Chinese hamster ovary cells were stably transfected with a human hepatic lipase (HL) cDNA. The recombinant enzyme was purified from culture medium in milligram quantities and shown to have a molecular weight, specific activity, and heparin affinity equivalent to HL present in human post-heparin plasma. The techniques of intensity light scattering, sedimentation equilibrium, and radiation inactivation were employed to assess the subunit structure of HL. For intensity light scattering, purified enzyme was subjected to size exclusion chromatography coupled to three detectors in series: an ultraviolet absorbance monitor, a differential refractometer, and a light scattering photometer. The polypeptide molecular weight (without carbohydrate contributions) was calculated using the measurements from the three detectors combined with the extinction coefficient of human HL. A single protein peak containing HL activity was identified and calculated to have a molecular mass of 107,000 in excellent agreement with the expected value for a dimer of HL (106.8 kDa). In addition, sedimentation equilibrium studies revealed that HL had a molecular mass (with carbohydrate contributions) of 121 kDa. Finally, to determine the smallest structural unit required for lipolytic activity, HL was subjected to radiation inactivation. Purified HL was exposed to various doses of high energy electrons at −135 °C; lipase activity decreased as a single exponential function of the radiation dose to less than 0.01% remaining activity. The target size of functional HL was calculated to be 109 kDa, whereas the size of the structural unit was determined to be 63 kDa. These data indicate that two HL monomer subunits are required for lipolytic activity, consistent with an HL homodimer. A model for active dimeric hepatic lipase is presented with implications for physiological function.

Through its ability to catalyze the hydrolysis of triglycerides and phospholipids, hepatic lipase (HL) influences the metabolism of chylomicron remnants, intermediate density lipoproteins, and high density lipoproteins (1). HL is synthesized by hepatocytes and has been shown to be catalytically active within endosomes (2), on the cell surface (3), and on the surface of the sinusoidal endothelium of the liver (4). In addition, there is evidence to suggest that, independent of its catalytic activity, HL can act as a ligand to facilitate the uptake of remnant lipoproteins through its interaction with cell surface proteoglycans and the low density lipoprotein receptor-related protein (5–7).

Although HL appears to have a number of roles in lipoprotein metabolism, it is evident in human HL deficiency that remnant metabolism is primarily affected (8). This observation is consistent with animal studies in which the inhibition of HL activity by the administration of HL antibodies decreased the rate of chylomicron remnant uptake by the liver (9, 10). However, there are several in vitro studies which demonstrate that HL also participates in the remodeling of HDL (11–13). This has been clearly demonstrated in gene-targeted and transgenic HL animals in which the metabolism of the predominant plasma lipoprotein species, high density lipoprotein, is significantly affected (14–16).

Mature human HL is a 476-amino acid glycoprotein with a calculated polypeptide molecular weight of 53,431, whereas the purified denatured enzyme has an apparent molecular weight of 65,000 reflecting the contribution of N-linked carbohydrate (17–19). HL is a member of a lipase gene family which also includes lipoprotein lipase (LPL) and pancreatic lipase (PL) (20–22). Amino acid homology, including the conservation of disulfide bridges among all three enzymes, suggests that they share similar three-dimensional structures (23). However, on comparison of their phylogenetic relationships, it is apparent that HL and LPL are more closely related to each other than either enzyme is to PL (21).

Radiation inactivation analysis of both rat (24) and bovine (25) LPL as well as sedimentation equilibrium studies (26) indicated that this enzyme is functionally active as a homodimer. By contrast, gel filtration techniques have suggested a tetrameric form for rat HL (27, 28) and a monomeric form for human HL (29). However, the construction and expression of HL-LPL chimera molecules that were catalytically active (30–32) suggests that HL, like LPL, may also exist as a homodimer.

To determine the subunit structure associated with the biochemical function of HL, light scattering, sedimentation equilibrium, and radiation inactivation analyses were performed. These studies demonstrate that HL exists as a homodimer and that this structure represents the smallest functional unit capable of lipolytic activity.

**EXPERIMENTAL PROCEDURES**

DNA Transfection and Production of Recombinant Human HL—Full-length human HL cDNA (17) was cloned into the HindIII and NotI sites of the expression vector pcDNANeo (Invitrogen). Chinese ham-
ster ovary (CHO)-Pro5 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. To mediate the transfection of CHO cells, co-precipitates of plasmid DNA and CaPO4 were prepared (33). The calcium phosphate/DNA mixture was incubated at room temperature for 30 min before it was added to a 50% confluent CHO monolayer. Stably transfected cells were selected by growth in the presence of genetin (G418 sulfate, 400 μg/ml) and surviving colonies were selected and expanded. Cell clones expressing maximal quantities of HL were identified by enzyme activity analysis.

