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Partial Purification and Identification of Hormone-Sensitive Lipase from Chicken Adipose Tissue

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HSL from chicken adipose tissue exhibits remarkable activation upon phosphorylation with cAMP-dependent protein kinase (cAMP-PK) compared to HSL from rat and human adipose tissue. In order to characterize the chicken HSL enzyme, it was purified 3500 fold from a chicken adipose tissue homogenate using pH 5.2 precipitation and anion-exchange chromatography. The purified chicken HSL was identified as an 86 kDa protein using Western blot analysis. The HSL diacylglycerol lipase activity was inhibited by 98% upon incubation with anti-rat HSL antiserum, and the specific activity of chicken HSL was estimated to be approximately the same as for the rat enzyme. Furthermore, the 86 kDa polypeptide was phosphorylated by cAMP-PK to about the same stoichiometry as for the recombinant rat enzyme. Hence, our results demonstrate that HSL from chicken adipose tissue is comparable in size and specific activity to HSL from mammalian species, and not a smaller 42 kDa polypeptide with 1000-fold lower specific activity as previously reported (Berglund, L., Khoo, J. C., Jensen, D., and Steinberg, D., 1980 *J. Biol. Chem.* 255, 5420–5428).

Hormone sensitive lipase (HSL; EC 3.1.1.3) is a key enzyme in mammalian lipolysis and fatty acid mobilization. It catalyzes the rate-limiting step in the hydrolysis of stored triacylglycerols in white adipose tissue, and also the subsequent hydrolysis of diacylglycerols (1, 2). HSL is unique among lipases in that it is regulated by reversible phosphorylation. Phosphorylation and concomitant activation is brought about by lipolytic hormones (such as catecholamines, glucagon, ACTH), and is mediated through cAMP-dependent protein kinase (cAMP-PK). cAMP-PK phosphorylates rat HSL at several serine residues, including Ser-563 (3, 4), and also at the recently identified Ser-659, Ser-660, which regulate the activity of HSL *in vitro* (5). Antilipolytic hormones, such as insulin cause a net dephosphorylation and deactivation of HSL. A second phosphorylation site, Ser-565 in rat HSL, termed the basal site, has been proposed to be phosphorylated by 5′AMP-activated kinase and to exert an antilipolytic effect (6, 7).

HSL has been cDNA cloned from rat, human and mouse adipose tissue (8, 9, 10). The sequence identity at the cDNA level is 92% between rat and mouse and 78% between the human and mouse sequences. The rat and mouse amino acid sequences share respectively 86 and 85% identity with the human HSL, whereas the rat and mouse proteins display 94% identity at the amino acid level. HSL is not significantly related to any other known mammalian lipase, but shows sequence similarity to the lipase 2 from *Moraxella TA144* (9) and to a few other bacterial and fungal proteins (11, 12, 13). Domain structure analyses have suggested that HSL is composed of two domains; an *N*-terminal, presumably lipid binding domain, and a *C*-terminal, catalytic domain, containing the catalytic triad and a regulatory module with the phosphorylation sites. The catalytic domain has been further analyzed by building a three-dimensional model; based on secondary structure homology between HSL and a superfamily of esterases and lipases that includes acetylcholinesterase, bile salt-stimulated lipase, and several fungal lipases. The model shows that the catalytic domain of HSL adopts the α/β-hydrolase fold typical of esterases and lipases, identifies the residues of the catalytic triad, and suggests an external location of the regulatory module (13, 14).

Hitherto, the mechanism behind activation of HSL upon phosphorylation by cAMP-PK is poorly understood, but seems to involve both translocation of HSL from the cytosol to the lipid droplet (15) and intrinsic changes in the HSL molecule. In fact, despite the ubiquitous use of reversible protein phosphorylation as a

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Abbreviations: cAMP-PK, cAMP-dependent protein kinase; HSL, hormone sensitive lipase; $C_{13}E_{12}$, an alkylpolyoxyethylene ether-type detergent; MOME, mono-oleoyl-2-O-mono-oleylglycerol; TO, trioleoylglycerol.

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principle for regulation of biological activity of proteins, glycogen phosphorylase (16), isocitrate dehydrogenase (17) and cyclin-dependent kinase (18) are the only proteins for which the molecular basis for this regulation of activity is known.

