Identical catalytic subunit in both molecular forms of hormone-sensitive cholesterol esterase from bovine adrenal cortex

Ulrich Sonnenborn, Günter Eiteljörge, Wieslaw H. Trzeciak* and Wolf-H. Kunau

Arbeitsgruppe für Bioorganische Chemie, Institut für Physiologische Chemie, Ruhr-Universität Bochum, FRG

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1. INTRODUCTION

The activity of neutral cholesterol esterase (sterol-ester acylhydrolase, EC 3.1.1.13) in the adrenal gland is enhanced by stress [1,2] or by administration of ACTH [3–5]. This increase of enzymatic activity is supposed to be due to phosphorylation of the enzyme by cAMP-dependent protein kinase [6–9]; for review see [10].

The actual number of neutral esterase species in the adrenal gland [7,11–14], and the identification of their constituent polypeptides [8,11,12] remains controversial. The existence of two molecular forms of cholesterol esterase in bovine [7,12] and rat adrenals [13] has been reported, but not generally recognized [8,9,11,14]. Although purification to near homogeneity of the enzyme from bovine adrenal cortex has been described [8,9], these results must be questioned in the light of recent reports [11,12]. Striking differences in M_r values of native cholesterol esterase (350 000 [9] or 173 000 [8]) as well as of its subunits (41 000 [8], 84 000 [11], or 92 000 [12]) have been reported. Beckett and Boyd [8] suggested composition of the enzyme of four identical subunits of M_r 41 000 and demonstrated their phosphorylation by cAMP-dependent protein kinase. On the contrary, Cook et al. [11] recently succeeded in separating cholesterol esterase activity from the M_r 41 000 polypeptide.

Therefore, since hormone-sensitive cholesterol esterase has not been purified to apparent homogeneity or characterized in molecular terms, it has been impossible to provide direct evidence for the proposed covalent modification of the enzyme.

In addition to cholesterol esterase, adrenal glands contain triacylglycerol lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) [13,14] activatable in response to ACTH in rats [15]. Yeaman et al. [14] described two distinct neutral lipases, one of them exhibiting significant cholesterol esterase activity. The same authors [11] suggested a possible relationship between the latter enzyme and the hormone-sensitive triacylglycerol lipase from adipose tissue [16,17].

This report describes identification and some characteristics of hormone-sensitive cholesterol esterase from bovine adrenal gland and its relation to triacylglycerol lipase.

2. MATERIALS AND METHODS

2.1. *Chemicals and radiochemicals*

Cholesteryl [1-^{14}C]oleate (58 mCi/mmol) and glycerol tri-[1-^{14}C]oleate (99.7 mCi/mmol) were from New England Nuclear, [1,3-^{3}H]di-isopropylfluorophosphonate ([^{3}H]DFP; 6.5 Ci/mmol) from Amersham International, the unlabeled substances from Serva, Heidelberg. Phenyl methane sulphonyl fluoride (PMSF) was obtained from E. Merck, Darmstadt. Sepharose CL-6B and Sephadex G25 were products of Deutsche Pharmacia, Freiburg. DEAE-cellulose (DE-52) was from Whatman Ltd. X-Omat AR X-ray films were from

* Permanent address: Department of Physiological Chemistry, Institute of Physiological Sciences, Academy of Medicine, 6 Swiecickiego St., 60-781 Poznan, Poland

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Eastman Kodak, and autoradiography enhancer (EN^{3}HANCE) from New England Nuclear. [γ-^{32}P]ATP was prepared according to Glynn and Chapell [18].

2.2. *Preparation of cholesterol esterase from bovine adrenal cortex*

A 100 000 × *g* supernatant (S_{100}) from bovine adrenal cortex was prepared in 50 mM potassium phosphate buffer, 0.25 M sucrose, 2 mM EDTA, 1 mM DTE, 0.5 mM benzamidine, pH 7.4 (buffer A), as described [7]. Cholesterol esterase was separated into two species (peak I and peak II) by gel filtration of the S_{100} on Sepharose CL-6B [12], equilibrated in 50 mM potassium phosphate buffer, 1 mM EDTA, 0.5 mM DTE, 0.5 mM benzamidine, pH 7.4 (buffer B). Peak II fractions were further purified by chromatography on a DE-52 column (2.2 × 10 cm), equilibrated in buffer B. A linear gradient of 0 to 0.6 M KCl in the same buffer was applied and cholesterol esterase eluted at about 0.2 M KCl.

