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Demonstration of 3α(17β)-hydroxysteroid dehydrogenase distinct from 3α-hydroxysteroid dehydrogenase in hamster liver

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NAD$^+$-linked and NADP$^+$-linked 3α-hydroxysteroid dehydrogenases were purified to homogeneity from hamster liver cytosol. The two monomeric enzymes, although having similar molecular masses of 38000, differed from each other in pI values, activation energy and heat stability. The two proteins also gave different fragmentation patterns by gel electrophoresis after digestion with protease. The NADP$^+$-linked enzyme catalysed the oxidoreduction of various 3α-hydroxysteroids, whereas the NAD$^+$-linked enzyme oxidized the 3α-hydroxy group of pregnanes and some bile acids, and the 17β-hydroxy group of testosterone and androstanes. The thermal stabilities of the 3α- and 17β-hydroxysteroid dehydrogenase activities of the NAD$^+$-linked enzyme were identical, and the two enzyme activities were inhibited by mixing 17β- and 3α-hydroxysteroid substrates, respectively. Medroxyprogesterone acetate, hexoestrol and 3β-hydroxysteroids competitively inhibited 3α- and 17β-hydroxysteroid dehydrogenase activities of the enzyme. These results show that hamster liver contains a 3α(17β)-hydroxysteroid dehydrogenase structurally and functionally distinct from 3α-hydroxysteroid dehydrogenase.

INTRODUCTION

Studies of mammalian hydroxysteroid dehydrogenases have shown that some of these enzymes exhibit oxidoreductase activity towards non-steroidal alcohols and carbonyl compounds. For example, dihydrodiol dehydrogenase and carbonyl reductase activities have been shown to be associated with purified 3α-hydroxysteroid dehydrogenases (EC 1.1.1.213; 3HSD) from rat [1,2], mouse [3] and rabbit liver [4,5]. 17β-Hydroxysteroid dehydrogenase from guinea pig liver has been also reported to possess dihydrodiol dehydrogenase and carbonyl reductase activities [6,7]. Moreover, several multiple forms of rabbit liver indanol dehydrogenase have been suggested to be those of 17β-hydroxysteroid dehydrogenase [5]. However, the cofactor and substrate specificities of these enzymes are different depending on the species. While 3HSD of rat [2] and rabbit liver [5] utilize both NAD$^+$ and NADP$^+$ as cofactor, that of mouse liver is NADP$^+$-dependent [3]. Rabbit liver 17β-hydroxysteroid dehydrogenase exhibits 3HSD activity [5,8], but the guinea pig liver enzyme does not [7,9].

We partially purified four multiple forms of dihydrodiol dehydrogenase from hamster liver, and suggested that two of the multiple forms of the enzyme were NAD$^+$-dependent and NADP$^+$-dependent 3HSDs [10]. In addition, the NAD$^+$-dependent enzyme preparation exhibited 17β-hydroxysteroid dehydrogenase activity [10], but there is no direct evidence which demonstrates the possibility that a single enzyme catalyses the oxidoreduction at two distinct positions on the steroid molecule. Therefore we purified the two 3HSDs from hamster liver cytosol to homogeneity, and compared their properties to elucidate the structural and metabolic relationship of the enzymes, and to establish whether the NAD$^+$-dependent enzyme exhibits a dual steroid specificity.

EXPERIMENTAL

Materials

Steroids were obtained from Sigma Chemical Co. and nicotinamide nucleotides and pI markers were from Oriental Yeast Co. Q-Sepharose, Sephadex G-100, ADP–Sepharose and molecular-mass standards were from Pharmacia Fine Chemicals and HA-Ultrogel was from LKB Produkter AB. Matrex Red A was purchased from Amicon Co. and *Staphylococcus aureus* V8 protease was from Pierce Chemical Co. Benzene dihydrodiol (*trans*-1,2-dihydroxycyclohexa-3,5-diene) was synthesized as described by Platt & Oesch [11]. Other chemicals of reagent grade were obtained from Wako Pure Chemical Industries.

Enzyme assay

During the purification, dehydrogenase activity was measured at 25 °C by monitoring NAD(P)H fluorescence at 450 nm (excitation at 340 nm). Each cuvette contained 2.0 ml of the reaction mixture, which consisted of 0.1 M-glycine/NaOH, pH 10.0, 2 mM-NAD$^+$ or 0.5 mM-NADP$^+$, 25 μM-5β-androstane-3α,17β-diol and enzyme. Substrate was omitted from the blank. The reaction was initiated by the addition of enzyme. Kinetic assays were performed similarly at least in duplicate with various amounts of hydroxysteroid added as specified in the text,

Abbreviations and trivial names used: 3HSD, 3α-hydroxysteroid dehydrogenase; glycolithocholic acid, *N*-(3α-hydroxy-5β-cholan-24-oyl)glycine; glycochenodeoxycholic acid, *N*-(3α,7α-dihydroxy-5β-cholan-24-oyl)glycine; lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; dehydrolithocholic acid, 3-oxo-5β-cholan-24-oic acid; testosterone, 17β-hyroxyandrost-4-en-3-one; 5β-dihydrocortisone, 17α,21-dihydroxy-5β-pregnane-3,11,20-trione.

