THE RELATIONSHIP BETWEEN CHOLESTEROL ESTER HYDROLASE AND TRIACYLGLYCEROL HYDROLASE FROM BOVINE ADRENAL CORTEX

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1. Introduction

One of the rate-limiting reactions in steroidogenesis in the adrenal gland is the supply of cholesterol from the stored cholesterol esters [1]. This step is catalysed by the enzyme cholesterol ester hydrolase which has been shown to be stimulated by hormones such as adrenocorticotropic hormone (ACTH). There is now considerable evidence that this effect is mediated by phosphorylation of the cholesterol ester hydrolase by cyclic AMP-dependent protein kinase [2,3].

The adrenal cortex also contains significant levels of triacylglycerol and of an activatable or 'hormone-sensitive' triacylglycerol hydrolase. This activity is increased in response to ACTH [4] and it has been postulated that the cholesterol ester hydrolase and triacylglycerol hydrolase activities are catalysed by the same protein. Both activities have a similar subcellular distribution, they co-migrate during gel filtration and sucrose-density centrifugation, and show concerted binding to the two different substrates. [5]. However, there is evidence that the cholesterol ester hydrolase can be selectively inactivated by treatment with the organophosphate, chlorpyrifos oxone, and also that cholesterol ester hydrolase precipitates at lower concentrations of ammonium sulphate than does triacylglycerol hydrolase [5]. The key enzyme in adipose tissue lipolysis is hormone-sensitive triacylglycerol hydrolase. This tissue also contains similar levels of cholesterol ester hydrolase activity and the two activities co-purify through a 36-fold purification procedure [6].

Here we demonstrate the existence of two distinct triacylglycerol hydrolases in bovine adrenal cortex, only one of which has significant cholesterol ester hydrolase activity.

2. Materials and methods

2.1. *Chemicals and radiochemicals*

Cholesteryl oleate (99% pure), glyceryl trioleate (99% pure), bovine albumin (fraction V, essentially fatty acid free), egg yolk phosphatidyl choline (Type V-E) and oleic acid (99% pure) were from Sigma; Sepharose 4B from Pharmacia; NE 260 Scintillation Cocktail from Nuclear Enterprises Ltd, Triton X-100 from BDH Chemicals Ltd, and cholesteryl [1-^{14}C]-oleate (25 mCi/mmol, 98% pure) and glyceryl tri-[1-^{14}C]oleate (55 mCi/mmol, 99% pure) from The Radiochemical Centre. They were used without further purification.

2.2. *Assay of cholesterol ester hydrolase and triacylglycerol hydrolase*

Both enzymes were routinely assayed by a modification of the method in [7]. Cholesteryl [1-^{14}C]oleate or glyceryl tri-[1-^{14}C]oleate (50 μCi in toluene) was added to unlabelled cholesteryl oleate or glyceryl trioleate, dried under a stream of nitrogen, and then redissolved in ethanol to 12.5 mM. This stock solution was used to make a substrate mixture containing bovine albumin (28 mg/ml), sodium phosphate (56 mM), ethanol (1.4%), cholesteryl oleate or glyceryl trioleate (180 μM), pH 7.0.

The assay was carried out in duplicate, by incubating substrate mixture (0.35 ml) with enzyme (0.05 ml) for 30 min at 30°C. The reaction was terminated by addition of the fatty acid extraction mixture (1.5 ml) and NaOH (1 N, 0.05 ml) and the fatty acid extracted as in [7]. Aliquots (0.5 ml) of the upper phase were added to scintillation fluid (4 ml) and radioactivity measured in a Beckman LS-255 Liquid Scintillation Counter. Where stated in the text, an

alternative method for preparing substrate mixture was used, based on that in [8]. Stock substrate (1.25 μmol) was mixed with phosphatidyl choline (21 μmol) then dried under nitrogen. Glycerol (0.7 ml) was added and the preparation sonicated (MSE 100 Watt Ultrasonic Disintegrator) for 10 min at 0°C. This preparation was then used to make a substrate mixture containing bovine albumin (28 mg/ml) sodium phosphate (56 mM) glycerol (10%), phosphatidyl choline (3 mM), cholesteryl oleate or glyceryl trioleate (180 μM) (pH 7.0). The assay was then done as above.

One unit of enzyme is that amount which catalyses the release of one nanomole of oleic acid per hour at 30°C.

