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RECONSTITUTION OF NADPH: 4-ENE-3-OXOSTEROID-5α-OXIDOREDUCTASE FROM SOLUBILIZED COMPONENTS OF RAT LIVER MICROSOMES

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SUMMARY

Rat liver microsomal NADPH: 4-ene-3-oxosteroid-5α-oxidoreductase was successfully solubilized by use of the non-ionic detergent Lubrol WX, sodium citrate and glycerol. It consists of 5 fractions which are necessary for maximal enzymatic activity: membraneous fragments, NADPH: cytochrome oxidoreductase, steroid-5α-reductase, phosphatidylcholine and coenzyme Q_{10}. The separation of 4 of these fractions was achieved by affinity chromatography on testosterone-linked Sepharose and Sepharose-testosterone mixture. The fraction containing steroid-5α-reductase was partially separated into a testosterone-5α-reductase and a progesterone-5α-reductase by isoelectric focusing. The participation of NADPH:cytochrome oxidoreductase could be proved by inhibition of the testosterone-5α-reduction by rabbit antibodies against NADPH:cytochrome oxidoreductase.

INTRODUCTION

NADPH:4-ene-3-oxosteroid-5α-oxidoreductase in rat liver, a membrane-bound enzyme system, is responsible for the conversion of 4-ene-3-oxosteroids to the corresponding 3-oxo-5α-dihydrosteroids. We succeeded in solubilizing this system by means of Lubrol WX, a non-ionic detergent [1]. Several fractions participate in the 5α-reduction of testosterone. The electrons are transferred from NADPH to coenzyme Q_{10} via NADPH:cytochrome oxidoreductase and then to testosterone by a testosterone-5α-reductase. The enzyme system has been partially resolved by affinity chromatography with a testosterone-17 linked Sepharose column [2]. We have continued these experiments and have found that further fractions participate in the 4-ene-3-oxosteroid-5α-reduction.

EXPERIMENTAL

Liver microsomes. Preparation of liver microsomes from female rats (Wistar) and chemical reagents have been described elsewhere [3, 4, 5]. Lubrol WX (Cirrasol Aln Wf) was obtained from ICI, D-6000 Frankfurt, Germany.

Enzyme assay. The activities of NADPH: 4-ene-3-oxosteroid-5α-oxidoreductase were determined with testosterone, progesterone and cortisone as substrates. The reaction mixture contained in a final vol. of 3.0 ml: 0.2 M potassium phosphate buffer pH 7.0, 0.4 mM NADPH or 0.1 mM reduced coenzyme Q_{10}, 0.1 mM steroid and a varying protein concentration. Incubation time 10–30 min at 37°C. Formed products were determined by gas-liquid chromatography [4, 5].

Preparation of substituted Sepharose. The methods for coupling of Sepharose 4B with octamethylenediamine as spacer and the reaction of substituted Sepharose with testosterone-17β-yl-hemisuccinate have been described in a previous communication [4]. The testosterone-linked Sepharose used for the separation of microsomal NADPH:4-ene-3-oxosteroid-5α-oxidoreductase contained 9.52 μg testosterone/mg dry Sepharose.

Solubilization. A suspension of rat liver microsomes (10 ml) containing 200–300 mg protein was stirred for 30 min at 0°C with 16 ml of glycerol, 4 ml 1 M KCl, 4 ml 1 M sodium citrate, 2 ml 10% Lubrol WX, 0.01 ml thioglycerol and 4 ml water. The supernatant obtained after centrifugation (1 h, 105 000 *g*) was used for affinity chromatography.

Affinity chromatography on testosterone linked Sepharose. The washing buffer contained 0.01 M potassium phosphate pH 7.0, 40% glycerol, 0.5% Lubrol, 10^{-4} M thioglycerol, 0.01 M EDTA and 1 mM $NADP^+$. The same buffer containing 1 M KCl was used as elution buffer. 40 ml of solubilized liver microsomes were applied to the column (3.3 × 13 cm) containing testosterone-linked Sepharose. The column was washed with 50 ml of washing buffer. Elution was carried out with 250 ml of elution buffer. Samples with a volume of 8 ml were collected.

