Hormone-sensitive Lipase of Rat Adipose Tissue

PURIFICATION AND SOME PROPERTIES*

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(Received for publication, February 9, 1981)

Hormone-sensitive lipase from rat adipose tissue was solubilized with nonionic detergent and purified 2,000-fold to 50% protein purity by repeated gradient sieving chromatography on diethyl-(2-hydroxypropyl)minoethylthio-Phedex and by affinity chromatography on triacylglycerol-containing polyacrylamide-agarose gel. The identity of the enzyme protein, a polypeptide with M₀ = 84,000 by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, was established by its copurification with lipase activity, selective labeling with [³H]diisopropyl fluorophosphate, and phosphorylation by the catalytic subunit of cAMP-dependent protein kinase from the same tissue and ATP-Mg. There was no indication for a role of a specific lipase kinase analogous to phosphorylase kinase. The phosphorylation enhanced the trioleoylglycerol lipase activity to 150% over that in control when approximately 0.5 mol of phosphate had been incorporated into each mol of M₀ = 84,000 enzyme protein. The purified lipase catalyzed the hydrolysis of emulsified tri-, di-, and monooleoylglycerol and cholesterol oleate at the relative rates of 1:10:4:1.5, with a molar specific activity of 600 mol/s/mol of M₀ = 84,000 enzyme protein toward dioleoylglycerol. Its specific activity was more than 1,000-fold higher than that of a lipase recently purified from hen adipose tissue (Berglund, Jensen, and Steinberg, 1980). The purified enzyme had a pKₐ optimum of 7.0, which was inhibited by micromolar diisopropyl fluorophosphate, Hg²⁺, and millimolar NaF, and was stable at neutral pH in detergent and high glycerol concentration. The results of this and previous work (Belfrage, Pr., Fredrikson, N. O., and Strålfors, P. 1980) FEBS Lett. 111, 120–124; Nilsson, N. O., Strålfors, P., Fredrikson, G. and Belfrage, P. 1980) FEBS Lett. 111, 125–130) demonstrate modulation of hormone-sensitive lipase activity by phosphorylation in vitro as well as in the intact fat cell and indicate that this is a significant mechanism for the hormonal short term regulation of adipose tissue lipolysis.

*This work was supported by grants from the following foundations: A. Påhlsson, Malmo; T. Segerfalk, Helsingborg; P. Håkansson, Eslov; Novo Insulin, Copenhagen; The Swedish Diabetes Association; and A. O. Swärd, Stockholm; it was also supported by the Medical Faculty, University of Lund, and the Swedish Medical Research Council (Project 3362). Preliminary reports of parts of this work have been presented at the INSERM-EMBO colloquium (1) and the 21st International Conference on the Biochemistry of Lipids, Cologne, West Germany. Phosphatidylinositol (Epicuron 510) was purified by ion exchange chromatography on CM-cellulose. [³²P]ATP was synthesized and purified as in Ref. 17. Bovine serum albumin (crystalline, defatted) was from Sigma; protease inhibitors, leupeptin, and pepstatin were from Peptide Institute, Inc. (Osaka, Japan); QAE-Sephadex A-25 and Sepharyl S-200 were from Pharmacia; hydroxyapatite (spheredead) was from BDH. The catalytic subunit of cAMP-dependent protein kinase was purified to near homogeneity from rat adipose tissue. (¹⁶⁵)Oleoylglycerol lipase (18), >95% protein purity, was prepared from rat adipose tissue by Dr. Hans Tornqvist. The specific inhibitor protein of cAMP-dependent protein kinase (19) was a generous gift from Dr. Philip Cohen, University of Dundee, Great Britain.

Fatty acid mobilization from adipose tissue is regulated by hormone-sensitive lipase, the enzyme catalyzing the rate-limiting step in the hydrolysis of stored triacylglycerol (2). The hormonal regulation of the enzyme (3–5) was early proposed to be mediated by cAMP-dependent phosphorylation in analogy with the hormonal regulation of glycogen degradation (6, 7). Supporting this, particular preparations of hormone-sensitive lipase were activated by cAMP-dependent protein kinase (8–10) and deactivated by protein phosphatases (11). However, purified lipase has not been available to experimentally verify this proposed mechanism.

We recently prepared hormone-sensitive lipase in detergent-solubilized, partially purified form, tentatively identified it with a polypeptide of M₀ = 86,000 by SDS-PAGE, and demonstrated its cAMP-dependent, protein kinase-catalyzed phosphorylation and activation (12). Extending these findings, we describe in this report a procedure for detergent solubilization and extensive purification of hormone-sensitive lipase from rat adipose tissue resulting in a stable enzyme of 50% protein purity. The previous identification of the enzyme protein is confirmed and enhancement of enzyme activity by phosphorylation with cAMP-dependent protein kinase is established. Substrate specificity, inhibition characteristics, and some additional properties of the purified enzyme are described and compared with those of other tissue lipases.

