Published in final edited form as:

Biochemistry. 2018 January 16; 57(2): 241–254. doi:10.1021/acs.biochem.7b01009.

We FRET So You Don't Have To: New Models of the Lipoprotein Lipase Dimer

Cassandra K. Hayne^{1,4}, Hayretin Yumerefendi^{1,4}, Lin Cao¹, Jacob W. Gauer², Michael J. Lafferty¹, Brian Kuhlman¹, Dorothy A. Erie^{2,3}, and Saskia B. Neher^{1,*}

¹Department of Biochemistry and Biophysics, University of North Carolina - Chapel Hill

²Department of Chemistry, University of North Carolina at Chapel Hill

³Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill

Abstract

Lipoprotein Lipase (LPL) is a dimeric enzyme responsible for clearing triglyceride rich lipoproteins from the blood. Although LPL plays a key role in cardiovascular health, an experimentally derived 3D structure has not been determined. Such a structure would aid in understanding mutations in LPL that cause familial LPL deficiency in patients and help develop therapeutic strategies to target LPL. A major obstacle to structural studies of LPL is that LPL is an unstable protein that is difficult to produce in the quantities needed for NMR or crystallography. We present updated LPL structural models generated by combining disulfide mapping, computational modeling, and data derived from single-molecule Förster resonance energy transfer (smFRET). We pioneer the technique of smFRET for use with LPL by developing conditions for imaging active LPL and identifying positions in LPL for the attachment of fluorophores. Using this approach, we measure LPL-LPL intermolecular interactions to generate experimental constraints that inform new computational models of the LPL dimer structure. These models suggest that LPL may dimerize using an interface that is different than the dimerization interface suggested by crystal packing contacts seen in structures of pancreatic lipase.

Graphical Abstract

*To whom correspondences should be addressed: Prof. Saskia Neher, Department of Biochemistry and Biophysics, University of North Carolina – Chapel Hill, 120 Mason Farm Rd., CB7260, Chapel Hill, NC 27599, Telephone: (919)-966-9550, Fax (919)-966-2852, neher@email.unc.edu.

⁴These authors contributed equally

AUTHOR CONTRIBUTIONS

CKH, HY, JWG, DAE, BK, and SBN designed experiments. CKH performed all human LPL experiments, including TIRF, and performed respective analysis. LC assisted with LPL mutagenesis and TIRF data collection. JWG optimized programs to streamline data analysis. MJL prepared mass spectrometry samples. HY performed all LPL modeling. CKH, HY, BK, and SBN wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.

INTRODUCTION

Lipoprotein lipase (LPL) is a dimeric lipase that hydrolyses triglycerides from triglyceride-rich lipoproteins to liberate free fatty acids for use in the tissues. LPL also enhances the clearance of remnant lipoprotein particles by hepatocytes¹. Homozygous mutations resulting in loss of LPL function cause familial LPL deficiency, a rare disorder resulting in severe hypertriglyceridemia². Over 100 missense mutations distributed throughout LPL's two domains can cause familial LPL deficiency³. The first of these two domains is the N-terminal domain, which is made up of an alpha-beta hydrolase fold that contains the catalytic triad responsible for its lipase activity. This active site is covered by a 22-residue helical lid, which opens upon interaction with lipid-rich substrates^{4, 5}. The N-terminal domain also contains a binding site for the LPL activator APOCII⁶. LPL's C-terminal domain is a beta sandwich important for substrate recognition and binding to partners such as lipoprotein receptors and LPL's anchor on the capillary endothelium, GPIHBP1^{7, 8}. No 3D structure of LPL has been solved, but the related, monomeric lipase known as pancreatic lipase (PL) is 30% identical and its crystal structure has been used as a starting model for LPL⁹.

Models of LPL's structure were described in two previous publications^{10, 11}. The first was reported in 1990, shortly after the crystal structure of PL was solved⁹. The sequences of LPL and PL were aligned, superimposed using the program TURBO and the energies were minimized using XPLOR¹⁰. LPL dimers were modeled by superimposing the LPL coordinates onto the crystallographic PL dimer with no optimization of the binding surfaces^{5, 10}. In this model, the two LPL molecules were placed together in a head to tail conformation, and rotated along their longitudinal axis to allow room for the lids to open. A later study, in 2002, generated LPL models using the molecular modeling system INSIGHT II¹¹. In this publication, LPL models with both open and closed lids were based on the structures of human, porcine and horse PL^{5, 11–13}. To generate a model of the LPL dimer, models of LPL monomers were again superimposed onto the crystallographic PL dimer structure, and then an energy minimization was performed using INSIGHT II^{5, 11}. The final dimer model shows the monomers in a head to tail configuration, and with both lids positioned on the same face of the complex¹¹. Neither study provides molecular coordinates for the LPL dimer model. However, by inspection the two models are generally similar, though the two LPL monomers appear more offset from each other in the first model. In the years since these LPL models were generated, protein structure prediction has improved due

to the development of new computational tools and the availability of greater processing power^{14, 15}.

Although experimental support for a head to tail configuration is strong, there is little evidence describing the precise molecular details of how the two LPL monomers come together. Early evidence for the head to tail configuration came from a study of LPL-HL chimeras, and this study indicated that the C-terminal domain of one monomer influenced substrate selection by the N-terminal domain of the other¹⁶. Further confirmation of the head to tail orientation came from a study in which LPL monomers with either N-terminal catalytic site mutations or C-terminal substrate binding mutations were inactive individually but gained activity when mixed together¹¹. Additionally, a tandem repeat variant of LPL generated using two LPL monomers connected by an eight amino acid linker was active, and the short linker would restrict this variant to a head to tail conformation¹⁷. Thus, it is widely accepted that the N-terminal catalytic domain of one LPL monomer contacts the C-terminal domain of the other LPL monomer. The next challenge is to gain a molecular understanding of the interface between these two monomers. Although many missense mutations negatively affecting LPL activity have been identified, LPL folding, dimerization and activity are intimately linked making it difficult to conclusively show that individual mutations affect the dimer interface¹⁸. For example, biochemical characterization of missense LPL mutations identified in patients with familial LPL deficiency revealed that most never folded into a stable, native monomer conformation and therefore could not form dimers¹⁹.

Understanding the LPL dimer interface could shed light on the reasons some LPL mutations result in loss of function, and it could also support efforts to stabilize LPL for use in gene therapy or protein replacement therapy. However, there is currently no experimentally derived LPL structure. One major challenge to using traditional biophysical methods to study LPL is that LPL undergoes extensive post-translational modification. LPL is both glycosylated and has ten cysteines that form five disulfide bonds. In addition, LPL requires interaction with lipase maturation factor 1 (LMF1), which assists in LPL folding within the endoplasmic reticulum^{20, 21}. These requirements limit LPL expression to mammalian systems. Purification from mammalian cell culture results in low yields, yet it is the only way to study relevant LPL mutations. Additionally, achieving the high protein concentrations of bovine or human LPL needed for X-ray crystallography, NMR, analytical ultracentrifugation, and isothermal titration calorimetry is challenging, limiting the use of these techniques to understand LPL structure-function relationships and LPL's interaction with regulatory factors.

Single-molecule techniques provide a novel approach to characterizing LPL by exponentially reducing the quantity of LPL necessary to conduct biophysical measurements. For example, surface plasmon resonance, which has been used to study LPL interactions^{22, 23}, requires several hundred-fold more protein per sample than single molecule experiments. We previously used atomic force microscopy (AFM) to show LPL and ANGPTL4 binding in a defined complex²⁴, but AFM only provides a snapshot of binding and it cannot provide information on dynamics or high resolution structural information. We thus turned to single molecule Förster resonance energy transfer (smFRET)

as an alternative approach for measuring LPL/partner binding and dynamics. smFRET allows estimation of distances within individual proteins or protein complexes that have been labeled with two different fluorophores (donor and acceptor) that can transfer energy by dipole-dipole interactions

smFRET provides insights into protein interactions that cannot be derived from bulk measurements, which yield ensemble averages. smFRET is a well-established technique for studying the dynamics of individual protein-DNA interactions^{25–28} and protein-protein interactions²⁵. Using smFRET, real-time measurements of dynamics, occurring in individual proteins in response to binding interactions²⁹, have been observed. smFRET is also particularly useful to identify and measure the lifetimes of different complexes and transition states²⁶, as well as conformational changes^{30, 31}. Here, we demonstrate the use of smFRET to study LPL structure-function relationships. FRET measurements can generate experimental constraints to support computational models of protein structures, and we apply this technique to inform new models for the LPL dimer structure^{32–34}.