2.3. *Protein determination*

Protein was determined by the method in [9], using bovine albumin as the standard.

2.4. *Preparation of pH 5.2 precipitate from bovine adrenal cortex*

Bovine adrenal glands were obtained from a local abattoir, placed on ice and processed within 60 min of slaughter. All surrounding fat was removed from the gland, and the cortex isolated from the medulla. The cortex was homogenised in 4 vol. ice-cold sucrose (0.25 M), Tris–HCl (10 mM) EDTA (1 mM) benzamidine (0.1 mM) pH 7.4. All subsequent operations were carried out at 4°C. The homogenate was centrifuged for 30 min at 6000 × *g* and the supernatant decanted through glass wool (1st supernatant). This supernatant was then centrifuged for 60 min at 100 000 × *g* and again the supernatant passed through glass wool (100 k supernatant). The pH of this supernatant was then carefully lowered to 5.2 by dropwise addition of acetic acid (1 M). After standing for 15 min, this fraction was centrifuged for 10 min at 17 000 × *g* and the pellet resuspended, using a glass homogeniser, in sodium phosphate (20 mM) EDTA (1 mM), β-mercaptoethanol (15 mM), benzamidine (0.1 mM) pH 7.0, to give finally ~20 mg/ml protein (pH 5.2 fraction).

3. Results

Bovine adrenal cortex contains both cholesterol ester hydrolase and triacylglycerol hydrolase. When these activities are assayed under identical conditions at pH 7.0 the 1st Supernatant routinely contains

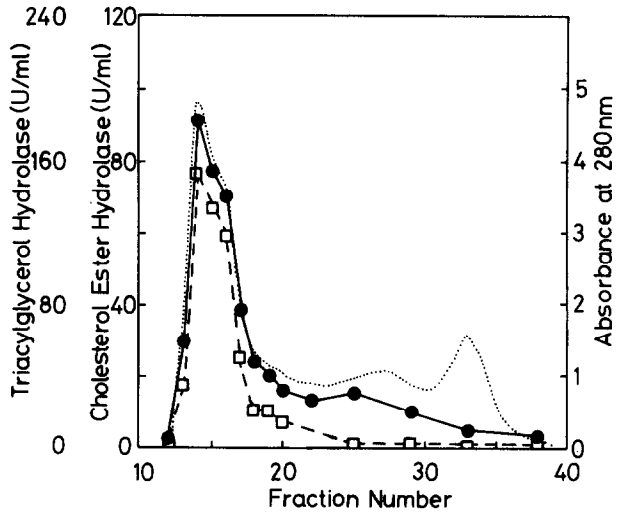

Fig.1. Gel filtration on Sepharose 4B of pH 5.2 fraction. The column (47 cm × 1.5 cm) was equilibrated in sodium phosphate (20 mM). EDTA (1 mM), β-mercaptoethanol (15 mM), benzamidine (0.1 mM) (pH 7.0). pH 5.2 fraction (1.0 ml), containing 740 U/ml cholesterol ester hydrolase and 1640 U/ml triacylglycerol hydrolase was applied. The flow rate was 5.5 ml/h and fractions of 2.1 ml were collected. The dotted line shows the A_{280}. Cholesterol ester hydrolase (□); triacylglycerol hydrolase (●).

1.5–3-fold higher levels of triacylglycerol hydrolase than of cholesterol ester hydrolase. The ratio of the two activities remains essentially unchanged following centrifugation at 100 000 × *g* and precipitation at pH 5.2. The mean ratio of triacylglycerol hydrolase/cholesterol ester hydrolase in the pH 5.2 fraction of 14 different preparations was 2.4:1.0.

When pH 5.2 fraction is subjected to gel filtration on Sepharose 4B both cholesterol ester hydrolase and triacylglycerol hydrolase are excluded from the gel ($K_{av} = 0$), indicating that they are present in a complex with an apparent molecular weight of several million (fig.1). If, however, the pH 5.2 fraction is incubated at 4°C in buffer containing Triton X-100 (0.02%, w/v), and then chromatographed on Sepharose 4B in the presence of the same concentration of Triton X-100, the apparent molecular weight of the two activities is reduced (fig.2). The cholesterol ester hydrolase elutes as a single peak with $K_{av} = 0.04$. The triacylglycerol hydrolase shows two major peaks, one corresponding in position with the cholesterol ester hydrolase with $K_{av} = 0.07$ and a second peak of lower apparent molecular weight ($K_{av} = 0.27$).

