HORMONE-SENSITIVE LIPASE OF RAT ADIPOSE TISSUE: IDENTIFICATION AND SOME PROPERTIES OF THE ENZYME PROTEIN

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1. Introduction

The cyclic AMP-dependent stimulation of hormone-sensitive lipase activity in adipose tissue preparations, originally described by Rizack [1] and confirmed by others [2,3] has been shown to be mediated via a protein kinase-catalyzed phosphorylation [4,5]. However, direct evidence for the molecular events taking place upon stimulation is lacking since the enzyme protein has not been isolated. We have recently purified monoacylglycerol lipase from rat adipose tissue by solubilization and fractionation in non-ionic detergent [6]. Using the same technique we have been able to separate solubilized hormone-sensitive lipase from other lipolytic enzymes of rat adipose tissue, to identify the enzyme protein and to demonstrate some of its properties, notably a protein kinase-catalyzed phosphorylation.

2. Methods

2.1. *Preparation of hormone-sensitive lipase*

Starting material for the preparation was 'pH 5.2 precipitate' obtained from rat epididymal fat pads [6]. All operations were performed at + 4°C. After suspension of the 'pH 5.2 precipitate' in 20 mM Tris–HCl, pH 7.4, 1 mM EDTA and 1 mM dithioerythritol (1 ml/g fat pad) the hormone-sensitive lipase was solubilized by sonication in 3 mM Nonipol TD 12*.

* Nonipol TD 12 (Rexoline Chemicals, Helsingborg, Sweden) is an alkyl polyoxyethylene derivative [9] with an average composition $C_{12\text{-}14}E_{12}$ (C=alkyl chain carbons, E=oxyethylene units).

All solutions in subsequent steps contained 3 mM detergent. Hormone-sensitive lipase was assayed as its 'diglyceridase' activity (see below), unless otherwise stated. The solubilized preparation was applied to a TEAE-cellulose column equilibrated with the above buffer and the hormone-sensitive lipase was eluted with a NaCl-gradient (fig.1). After dialysis and ultrafiltration (PM 10 Diaflo membrane) the combined enzyme fractions were electrofocused in an LKB 440 ml column (1% Ampholine, pH 3.5–10, 0–60% sucrose-gradient, 1200 V, 10 W for 65–80 h). The single peak of hormone-sensitive lipase (p*I* 6.7 at + 4°C) was electrofocused again in a 110 ml column under similar conditions in a pH 6–7 gradient (fig.2). The detergent was removed from a portion of the combined enzyme peak fractions by a third electrofocusing in the 110 ml column [7]. After electrofocusing in a pH 3.5–10 gradient sodium taurodeoxycholate was added in two portions (3 μmol each) near the cathode and the voltage was applied for another 24 h. The bile salt and the non-ionic detergent formed negatively charged mixed micelles, which moved to the anode. The enzyme peak obtained is referred to as the purified detergent-free enzyme. Another portion of the enzyme was chromatographed on Sephadex G-200 in the presence of α-methyl glucoside (fig.3). The over-all recovery after the second electrofocusing was approximately 10% and the total purification at least 100-fold. A detailed procedure for the preparation of the enzyme will be presented elsewhere.

2.2. *Enzyme assays*

The 'triglyceridase' activity of hormone-sensitive lipase was measured at pH 7.4 using di-*O*-oleyl-1(3)-

[^{3}H]oleoylglycerol or, in the activation experiments, tri[^{3}H]oleoylglycerol, emulsified with phospholipid as substrate [8]. Its 'diglyceridase' and 'monoglyceridase' activities were assayed, under analogous conditions, with 2.5 mM 1 (3)-[^{3}H]oleoyl-2-*O*-oleylglycerol (a monoester analogue of di-oleoylglycerol cf. [8]) or 2 mM mono-oleoyl[^{3}H]glycerol. In all assays of hormone-sensitive lipase activity the enzyme samples were diluted to reduce the inhibitory effect of Nonipol. Monoacylglycerol lipase and lipoprotein lipase were measured as described earlier [9,10]. Enzyme activity is expressed as nmol fatty acids produced/min at room temperature or, when so stated, 37°C.