MATERIALS AND METHODS

Molecular cloning

Human LPL was cloned into pCDNA5/FRT/TO (ThermoFisher Scientific) with the addition of either a 6× polyhistidine-tag or a V5 tag (GKPIPNPLLGLDST on the C-terminus). Single cysteines at positions S12, S36, S63, S97, S193, V224, S240, S251, S259, S323, S346, and S384 were introduced using Quickchange site directed mutagenesis.

LPL protein expression and purification

LPL was transiently or stably transfected into HEK 293 FRT Flip-In cells (ThermoFisher Scientific) using Fugene 6. Expression and purification of LPL was carried out as previously described³⁵. Washing with 75 column volumes was necessary to eliminate cleavage products. Protein was concentrated, aliquoted, flash frozen in liquid nitrogen, and stored at –80 °C until use or immediately labeled.

Preparation of fluorescent cysteine labeled LPL

Single cysteine mutants of LPL were purified (final buffer composition was 1.5 M NaCl, 10% glycerol, 20 mM Bis-Tris pH 6.5) and incubated with an approximately 10-fold excess of Alexa Fluor® Maleimide Dyes (Invitrogen). Label was added dropwise to the protein solution followed by gentle mixing. The reaction was allowed to continue, in light protected tubes, for 45 minutes at 4 °C or on ice. For samples in which biotinylated LPL was used, a 1–2 molar excess of Biotin-N-Hydroxysuccinimide ester (Sigma) was next added and the reaction was allowed to proceed on ice for another 10 minutes. For both biotinylated and non-biotinylated samples, LPL was then diluted to less than 500 mM NaCl with 20 mM Bis-Tris pH 6.5 and re-purified over a HiTrap Heparin Sepharose High Performance Column (GE Healthcare Life Sciences) to remove excess biotin and/or label. Excess label flowed through the heparin column whereas purified, labeled LPL bound and eluted normally. Alternatively, labeling could be followed by removal of excess dye by use of two tandem 7 kDa Zebra desalting columns (ThermoFisher Scientific). LPL samples were aliquoted and

stored at -80°C until use. If samples were concentrated, the samples were syringe filtered through a $0.2\text{ }\mu\text{m}$ PDVF filter to remove aggregates.

LPL Activity Assays

For LPL activity in conditioned media, plasmids bearing WT or mutant LPL variants were transiently transfected into HEK293 cells. LPL was released from the surface of cells through the addition of heparin, media was collected and the LPL secreted in media was assayed for activity using $10\text{ }\mu\text{M}$ DGGR essentially as previously described³⁵.

For LPL activity on a surface, a 96-well black-sided, clear-bottom plate, was functionalized by a brief incubation with biotinylated bovine serum albumin (Sigma, 1 mg/ml , 5 minutes), washed three times with PBS, incubated with streptavidin (Invitrogen, 0.1 mg/ml , 20 minutes) and washed again with PBS. Plates sat on ice with PBS in the wells until the experiment was ready to proceed. Wells were then treated as follows: A) No LPL (Buffer), B) biotinylated bovine LPL ($\sim 150\text{ nM}$, 5 minute incubation), C) Heparin-Biotin (Sigma, 1 mg/ml , 5 minutes) + bovine LPL (150 nM , 5 minutes). Wells were washed three more times with PBS and substrate was added (150 mM NaCl, 0.4% Triton-X, 20 mM Tris 8.0 , $14\text{ }\mu\text{M}$ DGGR). Wells were excited at 529 nm with a 590 nm cut-off and read for emission at 600 nm on a SpectaMax 5 plate reader.

Activity of fluorescently labeled LPL

Purified LPL^{S384C} was modified as described above with Alexa Fluor®647 dye and desalted through a 7 kDa Zebra desalting column (ThermoFisher Scientific). The protein was then quantified. The activity of equal concentrations of labeled and unlabeled LPL^{S384C} was measured as previously described²⁴, with the addition of 1 mM deoxycholate.

Total internal reflection microscopy (TIRF) microscopy slide preparation

Home-built quartz slides were prepared as previously described³⁶ with 5 minute epoxy (Staples) used for sealing the edges of the slide. The surface of the slides was pre-functionalized by brief incubation with biotinylated bovine serum albumin (Sigma, 1 mg/ml , 5 minutes), washing, and followed by incubating with streptavidin (Invitrogen, 0.1 mg/ml , 20 minutes) and washing. For slides utilizing heparin attachment, biotin-heparin (Sigma) was either added directly to the slides or pre-incubated with the LPL before addition. Immediately preceding data collection, LPL was deposited onto the slide surface in Buffer A (20 mM Tris, 300 mM sodium chloride, 5% glycerol, 1 mM deoxycholate, $\text{pH } 7.5$) and incubated for 5 minutes. Unbound LPL was then washed out with imaging buffer (Buffer A with the addition of $\sim 50\text{ }\mu\text{M}$ cyclooctatetraene (Sigma), a triplet state quencher to lessen fluorophore blinking.)

TIRF microscopy imaging

The slides were imaged using a through-prism TIRF laser microscope. Fluorophore excitation was achieved using constant illumination with 532 nm and 638 nm lasers. The fluorophore emissions were collected through a $60\times$ water immersion 1.2 N.A. objective, and the image was split using a DualView optical splitter (Chroma) equipped with a 645 nm dichroic mirror. The emission signals passed through optical filters before detection with a

Cascade 512 (Photometrics) emCCD camera: a 585/70 bandpass filter (Chroma) for the Alexa Fluor®555 emissions and a 655 nm longpass filter (Chroma) for the Alexa Fluor®647 emissions were used. Movies of approximately 850 seconds in length were collected at a 100 ms framerate, using software written in house.

LPL-LPL interactions visualized by smFRET using TIRF microscopy

Experiments were carried out as described above using the biotin-heparin mode of attachment. LPL molecules were fluorescently labeled, as described above, at specific cysteine residues either on or off the lid and mixed together immediately preceding imaging. Residual LPL was then washed off the slides. For some experiments shown, buffer without oxygen scavengers was used to promote fluorophore bleaching. During image capture, only the red (acceptor) laser was turned on for the first 10 frames and again after the 800th frame. From frames 10–800, only the green laser was turned on.

smFRET data analysis

The fluorescence intensities of single molecules were extracted from the movies using in house software, as previously described³⁷. Traces were analyzed only if LPL fluorescence intensity was present at the start of the movie. Time traces with intensities above or below threshold intensities (empirically determined) for single molecules were excluded from further analysis. The remaining intensity traces were then smoothed using a non-linear Chung-Kennedy filter that preserves edges^{38, 39}. LPL-LPL interactions were analyzed and the average FRET values of the bound complex were calculated.

Sample Preparation and Nano-Flow Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-MS/MS) Analysis

All protein sample preparation and mass spectrometry was carried out at the Duke proteomics facility. Following 1D-SDS-PAGE separation, the molecular weight region of interest was excised and subjected to an in-gel trypsin digestion with alkylation using 10 mM iodoacetamide for 30 min at room temperature but no reduction to reduce any disulfide shuffling during digestion⁴⁰. In addition, 10 mM Tris pH 5–6 was used instead of 50 mM ammonium bicarbonate. Extracted peptides were lyophilized to dryness and then resuspended in 20 μ L 2% acetonitrile, 0.1% formic acid prior to LC-MS/MS analysis. Chromatographic separation was performed on a Waters NanoAcquity UPLC equipped with a 1.7 μ m BEH130 C₁₈ 75 μ m I.D. $\times$ 250 mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 4 μ L injection, peptides were trapped for 5 min on a 5 μ m Symmetry C₁₈ 180 μ m I.D. $\times$ 20 mm column at 5 μ L/min in 99.9% A. The analytical column was then switched in-line and a linear elution gradient of 5% B to 40% B was performed over 90 min at 400 nL/min. The analytical column was connected to a fused silica PicoTip emitter (New Objective, Cambridge, MA) with a 10 μ m tip orifice and coupled to a Thermo QExactive Plus high resolution accurate mass tandem mass spectrometer through an electrospray interface. The instrument was operated in data dependent mode of acquisition with precursor MS scans from m/z 375–1600 and the top ten most abundant precursor ions being subjected to MS/MS fragmentation. For all experiments, charge dependent CID energy settings were

employed and a 20 s dynamic exclusion was employed for previously fragmented precursor ions.

Qualitative Identifications and Crosslinked Peptide Identification from Raw LC-MS/MS Data

To identify crosslinked species, Mascot distiller generated. MGF files were submitted to MassMatrix (v 2.4.2, Feb 2012) searches against a forward/reverse database containing only the proteins of interest⁴¹. Search tolerances were 5 ppm for precursor ions and 0.02 Da for product ions using trypsin specificity with up to two missed cleavages.

