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Identity of purified monoacylglycerol lipase, palmitoyl-CoA hydrolase and aspirin-metabolizing carboxylesterase from rat liver microsomal fractions

A comparative study with enzymes purified in different laboratories

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Two purified carboxylesterases that were isolated from a rat liver microsomal fraction in a Norwegian and a German laboratory were compared. The Norwegian enzyme preparation was classified as palmitoyl-CoA hydrolase (EC 3.1.2.2) in many earlier papers, whereas the German preparation was termed monoacylglycerol lipase (EC 3.1.1.23) or esterase pI 6.2/6.4 (non-specific carboxylesterase, EC 3.1.1.1). Antisera against the two purified enzyme preparations were cross-reactive. The two proteins co-migrate in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Both enzymes exhibit identical inhibition characteristics with Mg^{2+}, Ca^{2+} and bis-(4-nitrophenyl) phosphate if assayed with the two substrates palmitoyl-CoA and phenyl butyrate. It is concluded that the two esterase preparations are identical. However, immunoprecipitation and inhibition experiments confirm that this microsomal lipase differs from the palmitoyl-CoA hydrolases of rat liver cytosol and mitochondria.

INTRODUCTION

During a systematic study of the non-specific carboxylesterases in rat liver microsomal fractions (Mentlein *et al.*, 1980, 1984*a*; Mentlein & Heymann, 1984), it was found that two of the highly purified enzymes had a high palmitoyl-CoA-deacylating activity (Mentlein *et al.*, 1984*a*). These esterases, with pI values of 6.2 and 6.4, obviously represented different forms of a single protein (Mentlein *et al.*, 1984*b*), which is called 'Kiel esterase' in the present paper. Shortly before, one of us independently isolated and characterized a palmitoyl-CoA hydrolase from the same biological material (Berge, 1979, 1980; Berge *et al.*, 1980, 1981*a*); it is named 'Bergen esterase' in this paper. This enzyme hydrolysed long-chain thioesters with maximum activity for palmitoyl-CoA. However, the effect of this enzyme on xenobiotic esters and amides has not been investigated. Many features of the esterase isolated at Kiel and the acyl-CoA hydrolase prepared at Bergen seemed to be very similar, e.g. the M_r, but other features seemed to differ, especially the influence of Mg^{2+} and Ca^{2+} on the enzyme activity (Berge & Dossland, 1979; Mentlein *et al.*, 1984*a*). Thus, to find out whether the Kiel esterase and the Bergen enzyme might be identical proteins, it was necessary to compare both enzyme preparations directly. This was done in both laboratories, and the results of this study are presented here.

MATERIALS AND METHODS

Animals and tissue fractionation

In both laboratories, only adult male Wistar rats weighing 250–300 g were used. In Kiel the animals were of the non-inbred strain Han:WIST; in Bergen the non-inbred strain Mol:WIST was used.

To obtain cell fractions, liver homogenates were separated by differential centrifugation into nuclei, mitochondria, light mitochondria, microsomal fraction and cytosolic supernatant as described by Berge *et al.* (1983).

Enzymes and antisera

The Bergen esterase (Berge, 1979) and the Kiel esterase (Mentlein *et al.*, 1980) were purified from rat liver microsomal fractions as described in the references.

To remove a contaminating protein of M_r 80000, which occasionally occurred in Kiel esterase preparations and normally was present in the Bergen esterase, the isoelectric-focusing step of the original Kiel procedure (Mentlein *et al.*, 1980) was replaced by a chromatofocusing step: the fraction of the preceding ion exchange chromatography containing the esterases with pI 6.2 and 6.4 (1 mg of protein) was dialysed against 25 mM-imidazole/HCl buffer, pH 7.4, concentrated by ultrafiltration to a volume of 20 ml, and applied to a column (40 cm × 0.9 cm) filled with chromatofocusing gel PBE 94 (Pharmacia, Freiburg, Germany) equilibrated with the dialysis buffer. The column was eluted with 300 ml of Polybuffer 74 (Pharmacia), diluted 1:8 with water and adjusted to pH 5.5 with HCl. Fractions (2 ml) were collected. The esterase with pI 6.4 was found in fractions 115–125, that with pI 6.2 in fractions 130–140, and the contaminating protein in fractions 160–170.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified enzymes was performed on 10% -acrylamide slab gels as described by Mentlein *et al.* (1980).

