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KINETIC MECHANISM OF PORCINE TESTICULAR 17β-HYDROXYSTEROID DEHYDROGENASE

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SUMMARY

A study on product inhibition of 17β-hydroxysteroid dehydrogenase from porcine testes was carried out by measuring the initial velocities of NADPH formation using testosterone as the substrate steroid. Type of inhibition by NADPH against $NADP^+$ was competitive in both saturated and unsaturated concentrations of testosterone. In the saturated concentration of $NADP^+$, activity of the enzyme was not inhibited by NADPH against testosterone. In the unsaturated concentrations of $NADP^+$, however, NADPH brought mixed type inhibition against testosterone. The similar modes of inhibition by the product steroid, androstenedione were observed in the saturated and unsaturated concentrations of $NADP^+$ and testosterone.

The fluorescence of NADPH was increased in the presence of the enzyme, and fluorometric titration indicated that 1 mol of NADPH was bound to 1 mol of the 17β-hydroxysteroid dehydrogenase. Addition of testosterone to enzyme–NADPH complex reduced the intensity of fluorescence of NADPH, suggesting formation of testosterone–enzyme–NADPH complex as a ternary dead end complex.

From the analyses of product inhibition and spectral changes of NADPH, the kinetic mechanism of the enzyme was revealed as rapid equilibrium random system with two dead end complexes which consisted of the two reduced reactants bound to the enzyme and the two oxidized ones bound to it.

INTRODUCTION

Testicular 17β-hydroxysteroid dehydrogenase catalyzes testosterone synthesis from androstenedione in the following reaction:

Androstenedione + NADPH

$$\rightleftharpoons \text{Testosterone} + NADP^+$$

The dehydrogenase was purified from the microsomal fraction of porcine testes in this laboratory [1]. Molecular weight of the purified enzyme was estimated as about 35,000 daltons [1, 2], and active form of the dehydrogenase was demonstrated as a monomer by the sucrose gradient centrifugation of the enzyme in the presence of NADPH and androstenedione at an appropriate working temperature [3]. Michaelis constants of the dehydrogenase for NAD^+ and NADH were about 200 times larger than that for $NADP^+$ and NADPH [4]. Maximum velocities for the oxidative and the reductive reactions of the testicular 17β-hydroxysteroid dehydrogenase were almost the same for various concentrations of $NADP^+$ and NADPH [4].

Recently, we demonstrated that porcine testicular 17β-hydroxysteroid dehydrogenase was separable into at least four charged isomeric forms with the same molecular weight by using isoelectric focusing polyacrylamide gel electrophoresis [5]. The differences in isoelectric points of the enzyme were not caused by androstenedione, testosterone, or $NADP^+$ added to the enzyme, but by their amino acid compositions.

In rat testes, the presence of two distinct 17-ketosteroid reductases was reported, one in the interstitial tissue and the other in the seminiferous tubules [6]. Furthermore, a 17-ketosteroid reductase, which contain no activity of 17β-hydroxysteroid dehydrogenase was prepared from rat testicular microsomes [7].

In order to study the regulation of testosterone synthesis by some isomeric forms of the testicular 17β-hydroxysteroid dehydrogenase (and 17-ketosteroid reductases), it would be necessary to determine their localization within the tissue, to purify each of the isomers homogeneously, and to investigate their differences in molecular and kinetic properties.

In this report, we describe the detailed mechanism of conversion between androstenedione and testosterone by the purified testicular 17β-hydroxysteroid dehydrogenase (pI = 5.0) in the saturated and unsaturated concentrations of the substrates. Furthermore, binding of testosterone and NADPH to the dehydrogenase was analyzed by measuring the spectral changes in the fluorescence of NADPH.

MATERIALS AND METHODS

Materials

Androstenedione and testosterone were purchased from Steraloids (Wilton, NH), and NADPH and $NADP^+$ were obtained from Boeringer Mannheim GmbH (Germany). 17β-Hydroxysteroid dehydro-

This is paper 7 of the series "Porcine Testicular 17β-Hydroxysteroid Dehydrogenase". Paper 6 is Ref. [5].