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except that 0.1 M-potassium phosphate buffer, pH 7.5, was used instead of the glycine/NaOH buffer. The substrates were dissolved in methanol and 50 μl portions were added to the assay mixture. Reductase activity was assayed by recording the decrease of NAD(P)H absorbance at 340 nm in 2.0 ml of 100 mM-potassium phosphate, pH 7.5, containing 0.2 mM-NADH or 0.1 mM-NADPH, oxosteroid and enzyme at 25 °C. One unit of enzyme was defined as the amount that catalysed oxidation or reduction of 1 μmol of NAD(P)H/min. In a separate experiment, the assay was done at various temperatures (17–38 °C) with 6.3 and 25 μM-5β-pregnane-3α,21-diol-20-one as the substrates for the NAD^+- and $NADP^+$-dependent 3HSDs, respectively, and the activation energy for the reaction was calculated from the Arrhenius plot [12]. For determining thermal stability of the enzyme, the enzyme solution (0.1 mg/ml) in 20 mM-potassium phosphate, pH 7.5, containing 5 mM-2-mercaptoethanol, 0.5 mM-EDTA, 0.14 M-KCl and 1% bovine serum albumin was incubated at 43 or 45 °C; then 50 μl aliquots were removed for assay at a specific time.

Protein concentration was determined as described by Bradford [13].

Enzyme purification

All of the following procedures were performed at 4 °C. Livers (about 20 g) were excised from five adult male golden hamsters, immediately minced, and homogenized with 80 ml of 50 mM-Tris/HCl, pH 7.5, containing 5 mM-2-mercaptoethanol, 5 mM-EDTA and 0.14 M-KCl in a Teflon/glass homogenizer. The homogenate was centrifuged at 105000 *g* for 1 h. The cytosol fraction was fractionated by adding ammonium sulphate, and the proteins precipitated between 35 and 75% saturation were collected by centrifugation at 12000 *g* for 15 min, then dialysed against 10 mM-Tris/HCl, pH 8.0, containing 5 mM-2-mercaptoethanol and 0.5 mM-EDTA (Buffer A), which was changed three times during each 6 h period. The dialysed solution was applied to a 1.6 cm × 30 cm Q-Sepharose column equilibrated with Buffer A. The column was washed with 100 ml of the buffer, then eluted with a linear 0–0.12 M-NaCl gradient in Buffer A. 3HSD activity was resolved into two peaks, Peak 1 and Peak 2, which appeared at NaCl concentrations of about 0.05 and 0.09 M, respectively. Peak 1 showed high NAD^+-dependent 3HSD activity, whereas Peak 2 showed high $NADP^+$-dependent activity. The two enzyme fractions were separately applied to 1.6 cm × 7 cm Matrex Red A columns that had been equilibrated with Buffer A plus 20% glycerol. After the columns were washed with 50 ml of buffer, the NAD^+-dependent enzyme in Peak 1 was eluted with buffer containing 0.1 M-NaCl, whereas the $NADP^+$-dependent enzyme in Peak 2 was retained on the column and then eluted with buffer further supplemented with 0.5 mM-$NADP^+$. These enzyme fractions were separately concentrated by ultrafiltration using a YM-10 membrane, dialysed against 30 vol. of 10 mM-Tris/HCl, pH 8.0, containing 5 mM-2-mercaptoethanol and 20% glycerol, and then applied to 1.6 cm × 8 cm HA-Ultrogel columns equilibrated with the same buffer. The NAD^+-dependent 3HSD was eluted with a linear 0–80 mM-potassium phosphate gradient, and the $NADP^+$-dependent enzyme with a linear 0–20 mM-potassium phosphate gradient. The two enzyme fractions were separately concentrated by ultrafiltration, and passed through 2.5 cm × 90 cm Sephadex G-100 columns in Buffer A plus 20% glycerol. The enzyme-active fractions were separately concentrated by ultrafiltration. While the concentrate of $NADP^+$-dependent 3HSD (Peak 2) was stored at 4 °C, that of NAD^+-dependent 3HSD (Peak 1) was further purified with a 1.6 cm × 12 cm ADP–Sepharose column equilibrated with 10 mM-potassium phosphate (pH 7.0) containing 5 mM-2-mercaptoethanol and 0.5 mM-EDTA. The enzyme solution was dialysed against 30 vol. of the same buffer, then applied to the column. The enzyme was eluted out with a linear 0–0.2 mM-$NADP^+$ gradient in the buffer. The enzyme-active fractions were concentrated by ultrafiltration, dialysed against Buffer A plus 20% glycerol, and stored at 4 °C.