Carbamidomethylation (+57.0214 Da on C) was set as a fixed modification, whereas oxidation (+15.9949 Da on M) and deamidation (+0.984016 Da on NQ) were considered as variable modifications. The “advanced search” option was enabled to allow for inter- or intrapeptide disulfide crosslinking (−2.015650 Da). A crosslinked peptide match within MassMatrix was only considered if 1) the peptide scoring thresholds were above that required for a matching probability less than p-value <0.05 against a reversed protein sequence and 2) at least two peptide spectral matches were matched to the same predicted crosslinked species⁴¹. All putative crosslinked peptide mass spectra were manually inspected for quality.

LPL Modeling using the Rosetta Macromolecular Suite

Two alternative approaches were taken for generating models of the LPL dimer. Our first goal was to regenerate the original LPL homology model that was based on the crystallographic dimer observed for horse pancreatic lipase. In this first approach, the crystal structure of horse pancreatic lipase (HPL, PDB ID: 1hpl) was used as a homology modeling template for the LPL dimer. Despite being a functional monomer, HPL crystallizes as a dimer with extensive contacts between monomers¹². A second PL crystal structure, that of the porcine pancreatic lipase/colipase complex (PDB ID: 1eth), has two complex molecules in the asymmetric unit⁴². However, 1hpl is the only PL crystallographic dimer captured in a head to tail conformation. The comparative modeling package in Rosetta, RosettaCM, was used to thread the LPL sequence onto the HPL “dimer” and then refine intramolecular and intermolecular interactions⁴³. The 10 lowest scoring structures from 10,000 independent homology model trajectories each served as the starting structure for 5000 independent full atom docking and refinement trajectories, generating 50,000 models in total. Finally, a single model was chosen based on total Rosetta energy. Input files and command line options for this and other Rosetta simulations are supplied in the supplementary materials.

Our second approach for generating models of the LPL dimer was aimed at creating a more diverse set of models and consisted of the following stages: (1) homology modeling to predict the structure of the LPL monomer, (2) low-resolution global docking with loose distance constraints based on FRET data and previous structure/activity studies, (3) filtering based on biological factors, (4) clustering to identify the unique docking solutions, (5) local refinement to energy minimize contacts at the dimer interface, (6) and filtering based on calculated binding energies. To identify templates for homology modeling of the LPL monomer the PDB database was queried using blast with the lipoprotein lipase sequence (residues 28–475). Structures of rat (PDB ID: 1bu8), dog (1rp1), and human (2ppl) pancreatic lipases were chosen as templates for monomeric LPL in closed lid conformation.

Respectively, two structures of human (PDB ID: 1lpa and 1lpb) and pig (1eth) pancreatic lipases were chosen as templates for monomeric LPL in open lid conformation. 10,000 homology models were created using RosettaCM, and the 20 lowest scoring models were chosen as starting points for docking simulations. During the homology modeling, the disulfide linkages were fixed at the pairings observed in the LC-MS/MS Data. Disulfide bonds are mapped in Figure 5A, where they are numbered for bovine LPL (Bovine - 2 = human). Docking simulations were performed only for the homology models with a closed lid conformation.

Global docking (i.e. the partners start the simulation with random orientations relative to each other) was performed in centroid mode, which represents each amino acid side chain with a single point⁴⁴. C2 symmetry was enforced during all docking trajectories. Two separate sets of global docking simulations were performed with alternative sets of constraints. The first set of constraints (FRET only) was based on the FRET distances measured between two fluorophores at residue 224 and between two fluorophores at residue 384. Because side chains were not present at this stage of modeling, the constraints were applied to the C α atoms of each residue. To allow for the variable positions that the dyes could adopt relative to the C α atoms, a harmonic function with a large standard deviation, 20 Å, was used to model the constraints. The second set of constraints (FRET plus) added one additional distance constraints based on previous structure/activity studies with LPL. It was previously shown that an octasaccharide of heparin was the smallest length of heparin that could bind to the LPL dimer, suggesting that the heparin binding sites on the two chains of LPL are separated by ~38 Å^{45, 46}. Residues identified as important for heparin binding (279, 280, 282, 296, 297, 403, 407, 413 and 414) were constrained such that at least three of these residues was within 38 Å of the same residue on the opposing chain^{47, 48}. For each sets of constraints, 10,000 global docking trajectories were performed for each of the 20 selected homology models as input structures yielding a total of 200,000 docked solutions. The resulting decoys were filtered to remove models in which the constraint score ("atom_pair_constraints") from the FRET and heparin constraints was greater than 1 (the heparin constraint was only used as a filter for the models generated with heparin constraint), followed by filtering with a planarity score. The planarity score was constructed to favor models that place both lids and tryptophan clusters in the same plane so that they are well positioned to engage lipoprotein substrate. Using representative residues from each of the four structural elements (residue 225 in both chains for the lids and residue 393 in both chains for the tryptophan clusters), the planarity score function evaluates how far each residue (C α atoms) is from a plane constructed with the other three residues and takes the minimum of the four measured distances. The resulting decoys were clustered and filtered by interface interactions ("interchain_contact" <= -10). The aforementioned yielded 128 unique starting docking orientations for FRET only constraints modeling and 53 for FRET plus, modeling, giving a total of 181 starting models for full atom refinement.

Local refinement proceeded using full atom docking with rigid body perturbations and side chain repacking followed by two rounds of the Rosetta relax protocol. The relax protocol cycles between gradient-based minimization of backbone and side chain torsion angles and rotamer-based side chain sampling. Tighter constraints for the 2 FRET distances were employed constraining the respective C α atoms using a harmonic function with a standard

deviation of 10 Å. Each of the starting 181 models was used as the initial pose for 500 independent trajectories of refinement, resulting in 64,000 full atom models for the FRET only constraints and 26,500 for the FRET plus respectively. Binding energies were calculated for the full atom models by subtracting the energy of the complex from the energy of the separated dimer. Finally, the best 1000 models by binding energy were filtered and sorted by total Rosetta score (Rosetta's default REF15 score function is used throughout the entire course of the modeling)^{49, 50}. A chart showing total energy vs. RMSD for the top models is shown as supplemental figure 1. The lowest energy model was chosen as the final model for each set of constraints simulations. A flowchart describing the modeling is provided in as supplemental figure 2.

To test the compatibility of the final homodimer models with lid opening we used our lowest energy homology model in open lid conformation and superposed it onto each of the monomers in the final close lid models, thus creating a starting structure of the putative dimer in open conformation. Finally, the structure was refined with the Rosetta relax protocol with constraints designed to keep the model near its starting state ("coordinate constraints").

HAZARDOUS PROCEDURES

None

RESULTS

Production of an Original LPL Dimer Model

Because atomic coordinates were not available for the LPL dimer structure based on the PL crystallographic dimer, we first regenerated this model as the original model for comparison to subsequent models. Using the comparative modeling package in Rosetta, we threaded the LPL sequence onto the PL crystallographic dimer and used rotamer-based side chain packing coupled with backbone minimization to energy optimize the structure. Most, but not all, of the features of the original model are consistent with our current understanding of LPL function. As shown in Figure 1, the lids of both dimer partners are both oriented towards the same plane (representing a large, lipoprotein substrate such as a chylomicron), as mutational analysis suggests that both dimer partners are likely to be active on substrate at the same time¹¹. Next, the C-terminal tryptophan cluster of one monomer is implicated in the function of the N-terminal catalytic domain of its dimer partner¹¹. In the original model, the tryptophan cluster (blue) of one monomer is proximal and in a similar plane to the lid of its dimer partner. Furthermore, there is a C-terminal recognition motif in LPL for its anchor, GPIHBP1^{51, 52}. The original model has the GPIHBP1 interacting motif (black) on the opposite face of the LPL dimer than the lid, such that LPL is sandwiched between its anchor and substrate, as is expected.

Conversely, some features of the original model are difficult to reconcile with current knowledge of LPL biology. For example, the binding site for the LPL activator, APOCII, (shown in red) faces the dimer interface and is not exposed at the surface of LPL⁶. Additionally, two putative dimerization mutants, R75S and G188E (shown in purple), are

not localized to the dimerization interface in the original model^{53, 54}. These LPL mutations are reported to result in secreted LPL (indicating that it is sufficiently folded to pass ER quality control) that elutes as a monomer, rather than a dimer, from a heparin column^{53, 54}. As noted in the original LPL model, the lids of the LPL dimer partners clash when they are modeled based on the open lid conformation of pancreatic lipase (shown in Supplemental Figure 3)^{5, 10}.

