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Steroid Oxidoreductase Activity of Alcohol Dehydrogenases from Horse, Rat, and Human Liver

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Alcohol dehydrogenase from horse (isoenzyme SS and ES, but not EE), rat and human liver were found to catalyze the NAD-dependent oxidation of 3β-hydroxy groups in 5α- and 5β-steroids of the C_{19}, C_{21}, and C_{24} series. The enzymes from horse and rat liver were more active on 5β- than on 5α-steroids. This difference was most marked with the enzyme from rat liver, especially with 3β-hydroxyandrostan-17-ones and 3β-hydroxypregnan-20-ones as substrates. The K_m of isoenzyme ES from horse liver was lower for 3β-hydroxy-5α-cholanoic acid (0.4 μM) than for 3β-hydroxy-5β-cholanoic acid (0.9 μM). 3α-Hydroxysteroids were not substrates for the enzymes from horse and rat liver. Human liver alcohol dehydrogenase had low affinity for 3β-hydroxy-5α (and 5β)-cholanoic acids, but oxidation could be clearly demonstrated by gas chromatographic analysis of the products.

Alcohol dehydrogenase from horse,[1,2] rat,[3,4] and human [5] liver are known to catalyze the NAD-dependent oxidation of 3β-hydroxy-5β-steroids. In the original paper by Ungar,[1] the enzyme from horse liver was reported to be active also on 3β-hydroxy-5α-steroids, but in a more recent study [3] no activity was observed with 3β-hydroxy-5α-androstan-17-one as substrate. The latter study was carried out using an enzyme preparation mainly consisting of isoenzymes EE and ES [6] (at the time called $LADH_E$ and $LADH_S$), and earlier studies on steroid activity of horse liver alcohol dehydrogenase were made without separation of isoenzymes. Since the different isoenzymes (ES and SS) might differ with respect to specificity and kinetic constants, it was considered of interest to extend the previous studies to pure isoenzymes.

The enzymes from rat and human liver have only been tested with 5β-steroids: 3β-hydroxy-5β-cholanoate and 17β-hydroxy-5β-androstan-3-one. The steroid metabolism in human is markedly different from that in rats (see Ref. 7). The effects of ethanol consumption and alcohol dehydrogenase inhibitors on steroid metabolism are incompletely known. For an evaluation of the *in vivo* significance of the steroid activity of liver alcohol dehydrogenase, it should therefore be of value to have a more complete knowledge of species differences in specificity and kinetic constants.

EXPERIMENTAL PROCEDURES

Materials

Steroids. 3β-Hydroxy-5α (and 5β)-androstan-17-one and 17β-hydroxy-5β-androstan-3-one were from Sigma Chemical Company (St. Louis, Mo.). 3α-Hydroxy-5α (and 5β)-androstan-17-one, 3β-hydroxy-5β-pregnan-20-one and 3β-hydroxy-5α-cholanoic acid were obtained from Ikapharm (Ramat-Gan, Israel). 3α-Hydroxy-5β-pregnan-20-one was from Mann Research Laboratories (New York, N.Y.) and purified 3β-hydroxy-5α-pregnan-20-one was a gift from Dr. H. Eriksson. 3α-Hydroxy-5β-cholanoic acid was obtained from Calbiochem (Los Angeles, Calif.) and was recrystallized and served as starting material for synthesis of 3β-hydroxy-5β-cholanoic acid.[8] The purity of the steroids was checked by gas chromatography-mass spectrometry. The 3β-hydroxysteroids were purified for use as substrates in experiments with human LADH by chromatography of bile acid methyl esters on alumina columns, [8] and of neutral steroids on hydroxyalkoxypropyl Sephadex [9] (Lipidex®-5000, Packard, Downers Grove, Ill.)

Table 1. Rate of formation of NADH in incubations of horse liver alcohol dehydrogenase, isoenzyme SS [6] with 3β-hydroxy-5β-cholanoic acid as substrate together with various steroid solubilizers and 0.5 mM NAD^+.