Product identification

The reaction products from 5β-androstane-3α,17β-diol, 5β-pregnan-3α-ol-20-one and testosterone were analysed on t.l.c. The reaction mixture (4.0 ml), consisting of 0.1 mM-glycine/NaOH, pH 10.0, 2 mM-NAD^+ or 0.5 mM-$NADP^+$, one of the steroids (25 μM) and enzyme, was incubated for 15 min at 30 °C, and the products were extracted with 10 ml of ethyl acetate. The organic phase was concentrated by evaporating the solvent and then analysed on t.l.c. using silica gel plates in a solvent system of benzene/acetone (1:4, v/v) as described by Hara *et al.* [14]. The R_F values of 5β-androstane-3α,17β-diol, 5β-pregnan-3α-ol-20-one and testosterone were 0.20, 0.43 and 0.32, respectively, and those of the corresponding 3- and 17-oxosteroids were as follows: 5β-androstan-3α-ol-17-one (0.33), 5β-androstan-17β-ol-3-one (0.37), 5β-pregnane-3,20-dione (0.62) and androst-4-ene-3,17-dione (0.48).

Determination of M_r and pI values

The M_r of the native enzyme was estimated by gel filtration on a Sephadex G-100 column in Buffer A plus 20% glycerol, and that of the denatured enzyme by SDS/PAGE [15] using the molecular mass standards. The pI value of the enzyme was determined by isoelectric focusing on a 7% polyacrylamide disc gel [16] using the pI markers.

Peptide mapping

The enzyme solution (0.3 mg/ml) in 125 mM-Tris/HCl, pH 6.8, containing 1 mM-EDTA, 0.002% Bromophenol Blue, 0.1% SDS and 10% glycerol was heated at 100 °C for 5 min, and *Staphylococcus aureus* V8 protease (0.33 μg/ml) was added to the enzyme solution. The mixture (20 μl) was layered into the wells of a 4.5% stacking gel cast atop a 17.5–20% gradient polyacrylamide slab gel containing 0.1% SDS, then run at 15 mA. When electrophoresis was completed, the gel was stained using a silver stain kit (Daiichi Pure Chemicals Co., Tokyo, Japan).

RESULTS

Purification and purity

The results of a typical purification of NAD^+- and $NADP^+$-dependent 3HSDs from hamster liver are summarized in Table 1. The NAD^+-dependent enzyme activity in the cytosol and ammonium sulphate fraction could not be measured accurately, because the crude preparation showed high NAD^+ reductase activity with-

Table 1. Purification of NAD$^+$-dependent and NADP$^+$-dependent 3HSDs from hamster liver cytosol

Step	Protein (mg)	NADP$^+$-dependent 3HSD			NAD$^+$-dependent 3HSD		
		Activity (units)	Specific activity (units/mg)	Yield (%)	Activity (units)	Specific activity (units/mg)	Yield (%)
Cytosol	1500	8.5	0.0056	100	–	–	–
Ammonium sulphate fraction	1200	7.5	0.0062	88	–	–	–
Q-Sepharose							
Peak 1	160	1.1	0.0069	13	5.9	0.037	100
Peak 2	70	4.6	0.066	54	2.1	0.030	–
Matrex Red A							
Peak 1	20	0.90	0.045	10	4.5	0.23	76
Peak 2	4.5	3.3	0.73	39	0.0	–	–
HA-Ultrogel							
Peak 1	5.7	0.54	0.095	6.4	2.6	0.46	44
Peak 2	1.9	1.5	0.79	18	0.0	–	–
Sephadex G-100							
Peak 1	1.2	0.30	0.25	3.5	1.4	1.2	24
Peak 2	0.5	1.1	2.2	13	0.0	–	–
ADP–Sepharose							
Peak 1	0.31	0.22	0.71	2.6	1.3	4.2	22

out the steroid substrate. The unknown NAD$^+$ reductase activity was separated from the two 3HSDs (Peak 1 and Peak 2) in the Q-Sepharose step. The two 3HSDs appeared at the same position (M_r 35000) on the Sephadex G-100 filtration. The NADP$^+$-dependent enzyme was purified to homogeneity by the Sephadex G-100 chromatography, but the NAD$^+$-dependent 3HSD contained some other proteins when analysed on SDS/PAGE. The NAD$^+$-dependent 3HSD was further purified by chromatography on ADP–Sepharose. The NADP$^+$-