After growth to confluency in T-175 flasks, cells were washed with Dulbecco’s modified Eagle’s medium and fresh Dulbecco’s modified Eagle’s medium was supplemented with 1% Nutridoma (serum substitute) and 10 units/ml heparin was added to each flask. The medium was harvested and replaced every 24 h for a 10-day period. After centrifugation at 1000 × g for 10 min to remove cellular debris, the harvested medium was stored at −80 °C.

Purification of Recombinant Human HL—Three liters of thawed medium was mixed with NaCl and benzamidine to a final concentration of 0.5 mM and 0.5 mM, respectively. This mixture was applied to an octyl-Sepharose column (25 × 20 cm) previously equilibrated with 5 mM barbiturate buffer, pH 7.2, containing 0.35 mM NaCl and 20% glycerol (Buffer A). Following a wash with 800 ml of 0.5 mM NaCl, 20% glycerol, 5 mM barbiturate, pH 7.2, the lipase was eluted with 700 ml of Buffer A containing 1.2% Triton N-101 and applied to a heparin-Sepharose column (25 × 20 cm). This column was washed with 800 ml of Buffer A prior to elution with 500 ml of 1 mM NaCl, 20% glycerol, 5 mM barbiturate, pH 7.2. The eluted lipase was passed through a 1-ml hydroxylapatite column to remove contaminating proteins and then diluted with an equal volume of 20% glycerol, 5 mM barbiturate, pH 7.2, before it was loaded onto a dextran sulfate-Sepharose column (1 × 5 cm). The dextran sulfate-Sepharose column was washed with 50 ml of 0.5 mM NaCl, 5 mM barbiturate, pH 7.2, and then it was eluted with 50 ml of 1 mM NaCl, 5 mM barbiturate, pH 7.2. The collected eluant was concentrated in an Amicon filtration unit using a YM-30 membrane to a final volume of 1–2 ml. All purification steps were carried out at 4 °C.

Light Scattering/Size Exclusion Chromatography—Size exclusion chromatography was performed with a Superose 12 (Pharmacia) column equilibrated with 0.1 mM sodium phosphate buffer plus 0.5 mM NaCl buffer and a 500-μl sample loop operated at a flow rate of 0.5 ml/min. The eluant was monitored using three on-line detectors in series: a light scattering detector (Wyatt Minulab), a refractometric detector (Polymer Laboratories PLRI), and a UV absorbance monitor at 280 nm (Knauer A293). The polypeptide molecular weight of a glycoprotein was calculated using the equation:

\[ MW_g = K(UV/LS)\bar{c}^2/(RI)^2 \]  
(Eq. 1)

where UV, LS, and RI are the signals from the absorbance, 90° light scattering, and differential refractive index detectors, respectively, ε is the extinction coefficient (the absorbance of a solution containing 1 mg of polypeptide/ml for 1-cm path length), MWg is the polypeptide molecular weight, and K is a calibration constant that depends on instrument conditions. The calibration constant, K, was measured by running bovine serum albumin (Sigma) and ovalbumin (Sigma) molecular weight standards (34, 35). The extinction coefficient of human HL was calculated as 1.22 using Gill’s method (36).

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were performed in the Model E analytical ultracentrifuge (Beckman) equipped with a UV optical system and a photoelectric scanner interfaced to an IBM PC computer. Experiments were performed at 4–7 °C, at a rotor speed of 5,200 rpm over 3 days. During each run three sample chambers were monitored, two solutions of HL and one with a solution of PL. For the determination of molecular weights, partial specific volumes were calculated from amino acid composition and carbohydrate content of HL (r = 0.72) (18, 19). Slopes were calculated from least square lines of the log OD280 versus 1000 × r^2 plots, where r is the distance from the axis of rotation, ω is the angular velocity, R is the gas constant, and T is the absolute temperature.

Preparation and Irradiation of Samples—Samples of purified HL (0.3–0.6 mg/ml) were prepared for irradiation by adding 2000 units/ml (2.8 mg) glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Sigma). Aliquots of 80 μl in 0.5 ml microcentrifuge tubes were frozen immediately on dry ice and stored at −80 °C until shipped on dry ice for irradiation. These samples were irradiated at −135 °C with 13 MeV electrons for various times as described previously (37). Following irradiation the samples were returned on dry ice and stored at −80 °C until thawed for assays. Non-irradiated samples were assayed as controls.

