ISOLATION OF A 3-EQUATORIAL-HYDROXYSTEROID DEHYDROGENASE FROM RAT LIVER MICROSOMES

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1. Introduction

Saturated 3-oxosteroids are reduced to 3α- and 3β-hydroxysteroids by rat liver microsomes with NADH and NADPH as coenzymes [1–3]. The 3-hydroxysteroid dehydrogenases are most probably heterogeneous [4,5] and stereospecific with respect to 3α- and 3β-hydroxysteroids. Some reports, however, indicate a stereospecificity with respect to axial and equatorial 3-hydroxysteroids [6,7]. Recently we have solubilized the 3-hydroxysteroid dehydrogenase from rat liver microsomes and isolated a 3α-hydroxysteroid dehydrogenase which converts both 5α- and 5β-dihydrotestosterone (DHT) to the corresponding 3α-hydroxysteroids [7]. In the present paper we report on the isolation of an equatorial 3-hydroxysteroid dehydrogenase from liver microsomes of male rats.

2. Materials and methods

2.1. *Liver microsomes*

The preparation of liver microsomes from male rats (Wistar) and the solubilization with Lubrol, a non-ionic detergent, have been described [7].

2.2. *Enzyme assay*

The specific activities of 3α- and 3β-hydroxysteroid dehydrogenation in microsomes were determined with 5α-DHT and 5β-DHT as substrates. The reaction mixture contained in a final volume of 3.0 ml: 0.2 M Tris-HCl (optimal pH); 0.8 mM NADH (NADPH); 0.1 mM 5α-DHT (5β-DHT); 0.5 mg microsomal protein, and in the case of the inhibited reaction 10^{-4} M *p*-chloromercuribenzoate (PCMB). T = 37°C; 5 min incubation. The formed 3α- and 3β-hydroxysteroids were determined by gas-liquid chromatography [7]. The protein concentration was measured using to Folin method [8].

2.3. *Preparation of the substituted Sepharose*

Sepharose 4B was activated with cyanogen bromide and coupled with the spacer octamethylenediamine [7]. This modified Sepharose was used for the synthesis of 5α-DHT-Sepharose [7], PCMB-Sepharose [9] and 3β, 17β-dihydroxy-5α-androstane-Sepharose. For the synthesis of the latter 3-oxo-5α-androstane-17β-yl-hemisuccinate [7] was reduced with $NaBH_4$. The reaction product containing 91% 3β-hydroxy-5α-androstane-17β-yl-hemisuccinate and 9% of the 3α-hydroxysteroid derivative was coupled with octamethylenediamine-Sepharose according to the method used for the preparation of 5α-DHT-Sepharose [7].

2.4. *Affinity chromatography*

For washing and elution during affinity chromatography two buffer solutions were used: an undiluted buffer (0.01 M potassium phosphate buffer pH 7.0, containing 40% glycerol, 0.01 M EDTA, 0.5% Lubrol WX and 10^{-4} M thioglycerol) and a diluted buffer (1:4 diluted with water). Fractions of 16 ml were collected in all chromatographic procedures. The columns for affinity chromatography (5α-DHT-Sepharose, PCMB-Sepharose and 3β, 17β-dihydroxy-5α-androstane-Sepharose) were equilibrated with diluted buffer. 40 ml of solubilized liver microsomes [7] were diluted 1:4 with water and applied to the 5α-DHT-Sepharose column (4 × 13 cm). The column was washed with 130 ml of the diluted buffer. Frac-

Table 1
Influence of *p*-chloromercuribenzoate on the specific activities of microsomal 3-hydroxysteroid dehydrogenases at the pH optimum

