

# Functional molecular mass of rat hepatic lipase in liver, adrenal gland and ovary is different

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Lipoprotein lipase (LPL) is functionally active only as a dimer. It is also generally assumed that the highly homologous hepatic lipase functions as a dimer, but no clear evidence has been presented. A hepatic lipase-like activity, also indicated as L-type lipase, is present in adrenal and ovary tissues. This enzyme is thought to originate from the liver and to be identical to hepatic lipase. We determined the functional molecular mass of hepatic lipase in rat liver, adrenal gland and ovary by radiation inactivation, a method for determining the functional size of a protein without the need of prior purification. Samples were exposed to ionizing radiation at  $-135^{\circ}\text{C}$ . Hepatic lipase activity in liver homogenate showed a single exponential decay. The functional molecular mass was calculated to be  $63 \pm 10$  kDa.

Hepatic lipase activity in adrenal homogenate was found to have a functional molecular mass of  $117 \pm 16$  kDa. The functional molecular masses of the lipases partially purified from rat liver perfusate, adrenal homogenate or ovarian homogenate showed the same pattern, a target mass for the liver enzyme of  $56 \pm 6$  kDa and a target mass of  $117 \pm 14$  kDa for the enzyme from adrenal gland or ovary. In Western blot analysis the mass of the structural units of hepatic lipase in liver was 57 kDa and in adrenal and ovary tissue 51 kDa. We conclude that the functional unit of hepatic lipase in the liver is a monomer. The enzyme in adrenal gland and ovary is different from the liver and the functional unit may be a dimer.

## INTRODUCTION

Two extracellular lipases, lipoprotein lipase (LPL) and hepatic lipase (HL), serve important roles in lipoprotein metabolism [1–4]. Both enzymes are highly homologous.

LPL is found in extrahepatic tissues, i.e. heart, skeletal muscle and adipose tissue. Its main function is the hydrolysis of triacylglycerols in very-low-density lipoprotein and chylomicrons [5,6]. HL is present in the liver and is believed to play a role in the metabolism of high-density lipoprotein, intermediate-density lipoprotein and chylomicron remnants [7–11]. In addition to the liver, an immunologically related enzyme, sharing characteristics with HL, has been found in adrenal gland and ovary [12,13]. This enzyme is also indicated as L-type lipase.

After synthesis in the parenchymal cells LPL and HL are secreted and extracellularly bound to specific binding sites [14–19]. For adrenal gland and ovary the situation seems to be more complex. In these tissues the HL gene is transcribed into a truncated mRNA [20]. Function or catalytic properties of these HL gene products are not known. The full-length message of HL is missing in adrenal gland and ovary. It has been hypothesized that these tissues acquire catalytically active HL from the liver. In this model it is assumed that the enzyme, after being secreted by the liver, is transported to the adrenal gland and ovary, where it binds to specific binding sites [21,22]. Therefore the HL in adrenal gland and ovary is supposed to be identical to the liver enzyme.

It has been shown in studies of sedimentation equilibration, gel filtration and radiation inactivation studies that the homologous enzyme LPL is only catalytically active as a dimer [23–25]. The functional unit of HL has not been established yet.

Because of its close homology with LPL it is generally assumed that HL is also a dimer. Gel-filtration experiments yield conflicting results. Depending on the salt concentration molecular masses of the native enzyme of 65–200 kDa have been found [26–29]. HL easily aggregates when free in solution. Therefore we employed radiation inactivation to estimate the functional molecular mass of HL in liver, adrenal gland and ovary.

## MATERIALS AND METHODS

### Animals

Normally fed male Wistar rats, 200–250 g, were used for most experiments. Female Wistar rats were used for the experiments with ovaries. They were housed under controlled conditions: temperature 20–22 °C, light on 07:00–19:00 h. They had free access to Purina rat chow and water.

### Tissue preparations

Rats were killed by decapitation and 20% (w/v) liver homogenate and 10% (w/v) adrenal homogenate were prepared in PBS containing 10% (v/v) glycerol, pH 7.4 (15 s, Polytron, setting 4). Aliquots (400  $\mu\text{l}$ ) of homogenate were sealed in 1 ml glass ampoules, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use.