The different mammalian HSL sequences obtained up to date show high degree of homology. The major differences between the sequences consist of deletions of variable lengths, which are found immediately *N*-terminal of the phosphorylation sites in the regulatory module. However, no major differences in activation through *in vitro* phosphorylation have been found between rat and human HSL (19). In contrast, HSL from chicken adipose tissue has been shown to exhibit remarkable activatability upon phosphorylation compared to HSL from other species (20, 21). Phosphorylation by cAMP-PK increases the activity of chicken adipose tissue up to 800% *in vitro* as measured on triacylglycerol substrates, whereas the enzyme from rat is activated maximally 200%. This significant difference in activation upon phosphorylation by cAMP-PK indicates structural differences. In addition, it suggests specific regulatory properties for HSL from chicken adipose tissue, as can also be anticipated in view of the characteristic lipid metabolism of migratory birds with ability to rapidly store and, when needed, utilize the lipid storages (22). Determination of the primary structure of chicken HSL, where the sequence of the regulatory module is of particular interest, should in itself provide valuable information for the elucidation of the activation mechanism of HSL. Furthermore, after expression of the enzyme in a recombinant form, more extensive structural and functional studies of this highly activatable HSL enzyme will be feasible. Therefore, we have taken further steps to investigate chicken HSL.

All attempts to PCR-amplify chicken HSL cDNA using synthetic primers designed from presumably conserved sequences, including the active site serine (23), were unsuccessful. Also, full-length cDNA probes for HSL from rat and human adipose tissue, were not sufficiently similar to give significant hybridization to mRNA species in Northern blot analysis. Finally, using rat and human HSL cDNA probes, no true positive clones could be isolated upon screening a chicken adipose tissue cDNA library. In order to be able to generate partial protein sequences for the synthesis of chicken HSL specific probes, we therefore performed a partial purification of HSL from chicken adipose tissue. This work led to the identification of HSL from chicken adipose tissue as an 86 kDa protein. Consequently, HSL from chicken adipose tissue is comparable in size to HSL known from other species, and not a smaller 42 kDa polypeptide as previously reported (24).

MATERIALS AND METHODS

Preparation of homogenate and pH 5.2 precipitate. Abdominal fat from 75 laying hens was minced and homogenized for 2 minutes at 4°C in 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 10 μg/ml leupeptin, 2 μg/ml antipain and 1 μg/ml pepstatin at pH 7.4 (about 3 g fat per ml homogenization buffer). A fat-depleted infranatant was obtained by centrifugation at 10 000 xg at 4°C for 45 min, followed by filtration trough glass-wool. The infranatant was either analyzed directly, precipitated at pH 5.2 or frozen in liquid nitrogen and stored at −80°C until analyzed. The rat and human adipose tissue homogenates were prepared as described (25, 26).

Due to the low activity of HSL in the chicken adipose tissue homogenate (compared to the rat and human homogenates), a precipitation at pH 5.2 was performed in order to concentrate the activity. The pH 5.2 precipitate was prepared from the infranatant by addition of 0.2 M acetic acid to pH 5.2. The precipitate, formed over 30 min at 4°C, was collected by centrifugation at 10 000 xg for 30 min at 4°C and dissolved in 20 mM Tris-HCl, pH 7.0, with 1 mM EDTA, 1 mM dithioerythritol and, 80 μg/ml leupeptin, 8 μg/ml antipain and 4 μg/ml pepstatin to obtain 10-40 fold concentration. This fraction was stored at −80°C until used.

Detergent solubilization of the pH 5.2 precipitate and partial purification on Q-Sepharose. The pH 5.2 precipitate was solubilized with detergent (1% $C_{13}E_{12}$, a detergent from the alkyl polyoxyethylene group, Berol 058, Berol Kemi AB, Stenungsund, Sweden) and 10 mM sodium chloride using sonication with a Branson Sonifier 250 at setting 1-2 (3-4 min in 30 sec pulses; cooling with ice). Insoluble material was removed by centrifugation at 10 000xg for 10 min at 4°C. The clear supernatant was subjected to anion exchange chromatography on Q-Sepharose FF (Pharmacia Biotech), essentially as reported for the purification of recombinant rat HSL (27).

