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TESTICULAR AND ADRENAL 3β-HYDROXY-5-ENE-STEROID DEHYDROGENASE AND 5-ENE-4-ENE ISOMERASE

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Summary—The purified multifunctional enzyme, 3β-hydroxysteroid dehydrogenase with steroid 5-ene-4-ene isomerase from rat testes and adrenals showed similar catalytic properties. They exhibited the same molecular weight of 46,500. Either NAD^+ or NADH was required for steroid isomerizing activity, probably as an allosteric effector. It was clearly demonstrated by using the purified enzyme that without NAD(H) no isomerizing activity was detected. In the presence of NADH, or its analogue, 3β-hydroxysteroid dehydrogenase obtained from both tissues was inhibited; however, steroid isomerizing activity remained due to the allosteric effect. The results suggest that in these endocrine organs, both enzyme activities reside within the same protein.

INTRODUCTION

The activation effect of mammalian steroid 5-ene-4-ene isomerase (steroid isomerase) by NAD^+ or NADH was reported by Olenick and Koritz[1]. In their study, however, adrenal microsomes were used as the enzyme source. As the kinetic study of steroid isomerase in testicular or ovarian microsomes was difficult because of its low specific activity, characteristics of steroid isomerase in reproductive organs have not been well clarified until now.

By using adrenal microsomes, it was shown that p-chloromercuriphenylsulfonate (PCMS), and nucleotides structurally related to NAD(H), such as 2′,5′-adenosinediphosphoribose, cyclic AMP, FAD, and adenosine 5′-phosphoramidate inhibited the activation by NAD(H) of the steroid isomerase[1]. The inhibition by PCMS suggested involvement of sulfhydryl group(s) in the activation process[1]. However, the microsomal fraction used for the assay contained not only other proteins, but also substances with low molecular weights; both could modify the activity of steroid isomerase. Therefore in order to study steroid isomerase more exactly, the use of purified enzyme was essential.

3β-Hydroxysteroid dehydrogenase has been purified from ovine adrenal[2], and steroid isomerase activity was reported as not separable from the dehydrogenase during the purification procedure. In the case of mammalian tissue, it is widely accepted that dehydrogenation of 3β-hydroxy-5-ene-steroid and isomerization of 3-oxo-5-ene-steroid are catalyzed by a single enzyme. However, the relationship between NAD^+ binding to cofactor binding site and the activation of steroid isomerase by NAD^+ has not yet been clarified.

Recently we reported the purification of 3β-hydroxysteroid dehydrogenase with steroid isomerase from rat adrenal microsomes[3] and rat testicular microsomes[4], and demonstrated the requirement of NAD(H) for steroid isomerase activity. The enzymatic properties of the purified preparation from adrenal and testes were compared in this paper.

EXPERIMENTAL

Chemicals

[4-^{14}C]DHA (51.8 mCi/mmol) was obtained from New England Nuclear (U.S.A.). 5-Progesterone‡ and 5-androstenedione were obtained from Research Plus (U.S.A.), and other steroids were purchased from Steraloids (U.S.A.). All solvents were used after redistillation.

Analytical methods

Activity of 3β-hydroxysteroid dehydrogenase was assayed spectrophotometrically at 25°C as follows: 1 ml of the reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 20% glycerol, 0.1 mM EDTA, 1 mM NAD^+ and 0.1 mM DHA. DHA was added as 10 μl of the 10 mM steroid in ethanol solution. The time-dependent change of absorbance at 340 nm was recorded. The enzyme activity was expressed as nmol of NADH for-

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‡*Abbreviations*: 4-progesterone, pregn-4-ene-3,20-dione; 5-progesterone, pregn-5-ene-3,20-dione; 4-androstenedione, androst-4-ene-3,17-dione; 5-androstenedione, androst-5-ene-3,17-dione; DHA, 5-androsten-3β-ol-17-one; and FSBA, 5′-p-fluorosulfonylbenzoyladenosine.

med/min by using a molecular extinction coefficient of NADH of 6200.

Steroid isomerase activity was assayed at 25°C in 1 ml of the reaction mixture which contained 100 mM potassium phosphate buffer (pH 7.5), 20% glycerol, 0.1 mM EDTA, 0.1 mM NAD^+ and 0.02 mM 5-progesterone. The substrate was added as 10 μl of the 2 mM steroid in ethanol solution. Time-dependent increase of absorbance at 248 nm due to 4-progesterone formation was recorded. Enzyme activity was expressed as nmol of 4-progesterone formed/min by using a molecular extinction coefficient of 16,300.