Affinity chromatography on Sepharose/crystallized testosterone. A column (3.5 × 15 cm) was packed with a mixture of 40 ml Sepharose and 20 g testosterone and equilibrated with 0.01 M potassium-phosphate buffer pH 7.0 containing 40% glycerol, 0.5% Lubrol, 10^{-4} M thioglycerol and 0.01 M EDTA. 5 ml of liver microsomes were solubilized and applied to the

column. The column was washed with 50 ml of the same buffer and eluted with elution buffer which contained 1 M KCl.

Isoelectric focusing. The principal method has already been described [6]. Tubes No. 28 and 29 (16 ml) from affinity chromatography on testosterone linked Sepharose were used as protein solution for isoelectric focusing.

Determination of phosphatidylcholine in fractions of affinity chromatography. The samples obtained by affinity chromatography were extracted with 3 × 25 ml chloroform–methanol (2:1). Phosphatidylcholine was isolated from the extracts by thin layer chromatography on silica gel/magnesium silicate (20:2 w/w) in chloroform–methanol–water (97:37:6). The spots of pure phosphatidylcholine were colored by iodine and according to Dragendorff [7]. Phosphate in the eluate was determined by the method of Debuch [8].

Determination of coenzyme Q_{10}. Coenzyme Q_{10} was determined according to the method of Rokos [9]. The sample was dissolved in 5 ml 0.067 M KOH in freshly distilled ethanol:cyanoacetate (1:3, v/v). The stoppered tubes were kept in the dark at room temperature. Fluorescence was measured after six days (excitation 430 nm, emission 520 nm). For CoQ_{10} determination of fractions obtained by affinity chromatography, 3 ml samples were extracted with 3 × 3 ml of petrol ether (boiling range 40–60°C). CoQ_{10} was separated by thin layer chromatography on silica gel in chloroform–benzene (50:50, v/v) and its fluorescence was measured as described above.

Extraction of phosphatidylcholine and coenzyme Q_{10} from rat liver microsomes. 10 ml of a rat liver microsomal suspension (32 mg protein/ml) were frozen at −60°C and lyophilized for 2.5 h. One-hundred milligrams of lyophilized microsomes were stirred with 5 ml of acetone for 10 min in an ice bath and centrifuged at 10,000 *g* (10 min). After removing the supernatant the residue was suspended in 3 ml of 0.1 M potassium phosphate buffer pH 7.0 and homogenized in a Potter–Elvehjem homogenizer.

RESULTS

Solubilization

Solubilization of microsomal NADPH: 4-ene-3-oxosteroid-5α-oxidoreductase has been achieved by the non-ionic detergent Lubrol WX in the presence of high concentration of glycerol (40%), sodium citrate (0.1 M) and potassium chloride (0.1 M). The NADPH:4-ene-3-oxosteroid-5α-oxidoreductase remained in the supernatant after centrifugation at 105,000 *g*. It appears that sodium citrate is essential for successful solubilization [1].

Affinity chromatography on testosterone-linked Sepharose

NADPH:4-ene-3-oxosteroid-5α-oxidoreductase from rat liver microsomes consists of several fractions which were partially separated by affinity chromatography on testosterone-linked Sepharose (Fig. 1). The testosterone concentration bound to the