2.3. *Other methods*

Discontinuous polyacrylamide gel electrophoresis was performed in slab-gels (11.1 X 0.9%) in the presence of 0.1% sodium dodecylsulfate [11]. Samples were pretreated and gels stained with Coomassie Blue [6]. Protein was measured by a scaled-down version [12] of the Lowry method [13], or fluorimetrically with fluorescamine [14], using bovine serum albumin as standard.

3. Results

3.1. *Solubilization of hormone-sensitive lipase and separation from monoacylglycerol lipase and lipoprotein lipase*

The particulate 'protein–lipid' complexes of the 'pH 5.2 precipitate' contained both the hormone-sensitive lipase, monoacylglycerol lipase and lipoprotein lipase. After solubilization with detergent these enzymes were largely resolved by the ion-exchange chromatography (fig.1). The hormone-sensitive lipase (p*I* 6.7) was further separated from monoacylglycerol lipase (p*I* 7.2) and lipoprotein lipase (p*I* 5.0) by the two electrofocusings to give the isolated enzyme (fig.2).

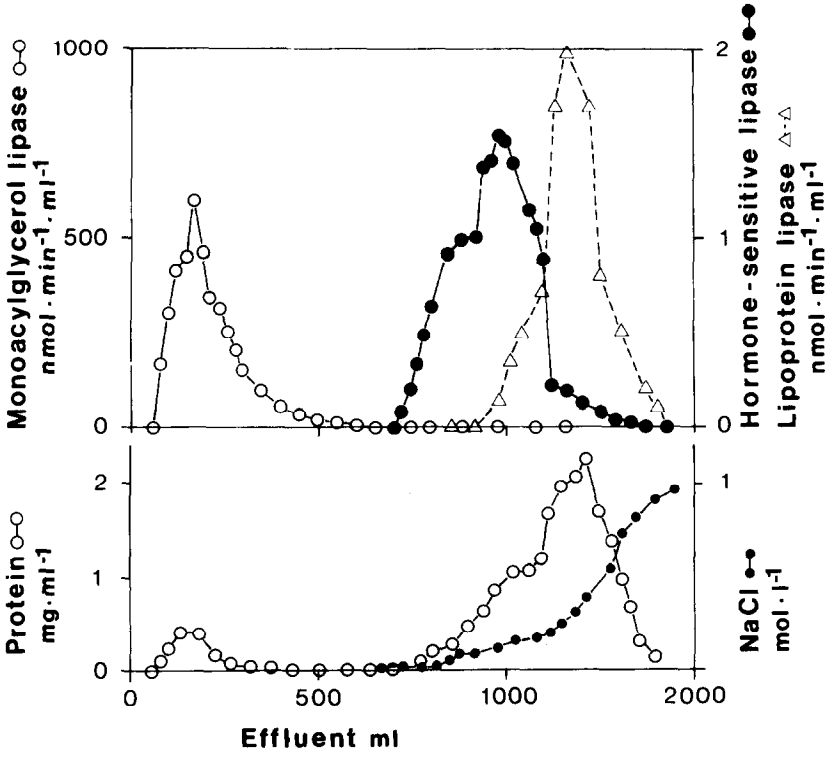

Fig.1. TEAE–Cellulose chromatography of solubilized 'pH 5.2 precipitate'. Protein, 700 mg obtained from 200 rats, was applied on a 5 X 20 cm column equilibrated with 20 mM Tris–HCl, pH 7.4, 1 mM dithioerythritol, 1 mM EDTA and 3 mM Nonipol and eluted with a NaCl-gradient.