In most experiments the pI 6.2 form of the Kiel esterase was used. In a few experiments the pI 6.4 form was additionally used and gave identical results. Antisera

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were raised in rabbits as described for the Kiel esterase (Mentlein *et al.*, 1984*b*). The anti-(Bergen esterase) serum was produced by a very similar procedure (Harboe & Ingild, 1973).

Immunoprecipitation

Immuno-double-diffusion was performed as described by Ouchterlony (1958). After diffusion, the plates were washed with phosphate-buffered 0.9% NaCl and stained for protein and/or esterase activity with 1-naphthyl acetate/Fast Red TR-salt (Mentlein *et al.*, 1984*b*). Enzymes or cell fractions were immuno-titrated with appropriate amounts of antisera in constant volumes of 150 mM-KCl/15 mM-Hepes, pH 7.4. In some titration experiments with purified enzymes, bovine serum albumin was added to obtain identical protein concentrations in all samples for the subsequent acyl-CoA hydrolase assay. Particulate cell fractions were lysed by mixing with 0.05% Triton X-100 (10 min) before the addition of antisera. The titration mixtures were allowed to react for 30 min at 20 °C, then centrifuged (16000 *g*, 5 min). Samples of the supernatants were assayed for palmitoyl-CoA hydrolase activity.

Enzyme assays

The hydrolysis of palmitoyl-CoA was monitored at 37 °C either spectrophotometrically (Berge & Farstad, 1979) or radiochemically with 50 μM-[1-^{14}C]palmitoyl-CoA (1 Ci/mol) in 15 mM-Hepes buffer, pH 7.4, containing 150 mM-KCl, 2 mM-EDTA and 0.01% Brij 58 (Berge *et al.*, 1983; Berge & Farstad, 1979). The spectrophotometric assay was conducted in the same buffer also containing 0.3 mM-5,5'-dithiobis-(2-nitrobenzoic acid), but without EDTA and Brij 58. Initial rates were measured with the spectrophotometric procedure. The radiochemical assay was stopped with Dole's reagent (Berge & Farstad, 1979) after incubation times of 2 min; during this period the activity was linear with time.

The hydrolysis of phenyl butyrate, 4-nitrophenyl acetate, aspirin and propanidid was determined as described previously (Mentlein *et al.*, 1984*a*; Mentlein & Heymann, 1984).

The reproducibility of two of the photometric assays was determined with various amounts ($n=20$) of a single Kiel esterase preparation. The S.D. was $\pm 4.5\%$ for the phenyl butyrate assay and $\pm 17\%$ for the palmitoyl-CoA assay.

The degradation of monoacylglycerols was monitored by enzymic determination of the released fatty acids. Appropriate amounts of monoacylglycerols were dissolved by ultrasonication (2 min, 150 W; sonifier from Branson, Danbury, CT, U.S.A) in 0.1 M-Tris/HCl buffer, pH 8.0, containing either bovine serum albumin (2 mg/ml) or taurocholate (20 mM) to give 0.1 mM solutions. These substrate solutions (500 μl) were made up to 1 ml with water and enzyme and were incubated for 5 min at 37 °C. The hydrolysis was stopped with 20 μl of 10 mM-paraoxon (diethyl 4-nitrophenyl phosphate), and the released fatty acid was measured enzymically with a commercial kit (NEFA-test; Wako Chemicals, Düsseldorf, Germany). Under these conditions, the hydrolysis of monoacylglycerol was linear with time if the amount of hydrolysed substrate did not exceed 15%.

Protein was determined with the Coomassie-Blue-binding method (Bradford, 1976), by using a commercial kit (Bio-Rad, München, Germany).