2.3. *Assay of cholesterol esterase and triacylglycerol lipase*

Incubation mixture for the estimation of cholesterol esterase activity contained 30 nmoles of cholesteryl [1-^{14}C]oleate (2600 cpm/nmol), added in 20 μl of acetone, 25–250 μg of enzyme protein, 0.5 mM DTE, and 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 0.5 ml. Assays were carried out in triplicate for 30 min at 37°C and liberated [1-^{14}C]oleate was determined as in [19].

Triacylglycerol lipase activity was assayed by the same method using glyceryl tri-[1-^{14}C]oleate as the substrate.

The reaction rates were linear over the incubation period and protein concentrations used.

Protein was determined as in [20] using bovine serum albumin as the standard.

2.4. *Incubation of cholesterol esterase preparations with [^{3}H]DFP*

Cholesterol esterase pools from gel filtration and DE-52 chromatography were concentrated about 10-fold by dialysis against 20% polyethylene glycol (PEG 20 000, Serva) in buffer B. Samples were incubated at 0°C or at 20°C for up to 3 h with 1.5 × 10^{-5} M [^{3}H]DFP (2.2 × 10^8 cpm, in 1,2-propanediol). Incubations were terminated by the addition of sample buffer for electrophoresis. The samples were treated at 60°C for 15 min and aliquots were subjected to SDS–PAGE. Prior to electrophoresis, protein-bound radioactivity was determined [21].

2.5. *Protein phosphorylation*

S_{100} (50 mg protein) was subjected to gel filtration on a Sephadex G25 column (1.5 × 30 cm), equilibrated with 50 mM Tris–HCl, 2 mM DTE, 1 mM theophylline, 0.5 mM benzamidine, pH 7.4 (buffer C). The flow-through fraction as well as concentrated cholesterol esterase pools from gel filtration and DE-52 chromatography were incubated with 5 mM $MgCl_2$ prior to phosphorylation [7,8].

The phosphorylation mixture contained between 0.3 and 2.2 mg of enzyme protein (in buffer C), 5 mM $MgCl_2$, 10 μM cAMP, and 0.3 mM [γ-^{32}P]ATP (29.4 μCi), pH 7.4, in a total volume of 0.5 ml. Incubations were done at 30°C for up to 15 min and terminated by the addition of sample buffer for electrophoresis. The samples were treated and subjected to electrophoresis as described in the previous section.

2.6. *SDS–polyacrylamide gel electrophoresis and autoradiography*

SDS–PAGE in 7.5% acrylamide slab gels was performed as described in [22]. Proteins were stained with Coomassie brilliant blue R-250. For autoradiography of ^{32}P-labeled samples, gels were dried using a Bio-Rad 224 Gel Slab Dryer, covered with X-ray films, and exposed at – 70°C. Slab gels containing [^{3}H]DFP-labeled proteins were soaked in EN^{3}HANCE and dried, prior to fluorography at −70°C.

3. RESULTS

3.1. *Effects of DFP and PMSF on cholesterol esterase and triacylglycerol lipase*

Gel filtration of bovine adrenal 100 000 × *g* supernatant (S_{100}) on Sepharose CL-6B columns repeatedly revealed two molecular forms of cholesterol esterase, one in the void volume (peak I) and the other between M_r 500 000–300 000 (peak II) with the highest activity at M_r about 350 000. Triacylglycerol lipase, however, was present mainly in the void volume (fig.1). Similar elution profile

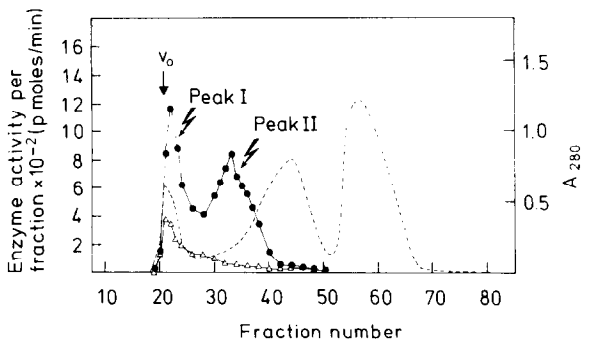

Fig.1. Gel filtration of S_{100} from bovine adrenal cortex on Sepharose CL-6B. The column (100 × 1.5 cm) was equilibrated in buffer B at a flow rate of 15 ml/h. Fractions of 2.4 ml were collected and assayed for cholesterol esterase (•) and triacylglycerol lipase (△). Peak I: fractions 20–25, peak II: fractions 30–36. The dotted line shows the A_{280}.