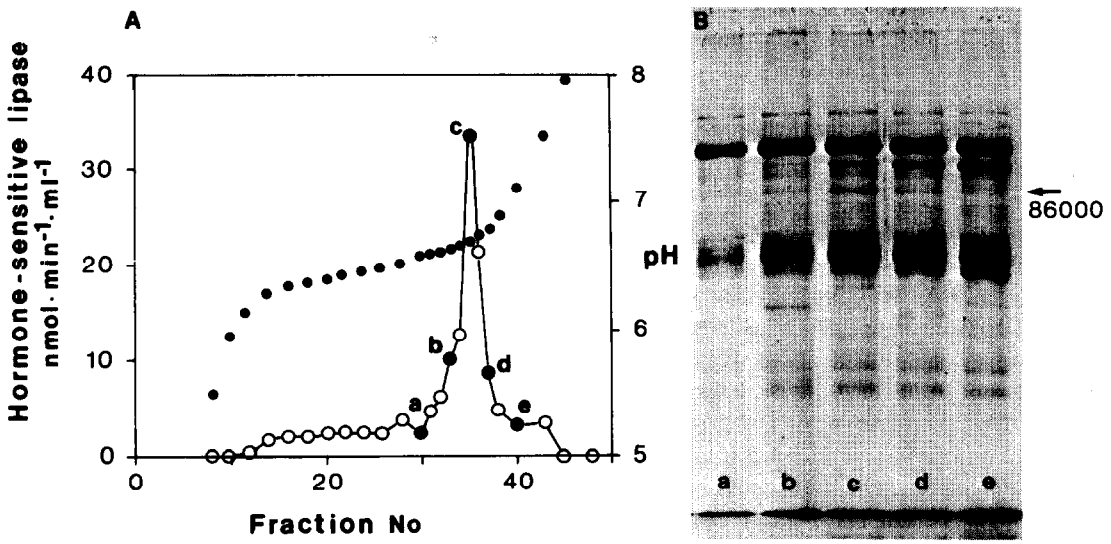

Fig.2A. Narrow pH-range electrofocusing of combined hormone-sensitive lipase peak fractions from the first electrofocusing. Conditions: 1% Ampholine, pH 6–7, 0–60% sucrose-gradient, 1 mM dithioerythritol, 1 mM EDTA, 3 mM Nonipol, 1200 V, 4 W for 80 h. No monoacylglycerol lipase or lipoprotein lipase could be detected. Fig.2B. Dodecylsulfate gel electrophoresis of the indicated fractions (a–e) from the electrofocusing.

Table 1
Activation of hormone-sensitive lipase

Activation medium	Enzyme activity (nmol·min^{-1}·ml^{-1})	Activation % P^a	
Complete	2.31 ± 0.03[b]	55	< 0.001
minus protein kinase	1.49 ± 0.04	0	
minus ATP, cyclic AMP	1.61 ± 0.04	8	n.s.
minus ATP, cyclic AMP, protein kinase	1.57 ± 0.08	5	n.s.
Complete	0.32 ± 0.01[c]	100	< 0.01
minus protein kinase	0.16 ± 0.03	0	

[a] Determined by Student's *t*-test for unpaired samples
[b] Mean ± SE, $n = 5$
[c] $n = 3$

Purified detergent-free enzyme was incubated for 10 min at 37°C in 10 mM sodium phosphate buffer, pH 8.0, with 1.5 mM ATP, 10 mM $MgCl_2$, 3 mM theophylline, 0.5 mM EGTA, 1 mM dithioerythritol, 10 μM cyclic AMP and 10 μg/ml protein kinase (cyclic AMP-dependent, beef heart, Sigma Chem. Co.). Lipase activity was then determined with (upper experiment) 0.25 mM or (lower experiment) 0.025 mM tri[^{3}H]oleoylglycerol as substrate in 0.1 M sodium phosphate buffer, pH 6.8, for 20 min at 37°C.