EXPERIMENTAL PROCEDURES

Materials—[¹³C]Oleoyl-labeled acyl- and aoylalkylglycerols, monooctoyl[¹³C]glycerol, and cholesterol [¹³C]oleate were synthesized and purified as previously described (13–16). Soybean phosphatidylcholine (Epicuron 200, >99% pure) was from L. Meyer (Hamburg, West Germany). Phosphatidylinositol (Epicuron 510) was purified by ion exchange chromatography on CM-cellulose. [³²P]ATP was synthesized and purified as in Ref. 17. Bovine serum albumin (crystalline, defatted) was from Sigma; protease inhibitors, leupeptin, and pepstatin were from Peptide Institute, Inc. (Osaka, Japan); QAE-Sephadex A-25 and Sephacryl S-200 were from Pharmacia; hydroxyapatite (spheredead) was from BDH. The catalytic subunit of cAMP-dependent protein kinase was purified to near homogeneity from rat adipose tissue. (¹⁶⁵)Oleoylglycerol lipase (18), >95% protein purity, was prepared from rat adipose tissue by Dr. Hans Tornqvist. The specific inhibitor protein of cAMP-dependent protein kinase (19) was a generous gift from Dr. Philip Cohen, University of Dundee, Great Britain.

Male Sprague-Dawley rats, 200–220 g, were from Anticimex (Swe-
den) and were fed a standard commercial pellet diet until killed. Fat pads were immediately removed and either processed directly or frozen in homogenization solution ("Results") in liquid N2 and stored at −70 °C until further use.

Detergents—Alkyl polyoxyethylene ethers with the general formula CnH2n+1(OCH2CH2)2OH, abbreviated to CnE1, were used. C13E12, from Berol Kemt AB (Stenungsund, Sweden); homogeneous C12E12 was from NIKKO Chemicals (Tokyo, Japan), while C13E12, a mixture of C12E12 (<25%), C13E12 (50%), and C14E12 (<25%), was synthesized as described in Ref. 20. C12E12 and C13E12 have a critical micellar concentration at 35 °C of approximately 0.1 mM and C13E12 has a critical micellar concentration of 10 mM (21). Heterogeneous C12[(CH2)13OH]-alkyl labeled C13E12 (or 1°C detergent) was synthesized and purified as will be described elsewhere. All detergents were deionized in concentrated aqueous solution on a mixed ion exchange resin and stored at −20 °C.

Affinity Chromatography Gel—Ultragel Aca 34 (manufactured by the Industrie Biologique Francaise, Gennevilliers, France) was obtained from LKB. Gel produced before 1977 contains soybean oil triacylglycerols, which were immediately removed and either processed directly or mined with tri[3H]oleylglycerol, di[3H]oleoylglycerol, 1(3)-monoo- [3H]oleoylglycerol (13) and lipoprotein lipase was determined with emulsified tri[3H]oleoylglycerol (18) was determined with micellar monooleoyl [3H]glycerol, respectively (equal amounts of triacylglycerol lipase measured in the assays for hormone-sensitive lipase with di- and trioleoylglycerol, respectively (equal amounts of triacylglycerol lipase activity of both enzymes were used). Unless otherwise stated, incubation was always 30 min at 37 °C. Assay of hormone-sensitive lipase activity was routinely assayed at concentrations 7.0 and 10 mM dithioerythritol, 10 μg/ml of leupeptin. It was then applied to a hydroxyapatite column (0.1 g/ml of enzyme solution) equilibrated in 10 mM potassium phosphate, pH 7.0, 30% (w/v) glycerol, 1 mM dithioerythritol, 0.2% (w/v) C12E12 or the same buffer but with 50% (w/v) glycerol, 2 mM C12E12. After sample application, the column was washed with 4 volumes of the equilibration buffer at a flow rate of 20–30 cm/h. The enzyme was eluted in the equilibration buffer with 0.5 or 0.3 M potassium phosphate, respectively.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed as described by Laemmli (28) with modifications (29). Enzyme samples were precipitated with trichloroacetic acid and extracted with solvex before electrophoresis (29). As reference proteins, phosphorylase a from rabbit muscle (M = 94,000), human transferrin (M = 76,700), bovine serum albumin (M = 67,000), and ovalbumin (M = 43,000) were used (all from Sigma). Protein bands were stained with Coomassie blue (Genaid blue; BDH) and roughly quantitated by scanning densitometry at 633 nm (soft laser densitometer, model SL 504; Biomedical Instruments, Chicago). The enzyme, the activity toward monoylalkylglycerol was equal to the diacylglycerol hydrolase activity of the hormone-sensitive lipase (see below).

Autoantibody and Fluorography—Stained and dried polyacrylamide gels were autoradiographed using Kodak X-Omat R film and an intensifying screen (DuPont) (30). For fluorography, stained gels were treated with sodium salicylate (31), dried, and autoradiographed as above.

Protein Determination—Protein was either monitored by absorbance at 280 nm or determined with a scaled down version (32) of the method of Lowry et al. (33) after trichloroacetic acid precipitation in detergent followed by extraction with diethyl ether:ethanol, 3:1 (v/v). Albumin was used as standard.

RESULTS

Hormone-sensitive lipase activity was routinely assayed with monoleoyl-2-O-oleoylglycerol, a monoether analogue of dioleoylglycerol (unless otherwise stated, enzyme activity refers to this monocacylglycerol hydrolyase activity), since the enzyme hydrolyzes this substrate 3-fold faster than triacylglycerols. Moreover, monoacylglycerol cannot be hydrolyzed to monoglycerides and thus cannot produce substrate for monoacylglycerol lipase. In preparations lacking this enzyme, the activity toward monocyacylglycerol was equal to the diacylglycerol hydrolyase activity of the hormone-sensitive lipase (see below).