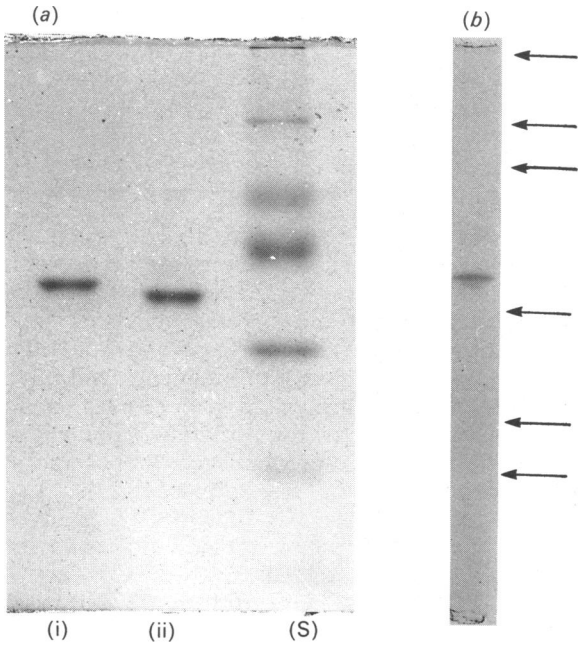

Fig. 1. Protein staining of hamster liver 3HSDs on polyacrylamide gels after SDS electrophoresis and isoelectric focusing

About 5 μg of the enzymes was analysed. (*a*) SDS/PAGE. Lanes: (i), NAD$^+$-dependent 3HSD; (ii), NADP$^+$-dependent 3HSD; (S) molecular mass standards of M_r 94000, 67000, 43000, 30000, 21100 and 14400 migrated from the top (cathode). (*b*) Gel focusing of NAD$^+$-dependent 3HSD. The arrows indicated the positions of pI markers with pI values of 4.1, 4.9, 6.3, 8.3, 9.7 and 10.6 migrated from the top (anode).

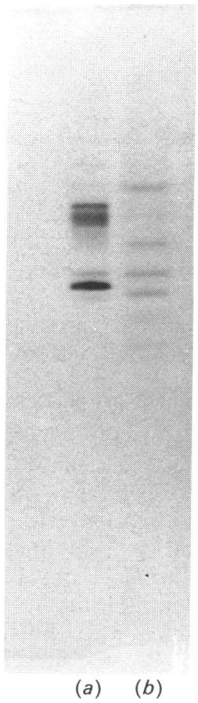

Fig. 2. Peptide mapping of hamster liver 3HSDs

The peptides of NAD$^+$-dependent 3HSD (*a*) and NADP$^+$-dependent 3HSD (*b*), after enzymic digestion, were resolved on a 17.5–20% gradient polyacrylamide gel containing SDS, and stained using a silver stain procedure. Details of the digestion were as described in the Experimental section.

Table 2. Catalytic and Michaelis constants for steroids of NAD$^+$-dependent and NADP$^+$-dependent 3HSDs from hamster liver

Kinetic constants were determined from Lineweaver–Burk analyses of assays performed in 0.1 M-potassium phosphate, pH 7.5, containing 2 mM-NAD$^+$ and steroids. The ranges of concentration of substrates were $0.1–1.5 \times K_m$ for bile acids and $0.2–2.0 \times K_m$ for other steroids. The catalytic constants were calculated assuming molecular masses for NAD$^+$-dependent and NADP$^+$-dependent 3HSDs of 38000. Values in parentheses were calculated from the activities with the indicated fixed concentration of the substrate.

