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SOLUBILIZATION, PURIFICATION AND CHARACTERIZATION OF FATTY ACYL-CoA REDUCTASE FROM DUCK UROPYGIAL GLAND

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Abstract. Membrane-bound fatty acyl-CoA reductase from the uropygial gland of duck has been solubilized from the microsomal preparation with 20% glycerol and 3 M NaCl and purified to homogeneity by Blue A agarose and Palmitoyl-CoA agarose affinity column chromatography followed by Suprose-6 gel filtration. The molecular mass of the enzyme was estimated by SDS-PAGE to be 56 kDa. The enzyme was stable in the presence of 20% glycerol and 1M NaCl and required NADPH for activity. The apparent Kms of the purified enzyme for palmitoyl-CoA and NADPH were 29 μM and 67 μM, respectively. The enzyme activity could be enhanced by the addition of lipid, and the presence of 2 mg/ml BSA enhanced the reductase activity by 5-fold.

Biological reduction of fatty acids provides fatty aldehydes for hydrocarbon biosynthesis (1,2,3,4) and fatty alcohols for the synthesis of ether lipids and wax esters (5,6,7). Ether lipids including the plasmalogens are widely distributed in the cellular lipids, especially the biological membranes. Wax esters are found widely distributed as components of surface lipids in animals (8) and plants (9) and as the major energy storage material in marine organisms (10).

Fatty acyl-CoA reduction was first demonstrated in cell free preparations from Euglena gracilis (7) and subsequently from many organisms in the plant and animal kingdoms (12,13,14,15,16). The soluble acyl-CoA reductase from acetone powder preparations of Brassica oleracea yielded free fatty aldehyde whereas the particulate preparations from wax ester synthesizing tissues yielded fatty alcohol. Even though fatty acyl-CoA reductase activity has been demonstrated in preparations solubilized from plant and animal sources, the enzyme has not been purified from any animal sources.

In the present paper we describe the first solubilization, purification and characterization of an acyl-CoA reductase from an animal tissue.

MATERIALS and METHODS

Reagents. Dithioerythritol, phenylmethysulfonyl fluoride, NADH and NADPH were purchased from Sigma Chemical CO. (St. Louis, Mo). [1-^{14}C] Palmitoyl-CoA was obtained from Du Pont-

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New England Nuclear, and silica gel TLC plates were purchased from Whatman Co. Protein inhibitors leupeptin and pepstatin were supplied by Boehringer Mannheim.

Solubilization of fatty acyl-CoA reductase. Uropygial glands of ducks were excised and kept frozen at -80°C as described (17); for each preparation, two glands were thawed at 4°C overnight. After removal of the adhering skin, fat, and muscle with a razor blade, the gland was sliced and suspended in 30 ml 0.1M sodium phosphate buffer, pH 6.8, containing 1 mM each of $MgCl_2$ and DTE, and three proteinase inhibitors, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride at 0.5 μg/ml, 0.5 μg/ml and 15 μg/ml respectively (P-buffer). The gland slices were ground by a Brinkmann Homogenizer for two minutes. The homogenate was centrifuged at 3,000 xg for 3 min at 4°C, and the supernatant was centrifuged at 105,000 xg for 90 min to pellet the microsomes. The pellet was resuspended in the solubilization buffer (P-buffer containing 20% glycerol and 3 M NaCl), and the suspension was homogenized by a glass homogenizer in an icebath for 5 min. The homogenate was shaken at 60 rpm for 1 hour at 4°C, and centrifuged at 105,000 xg for 90 min. The supernatant could either be frozen in liquid nitrogen and kept at -80°C for later use, or applied to Blue A agarose column for further purification.