Enzyme: 17β-Hydroxysteroid dehydrogenase or 17β-hydroxysteroid: $NADP^+$ 17β-oxidoreductase (EC 1.1.1.64).

genase was purified from porcine testes as described previously [5], except that hydroxyapatite column chromatography was carried out using 4 mM phosphate buffer instead of 5 mM phosphate buffer, and isoelectric focusing column electrophoresis was carried out without previous dialysis of the enzyme. The purity of the enzyme preparation was examined by a polyacrylamide gel electrophoresis in the presence or absence of 0.1% sodium dodecylsulfate [2]. About 90% of the enzyme employed in this study was contributed by one of the four charged isomers, the isoelectric point of which was 5.0 as assessed by isoelectric focusing column electrophoresis. Protein of the purified enzyme preparation was quantitated as described before [1]. 17β-Bromoacetoxytestosterone was prepared according to the method described by Sweet *et al.*[8] for synthesis of 16α-bromoacetoxyprogesterone.

All the reagents used were of analytical grade.

Enzyme assay

17β-Hydroxysteroid dehydrogenase activity was determined by measurement of absorbance at 340 nm due to NADPH formed during incubation of the enzyme with $NADP^+$ and testosterone, using Union Spectrophotometer SM-401 (Union Giken, Osaka, Japan) at 25°C. The incubation mixtures consisted of the enzyme (0.7 μM), and several concentrations of $NADP^+$ and testosterone as indicated in Figs 1–4, in 2 ml of 100 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 20% glycerol. Reaction rates were given in NADPH as nmol·min^{-1} formed by 1 nmol of the enzyme. Results were presented as double reciprocal plots, and lines were fitted to the points manually.

The reverse reaction was quantitated by measuring the oxidation of NADPH, in the medium to which NADPH and androstenedione were added, instead of $NADP^+$ and testosterone. The enzyme reactions were initiated by addition of the steroids.

Fluorometric analyses

A fluorescence spectrophotometer (Union Model FS-401) was employed for fluorometric measurement of NADPH at 20°C. NADPH (10 μM) in 1 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 20% glycerol was added to a cuvette, and emission spectrum of NADPH was recorded at 350 nm excitation wavelength, after correction of the reference to which no NADPH was added in the other cuvette. Then, the enzyme (0.5 μM) was added to the former cuvette, and the emission spectra of NADPH in the presence of the enzyme were recorded, after subtraction of the fluoresence of the reference cuvette in which the same amount of the enzyme was added in the absence of NADPH.

RESULTS

Product inhibition

Double reciprocal plots shown in Fig. 1 were the initial velocities for oxidation of testosterone versus $NADP^+$ concentrations in the presence of 0–20 μM NADPH as the product inhibitor with a fixed unsaturated concentration (10 μM) of testosterone, and a saturated concentration (100 μM) of it. At both 10 and 100 μM concentrations of testosterone, inhibitions of the 17β-hydroxysteroid dehydrogenase activity by NADPH were competitive type against $NADP^+$. The type of inhibition by NADPH in the presence of 10 μM $NADP^+$ was found to be a mixed type (both the slopes and apparent K_M were changed) against testosterone, as shown in Fig. 2. In the presence of a saturated concentration (100 μM) of $NADP^+$ as the cosubstrate and variable amounts of testosterone, however, the enzyme activity was not inhibited by NADPH (data not shown). Similar ex-

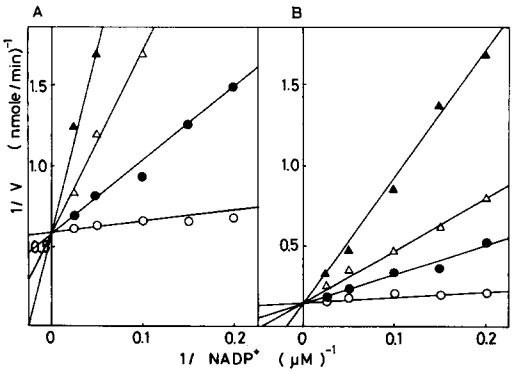


Fig. 1. Double reciprocal plots of initial velocities for the 17β-hydroxysteroid dehydrogenase activity with $NADP^+$ as the variable substrate and NADPH as the inhibitor. NADPH concentrations, 0 μM (○), 5 μM (●), 10 μM (△), and 20 μM (▲). Initial velocities are expressed as nmol of NADPH formed per min by 1 nmol of the enzyme, under the conditions of assay in Methods. Testosterone concentrations, (A) 10 μM, and (B) 100 μM.