Substrate	Concentration (μM)	NAD$^+$-dependent dehydrogenase			NADP$^+$-dependent dehydrogenase		
		$k_{cat.}$ (min^{-1})	K_m (μM)	$k_{cat.}/K_m$ (min$^{-1} \cdot \mu$M^{-1})	$k_{cat.}$ (min^{-1})	K_m (μM)	$k_{cat.}/K_m$ (min$^{-1} \cdot \mu$M^{-1})
Dehydrogenase activity							
Testosterone	25	25	2.2	11.4	(0)	–	–
5β-Androstane-3α,17β-diol		51	4.8	10.6	72	16	4.5
5α-Androstane-3α,17β-diol		27	3.1	8.7	80	15	5.3
5α-Pregnane-3α,21-diol-20-one	5	16	2.5	6.4	(14)	–	–
Lithocholic acid		3.0	0.5	6.0	13	4.0	3.1
Glycolithocholic acid		15	3.0	5.0	14	2.5	5.6
5β-Androstan-17β-ol-3-one	25	22	4.9	4.5	(0)	–	–
5β-Pregnane-3α,21-diol-20-one		22	6.2	3.6	38	5.5	6.9
5β-Pregnan-3α-ol-20-one		14	4.6	3.0	46	3.9	11.8
Glycochenodeoxycholic acid		1.2	7.5	0.2	12	2.5	4.8
5α-Androstan-17β-ol-3-one	5	(0.4)	–	–	(0)	–	–
17β-Oestradiol	5	(0.3)	–	–	(0)	–	–
5α-Androstan-3α-ol-17-one	25	(0)	–	–	163	47	3.5
5β-Androstan-3α-ol-17-one	25	(0)	–	–	80	12	6.7
Reductase activity							
5β-Pregnane-3,20-dione		9.5	3.8	2.5	25	0.8	31
5β-Androstane-3,17-dione		10	6.6	1.5	27	1.0	27
Dehydrolithocholic acid	10	(5.9)	–	–	44	1.5	29
5α-Pregnan-21-ol-3,20-dione	10	(3.0)	–	–	46	5.7	8.1
5α-Androstane-3,17-dione	10	(2.5)	–	–	34	3.0	11
5α-Androstan-17β-ol-3-one	10	(2.0)	–	–	21	1.3	16
5β-Dihydrocortisone	10	(2.0)	–	–	26	1.2	22
5β-Androstan-3α-ol-17-one	10	(0.4)	–	–	(0)	–	–
Oestrone 3-sulphate	10	(0.4)	–	–	(0)	–	–

dependent 3HSD was obtained in an overall activity recovery of 13% with 370-fold purification, and the recovery and purification factor of the NAD$^+$-dependent 3HSD from the Q-Sepharose step were 22% and 100-fold, respectively.

The two enzyme preparations showed single protein bands on SDS/PAGE and gel focusing (Fig. 1).

Comparison of structural properties

The NAD$^+$- and NADP$^+$-dependent 3HSDs gave identical molecular masses of 38000 ± 1000 on SDS/PAGE, whereas the respective pI values estimated by gel focusing were 8.1 and 5.0. The two enzymes also showed different thermostability: the times required for 50% inactivation at 45 °C were 2.3 and 17 min for the NAD$^+$- and NADP$^+$-dependent 3HSDs, respectively. Activation energy for 5β-pregnane-3α,21-diol-20-one oxidation by the NAD$^+$-dependent enzyme at pH 7.5 was 16 kJ/mol, whereas for that by the NADP$^+$-dependent enzyme it was 29 kJ/mol. Corroborative evidence of a primary difference in the two enzymes was obtained from peptide mapping, which revealed polypeptide fragments of distinct number and size for each enzyme (Fig. 2).

Comparison of catalytic properties

Although 5β-androstane-3α,17β-diol dehydrogenase activities of the NAD$^+$- and NADP$^+$-dependent 3HSDs were optimal at pH 10.4 and 10.2, respectively, we determined their kinetic constants for the steroids and benzene dihydrodiol at pH 7.5 to compare reactivity of the two enzymes to the steroids under physiological conditions (Table 2). The NAD$^+$-dependent 3HSD oxidized the 3α-hydroxy group of pregnanes and bile acids, but not of 3α-hydroxyandrostan-17-ones. However, 3α,17β-dihydroxy derivatives of androstane and several 17β-hydroxysteroids were oxidized more highly than 3α-hydroxypregnanes. Testosterone was the best substrate for this enzyme. The substrate specificity of the NAD$^+$-dependent enzyme was clearly different from that of the NADP$^+$-dependent enzyme, which was active only towards 3α-hydroxysteroids. The two enzymes also oxidized benzene dihydrodiol, but not 17α-oestradiol, 5β-pregnan-20α-ol-3-one, and 3β-hydroxysteroids such as 5β-androstan-3β-ol-17-one and 5β-pregnan-3β-ol-20-one.

The oxidized products of testosterone and 5β-pregnan-3α-ol-20-one formed by the NAD$^+$-dependent 3HSD were identified with androst-4-ene-3,17-dione and 5β-pregnane-3,20-dione, respectively, on t.l.c. The oxidation of 5β-androstane-3α,17β-diol yielded a single product with an R_F value of 0.33, which corresponds to that of authentic 5β-androstan-3α-ol-17-one but not to that of 5β-androstan-17β-ol-3-one, indicating that the 17β-hydroxy group of 5β-androstane-3α,17β-diol was

Table 3. Effects of various compounds on hamster liver 3HSDs

The 3α- and 17β-hydroxysteroid dehydrogenase activities of the NAD$^+$-dependent enzyme were assayed with 3.2 μM-5β-pregnane-3α,21-diol-20-one (PDO) and 6.3 μM-5β-androstan-17β-ol-3-one (AOO), respectively, as substrate, and the NADP$^+$-dependent enzyme activity with 19 μM-PDO. The inhibitors were dissolved in 50% (v/v) methanol, except that the thiol-blocking reagents were used as aqueous solutions, and 50 μl portions were added to the assay mixture just before the reaction was initiated by the addition of enzyme. The values represent the means ± S.D. of triplicate determinations.