### Isolation of HL

HL was isolated from heparin-containing rat liver perfusate or heparin-containing tissue extracts from rat adrenals and ovaries

as described previously [13] with minor modifications. The perfusate and tissue extracts were applied to a Sepharose–heparin column. After washing the column with 3–6 column volumes of 10 mM sodium phosphate buffer, pH 7.4, containing 0.2 M NaCl and 10% (v/v) glycerol, HL activity was eluted with the same buffer containing 1 M NaCl and 1% BSA in addition. The peak fractions were combined and the buffer was changed to PBS containing 1% BSA and 10% glycerol by gel filtration using PD10 columns. Aliquots (400  $\mu$ l) of partially purified HL from liver perfusate or tissue extracts of adrenals or ovaries were sealed in 1 ml glass ampoules and frozen in liquid nitrogen. Samples were stored at  $-80^{\circ}\text{C}$  until use. SDS/gel electrophoresis and Western blotting of the samples were performed as described previously [30]. To quantify the amount of protein, gels or blots were scanned with the Hewlett Packard Scan Jet IICX and analysed.

### Radiation inactivation and dosimetry

Samples were irradiated in a cryostat at  $-135^{\circ}\text{C}$ , employing a 3 MeV Van de Graaff electron accelerator. The duration of the electron pulse was 250 ns and the pulse had a 2 A peak current as described previously [31]. To improve dose homogeneity a 0.3-mm-thick aluminium scatter plate was used on top of the beam window of the cryostat. Dosimetry was performed before each irradiation experiment using FWT-60 radiochromic nylon film dosimeters (1 cm  $\times$  1 cm), between 3-mm-thick nylon plates. The dose received was determined from the change in absorbance at 510 nm and calculated from the formula:

$$\text{Dose (kGy)} = 151.6 \times (\Delta A)^{1.186}$$

obtained by dose calibration of the FWT-60 films using a  $^{60}\text{Co}$  source.

This resulted in a dose of 0.082 Gy/nC with an accuracy of  $\pm 5\%$ . Electron pulses were given at a rate of 5 Hz. The charge per pulse was determined before and after each irradiation and was found to be stable within 1%. The relationship between the molecular mass ( $M$ ) and the dose is given by the equation  $M = c \cdot K \cdot St$  where  $c = 6400$  kGy/kDa,  $St = 2.8$  (correction factor for the irradiation temperature of  $-135^{\circ}\text{C}$ ) and  $K =$  slope of the  $\ln$  (fractional activity  $A/A_0$ ) versus dose plot [32]. A least-square fit was calculated for the  $\ln$  (remaining activity versus radiation dose). The validity of our method was verified by determination of the target mass of yeast glucose-6-phosphate dehydrogenase [33]. We observed a monoexponential inactivation for this protein corresponding to a target mass of  $99 \pm 6$  kDa, which is in line with the molecular mass of the enzyme of 104 kDa.

Radiation inactivation of HL protein was followed by SDS/PAGE according to the method of Laemmli on a 3% stacking gel and a 10% resolving gel and subsequent Western blotting on nitrocellulose paper. HL was identified on the blot using polyclonal goat anti-HL IgG and an alkaline phosphatase-conjugated anti-(goat IgG) [30].

### HL assays

HL triacylglycerol hydrolase activity was measured using an artificial glycerol-[9,10( $n$ - $^3\text{H}$ )]trioleate emulsion in gum arabic, pH 8.5 [12]. To avoid any possible contamination with other neutral lipase activities in the adrenal and liver homogenates, HL activity was measured in samples preincubated with or without an anti-HL-specific polyclonal antibody [34].

### Materials

All chemicals used were of analytical grade. Heparin was purchased from Leo Pharmaceutical Products, Weesp, The Netherlands and BSA from Sigma (St. Louis, MO, U.S.A.). Glycerol [9,10( $n$ - $^3\text{H}$ )]trioleate was obtained from Amersham International, Amersham, Bucks., U.K. Alkaline phosphatase-conjugated anti-(goat IgG) was purchased from Tago, Burlingame, CA, U.S.A.

### RESULTS

#### HL in rat liver

HL activity in homogenate from rat liver, prepared in PBS containing 10% (v/v) glycerol and 1% BSA, is stable and can be frozen and thawed without loss of activity.

If frozen rat liver homogenate fractions were exposed to increasing doses of radiation triacylglycerol hydrolase activity showed a simple exponential decay. The functional molecular mass was calculated to be  $63 \pm 10$  kDa (Figure 1). A similar decay curve was observed on exposure of HL partially purified from rat liver heparin perfusate. The calculated target mass was  $56 \pm 6$  kDa.