Enzyme assays and phosphorylation by cAMP-PK protein kinase. HSL was routinely assayed using emulsified 1(3)-mono-[^{3}H]oleoyl-2-O-mono-oleyl-glycerol (a diacylglycerol ether analogue) as substrate (27, 28). For inhibition assays the homogenate or the pH 5.2 precipitate was preincubated with anti-rat HSL (whole antiserum or affinity-purified) for 25 min at 20°C before assaying. One unit (U) of enzyme activity is defined as 1 μmol of fatty acid released per min at 37°C. HSL in the pH 5.2 precipitate was phosphorylated and activated by cAMP-PK through incubation with 0.4 mM ATP, 5 mM $MgCl_2$, 1 mM dithioerythritol, 40 mM β-glycero-phosphate and the catalytic subunit of cAMP-PK (0.2 U/ml; Sigma) for 5-10 min at 37°C. In controls the protein kinase was omitted. Activated and control samples were assayed using a 0.5 mM trioleoyl glycerol substrate (29) at pH 8.3 (30). For analysis of protein phosphorylation, [γ-^{32}P]-ATP was added to a specific activity of 4400 cpm/pmol ATP. ^{32}P-labeled proteins were separated by SDS-PAGE and visualized by digital imaging of ^{32}P, using a Fujix BAS 2000 (Fuji). Radioactivity was quantified by digital imaging and volume integration of each band after subtraction of background ^{32}P.

SDS-PAGE and Western blotting. SDS-PAGE was performed in slab gels (14×14 cm) according to (31) with modifications (32). Reference proteins (molecular mass given in kDa), were: triosephosphate isomerase (26.6), lactic dehydrogenase (36.5), fumarase (48.5), pyruvate kinase (56), fructose 6-phosphate isomerase (84), β-galactosidase (116) and α_2-macroglobulin (180). SDS polyacrylamide gels were fixed in methanol and stained with silver following the method of (33). For Western blot analysis SDS polyacrylamide gels were blotted to nitrocellulose membranes (Amersham) and developed using the ECL system (Amersham) with chicken anti-rat HSL as primary antibody and horseradish peroxidase-conjugated anti-chicken IgG (Sigma) as secondary antibody.

Protein determination. Total protein was determined according to (34) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

In order to characterize the activation properties of chicken HSL in the pH 5.2 precipitate, this fraction

and a fat-depleted rat adipose tissue homogenate were incubated with the catalytic subunit of cAMP-PK. This resulted in an increase of the HSL triacylglycerol lipase activity by 495% (SD±143%; n=11) in the chicken pH 5.2 precipitate and 130% (SD±10%; n=10) in the fat-depleted rat adipose tissue homogenate. Thus, the difference in activatability of HSL from chicken and rat adipose tissue was in agreement with previous reports (20, 21). The phosphorylation and activation was maximal within 1-3 min (data not shown).

For the purpose of cloning HSL from chicken adipose tissue, attempts to utilize synthetic probes based on presumably conserved sequences for PCR-amplification of chicken cDNA (generated by reverse transcription or by extraction from an adipose tissue cDNA library), were unsuccessful. In contrast, by using primers designed from conserved regions in lipoprotein lipase (for which the chicken and rat cDNA sequences share 60% identities), chicken LPL sequences were successfully amplified from cDNA generated by reverse