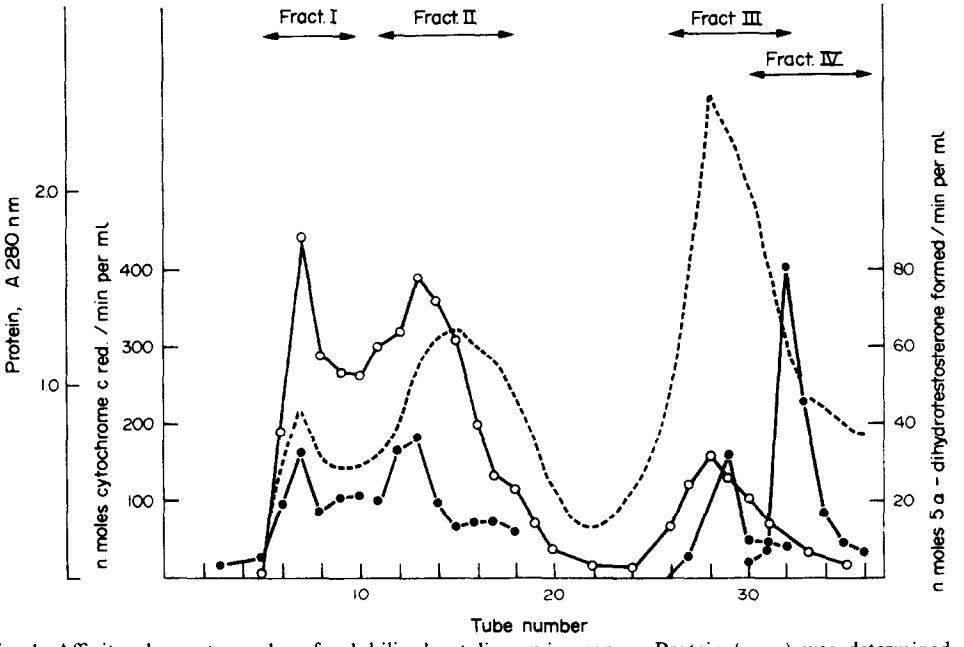

Fig. 1. Affinity chromatography of solubilized rat liver microsomes. Protein (-----) was determined by the absorption at 280 nm of the 1:6 diluted samples. NADPH:cytochrome-oxidoreductase activity (O——O) was estimated spectrophotometrically [3]. The NADPH: 4-ene-3-oxosteroid-5α-oxidoreductase activity (●——●) in the tubes of fraction I was determined in the presence of 0.05 ml aliquots of tubes No. 15, 28 and 34. The activity in the tubes of fraction II (+0.05 ml of No. 7, 28 and 34), fraction III (+0.05 ml of No. 7, 15 and 34) and fraction IV (+0.05 ml of No. 7, 15 and 28) was determined accordingly. Substrate:testosterone, time of incubation: 30 min, hydrogen donor: NADPH.

Sepharose (containing the spacer octamethylenediamine) was crucial for the reproducibility of the separation. Affinity chromatography on testosterone-linked Sepharose with a higher content of testosterone (15.57 μg steroid/mg dry Sepharose) yielded no usuable separation of NADPH:cytochrome oxidoreductase from testosterone-5α-reductase. After elution of the column 4 fractions were separated which contained NADPH:4-ene-3-oxosteroid-5α-oxidoreductase activity after recombination. All 4 fractions were necessary for maximal enzymatic activity. The first three fractions (I, II, III) were completely inactivated by incubation at 100°C for 10 min. The fourth fraction (IV) was not denatured by boiling. Two fractions (I and II) were not adsorbed to the column, but were separated from each other by the molecular sieve effect of the Sepharose.

Fraction I contained probably membraneous fragments which were necessary to restore the activity of NADPH:4-ene-3-oxosteroid-5α-oxidoreductase. An identification of this fraction was not carried out.

Fraction II contained NADPH:cytochrome oxidoreductase, because it was able to reduce cytochrome c with NADPH (Fig. 1). In recombination experiments it was possible to substitute fraction II by purified NADPH:cytochrome oxidoreductase [3]. We could prove the participation of NADPH:cytochrome oxidoreductase in testosterone-5α-reduction by use of rabbit antiserum against NADPH:cytochrome oxidoreductase. Testosterone-5α-reductase activity in rat liver microsomes was inhibited by addition of antibodies against NADPH:cytochrome oxidoreductase (Fig. 2). The coenzyme Q_{10}-reduction in rat liver microsomes was inhibited by these antibodies, too.