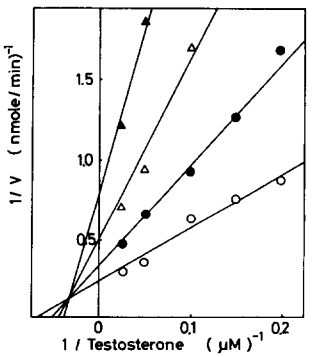


Fig. 2. Double reciprocal plots of initial velocities for the 17β-hydroxysteroid dehydrogenase activity with testosterone as the variable substrate and NADPH as the inhibitor. NADPH concentrations, 0 μM (○), 5 μM (●), 10 μM (△), and 20 μM (▲). $NADP^+$ concentration is fixed as 10 μM.

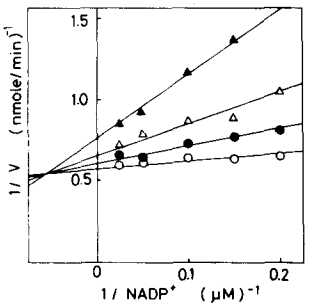


Fig. 3. Double reciprocal plots of initial velocities for the 17β-hydroxysteroid dehydrogenase activity with $NADP^+$ as the variable substrate and androstenedione as the inhibitor. Androstenedione concentrations, 0 μM (○), 5 μM (●), 10 μM (△), and 20 μM (▲). Testosterone concentration is fixed as 10 μM.

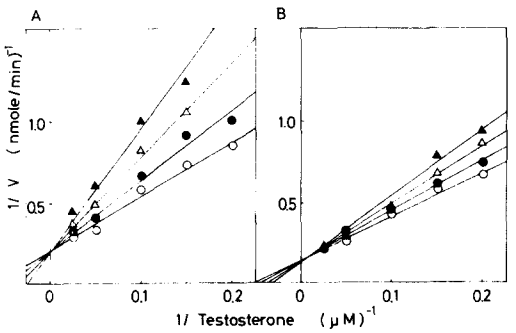


Fig. 4. Double reciprocal plots of initial velocities for 17β-hydroxysteroid dehydrogenase activity with testosterone as the variable substrate, and androstenedione as the inhibitor. Androstenedione concentrations, 0 μM (○), 5 μM (●), 10 μM (△), and 20 μM (▲). $NADP^+$ concentrations, (A) 10 μM, and (B) 100 μM.

periments on effect of androstenedione were carried out, using $NADP^+$ as the varied substrate with the fixed concentrations of testosterone. At the high concentration (100 μM) of testosterone, androstenedione did not inhibit the enzyme activity against $NADP^+$ (data not shown), while at the low concentration (10 μM) of testosterone, mixed type of inhibition of the enzyme activity was confirmed (Fig. 3). When testosterone was used in variable amounts in the presence of 10 μM and 100 μM of $NADP^+$, the inhibitions of the enzyme by androstenedione against testosterone were of competitive type as shown in Fig. 4, (A) and (B).

From these results, the inhibitions by the product steroid and the product pyridine nucleotide were competitive against the substrate steroid and pyridine nucleotide, respectively. No inhibition of the enzyme activity was observed by the product steroid and pyridine nucleotide against the substrate pyridine nucleotide and steroid, respectively, at the saturated concentration of the cosubstrates. At the unsaturated concentration of the cosubstrates, however, inhibitions by the product steroid and pyridine nucleotide were of mixed type against the substrate pyridine nucleotide and steroid, respectively. These patterns of inhibition obtained from Figs 1–4 were summarized in Table 1.

Product inhibitions by $NADP^+$ or testosterone of the reverse reaction were examined with unsaturated concentrations of the cosubstrates against NADPH and androstenedione, respectively. The inhibition patterns (which are not shown here) were similar to those as shown in Table 1.