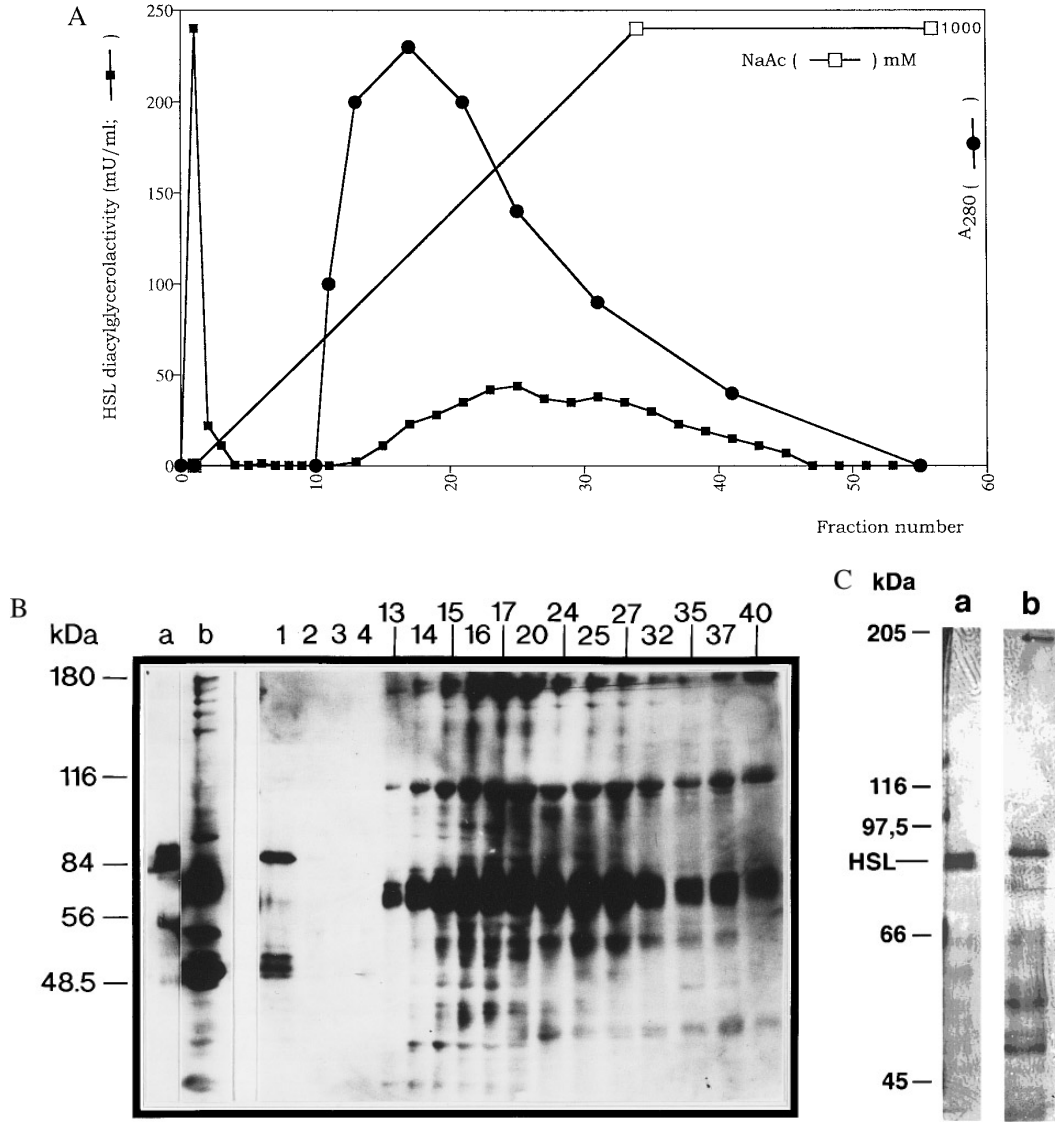

FIG. 1. (a) Anion-exchange chromatography. Q-Sepharose FF was equilibrated at 10°C with 10 volumes of buffer A (50 mM Tris-Acetate, pH 7.5, 1 mM dithioerythritol, 0.2% $C_{13}E_{12}$, 20% glycerol). The sample, typically 50 ml of detergent-solubilized pH 5.2 precipitate, was applied (flow rate, 0.35 ml/cm^2 min) and the column was washed with 10 volumes of buffer A. A linear gradient from 0 to 1 M NaAc in buffer A, pH 7.0 was then applied. Fractions were collected an analyzed for diacylglycerol lipase activity. (b) Western blotting of recombinant rat HSL and chicken HSL. Recombinant rat HSL (a), pH5.2 precipitate (b) and selected fractions from the anion exchange column were subjected to Western blot analysis using a chicken anti-rat HSL antibody. Three and 6 mU of enzyme activity were applied of the pH 5.2 precipitate and material from peak 1, respectively. For fractions 2-40, between 0 and 2.5 mU were applied. (c) SDS-PAGE of recombinant rat HSL and chicken HSL. Recombinant rat HSL (a; 22 mU) and material corresponding to peak 1 from the anion exchange column (b; 5.2 mU) were subjected to SDS-PAGE followed by silver staining of the gel.