Concentrations in incubation mixture					Rate of
Enzyme μN	Substrate μM	Tween 80 μg/ml	Na glyco-deoxycholate mM	Acetone mM	NADH formation μM/s
0.72	0	0	0	0	17
0.72	0	0.5	0	0	67
0.72	0	0	0.44	0	50
0.72	0	0	0	450	317
0	0	0	0	450	317
0.72	87	0	0	0	1620
0.72	87	0.5	0	0	1820
0.72	87	5	0	0	2640
0.72	87	0	0.44	0	1690
0.72	87	0	0.88	0	1590
0.72	87	0	0	90	1950
0.72	87	0	0	450	2400
0.01	0	0	0	0	2.0[a]
0.01	0	0.5	0	0	3.8[a]
0.01[b]	0	0	0	0	2.3[a]
0.01[b]	0	0.5	0	0	6.5[a]

[a] Measurements carried out in the fluorimeter, otherwise in the spectrophotometer. [b] Isoenzyme ES.[6]

in the solvent system hexane/chloroform, 9:1 (v/v). Several other steroids were obtained from the reference collection of the laboratory.

Sodium glycodeoxycholate was a gift from Dr. A. Norman. Tween 80 (polyoxyethylenesorbitane monooleate) was purchased from Kebo (Stockholm, Sweden). NAD^+, NADH, and $NADP^+$ were obtained from Sigma Chemical Company.

Enzymes. Alcohol dehydrogenase was isolated from livers of horse (Åkeson, Å. and Lundqvist, G., to be published), rat,[4] and human [5] as described elsewhere. The enzyme preparation from human liver contained all isoenzymes.

Methods

Incubations and reactions. The reactions were carried out in 1 cm cuvettes at 23.5 °C in a total volume of 3.0 ml using NAD^+ or $NADP^+$, 0.5 mM in glycine buffer, pH 10.0 and ionic strength 0.1. In some cases the incubations were carried out using NADH, 0.15 mM in phosphate buffer, pH 7.0 and ionic strength 0.1. Ethanol was used in a final concentration of 8.6 mM. Bile acid solutions, 200 – 400 μM in 0.1 M NaOH, were added to give the desired final concentration. All neutral steroids were dissolved in acetone, 1 mg/ml. Appropriate volumes of this stock solution were mixed with a solution of Tween 80 in acetone, and evaporated to give an oily residue. This was dissolved in buffer and added to the incubation mixture to give a final concentration of Tween 80 of 0.5 μg/ml. Enzyme was added in 5 – 20 μl of 0.05 M Tris-HCl buffer, pH 8.6. Measurements were made on a Beckman DK2 recording spectrophotometer at 340 nm, and a recording fluorimeter [10] modified to yield enhanced stability and sensitivity.[11] Rates of reaction were calculated as mol of NADH formed per mol of active sites per second. K_m and $\bar{V}$ values were determined from Lineweaver-Burk plots.

Steroid analysis by gas chromatography and gas chromatography-mass spectrometry. The reactions were stopped 5 – 20 min after the addition of enzyme by addition of 0.2 ml 3 M H_2SO_4, and the steroids were extracted with 3 × 5 ml of ethyl acetate (methylene chloride in experiments with human LADH). The organic phases were pooled and washed until neutral with 1 ml portions of water. The solvents were evaporated, and bile acids were methylated by diazomethane in ether/methanol, 9:1 (v/v). The steroids were converted into trimethylsilyl ethers [12] or *O*-methyloxime trimethylsilyl derivatives [13] which were analyzed by gas chromatography on a 3 % QF-1 column.

Gas chromatography-mass spectrometry was carried out using an LKB 9000 gas chromatography-mass spectrometry instrument (LKB-Produkter AB, Bromma, Sweden), equipped with a 1.5 % SE-30 or a 3 % QF-1 column. The electron energy was 22.5 eV and the ion source temperature was 290 °C.