Substrate	Formed products	NADPH pH optimum		Specific activity (nmoles/min·mg prot.)		NADH pH optimum		Specific activity (nmoles/min·mg prot.)	
		without PCMB	with PCMB	without PCMB	with PCMB	without PCMB	with PCMB	without PCMB	with PCMB
5α-DHT	3α-OH-steroids	6.0	6.0	27.8	7.0	6.0	7.0	41.2	18.2
5α-DHT	3β-OH-steroids	6.5	6.5	23.6	16.8	7.0	7.0	5.4	4.6
5β-DHT	3α-OH-steroids	6.5	6.5	29.0	7.3	6.5	7.0	44.8	9.3
5β-DHT	3β-OH-steroids	6.5	6.5	12.0	3.3	8.5	7.5	7.7	5.2

tions 7–14 (enzymes not adsorbed to the column) were pooled and applied to the PCMB-Sepharose column (4 X 3.5 cm) which was subsequently washed with 50 ml of the diluted buffer. The combined fractions (Nos. 3–10) were applied to the 3β, 17β-dihydroxy-5α-androstane-Sepharose column (3.5 X 6 cm) which was subsequently washed with 80 ml of the diluted buffer. Elution was carried out with 200 ml undiluted buffer containing 1 mM 5α-DHT and 1 M KCl.

3. Results and discussion

The various 3-hydroxysteroid dehydrogenases in rat liver microsomes have probably different pH optima and show a different behaviour towards PCMB. The 3α-hydroxysteroid dehydrogenation by microsomes with NADH and NADPH as coenzymes as a pH optimum at 6.0–6.5 (table 1), whereas the 3α-hydroxysteroid dehydrogenase recently isolated by us has a pH optimum at 7.0. The formation of 3β-hydroxysteroids by rat liver microsomes is best carried out at pH 6.5–7.0 (with 5α-DHT as substrate and NADH and NADPH as coenzymes) or at pH 8.5 (5β-DHT, NADH) or at pH 6.5 (5β-DHT, NADPH). With intact microsomes a broad pH optimum curve is obtained due to the overlapping pH optima of the different 3-hydroxysteroid dehydrogenases.

The 3-hydroxysteroid dehydrogenases in rat liver microsomes are differently sensitive towards PCMB. Obviously there are 3-hydroxysteroid dehydrogenases which are inhibited by PCMB and others which are not inhibited. The 3β-hydroxysteroid dehydrogenation is much less inhibited than the 3α-hydroxysteroid dehydrogenation (table 1). In contrast the purified 3α-hydroxysteroid dehydrogenase [7] is not inhibited by 10^{-4} M PCMB. In the presence of PCMB the PCMB-sensitive 3-hydroxysteroid dehydrogenases are inhibited and the pH optimum curve represents the pH optimum of the PCMB-insensitive 3-hydroxysteroid dehydrogenases.

We have used the different behaviour of the 3-hydroxysteroid dehydrogenases towards PCMB for their separation by affinity chromatography on PCMB-Sepharose. After separation of the PCMB-insensitive 3α-hydroxysteroid dehydrogenase [7] on 5α-DHT-Sepharose a further separation is achieved by affinity chromatography on PCMB-Sepharose. Most of the 3α-hydroxysteroid dehydrogenases are not adsorbed on the PCMB-Sepharose and may be further purified by affinity chromatography on 3β, 17β-dihydroxy-5α-androstane-Sepharose. After elution with KCl (fig.1) an enzyme fraction is obtained which residuces 5α-DHT to 3β, 17β-dihydroxy-5α-androstane and 5β-DHT to 3α, 17β-dihydroxy-5β-androstane with NADPH as coenzyme, i.e. from both substrates only 3-hydroxysteroids with equatorial 3-hydroxy group are formed. Steroids with axial 3-hydroxy group are not formed under these conditions. The data of the purification of the 3-equatorial hydroxysteroid dehydrogenase are represented in table 2.

An equatorial 3-hydroxysteroid dehydrogenase has been presumed, but could not yet be isolated. The purified enzyme has a pH optimum at 6.3 (fig.1) and is not inhibited by 10^{-4} M PCMB. The elution diagram (fig.2) shows that there are possibly two further enzymes for the 3-hydroxysteroid dehydrogenation in the front fractions of the 3β, 17β-dihydroxy-5α-androstane-Sepharose column which are to be studied in further investigations.