In order to examine whether or not other steroidal metabolites are actually formed during the incubation, the enzyme was separately incubated with 40,000 d.p.m. of ^{14}C-labeled testosterone (200 μM) in the presence of $NADP^+$ (1,000 μM), NADPH (1,000 μM), or both $NADP^+$ (500 μM) and NADPH (500 μM), in 1 ml of the assay medium at 37°C for 10 min. Also the enzyme was incubated with ^{14}C-labeled androstenedione in the presence of the above cofactors. After the incubation, the steroids were extracted from the incubation medium and were chromatographed on thin layer plates of silica gel (E. Merck, Darmstadt, Germany). Autoradiographic detection of the radioactive steroids on the chromatograms was carried out as described previously [1]. Throughout the experiments, no radioactive steroids other than testosterone and androstenedione were detected.

Binding of NADPH to the 17β-hydroxysteroid dehydrogenase

Influence of the 17β-hydroxysteroid dehydrogenase upon the fluorescence of NADPH was studied. As shown in Fig. 5, addition of the enzyme to NADPH caused intensification of the fluorescence maximum and the shift to the shorter wavelength in the emission spectrum. The enzyme in the same medium was added by small amounts to NADPH solution, so that the dilution effect on the fluorescence of NADPH

Table 1. Summarized patterns of product inhibition of 17β-hydroxysteroid dehydrogenase

	Varied substrate			
	$NADP^+$		Testosterone	
Product inhibitor	with testosterone		with $NADP^+$	
	10 μM	100 μM	10 μM	100 μM
Androstenedione	Mixed type	No inhibition	Competitive	Competitive
NADPH	Competitive	Competitive	Mixed type	No inhibition

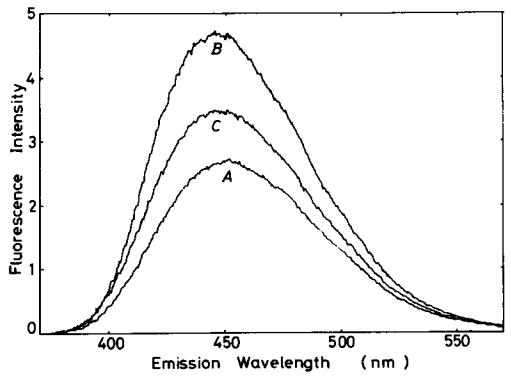


Fig. 5. Effects of the enzyme and testosterone on the fluorescence of NADPH. The fluorescence emission spectra of NADPH were measured at 350 nm as excitation wavelength. The cuvette contained, in a final vol. of 1.0 ml. 10 μM NADPH in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 20% glycerol. (A) NADPH alone, (B) NADPH with the enzyme (0.5 μM), and (C) NADPH with the enzyme (0.5 μM) in the presence of 100 μM testosterone.

was negligible. When $NADP^+$ was added to the mixture of NADPH and the enzyme, the increased fluorescence due to the addition of the enzyme was reduced to the initial level of NADPH alone. When, 10 μl of 10 mM testosterone in ethanol was added to the mixture of NADPH and the enzyme, the fluorescence of NADPH intensified by the addition of the enzyme was suppressed. In the control experiment, in which only the same volumes of ethanol were added, no change in intensities of the fluorescence due to the mixture of NADPH and the enzyme was observed. In the absence of the enzyme, testosterone showed no effect upon the fluorescence of NADPH.

The fluorometric titration of the 17β-hydroxysteroid dehydrogenase with NADPH was carried out. Figure 6 shows that the equivalence point is 0.40 μM for NADPH which corresponded to binding for 0.33 μM of the enzyme, indicating that 1 mol of NADPH is bound to 1 mol (Mr = 35,000) of the enzyme.

Testicular 17β-hydroxysteroid dehydrogenase was inactivated by modification with the affinity ligand, 17β-bromoacetoxytestosterone, time-dependently. Using 17β-bromo[1'-^{14}C]-acetoxy[1,2-^{3}H]-testosterone as the ligand, we observed that the complete inactivation of the enzyme occurred, when equimolar of the ligand bound to the enzyme (unpublished data).

In the presence of 20-fold excess of the affinity ligand, 0.8 μM of the enzyme was left for 2 h in 100 mM phosphate buffer (pH 7.5) containing 1 mM EDTA and 20% glycerol in order to modify the enzyme completely, then the fluorometric titration by NADPH was carried out as described above. As shown in Fig. 7, the maximum fluorescence intensity of NADPH caused by the presence of the modified enzyme was significantly reduced in comparison with that of the native enzyme.

