PURIFICATION AND PROPERTIES OF A NAD:3α-HYDROXY-5α-PREGNAN-20-ONE-OXIDOREDUCTASE FROM RAT LIVER MICROSOMES

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ABSTRACT

From rat liver microsomes a NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase was isolated and purified up to a specific activity of 73 nmol/min.mg by affinity chromatography and DEAE-cellulose chromatography. Various Km-values have been determined. The enzyme exhibits highest affinity for 5α-pregnane-3,20-dione and NADH. The 3-oxo group of 5α-dihydrocortisone (17,21-dihydroxy-5α-pregnane-3,11,20-trione) was not reduced by the purified enzyme preparation and NADH and no dehydrogenation with NAD was observed of 3α,11β,17,21-tetrahydroxy-5α-pregnan-20-one. The optimal pH for the hydrogenation of the 3-oxo group was at pH 5.3 and for the dehydrogenation at pH 8.9. Disc gel electrophoresis in presence of 0.1% sodium dodecylsulfate yielded a homogeneous preparation.

INTRODUCTION

Various 3α- and 3β-hydroxysteroid oxidoreductases have been isolated and partially purified from rat liver microsomes (1,2,3). Disc gel electrophoresis of liver homogenate yielded four isoenzymes specific for 3α-hydroxy groups of steroids with varying configuration and varying coenzyme (4). The presence of multiple forms of enzymes is closely related to the cellular regulation of various metabolic activities. The intermediary metabolism of C_{19}- and C_{21}-steroids is essentially dependent on the hepatic 3-hydroxysteroid oxidoreductases which are moreover concerned with the inactivation and excretion of the steroids.

Reduction of the 3-oxo group of C_{19}- and C_{21}-steroids by rat liver microsomal 3-hydroxysteroid oxidoreductases yields mainly 3α-hydroxysteroids and to a lesser extent 3β-hydroxysteroids (5). The overlapping of different activities, however, is a difficult obstacle for the characterization of enzyme specificity. Specificities for cis and trans A/B ring junctures and the 3α-hydroxy or 3β-hydroxy configuration have been discussed (6). Several enzymes have been characterized following their purification in our laboratory. A 3α-hydroxysteroid oxidoreductase with broad affinity towards steroid and cofactor has been separated (1) as well as a highly specific enzyme accepting only 5β-dihydrotestosterone (17β-hydroxy-5β-androstan-3-one) as substrate, which is then converted to both 5β-androstane-3α,17β-diol and 5β-androstane-3β,17β-diol (3). In this report an enzyme is described with a high affinity towards 5α-pregnane-3,17-dione and NADH converting the 3-oxo group into a 3α-hydroxy configuration.

MATERIALS AND METHODS

Liver microsomes: The preparation of liver microsomes from male rats (Wistar) has been described earlier (1).

Solubilization: 10 ml suspension of rat liver microsomes (20-30 mg/ml protein) and 30 ml of a solubilization mixture containing 53.3% (v/v) glycerol, 2% Lubrol WX (w/v) (Cirrasol ALN WF, ICI, Frankfurt, Germany), 0.13 M sodium citrate, 0.13 M potassium chloride and 1.33×10^{-4} M thioglycerol were incubated for 30 minutes at 0°C. The clear solution thus obtained was directly used for further purification.

Enzyme assay: The specific activities in microsomes and in purified fractions obtained by DEAE-cellulose chromatography and affinity chromatography were determined with 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) and 5α-pregnane-3,20-dione as substrates and NADH or NADPH as cosubstrates. The reaction mixture contained in a final volume

of 3.0 ml 0.2 M potassium phosphate buffer pH 5.3 (or varying pH), 0.8 M NADH (NADPH), 0.1 M steroid and varying amounts of microsomal protein. For dehydrogenation of 3α-hydroxy-5α-androstan-17-one and 3α-hydroxy-5α-pregnan-20-one NADP and NAD were used in above concentrations. Incubations were carried out for 5-30 minutes at 37°C. Steroid products were determined by gas-liquid chromatography (1). The nature of formed steroid products was established by comparison of Kovats-indices of pure steroids and by thin-layer chromatography on silica gel G in chloroform/ethanol 95:5 (v/v). Protein concentration was measured using the biuret (7) or Folin (9) method.

Preparation of substituted Sepharose: Sepharose 4B was activated with cyanogen bromide and coupled with the spacer octamethylenediamine. This modified Sepharose was used for the synthesis of 3-oxo-5α-androstan-17ß-yl-succinyl-Sepharose (3).