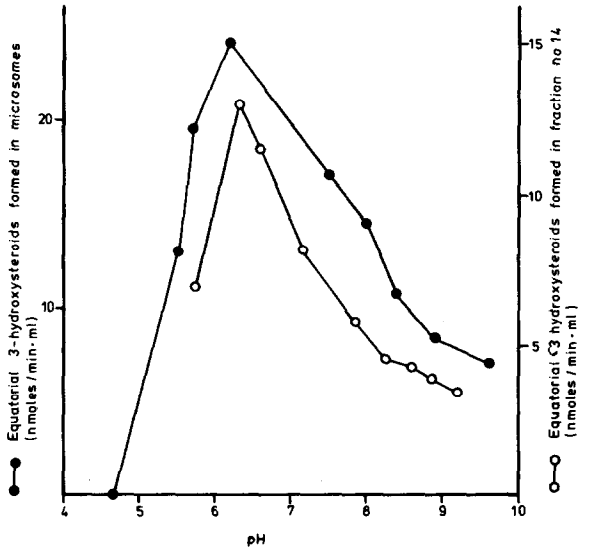

Fig.1. Influence of pH on the activity of the 3-equatorial hydroxysteroid dehydrogenase in liver microsomes; (●——●) 3.0 ml 0.2 M Tris-HCl, 0.8 mM NADPH, 0.1 mM 5β-DHT, 0.5 mg microsomal protein and in fraction No. 14 of the 3β, 17β-dihydroxy-5α-androstane-Sepharose column (○——○); 3.0 ml potassium phosphate buffer, 0.8 mM NADPH, 0.1 mM 5α-DHT, 0.5 ml of fraction No. 14; 37°C, 5 min.

Table 2
Purification of the microsomal 3-equatorial hydroxysteroid dehydrogenase

Fraction	Vol (ml)	Protein (mg/ml)	Specific activity (nmoles/min·mg prot.)	Purification	Yield (%)	Ratio $\frac{\text{equatorial steroids}}{\text{axial steroids}}$
Microsomes	10	28.0	3.02	1	100	0.21
Solubilized microsomes diluted 1:4	112	2.50	3.50	1.16	116.1	0.34
5α-DHT-Sepharose column, pooled front fractions	135	0.54	10.3	3.41	91.0	2.95
PCMB-Sepharose column, pooled front fractions	138	0.29	15.1	5.00	74.6	8.37
5α-androstane-3β-ol-Sepharose column, eluate fraction 14	15	0.007	634.7	209	8.4	∞

The purification data were obtained with 5α-DHT (0.1 mM) as substrate and NADPH (0.8 mM) as cosubstrate in 0.2 M potassium phosphate buffer pH 7.0.

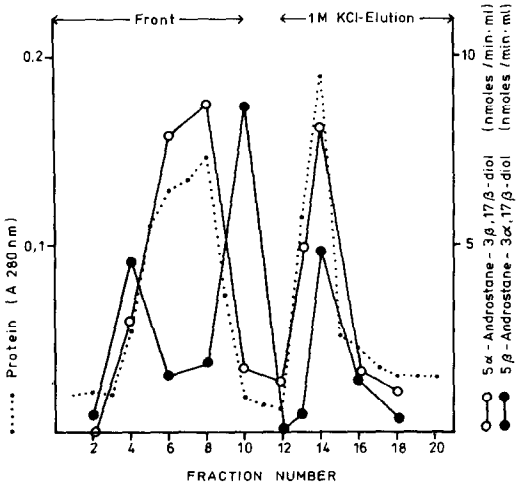

Fig.2. Affinity chromatography on the 3β, 17β-dihydroxy-5α-androstane-Sepharose column. Incubation conditions are the same as in fig.1.

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