Compound	Concentration (μM)	Inhibition (%)		
		NAD$^+$-dependent 3HSD		NADP$^+$-dependent 3HSD
		PDO	AOO	PDO
p-Chloromercuribenzenesulphonate	1	0	3±3	72±2
p-Chloromercuribenzoate	1	0	0	66±5
5β-Androstan-3β-ol-17-one	5	87±2	82±1	0
5β-Pregnan-3β-ol-20-one	5	81±6	86±2	15±2
5α-Androstan-3β-ol-17-one	5	55±3	50±4	0
5α-Pregnan-3β-ol-20-one	5	52±4	50±4	0
21-Hydroxypregn-4-ene-3,20-dione	10	73±3	70±2	53±7
Hexoestrol	10	62±2	68±3	30±1
Stilboestrol	10	61±1	65±3	36±3
Dienstrol	10	60±8	66±2	28±8
Medroxyprogesterone acetate	10	22±2	30±2	91±1
Indomethacin	100	67±4	64±2	86±1
Dexamethasone	100	39±1	37±4	46±3

selectively oxidized by the enzyme. In contrast, the NADP$^+$-dependent 3HSD formed only the 3-oxosteroid as the product from 5β-androstane-3α,17β-diol.

The NAD$^+$-dependent 3HSD reduced several 3- and 17-oxosteroids, but the reductase activity was lower than the dehydrogenase activity. Conversely, the NADP$^+$-dependent 3HSD showed higher $k_{cat.}/K_m$ values for 3-oxosteroids than for 3α-hydroxysteroids.

The NAD$^+$-dependent 3HSD preferred NAD(H) to NADP(H) as cofactor. The 5β-androstane-3α,17β-diol dehydrogenase activity with 2 mM-NADP$^+$ was 15% of that with 2 mM-NAD$^+$, and the 5β-androstane-3,17-dione reductase activity with 0.2 mM-NADPH was 27% of that with 0.2 mM-NADH. The K_m values of the enzyme for NAD$^+$ and NADH were 270 and 20 μM, respectively. On the other hand, the NADP$^+$-dependent 3HSD utilized NADP(H) as the preferable cofactor, and the K_m values for NADP$^+$ and NADPH were 3 and 5 μM, respectively.

Synthetic oestrogens, Δ^4-3-oxosteroids and anti-inflammatory agents inhibited both the NAD$^+$- and NADP$^+$-dependent 3HSDs. In addition, the NAD$^+$-dependent enzyme was selectively and potently inhibited by 3β-hydroxysteroids, whereas the NADP$^+$-dependent enzyme was highly sensitive to thiol group reagents (Table 3).

To confirm that the NAD$^+$-dependent 3HSD exhibits dual substrate specificity, we first performed mixed substrate experiments. When two of 5β-androstane-3α,17β-diol, 5β-pregnane-3α,21-diol-20-one and 5β-androstan-17β-ol-3-one were mixed at their respective K_m values, the dehydrogenation rates were about 60–65% of the sum of the rates obtained with the individual substrates. Second, the dehydrogenase activities for 5β-pregnane-3α,21-diol-20-one and 5β-androstan-17β-ol-3-one were similarly inactivated during incubation of this enzyme at 43 °C (Fig. 3). Third, the various inhibitors similarly inhibited both 5β-pregnane-3α,21-diol-20-one and 5β-androstan-17β-ol-3-one dehydrogenase activities of the enzyme (Table 3). The representative inhibitors, medroxyprogesterone acetate, hexoestrol and 5β-androstan-3β-ol-17-one, competitively inhibited with respect to the 3α- and 17β-hydroxysteroids, and the K_i values for the respective inhibitors, which were determined with the two substrates, were similar

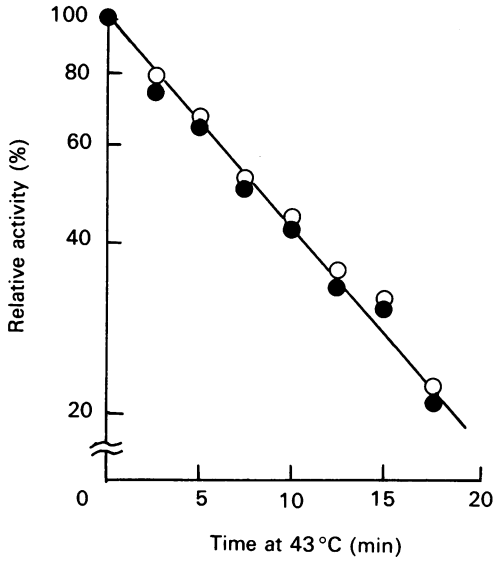

Fig. 3. Thermal stability of 3α- and 17β-hydroxysteroid dehydrogenase activities of NAD$^+$-dependent 3HSD

The enzyme activities were assayed with 6.3 μM-5β-pregnane-3α,21-diol-20-one (○) and 6.3 μM-5β-androstan-17β-ol-3-one (●) as substrate.