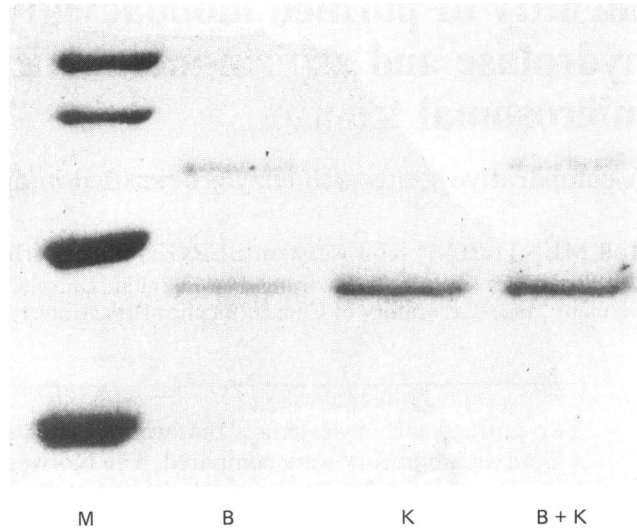

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified esterases

The 10% -polyacrylamide gels were stained with Coomassie Brilliant Blue R250. B, Bergen esterase containing some transferrin; K, Kiel esterase with pI 6.2; B + K, mixture of both esterases; M, marker proteins [from top: β-galactosidase from *Escherichia coli* (M_r 116000), phosphorylase *b* from rabbit muscle (M_r 94000), bovine serum albumin (M_r 68000), ovalbumin (M_r 45000)].

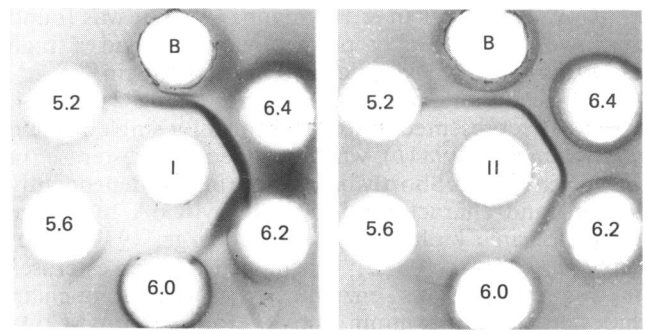

Fig. 2. Ouchterlony double diffusion with purified rat liver microsomal carboxylesterases and antisera to (I) Kiel esterase (pI 6.2) and (II) Bergen esterase

The gels are stained for 1-naphthyl acetate-hydrolysing activity. Centre wells, 5 μl of antiserum; B, Bergen esterase; the Kiel esterases are indicated by their isoelectric points (Mentlein *et al.*, 1980). To each well 1 μg of purified esterase was applied.

Inhibition experiments

To evaluate the influence of the covalently reacting inhibitor bis-(4-nitrophenyl) phosphate, we preincubated approx. 5 μg of purified esterase or 100 μg of cell fraction with this inhibitor in 50 mM-Tris/HCl buffer, pH 8.0, for 30 min (60 min for cell fractions) at 25 °C. The inhibitor was diluted 50-fold in the subsequent enzyme assay. The other inhibitors were applied directly in the assay mixture. EDTA was omitted in the experiments with bivalent cations.

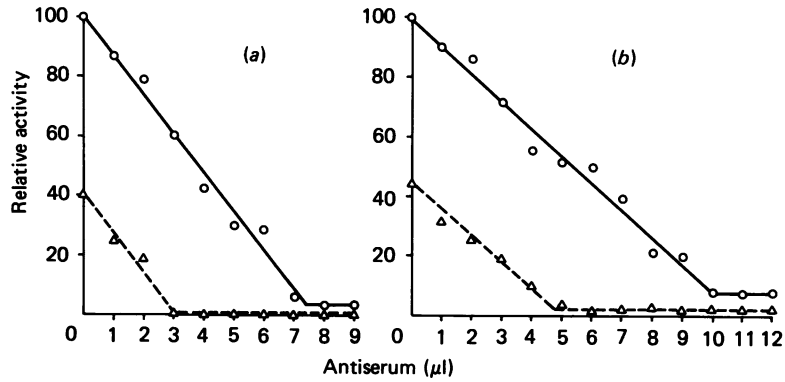

Fig. 3. Immunotitration of purified Bergen esterase (△) and Kiel esterase (○) with (*a*) anti-(Kiel esterase) and (*b*) anti-(Bergen esterase) antisera

Each point represents 2 μg of hydrolase that was treated for 30 min at 25 °C with the given volume of antisera; palmitoyl-CoA-hydrolysing activity was measured in the supernatants after centrifugation at 16000 *g* for 5 min. The activity of 2 μg of Kiel esterase corresponds to 100%. The titration curves were measured with the enzyme preparations shown in Fig. 1 (B and K); each point represents a single determination. The titration with anti-(Kiel esterase) was repeated with two other esterase preparations and gave almost identical results.