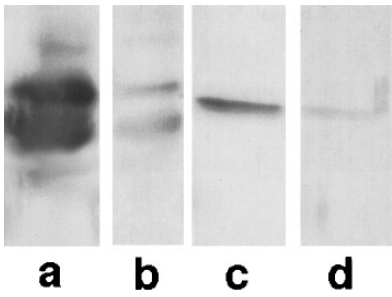

FIG. 2. Western blotting of fat-depleted infranatant from rat adipose tissue and chicken HSL. About 3 mU HSL activity of a fat-depleted infranatant from rat adipose tissue (a and b) and peak 1 (c and d) were analyzed by SDS-PAGE and Western blotting. The anti-rat HSL antibodies used for developing the blot had been preincubated without (a and c) or with (b and d) recombinant rat HSL (10 μg).

transcription, from the commercial adipose tissue cDNA library (Stratagene) and also from genomic chicken DNA (Clontech). Furthermore, the use of full-length rat and human HSL nucleotide probes for screening of the chicken adipose tissue cDNA library (Stratagene) did not result in any true positive clones. Consequently, a partial purification of HSL from chicken adipose tissue was performed, in order to be able to generate peptide sequences from the chicken HSL protein. The pH 5.2 precipitate was detergent-solubilized, subjected to anion exchange chromatography and eluted with sodium acetate as shown in Fig. 1a. HSL diacylglycerol activity eluted in two peaks, with a total recovery of 60%. The first peak had high HSL activity and was more distinct than the second peak, which had a broader elution pattern that essentially followed the total protein profile. Hereafter, these peaks will be referred to as peak 1 and peak 2, respectively. From several repeated purifications it was observed that the amount of HSL activity in peak 1 relative to peak 2 varied. Although HSL in the two peaks showed identical migration patterns upon SDS-PAGE analysis, we cannot exclude that HSL in the two peaks represents two different isoforms or populations of the enzyme, causing different elution patterns. Western blot analyses of the pH 5.2 precipitate and different fractions obtained during the anion exchange chromatography is shown in Fig. 1b. A major immunoreacting protein at about 86 kDa appeared in peak 1. Additional evidence for the identity of the 86 kDa protein band as chicken HSL was obtained through the observation of a specific decrease in the intensity of the 86 kDa polypeptide in Western blotting after preincubation of the anti-rat HSL with surplus amounts of recombinant rat HSL (Fig. 2). The molecular size of 86 kDa is in contrast to Berglund *et al.* (24), who partially purified HSL from chicken adipose tissue and identified the chicken HSL protein as a 42 kDa protein. Slight heterogeneity in the molecular mass of HSL from different species, ranging from 82-88 kDa, has previously been reported (35). In peak 2, the 86 kDa protein was not the dominating immunoreacting protein and several proteins were detected in addition to the 86 kDa protein (Fig. 1a). The strong band at about 76 kDa in peak 2 arises from reaction with the secondary antibody (rabbit anti-chicken IgG), as it appeared even if the primary antibody was not included in the ECL developing procedure (data not shown).

In order to characterize the 86 kDa protein, material corresponding to peak 1 was chosen for further investigations (due to its higher purity relative to peak 2). After SDS-PAGE analysis of this material, followed by silver staining, a major protein band of 86 kDa appeared (Fig. 1c). By loading equal amounts of HSL diacylglycerol lipase activity and comparing the intensities of the bands corresponding to chicken HSL and recombinant rat HSL, the specific activity of chicken HSL was estimated to be approximately the same as for the rat (215 U/mg; 1, 27), human and bovine enzymes, and not thousand-fold lower as previously reported (1). From the estimated specific activity, it was calculated that the fold purification of HSL (peak 1) from the tissue homogenate, having an HSL activity of about 30 mU/mg total protein, was about 3500.