It had been shown in [5] that both the cholesterol

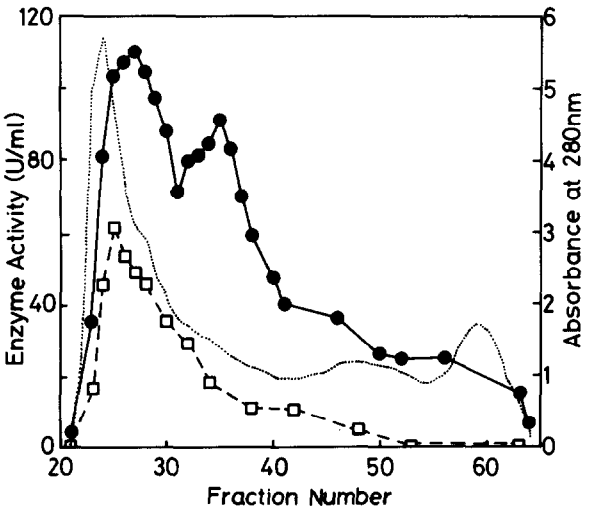

Fig.2. Gel filtration of pH 5.2 fraction on Sepharose 4B in the presence of Triton X-100. The column (87 cm × 1.5 cm) was equilibrated in sodium phosphate (20 mM), EDTA (1 mM), β-mercaptoethanol (15 mM), benzamidine (0.1 mM), Triton X-100 (0.02%, w/v) (pH 7.0). pH 5.2 fraction (2.0 ml) containing 740 U/ml cholesterol ester hydrolase and 1640 U/ml triacylglycerol hydrolase was incubated overnight at 4°C in Triton X-100 (0.02%) and then applied to the column. The flow rate was 6.0 ml/h and fractions of 3.0 ml were collected. The dotted line shows A_{280}. Cholesterol ester hydrolase (□); triacylglycerol hydrolase (●).

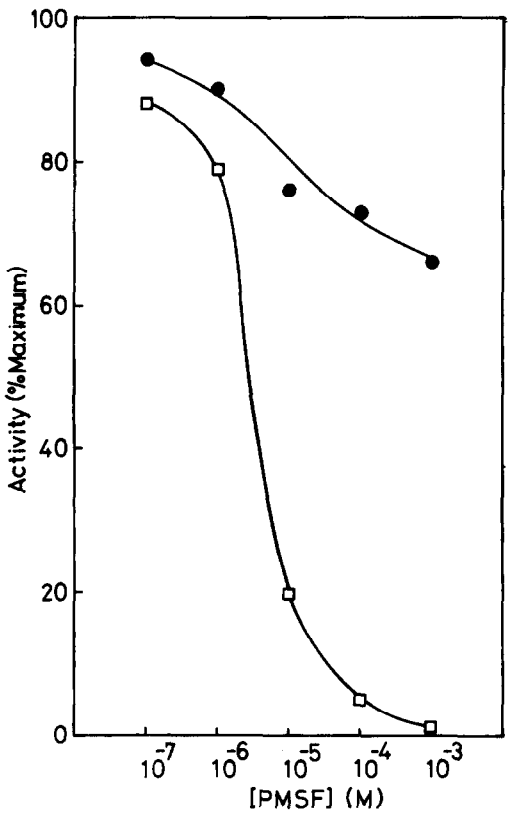

Fig.3. Effect of phenyl methane sulphonyl fluoride on cholesterol ester hydrolase (□) and triacylglycerol hydrolase (●) in pH 5.2 fraction. Phenyl methane sulphonyl fluoride was dissolved in absolute ethanol. pH 5.2 fraction, (100 μl, containing 765 U/ml cholesterol ester hydrolase and 2280 U/ml triacylglycerol hydrolase) was diluted 5-fold with sodium phosphate (20 mM) EDTA (1 mM), β-mercaptoethanol (15 mM) pH 7.0. Phenyl methane sulphonyl fluoride (5 μl of the appropriate concentration) was added and the sample incubated for 30 min at 4°C. Aliquots were then removed and assayed. Maximum activity was determined in a sample preincubated with ethanol (5 μl). The alternative assay method was used.