Enzyme purification. The reductase solubilized from the microsomes was dialyzed overnight against P-Buffer containing 20% glycerol and applied to a 25 ml column (2.5 cm diameter) of Blue A agarose. The bound acyl-CoA reductase was eluted with 50 ml P-Buffer containing 1 M NaCl and 20% glycerol. The acyl-CoA reductase eluted from this column was concentrated by Centricon (Amicon) and dialyzed against P-Buffer containing 20% glycerol. The dialyzed enzyme was subsequently applied to a palmitoyl-CoA agarose affinity column (6 ml, 1.5 cm diameter), which was equilibrated with P-Buffer containing 20% glycerol. The enzyme was eluted with 10 ml NADPH (10 mM) at a flow rate of 10 ml/hr. Fractions containing enzyme activity were concentrated to 200 μl and applied to a FPLC Suprose-6 gel filtration column (HR 10/30, 25 ml) which had been equilibrated with P-buffer containing 1% glycerol and 0.2 M NaCl. The fractions were tested for enzyme activity.

Assay for fatty acyl-CoA reductase activity. Fatty acyl-CoA reductase activity was assayed by measuring the formation of radioactive hexadecanol from [1-^{14}C] palmitoyl-CoA. A mixture containing 20 μM palmitoyl-CoA (10 Ci/mole), and 300 μM NADPH in a final volume of 0.5 ml in P-buffer was placed in a 16 x 125 mm tube, sealed with serum stopper with one polypropylene cup attached. The sealed tubes were flushed with nitrogen for 30 min, then 200μl of a freshly prepared solution containing 12.5% pyrogallol and 20% KOH was injected into the cup to remove any remaining oxygen. This anaerobic condition reduced background. The mixture was incubated at 30°C for 10 min before injection of the enzyme. After incubating the reaction mixture for 30 min, 200 μl of 2 M HCl was added to stop the reaction, the lipids were extracted by the Folch procedure (18) and the solvent was removed by a stream of nitrogen. The residue was dissolved in hexane, and an aliquot was applied to a TLC plate. The alcohol product that co-migrated with nonradioactive authentic hexadecanol was scraped from the TLC plate and assayed for radioactivity in a Beckman LS3801 scintillation counter.

RESULTS and DISCUSSION

Solubilization and purification of acyl-CoA reductase. The major portion of acyl-CoA reductase activity was found in the microsomal preparation of the duck uropygial gland. Four different solubilization procedures were tested: 0.1% ß-octyl glucoside, 0.15% deoxycholate, 3 M NaCl and 3 M NaCl plus 20% glycerol. Even though all the solutions tested could release some acyl-CoA reductase from the membranes, the combination of 3 M NaCl and 20% glycerol achieved the best solubilization; about 40% of the reductase activity of the microsome preparation was found in the soluble form (data not shown). This condition had been used to solubilize acyl-CoA reductase from the microsomes of Euglena gracilis (19), but the enzyme was not purified. The solubilized enzyme was found to be stable if kept at -80°C. When the solubilized enzyme was applied to a Blue A agarose column, most of the reductase activity was retained in the column and 55% of the total enzyme activity was recovered by elution with P-buffer containing 1 M NaCl and 20% glycerol (Fig. 1A). The fractions containing enzyme activity were concentrated and dialyzed against P-buffer containing 20% glycerol, followed by application to a palmitoyl-CoA agarose affinity column. About 40% of the reductase activity was not bound to the column. The