Suppression by testosterone of the fluorescence due to the mixture of NADPH and the enzyme was demonstrated as double reciprocal plots of the fluorescence increment versus NADPH concentrations (Fig. 8). Apparent dissociation equilibrium constant for NADPH to the 17β-hydroxysteroid dehydrogenase was 0.5 μM in the presence and absence of testosterone. Testosterone inhibited the increment of the fluorescence noncompetitively, and K_i was estimated as about 50 μM.

DISCUSSION

In the presence of several dehydrogenases, fluorescence of NAD(P)H was increased and emission spectrum of NAD(P)H shifted to the shorter wavelength, suggesting the interaction of NAD(P)H with NAD(P)H-binding site of the dehydrogenases [9–12].

In the present study, porcine testicular 17β-hydroxysteroid dehydrogenase intensified the fluorescence of NADPH, indicating a formation of the binary

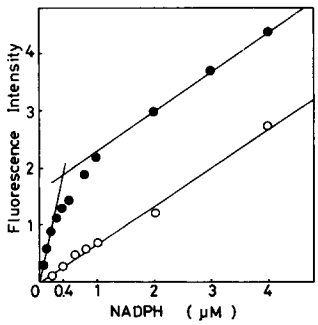


Fig. 6. Fluorometric titration of the 17β-hydroxysteroid dehydrogenase with NADPH. The cuvette contained 0.33 μM of the enzyme (●), or without enzyme (○), in 1 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 20% glycerol. The variable amounts (0–4 μM) of NADPH were added to the mixture. Excitation wavelength was at 350 nm, and the emission was at 450 nm.

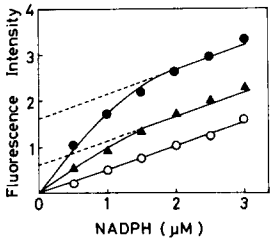


Fig. 7. Fluorometric titration of the affinity ligand treated enzyme. The cuvettes contained 0.8 μM of the enzyme in the presence (▲) and absence (●) of 16 μM of 17β-bromoacetoxytestosterone in 1 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 20% glycerol were allowed to stand at 25°C for 2 h. The variable amounts (0–3 μM) of NADPH were added to the mixture, and emissions at 450 nm were measured. The values of emission of NADPH alone in the absence of the enzyme are shown (○). Excitation wavelength was at 350 nm.

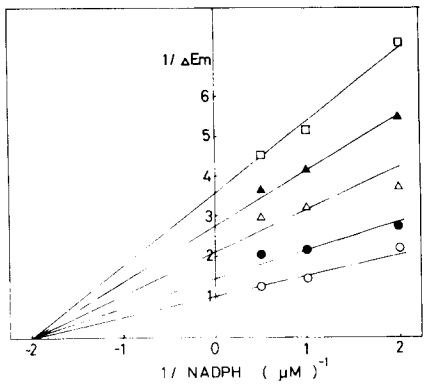


Fig. 8. Effects of testosterone on increment of NADPH fluorescence by the enzyme. The cuvette contained 0.5, 1.0 or 2.0 μM of NADPH and 0.33 μM of the enzyme in 1 ml of 100 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 20% glycerol. Testosterone was then added, and fluorescence intensity at 450 nm was measured; the excitation wavelength was 350 nm. Increase of the fluorescence (ΔEm) was obtained after subtraction of the values of fluorescence of the enzyme and NADPH. Values were shown as double reciprocal plots of 1/ΔEm against 1/NADPH. Testosterone concentrations, 0 μM (○), 20 μM (●), 50 μM (△), 100 μM (▲), and 150 μM (□).