RESULTS AND DISCUSSION

Molecular and immunological relationship

The Kiel esterase (monoacylglycerol lipase) corresponds to ES 4 (O. von Deimling & E. Heymann, unpublished work) according to the new genetical nomenclature for rat esterases (Van Zutphen, 1983). It is known that multiple genetical variants of this esterase exist (Bender *et al.*, 1984). Although in both Bergen and Kiel only male Wistar rats are used for the enzyme purification, the strains used are genetically variant. Thus a strictly chemical identity of the purified esterases cannot be expected. However, if the two esterases are identical in a biochemical sense, i.e. both represent ES 4, the divergence should not be greater than that of successive enzyme preparations obtained in the same laboratory.

Very similar subunit M_r values have been reported for the Bergen esterase (59000; Berge, 1980) and the Kiel esterase (61000; Mentlein *et al.*, 1980, 1984*b*). This is not a real difference, since it now turns out that a mixture of the two purified enzymes gives a single sharp band of M_r around 60000 in polyacrylamide-gel electrophoresis (Fig. 1). In addition, different preparations of the two esterases contained different amounts of a contaminating protein of M_r 80000. This contaminant is very efficiently removed by the chromatofocusing step described in the Materials and methods section. The contaminating protein is immunologically cross-reactive with rat transferrin (G. Kreibich, unpublished work) and has been identified as such by its visible spectrum (maximum at 470 nm). Preparative isoelectric focusing as described for the Kiel esterase (Mentlein *et al.*, 1980) normally removes most of the transferrin. The Bergen esterase, which is prepared without such a step, normally contains some (lane B in Fig. 1).

The two esterases are immunologically cross-reactive (Fig. 2). In double-diffusion experiments both the anti-(Bergen esterase) and the anti-(Kiel esterase) antisera precipitated all corresponding purified enzyme forms, namely the Kiel esterases with pI 6.2 and 6.4, and the Bergen esterase. In addition, the anti-(Bergen esterase) serum also gives a faint precipitation with the purified rat liver esterase of pI 6.0, a different protein (Mentlein *et al.*, 1984*b*). We interpret this as a contamination of the antigen used for the production of this antiserum. The precipitation bands with Bergen esterase and Kiel esterase are directly visible in the gel, whereas the precipitation line with pI 6.0 esterase only becomes visible after staining for esterase activity (Fig. 2). Thus the purified Bergen esterase seems to contain a trace of pI 6.0 esterase. Other purified carboxylesterases from rat liver microsomal fractions are not cross-reactive with the two antisera.

Table 1. Relative activities of the purified Bergen and Kiel esterases with typical substrates (37 °C, pH 8.0)

Means of values obtained with two different preparations of each esterase are shown; the individual values differed by less than 15%, except for palmitoylglycerol in the presence of albumin. With this substrate the activities of the two Bergen esterase preparations differed by 27%.

Substrate	Concn. (mM)	Relative activities (palmitoyl-CoA = 100)		
		Bergen esterase	Kiel esterase	
			pI 6.2	pI 6.4
Palmitoyl-CoA	0.05	100	100	100
1-Palmitoylglycerol*	0.05	20	31	39
1-Palmitoylglycerol†	0.05	26	38	39
1-Lauroylglycerol*	0.05	261	312	300
4-Nitrophenyl acetate	1	916	820	1090
Phenyl butyrate	1	1370	1300	1330
Aspirin	1	15	20	42
Propanidid	2	453	414	448

* Emulsified with bovine serum albumin.
† Emulsified with 10 mM-taurocholate.

Table 2. Effects of inhibitors on the palmitoyl-CoA hydrolase and arylesterase activities of Bergen esterase and Kiel esterase

The data (means of duplicates) were obtained with a single esterase preparation of each type. The variation between the individual determinations was less than 10%, except for the low activities in line 3; there the variation was less than 30%.