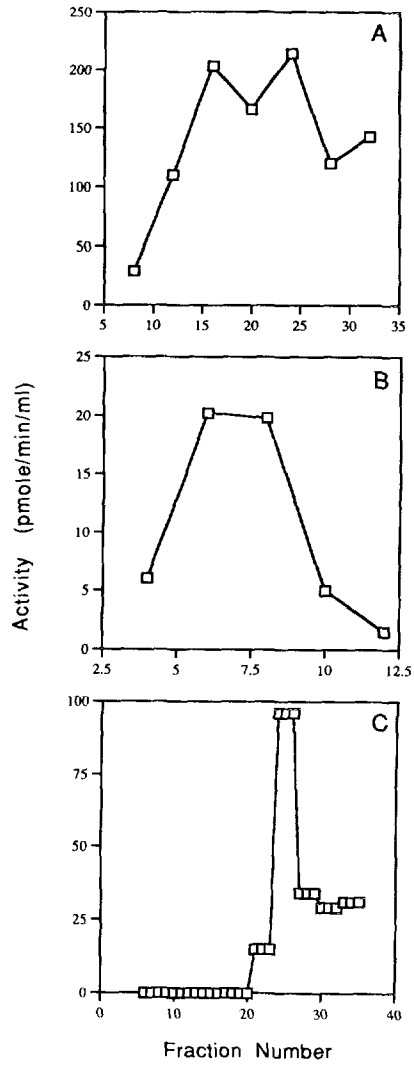

Fig. 1. Elution of fatty acyl-CoA reductase from chromatography columns. A: elution profile of the reductase from Blue A agarose. 4 ml was collected and 100-µl aliquots were used for the enzyme assay. B: Elution profile from a palmitoyl-CoA agarose column. The enzyme from the step shown in A was applied to the palmitoyl-CoA agarose column in P-buffer containing 20% glycerol and acyl-CoA reductase was eluted with 10 ml of 10 mM NADPH. C: Suprose-6 FPLC gel filtration column chromatography. The reductase from the palmitoyl-CoA agarose column was applied to FPLC, and the enzyme was eluted at a flow rate of 0.25 ml/min.

bound reductase was eluted with 10 mM NADPH (Fig. 1B); only 14% of the total activity was recovered. The acyl-CoA reductase recovered from this step was subjected to a FPLC Suprose-6 gel filtration; the reductase eluted after the column volume (Fig. 1C), indicating that the enzyme interacted with column matrix. The two affinity column steps and FPLC gel filtration achieved a 200-fold purification with 6.2% recovery of the acyl-CoA reductase (table 1). A major loss of enzyme activity occurred in the palmitoyl-CoA column chromatography. Besides the incomplete binding of the enzyme as mentioned above, another possible reason for the low recovery of the

Table I

Purification procedure of fatty acyl-CoA reductase from Botrycoccus braunii

Procedure	Total activity nmole/min	Total protein mg	Specific activity nmole/min/mg	Recovery %
Homogenate	28.8	139	0.208	100
Microsome solubilized	16.8	10	1.68	58
Blue A column	4.0	0.85	4.70	14
Palmitoyl-CoA column	0.102	0.0031	33	0.35

enzymatic activity is that at the low concentration of protein encountered in these procedures, the enzyme might have undergone inactivation. The enzyme from the FPLC column lost activity rapidly, presumably due to the low concentration of glycerol and salt (high glycerol concentration could not be applied to the column because of the high back pressure generated from it); therefore the enzyme assay was performed immediately following the elution step. SDS-PAGE of the enzyme preparation from the final step showed that the enzyme had been purified to near homogeneity. This protein showed a single band at molecular mass of 56 kDa (Fig. 2). Attempts to determine the N-terminal amino acid sequence of the enzyme failed most probably because it is blocked (data not shown).

Product identification and substrate specificity. The product generated by the enzyme in the presence of palmitoyl-CoA and NADPH when analyzed by TLC revealed only one component and it co-chromatographed with authentic hexadecanol. No labeled aldehyde was detected. When the reductase was solubilized from the microsome, it could use both NADH and NADPH as the reductant to generate fatty alcohol; NADH gave almost 60% of the activity obtained with NADPH. However after the palmitoyl-CoA affinity column purification, the alcohol production was absolutely dependent on NADPH; NADH was totally ineffective (data not shown).

Km determination. The effects of the concentration of palmitoyl-CoA and NADPH on the reductase showed typical hyperbolic pattern (data not shown). The Kms calculated from the linear Lineweaver-Burk plots were 29 μM and 67 μM for palmitoyl-CoA and NADPH, respectively. The values are close to those obtained with the reductase from Euglena gracilis (7).