ester hydrolase and triacylglycerol hydrolase from rat adrenal can be inactivated by treatment with the organophosphate, chlorpyrifos oxone. Similarly the hormonesensitive triacylglycerol hydrolase from rat adipose tissue reacts selectively with diisopropylphosphofluoridate [10] and the same enzyme from chicken adipose tissue can be inactivated by phenyl methane sulphonyl fluoride (PMSF) [11]. The effect of differing concentrations of PMSF on the activity of bovine adrenal cholesterol ester hydrolase and triacylglycerol hydrolase in the pH 5.2 fraction is shown in fig.3. At $>10^{-4}$ M PMSF the cholesterol ester hydrolase is >90% inactivated. However, even at 10^{-3} M PMSF, over 60% of the triacylglycerol hydrolase activity remains. Essentially identical results are obtained if PMSF is added directly to the assay mixture, indicating that the inactivation is exceedingly rapid and that the presence of substrate does not protect against inactivation.

One possible explanation of these findings is that adrenal cortex contains two distinct enzymes possessing triacylglycerol hydrolase activity, only one of which hydrolyses cholesterol esters and can be inactivated by pre-treatment with PMSF. In view of this possibility the effect of 10^{-3} M PMSF on the triacylglycerol hydrolase activity was tested in several of the fractions obtained from the Sepharose 4B column run in the presence of Triton X-100. These results are summarised in table 1. They indicate that the second peak of triacylglycerol hydrolase activity is essentially unaffected by treatment with PMSF, but that the activity in the first peak against triacylglycerol is largely abolished by PMSF. As shown in fig.3, cholesterol ester hydrolase in bovine adrenals is >95% inactivated by 10^{-3} M PMSF.

Table 1
Effect of phenyl methane sulphonyl fluoride on triacylglycerol hydrolase activity in fractions from Sepharose 4B

Fraction no.	Triacylglycerol hydrolase in presence of PMSF (% control)
25	24
27	29
31	31
37	50
46	84

Fractions from the Sepharose 4B column run in the presence of Triton X-100 (fig.2) were assayed for triacylglycerol hydrolase activity in the presence of phenyl methane sulphonyl fluoride (1 mM). The activity is expressed as a percentage of that measured in the absence of PMSF

4. Discussion

This work demonstrates that the cytosol of bovine adrenal cortex contains two distinct triacylglycerol hydrolase activities. They can be resolved physically by gel filtration in the presence of low levels of non-ionic detergent. In the absence of detergent both species are excluded from Sepharose 4B, indicating a molecular weight of several million. This excluded fraction is rich in phospholipids (F. T. L. and S. J. Y., unpublished) suggesting that the enzymes are associated in a proteinphospholipid complex. A similar finding has been reported for the hormone-sensitive triacylglycerol hydrolase from rat adipose tissue [12]. Whether this complex is of physiological importance, or is generated by the homogenisation procedure is not yet known.

The two triacylglycerol hydrolase activities can also be distinguished chemically, by pre-treating them with phenylmethane sulphonylfluoride. The activity of the enzyme with higher apparent molecular weight is essentially abolished by phenylmethane sulphonylfluoride, whereas the activity of the smaller species is unaffected by this pre-treatment. This provides strong evidence that the two enzymes are distinct, and that the smaller species is not derived from the larger one, either by limited proteolysis or loss of dissociable subunits, since the result suggests the two enzymes have different active sites. This is also indicated by their different substrate specificities, the larger having ~1.6-fold higher activity towards glyceryl trioleate than towards cholesteryl oleate whereas the smaller species has a 7-fold higher activity towards glyceryl trioleate than towards cholesteryl oleate (fig.3).

The existence of two different triacylglycerol hydrolases may at least partly explain the finding that, in rat adrenals, the ratio of triacylglycerol hydrolase to cholesterol ester hydrolase differed 6-fold in fractions salted out at different concentrations of ammonium sulphate [5]. However, in bovine adrenals such a differential fractionation of the two activities was not obtained [13].

The physiological role of this second triacylglycerol hydrolase is not yet known, nor is it known whether it is under hormonal influence. It is not an acid lipase, having a pH optimum above neutrality (K. G. C. and S. J. Y., unpublished). It was reported in [14] that rat adrenals contain a heparin-releasable triacylglycerol hydrolase, thought to be involved in the uptake of high density lipoprotein phospholipids and cholesterol from the plasma. However, we have been unable to detect any increase in triacylglycerol hydrolase activity in the high-speed supernatant following homogenisation in the presence of heparin (K. G. C. and S. J. Y., unpublished).

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