20β-Hydroxysteroid Dehydrogenase of Neonatal Pig Testis: Cofactor Requirement and Stereospecificity of Hydrogen Transfer from Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form

Shizuo NAKAJIN,*,[a] Shuji OHNO,[a] Masatada AOKI,[b] and Masato SHINODA[a]

Faculty of Pharmaceutical Sciences, Hoshi University,[a] 2-4-41, Ebara, Shinagawa-ku, Tokyo 142, Japan and College of Pharmacy, Nihon University,[b] 7-7-1, Narashinodai, Funabashi-shi, Chiba 274, Japan. *Received June 27, 1988*

The cofactor requirement of purified 20β-hydroxysteroid dehydrogenase from cytosol fraction of neonatal pig testis, in the reduction of 17α-hydroxyprogesterone was investigated. The enzyme required β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH) as the preferred cofactor, with an apparent K_m value of 17 μM. Furthermore, α-nicotinamide adenine dinucleotide phosphate, reduced form (α-NADPH), β-3′-NADPH and β-nicotinamide adenine dinucleotide (β-NADH) were also utilized as hydrogen donors in the reduction at relatively high concentration with apparent K_m values of 85.2 μM, 179.2 μM and 1.00 mM, respectively. The optimum pH was 5.5 when β-NADPH was used as the cofactor, while it was 6.0 when β-NADH was used. The hydrogen transfer from the β-NADPH to the product, 17α,20β-dihydroxypregn-4-en-3-one catalyzed by 20β-hydroxysteroid dehydrogenase was stereospecific, and the 4-*pro*-S-hydrogen of the nicotinamide moiety was transferred to the product.

Keywords 20β-hydroxysteroid dehydrogenase; neonatal pig; testis; cofactor requirement; pyridine nucleotide; hydrogen transfer; optimum pH

It is well known that 20α-hydroxysteroid dehydrogenase (20α-HSD; EC 1.1.1.149), which catalyzes the stereospecific and reversible interconversions of hydroxyl and carbonyl groups of C_{21}-steroid, is present in pig testis and other organs.[1] In contrast, little is known about 20β-hydroxysteroid dehydrogenase (20β-HSD; EC 1.1.1.53) of pig testis, though the enzyme from the mycelium of *Streptomyces hydrogenans* has been studied.[2]

We have recently reported that a large quantity of 20β-HSD is localized in the cytosol fraction of the testes from neonatal pig as compared with that from mature pig.[3] Furthermore, purification of 20β-HSD from neonatal pig testes was accomplished, and some of its properties were characterized.[4] We are interested in the synthesis and regulation of hormonal steroids in the testis, but at the present stage, the physiological significance of 20β-HSD in pig testis is not clear.

In the present study, as a part of our research on 20β-HSD, the cofactor requirement and stereospecificity of hydrogen transfer from nicotinamide adenine dinucleotide phosphate, reduced form, were investigated.

Experimental

Chemicals β-Nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), α-nicotinamide adenine dinucleotide phosphate, reduced form (α-NADPH), β-nicotinamide adenine dinucleotide 3′-phosphate, reduced form (β-3′-NADPH), β-nicotinamide adenine dinucleotide, reduced form (β-NADH) and α-nicotinamide adenine dinucleotide, reduced form (α-NADH), all as the sodium salts, were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Adenosine 5′-triphosphate (ATP), nicotinamide adenine dinucleotide (NAD^+)-kinase, isocitric acid and isocitric dehydrogenase were also purchased from Sigma Chemical Co. Hydroxyapatite (Bio Gel HTP) was obtained from Bio-Rad Laboratories (Richmond, CA., U.S.A.).