Effect of lipids and BSA on acyl-CoA reductase. Since this acyl-CoA reductase is a membrane localized protein, we tested the effect of lipids on the activity of the purified enzyme. Addition of total lipids enhanced acyl-CoA reductase by more than 150% (data not shown). Because a high concentration of salt was used in the solubilization and purification, the effect of NaCl was tested on the enzyme activity; no effect was observed up to 4 M NaCl (data not shown). BSA has been known to enhance fatty acyl-CoA reductase activity from other source

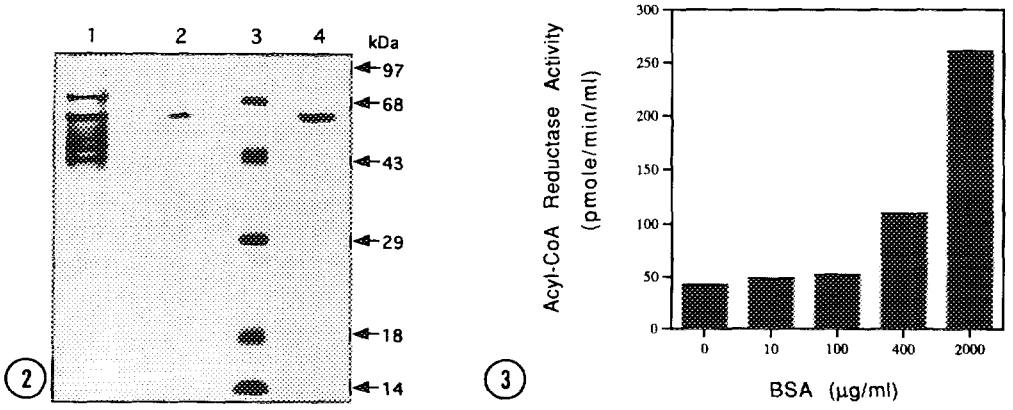

Fig. 2. SDS-PAGE of fatty acyl-CoA reductase at different stages of purification. Lane 1, solubilized proteins from microsomes; lane 2, purified by Blue A agarose column; lane 3, protein markers; lane 4, reductase from FPLC Suprose-6 gel filtration column.

Fig. 3. Effect of BSA on fatty acyl-CoA reductase activity. Freshly prepared BSA (10 mg/ml) was diluted to proper concentration and tested for the effect on the reductase activity.

(20,21). Results presented in Fig. 3 indicate that the addition of BSA at 2 mg/ml increased the purified acyl-CoA reductase activity by more than 5 times.

Since the first report of the enzymatic formation of fatty alcohol in the early seventies (7), significant progress has been made in the understanding of the synthesis of fatty alcohol. It is a well-established process that fatty acyl-CoA is the direct precursor of the corresponding fatty alcohol (7,12), but the mechanism of conversion from fatty acyl-CoA to alcohol shows diversity among different organisms. Even though the reduction of fatty acid to alcohol must be a two step process involving an aldehyde intermediate, such an intermediate does not accumulate in the free form in cases involving microsomal reductases such as the present case and others (14,15,16). But in cases where soluble enzyme preparations catalyze the reduction, free aldehyde can be detected (7). In microsomal preparations, the synthesis of the aldehyde and its reduction to alcohol are tightly coupled as both steps are catalyzed probably by the same enzyme as found in the present case. In vivo fatty alcohols do not often accumulate as they are effectively esterified to wax esters or converted to ether lipids. Tight coupling between synthesis of acyl chains, reduction and esterification to wax esters has been found in microsomes of dark-grown Euglena gracilis (7). In other cases, such as the epidermal cells of pea leaves (1), uropygial glands of certain birds (3), diapausing flesh flies (23) and green alga Botryococcus braunii (4,22), the acyl-CoA reduction to the aldehyde is probably coupled to the decarbonylation of the aldehyde to yield hydrocarbon (1,4). Thus, the reductase can be functionally coupled to other reactions in a tissue specific manner to generate the desired end products for the tissue.

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