RESULTS

Horse liver alcohol dehydrogenase

Studies on steroid solubilizers for use in the enzymatic experiments. The reaction rate was constant at least during the first 5 min after addition of enzyme, and initial velocities were measured in the spectrophotometer (Table 1). Without solubilizers or substrate, the rate of formation of NADH was insignificant, and addition of Tween 80 or sodium glycodeoxycholate resulted in very slow rates of NADH formation. Addition of acetone resulted in much higher rates of NADH formation, and this reaction was not dependent on enzyme. None of the solubilizers tested inhibited the oxidation of 3β-hydroxy-5β-cholanoic acid. Acetone and sodium glycodeoxycholate were not sufficiently effective as solubilizers for the neutral steroids. The reaction of NAD^+ and acetone disturbed the measurements in the fluorimeter, as did production of NADH at concentrations of Tween 80 above 0.5 μg/ml. Thus, Tween 80 at a concentration of 0.5 μg/ml was chosen as solubilizer for the neutral steroids.

Activity of horse liver alcohol dehydrogenase isoenzymes with steroids hydroxylated in various positions. Neutral steroids were solubilized in Tween 80, and bile acids were dissolved in sodium hydroxide. Using the spectrophotometer, the steroids were tested at a concentration of 30 μM. Oxidation of the following steroids was not catalyzed by the SS isoenzyme (1.2 μN): 3α,6α-dihydroxy-5β-cholanoate, 3α,7β-dihydroxy-5β-cholanoate, 20β-hydroxy-4-pregnen-3-one, 6β-hydroxy-4-pregnene-3,20-dione, 11β-hydroxy-4-pregnene-3,20-dione, 6β,11α-dihydroxy-4-pregnene-3,20-dione, 20α-hydroxy-4-pregnen-3-one, and 15α-hydroxy-4-androstene-3,17-dione. Under the same conditions, oxidation of the following steroids was catalyzed by the SS isoenzyme (but not by the EE isoenzyme, 0.8 μN): 3β,12β-dihydroxy-5α-androstan-17-one and 5α-androstane-3β,17β-diol. The products were analyzed by gas chromatography-mass spectrometry of trimethylsilyl ethers and *O*-methyloxime-trimethylsilyl ethers on an SE-30 column, and were identified as an androstanoldione and an androstanolone, respectively. Neither the SS isoenzyme (0.1 μN) nor the EE isoenzyme (0.08 μN) catalyzed the oxidation of the following steroids: 17α,21-dihydroxy-4-pregnene-3,11,20-trione, 17α,19-dihydroxy-4,6-preg-

Table 2. Rate of formation of NADH in incubations of horse liver alcohol dehydrogenase isoenzymes (0.01 μN) with 3-hydroxysteroids. The NAD^+ concentration was 0.5 mM.

Isoenzyme	Substrate [a]	Substrate concentration μM	Rate of NADH formation per active site s^{-1}
EE	5βA-3β-ol-17-one	5.75	0
EE	5αA-3β-ol-17-one	5.75	0
EE	5βP-3β-ol-20-one	5.25	0
EE	5αB-3β-ol	1.77	0
ES	5βA-3β-ol-17-one	5.75	0.88
ES	5αA-3β-ol-17-one	5.75	0.49
ES	5βA-3α-ol-17-one	5.75	0.05[b]
ES	5βP-3β-ol-20-one	5.25	0.53
ES	5αP-3β-ol-20-one	5.25	0.34
ES	5βB-3β-ol	8.80	0.22
ES	5αB-3β-ol	8.85	0.15
ES	5βB-3α-ol	8.80	0.01[b]
SS	5βA-3β-ol-17-one	5.75	1.40
SS	5αA-3β-ol-17-one	5.75	0.74
SS	5βP-3β-ol-20-one	5.25	0.98
SS	5αP-3β-ol-20-one	5.25	0.42
SS	5βB-3β-ol	8.79	0.37
SS	5αB-3β-ol	8.85	0.22

[a] Abbreviations: A=androstane, P=pregnane, B=cholan-24-oic acid; greek letters denote configuration of hydroxyl groups. [b] Values not higher than corresponding blank values (Table 1).

nadiene-3,20-dione 17-acetate, and 19-hydroxy-18-nor-4-androstene-3,17-dione. Reduction of 3α,7α-dihydroxy-12-oxo-5β-cholanoate and 3β-hydroxy-5-androsten-17-one was not catalyzed by the SS isoenzyme (1.2 μN).