Inhibitor	Concn. (mM)	Palmitoyl-CoA hydrolase activity† (% of control)		Arylesterase activity‡ (% of control)	
		Bergen esterase	Kiel esterase	Bergen esterase	Kiel esterase
Bis-(4-nitrophenyl) phosphate	0.01*	77	67	75	69
	0.10*	33	29	38	31
	1.0*	12	9	11	2
Mg^{2+}	0.25	146	141	96	97
	5.0	72	87	99	96
Ca^{2+}	0.25	132	155	95	96
	5.0	48	54	98	100

* Enzyme was preincubated at this inhibitor concentration for 30 min at pH 7.4 and 25 °C. In the subsequent assay the inhibitor was diluted 50-fold.
† Standard spectrophotometric assay without EDTA.
‡ Standard spectrophotometric assay with 1 mM-phenyl butyrate.

The results of immunotitrations with both antisera confirm the double-diffusion experiments: if constant amounts of the two esterases are titrated with increasing amounts of both antisera, all curves reach an equivalent point, with almost no acyl-CoA hydrolase activity remaining in the supernatant fluid (Fig. 3). Again, the low activities remaining beyond the equivalent points in some of the curves may represent a contamination of the purified esterases with some pI 6.0 esterase, which also cleaves palmitoyl-CoA (Mentlein *et al.*, 1984*a*).

Substrate specificity and effect of inhibitors

In the past, the Bergen esterase has only been assayed with CoA esters (Berge, 1979, 1980; Berge *et al.*, 1980, 1981*a*) which are hydrolysed with complex kinetics, depending on micelle formation (Berge *et al.*, 1980, 1981*a*). In contrast, the substrate specificity of the Kiel esterase has been studied extensively (Mentlein *et al.*, 1984*a*; Mentlein & Heymann, 1984).

In Table 1 the substrate specificity of the purified Bergen and Kiel esterases is compared. In general, the specificity patterns of both enzymes are very similar. The Bergen esterase has the same high activities with the xenobiotic esters 4-nitrophenyl acetate, phenyl butyrate and propanidid as were already known for the two purified forms of the Kiel esterase (Mentlein *et al.*, 1984*a*; Mentlein & Heymann, 1984). Further, the Bergen esterase cleaves monoacylglycerols. Although the hydrolysis rates of 1-laurylglycerol are similar in all esterase preparations, the activity of the Bergen esterase is relatively lower with 1-palmitoylglycerol. This discrepancy was reproducible with two independent enzyme preparations from each laboratory; it might point to a genetic divergence between the two rat strains used.

Earlier experiments with bivalent cations seemed to show a major difference between the Bergen and Kiel esterases (Mentlein *et al.*, 1984*a*; Berge & Dossland, 1979). However, these inhibition experiments were performed with different substrates and assay conditions. As it turns out now, both esterases behave identically if assayed under identical conditions with these inhibitors

Table 3. Selective inhibition of palmitoyl-CoA hydrolase activity in liver cell fractions

The particulate cell fractions were disintegrated by stirring with 1% (v/v) Triton X-100 before the inhibition experiments. Values are means of duplicates from experiments with fractions from two individual rats each.

Cell fraction	Palmitoyl-CoA hydrolase activity (% of control) after:	
	Organophosphate inhibition*	Immunotitration†
Mitochondria	91	92
Microsomal fraction	5	8
Cytoplasm	98	92

* Preincubation with 1 mM-bis-(4-nitrophenyl) phosphate for 60 min at 25 °C.
† With 20 μl of anti-(Bergen esterase) serum/200 μg of sample protein; activity of the supernatant after centrifugation. Similar results were obtained with anti-(Kiel esterase) serum.

(Table 2). Mg^{2+} and Ca^{2+} only inhibit the palmitoyl-CoA-hydrolysing activity of both esterases, and not their xenobiotic arylesterase activity. Moreover, low concentrations of these cations stimulate the palmitoyl-CoA hydrolase activity of both esterases. Thus the effect of Mg^{2+} and Ca^{2+} is directed against the thiolester substrate and not against the enzymes as such. In previous reports (Berge & Dossland, 1979; Kawashima *et al.*, 1982), probably the effects of Mg^{2+} or Ca^{2+} on palmitoyl-CoA micelle formation (Berge *et al.*, 1980, 1981*a*) had been measured.