A representative purification was summarized in Table I.

Table I. Initial Fractionation of Crude Tissue Extracts—Epididymal adipose tissue (approximately 1.1 g of tissue/rat) was homogenized in 2 volumes of 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 20 μg/ml of leupeptin, 1 μg/ml of peptatin at pH 7.0 and 10 °C. The use of protease inhibitors was necessary when frozen fat was used to prevent rapid proteolytic degradation of the enzyme. Floating fat and
TABLE I
Purification of hormone-sensitive lipase from rat adipose tissue

Enzyme was purified from 220 g of fat pads obtained from 200 rats. Enzyme activity was measured toward mono[3H]oleoylalkylglycerol substrate ("Experimental Procedures"). One unit of enzyme activity is equivalent to 1 pmol of fatty acid produced/min at 37 °C.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Recovery</th>
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<tbody>
<tr>
<td></td>
<td>units</td>
<td>units/mg protein</td>
<td>%</td>
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<tr>
<td>I. Tissue homogenate</td>
<td>160</td>
<td>0.049</td>
<td>100</td>
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<tr>
<td>II. 110,000 × g supernatant</td>
<td>100</td>
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<td>62</td>
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<tr>
<td>III. pH 5.2 precipitate</td>
<td>93</td>
<td>0.22</td>
<td>58</td>
</tr>
<tr>
<td>IV. Solubilized pH 5.2 precipitate</td>
<td>88</td>
<td>0.21</td>
<td>55</td>
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<tr>
<td>V. First gradient sievrotive chromatography</td>
<td>44</td>
<td>3.1</td>
<td>28</td>
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<tr>
<td>VI. Second gradient sievrotive chromatography</td>
<td>23</td>
<td>7.4</td>
<td>14</td>
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<tr>
<td>VII. Concentration on hydroxyapatite</td>
<td>14</td>
<td>7.2</td>
<td>8.8</td>
</tr>
<tr>
<td>VIII. Affinity chromatography</td>
<td>7.2b</td>
<td>100b-c</td>
<td>4.6b</td>
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</table>

* Measured by pH-stat titration using unlabeled monoacylalkylglycerol ("Experimental Procedures").
* Represents the combined enzyme from two identical chromatographies.
* Protein was measured after concentration of the enzyme on hydroxyapatite.

The enzyme was solubilized with detergent as follows: 13% (w/v) sucrose, 65 mM NaCl, 1 mM EDTA, 1 mM dithioerythritol (final concentrations) dissolved in 50 ml of pH 5.2 ppt. This solution was added in portions, with light sonication, to concentrated detergent solution: 69 ml of 12% (w/v) C12E12 and 15 mM NaCl, all pH 7.97. The sample, 130 ml of solubilized pH 5.2 ppt, was applied (flow rate, 300 ml/h) followed by 200 ml of final equilibration buffer but with 65 mM NaCl, 5% (w/v) sucrose and eluted with the same buffer without sucrose. A 65-1000 mM NaCl gradient was then applied. Glyceral with 25 mM KH2PO4, 1 mM dithioerythritol, 10 μg/ml of leupeptin (5.7 ml) was added continuously to each column fraction (14.4 ml). TG, MG, CE, emulsified tri., monoacylglycerol, and cholesterol ester used as substrates (detergent inhibition prevented the measurement of absolute values); MG lipase, monoacylglycerol lipase; kinase, cAMP-dependent protein kinase; phosphatase, protein phosphatase; lipid P, lipid phosphorous. Inset, same chromatography (scaled down to a column of 2.6 × 90 cm) of solubilized pH 5.2 ppt containing 125I-lipoprotein lipase. Conditions as above. Except for 125I-lipoprotein lipase, illustrated parameters can be identified from comparison with A.
The mixture was then sonicated at 10 °C until almost clear visually. Insoluble material was removed by centrifugation at 15,000 \( \times g \) for 20 min. The final detergent concentration (6.5%, w/v) was chosen to correspond to at least 5 detergent micelles/protein molecule (cf. Ref. 21). Detergent was included in all solutions in subsequent purification steps.

**Step V: First Gradient Sievorrptive Chromatography**—Conventional ion exchange chromatography resulted in large losses of enzyme, probably due to irreversible aggregation of proteins concentrated by adsorption to the matrix. This problem was overcome by fractionation with gradient sievorrptive ion exchange chromatography (detailed description of this technique in Ref. 35), which allowed the proteins to interact with the matrix without being bound and concentrated.

The solubilized pH 5.2 ppt was chromatographed according to this technique on a short QAE-Sephadex column (Fig. 1). The column was developed by the inherent 15–65 mM NaCl gradient, formed by applying sample and eluting in 65 mM NaCl (cf. Ref. 35). Hormone-sensitive lipase was eluted at 40 mM NaCl (Fig. 1A). The elution patterns for all acylhydrolase activities were the same except for the major part of the activity toward monoacylglycerols, due to monoacylglycerol lipase that eluted with the unretained protein peak (Fig. 1B).