for cytosolic cholesterol esterase from rat adrenals was reported, but in contrast to our results in bovine adrenals the rat triacylglycerol lipase, too, was separated into two peaks [13].

Bovine adrenal cholesterol esterase and triacylglycerol lipase, could also be distinguished by their different inhibition with DFP and PMSF (fig.2), organophosphates known to modify selectively serine residues at the active site of some enzymes [23]. At 10^{-4} M concentration, these reagents inhibited cholesterol esterase activity in the S_{100} almost completely, whereas the inhibitory effect on triacylglycerol lipase was considerably smaller and amounted to only 50% at 10^{-3} M concentration.

Almost identical results were obtained when pooled peak I or peak II fractions from the gel filtration step were used for the inhibition studies (results not shown).

3.2. *Identification of the cholesterol esterase polypeptide by labeling with [³H]DFP*

Labeling of the S_{100} with [³H]DFP, followed by SDS–polyacrylamide gel electrophoresis and fluorography, showed incorporation of radioactivity into five protein bands, three of them of M_r < 30 000 and the others corresponding to M_r 87 000 and 78 000 (fig.3A). However, in cholesterol esterase pools from gel filtration or from DE-52 chromatography, usually only one [³H]DFP labeled polypeptide was detected (fig.3B–D). Radioactivity co-migrated with a faint protein band of apparent M_r 87 000 ± 2000, present in both the high- and the low-M_r forms of cholesterol esterase. In addition, a second labeled protein band with twice the M_r of the M_r 87 000 polypeptide was occasionally observed.

A clear-cut separation of the M_r 87 000 polypeptide, labeled with [³H]DFP, from the M_r 92 000 protein [12] could be seen only in the material from peak I (fig.3B), whereas in pooled peak II fractions, the polypeptide of M_r 87 000 was always covered by the predominant protein band of M_r

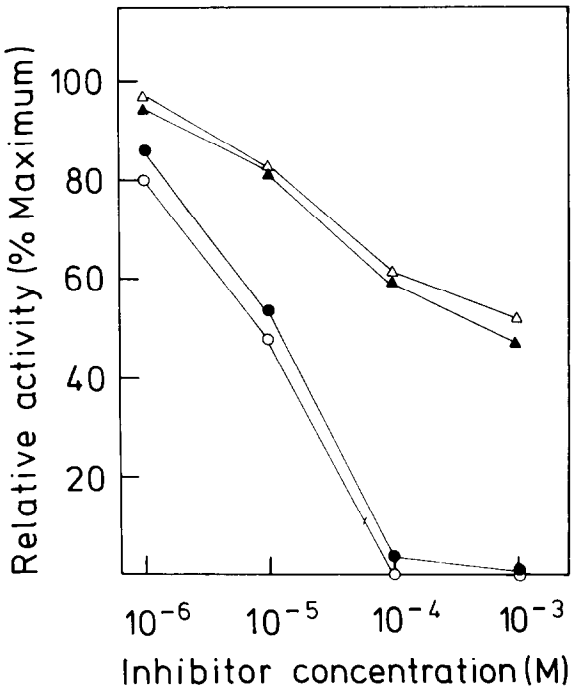

Fig.2. Effects of DFP and PMSF on cholesterol esterase and triacylglycerol lipase activities in the S_{100} from bovine adrenal cortex. Appropriate concentrations of the inhibitors (DFB in 10 μl of 1,2-propanediol, PMSF in 10 μl of ethanol) were included in the standard assay mixture and the samples incubated at 0°C for 30 min prior to the addition of substrates. Maximum activities (100% values) were determined after preincubation of S_{100} with 10 μl of the respective solvents. Cholesterol esterase activity in the presence of DFP (•) or PMSF (○), triacylglycerol lipase activity in the presence of DFP (▲) or PMSF (△).