Table 4. Inhibition patterns and constants for hexoestrol, 5β-androstan-3β-ol-17-one and medroxyprogesterone acetate

The 3α- and 17β-hydroxysteroid dehydrogenase activities of NAD^+-dependent 3HSD were assayed with 0.8–6.3 μM-5β-pregnane-3α,21-diol-20-one (PDO) and 5β-androstan-17β-ol-3-one (AOO), respectively, as substrate at pH 7.5. The values represent the means ± S.D. of triplicate determinations.

Inhibitor (concentrations, μM)	Varied substrate	Inhibition pattern	K_i (μM)
Hexoestrol	PDO	Competitive	0.11 ± 0.01
(0, 0.1, 0.3, 0.7)	AOO	Competitive	0.17 ± 0.01
5β-Androstan-3β-ol-17-one	PDO	Competitive	0.92 ± 0.34
(0, 0.08, 0.16, 0.3)	AOO	Competitive	0.62 ± 0.07
Medroxyprogesterone acetate	PDO	Competitive	5.3 ± 0.2
(0, 5, 10, 25)	AOO	Competitive	6.2 ± 0.1

(Table 4). These results indicate that a single enzyme protein catalyses the oxidation of 3α- and 17β-hydroxysteroids.

DISCUSSION

The homogeneous NAD^+-dependent and $NADP^+$-dependent 3HSDs from hamster liver oxidized benzene dihydrodiol. The structural and catalytic properties of the $NADP^+$-dependent enzyme are identical with those of one of four dihydrodiol dehydrogenases (F4 in [10]) previously purified from this tissue, and the NAD^+-dependent 3HSD showed cofactor requirement, optimal pH and substrate specificity similar to those reported with another partially purified hamster liver dihydrodiol dehydrogenase (F3-1 in [10]). Thus, we here demonstrate that the two forms of dihydrodiol dehydrogenase in hamster liver are 3HSDs with different specificities for cofactor and steroid.

The $NADP^+$-dependent 3HSD of hamster liver has been reported to cross-react immunologically with mouse liver 3HSD, but not with hamster liver NAD^+-dependent 3HSD [10]. The present studies further reveal the differences of the two hamster liver enzymes in thermal stability, activation energy and peptide mapping, which indicates that the two enzyme proteins are genetically distinct. In addition, the dual substrate specificity of the NAD^+-dependent 3HSD and its selective competitive inhibition by 3β-hydroxysteroids may also reflect a structural difference in active centre between the two enzymes.

It is clear from our studies with the homogeneous preparation that the NAD^+-dependent 3HSD possesses 17β-hydroxysteroid dehydrogenase activity. Since the 3α- and 17β-hydroxysteroid dehydrogenase activities of the enzyme were depressed to similar degrees by various inhibitors, some of which competitively inhibited with respect to both 3α- and 17β-hydroxysteroids, the enzyme may catalyse the dehydrogenation of the different positions of the steroid molecules at the same catalytic site. The enzyme oxidized the 3α-hydroxy group for pregnanes and bile acids, and the 17β-hydroxy group for androstanes. This suggests that the hydroxy group at the A and D rings of the steroid molecule may be able to bind to the active centre of the enzyme, although the enzyme preferentially binds to the hydroxy group of the D ring of androstanes, as demonstrated by the high $k_{cat.}/K_m$ values for testosterone and androstane-3,17-diols. The substrate specificity of hamster liver NAD^+-dependent 3HSD differs from cystolic 3HSD in other mammalian tissues [1–5,17–20], but similar bifunctional enzyme activity has been observed with rabbit liver 17β-hydroxysteroid dehydrogenase, which oxidizes 3α-hydroxyandrostanes in the presence of $NADP^+$ [4,5]. However, hamster liver NAD^+-dependent 3HSD did not oxidize 3α-hydroxyandrostanes and preferred NAD^+ to $NADP^+$ as a cofactor. Although further studies comparing properties of the NAD^+-dependent 3HSD and 17β-hydroxysteroid dehydrogenase in this animal tissue will be necessary before deciding whether the NAD^+-dependent 3HSD is called a 3α(17β)-hydroxysteroid dehydrogenase, an earlier work on 17β-hydroxysteroid dehydrogenase, which reported that the $NADP^+$-linked enzyme activity in hamster liver cytosol was about 2-fold higher than the NAD^+-linked activity [21], suggests that the present enzyme is also distinct from $NADP^+$-dependent 17β-hydroxysteroid dehydrogenase.