No other acylhydrolase activity was detected when the entire column eluate was tested with all four acylhydrolase substrates at neutral and acid pH. Total enzyme recovery was more than 70% at this step. The enzyme was stable only at neutral pH. By lowering the pH of the chromatography toward neutrality, enzyme recovery could be increased to almost 100% at the expense of decreased resolution. Additional inactivation was caused by the pH increase due to salt gradient effects on the ion exchange gel matrix (Fig. 1C; not illustrated in Fig. 1). The pH inactivation was minimized by continuously bringing column effluent to pH 7 in 50% (w/v) glycerol.

At this step, the enzyme was separated from the bulk of sample detergent (1°C detergent; Fig. 1C) and phospholipids (Fig. 1D) (neutral lipids also eluted with unretained proteins). Lipoprotein lipase activity, known to be present in the pH 5.2 ppt, could not be measured in the column eluate, probably due to detergent inhibition. \( ^{125} \text{I}-\text{Lipoprotein lipase added to the pH } 5.2 \text{ ppt, solubilized and subjected to gradient sievorrptive chromatography, was partly eluted with the unretained proteins (Fig. 1D, inset). Most of the enzyme, however, was eluted over a whole column volume, presumably aggregated (total yield was 87%). The small amount (<10% of initial) eluting with hormone-sensitive lipase at this step was negligible (<0.5% of initial) after the second gradient sievorrptive chromatography.}

Cyclic AMP-dependent protein kinase and protein phosphatase activities were eluted well separated from hormone-sensitive lipase (Fig. 1, B and C). Very low levels of the catalytic subunit of cAMP-dependent protein kinase and a cAMP-independent protein kinase activity contaminated hormone-sensitive lipase fractions but were completely removed after the second gradient sievorrptive chromatography (not illustrated).

**Step VI: Second Gradient Sievorrptive Chromatography**—Indicated fractions from the first gradient sievorrptive chromatography (Fig. 1A, shaded area) were concentrated approximately 10-fold by pressure ultrafiltration (“Experimental Procedures”) and dialyzed at 10 °C against the elution buffer with 50 mM NaCl used at the second gradient sievorrptive chromatography (legend for Fig. 2). The enzyme was then immediately (due to the pH inactivation) applied to a long QAE-Sephadex column and eluted with a preformed salt gradient of gentle slope (Fig. 2).

Hormone-sensitive lipase eluted at 40 mM NaCl in 70% total yield (Fig. 2A). The elution patterns for all acylhydrolase activities were similar (Fig. 2). The enzymological properties of the second enzyme peak, including its phosphorylatability, could not be distinguished from those of the main enzyme peak. Thus, it probably reflected enzyme charge heterogeneity.

The protein purity of the enzyme after this step was 3–4%. At this stage of purification, the enzyme preparation completely lacked monoacylglycerol lipase, lipoprotein lipase, and the activities potentially involved in the regulation of hormone-sensitive lipase, those of protein kinase and protein phosphatase.

**Step VII: Affinity Chromatography**—Indicated fractions from the second gradient sievorrptive chromatography were dialyzed and concentrated on hydroxyapatite (“Experimental Procedures”) in a total yield of 75%. The most concentrated enzyme (80% of total) was then subjected to affinity chromatography on a triacylglycerol-containing polyacrylamide-agarose gel (Ultrogel AcA 34; “Experimental Procedures”) in CsE6 detergent (Fig. 3).

The enzyme was eluted (70% total yield) immediately before the column total volume and the high phosphate applied with the sample (Fig. 3A). The elution patterns for all four acylhydrolase activities were again the same (Fig. 3), indicating...
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that they were all due to hormone-sensitive lipase. The pattern of eluted protein could not be monitored by its absorbance at 280 nm due to its low concentration and interfering trace impurities in the C₈E₆ detergent. Protein values given in Fig. 3A (measured according to Lowry et al. (33) as modified by Tornqvist and Belfrage (32)) should only be considered as approximate since the high concentration of C₈E₆ detergent partly prevented precipitation of the protein. This precluded a direct quantitation of the minute amounts of protein in the enzyme fractions. However, the mean protein concentration of the pooled enzyme (Fig. 3A, shaded area) was calculated to approximately 1 µg/ml by protein determination after concentration and C₈E₆ removal on hydroxyapatite (see below).

In the pooled enzyme, the predominant protein species, the $M_r = 84,000$ polypeptide identified below with the lipase, was enriched from approximately 4 to 45% of stabilable protein by the affinity chromatography step (Fig. 3A, Insets a and b). SDS-PAGE performed on consecutive fractions over the enzyme peak showed that this polypeptide selectively coeluted with enzyme activity (cf. Ref. 12), thereby separating from the main protein contaminant with slightly higher electrophoretic mobility (data not shown).

The mechanism of adsorption of the enzyme on the Ultrogel AcA 34 is not clear. The presence of triacylglycerol in the gel matrix was, however, necessary, as was equilibration in C₈E₆ detergent instead of the usual C₁₃E₁₂, possibly reflecting the difference in critical micellar concentration between these two detergents. True affinity interaction between enzyme and immobilized substrate is certainly an important part of the overall process, but nonspecific hydrophobic interactions and polar interactions probably also determine the behavior of the enzyme and contaminating proteins eluting with the enzyme (cf. Ref. 36).

