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SHORT COMMUNICATION

SOLUBILIZATION OF NADH: 4-ENE-3-OXOSTEROID-5α-REDUCTASE FROM RAT LIVER MICROSOMES

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SUMMARY

The conditions for the solubilization of NADH: 4-ene-3-oxosteroid-5α-reductase from a rat liver microsomal preparation were studied. The optimal solubilization mixture contained 0.2 M potassium phosphate buffer pH 7.0, 0.25% of the non-ionic detergent Lubrol (Cirrasol ALN-WF), 1 mM NADH, 0.2 M sodium citrate, 0.5 mM thioglycerol and 2.5–5 mg microsomal protein/ml. Phosphatidylcholine micelles (20 mg/mg protein) were essential for enzyme activity.

INTRODUCTION

In rat liver microsomal preparations the 4,5-double bond of testosterone and other 4-ene-3-oxosteroids is reduced with NADPH as hydrogen donor. Leybold and Staudinger[1, 2] found that in rat liver microsomal preparations the 4,5-double bond of 4-ene-3-oxo-steroids can also be reduced with NADH. This reaction is possible only in the presence of phosphate ions. Recently we obtained evidence that the steroid 5α-reduction with NADPH and NADH is catalysed by two different enzymes [3]. For the further investigation of NADH: 4-ene-3-oxosteroid-5α-reductase it was necessary to solubilize this enzyme from a rat liver microsomal preparation. Whereas we were able to solubilize the NADPH dependent 5α-reductase with a mixture containing 40% glycerol, 0.5% of the non-ionic detergent Lubrol, 0.1 M sodium citrate, 0.1 M KCl and 2.5 mM thioglycerol [3], the solubilization of the active NADH dependent 5α-reductase was not possible under these conditions. The enzymatic activity was almost completely destroyed when the microsomal preparation was treated with this mixture. In the present paper we have studied the conditions for the solubilization of NADH: 4-ene-3-oxosteroid-5α-reductase.

EXPERIMENTAL

Reagents

Lubrol (Cirrasol ALN-WF) was obtained from ICI, Frankfurt. Tween 20, phosphatidylcholine and other phospholipids were provided by Serva, Heidelberg.

Solubilization

Female rats (Wistar) were used for preparation of a microsomal fraction [4]. 2 ml of the solubilization mixture were stirred under N_2 for 30 min at 0°C and then centrifuged at 105,000 *g* for 1 h at 0°C. Supernatant and sediment were separated and the pellet was suspended in 2 ml of 0.2 M potassium phosphate buffer pH 7.0.

Enzyme assay

NADH: 4-ene-3-oxosteroid-5α-reductase activity was assayed in 3.0 ml reaction mixtures containing 0.2 M potassium phosphate buffer pH 7.0, 0.1 ml supernatant or sediment-suspension, 0.2 mM testosterone, 0.8 mM NADH and 20 mg phosphatidylcholine/mg protein in micellar distribution. Sonication of phosphatidylcholine in 0.2 M phosphate buffer pH 7.0 with cooling was carried out with a Branson "sonic power" sonifier (Branson Instruments Inc. Danbury, CT., U.S.A.) at position 3–4. Enzyme activity of crude microsomal preparations (0.2–0.3 mg protein) was assayed without phosphatidylcholine. Incubation temperature 37°C, incubation time 10 min. The reaction was stopped by extraction with ether (2 × 4 ml) and the combined ether extracts were evaporated under N_2. 0.1 ml of acetone, 1 ml of CrO_3 solution (7% in H_2O) and 4 drops of H_2SO_4 (65%) were added. After 10 min the solution was made alkaline by adding 1 ml of 5 M NaOH and then extracted with ether (2 × 4 ml). The combined extracts were evaporated under N_2. Cholesterol acetate was added as internal standard and an aliquot of the solution was analysed by gas–liquid chromatography (Beckman GC-M), using a glass column (2 m) with 3% OV 17 on Gas-Chrom Q (Serva, Heidelberg), (column temperature 275°C, detector 300°C). 5α-Androstane-3,17-dione was measured. The recovery was 90.4% ± 1.5%.

RESULTS AND DISCUSSION

The enzymatic activity of NADH: 4-ene-3-oxosteroid-5α-reductase in rat liver microsomal preparations, measured in Tris buffer pH 7.0 in the absence of phosphate, was very low (3 nmol/min·mg protein). The activity was enhanced with increasing concentrations of phosphate up to 40 nmol/min·mg protein. A phosphate concentration of 0.2 M was optimal. The temperature optimum of the reaction shifts from 37°C to 27°C when the phosphate concentration is decreased from 0.1 M to 0.01 M. It was found that phosphate is necessary for stabilizing and activating the enzyme. In the first attempts to solubilize the NADH: 4-ene-3-oxosteroid-5α-reductase the conditions for successful solubilization of the NADPH: 4-ene-3-oxosteroid-5α-reductase were used. The medium consisted of 10 ml of a microsomal suspension in 0.1 M potassium phosphate buffer pH 7.0, 16 ml of glycerol, 6 ml of 10% detergent solution (Lubrol, Tween 20, sodium desoxycholate, Lensodel NP 40, Lensodel NP 80, Triton X 100, Triton N 101 or Triton N 111), 4 ml 1 M KCl, 4 ml 1 M sodium citrate and 0.02 ml of thioglycerol. Microsomal proteins were

* The results presented are part of G. Rempeter's doctoral thesis.

solubilized by this treatment, but the NADH: 4-ene-3-oxosteroid-5α-reductase was inactivated. Further attempts to minimize inactivation and maximize solubilization are listed in Table 1.