Enzyme Assays—HL activity was monitored as described previously (38) using radio-labeled triolein substrate. The apparent KM and Vmax values for a triolein substrate were determined from a linear regression analysis of a double reciprocal plot of lipolytic activity (nmol/min/mg) versus triolein concentration (mM). Glucose-6-phosphate dehydrogenase activity was assayed by measuring absorption at 340 nm at 25 °C and pH 7.8 to monitor the production of NADPH in the presence of glucose-6-phosphate and NADP (39). Protein concentration was determined by the Bradford method (40).

Electrophoresis and Protein Staining—in order to monitor the radiation-induced degradation of HL monomers, irradiated samples were analyzed by SDS-PAGE. Sample aliquots were mixed with a half-volume of buffer containing 2% SDS, 0.1 mM Tris-HCl, pH 6.8, 50 mM glycerol, 10% β-mercaptoethanol, 0.05% bromphenol blue. The mixture was placed in boiling water for 5 min prior to loading onto a 10% acrylamide gel. After fixation in 40% methanol, 10% acetic acid, gels were stained overnight with a fluorescent dye (SYPRO orange, Molecular Probes Inc.) in the presence of 7.5% (v/v) acetic acid. Protein bands were visualized using a standard UV transilluminator. The relative intensity of the signal for individual bands was quantitated using the AIDA/OS Imaging System and software. Protein concentration standards were produced using serial dilutions of non-irradiated protein samples.

RESULTS

Purification and Characterization of Recombinant Human HL—The results of a purification from 3300 ml of medium from transfected CHO cells is shown in Table I. As described under “Experimental Procedures,” a four-step procedure was utilized consisting of octyl-Sepharose, heparin-Sepharose, hydroxylapatite, and dextran sulfate-Sepharose. Purity of the enzyme preparation was assessed by SDS-PAGE silver staining and capillary electrophoresis both of which indicated the presence of only a single protein species (data not shown). Following concentration of the final eluant, the overall yield based on activity was approximately 40%, with a calculated HL specific activity of about 150 μmol/min/mg. This specific activity is similar to previous reports of HL purified from human postheparin plasma (29, 41) and recombinant human HL made by stably transfected rat hepatoma McA-RH7777 cells (42). Kinetic analysis of the purified enzyme with a triolein emulsion substrate indicated an apparent Vmax of 1.4 ± 0.3 (mean ± S.D.) μmol/min/mg and an apparent KM of 16 ± 2 mM (n = 3). The purified HL bound to heparin-Sepharose and eluted as a single peak with maximum elution at 0.7 mM NaCl (data not available).

TABLE I

| Volume | Protein | Specific activity | Purification | Recovery |
|--------|---------|------------------|--------------|----------|
| ml     | mg      | μmol/min/mg      | fold         | %        |
| Culture medium | 3300 | 131 | 1.5 | 1 |  
| Hydroxylapatite flow-through | 500 | 1.10 | 109 | 73 | 61 |
| Dextran sulfate-Sepharose eluent | 50 | 0.62 | 152 | 101 | 48 |
| HL concentrate | 1 | 0.55 | 146 | 97 | 41 |
on-line detectors (A) and activity assay of corresponding fractions (exclusion chromatography system). The chromatograms from all three on-line detectors (A) and activity assay of corresponding fractions (B) are shown. LS (solid line), RI (dashed line), and UV (dotted line) are signals from 90° light scattering, refractive index, and absorbance detectors, respectively. The first peak, eluting at about 6.4 ml, has considerable light scattering but lacked absorbance indicating that there is a very small amount of aggregated protein. The second peak at about 9.6 ml has a molecular weight which was calculated to be 107,000 ± 3,000 (mean ± S.D.).

shown. This affinity for heparin is consistent with previous reports for human HL (32, 41). Thus, the properties of the recombinant enzyme were the functional equivalent of the native enzyme.