Overall recovery of enzyme activity after this final purification step was 4–5% (range of six different purifications) but, as discussed below, due to pH inactivation, enzyme protein yield was higher. The specific activity of the final enzyme preparation was 100 units/mg of protein (Table I), varying between 83–113 in three different purifications.

Enzyme activity was labile in C₈E₆, in which it was eluted from the affinity chromatography column. A stable working preparation, referred to as purified enzyme in the following, was obtained by concentration with detergent exchange of indicated fractions (Fig. 3A) on hydroxyapatite (“Experimental Procedures”). After this procedure, i.e. in 0.3 M potassium phosphate, pH 7.0, 50% (w/v) glycerol, 1 mM dithioerythritol, the concentrated enzyme from the second gradient sievoprtive chromatography was fractionated one-half at a time. The sample, 8.8 ml, was followed by 12 ml of sample buffer (0.5 M potassium phosphate, pH 7.0, 30% (w/v) glycerol, 1 mM dithioerythritol, 0.15 M NaCl, 0.2% (w/v) C₁₃E₁₂), followed by 2 volumes of the same buffer with 0.8% (w/v) C₈E₆ instead of C₁₃E₁₂. Column dimensions and other conditions were chosen for optimal resolution; therefore, the concentrated enzyme from the second gradient sievoprtive chromatography was fractionated one-half at a time. The sample, 8.8 ml, was followed by 12 ml of sample buffer (0.5 M potassium phosphate, pH 7.0, 30% (w/v) glycerol, 1 mM dithioerythritol, 0.2% (w/v) C₁₃E₁₂), and the enzyme eluted with the equilibration buffer with C₈E₆. Flow rate was 23 ml/h, fraction volume, 6.1 ml and temperature, 4 °C. C₈E₆ was added to each column fraction to 0.1% (w/v) to stabilize the enzyme. Abbreviations are as in Fig. 1. Inset, Coomassie blue-stained SDS-PAGE slab gels of (a) pooled enzyme from Step VII (approximately 30 µg) and (b) pooled enzyme from Step VIII concentrated on hydroxyapatite (approximately 4 µg); β₂-microglobulin (10 µg) was added before precipitation of sample.

Purity of Enzyme—The inhibition of hormone-sensitive lipase by DFP (see below) implies a covalent modification of a catalytically essential serine residue (37). The selective enrichment, during the purification, of [¹⁹F]DFP label in the $M_r = 84,000$ polypeptide (Fig. 4A) and its exclusive labeling in the purified enzyme preparation (Fig. 4A) confirmed its identification with the hormone-sensitive lipase (12). In addition, lipase-substrate binding was directly related to the $M_r = 84,000$ polypeptide because this binding selectively and reversibly protected it from modification by DFP. In the presence of emulsified acylglycerol, enzyme from Step VII was incubated with nonlabeled DFP. After successive removal of the DFP and substrate by microcentrifuge desalting (38) and flotation, respectively, [¹⁹F]DFP incorporation into the other polypeptides decreased to less than 10%, whereas in the $M_r = 84,000$ polypeptide, the incorporation was unchanged when compared to controls pretreated without nonlabeled DFP.

Furthermore, the functional relationship between the enzyme and the $M_r = 84,000$ polypeptide was demonstrated by its selective phosphorylation with enhancement of enzyme activity (see below). In the second gradient sievoprtive chromatography and subsequent steps, only this polypeptide was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase from rat adipose tissue and [γ-³²P]ATP-Mg (Fig. 4B).

The purity of the enzyme protein, the $M_r = 84,000$ polypeptide, estimated by scanning densitometry of Coomassie blue-stained proteins after SDS-PAGE, was 45% (Fig. 4C), varying between 40–60% with six different batches of purified enzyme.

Fig. 3. Affinity chromatography. Ultrogel AcA 34 (2.6 × 89 cm) containing triacylglycerol was equilibrated in 1 volume of 5 mM potassium phosphate, pH 7.0, 30% (w/v) glycerol, 1 mM dithioerythritol, 0.15 M NaCl, 0.2% (w/v) C₁₃E₁₂, followed by 2 volumes of the same buffer with 0.8% (w/v) C₈E₆ instead of C₁₃E₁₂. Column dimensions and other conditions were chosen for optimal resolution; therefore, the concentrated enzyme from the second gradient sievoprtive chromatography was fractionated one-half at a time. The sample, 8.8 ml, was followed by 12 ml of sample buffer (0.5 M potassium phosphate, pH 7.0, 30% (w/v) glycerol, 1 mM dithioerythritol, 0.2% (w/v) C₁₃E₁₂) and the enzyme eluted with the equilibration buffer with C₈E₆. Flow rate was 23 ml/h, fraction volume, 6.1 ml and temperature, 4 °C. C₈E₆ was added to each column fraction to 0.1% (w/v) to stabilize the enzyme. Abbreviations are as in Fig. 1. Inset, Coomassie blue-stained SDS-PAGE slab gels of (a) pooled enzyme from Step VII (approximately 30 µg) and (b) pooled enzyme from Step VIII concentrated on hydroxyapatite (approximately 4 µg); β₂-microglobulin (10 µg) was added before precipitation of sample.
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FIG. 4. Scanning densitograms from SDS-PAGE of hormone-sensitive lipase. Hormone-sensitive lipase from Step VII, 60 µg of protein (upper), and the purified enzyme, 4 µg of protein (lower), were analyzed by SDS-PAGE and scanning densitometry after (A) ['H]DFP labeling and fluorography ("Experimental Procedures"), (B) phosphorylation and autoradiography ("Experimental Procedures"), and (C) Coomassie blue staining of protein. Migrating positions of reference proteins and hormone-sensitive lipase are indicated (k = 10^8).