For the activity of the 3β-hydroxysteroid dehydrogenase together with that of the isomerase, radioactive 4- plus 5-androstenediones *in vitro* produced from [4-^{14}C]DHA at 37°C were quantitated as previously reported [4]. Protein concentration was measured by the method by Bradford[5], using bovine serum gamma-globulin as the standard.

Purification of 3β-hydroxysteroid dehydrogenase and steroid isomerase from microsomal fractions of testes and adrenals

Testes and adrenal glands from male rats of the Wistar strain (90-days old) were collected and their microsomes were prepared by the method described before [4], using 10 mM potassium phosphate buffer (pH 7.5) containing 0.25 M sucrose, 0.1 mM EDTA and 0.1 mM dithiothreitol. All the media used for preparation of microsomes and enzyme purification contained 2 μg/ml each of leupeptin and pepstatin in order to minimize proteolytic influence upon the enzyme. The microsomes were suspended in 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol.

Concentration of the microsomal protein was adjusted to 10 mg/ml. 3β-Hydroxysteroid dehydrogenase and isomerase were solubilized from the microsomes by addition of sodium cholate (final concentration, 0.6%). The solubilized proteins were obtained by centrifugation at 105,000 *g* for 1 h.

The purification was carried out as previously described [3]. The solubilized fraction was applied to a column of TSK gel-DEAE Toyopearl 650 S (Toyo Soda, Japan), which had been equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.01 mM NAD^+ and 0.2% sodium cholate. The column was washed with the same buffer as that used for the equilibration. The fractions containing the enzyme activities were eluted with the same buffer containing 0.4% Emulgen 913 (Kao Atlas, Japan), were pooled and then diluted with the same volume of a 20% glycerol solution. The preparation was applied to a column of hydroxylapatite (Bio-Rad, F.R.G.), which had been equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.01 mM NAD^+ and 0.2% Emulgen 913. Elution was carried out with a linear concentration gradient from 10 to 70 mM potassium phosphate buffer (pH 7.5).

The enzyme preparation obtained from the hydroxylapatite chromatography was concentrated by Amicon ultrafiltration apparatus with membrane filter PM 10 (Amicon, U.S.A.). The enzyme preparation was applied to a column of Sepharose 6B (Pharmacia, Sweden) which had been equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol and 0.2% Emulgen 913. Gel filtration was carried out with the same buffer to remove NAD^+ from the enzyme preparation. The enzyme activities emerged around 2.62 void volume.

Then, the partially purified preparation was applied to a column of 5′-AMP-Sepharose 4B (Pharmacia) which had been equilibrated with 10 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol and 0.2% Emulgen. The enzyme was eluted with 100 mM potassium phosphate buffer (pH 7.5).

Purity of the final enzyme preparation was determined by SDS–PAGE which was carried out by the method of Laemmli[6], using 7.5% polyacrylamide as a separation gel. After the electrophoresis, the gel was treated with 0.2% glutalaldehyde. Protein in the gel was stained by using silver stain kit (Dai-ichi Pure Chem. Co. Japan).

RESULTS AND DISCUSSION

The properties of testicular and adrenal 3β-hydroxysteroid dehydrogenase were compared as described in Table 1. It is strongly suggested that both enzymes were the same protein. From the differential centrifugation study, conversion of [4-^{14}C]DHA to androstenedione was observed in both microsomal and mitochondrial fractions. The presence of the enzyme on inner mitochondrial membrane was suggested [7], but the molecular properties of mitochondrial 3β-hydroxysteroid dehydrogenase have not yet been well established. We did not determine the further location of the enzyme within organelles, but would like to study this problem in future by using the specific antibody against the purified microsomal enzyme.

The SDS–PAGE of the purified enzyme preparations from testes and adrenals were carried out, with the result that two preparations were indistinguishable by their mobilities. Also, the kinetic characteristics of these preparations were compared. The activity of 3β-hydroxysteroid dehydrogenase was assayed with [4-^{14}C]DHA as the substrate and quantitated from sum of 5- and 4-androstenediones formed by the enzyme. Through the conversion of DHA to 4-androstenedione by the purified enzyme, a limited amount of an intermediate, 5-androstenedione [4], was formed as described in Fig.