The target mass was also evaluated by SDS/gel electrophoresis and immunoblotting. On Western blots HL had an apparent molecular mass of 57 kDa. Upon radiation, immunoreactivity at this position in the gel was lost. In parallel immunoreactive material increased at the front of the gel, suggesting that irradiation resulted in fragmentation of the protein (Figure 2). The loss in HL-immunoreactive material at 57 kDa on the Western blot upon irradiation yielded a target mass of  $53 \pm 5$  kDa (Figure 3). We also followed the decrease in the amount of albumin during irradiation at 66 kDa on the gel. For albumin a target mass of  $70 \pm 6$  kDa was found.

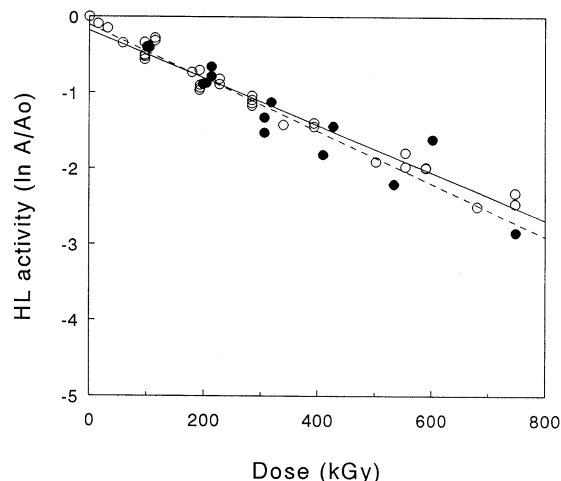
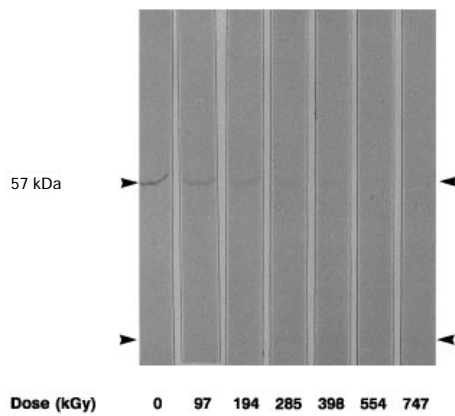


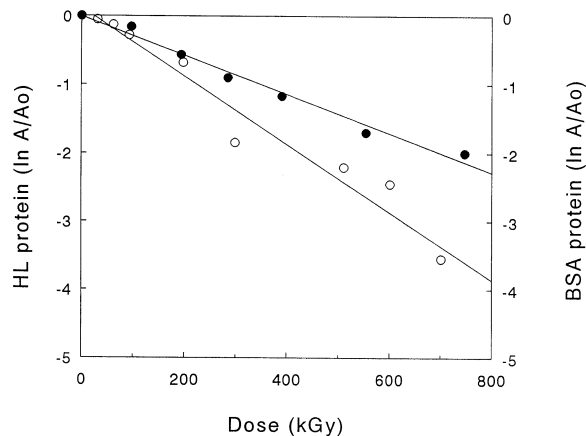
Figure 1 Radiation inactivation of HL in liver

HL triacylglycerol hydrolase activity was measured in irradiated liver homogenate ( $\bullet$ , broken line) or in irradiated hepatic lipase (HL) purified from liver heparin perfusate ( $\circ$ , solid line) as described in the Materials and methods section. Data are expressed as  $\ln$  [activity measured in the samples after irradiation ( $A$ ) divided by the HL activity of non-irradiated samples ( $A_0$ )]. The fitted line is from least-square analysis. Values plotted are the means of three determinations from five separate experiments.



**Figure 2** Western blot of irradiated partly purified HL

Partly purified HL was irradiated as described in the Materials and methods section. After radiation the samples were mixed with SDS/PAGE sample buffer, electrophoresed on SDS/PAGE and subjected to Western blot analysis.