Radioactive [4-^{3}H]NAD^+ (2.0 Ci/mmol, TRA-298 batch 24) was purchased from Amersham International plc (Buckinghamshire, England). The sources of other chemicals have been described in a previous paper.[3]

Purification of 20β-HSD from Testes The method for preparing purified 20β-HSD from neonatal pig testes was described in a previous paper.[4] A slight modification was employed to prevent the decrease of enzyme activity at the final step of the purification procedures. The 20β-HSD fraction from rechromatography on a diethylaminoethyl (DEAE)-cellulose column was dialyzed against 1 mM potassium phosphate buffer (KPB), pH 7.4–0.1 mM ethylenediaminetetra acetic acid (EDTA) and then applied to a hydroxyapatite column which had been equilibrated with the same buffer, instead of density-gradient isoelectric focusing. Elution of 20β-HSD at breakthrough yielded a single protein band which was homogeneous on polyacrylamide gel electrophoresis and isoelectric focusing as described previously.[4] The specific activity of the final preparation used was 5.1 nmol/min/mg protein.

Assay of the 20β-HSD Activity The radioactive steroid, 17α-hydroxy-[4-^{14}C]progesterone (0.01 μCi/20 nmol/10 μl ethanol), was incubated with the purified 20β-HSD in the presence of β-NADPH or other pyridine nucleotide cofactors in a total volume of 1.0 ml of KPB (pH 7.4) for 20 min at 37 °C. The enzyme reaction was initiated by addition of 100 μl of cofactor solution to the assay mixture. All incubations were carried out in duplicate. After the incubation, 10 ml of CH_2Cl_2 was added to the incubation medium and the mixture was shaken vigorously. After the addition of carrier steroids (17α-hydroxyprogesterone and 17α,20β-dihydroxypregn-4-en-3-one, 5 μg each), the mixture was again shaken vigorously. After the removal of the aqueous phase, the organic extract was dried with anhydrous sodium sulfate and evaporated under a nitrogen stream at 40°C. The residue obtained was applied to a thin-layer chromatography (TLC) plate (2 × 8 cm, Kodak; 13181 Silica gel with fluorescent indicator), which was developed with a benzene–acetone mixture (8 : 2, v/v). Under these conditions, the *Rf* values of 17α-hydroxyprogesterone and 17α, 20β-dihydroxypregn-4-en-3-one were 0.72 and 0.39, respectively. After the development, spots of the carrier steroids on the chromatogram were enhanced under an ultraviolet light at 254 nm. The relevant spots and radioactive areas of chromatogram were cut out and placed in vials. The ^{14}C radioactivity of each spot was counted directly in 10 ml of toluene containing 2,5-diphenyloxazole (0.4%, w/v) and 2,2′-*p*-phenylene-bis-(5-phenyloxazole) (0.01%, w/v) with a liquid scintillation counter (Packard Tri-Carb 460). In the case of the counting of ^{3}H radioactivity, methanol (1.0 ml) was added to the portion of TLC plate in the vial to release the steroid and then the toluene-based scintillation fluid was added. The 20β-HSD activity was determined from the radioactivity of the area corresponding to 17α, 20β-dihydroxypregn-4-en-3-one on the TLC plate. The enzyme activity was corrected for recovery, which was calculated from the ratio of total radio activity of the areas of the substrate and product on the TLC plate to that of the radioactive steroid added.

Preparation of [4-*pro*-*R*-^{3}H]NADPH and [4-*pro*-*S*-^{3}H]NADPH [4-^{3}H]-NAD^+ (17.2 μCi/8.6 nmol) was phosphorylated by incubation with NAD^+-kinase (10 unit) in the presence of ATP (10 μmol) in 1.0 ml of 50 mM KPB–10 mM $MgCl_2$ for 30 min at 37 °C and the mixture was divided into two equal parts.

For the preparation of [4-*pro*-*R*-^{3}H]NADPH, glucose-6-phosphate (5 μmol) and glucose-6-phosphate dehydrogenase (1 unit) were added to one part of the above mixture. [4-^{3}H]$NADP^+$ was then reduced to [4-*pro*-*R*-^{3}H]NADPH by incubation for 30 min at 37 °C. By this procedure, a hydrogen of the substrate is transferred to the 4-*pro*-*S* position of the nicotinamide moiety.[5]

The other part of the mixture was used for preparation of [4-*pro*-*S*-

^{3}H]NADPH. It was prepared by the reduction with isocitric acid (5 μmol) and isocitric dehydrogenase (1 unit) by incubation for 30 min at 37 °C, which catalyzed the transfer of hydrogen from the substrate to the 4-*pro-R* position.[6] [4-*pro-R*-^{3}H]NADPH and [4-*pro-S*-^{3}H]NADPH supported with NADPH-generating systems, were diluted appropriately with non-radioactive NADPH. Aliquots were used without delay as the cofactor to determine the stereospecificity of hydrogen transfer.