Table 1. Molecular properties of testicular and adrenal 3β-hydroxysteroid dehydrogenase with steroid isomerase

	Testicular	Adrenal
Localization	Microsomes (mitochondria)	Microsomes (mitochondria)
Molecular weight	46.5 kDa	46.5 kDa
3β-Hydroxysteroid dehydrogenase activity		
K_m for NAD^+	33 μM	16 μM
K_m for DHA	0.1 μM	Not done
Effect of NADH*	Inhibition	Inhibition
Effect of FSBA*	Inhibition	Inhibition
Steroid isomerase activity		
K_m for NAD	10 μM	12 μM
K_m for 5-progesterone	0.7 μM	1 μM
Effect of NADH†	Activation (~90%)	Activation (~90%)
Effect of FSBA†	Activation (~10%)	Activation (~10%)

*The enzyme activity was assayed with NAD^+.
†The enzyme activity was assayed without NAD^+.

1. As shown in Table 1, in the presence of NADH, 3β-hydroxysteroid dehydrogenase was severely diminished, but steroid isomerase was activated. Maximum activating effect by NADH was about 90% of that by NAD^+. Also, addition of NADH did not decrease the activation effect of steroid isomerase by NAD^+.

FSBA was known to act as an electrophilic agent in covalent reactions with lysine, tyrosine, histidine, serine and cysteine residues at the ATP-, NADH- or NADPH-binding site of various enzymes [8]. FSBA caused inactivation of 3β-hydroxysteroid dehydrogenase. In the case of steroid isomerase, however, the modification by FSBA was more complicated. About 10% of the activity of steroid isomerase remained under any condition of the FSBA treatment, even after 3β-hydroxysteroid dehydrogenase was completely inactivated. Furthermore, the FSBA-treated protein showed about 10% of steroid isomerase activity when the assay was carried out in the absence of NAD^+. In this regard, it should be noted that the initial enzyme, in the absence of NAD^+, showed no isomerizing activity. NAD^+ partially protected the enzyme from the inactivation by FSBA. It is possible that binding of FSBA to the NAD^+ binding site caused partial allosteric activation of steroid isomerase.

By differential modification with DTNB, FSBA and dithiothreitol, amino acid residue(s) other than cysteine at or near the cofactor-binding site of the enzyme were modified [3], resulting in inactivation of 3β-hydroxysteroid dehydrogenase and modification of steroid isomerase activity.

Finally, the catalytic mechanism of 3β-hydroxysteroid dehydrogenase with steroid isomerase upon 5-ene-3β-hydroxysteroid was summarized as shown in Fig. 2(1)–(4).

Formation of 4-androstenedione from DHA was described in Fig. 2(1). The relative specific activity of steroid isomerase was about 10 times higher than that of 3β-hydroxysteroid dehydrogenase, so that the intermediate, 5-androstenedione, may be hardly detected under normal condition. Also, NAD(H) bound to the enzyme modified conformation of the enzyme, and stimulated steroid isomerase. Therefore 5-androstenedione could not be reduced by the reverse reaction of 3β-hydroxysteroid dehydrogenase. But no steroid isomerase activity was detectable in the absence of enzyme reaction of 3β-hydroxysteroid dehydrogenase, as shown in Fig. 2(2).

When various 3β-hydroxy-5-ane-steroids were used as the substrates instead of DHA, NAD: 3β-hydroxysteroid oxidoreductase activity was observed [Fig. 2(3)]. When the substrate specificity of 3β-hydroxysteroid dehydrogenase was examined [3], the enzyme prefered C-21 steroids to C-19 steroids, and 5α(β)-reduced steroids to 4- or 5-ene steroids. Hydroxy groups at 17β of C-19