Hamster liver NAD^+-dependent 3HSD showed high $k_{cat.}/K_m$ values for 3α-hydroxysteroids which are comparable with the values for the $NADP^+$-dependent 3HSD from the tissue, but the active 3α-hydroxysteroids for the NAD^+-dependent enzyme were limited to pregnanes and some bile acids, and its reverse reaction rates were low. The reductive reaction by 3HSD is important in the biosynthesis of bile acids [22] and catabolism of steroid hormones in liver [23]. In addition, the ratio of NAD^+/NADH is high in mammalian liver cytosol, whereas that of $NADP^+$/NADPH is low. Therefore the contribution of the NAD^+-dependent enzyme as 3HSD to hepatic steroid metabolism may be less than that of the $NADP^+$-dependent enzyme, which exhibited high reductase activity towards various types of 3-oxosteroids. However, the high reactivity of the NAD^+-dependent enzyme to 17β-hydroxy-C_{19} steroids, together with its inhibition by 3β-hydroxy metabolites of androgen, indicates that the enzyme acts as 17β-hydroxysteroid dehydrogenase rather than as 3HSD in regulating the physiological action of androgenic steroids.

REFERENCES

1. Penning, T. M., Mukharji, I., Barrows, S. & Talalay, P. (1984) Biochem. J. **222**, 601–611
2. Ikeda, M., Hattori, H., Ikeda, N., Hayakawa, S. & Ohmori, S. (1984) Hoppe-Seylers Z. Physiol. Chem. **356**, 377–391

3. Hara, A., Inoue, Y., Nakagawa, M., Naganeo, F. & Sawada, H. (1988) J. Biochem. (Tokyo) **103**, 1027–1037
4. Sawada, H., Hara, A., Nakayama, T. & Kato, F. (1979) J. Biochem. (Tokyo) **87**, 1153–1165
5. Hara, A., Kariya, K., Nakamura, M., Nakayama, T. & Sawada, H. (1986) Arch. Biochem. Biophys. **249**, 225–236
6. Sawada, H., Hara, A., Hayashibara, M. & Nakayama, T. (1979) J. Biochem. (Tokyo) **86**, 883–892
7. Hara, A., Hasebe, K., Hayashibara, M., Matsuura, K., Nakayama, T. & Sawada, H. (1986) Biochem. Pharmacol. **35**, 4005–4012
8. Antoun, G. R., Brglez, I. & Williamson, D. G. (1985) Biochem. J. **224**, 383–390
9. Kobayashi, K. & Kochakian, C. D. (1978) J. Biol. Chem. **253**, 3635–3642
10. Sawada, H., Hara, A., Nakagawa, M., Tsukada, F., Ohmura, M. & Matsuura, K. (1989) Int. J. Biochem. **21**, 367–375
11. Platt, K. & Oesch, F. (1977) Synthesis **7**, 449–450
12. Dixon, M., Webb, E. C., Thorne, C. J. R. & Tipton, K. F. (1979) Enzymes, 3rd edn., pp. 164–182, Longman, London
13. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
14. Hara, A., Deyashiki, Y., Nakagawa, M., Nakayama, T. & Sawada, H. (1982) J. Biochem. (Tokyo) **92**, 1753–1762
15. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
16. Hara, A., Kariya, K., Nakamura, M., Nakayama, T. & Sawada, H. (1986) Arch. Biochem. Biophys. **249**, 225–236
17. Koide, S. S. (1969) Methods Enzymol. **15**, 651–656
18. Taurog, J. D., Moore, R. J. & Wilson, J. D. (1975) Biochemistry **14**, 810–817
19. Krause, J. E. & Karavolas, H. J. (1981) J. Steroid Biochem. **14**, 63–69
20. Penning, T. M., Sharp, R. B. & Krieger, N. R. (1985) J. Biol. Chem. **260**, 15266–15272
21. Aoshima, Y. & Kockakian, C. D. (1963) Endocrinology (Baltimore) **72**, 106–114
22. Tchen, T. T. (1960) in Metabolic Pathways (Greenberg, D. M., ed.), pp. 389–429, Academic Press, New York
23. Gower, D. B. (1984) in Biochemistry of Steroid Hormones (Makin, H. L. J., ed.), 2nd edn., pp. 349–382, Blackwell Scientific Publications, Oxford

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