Purification of NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase Solubilized microsomes (40 ml) were applied to a column of 3-oxo-5α-androstan-17ß-yl-succinyl-Sepharose 4B (8 x 4 cm) which had been equilibrated with 0.01 M potassium phosphate buffer pH 7.0, 40% glycerol, 0.5% Lubrol and 10^{-4} M thioglycerol (buffer A). The microsomal fractions not retained by the Sepharose were located by measuring the absorbance at 280 nm. They were pooled and applied to a DEAE-cellulose column (15 x 5 cm), previously equilibrated with buffer A. After washing the column with 100 ml of buffer A the enzyme in question was eluted with a linear KCl-gradient from 0 to 0.8 M in 800 ml of buffer A. The enzyme was located by measuring its activity. The combined fractions with 3-hydroxysteroid oxidoreductase activity were pooled and dialyzed for 18 hours against buffer A. One fourth of the dialyzed fraction was then applied to above 3-oxo-5α-androstan-17ß-yl-succinyl-Sepharose column in presence of 1 mM NADH at pH 7.0. Washing was carried out with 100 ml of buffer A and 150 ml of buffer A containing 0.1 mM 5α-dihydrotestosterone. Elution was performed with 200 ml of buffer A containing 1 M KCl.

Regeneration of substituted Sepharose: 100 ml of 3-oxo-5α-androstan-17ß-yl-succinyl-Sepharose converted to 3α-hydroxy-5α-androstan-17ß-yl-succinyl-Sepharose by the enzyme preparation was adjusted to pH 8.9 and incubated for 24 hours at room-temperature in presence of 20 ml of undialyzed preparation IV and 1mM NAD. Completion of oxidation of 3-hydroxy groups was confirmed by gas-chromatography of the hydrolyzed product.

Disc gel electrophoresis was carried out according to Maurer (9) in 7% gel in presence or without 0.1% sodium dodecyl sulfate and 4 M urea.

RESULTS AND DISCUSSION

Rat liver microsomal NAD(P):3α-hydroxysteroid oxidoreductases were solubilized by the non-ionic detergent Lubrol WX, were subsequently separated from each other and clearly characterized (1,2,3). As formerly reported (14) the NAD:3α-hydroxysteroid oxidoreductase was activiated by the detergent whereas the activity of NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase was not altered by Lubrol (Table 1). Purification of NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase started with affinity chromatography of solubilized microsomes on 3-oxo-5α-androstan-17ß-yl-succinyl-Sepharose 4B. The NAD(P):3α-hydroxysteroid oxidoreductases active on 5α-di-

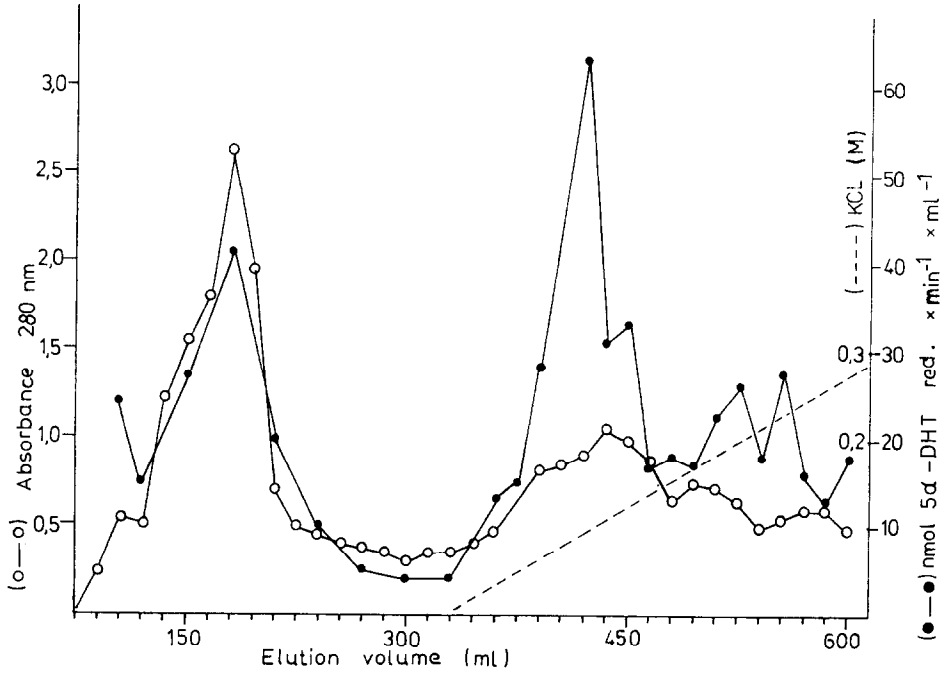

Fig. 1. DEAE-cellulose chromatography with KCl-gradient elution of NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase

hydrotestosterone (1) and other enzymes were adsorbed to the substituted Sepharose. The NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase was not retained and appeared in the front fractions. These fractions contained the 3-equatorial-hydroxysteroid oxidoreductase, too (2). Further purification of NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase included an unselective elution of the enzyme from DEAE-cellulose by a KCl-gradient (Fig. 1). After dialysis the affinity chromatography was repeated on 3-oxo-5α-androstan-17β-yl-succinyl-Sepharose in presence of NADH. The bound 5α-dihydrotestosterone was reduced by the microsomal fraction and NADH to the corresponding 3α-hydroxysteroid derivative. The enzyme was strongly adsorbed to the substituted Sepharose. Unspecific protein was washed out by equilibration buffer. 3-Hydroxysteroid oxidoreductases specific for 5α-dihydrotestosterone still present were eluted by washing with buffer containing 5α-dihydrotestosterone. The NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase was eluted by buffer containing 1 M KCl.

As shown in Table 1 the enzyme specific for 5α-pregnane-3,20-dione could be separated from those enzymes specific for 5α-dihydrotestosterone. During the three steps of purification the ratio of activities of 3-dehydrogenation of 5α-pregnanedione/5α-dihydrotestosterone increased from 1.54 to 15.14. The yield after the second affinity chromatography step was 26%. This is rather high and reflects activation of NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase due to the purification procedure, as is seen in Table 1.

Table 1. Purification of NAD:3α-hydroxy-5α-pregnan-20-one oxidoreductase

Preparation	Vol (ml)	Protein (mg/ml)	Spec. activity (nmol/min·mg) substrate 5α-DHT	substrate 5α-P-dione	Purification (-fold)	Yield (%)	Ratio spec.act. 5α-P-dione/ 5α-DHT
I. Microsomes	10	25.6	8.62	13.27	1	100	1.54
II.Solubilized Microsomes	40	6.4	23.13	13.08	1	100	0.57
III. First affinity chromatography	56	4.0	11.61	17.82	1.3	115	1.53
IV. DEAE-cell. column	120	0.24	20.13	57.88	4.4	49	2.88
V. Second affinity chromatography	200	0.06	4.80	72.67	5.5	26	15.14

Abbreviations: 5α-DHT = 5α-dihydrotestosterone, 5α-P-dione = 5α-pregnane-3,20-dione

Disc gel electrophoresis of purified preparation V showed 3 protein bands in absence and one band in presence of sodium dodecylsulfate. The purified enzyme exhibits an optimal pH for the reduction of the 3-oxo group at pH 5.3 and at pH 8.9 for the oxidation of the 3-hydroxy group. The physiological meaning of such differences in optimal pH for forward and reverse reaction implies a dominance in hydrogenation of 5α-pregnane-3,20-dione in acidic environment so that no glucuronidation is possible since microsomal glucuronyltransferases exhibit an optimal pH at slightly alkaline pH (7.6) with no activity at pH 5.3 (15).

Table 2. Km-values and Vmax for various substrates and cosubstrates of purified NAD:3 hydroxy-5α-pregnan-20-one oxidoreductase (Vmax = nMol/min·ml)

Substrate	Cosubstrate	Km (μM)	Vmax	Correlation coefficient
NADPH	5α-dihydrotestosterone	3.32 ± 0.055	5.50	0.968
NADPH	5α-pregnan-3,20-dione	2.73 ± 0.008	5.50	0.998
NADH	5α-pregnan-3,20-dione	0.31 ± 0.002	6.32	0.992
NADH	5α-dihydrotestosterone	4.20 ± 0.005	1.84	0.960
3α-hydroxy-5-pregnan-20-one	NAD (pH 8.9)	2.55 ± 0.100	2.08	0.982
3α-hydroxy-5-androstan-17-one	NAD (pH 8.9)	0.18 ± 0.002	3.49	0.992
5α-dihydrotestosterone	NADH	0.78 ± 0.012	1.79	0.998
5α-pregnane-3,20-dione	NADH	0.50 ± 0.001	15.2	0.996

The 3-oxo group of 5α-dihydrocortisone was not reduced by the purified enzyme preparation and NADH. No oxidoreduction

with NAD was observed of 3α,11ß,17,21-tetrahydroxy-5α-pregnan-20-one.