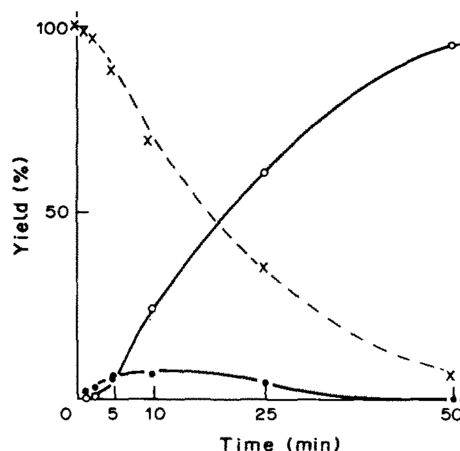
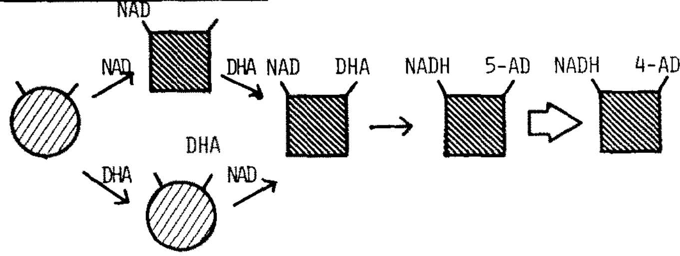
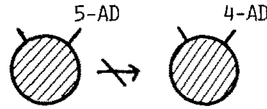


Fig. 1. Conversion of DHA to 5- and 4-androstenedione by purified testicular 3β-hydroxysteroid dehydrogenase with steroid isomerase. ●—●, 5-Androstenedione; ○—○, 4-androstenedione; ×---×, DHA.

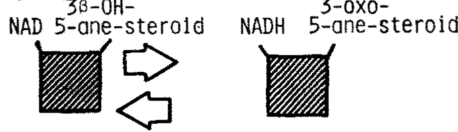
(1) Formation of 4-AD from DHA



(2) Without NAD(H)



(3) 5-Reduced steroids as the substrate



(4) FSBA-Bound enzyme

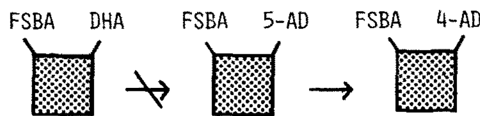


Fig. 2. Enzyme reaction of 3β-hydroxysteroid dehydrogenase with steroid isomerase. (1) Formation of 4-androstenedione from DHA. NAD(H) bound to the cofactor binding site modified the protein conformation to stimulate steroid isomerase. (2) Without NAD(H). No steroid isomerase activity was observed. (3) 5-Reduced steroids as substrates. NAD: 3β-hydroxysteroid oxidoreductase activity was observed. (4) The enzyme which was modified by covalently bound FSBA. The partial activity of steroid isomerase was detected but that of 3β-hydroxysteroid dehydrogenase was not.

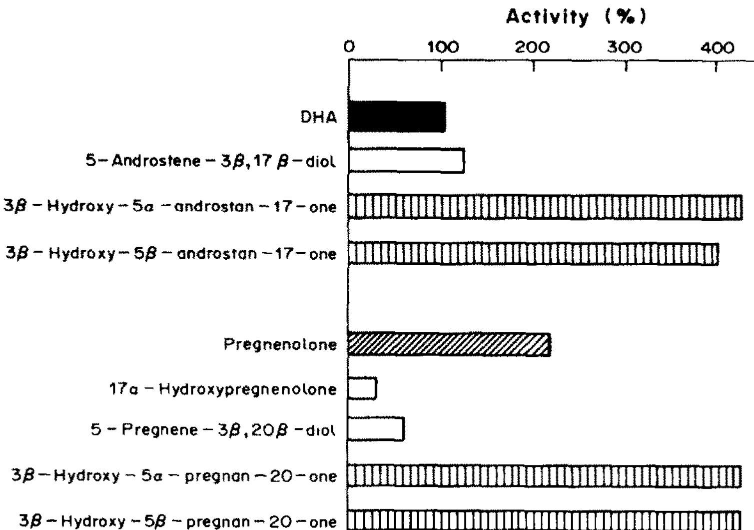


Fig. 3. Substrate specificity of 3β-hydroxysteroid dehydrogenase.

steroids were not inhibitory, where hydroxy groups at 17α or 20β of C-21 steroids exhibited steric inhibition on 3β-hydroxysteroid dehydrogenase activity. These results are summarized as shown in Fig. 3.

Covalently bound FSBA at the cofactor site of the enzyme resulted in the loss of 3β-hydroxysteroid dehydrogenase activity, but still the partial activity of steroid isomerase was detected, which may be explained through the allosteric effect as discussed above and shown in Fig. 2(4).

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