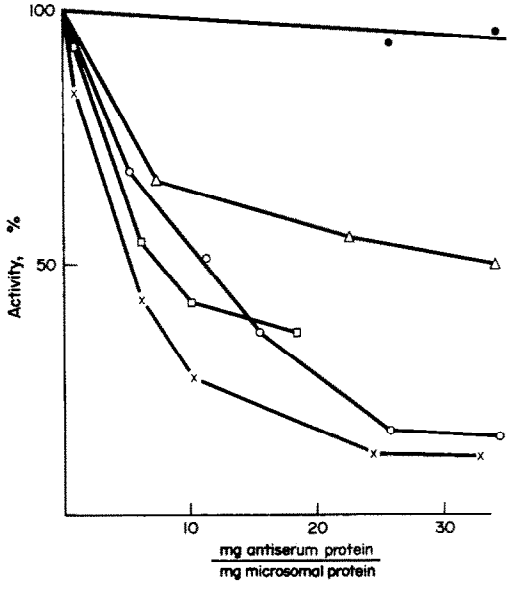

Fig. 2. Inhibition of rat liver microsomal enzyme activities by antiserum against NADPH:cytochrome oxidoreductase. O——O NADPH:testosterone-5α-reduction, pH 7.0, 11.4 nmol·min^{-1}·mg^{-1} = 100%. ●——● NADPH:testosterone-5α-reduction with non-immune antiserum. ×——× NADPH:cytochrome c reduction, pH 7.7, 168.6 nmol·min^{-1}·mg^{-1} = 100%. □——□ NADPH:cytochrome c reduction, pH 7.0, 128.7 nmol·min^{-1}·mg^{-1} = 100% △——△ NADPH:coenzyme Q_{10} reduction, pH 7.7, 27.3 nmol·min^{-1}·mg^{-1} = 100%.

Table 1. Steroid-5α-reductase activity of the protein peak fractions from isoelectric focusing

Steroid substrates	Peak fractions from isoelectric focusing (nmol reduced steroid/min/ml) III A	III B	III C
(a) with NADPH			
Testosterone	31.93	13.66	0.63
Progesterone	4.30	34.18	6.76
Cortisone	7.13	32.55	0
(b) with reduced CoQ_{10}			
Testosterone	55.83	29.75	0

The reaction mixture contained in a total volume of 3.0 ml (a) 0.05 ml of fraction III A, III B and III C, respectively, obtained by isoelectric focusing, 0.05 ml of fractions I, II and IV from affinity chromatography, 0.1 mM steroid and 0.4 mM NADPH as hydrogen donor. The reaction mixture (b) contained 0.05 ml of fraction III A, III B or III C, 0.1 mM testosterone and 0.1 mM reduced coenzyme Q_{10}.

Fraction III, which contained testosterone-5α-reductase, was completely eluted by buffer containing 1 M KCl. A complete separation of NADPH:cytochrome oxidoreductase and testosterone-5α-reductase was not possible by affinity chromatography on testosterone-linked Sepharose.

For further purification fraction III was subjected to isoelectric focusing. A separation into 3 peaks was obtained. The peak fractions III A, III B and III C were tested for different specificity toward testosterone, progesterone and cortisone as substrates. Each one of these protein fractions was by itself inactive toward reduction of 4-ene-3-oxosteroids in the presence of NADPH, however in the presence of fractions I, II and IV considerable NADPH:4-ene-3-oxosteroid-5α--oxidoreductase activity was shown (Table 1). A partial separation of testosterone- and progesterone-5α-reductase activity was observed. While testosterone-5α-reductase activity was mainly found in fraction III A, progesterone- and cortisone-5α-reductase activities were highest in fraction III B. The fractions obtained by isoelectric focusing were able to reduce testosterone by reduced coenzyme Q_{10}.

Fraction IV, which was necessary to obtain maximal enzymatic activity of NADPH:4-ene-3-oxosteroid-5α-oxidoreductase, was heat resistant. Sonicated phosphatidylcholine [10] was able to replace this factor (Table 2). We found that 20 mg phosphatidylcholine/mg protein were most effective. It is noted that exogenic phospholipid micelles at least partially restore the activity of NADPH:4-ene-3-oxosteroid-5α-oxidoreductase even in the absence of fraction I. The eluate fractions obtained after affinity chromatography were analyzed for phosphatidylcholine content. It was found that two fractions of the eluate

Table 2. Influence of phosphatidylcholine (PC) on solubilized and recombined NADPH:4-ene-3-oxosteroid-5α-oxidoreductase

mg PC/mg protein	S.A. of NADPH:4-ene-3-oxosteroid-5α-oxidoreductase (nmol/min·mg) Solubilized microsomes	Recombined steroid-5α-reductase (fractions I, II and III)	Recombined steroid-5α-reductase (fractions II and III)
—	5.69	22.00	10.90
10	13.65	26.09	10.12
20	15.49	42.04	24.65
80	9.16	6.40	4.71

The reaction mixture was incubated for 10 min at room temperature before starting the reaction by addition of NADPH.