When microsomal preparations were stirred in an aqueous Lubrol solution the supernatant contained no 5α-reductase activity and the enzyme in the sediment was largely inactivated. After addition of phosphate the supernatant contained little enzymatic activity and the activity in the sediment was increased. NADH caused a further increase of activity in both fractions. These results show that phosphate and NADH stabilize the 5α-reductase. Sodium citrate improved the solubilization of microsomal proteins and again increased the activity of solubilized NADH: 4-ene-3-oxosteroid-5α-reductase. By addition of thioglycerol the activity of the solubilized enzyme was further enhanced. Concentrations of 0.2 M sodium citrate and 0.29–0.58 mM thioglycerol were optimal.

In further experiments the effect of the detergents Lubrol and Tween 20 was compared: Lubrol solubilized microsomal proteins better and inactivated the enzyme less than Tween 20.

Table 1. Solubilization of NADH: 4-ene-3-oxosteroid-5α-reductase from a rat liver microsomal preparation

	Distribution of recovered enzyme activity*					
	Supernatant			Sediment*		
Solubilization mixture	Protein (%)	S.A. (nmol/min·mg)	Act. recov. (%)	Protein (%)	S.A. (nmol/min·mg)	Act. recov. (%)
Lubrol 0.5% Micr. protein 2.5–5 mg/ml in bidest. water	70	0	0	30	3.8	3
Lubrol 0.5% Micr. protein 2.5–5 mg/ml Potassium phosphate buffer pH 7.0, 0.2 M	68	1.3	2	32	9.6	8
Lubrol 0.5% Micr. protein 2.5–5 mg/ml Potassium phosphate buffer pH 7.0, 0.2 M NADH 1 m	65	4.3	7	35	19.9	18
Lubrol 0.5% Micr. protein 2.5–5 mg/ml Potassium phosphate buffer pH 7.0, 0.2 M NADH 1 mM Sodium citrate 0.1 M						
(a) Thioglycerol 0	79	10.6	22	21	32.1	18
(b) Thioglycerol 0.12 mM	73	16.9	32	27	41.0	29
(c) Thioglycerol 0.29 mM	75	22.2	43	25	31.0	20
(d) Thioglycerol 0.58 mM	74	21.4	41	26	31.2	21
Lubrol 0.5% Micr. protein 2.5–5 mg/ml Potassium phosphate buffer pH 7.0, 0.2 M NADH 1 mM Thioglycerol 0.58 mM						
(a) Sodium citrate 0.1 M	74	21.4	41	26	31.2	21
(b) Sodium citrate 0.2 M	77	28.2	57	23	26.7	16
(c) Sodium citrate 0.3 M	78	24.7	50	22	39.0	22
(d) Sodium citrate 0.4 M	78	20.4	41	22	67.0	38
Micr. protein 2.5–5 mg/ml Potassium phosphate buffer pH 7.0, 0.2 M NADH 1 mM Thioglycerol 0.58 mM Sodium citrate 0.2 M						
(a) Lubrol 0.25%	74	32.5	63	26	25.4	17
(b) Lubrol 0.5%	77	28.2	57	23	26.7	16
(c) Lubrol 1.0%	75	23.6	46	25	22.4	15
(d) Tween 20 0.25%	33	10.8	9	67	47.0	82
(e) Tween 20 0.5%	48	15.2	19	52	35.8	48
(f) Tween 20 1.0%	68	5.3	9	32	12.0	10

* Enzyme activity was assayed in presence of 20 mg phosphatidylcholine/mg protein.
Specific activity of crude microsomal preparation: 38.4 nmol/min·mg.

Table 2. Effect of phosphatidylcholine upon enzymatic activity in enzyme assay

	Phosphatidylcholine (mg/mg protein)	S.A. (nmol/min·mg)
Crude microsomal preparation	0	38.4
	20	51.8
Microsomal preparation with optimal solubilization mixture	0	5.7
	5	25.8
	10	29.6
	20	30.7
	40	23.4

The following mixture was found to be best suited for solubilization of NADH: 4-ene-3-oxosteroid-5α-reductase: 0.2 M potassium phosphate buffer pH 7.0, 0.25% Lubrol, 1 mM NADH, 0.2 M sodium citrate, 0.5 mM thioglycerol and 2.5–5 mg microsomal protein/ml. The specific activity of 5α-reductase was increased 20% by solubilization under N_2. When microsomal preparations were solubilized under these conditions and reactivated by phosphatidylcholine in enzyme assay the supernatant contained 63% of the activity of the crude microsomes used. The purification factor was 0.85.

The above mentioned effect of phosphatidylcholine in enzyme assay was studied (Table 2). The activity of microsomal preparations treated with the optimal solubilization mixture was increased 5–6 fold by addition of 20 mg micellar phosphatidylcholine/mg microsomal protein. Further experiments showed that the reactivation by phosphatidylcholine is similar in supernatant and sediment. The activity of crude microsomal preparations was enhanced only slightly by addition of 20 mg micellar phosphatidylcholine/mg protein. Other phospholipids and glycerophosphates (phosphatidylethanolamine, lysolecithine, glycerophosphorylcholine, glycerolphosphate and phosphoethanolamine) were not able to reactivate the enzyme.

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