Relative Acylhydrolase Activities—The four acylhydrolase activities eluted almost identically in the chromatographies (Figs. 1–3), indicating that they were all due to hormone-sensitive lipase. However, only the activity toward monoacylalkylglycerol (and diacylglycerol) could be measured under conditions of minimal detergent inhibition. Enzyme from different stages of purification was therefore concentrated on hydroxyapatite, to allow dilution to noninhibitory detergent concentration (approximately 0.0002% (w/v) C_{12}E_{12} or 3 µM C_{12}E_{0}).

The enzyme activity "profile," approximately 1:10:4:1.5 for tri-, di-, and monoacylglycerol and cholesterol ester substrate, was quite similar over the successive chromatographies, monoacylalkylglycerol and diacylglycerol lipase activities being equal (Fig. 5B). At the stages of purification prior to the chromatographies, including the tissue homogenate (measured with pH-stat titration), activities toward tri-, di-, and especially monoacylglycerol were relatively higher (Fig. 5A). The higher activity toward the last substrate was due to monoacylglycerol lipase (cf. Fig. 1, B and C). However, this enzyme was also responsible for increased fatty acid release from tri- and diacylglycerol with crude tissue preparations (Fig. 5A) by hydrolysis of labeled monoacylglycerols formed as reaction products after hormone-sensitive lipase action on the first ester bonds. This was indicated by the discrepancy between the diacylglycerol and monoacylalkylglycerol hydrolase activities in crude tissue fractions (Fig. 5A) and by the fact that labeled monoacylglycerol was found, by thin layer chromatography, to be a large part of the reaction products during hydrolysis of tri- and diacylglycerol with purified enzyme (up to 50% with the latter substrate) but much less with enzyme preparations containing monoacylglycerol lipase (only unesterified H-fatty acids are isolated and quantitated by the assay procedure used). Addition of pure monoacylglycerol lipase to enzyme preparations at different stages of purification, as expected, had little additional effect on hydrolysis of tri- and diacylglycerol by crude enzyme (Fig. 5, Step III enzyme) but increased these activities with purified enzyme (Fig. 5, Steps V and VIII enzymes) to almost the same level as with crude enzyme. Labeled monoacylglycerol no longer accumulated. Thus, the stated enzyme activity profile is likely to be valid for hormone-sensitive lipase per se.

Effect of Inhibitors—Enzyme activity toward the illustrated substrates (Table II) was 50% inhibited by DFP, NaF, and Hg^{2+} at approximately the same inhibitor concentrations, both

Fig. 5. Relative acylhydrolase activities at different steps of purification. Hormone-sensitive lipase was measured toward the indicated substrates (30 min at 37 °C). Enzyme was from the indicated purification steps (see Table I). Steps V and VIII enzymes were first concentrated on hydroxyapatite ("Experimental Procedures"). When required, enzyme samples were diluted to 3 µM detergent (final concentration). Monoacylglycerol lipase (approximately 4 ng/incubation) was added to incubations with tri- and dioleoylglycerol and monoacylalkylglycerol substrates (as indicated by *) with Steps III, V, and VIII enzymes. Vertical bars indicate S.E. (n = 5). Enzyme activity against monoacylglycerol was set to 100 in each step, and other activities were expressed relative to this. Abbreviations are as in Fig. 1.
with the purified lipase and with the pH 5.2 ppt preparation. The only exception was due to presence of monoacylglycerol lipase in the latter preparation, since this enzyme is not inhibited by NaF (18). The varying effect of the NaF inhibition with different substrates may indicate that it did not interact directly with the enzyme protein. Inhibition by DFP was dependent on the conditions of incubation with enzyme. Endogenous substrate (triacylglycerol) in crude tissue preparations, e.g. pH 5.2 ppt, protected the enzyme catalytic site from DFP modification; thus, millimolar DFP concentrations were required to significantly inhibit the enzyme when incubated in concentrated form (the pH 5.2 ppt data in Table II reflect some remaining protection, although the preparation was diluted 500-fold). Also with purified enzyme, dilution affected the sensitivity to DFP inhibition; with more concentrated enzyme, severalfold higher DFP concentrations were needed for the same effect (see below; Fig. 6).

While it was difficult to inhibit the enzyme in the crude tissue homogenate with DFP for the reasons described above, it was more than 90% inhibited by 200 μM HgCl₂, as measured by pH-stat titration of fatty acid release from endogenous lipids or added emulsified monoacylglycerol or diacylglycerol. Preincubation of enzyme in 1.5 M NaCl at 37 °C had no significant effect on crude or purified enzyme. These inhibition characteristics, which agree with previous findings (7, 12, 39), indicate that hormone-sensitive lipase sustained by monoacylglycerol lipase accounted for most, possibly all, enzyme activity measured in the crude tissue preparations.