(III and IV). contained phosphatidylcholine (Fig. 3). During solubilization microsomal specific enzyme activity decreased from 30.00 ± 1.71 nmol/min/mg to 5.69 ± 0.5 nmol/min/mg. The activity increased again by addition of sonicated phosphatidylcholine (20 mg/mg protein) to 15.49 nmol/min/mg. Other phospholipids (phosphatidylethanolamine and phosphatidylserine) were unable to substitute phosphatidylcholine.

Affinity chromatography on Sepharose/crystallized testosterone

As shown in Fig. 1 the separation of NADPH:cytochrome oxidoreductase from steroid-5α-reductase by affinity chromatography on testosterone-linked Sepharose was incomplete. The eluate fraction (III) contained NADPH:cytochrome oxidoreductase and steroid-5α-reductase. Even by variation of chromatography conditions the complete separation of both enzymes was not possible.

Finally we succeeded in obtaining a preparation of steroid-5α-reductase which contained no NADPH:cytochrome oxidoreductase. Solubilized microsomes were applied to a column filled with a mixture of Sepharose 4B and crystallized testosterone. By washing with buffer solution all NADPH:cytochrome oxidoreductase was removed (Fig. 4). The elution was performed with buffer containing 1 M KCl. The eluate tube No. 15 contained steroid-5α-reductase. Testosterone was not reduced by this sample in the presence of NADPH, however, by addition of sample No. 5 or purified NADPH:cytochrome oxidoreductase testosterone was reduced.

Participation of coenzyme Q_{10} in the 5α-reduction of testosterone

Coenzyme Q_{10} was isolated from rat liver microsomes by extraction with *n*-hexane [12] and subsequent thin layer chromatography. The isolated

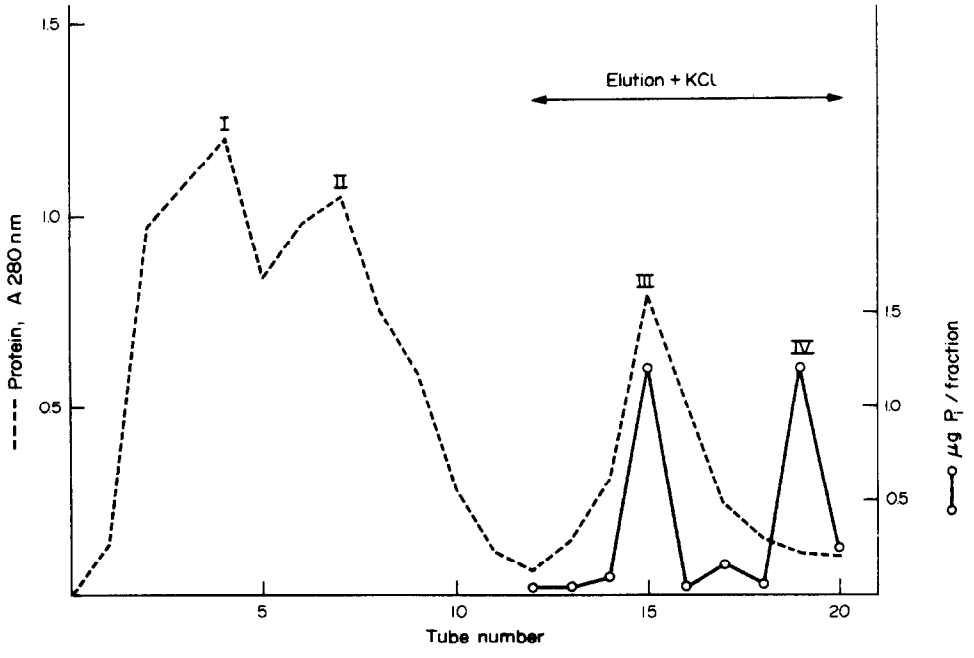

Fig. 3. Phosphatidylcholine content (expressed in μg inorganic phosphorus) in fractions of affinity chromatography of solubilized rat liver microsomes.