Results

Cofactor Requirement Several pyridine nucleotide cofactors (reduced forms) were examined as the hydrogen donor for the reduction of 17α-hydroxyprogesterone catalyzed by purified 20β-HSD from testes. As shown in Fig. 1, β-NADPH was utilized for the reduction of 17α-hydroxyprogesterone to 17α,20β-dihydroxypregn-4-en-3-one at the lowest concentration. Although β-NADH, α-NADPH and β-3′-NADPH were also utilized for the reduction of 17α-hydroxyprogesterone with 20β-HSD at relatively high concentrations (1.9—3.8 mM), α-NADH was not entirely utilized at the concentration of 1.9 mM. On the other hand, the V_{max} value for β-NADPH is not as large as that for β-NADH, but the parameter V_{max}/K_m was about 30 times larger than that for β-NADH (Table I). These results show that β-NADPH was the most effective cofactor in the reduction by testicular 20β-HSD.

pH Optimum The effect of pH on the enzyme activity was examined with β-NADPH and β-NADH as the cofactor. The rate of reduction of 17α-hydroxyprogesterone at pH between 5.5 and 8.5 was determined as shown in Fig. 2. When β-NADPH was used, the maximum rate was observed at pH 5.5. The maximum rate with β-NADH was observed at pH 6.0. A clear difference in optimum pH was observed.

Stereospecificity of Hydrogen Transfer from NADPH The results of the incubation of 17α-hydroxy[4-^{14}C] progesterone with [4-*pro-R*-^{3}H]NADPH or with [4-*pro-S*-^{3}H]NADPH in the reduction catalyzed by testicular 20β-HSD are shown in Table II. A significant incorporation of tritium into the product, 17α, 20β-dihydroxypregn-4-en-3-one, was observed when 17α-hydroxyprogesterone was incubated with [4-*pro-S*-^{3}H]NADPH, as compared to the incubation with [4-*pro-R*-^{3}H]NADPH. Incorporation of ^{3}H into 17α,20β-dihydroxypregn-4-en-3-one was about 43 times larger than that in the case of [4-*pro-R*-^{3}H]NADPH as expressed in terms of the ratio of ^{3}H to ^{14}C.

These results demonstrated that the hydrogen transfer from β-NADPH to 17α,20β-dihydroxypregn-4-en-3-one catalyzed by testicular 20β-HSD is stereospecific and the 4-*pro-S* hydrogen is transferred to the product.

Discussion

The present study showed that the pig testicular 20β-

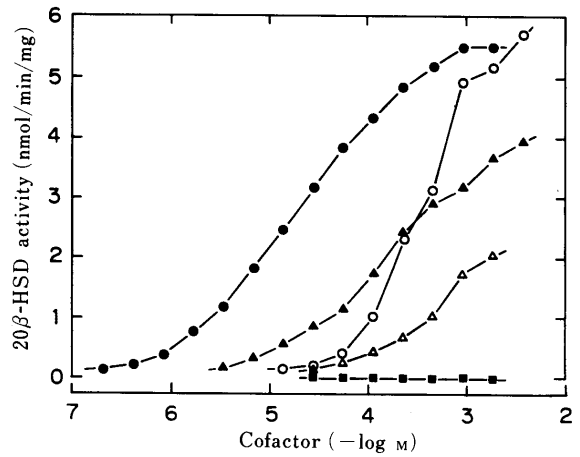

Fig. 1. Cofactor Requirement of Testicular 20β-HSD

17α-Hydroxy[4-^{14}C]progesterone (20 nmol/10 μl of ethanol) was incubated with the purified 20β-HSD (44 μg) in the presence of various concentrations of cofactor in 1.0 ml of 50 mM KPB (pH 7.4) for 30 min at 37 °C. Cofactors used were β-NADPH (●), β-NADH (○), α-NADPH (▲), β-3′-NADPH (△) and α-NADH (■).