Km-Values for various substrates and cosubstrates are shown in Table 2. The low Km-values for NADH and 5α-pregnane-3,20-dione explain the separation of the enzyme from other 3-hydroxysteroid oxidoreductases by its strong retention to the 3-oxo-5α-androstan-17-yl-succinyl-Sepharose 4B column, because 5α-dihydrotestosterone is fixed to the Sepharose by means of the 17-yl-hemisuccinate resulting a structure similar to that of 5α-pregnane-3,20-dione(14). A high Km of the purified enzyme for 3α-hydroxy-5α-pregnan-20-one in presence of NAD and a low Vmax at pH 8.9 if compared to the Km and Vmax values in presence of NADH and 5α-pregnane-3,20-dione support the assumption that reduction of 5α-pregnane-3,20-dione rather than oxidation of 3α-hydroxy-5α-pregnan-20-one is the physiological role of the enzyme.

Microsomal 3-hydroxysteroid oxidoreductases are concerned with the intermediary metabolism of C_{19}- and C_{21}-steroids as well as with their inactivation and excretion. The presence of multiple forms of enzymes has been related to the cellular regulation of metabolic activities. An association of hepatic 3-hydroxysteroid oxidation or 3-oxosteroid reduction of a given steroid with a specific enzyme so far has been impossible since most data have been obtained by whole microsomes (6,10,11,12). It was shown that microsomal 3-hydroxysteroid oxidoreductases are active on different C_{19}-3-oxosteroids (12), C_{21}-3-oxosteroids (13) and C_{27}-3-oxosteroids (13).

Enzymes active on C_{21}-3-oxosteroids are different from those active on C_{24}-, C_{27}- and C_{29}-steroids (6,10). NADH is the preferred donor for the hydrogenation of a 3-oxosteroid towards its corresponding 3α-hydroxy derivative (11) and NADPH for the conversion to the corresponding 3ß-hydroxy derivative (10). Triton extracts of microsomes indicated the presence of several different 3α- and 3ß-hydroxysteroid oxidoreductases in rat liver microsomes with different specificity towards pyridinnucleotides and with overlapping steroid specificity (13). Final characterization of microsomal 3-hydroxysteroid oxidoreductases,however, will only be possible by purification of the enzymes. Three enzymes have been characterized so far. One has a broad specificity towards nucleotide and substrate configuration, because it can use NADPH and NADH as well as 5α- and 5ß-dihydrotestosterone (1). On the other hand an enzyme has been isolated from rat liver microsomes (2) which reduces 3-oxo-5α-steroids to 3ß-hydroxy-5α-steroids and 3-oxo-5ß-steroids to 3α-hydroxy-5ß-steroids by use of NADH or NADPH. A rather specific enzyme is present in microsomes, too, which reduces only 5ß-dihydrotestosterone by NADH to the corresponding 3α(ß)-hydroxy derivative (3). The NADH:3α-hydroxy-5α-pregnan-20-one oxidoreductase described here is another example for the heterogeneity of microsomal 3-hydroxysteroid oxidoreductases.

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REFERENCES

1.) Golf, S.W., Graef, V. and Nowotny, E., Hoppe-Seyler's Z. physiol. Chem. 357, 35 (1976).
2.) Golf, S.W. and Graef, V., FEBS-Letters 64, 215 (1976).
3.) Golf, S.W. and Graef, V., J. Steroid Biochem. 10, 201 (1979).
4.) Matsuzawa, T., Endocrinol. Japon. 25, 321 (1978).
5.) Graef, V., Golf, S.W. and Goerz, G., Arch. Toxicol. 43, 115 (1979).
6.) Björkhem, I. and Gustafsson, J., Eur. J. Biochem. 18, 207 (1971).
7.) Gornall, A.G., Bardawill, C.J. and David, M.M., J. Biol. Chem. 177, 751 (1949).
8.) Wallenfels, K., Zarnitz, M.L., Laule, G., Bender, H. and Keser, M., Biochem. Z. 331, 459 (1959).
9.) Maurer, H.R.: Disc Electrophoresis and related techniques of polyacrylamide gel electrophoresis. Walter de Gruyter, Berlin (1971) p. 144.
10.) Björkhem, I., Einarsson, K., Hellers, G. and Wikvall, K., Acta Endocrinol. 81, 530 (1976).
11.) Björkhem, I., Einarsson, K., Gustafsson, J.-Å. and Somell, A., Acta Endocrinol. 71, 569 (1972).
12.) Shefer, S., Hauser, S. and Mosbach, E.H., J. Lipid Res. 7, 763 (1966).
13.) Björkhem, I. and Einarsson, K., Eur. J. Biochem. 13, 174 (1970).
14.) Graef, V. and Golf, S.W., Z. klin. Chem. klin. Biochem. 13, 333 (1975).
15.) Isselbacher, K.J., Rec. Progr. Horm. Res. XII, 134 (1956).