**Figure 3** Irradiation fragmentation of HL protein

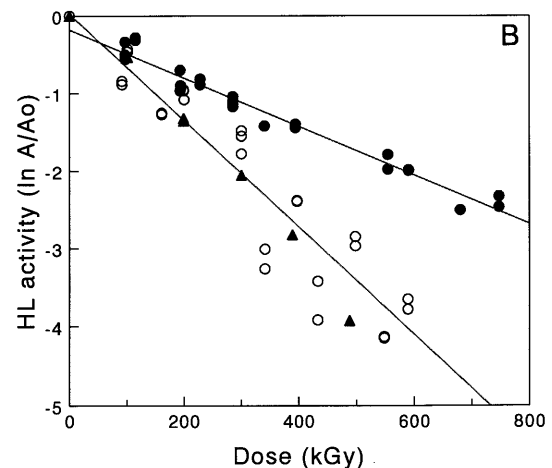
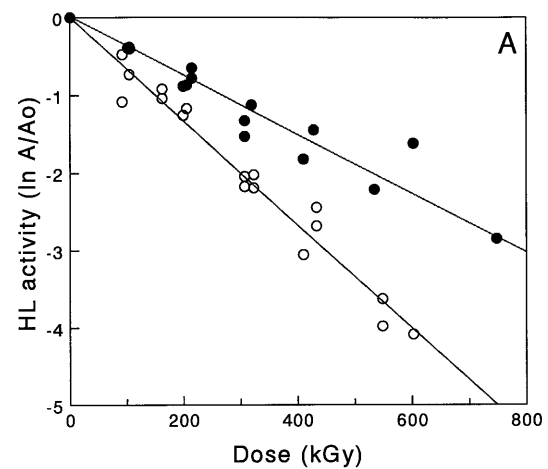
The fraction of surviving immunoreactive protein of hepatic lipase (HL, ●) or BSA (○) [ $\ln(A/A_0)$ ] is plotted as a function of radiation dose. HL-immunoreactive protein was measured at 57 kDa on Western blots. The amount of BSA was measured as the amount of protein at 66 kDa on SDS-gels. To quantify the amount of protein, gels or blots were scanned with the Hewlett Packard Scan Jet IICX and analysed.

### HL in rat adrenal gland and ovary

Tissue homogenate of rat adrenals was exposed to increasing doses of radiation. A target mass of  $117 \pm 16$  kDa was found, which is much larger than in liver (Figure 4A). Because the HL activity in ovary was not sufficient to employ radiation inactivation in the homogenate, we purified the enzyme from ovaries and adrenals. The same target mass as for the lipase activity in the adrenal homogenate was obtained for the purified HL from rat adrenal gland and ovary, i.e.  $117 \pm 14$  kDa (Figure 4B).

### Structural unit of HL

Besides the size of the functional unit, we also compared the size of the structural unit of the liver enzyme and the enzyme in adrenal gland and ovary with SDS/gel electrophoresis and



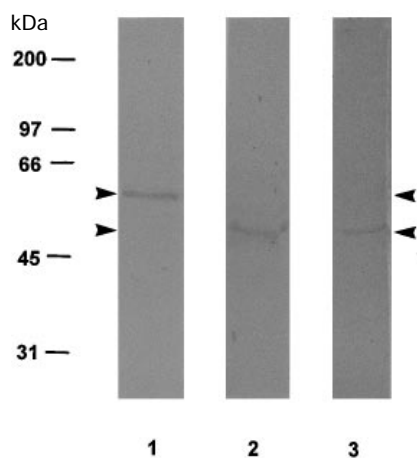
**Figure 4** Radiation inactivation of HL in liver, adrenal gland and ovary

HL triacylglycerol hydrolase activity was measured in (A) irradiated liver homogenate (●) or adrenal homogenate (○); or (B) irradiated HL partly purified from liver heparin perfusate (●), or heparin-containing homogenates of adrenals (○) or ovaries (▲) as described in the Materials and methods section. Data are expressed as  $\ln$  [activity measured in the samples after irradiation (A) divided by the activity in non-irradiated samples ( $A_0$ )]. The fitted line is from least-square analysis. The values plotted are the means from 3–5 different experiments.

immunoblotting. In liver a structural unit of 57 kDa was found compared with a structural unit of 51 kDa in adrenal gland and ovary (Figure 5).