Stereospecificity of horse liver alcohol dehydrogenase isoenzymes for the configuration at C-3 and C-5 in 3-hydroxysteroids. The following experiments were carried out using the fluorimeter. The fluorescence increase in the absence of substrate was usually small (Table 1) and was not substracted from the values obtained (Table 2). Isoenzyme EE was not active on the steroids studied, which had a 3β,5β or a 3β,5α configuration. Isoenzymes ES and SS both catalyzed the oxidation of steroids having a 3β,5β or a 3β,5α configuration, but not of steroids having a 3α,5β configuration. The ratio between the activities on steroids with these configurations were approximately the same (3β,5β/3β,5α: 1.8 ± 0.3) for both isoenzymes and with the 3β-hydroxyandrostanes, 3β-hydroxypregnanes, and 3β-hydroxycholanoates as substrates. The ratio between the activities of the SS and ES isoenzymes was about the same for the steroids tested (1.6 ± 0.2). The activities were highest with 3β-hydroxyandrostanes and lowest with 3β-hydroxycholanoates.

3α-Hydroxy-5β-cholanoate (44 or 88 μM) inhibited the oxidation of 3β-hydroxy-5β-cholanoate (3.51 μM) completely.

Determinations of K_m *and* V *of isoenzyme ES and SS for 3β-hydroxy-5α(and 5β)-cholanoate.* The K_m values were at 2 μM or below with the two isoenzymes, and at least with isoenzyme ES it was lower with the 3β,5α than with the 3β,5β epimer (Table 3). The *V* values were higher with the 3β,5β epimer than with the 3β,5α epimer. With the ES isoenzyme, substrate inhibition was observed at concentrations of both epimers above 10 μM.

Table 3. Kinetic parameters from Lineweaver-Burk plots for activities of horse liver alcohol dehydrogenase isoenzymes with 3β-hydroxysteroids.

Isoenzyme [a]	Substrate [b]	K_m μM	*V* s^{-1}
ES	ethanol	1700	2.17
ES	5βB-3β-ol	0.9	0.25
ES	5αB-3β-ol	0.4	0.18
SS	ethanol	8000	3.67
SS	5βB-3β-ol	< 2	0.42
SS	5αB-3β-ol	< 0.4	0.23

[b] Abbreviations see Table 2.

Table 4. Rate of formation of NADH in incubations of rat liver alcohol dehydrogenase (0.01 μN) with 3-hydroxysteroids. The NAD^+ concentration was 0.5 mM.

Substrate [b]	Substrate concentration μM	Rate of NADH formation per active site s^{-1}
5βA-3β-ol-17-one	5.75	0.438
5αA-3β-ol-17-one	5.75	0.060
5βA-3α-ol-17-one	5.75	0.047
5αA-3α-ol-17-one	5.75	0.027
5βP-3β-ol-20-one	5.25	0.480
5αP-3β-ol-20-one	5.25	0.050
5βP-3α-ol-20-one	5.25	0.028
5βB-3β-ol	7.4	0.820
5αB-3β-ol	7.4	0.180
5βB-3α-ol	8.9	0.038
—[c]	0.0	0.042
—[d]	0.0	0.022

[b] Abbreviations see Table 2. [c] Blank with Tween 80. [d] Blank without Tween 80.

Rat liver alcohol dehydrogenase

Studies on the stereospecificity for configuration at C-3 and C-5 were carried out using the fluorimeter. The results are shown in Table 4. The enzyme did not catalyze the oxidation of 3α-hydroxysteroids. The activity on 3β,5β-steroids was higher than on 3β,5α-steroids, the difference being most marked with the neutral steroids. In order to confirm that 3β-hydroxy-5α-androstan-17-one and 3β-hydroxy-5α-pregnan-20-one were oxidized, these steroids were incubated for 2 h at concentrations of 12 μM and 31 μM, respectively, using 0.09 μN enzyme and 0.5 mM NAD^+. Gas-liquid chromatography showed that 70 % of the 3β-hydroxy-5α-androstan-17-one and 30 % of the 3β-hydroxy-5α-pregnane-20-one were oxidized.

The reverse reaction was tested with 17β-hydroxy-5α-androstan-3-one (12.6 μM) and 17β-hydroxy-5β-androstan-3-one (12.3 μM), dissolved in methanol (10 μl). The reaction was

followed in the spectrophotometer, and it was observed that the activity was higher with the 3β,5β epimer (rate of NADH decrease per active site: 0.75 s^{-1}) than with the 3β,5α epimer (rate of NADH decrease per active site: less than 0.05 s^{-1}).