Another problematic feature of the original model comes from surface plasmon resonance studies of the LPL-heparin interaction that showed that heparin octa- and decasaccharides could bind to LPL dimers⁴⁶. Because heparin bound LPL monomers far less tightly, and a 1:1 ratio of heparin decasaccharides:LPL dimers was sufficient for maximal stabilization of LPL, the authors concluded that a single heparin octa- or decasaccharide could bind to both LPL dimer partners simultaneously⁴⁶. The length of a heparin decasaccharide is approximately 41.5 Å, but in the original dimer model, the distance between the C-terminal heparin binding sites in the two LPL dimer partners (measured at various points between K403–K414) ranged from 50–75 Å^{45, 47, 48}. We thus set out to verify and enhance the original model using experimentally derived constraints.

Generation of smFRET distance constraints between LPL dimer partners

To perform smFRET measurements on LPL, we first established methods for attaching active LPL to the surface of slides for TIRF imaging. Methods for attaching macromolecules to slides include protein-specific binding reagents, antibodies, liposomes, and biotin linkages. Each of these methods could be applied to LPL studies, except for liposomes. Liposomes circumvent the need to use protein linkages to attach proteins to slides, but would not be strategic to use with an active lipase. We devised two approaches to attach LPL to slides, both using biotinylated bovine serum albumin (BSA) bound to streptavidin. BSA blocks nonspecific binding of target proteins and the biotin-streptavidin provides a means to orient biotinylated proteins or conjugates on the surface of the slides. In one approach, we took advantage of LPL's strong affinity for heparin and utilize biotin-conjugated heparin to bridge LPL to functionalized slides (Figure 2A, right panel). Heparin binds to LPL's C-terminus and has been shown to stabilize LPL against inactivation⁵⁵. In the second approach, we use LPL that is directly biotinylated and allow the protein to bind the BSA-biotin-streptavidin functionalized slide (Figure 2A, left panel). Biotinylation of LPL leaves its heparin binding site free and thus may be particularly useful for monitoring LPL interactions that utilize LPL's heparin binding site.

Both biotinylated LPL and the heparin-biotin conjugate are suitable methods for attaching active LPL to slides, as LPL maintains activity under both conditions (Figure 2B). However, the biotin ester can covalently react with any available amine. Thus, excessive biotinylation of LPL reduces its activity, and so a very small molar excess of biotin to LPL, along with a short incubation period, is necessary to maintain active LPL as shown (Figure 2B). The heparin-biotin conjugation does not have this undesirable effect. In fact, heparin stabilizes LPL and represents a more physiological mode of attachment⁵⁵. Accordingly, we use the heparin method for monitoring LPL-LPL interactions and the biotin approach when

monitoring binding interactions that may require LPL's heparin binding site. Notably, both methods can be used with LPL purified from milk, plasma, or tissue culture.

Specific labeling of LPL with fluorophores

After establishing that active LPL could be attached to slides, we determined methods for adding fluorophores to different locations on LPL. We specifically labeled LPL using maleimide fluorophores that react with free cysteines. We took advantage of the fact that although LPL contains ten cysteine residues, they are incorporated into five critical disulfide bonds, leaving no native cysteines available for labeling. Consequently, we engineered specific labeling sites by introducing additional cysteines. We added an additional cysteine residue either on the lid that covers the active site or elsewhere on LPL (Figure 3A, top cartoon). To add non-lid cysteine residues, we targeted serine residues to minimize potential structural and functional changes. We mapped LPL's 40 serine residues on a computational model of the LPL monomer to identify serine residues in distinct regions (near the lid, C-terminus, and N-terminus, as shown in Figure 3A). In total, twelve positions were selected for mutation to cysteine. All serine to cysteine mutants, except LPL^{S251C}, were secreted as shown in the Western blots in the media panel in Figure 3B. Comparison of the lipase activity of wild-type and C→S mutants from conditioned media showed that many of the mutants exhibit activities similar to wild-type LPL (Figure 3B). As expected, LPL with an introduced cysteine are readily labeled with the maleimide dye Alexa Fluor®647, whereas wild-type LPL is not labeled under the same conditions (Figure 3C, top panel). In addition, we show that maleimide labeling does not hinder the activity of a representative, purified mutant, LPL^{S384C} (Figure 3D).

Use of Intermolecular FRET to measure distances between dimer subunits

With multiple, active LPL variants labeled at unique positions in hand, we set out to use smFRET to estimate the intermolecular distances between these positions. LPL is a homodimer in which the subunits undergo rapid exchange⁵⁶. This rapid exchange ensures that when LPL dimers labeled with different fluorophores are mixed, they form LPL dimers containing both fluorophores. The FRET efficiency between the differently labeled monomers in the LPL dimer can then be used to estimate the distances between dyes^{57, 58}. The Förster distance (R_0 , or distance at which half of the energy is transferred) is approximately 51 angstroms for the fluorophores (Alexa Fluor®647 and Alexa Fluor®555) used here⁵⁹. We tested multiple different pairwise combinations of fluorescently labeled LPL dimers, but not all combinations yielded usable data. However, smFRET distributions were successfully determined from the pairs V224C to V224C, S384C to S384C, and S36C to S384C, and these FRET values were used to estimate the distances between pairs.

To monitor FRET, an automatic alternating laser system was used to toggle lasers (see methods). Time traces for individual molecules were extracted and analyzed. Traces were then manually sorted and only FRET events that involved one acceptor and one donor, as evidenced by single step changes in intensity to zero from blinking or bleaching events, were analyzed. Notably, if these measurements were done in bulk the FRET values would be artificially low because populations with no acceptor would give off high donor fluorescence and thus reduce the apparent FRET. To generate a distance between each pair, hundreds of

individual FRET measurements were collected and plotted as a population in a histogram. Based on a Gaussian fit, an average FRET value for each mutant pair was determined and then distance calculated based on an R_0 value of 51 Å. An in-depth discussion of the analysis methods can be found in previous literature^{29, 60}.

Figure 4A shows schematics of the positions of each of the three FRET pairs being tested on the original model of the LPL dimer. Below the models, in figures 4B–C, example data show the fluorescence intensity and FRET traces for each FRET pair. These data show the protein dynamics that smFRET can capture. For example, the first panel shows the interaction between two lids (LPL^{V224C} with one monomer labeled with acceptor (Alexa Fluor®647) and one monomer labeled with donor (Alexa Fluor®555)). Inspection of the traces shows that at approximately 4 seconds, there is a slight decrease in FRET, suggesting that the lids are somewhat dynamic (Figure 4C, first panel). Stronger changes in FRET, indicating a larger protein conformational change, are shown in figure 4B–C, panel three. In this panel, acceptor labeled LPL^{S384C} is combined with donor labeled LPL^{S36C}. This trace shows an initial FRET near 1.0 when the laser turns on at the start of the trace, then a conformation change occurs at approximately 3 seconds causing the FRET to drop to just under 0.4, then another change occurs at approximately 6 seconds and the FRET increases to just above 0.4. In addition to conformational changes, all the traces shown also exhibit a loss of FRET when the donor fluorophores bleach.

The histogram of FRET values for LPL^{V224C} to LPL^{V224C} (figure 4D, first panel) shows a single peak, indicating one major conformational state with a mean FRET efficiency of 0.62. This value was used to calculate the distance between the two fluorophores (47 Å), which is used in our computational dimer model. By contrast, the histograms of FRET efficiencies between LPL^{S384C} and LPL^{S384C} as well as between LPL^{S384C} and LPL^{S36C}, show one major and one minor peak, indicating that at least one position adopts two conformational states. We used distances derived from the most populated, major peak for modeling (mean FRET efficiencies of 0.43 for 384-384 and 0.50 for 384-36). Additionally, we determined the FRET efficiencies at half the maximum amplitude and calculated their associated distances to provide a range of distances to use to analyze molecular models of the LPL dimers (ranges listed in Table 1).

Clarification of LPL Disulfide Bonds

LPL has 10 cysteine residues that are paired in 5 disulfide bonds. Based on the structure of PL, in which disulfide bonds form sequentially, the first cysteine in LPL is expected to bond with the second, etc., as shown in Figure 5A¹³. However, published data on the disulfide bonding of bovine LPL derived from sequencing its tryptic peptides suggests a non-sequential pattern of disulfide bonding in which the fifth cysteine (residue 266 in bovine LPL) forms a disulfide with the eighth (residue 285), and the sixth with the seventh (277 and 280, respectively), as shown in Figure 5B⁶¹. To identify the correct disulfide bonding arrangement to use in our computational model of LPL, we performed a mass spectrometry analysis of the disulfide bonds in bovine LPL.