### DISCUSSION

Employing radiation inactivation, we show that the functional target mass of HL in rat liver is 63 kDa, in the adrenal gland and in the ovary it is 117 kDa. The structural unit in liver is 57 kDa and in the adrenal and ovary 51 kDa, as measured by Western blotting. Using radiation target mass analysis the molecular mass of the enzyme can be determined in its native state, bound to its binding site, without prior purification. In this way purification artefacts are avoided. Moreover, radiation inactivation reveals functional interactions among enzyme complexes with receptors or in oligomeric enzyme systems. After purification the sizes of the functional units of the liver and the adrenal or ovarian enzyme were respectively 56 and 117 kDa, similar to the values observed in crude homogenate. Therefore it seems that interaction of the enzyme with its binding site does not influence



**Figure 5** Determination of the size of the structural unit of HL in adrenal gland and ovary

Partly purified HL from heparin-containing liver perfusate (lane 1) or from heparin-containing homogenate of adrenals (lane 2) or ovaries (lane 3) were subjected to SDS/PAGE and Western blotting to determine the size of the structural unit.

the activity of the enzyme. The reported molecular mass of HL in liver, as estimated by SDS/gel electrophoresis, is in the range 55–66 kDa [28,29,35,36]. The value obtained with gel filtration varies between 65 and 200 kDa [26–29]. The variation in the observed values of the enzyme in its native state may be due to association of the enzyme with specific or non-specific HL-binding proteins or to aggregation of the enzyme. The monomeric value of the molecular mass of HL after gel filtration as observed by Ikeda et al. [29] gives no information about the size of the functional unit; formation of dimeric HL in the lipase assay cannot be excluded, because for LPL also, which is clearly a dimer, Ikeda observed a monomeric value of the molecular mass after gel filtration. The target size of both the native liver enzyme in the homogenate and the purified liver enzyme as measured by radiation inactivation indicates that the functional unit of HL in liver is a monomer. This is in contrast to LPL. Using a number of techniques including radiation inactivation the functional unit of LPL has been shown to be a dimer [23–25]. The dimeric functional unit of LPL refers to its triacylglycerol hydrolase activity, for its esterase activity there is no need for a dimeric structure [37]. For LPL it is assumed that the dimeric structure is a prerequisite for the binding of the water-insoluble triacylglycerol. The proposed model for the LPL structure is a model consisting of two monomeric LPL units arranged in such a manner that the C-terminal domain, the lipid-binding domain of one subunit, is close to the N-terminal domain, the catalytic domain of the other subunit, in a so called head-to-tail arrangement [38,39]. Based on the close relationship between HL and LPL it is assumed that the functional unit of HL is also dimeric. However, it appears that HL in liver is a monomer rather than a dimer. Therefore, it may well be that the interaction of HL, or at least the liver enzyme, with its substrate triacylglycerol is different from the proposed interaction of LPL with its substrate.

For the size of the functional unit of the enzyme from adrenal gland or ovary a much higher target mass was found than for the liver enzyme, i.e. 117 kDa. Despite this higher target size, we found a smaller size of structural unit of the HL from adrenal gland and ovary, 51 kDa. This is lower than for the liver enzyme,

but similar to the size that was reported for the adrenal gland by Doolittle et al. [21]. Since we found a functional unit of 117 kDa for the adrenal and ovarian enzyme, both in the crude homogenate and after purification, we conclude that the enzyme in adrenal gland and ovary is in a dimeric form. The difference between the liver enzyme and the enzyme in the adrenal gland or ovary is unexpected as it was assumed that the enzyme in these tissues is derived from the liver [21,22]. This suggests that HL is present in the liver as a monomer and is dimerized after or during its transport to the adrenals and ovaries. Radiation inactivation of the liver enzyme would lead to inactivation of only one monomeric subunit, radiation inactivation of the adrenal or ovarian enzyme on the other hand, would lead to inactivation of both subunits because of energy transfer between the two monomeric subunits. The size of the structural unit in the adrenal gland and ovary is also different from that in the liver, indicating that post-translational modification of the enzyme occurs in the liver or during its transport from the liver to the adrenal. Liver and adrenal enzymes differs also in binding characteristics. The release of HL from the liver and from the adrenal gland by protamine is different [40]. No significant release of HL was found in the adrenal gland after injection of protamine, while protamine was as effective as heparin in the release of HL from liver. It was concluded that this may be due to a difference in the binding site for HL in liver and adrenal gland. However, the observed difference in the release of HL could also indicate that the adrenal enzyme is more tightly bound because of its dimeric functional unit. The release of the homologous dimeric enzyme LPL by protamine injection was also very low and only 10% of the amount of LPL released by heparin.

In this paper we have shown that HL in liver is in the monomeric form and different from HL in the adrenal gland and ovary, which is in the dimeric form. Whether the size of the functional and structural unit as well as the substrate specificities or physiological function of HL in the adrenal gland and ovary are different is currently under investigation.

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