Detergents inhibited all acylhydrolase activities but to a variable degree, being more pronounced with tri- than with di- and monacoylated substrates. Inhibition also varied with the specific conditions in each assay (glycerol concentration, type of enzyme preparation). Therefore, determination of enzyme activity required careful attention to detergent concentration in the assay (see above).

**Specific Activity**—From Table I and Fig. 4C, the specific activity was calculated to be 220 units/mg of enzyme protein. However, this value was probably underestimated due to enzyme inactivation, mainly pH inactivation during the gradient sievortive chromatographies. Catalytically active enzyme has therefore been estimated. DFP inhibition and [³H]DFP labeling of purified enzyme varied similarly with DFP concentration (Fig. 6), indicating that only catalytically active enzyme molecules were modified (cf. Ref. 37). From the enzyme activity inhibited by the incorporation of a particular amount of [³H]DFP (Fig. 6) and assuming that a single serine residue per enzyme molecule was modified (37), a molar specific activity of approximately 800 mol/s/mol of M₉₈₈, 48,000 polypeptide at 37 °C could be calculated. This corresponds to a specific activity of 400-500 units/mg of enzyme protein. These data and the final yield of enzyme activity of 4.5% (Table I) thus suggest an overall yield of enzyme protein around 10%.

**Phosphorylation and Activation of Hormone-sensitive Lipase**—It was demonstrated above (Fig. 4B) that purified hormone-sensitive lipase was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase and ATP-Mg. Under the same conditions, enzyme activity increased approximately 2-fold (Fig. 7), with a similar degree of activation at all stages of enzyme purification. In the pH 5.2 ppt, an endogenous cAMP-dependent protein kinase activated the
enzyme but addition of protein kinase catalytic subunit enhanced enzyme activity further. At all subsequent stages of purification, enzyme activation was completely dependent on exogenous protein kinase. Endogenous phosphorylation and activation were completely inhibited by the specific protein inhibitor of CAMP-dependent protein kinase.

To establish that activation was caused by phosphorylation, the time course of these parameters was followed with purified enzyme (Fig. 8). They closely correlated, with half-maximal effects on both after 35 min. The maximal activation of the enzyme approached 150% over control when approximately 0.5 mol of phosphate was incorporated/mol of enzyme protein ($M_r = 84,000$). Addition of the inhibitor of CAMP-dependent protein kinase caused a prompt arrest of further phosphorylation of the purified enzyme (Fig. 8).

The ATP γ-phosphate incorporated was found to be stable in boiling acid and labile in boiling alkali (40), indicating phosphorylation of serine or threonine residues.

Other Properties of the Enzyme—The purified enzyme had a broad pH optimum around pH 7. The activity against monoa.aryl glycerol and cholesterol ester but not toward triacylglycerol was still high at pH 8, while below pH 6, the activity against all substrates declined quickly and disappeared below pH 5.

Its apparent $M_r$ was 84,000 by SDS-PAGE under the conditions used (see “Experimental Procedures”). The apparent Stokes radius of the enzyme was 50 Å, as determined by gel chromatography on Sephacryl S-200 in CsCl which might indicate an enzyme monomer in a detergent micelle or an enzyme dimer. The enzyme lost 50% of its activity by incubation for 30 min in 50% glycerol, 2 mM Ca$^{2+}$ at 70 °C but lost 30% of its activity at 30 °C when the glycerol was diluted 100-fold.

**DISCUSSION**

The enzyme, extensively purified in this work, was identified with hormone-sensitive lipase on the basis of several lines of evidence. It was purified from an adipose tissue preparation, the pH 5.2 ppt, the neutral lipase activity of which has been amply demonstrated to be due to this enzyme (10, 39, 41). The acylhydrolase activities copurified (Figs. 1–3) with a constant relation between the activities (Fig. 5), and no other lipases other than monoa.aryl glycerol lipase and lipoprotein lipase were found to separate during the purification. Loss of enzyme can be accounted for by the pooling of enzyme fractions and by inactivation, mainly pH inactivation. Moreover, the purified enzyme had the same inhibition characteristics as the pH 5.2 ppt lipase activity (Table II). The lipase could be activated by cAMP-dependent protein kinase (Fig. 7), a most important characteristic of hormone-sensitive lipase (8), and it had the inhibition properties of this enzyme (7). Most of the lipase activity of the crude homogenate was recovered in the pH 5.2 ppt (Table I), the only significant loss being that to the floating fat. However, this activity is likely also to be due to the same enzyme since $^{32}$P-labeled hormone-sensitive lipase (from in vivo $^{32}$P-labeled intact adipocytes (42)) distributed similarly (25–30% in the floating fat) and the inhibition properties were the same (90% inhibition by 200 μM Hg$^{2+}$; substrate protected from DFP modification). The original report characterizing hormone-sensitive lipase activity (7) did not exclude the possibility that this activity would be due to more than one lipase. The present work indicates that this may indeed be the case, since monoa.aryl glycerol lipase contributed to the free fatty acid release measured in crude tissue preparations by hydrolyzing the monoa.aryl glycerol formed.