Our mass spectrometry data show that LPL's disulfide bonds are sequential (as in Figure 5A), comparable to the disulfide bonding arrangement in PL. The disulfide search algorithm

in MassMatrix was used to identify intact disulfide bonds in peptides resulting from tryptic digest of bovine LPL⁶². Several spectra from a disulfide linked tripeptide containing cysteine residues 266, 277, 280 and 285 were found. The tripeptide is diagrammed in Figure 5C. This disulfide linked tripeptide would not exist if the arrangement of disulfide bonding were nonsequential. An example spectrum in Figure 5C shows the parent tripeptide m/z peak (M +4, boxed). Additionally, it shows a boxed m/z peak (B_{Y2'}+) resulting from fragmentation of the B (middle) chain between its two cysteines. The fragmentation table for this spectrum is shown as supplemental figure 4, and a model of the disulfide bonds in LPL is shown as supplemental figure 5. We also identified many of the other, uncontested disulfide bonds in LPL. Figure 4D shows a heat map from MassMatrix that indicates the confidence in identification of each disulfide bond⁶².

Application of Experimentally Derived Data to New LPL Dimer Models

With the pattern of disulfide bonding in the LPL monomers resolved, and new, experimentally derived distance constraints between dimer partners in hand, we set out to improve models of the LPL dimer structure. To produce new LPL dimer models, we performed constrained molecular docking simulations with Rosetta. Two alternative sets of models were generated by varying the constraints enforced during docking. The first set of constraints (FRET only) was composed of two FRET-derived distance constraints, specifically those between the two lid fluorophores at position 224 in each dimer partner, and between the two fluorophores at position 384 in each dimer partner (diagrammed in Figure 4A, panels 1–2). We excluded the third FRET-derived distance constraint (36–384) from the modeling to use later to test the model. The second set of constraints (FRET plus) included the two FRET-derived constraints combined with one additional restraint: the heparin-binding residues in each monomer should be close enough for the dimer to engage a single heparin octa- or decasaccharide molecule⁴⁶. Global docking simulations were performed in order to identify unique binding orientations compatible with the specified constraints. A flow chart showing the modeling process is included as supplemental figure 1. Prior to full atom refinement, the models were filtered with a planarity score. The planarity score (see methods) favored the placement of both lids and the tryptophan clusters on the same face of the dimer, allowing for simultaneous engagement of a lipoprotein substrate. Next, the unique solutions from the previous step were further refined and the final model for each set of simulations (FRET only and FRET plus) was selected by filtering out the best 1000 models by calculated binding energy and selecting the model with the lowest total energy for the complex.

Features of the new LPL Dimer Models

Figure 6 shows side, top, and bottom views of dimer models generated by both sets of constraints. Overall, the top scoring model generated from the FRET only constraints resembles the original model, with an RMSD of 5 Å. By contrast, in the FRET plus model the dimerization interface is not the same as the interface in the original model, and it is in fact switched such that the outer face in the original model is the interface in the new model. The binding energies of these interfaces are nearly equivalent, as shown in Table 1. This switch of the binding interface has four important consequences. First, it moves the APOCII binding site from the dimerization interface to the outside of the dimer (shown in red in

Figures 6). Second, it moves the putative dimerization mutant G188E into the dimerization interface (see residues shown in purple spacefill in Figure 6). Unlike the G188E mutation, the putative dimer mutant R75A does not make dimerization contacts in any of the models. Next, the lids can open without clashing (see supplemental figure 3). Finally, the heparin binding residues in the FRET plus model are in close proximity and form a grove that appears compatible with binding of an octasaccharide molecule.

Next, we analyzed the new models in the context of the experimental distances estimated from the average FRET values. Two FRET distances, from positions 224-224 and 384-384, were used to generate the model. A third distance, from 36-384, was excluded from the modeling in order to have an independent constraint to verify the models. We measured all three distances in the two new models. For both the FRET only and the FRET plus models, the distances measured from at positions 224-224 and 384-384 fell within the range of experimental FRET distances measured at the half maximum amplitude. With respect to the distance between FRET positions 36 and 384, FRET model was the best fit, at 49.5 Å in the model vs 51 Å measured experimentally. By contrast, the distance between positions 36 and 384 in the FRET only model did not fall within the half maximum experimental range (see Table 1). Overall, the RMSD between the experimental and model distances were 9.7 Å for the FRET only model and 1.7 Å for the FRET plus model.

DISCUSSION

Ultimately, a precise molecular description of LPL will be obtained only once the structure is solved through NMR, crystallography, or cryo-electron microscopy. However, LPL is challenging to characterize because it is an unstable protein that is difficult to produce at high concentrations, and therefore such a structure may not be soon forthcoming. Consequently, we generated models of the LPL dimer structure using the molecular modeling program Rosetta constrained by biological data, experimentally derived distances, and experimentally verified disulfide connectivity. Our goal was to provide atomic coordinates of these models to researchers to facilitate further study of LPL structure-function relationships. Although models of the LPL dimer have previously been reported, atomic coordinates for these models were not published. One consideration is that details of the models may prove incorrect if an experimental structure of LPL is obtained. However, we appreciate that not all groups have access to the modeling software, expertise and computational power needed to generate these models, and therefore, with the caveat that they are only models, they are provided as supplemental files.

The original model and the FRET only model are similar, and both share a major difference compared to the FRET plus model in that the dimerization interfaces are switched. This is unexpected, especially for those accustomed to viewing LPL as it appears in original model, which was generated from the crystallographic dimer observed in some PL structures. However, it is important to note that PL is a monomeric protein, and the crystal-packing contacts observed in its structure may not represent a legitimate dimerization interface. In fact, a comparison of protein-protein contacts stemming from crystal-packing versus those naturally occurring in multimeric proteins suggested that crystal-packing contacts occur randomly, and likely share little in common with physiological oligomerization interfaces⁶³.

Furthermore, another PL crystal structure with two PL molecules in the asymmetric unit shows a completely different interface⁴². Based on binding energies, both the original and the new models are possible. All models fit with the known biology of LPL in that they place LPL between its substrate and its membrane anchor. The FRET plus model has the additional benefits in that the lid appears to be able to open unobstructed, and the APOCII binding site is on the outside surface of the dimer structure, where it is likely to be more accessible. Additionally, in the FRET plus model the G188E mutation, a putative dimerization mutant and one of the most prevalent inactivating LPL mutations, is positioned at the dimer interface⁶⁴.

One question raised by the presence of two potential dimerization interfaces is if LPL dimers undergo biologically significant oligomerization; such that one interface mediates dimer formation while the other facilitates assembly of dimers to higher order molecular structures. All LPL dimer models filled an area of roughly 100 Å by 60 Å. VLDL particles are 300–800 Å in diameter, and chylomicrons range in size from 750–6000 Å in diameter⁶⁵. Many LPL dimers thus could, in theory, fit on one lipoprotein particle. In fact, a previous study showed that LPL most efficiently hydrolyzed chylomicrons when 43 molecules of LPL were present per molecule of chylomicron⁶⁶. Taken together, these data raise the question of whether dimers of LPL may function in arrays *in vivo*.

An intriguing feature shared by all of the models is that they feature a doughnut-like hole in the middle. Energetically, a cavity between dimer partners is not ideal, and so it raises the question of whether or not this cavity has an important biological function. Could free fatty acids pass through this cavity, such that they are directly channeled to the endothelial surface? Measurement of the narrowest cavity, in the FRET plus model, revealed that the cavity was approximately 6 Å in each direction. Ultimately, experimental data will be needed to answer this and other important questions raised by these new LPL models. It is our hope that they prove useful in generating and answering questions that advance our understanding of LPL structure-function relationships in lipid metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of both the Neher and Erie labs for thoughtful discussions of this work.

FUNDING INFORMATION

This work was supported by American Heart Association Grant 14BGIA20370000 and NIH Grants R01-HL125654 to SBN, R01-GM073960 to BK and R01-GM079480 to DAE. CKH is supported by a Dissertation Completion Fellowship from the University of North Carolina – Chapel Hill Graduate School and by the National Science Foundation Graduate Research Fellowship DGE-1144081. JWG was supported by a Pre-Doctoral Fellowship from the American Heart Association, Grant 13PRE16940085. Any opinion, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of any of the funding agencies. The authors declare no competing financial interest.