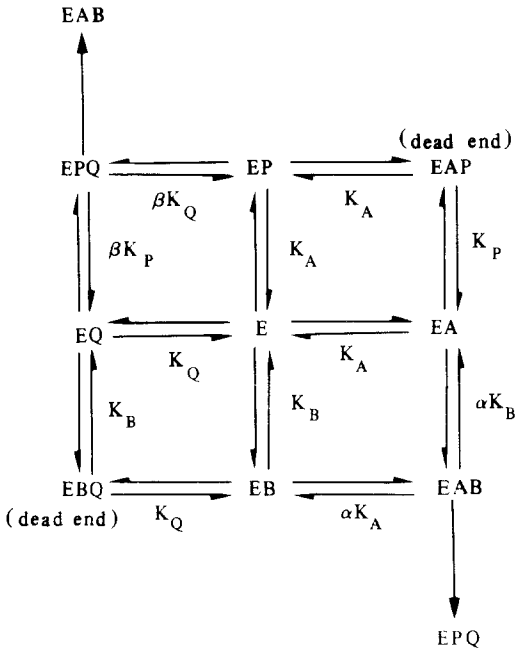
complex of the enzyme and NADPH. This was so since $NADP^+$ which competed with NADPH for the same binding site of the enzyme, decreased the fluorescence of NADPH intensified by the enzyme to the level of NADPH alone in a dose-dependent manner (data not shown). The enzyme also intensified the fluorescence of NADH to a lesser extent than that of NADPH. This result seems agreeable with the result that the K_M value of porcine testicular 17β-hydroxysteroid dehydrogenase for NADH was larger than that for NADPH [4].

Testosterone reduced the fluorescence increment of NADPH due to the addition of the 17β-hydroxysteroid dehydrogenase. Furthermore, the modified enzyme which bound covalently with an equimolar amount of the analogue of testosterone was not able to increase fluorescence of NADPH to such an extent as the native enzyme. This result suggests the presence of the ternary complex in which the fluorescence of NADPH was intensified less than in the binary complex. Quantitatively, the increase in emission of NADPH was depressed noncompetitively by testosterone, suggesting the binding of testosterone to the binary complex of enzyme–NADPH rather than the dissociation of NADPH from the enzyme–NADPH complex due to the formation of an enzyme–testosterone complex.

The kinetic mechanisms of enzyme-catalyzed reactions with two substrates and two products were categorized on the basis of the types of product inhibitions at saturated and unsaturated concentrations of cosubstrates [13–15]. The characteristics of product inhibition indicated the kinetic mechanism of testicular 17β-hydroxysteroid dehydrogenase of the following mechanisms; rapid equilibrium random system with two dead end complexes, Theorell–Chance mechanism, and ping pong mechanism, all of which showed similar modes of inhibition [13]. However, in the case of the latter two mechanisms, no ternary abortive (dead end) complex is to be observed. From our present results, the kinetic mechanism of the testicular 17β-hydroxysteroid dehydrogenase was defined as a rapid equilibrium random system with two dead end complexes which were testosterone–enzyme–NADPH and androstenedione–enzyme–$NADP^+$ as shown in Scheme 1. The latter of the two dead end complexes was not detected in this report.

17β-Hydroxysteroid dehydrogenase activity in human testicular microsomes was reported to be activated by the product [16, 17]. In rat testes, 17β-hydroxysteroid dehydrogenase activity in seminiferous tubules was activated by the presence of the product, while the activity in Leydig cells which are the major site of testosterone synthesis was inhibited by the product [6]. In the present study, the purified enzyme was not activated by the steroidal product, as described previously [1], though we could not eliminate the possibility that the dehydrogenase investigated in this study is regulated *in vivo* by other factors on the membrane of endoplasmic reticulum.

The kinetic mechanism of human placental 17β-hydroxysteroid dehydrogenase (estradiol 17β-dehydrogenase) which was distinct from the testicular 17β-hydroxysteroid dehydrogenase in respect of molecular properties and biological significance [18, 19] was concluded by the method of isotope exchange as rapid equilibrium random system without formation of dead end complexes [20]. Sheep ovarian 17β-hydroxysteroid dehydrogenase which has close analo-



Scheme 1

gies with human placental 17β-hydrosteroid dehydrogenase in terms of molecular weight, pH optimum, and substrate binding site requirement has been suggested as an ordered mechanism with binding pyridine nucleotide as the first substrate [21]. Therefore, the reaction mechanism for the porcine testicular 17β-hydroxysteroid dehydrogenase which was investigated in the present study was concluded as distinct from human placental and sheep ovarian 17β-hydroxysteroid dehydrogenases, with regard to the kinetic mechanism.

Investigation of the substrate binding site in the molecule of 17β-hydroxysteroid dehydrogenase by the affinity ligands is in progress in this laboratory, and further information on the catalytic mechanism of the testicular 17β-hydroxysteroid dehydrogenase is being studied in this laboratory.

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