Light Scattering/Size Exclusion Chromatography of HL—To determine the subunit structure of HL, size exclusion chromatography was used in combination with an ultraviolet detector, a differential refractometer, and a light scattering photometer, as described by Takagi (34). The elution characteristics of purified HL were analyzed using a Superose 12 gel filtration column in the presence of 0.1 M sodium phosphate and various NaCl concentrations. A single protein peak was obtained with the salt concentration ranging from 0.15 to 1 M NaCl (data not shown). Following calibration of the light scattering/size exclusion chromatography system using molecular weight standards, a 500-μl sample of purified enzyme (150 μg) was injected to assess the subunit structure of HL. The chromatograms from all three on-line detectors and corresponding activity assay of each fraction are shown in Fig. 1. The first peak, eluting at about 6.4 ml, had considerable light scattering but lacked absorbance indicating that there was a very small amount of aggregates (Fig. 1A). The second peak at about 9.6 ml had a molecular weight calculated from light scattering of 107,000 in excellent agreement with the expected value for a dimer of hepatic lipase (the monomer polypeptide formula weight of hepatic lipase is 53,431). Also, this second light scattering peak corresponded to fractions determined to be lipolytically active (Fig. 1B).

Analytical Ultracentrifugation of HL—Sedimentation equilibrium experiments of HL were analyzed simultaneously with human PL, which is recognized as a monomer (43) and thus acts as an internal control. The slopes of the least square lines of the log_e OD versus 1000 × r²/2RT plots obtained at 5200 rpm of two different concentrations of HL were similar, whereas the slope observed for PL was clearly distinct and approximately one-half the magnitude in comparison to HL (Fig. 2A). The molecular mass of PL was confirmed to be 54 kDa, and indicated that HL was at least twice as large. The observed “tailing” of the HL data at higher OD readings (Fig. 2A) is indicative of sample heterogeneity and consistent with the light scattering findings which showed the presence of a small amount of aggregates and asymmetry in the main protein peak (Fig. 1A). To improve the quality of the data, only fractions including and immediately adjacent to the protein peak obtained from Superose 12 chromatography was subjected to sedimentation equilibrium analyses (Fig. 2B). Measured at two wavelengths (230 and 235 nm), these data show greater homogeneity and are consistent with a single species with an average molecular mass of 121 kDa. Unlike the light scattering analysis which does not represent the contribution of HL carbohydrates, sedimentation equilibrium results represent the total glycoprotein molecular mass of the dimer (~130 kDa based on SDS-PAGE analysis). The accuracy of the sedimentation equilibrium result (121 versus 130 kDa) as well as the total glycoprotein mass of the dimer (~130 kDa based on SDS-PAGE analysis) agree well with that obtained from light scattering experiments (107 versus 106.8 kDa) clearly demonstrate that HL is a dimeric molecule in solution.

Radiation Inactivation of HL—In order to ascertain whether the HL dimer was responsible for catalytic activity, radiation inactivation studies were performed. Radiation inactivation of a molecule enables the determination of the molecular mass or “target size” of the functional unit. Target theory states that
irradiated molecules are physically degraded and rendered totally inactive by a single electron hit whereas undamaged molecules remain completely active (37). Thus, by irradiating an enzyme with different doses, enzymatic inactivation curves are produced from which the target size and hence functional molecular weight is calculated.

Prior to irradiation, an internal standard, glucose-6-phosphate dehydrogenase from *L. mesenteroides* was added to purified preparations of HL to establish the validity of the experimental protocol. Measurement of glucose-6-phosphate dehydrogenase activity after different doses of radiation led to calculation of a functional molecular mass of 121 ± 13 kDa (Table II) consistent with previously reported values for the molecular mass of the dimeric enzyme (37). Following irradiation of purified HL with different doses, enzymatic inactivation curves were obtained. The enzyme activity decayed as a single exponential function of radiation dose to less than 0.01% remaining activity (Fig. 3). The functional molecular weight for HL, determined from four independent experiments, was calculated to be 109 ± 24 kDa (Table II). Like light scattering, radiation inactivation analyses do not include the contribution of carbohydrate to the molecular mass of the functional unit. Therefore, the 109-kDa value obtained compares favorably to the expected 106.8 kDa of a theoretical dimer. To determine that the affinity of HL for its triolein substrate was not affected by radiation exposure, the apparent *Km* of the enzyme was determined in two separate experiments for both non-irradiated and exposed samples with a 50% reduction in activity. The values determined were not significantly different (data not shown), indicating that there were no HL molecules with altered activity as a result of irradiation.