The hormone-sensitive lipase showed 'tri- and monoglyceridase' activities in addition to the 'diglyceridase' activity, used for practical reasons to monitor the enzyme. The relative proportions of the 'tri-, di- and monoglyceridase' activities of the enzyme were approximately 1:10:15.

3.2. *Activation of the hormone-sensitive lipase*

The activity of the purified, detergent-free enzyme increased on incubation with protein kinase, ATP–Mg^{2+} and cyclic AMP (table 1). The magnitude of the activation was inversely related to the substrate concentration used in the enzyme assay (table 1) (cf. [5]). The 'triglyceridase' was stimulated more than the 'diglyceridase' activity while no significant activation of the 'monoglyceridase' could be demonstrated (cf. [5,15]). Crude preparations could also be activated but with less dependency on added protein kinase.

3.3. *Identification of the enzyme protein*

Dodecylsulfate gel electrophoresis of several fractions from the second electrofocusing showed that only one stainable protein with apparent mol.

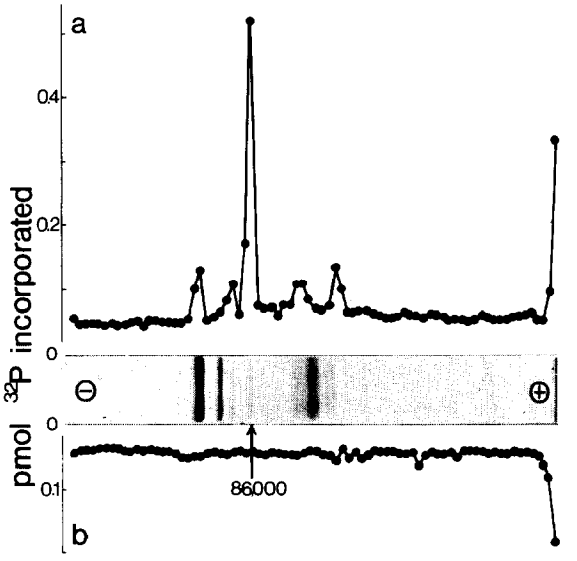

Fig.4. Dodecylsulfate gel electrophoresis of hormone-sensitive lipase after phosphorylation with [γ-^{32}P]ATP. Purified detergent-free enzyme was incubated as described in table 1, but with [γ-^{32}P]ATP (450 mCi/mmol). Gels were cut in 1 mm slices, dissolved in 30% H_2O_2 and ^{32}P was determined by liquid scintillation counting. (a) Complete activation system, (b) control without lipase.