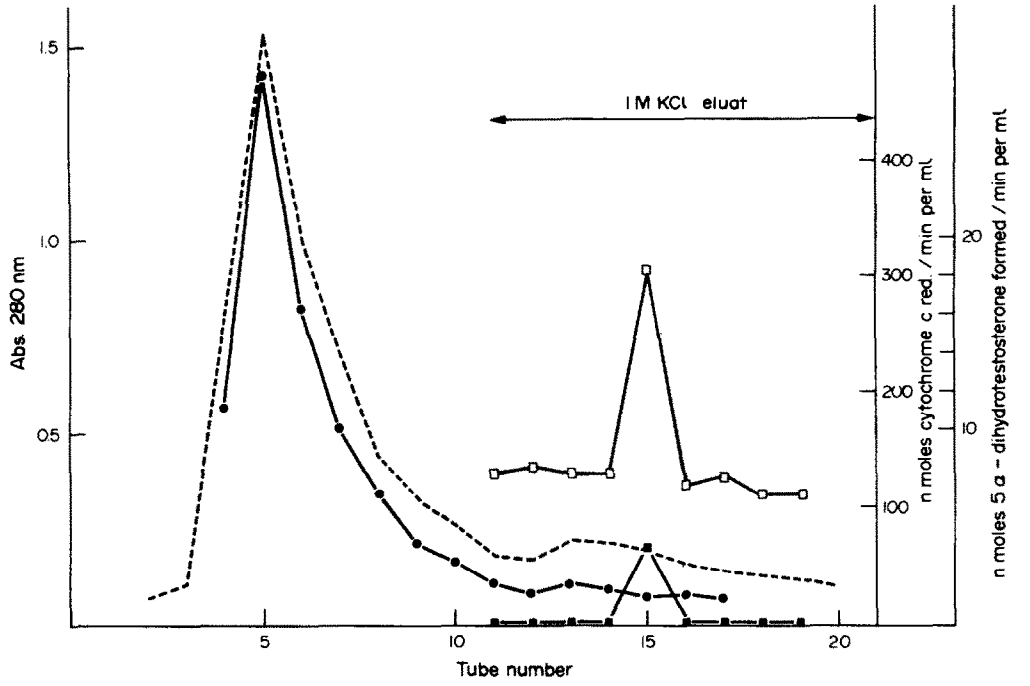

Fig. 4. Affinity chromatography on Sepharose/cryst. testosterone of solubilized rat liver microsomes. Protein (------) was determined by the absorption at 280 nm. NADPH:cytochrome oxido-reductase activity (●——●) was determined spectrophotometrically [3]. NADPH: 4-ene-3-oxosteroid-5α-oxido-reductase activity was measured in presence of 0.05 ml of tube 5 (□——□) and in presence of purified NADPH:cytochrome oxidoreductase [3] (■——■) and phosphatidylcholine (20 mg/mg protein).

coenzyme Q_{10} had the same R_F as pure coenzyme Q_{10} in two solvent systems (chloroform–benzene (1:1) and benzene) and produced a blue color after spraying the chromatogram with leucomethyleneblue. The microsomal fraction contained 0.207 nmol coenzyme Q_{10}/mg protein. All fractions obtained by affinity chromatography of solubilized microsomes on testosterone-linked Sepharose were analyzed for coenzyme Q_{10} content. Coenzyme Q_{10} was uniformly distributed in all fractions. Each of them contained 3.8–6.6 μg coenzyme Q_{10}.

The participation of coenzyme Q_{10} in testosterone 5α-reduction was proven by the following experiment: Lyophilized microsomes were extracted with acetone. By this procedure phospholipids and coenzyme Q_{10} were removed. The extracted microsomes had lost testosterone-5α-reductase activity (Table 3) and remained inactive after addition of coenzyme Q_{10}. The activity of extracted microsomes was very low, too, after addition of phosphatidylcholine, but increased markedly in the presence of phosphatidylcholine and coenzyme Q_{10}.

DISCUSSION

In previous papers [2, 6] we have found that NADPH:4-ene-3-oxosteroid-5α-oxidoreductase in rat liver microsomes is an enzyme system which consists of several fractions. In the reduction of the double bond the electrons are transferred from NADPH to coenzyme Q_{10} via a NADPH:cytochrome oxido-reductase and then to testosterone.