Further, both esterases are inhibited in parallel by different concentrations of bis-(4-nitrophenyl) phosphate (Table 2). This organophosphate is a useful tool for discrimination between serine-type esterases (Heymann & Krisch, 1967; Von Deimling & Böcking, 1976).

Experiments on cell compartmentation

Treatment of rat liver microsomal fraction with anti-(Bergen esterase) or anti-(Kiel esterase) antisera, or with 1 mM-bis-(4-nitrophenyl) phosphate, gives over 90% inhibition of the microsomal palmitoyl-CoA hydrolase activity (Table 3). These effects confirm the observations made when isolating the esterases (Berge, 1979; Mentlein *et al.*, 1984*a*), namely that the two esterases represent the dominant palmitoyl-CoA hydrolases of this organelle.

In contrast, the corresponding activities of mitochondria and cytoplasm are not altered by treatment with these antisera or with the organophosphate (Table 3). This confirms previous studies showing that the mitochondrial (Berge & Farstad, 1979) and cytosolic (Kawashima *et al.*, 1982; Berge *et al.*, 1981*b*; Miyazawa *et al.*, 1981) palmitoyl-CoA hydrolases differ from the microsomal enzyme.

Conclusions

We conclude from the data described here that the esterases isolated in Bergen and in Kiel are biochemically identical, i.e. both represent ES 4 according to the current genetical nomenclature for rat esterases (Van Zutphen, 1983).

As previously shown (Mentlein *et al.*, 1984*b*), the Kiel esterase also corresponds to the monoacylglycerol lipase isolated by Oerlemans *et al.* (1977). It exhibits highest specific activities with xenobiotic esters (Mentlein *et al.*, 1984*a*; Mentlein & Heymann, 1984), and it represents the main aspirin- and propanidid-cleaving esterase of rat liver (Mentlein & Heymann, 1984). Further, the Kiel esterase is the microsomal retinol palmitate-hydrolysing enzyme (R. Mentlein, unpublished work). Its possible identity with other purified carboxylesterases (Mentlein *et al.*, 1980), lipases (Mentlein *et al.*, 1984*a*) and drug-metabolizing hydrolases (Mentlein & Heymann, 1984) has previously been discussed. Combining these reports and others from the Bergen (Berge, 1979, 1980) and Kiel (Mentlein *et al.*, 1984*b*) groups, a wealth of information is now available on the purified non-specific esterase/palmitoyl-CoA hydrolase from rat liver microsomes.

The enzyme should be named according to its action on natural substrates and not according to its highest specific activities with xenobiotic esters. However, we cannot decide yet whether the esterase be termed monoacylglycerol lipase (EC 3.1.1.23) or palmitoyl-CoA hydrolase (EC 3.1.2.2). One should remember that a different monoacylglycerol lipase preferring medium-chain monoacylglycerols exists at the same cellular site. This lipase, also called 'hydrolase pI 6.0', has a much higher specific activity with octanoylglycerol (Mentlein *et al.*, 1984*a*), and clearly is a different protein (Mentlein *et al.*, 1984*b*). It corresponds to ES 10 (O. von Deimling & E. Heymann, unpublished work) and at present is usually classified as non-specific esterase (EC 3.1.1.1).

The lipase described in the present paper is not identical with heparin-releasable liver lipase (Mentlein *et al.*, 1984*b*), which also has a subunit M_r of 60000 (Kuusi *et al.*, 1979) and also cleaves monoacylglycerols and palmitoyl-CoA (Jansen & Hülsmann, 1980). Further, the mitochondrial (Berge & Farstad, 1979) and cytosolic (Kawashima *et al.*, 1982; Berge *et al.*, 1981*b*; Miyazawa *et al.*, 1981) palmitoyl-CoA hydrolases of rat liver clearly differ from the lipase described here. However, we cannot exclude the possibility that the microsomal hydrolase may be a precursor of the inducible cytosolic palmitoyl-CoA hydrolase.

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