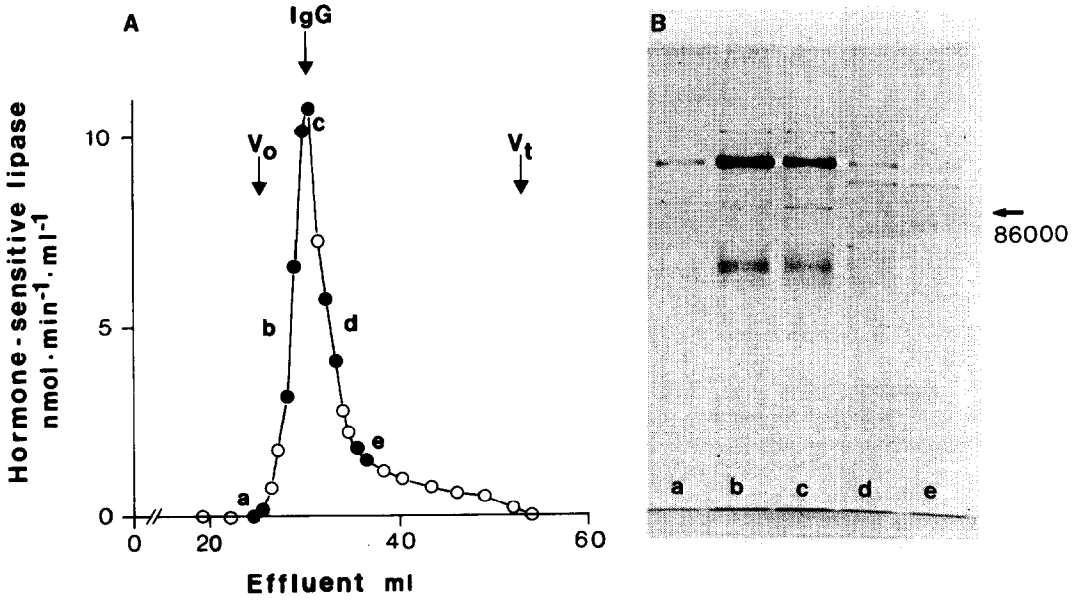

Fig.3A. Chromatography on Sephadex G-200 superfine of the enzyme peak fraction of the second electrofocusing. The column (1.6 × 27 cm) was equilibrated and eluted (1.1 ml/h) with 20 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl, 20% glycerol, 1 mM dithioerythritol, 1 mM EDTA, 0.5 M α-methyl glucoside and 3 mM Nonipol. Fig.3B. Dodecylsulfate gel electrophoresis of indicated fractions (a–e) from the gel chromatography (two adjacent fractions have been combined in each case).

wt 86 000 coincided with enzyme activity (fig.2). Also, only one protein, with the same apparent molecular weight, co-chromatographed with lipase activity on Sephadex G-200 (fig.3). On the basis of these results this protein band was tentatively identified as the hormone-sensitive lipase.

Further support for the identification was the fact that a protein with the same apparent molecular weight was the only protein which extensively incorporated [^{32}P]phosphate when the detergent-free, purified lipase was incubated with protein kinase, [γ-^{32}P]ATP and cyclic AMP (fig.4).

3.4. *Properties of the enzyme*

The solubilized enzyme had a p*I* of 6.7 (fig.2) and the same Stokes' radius as IgG (55 Å), presumably as a protein–detergent complex (fig.3). The enzyme activity ('diglyceridase') was inhibited by 50% after a 30 min preincubation at 37°C in NaF (10 mM), diisopropyl fluorophosphate (50 μM), $HgCl_2$ (10 μM), *p*-chloromercuribenzoic acid (20 μM) or Nonipol (0.6 mM).

4. Discussion

Our results provide strong evidence for a first identification of the enzyme protein of hormone-sensitive lipase, namely as a protein with apparent minimum mol. wt 86 000. First, it seems unlikely that any other protein than the lipase would co-fractionate with the enzyme activity in two different fractionation systems, one based on charge properties and the other on molecular size. Second, a protein with the same apparent molecular weight was phosphorylated by ATP, in a protein kinase-catalyzed reaction, as has also been shown to occur for a partially purified hormone-sensitive lipase preparation from rat adipose tissue [4]. The presented results may also indicate that the activation of hormone-sensitive lipase occurs via a direct protein kinase-catalyzed phosphorylation of the enzyme, without an intermediary 'lipase kinase' analogous to phosphorylase b kinase [16].

Our results also show that hormone-sensitive lipase can be physically resolved from monoacylglycerol lipase, and confirm the separation from lipoprotein lipase [5]. Like the latter enzyme [17] and pancreatic lipase [18] the isolated hormone-sensitive lipase hydrolyzed both tri-, di- and monoacylglycerols. The monoacylglycerol-hydrolyzing activity, however, was low compared to that of monoacylglycerol lipase [6]. The isolated enzyme had the same inhibition properties as monoacylglycerol lipase [6] but in addition it was inhibited by non-ionic detergent and NaF (cf. [19]).

Partially purified hormone-sensitive lipase from rat adipose-tissue has been shown to reside in high molecular weight protein–lipid complexes, which have been suggested to represent a multi-enzyme triacylglycerol-degrading system [4]. However, since an active hormone-sensitive lipase was obtained in a solubilized form with the same K_{av} as IgG (fig.3) the native form of the enzyme has yet to be determined.

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