ABBREVIATIONS

AFM	Atomic force microscopy
DGGR	1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester)
DMEM	Dulbecco's Modified Eagle Medium
FFA	Free fatty acid
FRET	Fluorescence resonance energy transfer
GPIHBP1	Glycosylphosphatidylinositol anchored high-density lipoprotein binding protein 1
LPL	Lipoprotein lipase
PL	Pancreatic lipase
PBS	Phosphate buffered saline
smFRET	single-molecule Förster resonance energy transfer
TG	Triglyceride
TRLs	Triglyceride rich lipoproteins
VLDL	Very low density lipoprotein

References

1. Beisiegel U, Weber W, Bengtsson-Olivecrona G. Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein. *Proc Natl Acad Sci U S A*. 1991; 88:8342–8346. [PubMed: 1656440]
2. Murthy V, Julien P, Gagne C. Molecular pathobiology of the human lipoprotein lipase gene. *Pharmacol Ther*. 1996; 70:101–135. [PubMed: 8843465]
3. Burnett, JR., Hooper, AJ., Hegele, RA. Familial Lipoprotein Lipase Deficiency. In: Pagon, RA, Adam, MP, Ardinger, HH, Wallace, SE, Amemiya, A, Bean, LJH, Bird, TD, Ledbetter, N, Mefford, HC, Smith, RJH., Stephens, K., editors. *GeneReviews(R)*. Seattle (WA): 1993.
4. Dugi KA, Dichek HL, Santamarina-Fojo S. Human hepatic and lipoprotein lipase: the loop covering the catalytic site mediates lipase substrate specificity. *The Journal of biological chemistry*. 1995; 270:25396–25401. [PubMed: 7592706]
5. van Tilbeurgh H, Egloff MP, Martinez C, Rugani N, Verger R, Cambillau C. Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography. *Nature*. 1993; 362:814–820. [PubMed: 8479519]
6. McIlhargey TL, Yang Y, Wong H, Hill JS. Identification of a lipoprotein lipase cofactor-binding site by chemical cross-linking and transfer of apolipoprotein C-II-responsive lipolysis from lipoprotein lipase to hepatic lipase. *J Biol Chem*. 2003; 278:23027–23035. [PubMed: 12682050]
7. Gin P, Goulbourne CN, Adeyo O, Beigneux AP, Davies BS, Tat S, Voss CV, Bensadoun A, Fong LG, Young SG. Chylomicronemia mutations yield new insights into interactions between lipoprotein lipase and GPIHBP1. *Hum Mol Genet*. 2012; 21:2961–2972. [PubMed: 22493000]
8. Nielsen MS, Brejning J, Garcia R, Zhang H, Hayden MR, Vilaro S, Gliemann J. Segments in the C-terminal folding domain of lipoprotein lipase important for binding to the low density lipoprotein receptor-related protein and to heparan sulfate proteoglycans. *The Journal of biological chemistry*. 1997; 272:5821–5827. [PubMed: 9038197]

9. Winkler FK, D'Arcy A, Hunziker W. Structure of human pancreatic lipase. *Nature*. 1990; 343:771–774. [PubMed: 2106079]
10. van Tilbeurgh H, Roussel A, Lalouel JM, Cambillau C. Lipoprotein lipase. Molecular model based on the pancreatic lipase x-ray structure: consequences for heparin binding and catalysis. *J Biol Chem*. 1994; 269:4626–4633. [PubMed: 8308035]
11. Kobayashi Y, Nakajima T, Inoue I. Molecular modeling of the dimeric structure of human lipoprotein lipase and functional studies of the carboxyl-terminal domain. *Eur J Biochem*. 2002; 269:4701–4710. [PubMed: 12230584]
12. Bourne Y, Martinez C, Kerfelec B, Lombardo D, Chapus C, Cambillau C. Horse pancreatic lipase. The crystal structure refined at 2.3 Å resolution. *J Mol Biol*. 1994; 238:709–732. [PubMed: 8182745]
13. van Tilbeurgh H, Sarda L, Verger R, Cambillau C. Structure of the pancreatic lipase-procolipase complex. *Nature*. 1992; 359:159–162. [PubMed: 1522902]
14. Leaver-Fay A, Tyka M, Lewis SM, Lange OF, Thompson J, Jacak R, Kaufman K, Renfrew PD, Smith CA, Sheffler W, Davis IW, Cooper S, Treuille A, Mandell DJ, Richter F, Ban YE, Fleishman SJ, Corn JE, Kim DE, Lyskov S, Berrondo M, Mentzer S, Popovic Z, Havranek JJ, Karanicolas J, Das R, Meiler J, Kortemme T, Gray JJ, Kuhlman B, Baker D, Bradley P. ROSETTA3: an object-oriented software suite for the simulation and design of macromolecules. *Methods Enzymol*. 2011; 487:545–574. [PubMed: 21187238]
15. Moulton J, Fidelis K, Krysztofowicz A, Schwede T, Tramontano A. Critical assessment of methods of protein structure prediction (CASP)–round x. *Proteins*. 2014; 82(Suppl 2):1–6.
16. Davis RC, Wong H, Nikazy J, Wang K, Han Q, Schotz MC. Chimeras of hepatic lipase and lipoprotein lipase. Domain localization of enzyme-specific properties. *J Biol Chem*. 1992; 267:21499–21504. [PubMed: 1400461]
17. Wong H, Yang D, Hill JS, Davis RC, Nikazy J, Schotz MC. A molecular biology-based approach to resolve the subunit orientation of lipoprotein lipase. *Proc Natl Acad Sci U S A*. 1997; 94:5594–5598. [PubMed: 9159117]
18. Osborne JC Jr, Bengtsson-Olivecrona G, Lee NS, Olivecrona T. Studies on inactivation of lipoprotein lipase: role of the dimer to monomer dissociation. *Biochemistry*. 1985; 24:5606–5611. [PubMed: 4074716]
19. Peterson J, Ayyobi AF, Ma Y, Henderson H, Reina M, Deeb SS, Santamarina-Fojo S, Hayden MR, Brunzell JD. Structural and functional consequences of missense mutations in exon 5 of the lipoprotein lipase gene. *J Lipid Res*. 2002; 43:398–406. [PubMed: 11893776]
20. Doolittle MH, Ehrhardt N, Peterfy M. Lipase maturation factor 1: structure and role in lipase folding and assembly. *Curr Opin Lipidol*. 2010; 21:198–203. [PubMed: 20224398]
21. Doolittle MH, Neher SB, Ben-Zeev O, Ling-Liao J, Gallagher CM, Hosseini M, Yin F, Wong H, Walter P, Peterfy M. Lipase maturation factor LMF1, membrane topology and interaction with lipase proteins in the endoplasmic reticulum. *J Biol Chem*. 2009; 284:33623–33633. [PubMed: 19783858]
22. Boren J, Lookene A, Makoveichuk E, Xiang S, Gustafsson M, Liu H, Talmud P, Olivecrona G. Binding of low density lipoproteins to lipoprotein lipase is dependent on lipids but not on apolipoprotein B. *J Biol Chem*. 2001; 276:26916–26922. [PubMed: 11331277]
23. Reimund M, Larsson M, Kovrov O, Kasvandik S, Olivecrona G, Lookene A. Evidence for Two Distinct Binding Sites for Lipoprotein Lipase on Glycosylphosphatidylinositol-anchored High Density Lipoprotein-binding Protein 1 (GPIHBP1). *J Biol Chem*. 2015; 290:13919–13934. [PubMed: 25873395]
24. Lafferty MJ, Bradford KC, Erie DA, Neher SB. Angiopoietin-like protein 4 inhibition of lipoprotein lipase: evidence for reversible complex formation. *J Biol Chem*. 2013; 288:28524–28534. [PubMed: 23960078]
25. DeRocco V, Anderson T, Piehler J, Erie DA, Weninger K. Four-color single-molecule fluorescence with noncovalent dye labeling to monitor dynamic multimolecular complexes. *Biotechniques*. 2010; 49:807–816. [PubMed: 21091445]
26. Sass LE, Lanyi C, Weninger K, Erie DA. Single-molecule FRET TACKLE reveals highly dynamic mismatched DNA-MutS complexes. *Biochemistry*. 2010; 49:3174–3190. [PubMed: 20180598]