Human liver alcohol dehydrogenase

Since it was found that the steroid activity of human liver alcohol dehydrogenase was much lower than that of horse and rat liver alcohol dehydrogenases, it was difficult to make activity measurements with the fluorimeter. However, formation rates of NADH above blank values with Tween 80 were observed with 3β-hydroxy-5β-androstan-17-one (17.2 μM) and 3β-hydroxy-5β-pregnan-20-one (15.7 μM) with an enzyme concentration of 0.7 μN and 0.5 mM NAD^+. There was no reduction of $NADP^+$ (0.5 mM) when incubations were carried out as with NAD^+ and with the substrates listed in Table 4 at concentrations of about 15 μM. However, this lack of activity may be due to the presence of NAD^+ in the commercial $NADP^+$ preparation used.[14]

In a study of the kinetics of the oxidation of 3β-hydroxycholanoic acids by human liver alcohol dehydrogenase, a gas chromatographic method was used to measure conversions. The reaction was stopped after 10 and 20 min, and the mean conversion rate was calculated. With 3β-hydroxy-5β-cholanoic acid as substrate, K_m was about 40 μM and V was about 0.004 s^{-1}. Negative values for these parameters were obtained with 3β-hydroxy-5α-cholanoic acid as substrate. This is probably due to errors in the measurements, but the results indicate that K_m is at least as high as with 3β-hydroxy-5β-cholanoic acid.

DISCUSSION

In previous investigations,[2,3] horse liver alcohol dehydrogenase was found to catalyze the oxidation of 3β-hydroxysteroids, but hydroxyl groups in the 3α-, 7α-, 7β-, 11α-, 12α-, and 17β-positions were not oxidized. In the reverse reaction, 3-oxosteroids yielded 3β-hydroxysteroids, and no reduction was seen with oxo-groups in the 7-, 11-, and 12-positions. In the present study, purified isoenzyme SS was found to be specific for the 3β-hydroxy group and no oxidation was observed with hydroxyl groups in the 3α-, 6α-, 6β-, 7β-, 11α-, 11β-, 15α-, and 20β-positions. The reduction of oxo-groups in the 12- and 17-positions was not catalyzed by the SS isoenzyme.

Since horse liver alcohol dehydrogenase has been observed to catalyze the oxidation of primary hydroxyl groups, some neutral steroids with hydroxyls in the 19- and 21-positions were tried as substrates, but neither the SS nor the EE isoenzyme was active.

Both isoenzyme ES and SS, but not EE, catalyzed the oxidation of 3β-hydroxygroups in 5α- and 5β-steroids of the C_{19}, C_{21}, and C_{24} series. This result differs from those previously obtained,[3] where only steroids of the 5β-series were found to be substrates. The reason for this discrepancy is not known, but it appears likely that it is due to the use in the previous study of a preparation containing only small amounts of isoenzymes active on steroids. With both isoenzymes, V was higher with 5β-cholanoates. At least with isoenzyme ES, K_m was higher with 5β-cholanoates, and these results indicate that at low concentrations of steroids, such as may be present *in vivo*, this enzyme is more active on the 5α-steroids. The ratio between the activities towards C_{19}, C_{21}, and C_{24} steroids and towards 5α- and 5β-steroids were the same with both isoenzymes, indicating that the E subunit does not change the steroid activity of the S subunit.

Alcohol dehydrogenase from rat liver was also found to catalyze the oxidation of 3β-hydroxy groups in 5α- and 5β-steroids of the C_{19}, C_{21}, and C_{24} series, and the activity was higher with 5β-steroids. The difference in activity towards 5β- and 5α-steroids was more marked with the rat enzyme and this difference was also observed in the reverse reaction.

Alcohol dehydrogenase from human liver had a much lower steroid activity than those from horse and rat liver. This was mainly due to high K_m values, both with 3β-hydroxy-5α-cholanoate and with 3β-hydroxy-5β-cholanoate. Thus, alcohol dehydrogenase may be of minor significance in human steroid metabolism.

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