As a control, the size of the HL structural unit was determined by measuring the reduction in full-sized HL monomers observed by protein staining in SDS-PAGE gels. To ensure that the observed staining was proportional to the quantity of the protein, standard curves were produced using serial dilutions of non-irradiated protein samples. Intact HL monomer decayed as a simple exponential function of radiation dose (Fig. 3) revealing a target size of 63 ± 11 kDa (Table II) compared to an expected value of 53.4 kDa. These data suggest that one radiation hit destroys a single monomer subunit of HL and that two of these units are required for lipolytic activity.

**DISCUSSION**

We have established a purification procedure to obtain milligram quantities of recombinant HL protein from stably transfected CHO cells. The properties of the purified enzyme closely resemble those of human post-heparin plasma HL with respect to molecular weight, specific activity, and heparin affinity. For the first time, sufficient quantities of HL have been purified to enable a more accurate assessment of its subunit structure and functional size.

The only previous analyses of the functional molecular weight of HL have relied on conventional size exclusion chromatography methods (27–29). A disadvantage of this procedure when used to estimate the molecular weight of a protein is that...
Subunit Structure of Hepatic Lipase

Catalytic domain

C-terminal domain

C-terminal domain

Product

Substrate

Substrate entry

Active site

Product

Substrate entry

Active site

Fig. 4. Model for active, dimeric hepatic lipase. HL monomers are arranged in a head-to-tail fashion, with a 2-fold rotational axis of symmetry perpendicular to the plane of the page (single dot) which positions the COOH-terminal domain of one subunit in close proximity to the catalytic domain from the other subunit. The catalytic domain contains the active site residues which in the absence of substrate are covered by a lid domain. As indicated, lipid substrates interact with elements in the COOH-terminal domain for presentation to the catalytic cleft and subsequent hydrolysis.

Conformational and structural target sizes of human HL. The smallest active enzyme unit of HL was determined to be 109 kDa whereas the structural target size was 63 kDa. Since the carbohydrate moiety of glycoproteins do not contribute to the target size (37), these values are in close agreement with the theoretical target size of HL dimer and monomer, 106.8 and 53.4 kDa, respectively. These results strongly indicate that two monomers form the active HL dimer.

Radiation inactivation studies of a closely related lipase, bovine LPL, demonstrated that the active dimeric structure of this lipase was observed in the presence or absence of heparin or lipid substrate (25). In the same manner, we believe that the dimeric target size of purified HL reported here strongly suggests that the same subunit structure exists whether in the presence of lipoprotein substrate or on the surface of the liver endothelium. Thus, it appears that HL and LPL are each functionally active as a homodimer.

The crystal structure of PL has revealed a two-domain structure composed of a NH₂-terminal domain containing the catalytic site joined by a short spanning region to a smaller COOH-terminal domain (44). Based on sequence homology, the conservation of disulfide bridges, and similarity of lipolytic function, LPL and HL are believed to have a similar three-dimensional structure. Multiple functional regions of HL have been identified including the catalytic site, lid domain, heparin-binding site, and COOH-terminal domain (45, 46). Characterization of functional HL-LPL chimeras suggested that HL and LPL were both active dimers and that the COOH-terminal domain of HL was necessary for catalyzing lipid substrate hydrolysis and contained binding sites for heparin (31, 32). Recently, the COOH-terminal domain of LPL has been shown to bind to the low density lipoprotein receptor-related protein (47), but such binding remains to be established for HL.

These HL functions must now be considered in the context of a dimer conformation. As we have previously proposed for LPL (48), a head-to-tail arrangement of two HL monomer subunits is in agreement with both structural and functional studies of the enzyme (Fig. 4). The model predicts that the COOH-terminal domain of one subunit is juxtaposed to the NH₂-terminal or catalytic domain of the opposing subunit. The initial interaction of the enzyme with lipid substrates may be facilitated by requisite contact with the COOH-terminal domain which enables the catalytic reaction to take place at the active site found within the neighboring catalytic domain originating from the other subunit. The presence of multiple functional domains proposed by this model is consistent with the putative role of HL in simultaneous interactions with circulating lipoproteins and cell surface proteoglycans (or to the low density lipoprotein receptor-related protein) (7). In the absence of crystal structure information, this model provides the opportunity to test hypotheses regarding function location and inter-domain interaction.

An in-depth analysis of the structure and function of human HL has often been limited by the inability to purify sufficient quantities of protein for analysis. The discovery of the properties of HL subunit structure reported here is essential to further probe the nature of the specific structure-function relationships of this important enzyme of lipoprotein metabolism.

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