The identification of the purified enzyme is further supported by quantitative considerations regarding its functional importance. Its activity in crude tissue homogenates can be compared with the lipolysis rates of intact tissue or fat cells. Glycerol release from whole fat pads (fed rats (220 g), we find a fatty acid release of less than 0.03 μmol/min/μl of packed cells before and approximately 0.7 after maximal hormonal activation (cf. below). The rate of endogenous triacylglycerol hydrolysis in the crude homogenate used as the enzyme source in the purification described in this report (Table I) was 0.15 μmol of fatty acid/min/g of adipose tissue. Thus, the lipase which has been purified may well account for the lipolysis rates obtained in intact tissue and fat cells.

The identification of the hormone-sensitive lipase with the protein of apparent $M_r$ of approximately 84,000 is established following several lines of evidence: (a) It was enriched through the successive stages of purification to almost 50% purity in the final preparation and selectively eluted together with enzyme activity in the affinity chromatography; (b) in the purified enzyme preparation, only this polypeptide was labeled with $[^3H]$DFP, an inhibitor of the lipase activity, and this labeling and inhibition was shown to be modified by enzyme-substrate interaction; (c) only the $M_r = 84,000$ polypeptide was $^{32}$P phosphorylated by incubation with the catalytic subunit of cAMP-dependent protein kinase and [γ-$^{32}$P]ATP-Mg in close correlation with enhancement of enzyme activity. These results confirm our previous results (12) and are further supported by the findings that hormone-sensitive lipase activity was associated with $^{32}$P-labeled protein of $M_r = 84,000$ after extensive purification of the enzyme from $^{32}$P-labeled intact fat cells (42).

Previous attempts to purify hormone-sensitive lipase (41) have been only partly successful due to interaction of the enzyme with lipid, resulting in large lipid-protein aggregates, and to difficulties encountered when trying to extract the lipids with solvents (45, 46) or detergents. However, with techniques for detergent solubilization of adipose tissue preparations developed in this laboratory and used for purification of monoa.aryl glycerol lipase (18), hormone-sensitive lipase was solubilized and partially purified (12). Recently, extensive purification of hormone-sensitive lipase from hen adipose
tissue was reported by Berglund et al. (47). They identified the enzyme with a protein of apparent M, =42,000 by SDS-PAGE. Although this finding may reflect a species difference, the characteristics of this purified lipase are so different that its relation to hormone-sensitive lipase must be questioned. The purified enzyme, the predominant protein by SDS-PAGE in the final preparation, had a maximal specific enzyme activity of approximately 0.1 μmol of fatty acid/min/mg of protein, less than 1/1000 of that calculated for the rat enzyme in this report. The total amount of enzyme purified was also quite low (0.033 unit from 275 g of hen adipose tissue as compared with 7 units from 225 g of rat adipose tissue), reportedly (47) partially due to its marked instability. Moreover, although the M, = 42,000 polypeptide could be phosphorylated by incubation with cAMP-dependent protein kinase and ATP-Mg, enzyme activatability was rapidly and completely lost during the purification. The recent observations by the same workers that the M, was 45,000 (47) or, after cautious sample pretreatment, 84,000 by SDS-PAGE (48), may indicate that the purified enzyme represented a proteolytically modified hormone-sensitive lipase.

In the present work, the affinity chromatography accounted for much of the enzyme purification. Although, as discussed above, the mechanism for enzyme binding and elution at this step is not clear, under the conditions used, it was quite reproducible. Interestingly, carboxymethyl-Sepharose substituted with diocetanin has been used in a similar manner for the purification of a glycerol ester hydrolase of rat intestinal mucosa (36). Also, elution of milk lipoprotein lipase from heparin-substituted agarose gel with detergent has been observed (49). At present we have found no way to continue purification of the small amount of enzyme protein (30 μg from 200 rats) to homogeneity. Molecular size fractionation has provided little further separation from the remaining protein contaminants.

The specific enzyme activity of the purified hormone-sensitive lipase toward its optimal substrate approached that found with other mammalian tissue lipases such as lipoprotein lipase (50), hepatic (salt-resistant) lipase (51), and monoacylglycerol lipase (18). The DPF inhibition and [H]JDFP-labeling experiments demonstrated that the molar specific activity with this substrate was in the same order of magnitude as that found in bovine milk lipoprotein lipase (50). The substrate specificity of the enzyme was distinctly different from that of other mammalian tissue lipases (50-52), with much higher relative activity toward diacylglycerol and with activity toward cholesterol esters (Fig. 5). The latter finding probably explains the reported presence of cholesterol esterase activity which could be enhanced with cAMP-dependent protein kinase and ATP-Mg in particulate hormone-sensitive lipase preparations (53) and the recent observation (54) that phenylmethylsulfonyl fluoride inhibited both triacylglycerol and cholesterol ester hydrolase activity to the same extent.

The hallmark of hormone-sensitive lipase is its activatability. In this work, we have shown that the enzyme can be phosphorylated and activated by a cAMP-dependent protein kinase, confirming and extending our previous findings (12). The dependence of activation on phosphorylation demonstrated by their close time relationship clearly indicates the significance of the process. The prompt arrest of phosphorylation by the specific inhibitor protein of cAMP-dependent protein kinase supports the notion of no obligatorily intervening "lipase-kinase" (55), analogous to phosphorylase kinase. There may, however, be other protein kinases, acting independently of cAMP and cAMP-dependent protein kinase, that operate in the regulation of hormone-sensitive lipase.

The physiologic relevance of phosphorylation as a regula-
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