TABLE I. Kinetic Parameters of Testicular 20β-HSD with Various Cofactors

Cofactor	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m ($\times 10^{-3}$)
β-NADPH	17.0	5.0	290
α-NADPH	85.2	3.0	35
β-NADH	1003.0	9.8	9.8
β-3′-NADPH	179.2	1.2	6.7

17α-Hydroxy[4-^{14}C]progesterone (20 nmol/10 μl of ethanol) was incubated with the purified 20β-HSD (44 μg) in the presence of several kinds of cofactor in 1.0 ml of 50 mM KPB (pH 7.4) for 30 min at 37 °C. Correlation coefficients for Lineweaver–Burk plots were $r=0.9974$ (β-NADPH), $r=0.9960$ (α-NADPH), $r=0.9922$ (β-NADH) and $r=0.9972$ (β-3′-NADPH).

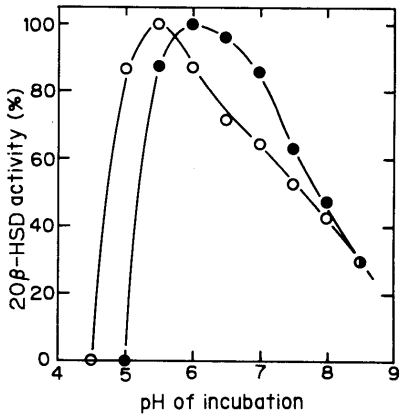

Fig. 2. Effect of pH on Enzyme Activity of 20β-HSD

17α-Hydroxy[4-^{14}C]progesterone (20 nmol/10 μl of ethanol) was incubated with the purified 20β-HSD (44 μg) in the presence of β-NADPH (○) and β-NADH (●). The incubation was carried out in 1.0 ml of 50 mM KPB at various pH for 30 min at 37 °C. The incubation buffers used were 50 mM acetate buffer (pH 4.0—5.5) and 50 mM KPB (pH 5.5—8.5). Enzyme activity was expressed as a percentage of the maximum activity.

TABLE II. Stereospecificity of Hydrogen Transfer from NADPH to 17α,20β-Dihydroxypregn-4-en-3-one

Cofactor	Exp. No.	20β-HSD activity (nmol/min/mg)	^{3}H/nmol[a] (dpm)	^{3}H/^{14}C
[4-*pro-R*-^{3}H] NADPH	1	3.56	135.2	3.1
	2	3.93	122.4	2.5
	3	3.95	112.6	2.8
	Mean	3.81	123.4	2.8
	±S.E.	±0.13	±6.5	±0.2
[4-*pro-S*-^{3}H] NADPH	1	2.64	1816.4	106.2
	2	2.22	2497.4	133.1
	3	2.39	2491.2	122.8
	Mean	2.42	2268.3	120.7
	±S.E.	±0.12	±226.0	±7.8

a) dpm of tritium incorporated per nmol of product.

HSD prefers β-NADPH to β-NADH or other pyridine nucleotides in the reduction of 20-ketopregnane steroids to the 20β-hydroxy derivatives. 20β-HSD of *Streptomyces hydrogenans* was reported to require specifically β-NADH, and β-NADPH was much less effective than β-NADH.[7] 20α-HSD activity has been reported to be present in various organs (liver, ovary, testis, adrenal, placenta) of several mammalian species.[1] The testicular 20α-HSD in rat,[8] pig[9] and bull[10] utilizes β-NADPH as the preferred cofactor. The cofactor preference of the testicular 20β-HSD is similar to that of pig testicular 20α-HSD.

The enzyme showed a pH optimum at 5.5 or 6.0 when β-NADPH or β-NADH was used, respectively. It is reasonable that the enzyme activity increased gradually from pH 8.5 to pH 5.5 or 6.0 (Fig. 2), because the reduction rate of 17α-hydroxyprogesterone is dependent on the concentration of H^+ in the medium: the reaction is 17α-hydroxyprogesterone + NAD(P)H + H^+ → 17α,20β-dihydroxypregn-4-en-3-one + $NAD(P)^+$.