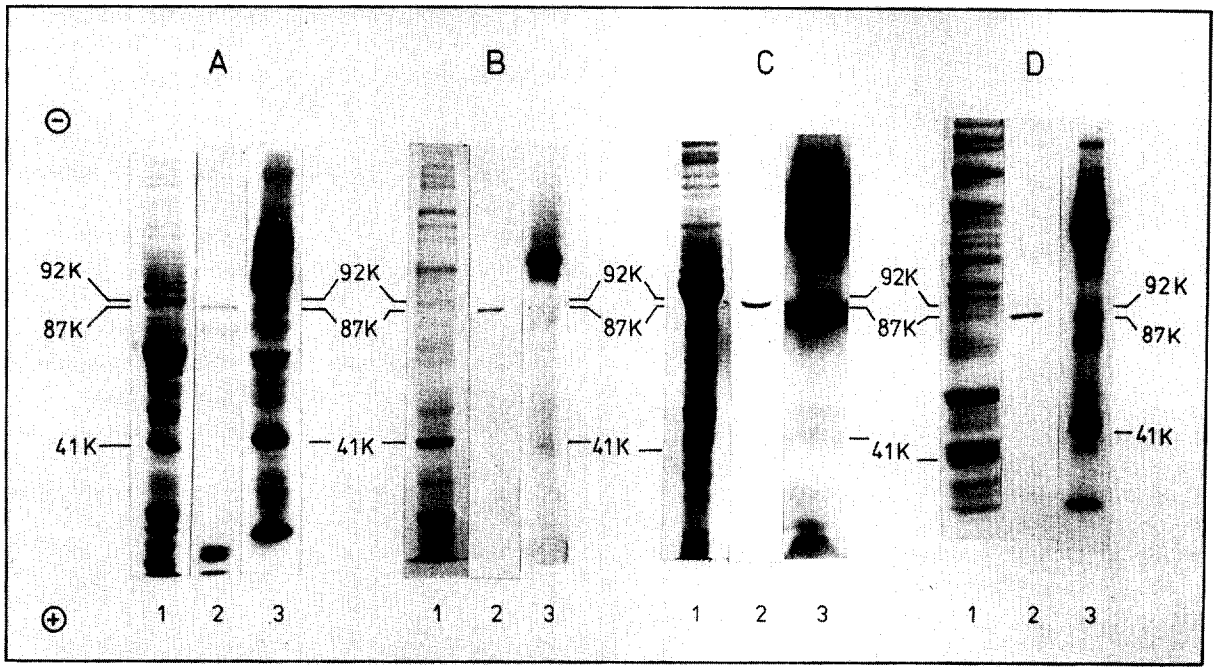

Fig.3. SDS–polyacrylamide gel electrophoresis of [^{3}H]DFP- and ^{32}P-labeled cholesterol esterase preparations: slots 1, protein staining; slots 2, fluorography of [^{3}H]DFP-labeled samples; slots 3, autoradiography of ^{32}P-labeled samples. Amount of protein and radioactivity applied are given in brackets. (A) S_{100}: slot 1 (132 μg), 2 (132 μg, 10 500 cpm), 3 (132 μg, 12 800 cpm). (B) Sepharose CL-6B peak I: slot 1 (40 μg), 2 (40 μg, 3400 cpm), 3 (32 μg, 4800 cpm). (C) Sepharose CL-6B peak II: slot 1 (116 μg), 2 (116 μg, 12 300 cpm), 3 (38 μg, 9000 cpm). (D) DE-52 cholesterol esterase: slot 1 (96 μg), 2 (96 μg, 5500 cpm), 3 (96 μg, 43 800 cpm). Molecular weight markers used were ^{14}C-labeled *E. coli* RNA polymerase (subunit M_r 165 K, 155 K, 89 K, 40 K), ^{32}P-labeled phosphorylase *a* (M_r 97.5 K), bovine serum albumin (M_r 68 K), ovalbumin (M_r 43 K), and lactate dehydrogenase (subunit M_r 36 K). Direction of electrophoresis is from top (⊖) to bottom (⊕).

92 000 (fig.3C). Evidence for the existence of the M_r 87 000 polypeptide in peak II could be provided after partial separation of both proteins by ion-exchange chromatography on DE-52 (fig.3D).