We succeeded in solubilizing this enzyme system by means of Lubrol, a non-ionic detergent, in the presence of sodium citrate. The solubilized NADPH:4-ene-3-oxosteroid-5α-oxidoreductase was partially separated by affinity chromatography on testosterone-linked Sepharose. Our experiments show that five fractions are necessary for the maximal enzymatic activity: membraneous fragments, NADPH:cytochrome oxidoreductase, steroid-5α-reductase, phosphatidylcholine and coenzyme Q_{10}. Exogenic phosphatidylcholine at least partially restores the activity of recombined NADPH:4-ene-3-oxosteroid-5α-oxido-

Table 3. Testosterone-5α-reduction with lyophilized and acetone-extracted microsomes

Enzyme source	Additions	S.A. (nmol/min·mg)
Lyophilized microsomes	—	6.44
Lyophilized and acetone-extracted microsomes	—	0
	0.1 mM CoQ_{10}	0
	Phosphatidylcholine (20 mg/mg protein)	0.58
	Phosphatidylcholine (20 mg/mg protein) and 0.1 mM CoQ_{10}	2.12

reductase even in the absence of fraction I (membraneous fragments).

Fraction II (NADPH:cytochrome oxidoreductase) could be substituted by purified NADPH:cytochrome oxidoreductase in the recombination experiments. The participation of this enzyme could be proved by inhibition of testosterone-5α-reduction by rabbit antibodies against NADPH:cytochrome oxidoreductase. As expected the coenzyme Q_{10} reduction by NADPH in rat liver microsomes was inhibited by these antibodies, too.

The third fraction which was separated from solubilized NADPH:4-ene-3-oxosteroid-5α-oxidoreductase by affinity chromatography was a steroid-5α-reductase. This fraction was separated into two 5α-reductases by isoelectric focusing: a testosterone-5α-reductase and another fraction responsible for progesterone and cortisone reduction. The testosterone-5α-reductase was capable to reduce testosterone with reduced coenzyme Q_{10}. When solubilized microsomes were separated by affinity chromatography on testosterone-linked Sepharose the fraction of steroid-5α-reductase contained some NADPH:cytochrome oxidoreductase. Even by variation of the experimental conditions a complete separation of both enzymes was not possible. However, by affinity chromatography on Sepharose/crystallized testosterone a steroid-5α-reductase was obtained which was free from NADPH:cytochrome oxidoreductase.

By affinity chromatography we isolated an additional fraction containing phosphatidylcholine which was also required for maximal activity of NADPH:4-ene-3-oxosteroid-5-oxidoreductase. Lumper *et al.* [11] found that phospholipids probably participate in the 4-ene-3-oxosteroid-5α-reduction of rat liver microsomes. By treatment of rat liver microsomes with phospholipase C the NADPH:4-ene-3-oxosteroid-5α-oxidoreductase was completely inactivated. In our recombination experiments we found that other phospholipids were not capable to substitute phosphatidylcholine. A concentration of 20 mg phosphatidylcholine/mg protein was optimal for the testosterone-5α-reduction.

The participation of coenzyme Q_{10} in NADPH:4-ene-3-oxosteroid-5α-reduction could not be proved by recombination experiments with fractions obtained by affinity chromatography, because coenzyme Q_{10} was uniformely distributed in all fractions. But the participation of coenzyme Q_{10} could be shown by another experiment. Lyophilized and with acetone extracted microsomes had no NADPH:4-ene-3-oxosteroid-5α-reductase activity. Coenzyme Q_{10} and phosphatidylcholine were removed by acetone extraction. By addition of phosphatidylcholine and coenzyme Q_{10} the testosterone-5α-reduction was possible again. However, the activity was lower than in original microsomes, because the enzymes were partially inactivated by treatment with acetone. Extraction of lyophilized microsomes with acetone for more than 10 min resulted in a complete inactivation and testo sterone-5α-reduction was not restored by addition of phosphatidylcholine and coenzyme Q_{10}. Another indication for the participation of coenzyme Q_{10} in the NADPH:4-ene-4-oxosteroid-5α-oxidoreductase was the observed 5α-reduction of testosterone in the presence of reduced coenzyme Q_{10} and testosterone-5α-reductase which was obtained by isoelectric focusing.

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