In the present experiment, the pig testicular 20β-HSD was found to transfer the hydrogen at the 4-*pro*-*S*-position in the nicotinamide moiety of the β-NADPH to the product, 17α,20β-dihydroxypregn-4-en-3-one. These results conform with the stereospecificity of hydrogen transfer from β-NADH catalyzed by 20β-HSD of *Streptomyces hydrogenans*.[11] The reaction catalyzed by NAD(P)H-dependent hydroxysteroid dehydrogenase has been shown to be stereospecific for the transfer of one of the two hydrogens at C-4 in the nicotinamide moiety. As enzymes which utilize the hydrogen at the 4-*pro*-*S*-position of NADPH for reduction, 17β-HSD of human placenta[12] and pig testis,[13] 3α-HSD of *Pseudomonas testosteroni*,[12] rooster comb[14] and rat seminal vesicle,[15] and 3β-HSD of *Pseudomonas testosteroni*[16] are already known. On the other hand, 20α-HSD of rat ovary[17] and pig testis[18] and 3α-HSD of rat liver[19] were reported to transfer the hydrogen at the 4-*pro*-*R* position to the steroid for the reduction.

Concerning the stereospecificity of hydrogen transfer, Akhtar *et al.*[20] proposed that the hydrogen at the 4-*pro*-*S* position of the cofactor was transferred to the α-face of the steroid molecule, whereas the 4-*pro*-*R* hydrogen of the cofactor was transferred to the β-face. Our experimental results confirm their hypothesis.

Acknowledgement This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

1) D. B. Gower, "Biochemistry of Steroid Hormones," ed. by H. L. J. Markin, Blackwell Scientific Publications, Oxford, 1984, pp. 230—292.
2) H. J. Hübener, F. G. Sahrholz, J. Schmidt-Thomé, G. Nesemann, and R. Junk, *Biochim. Biophys. Acta*, **35**, 272 (1959).
3) S. Nakajin, S. Ohno, M. Takahashi, K. Nishimura, and M. Shinoda, *Chem. Pharm. Bull.*, **35**, 2490 (1987).
4) S. Nakajin, S. Ohno, and M. Shinoda, *J. Biochem.* (Tokyo), **104**, 565 (1988).
5) B. K. Stern and B. Vennesland, *J. Biol. Chem.*, **235**, 205 (1960).
6) T. Nakamoto and B. Vennesland, *J. Biol. Chem.*, **235**, 202 (1960).
7) H. J. Hübener and C. O. Lehmann, *Z. Physiol. Chem.*, **313**, 124 (1958).
8) M. Shikita, H. Inano, and B. Tamaoki, *Biochemistry*, **6**, 1760 (1967).
9) F. Sato, Y. Takagi, and M. Shikita, *J. Biol. Chem.*, **247**, 815 (1972).
10) J. A. Pineda, M. E. Salinas, and J. C. Warren, *J. Steroid Biochem.*, **23**, 1001 (1985).
11) G. Betz and J. C. Warren, *Arch. Biochem. Biophys.*, **128**, 745 (1968).
12) J. Jarabak and P. Talalay, *J. Biol. Chem.*, **235**, 2147 (1960).
13) H. Inano and B. Tamaoki, *Eur. J. Biochem.*, **53**, 319 (1975).
14) M. Mori, K. Suzuki, and B. Tamaoki, *Biochim. Biophys. Acta*, **337**, 118 (1974).
15) K. Suzuki and B. Tamaoki, *J. Steroid Biochem.*, **5**, 249 (1974).
16) P. Talalay, F. A. Loewus, and B. Vennesland, *J. Biol. Chem.*, **212**, 801 (1955).
17) W. H. Kersey and R. B. Wilcox, *Biochemistry*, **9**, 1284 (1970).
18) F. Hatano-Sato, Y. Takagi, and M. Shikita, *J. Biochem.* (Tokyo), **74**, 1065 (1973).
19) I. Bjorkhem and H. Danielson, *Eur. J. Biochem.*, **12**, 80 (1970).
20) M. Akhtar, D. C. Wilton, I. A. Walkinson, and A. D. Rahintula, *Proc. R. Soc. London, Ser. B*, **180**, 167 (1972).