3.3. *Phosphorylation of cholesterol esterase fractions with [γ-^{32}P]ATP*

Endogenous phosphorylation with [γ-^{32}P]ATP of cofactor-depleted S_{100} as well as of cholesterol esterase pools from gel filtration and DE-52 chromatography, resulted in incorporation of radioactive phosphate into numerous proteins (fig.3A–D). In all cases, one of the ^{32}P-labeled polypeptides co-migrated with the M_r 87 000 protein band which could be labeled by [^{3}H]DFP.

Another protein band containing considerable amount of ^{32}P, corresponded to an M_r of 41 000 ± 2000 [8]. However, no radioactive phosphate seemed to be associated with the M_r 92 000 polypeptide, which on the basis of protein staining comprised more than 95% of a cholesterol esterase preparation, purified from the low-M_r species of the enzyme [12].

4. DISCUSSION

The high- as well as the low-M_r forms of cholesterol esterase (fig.1) are inhibited by DFP and PMSF in a nearly identical manner, and share the same polypeptide of M_r 87 000 which is the only [^{3}H]DFP-labeled protein present in both species (fig.3B–D). Incorporation of [^{3}H]DFP is detected neither in the M_r 92 000 nor in the M_r 41 000 polypeptide (fig.3), described as cholesterol esterase monomers [12,8]. Separation of cholesterol es-

terase from the M_r 92 000 (fig.3D), as well as from the M_r 41 000 polypeptide [11] provides additional evidence that these proteins are not subunits of both forms of the enzyme. From these results we conclude that the M_r 87 000 polypeptide represents the hormone-sensitive cholesterol esterase monomer, since we show that this is one of the endogenous substrates for phosphorylation (fig.3). Phosphorylation of this protein has not been reported so far [24–26].

Present results on [^{3}H]DFP labeling of cholesterol esterase are consistent with those of Cook et al. [11] who independently showed incorporation of [^{3}H]DFP into a polypeptide of M_r 84 000, derived from the high-molecular weight form.

Apart from the M_r 87 000, another [^{3}H]DFP-labeled protein band of M_r 174 000 is occasionally observed after SDS–polyacrylamide gel electrophoresis under mild denaturating conditions, and this may represent the dimeric form of cholesterol esterase. The suggestion that the enzyme may have a quaternary structure is supported by M_r estimations on Sepharose CL-6B, and by reports from others [8,9,13]. The esterase species constituting peak II (M_r about 350 000) may thus represent the tetrameric form of the enzyme (fig.1 and [9]). The large difference in M_r between both forms of cholesterol esterase may simply reflect different amount and/or composition of the associated lipids [7,13]. Whether both forms of the enzyme are present in the intact cell or arise during homogenization remains to be established.

It has been proposed [11,15] that adrenal cholesterol esterase and lipase activities are located on the same polypeptide chain, or that the enzymes at least are 'related proteins' [15]. These views, however, are not substantiated by (i) different behaviour of both enzymatic activities on Sepharose CL-6B (fig.1), and (ii) different inhibition by organophosphate compounds (fig.2 and [13]). In addition, another neutral lipase which is not inhibited by PMSF and does not exhibit cholesterol esterase activity, has been found in bovine adrenal cortex [14]. This activity may be due to 'liver-like' lipase [27], although no increase in total lipase activity has been observed upon homogenization of adrenal tissue in the presence of heparin [14].

On the other hand, by analogy to hormone-sensitive lipase from adipose tissue [16,17] and to triacylglycerol lipase from liver [28], adrenal cholesterol esterase may also hydrolyze trioleyl glycerol, and this will explain concomitant activation of cholesterol esterase and triacylglycerol lipase by ACTH [15], and also inhibition of the enzymes by competing substrates [13].

Whether or not cholesterol esterase and one of the triacylglycerol lipase activities are located on the same polypeptide chain can be elucidated only when the enzyme(s) will be purified, and this will eventually allow more detailed studies of the effects of phosphorylation and dephosphorylation on their molecular structure(s). This task may be as difficult as the purification of hormone-sensitive lipase from adipose tissue [17,29], since the esterase subunit of M_r 87 000 represents only a minor component of the soluble fraction from the adrenal cortex (fig.3).

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