27. Ha T, Kozlov AG, Lohman TM. Single-molecule views of protein movement on single-stranded DNA. *Annu Rev Biophys.* 2012; 41:295–319. [PubMed: 22404684]
28. Erie DA, Weninger KR. Single molecule studies of DNA mismatch repair. *DNA Repair (Amst).* 2014; 20:71–81. [PubMed: 24746644]
29. Liao JY, Song Y, Liu Y. A new trend to determine biochemical parameters by quantitative FRET assays. *Acta Pharmacol Sin.* 2015; 36:1408–1415. [PubMed: 26567729]
30. Piraino MS, Kelliher MT, Aburas J, Southern CA. Single molecule Forster resonance energy transfer studies of the effect of EndoS deglycosylation on the structure of IgG. *Immunol Lett.* 2015; 167:29–33. [PubMed: 26112419]
31. Sadler EE, Kapanidis AN, Tucker SJ. Solution-Based Single-Molecule FRET Studies of K(+) Channel Gating in a Lipid Bilayer. *Biophys J.* 2016; 110:2663–2670. [PubMed: 27332124]
32. Choi UB, Strop P, Vrljic M, Chu S, Brunger AT, Weninger KR. Single-molecule FRET-derived model of the synaptotagmin 1-SNARE fusion complex. *Nat Struct Mol Biol.* 2010; 17:318–324. [PubMed: 20173763]
33. Brunger AT, Strop P, Vrljic M, Chu S, Weninger KR. Three-dimensional molecular modeling with single molecule FRET. *J Struct Biol.* 2011; 173:497–505. [PubMed: 20837146]
34. Ross J, Buschkamp P, Fetting D, Donnermeyer A, Roth CM, Tinnefeld P. Multicolor Single-Molecule Spectroscopy with Alternating Laser Excitation for the Investigation of Interactions and Dynamics. *The Journal of Physical Chemistry B.* 2007; 111:321–326. [PubMed: 17214479]
35. Hayne CK, Lafferty MJ, Eglinger BJ, Kane JP, Neher SB. Biochemical Analysis of the Lipoprotein Lipase Truncation Variant, LPLS447X, Reveals Increased Lipoprotein Uptake. *Biochemistry.* 2017; 56:525–533. [PubMed: 27984852]
36. Weninger K, Bowen ME, Chu S, Brunger AT. Single-molecule studies of SNARE complex assembly reveal parallel and antiparallel configurations. *Proc Natl Acad Sci U S A.* 2003; 100:14800–14805. [PubMed: 14657376]
37. Bowen ME, Weninger K, Ernst J, Chu S, Brunger AT. Single-molecule studies of synaptotagmin and complexin binding to the SNARE complex. *Biophys J.* 2005; 89:690–702. [PubMed: 15821166]
38. Chung SH, Kennedy RA. Forward-backward non-linear filtering technique for extracting small biological signals from noise. *J Neurosci Methods.* 1991; 40:71–86. [PubMed: 1795554]
39. Haran G. Noise reduction in single-molecule fluorescence trajectories of folding proteins. *Chemical Physics.* 2004; 307:137–145.
40. Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T, Mann M. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature.* 1996; 379:466–469. [PubMed: 8559255]
41. Xu H, Freitas MA. A mass accuracy sensitive probability based scoring algorithm for database searching of tandem mass spectrometry data. *BMC Bioinformatics.* 2007; 8:133. [PubMed: 17448237]
42. Hermoso J, Pignol D, Kerfelec B, Crenon I, Chapus C, Fontecilla-Camps JC. Lipase activation by nonionic detergents. The crystal structure of the porcine lipase-colipase-tetraethylene glycol mono-octyl ether complex. *J Biol Chem.* 1996; 271:18007–18016. [PubMed: 8663362]
43. Song Y, DiMaio F, Wang RY, Kim D, Miles C, Brunette T, Thompson J, Baker D. High-resolution comparative modeling with RosettaCM. *Structure.* 2013; 21:1735–1742. [PubMed: 24035711]
44. Gray JJ, Moughon S, Wang C, Schueler-Furman O, Kuhlman B, Rohl CA, Baker D. Protein-protein docking with simultaneous optimization of rigid-body displacement and side-chain conformations. *J Mol Biol.* 2003; 331:281–299. [PubMed: 12875852]
45. Khan S, Gor J, Mulloy B, Perkins SJ. Semi-rigid solution structures of heparin by constrained X-ray scattering modelling: new insight into heparin-protein complexes. *J Mol Biol.* 2010; 395:504–521. [PubMed: 19895822]
46. Lookene A, Chevreuil O, Ostergaard P, Olivecrona G. Interaction of lipoprotein lipase with heparin fragments and with heparan sulfate: stoichiometry, stabilization, and kinetics. *Biochemistry.* 1996; 35:12155–12163. [PubMed: 8810923]

47. Sendak RA, Bensadoun A. Identification of a heparin-binding domain in the distal carboxyl-terminal region of lipoprotein lipase by site-directed mutagenesis. *J Lipid Res.* 1998; 39:1310–1315. [PubMed: 9643364]
48. Lutz EP, Merkel M, Kako Y, Melford K, Radner H, Breslow JL, Bensadoun A, Goldberg IJ. Heparin-binding defective lipoprotein lipase is unstable and causes abnormalities in lipid delivery to tissues. *J Clin Invest.* 2001; 107:1183–1192. [PubMed: 11342582]
49. Alford RF, Leaver-Fay A, Jeliazkov JR, O'Meara MJ, DiMaio FP, Park H, Shapovalov MV, Renfrew PD, Mulligan VK, Kappel K, Labonte JW, Pacella MS, Bonneau R, Bradley P, Dunbrack RL Jr, Das R, Baker D, Kuhlman B, Kortemme T, Gray JJ. The Rosetta All-Atom Energy Function for Macromolecular Modeling and Design. *J Chem Theory Comput.* 2017; 13:3031–3048. [PubMed: 28430426]
50. Park H, Bradley P, Greisen P Jr, Liu Y, Mulligan VK, Kim DE, Baker D, DiMaio F. Simultaneous Optimization of Biomolecular Energy Functions on Features from Small Molecules and Macromolecules. *J Chem Theory Comput.* 2016; 12:6201–6212. [PubMed: 27766851]
51. Voss CV, Davies BS, Tat S, Gin P, Fong LG, Pelletier C, Mottler CD, Bensadoun A, Beigneux AP, Young SG. Mutations in lipoprotein lipase that block binding to the endothelial cell transporter GPIHBP1. *Proceedings of the National Academy of Sciences of the United States of America.* 2011; 108:7980–7984. [PubMed: 21518912]
52. Goulbourne CN, Gin P, Tatar A, Nobumori C, Hoenger A, Jiang H, Grovenor CR, Adeyo O, Esko JD, Goldberg IJ, Reue K, Tontonoz P, Bensadoun A, Beigneux AP, Young SG, Fong LG. The GPIHBP1-LPL complex is responsible for the margination of triglyceride-rich lipoproteins in capillaries. *Cell Metab.* 2014; 19:849–860. [PubMed: 24726386]
53. Wilson DE, Hata A, Kwong LK, Lingam A, Shuhua J, Ridinger DN, Yeager C, Kaltenborn KC, Iverius PH, Lalouel JM. Mutations in exon 3 of the lipoprotein lipase gene segregating in a family with hypertriglyceridemia, pancreatitis, and non-insulin-dependent diabetes. *J Clin Invest.* 1993; 92:203–211. [PubMed: 8325986]
54. Emi M, Wilson DE, Iverius PH, Wu L, Hata A, Hegele R, Williams RR, Lalouel JM. Missense mutation (Gly---Glu188) of human lipoprotein lipase imparting functional deficiency. *J Biol Chem.* 1990; 265:5910–5916. [PubMed: 1969408]
55. Bengtsson-Olivecrona G, Olivecrona T. Binding of active and inactive forms of lipoprotein lipase to heparin. Effects of pH. *Biochem J.* 1985; 226:409–413. [PubMed: 3994667]
56. Lookene A, Zhang L, Hultin M, Olivecrona G. Rapid subunit exchange in dimeric lipoprotein lipase and properties of the inactive monomer. *J Biol Chem.* 2004; 279:49964–49972. [PubMed: 15385564]
57. Joo C, Balci H, Ishitsuka Y, Buranachai C, Ha T. Advances in single-molecule fluorescence methods for molecular biology. *Annu Rev Biochem.* 2008; 77:51–76. [PubMed: 18412538]
58. Joo C, Ha T. Single-molecule FRET with total internal reflection microscopy. *Cold Spring Harb Protoc.* 2012; 2012:1223–1237.
59. The Molecular Probes Handbook.
60. Floyd DL, Harrison SC, van Oijen AM. Analysis of kinetic intermediates in single-particle dwell-time distributions. *Biophys J.* 2010; 99:360–366. [PubMed: 20643053]
61. Yang CY, Gu ZW, Yang HX, Rohde MF, Gotto AM Jr, Pownall HJ. Structure of bovine milk lipoprotein lipase. *J Biol Chem.* 1989; 264:16822–16827. [PubMed: 2674142]
62. Xu H, Hsu PH, Zhang L, Tsai MD, Freitas MA. Database search algorithm for identification of intact cross-links in proteins and peptides using tandem mass spectrometry. *J Proteome Res.* 2010; 9:3384–3393. [PubMed: 20469931]
63. Carugo O, Argos P. Protein-protein crystal-packing contacts. *Protein Sci.* 1997; 6:2261–2263. [PubMed: 9336849]
64. Gilbert B, Rouis M, Griglio S, de Lumley L, Laplaud P. Lipoprotein lipase (LPL) deficiency: a new patient homozygote for the preponderant mutation Gly188Glu in the human LPL gene and review of reported mutations: 75 % are clustered in exons 5 and 6. *Ann Genet.* 2001; 44:25–32. [PubMed: 11334614]

