2-Nonanone	0.064	46.0	719
35	2-Decanone	0.100	40.2	403
36	2-Methylglyoxal	0.470	152	324
37	2,3-Butanedione	0.070	214	3051
Aliphatic aldehydes				
38	Ethanal	4.52	21.9	4.85
39	1-Propanal	1.91	60.9	31.9
40	1-Butanal	0.928	107	116
41	1-Pentanal	0.761	168	220
42	1-Hexanal	0.373	186	499
43	1-Heptanal	0.056	162	2890
44	1-Octanal	0.036	157	4361
45	1-Nonanal	0.019	159	8368
46	1-Decanal	0.010	71.9	7190
47	1-Undecanal	0.028	127	4521
48	1-Dodecanal	0.020	101	5036

Values in parentheses indicate the activity with 625 μM substrate. n.d.: not determined.

values (σ_I)[23] ($r = 0.8522$). The three-dimensional structure of 20β-HSD from *S. hydrogenans* was recently established, and a possible mechanism for stereospecific hydride ion transfer and proton relay during the 20-carbonyl to 20β-hydroxyl conversion was proposed.[24] As a general rule, a carbonyl group on the substrate must attacked with the hydride ion from NAD(P)H in the reduction catalyzed by oxidoreductase. In this experiment, compound **17**, which has a nitro group as a powerful electro-drawing group, showed the highest V_{max}/K_m values in the aromatic aldehydes, whereas 4-dimethylaminobenzaldehyde (**13**), which has a strong electro-donating group, is not enzymatically catalyzed. These results show that the carbon atom on the aldehyde group was more positively charged by the powerful electro-drawing nitro group and the aldehyde group was effectively attacked with the hydride ion, thus being effectively reduced by the enzyme; this result seems reasonable. Like aromatic aldehydes, an aromatic ketone such as acetophenone was also reduced by 20β-HSD, and substitution of a nitro group for the 4-hydrogen resulted in a high V_{max}/K_m value. This result also might have been expected and seems reasonable. The substitution of a propyl, butyl or benzyl group for the aromatic aldehyde hydrogen, however, remarkably decreased the V_{max}/K_m value, while the substitution of a pyridyl group increased this value. Compound **25** was shown to have the highest value because the 2-pyridyl group is an electro-drawing group. We have found that aliphatic aldehydes with straight-chain alkyl groups also serve as an efficient substrate of 20β-HSD. The V_{max}/K_m values for aliphatic aldehydes increased with increasing number of carbon atoms in a straight chain of the alkyl group reaching a maximum value against compound **45**, and then decreased with higher chain lengths. From these results it can be postulated that a hydrophobic pocket is located in the substrate-binding domain of the enzyme, and that this pocket best fits a chain nine carbon atoms in length. Pig testicular 20β-HSD is known to catalyze the reduction of not only the 20 carbonyl group of C_{21}-steroids, but also the 3-carbonyl group of C_{19}-steroids at low K_m values.[7] It is very interesting that the length of the carbon chain of these steroids approximately corresponds to that of compound **45**.

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REFERENCES

1) Hübener H. J., Lehmann C. O., *Hoppe-Seyler's Z. Physiol. Chem.*, **313**, 124—129 (1958).
2) Nakajin S., Ohno S., Takahashi M., Nishimura K., Shinoda M., *Chem. Pharm. Bull.*, **35**, 2490—2494 (1987).
3) Ohno S., Nakajin S., Shinoda M., *J. Steroid Biochem. Molec Biol.*, **42**, 17—21 (1992).
4) Nakajin S., Ohno S., Shinoda M., *J. Biochem. (Tokyo)*, **104**, 565—569 (1988).
5) Nakajin S., Ohno S., Aoki M., Shinoda M., *Chem. Pharm. Bull.*, **39**, 972—975 (1991).
6) Nakajin S., Ohno S., Aoki M., Shinoda M., *Chem. Pharm. Bull.*, **37**, 148—150 (1989).
7) Ohno S., Nakajin S., Shinoda M., *J. Steroid Biochem. Molec. Biol.*, **38**, 787—794 (1991).
8) Tanaka M., Ohno S., Adachi S., Nakajin S., Shinoda M., Nagahama Y., *J. Biol. Chem.*, **267**, 13451—13455 (1992).
9) Nakajin S., Nakajima T., Uchida M., Ohno S., Shinoda M., *J. Steroid Biochem. Molec. Biol.*, **54**, 257—264 (1995).
10) Wermuth B., Bohren K.M., Heinemann G., von Wartburg J. P., Gabbay K. H., *J. Biol. Chem.*, **263**, 16185—16188 (1988).
11) Forrest G. L., Akman S., Krutzik S., Paxton P. J., Aparkes R. S., Doroshow J., *Biochim. Biophys. Acta*, **1048**, 149—155 (1990).

12) Wermuth B., Heinemann G., Ernst E., *Eur. J. Biochem.*, **228**, 473—479 (1995).
13) Lowry O.H., and Rosenbrough N. J., Far A. L., Randal R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
14) Laemmli U. K., *Nature* (London), **27**, 680—685 (1970).
15) Wermuth B., *J. Biol. Chem.*, **256**, 1206—1213 (1981)
16) Jarabak J., Luncsford A., Berkowitz D., *Prostaglandins*, **26**, 849—868 (1983).
17) Nakayama T., Hara A., Yashiro K., Sawada H., *Biochem Pharmacol.*, **34**, 107—117 (1985).
18) Inazu N., Ruepp B., Wirth H., Wermuth B., *Biochim. Biophys. Acta*, **1116**, 50—56 (1992).
19) Iwata N., Inazu N., Satoh T., *J. Biochem.* (Tokyo), **105**, 556—564 (1989).
20) Iwata N., Inazu N., Takeo S., Satoh T., *Eur. J. Biochem.*, **193**, 75—91 (1990).
21) Inazu N., Nagashima Y., Satoh T., Fujii T., *J. Biochem.*, **115**, 991—999 (1994).
22) McDaniel D. H., Brown H. C., *J. Org. Chem.*, **23**, 420—427 (1958).
23) Charton, M., Progr. *Phys. Org. Chem.*, **13**, 119—251 (1981).
24) Ghosh D., Wawrzak Z., Weeks C. M., Duax W. L., Erman M., *Structure*, **15**, 629—640 (1994).