Also, upon immunoinhibition of the enzyme activity in peak 1 with anti-rat HSL, an inhibition of HSL diacylglycerol lipase activity of 98% was observed under conditions where HSL activity in the recombinant rat protein was inhibited by 99.5%. The immunoinhibition experiments also disclosed difficulties in achieving cross-reactivity between chicken HSL and anti-rat HSL when working with impure chicken adipose fractions. For instance, incubation of the pH 5.2 precipitate with anti-rat HSL caused only about 5% inhibition of HSL diacylglycerol activity when using conditions which caused 65 and 30% inactivation of HSL-diacylglycerol activity in rat and human adipose tissue homogenates, respectively. Moreover, it was not possible to immunoprecipitate chicken HSL from the pH 5.2 precipitate. In the immunoinhibition experiments, it was observed that the chicken homogenate without added anti-rat HSL lost considerably more diacylglycerol activity upon incubation at 20°C than the corresponding human and rat homogenate. This indicates that chicken HSL is more prone to inactivation by either proteolytic degradation or thermal denaturation than the human and rat enzyme. It is possible that the 42 kDa protein, previously reported to be chicken HSL (24), represented a proteolytic fragment of this enzyme. The Western blot in Fig. 1c also illustrates the difficulties in detecting chicken HSL in impure fractions, as the 86 kDa band could not be detected in the pH 5.2 precipitate fraction.

Incubation of material from different fractions obtained during the anion exchange chromatography with cAMP-PK, resulted in phosphorylation of a poly-

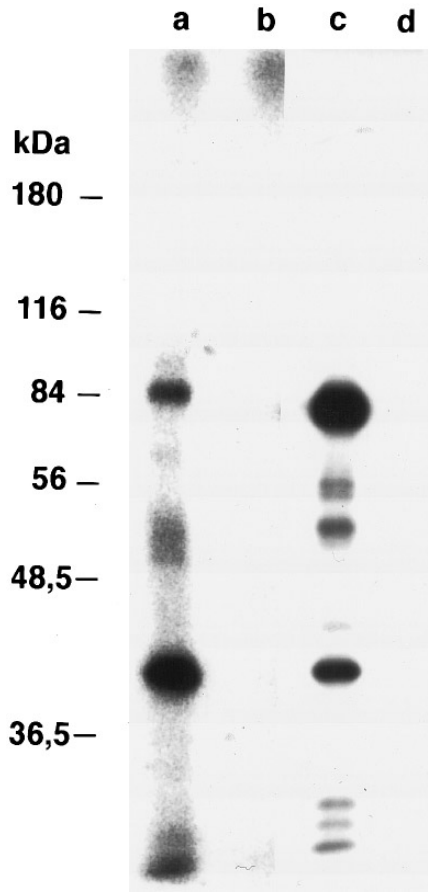

FIG. 3. Phosphorylation of recombinant rat HSL and chicken HSL. Recombinant rat HSL (50 mU) and material corresponding to peak 1 from the anion-exchange column (1 mU) were incubated with cAMP-PK and subjected to SDS-PAGE and digital imaging of ^{32}P. Lanes a and c display phosphorylated recombinant rat and chicken HSL from peak 1, respectively. In controls (b; recombinant rat HSL and d; chicken HSL), cAMP-PK was omitted. A shorter exposure time is shown for the images of recombinant rat HSL.

peptide with molecular mass of 86 kDa in peak 1 (Fig. 3). The phosphorylated protein at about 40 kDa probably represents autophosphorylated cAMP-PK. The stoichiometry of phosphorylation of the 86 kDa band, despite the higher degree of activation of the chicken enzyme, was similar to that of the recombinant rat enzyme. In material from peak 2, there were several phosphorylated proteins (not shown) and it was not possible to identify chicken HSL in these fractions.

Hence, our results clearly demonstrate that HSL from chicken adipose tissue can be identified as an 86 kDa polypeptide having about the same specific activity as the corresponding rat enzyme, instead of a 42 kDa protein having thousand-fold less specific activity, as reported previously (24). Furthermore, our results indicate that the stoichiometry of phosphorylation of chicken HSL is similar to that of the recombinant rat protein, and hence, the higher activation of the chicken enzyme upon phosphorylation with cAMP-PK cannot be ascribed to a higher phosphorylation state.

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