65. Lossow WJ, Lindgren FT, Murchio JC, Stevens GR, Jensen LC. Particle size and protein content of six fractions of the Sf 20 plasma lipoproteins isolated by density gradient centrifugation. *J Lipid Res.* 1969; 10:68–76. [PubMed: 5764118]
66. Scow RO, Olivecrona T. Effect of albumin on products formed from chylomicron triacylglycerol by lipoprotein lipase in vitro. *Biochim Biophys Acta.* 1977; 487:472–486. [PubMed: 884124]
67. Lutz EP, Kako Y, Yagyu H, Heeren J, Marks S, Wright T, Melford K, Ben-Zeev O, Radner H, Merkel M, Bensadoun A, Wong H, Goldberg IJ. Mice expressing only covalent dimeric heparin binding-deficient lipoprotein lipase: muscles inefficiently secrete dimeric enzyme. *J Biol Chem.* 2004; 279:238–244. [PubMed: 14570890]
68. Hata A, Ridinger DN, Sutherland S, Emi M, Shuhua Z, Myers RL, Ren K, Cheng T, Inoue I, Wilson DE, et al. Binding of lipoprotein lipase to heparin. Identification of five critical residues in two distinct segments of the amino-terminal domain. *J Biol Chem.* 1993; 268:8447–8457. [PubMed: 8473288]
69. Lookene A, Groot NB, Kastelein JJ, Olivecrona G, Bruin T. Mutation of tryptophan residues in lipoprotein lipase. Effects on stability, immunoreactivity, and catalytic properties. *J Biol Chem.* 1997; 272:766–772. [PubMed: 8995362]

Figure 1. Original Model of the LPL dimer

Top, side, and bottom views of an LPL dimer modeled on the crystallographic dimer from the PL structure (PDB ID 1HPL). This model allows for the lid (blue) to contact substrate and GPIHBP1 (black) to anchor LPL to the capillary endothelium, placing LPL in between these surfaces. However, much of the published binding site for APOCII (red) appears occluded by the dimer interface. Furthermore, two mutations (R75A and G188E, purple) that are secreted but do not dimerize do not appear to make dimerization contacts. The bottom view shows residues known to play a role in heparin binding (yellow), and the distances between some of these residues are listed.

Figure 2. Attachment of Active LPL to a Slide Using Two Methods

A) Quartz slides were functionalized for LPL attachment with BSA-biotin and streptavidin. LPL was added either after being conjugated to biotin (left) or after the addition of biotinylated heparin⁶⁷ to the slide. LPL avidly binds to heparin. **B)** LPL remains active on a functionalized surface. LPL was bound to a BSA-biotin-streptavidin coated 96-well plate. The activity of the LPL remaining in the wells after washing was read after the addition of a substrate mixture containing DGGR. Both biotinylated LPL and LPL bound to the surface functionalized with heparin-biotin were active.

Figure 3. Strategies for Fluorescently Labeling LPL

A) All serine residues on LPL are colored cyan, and the three residues used for FRET measurements in this work (36, 224, and 384) are colored red. **B)** LPL serine to cysteine mutants with a V5 tag were transiently expressed in HEK293 cells and the media (secreted fraction) was tested for activity (top panel). Error bars represent the standard deviation for triplicate measurements. A Western blot shows protein expression in the media and lysate fractions. GAPDH was a loading control for the lysate. **C)** Wildtype LPL and LPL^{S97C} were labeled with Alexa Fluor®647, resolved by SDS-PAGE and fluorescence was imaged. A Western blot against LPL (second panel, α -4-1a) shows total protein. **D)** Purified LPL^{S384C} retains activity when labeled with Alexa Fluor®647 maleimide dye (dashed versus solid line).

Figure 4. LPL-LPL Intermolecular FRET for distance measurements

A) The original dimer model is used to show the positions of fluorophore on LPL. **B)** Example fluorescence intensity time traces for dual-labeled LPL dimers bound to the surface. Traces show both protein dynamics and donor and acceptor bleaching events. **C)** FRET values corresponding to the time traces in B. **D)** Histograms showing the distribution of FRET values.

Figure 5. Disulfide bonding pattern of LPL

A and B. The two potential arrangements of disulfide bonds in LPL are sequential and nonsequential, respectively. Numbering is per bovine LPL (which is offset by 2 residues as compared to human LPL used in models) starting after the signal peptide is cleaved off. **C. Top:** An LPL tripeptide that could only result from a sequential arrangement of disulfide bonds. Possible positions of fragmentation of each peptide chain (A is the bottom chain, B the middle, and C, the top) are marked. Positions of disulfide bonds are shown by gray bars. **Bottom:** Example MS/MS spectra from this tripeptide. The position of the parent peptide, and a fragment of chain B separated between its two cysteines, are boxed. **D.** A heat map of all potential disulfide bonds from MassMatrix shows that 4 out of 5 disulfides in LPL were identified with high confidence⁶². SP indicates a cysteine in the signal peptide that was not present because it was cleaved off. The confidence of identification of each disulfide bond is indicated by the color code shown below the heat map.

Figure 6. New models of the LPL dimer

Top, side, and bottom views of the three new LPL dimer models. Lids and tryptophan clusters are in blue, the GPIHBP1 binding site is in black, the APOCII binding site is in red and two mutations (R75A and G188E) that are secreted but do not dimerize are in purple. The bottom view shows residues 403, 405, 407, and K414, which are involved in heparin binding in yellow, and the distances between some of these residues are listed.

Table 1

Features of the models

Feature	Description	Reference	Original Model	FRET Only	FRET plus
ApoCII Binding	Residues 69–83	6	Not on surface	Not on surface	On surface
Heparin Binding Residues	R279, K280, R282, K296, R297, K403, R405, K407, K413, K414	47, 48, 68			
Heparin binding distance	One heparin octamer/dodecamer can bind one LPL dimer	46			
Size of a heparin octamer/dodecamer	33Å/41 Å	45			
Shortest Heparin Distance, Å		Current work	53.2	56.5	29.5
Tryptophan cluster	W390, W393, W394	11, 69	On surface	On surface	On surface
GP1HBP1 binding site	Residues 419–433	51	On surface	On surface	On surface
Head to tail arrangement	LPL monomers connected C to N by a linker are active. Mixed monomers (catalytic site mutant and tryptophan cluster mutant) are active.	11, 17	Satisfied	Satisfied	Satisfied
Dimer mutations	G188E and R755S are secreted, but runs as a monomers on heparin chromatography.	19, 53	Neither at interface	Neither at interface	G188E, but not R755S, at interface
Native Disulfide Bonds	Residues 27⇌40, 216⇌239, 264⇌275, 278⇌283, ⇌418⇌438 ⇌ All numbering is for human LPL, +2 for bovine.	Current work, ⁶¹	All models use this disulfide bonding pattern.	All models use this disulfide bonding pattern.	All models use this disulfide bonding pattern.
Total Energy	Rosetta total energy	Current work	–2496.04	–2646.15	–2635.13
Binding Energy	The difference between the energy of the complex and the energy of the unbound.	Current work	–65.465	–67.845	–43.206
Mean 224–224 Ca Distance, and range at half maximum amplitude, Å	47.1, 42–52	Current work	36.4	46.4	49.6
Mean 384–384 Ca Distance, and range at half maximum amplitude, Å	53.6, 48–61	Current work,	56.3	49.8	54.0
Mean 36–384 Ca Distance, and range at half maximum amplitude, Å	51, 43–59	Current work	25.0	34.7	49.5
RMSD Model vs. FRET Distances, Å		Current work	15.9	9.7	1.7