Lipoprotein lipase (LPL), a key enzyme in the metabolism of triglyceride-rich plasma lipoproteins, is a homodimer. Dissociation to monomers leads to loss of activity. Evidence that LPL dimers rapidly exchange subunits was demonstrated by fluorescence resonance energy transfer between lipase subunits labeled with Oregon Green and tetramethylrhodamine, respectively, and also by formation of heterodimers composed of radiolabeled and biotinylated lipase subunits captured on streptavidine-agarose. Compartmental modeling of the inactivation kinetics confirmed that rapid subunit exchange must occur. Studies of activity loss indicated the existence of a monomer that can form catalytically active dimers, but this intermediate state has not been possible to isolate and remains hypothetical. Differences in solution properties and conformation between the stable but catalytically inactive monomeric form of LPL and the active dimers were studied by static light scattering, intrinsic fluorescence, and probing with 4,4’-dianilino-1,1’-binaphthyl-5,5’-disulfonic acid and acrylamide. The catalytically inactive monomer appeared to have a more flexible and exposed structure than the dimers and to be more prone to aggregation. By limited proteolysis the conformational changes accompanying dissociation of the dimers to inactive monomers were localized mainly to the central part of the subunit, probably corresponding to the region for subunit interaction.

Lipoprotein lipase (LPL)\(^1\) plays a fundamental role in the metabolism of blood lipoproteins (1–3). Lowered LPL activity results in dyslipidemia (4). The catalytically active form of LPL is a noncovalent homodimer of 55 kDa subunits (5, 6). The functional site of LPL is at the luminal surface of the vascular endothelium, where the enzyme is bound to heparan sulfate proteoglycans (1–4). Inactive monomers are less tightly bound to heparan sulfate (7) and are therefore the dominant form of LPL in the circulating blood (8).

In vitro studies show that the dimeric state of LPL is unstable (9). Under physiological conditions (pH, temperature, and concentration of salt), the dimers spontaneously dissociate into monomers within a few minutes. The formed monomers may reassociate but into inactive aggregates (9). It is believed that this process is irreversible. The inactivation is strongly prevented by heparan sulfate and heparin (7). The dimeric form of LPL is also relatively stable in the presence of high concentrations of salt (at low temperature) (9, 10). In the early 1970s this discovery allowed purification of the active enzyme from bovine milk by using adsorption to heparin-Sepharose followed by elution with a salt gradient (5, 11). Osborne et al. (6) showed that the inactivation rate is dependent on the concentration of LPL. On this basis, they proposed that inactivation of LPL includes a step of reversible dissociation of the dimer followed by a step of irreversible unfolding of the dissociated monomers (6).

LPL, pancreatic lipase, hepatic lipase, and endothelial lipase share sequence homologies and together form the mammalian triacylglycerol lipase gene family (12). A model of the three-dimensional structure of LPL has been constructed based on the x-ray structure of pancreatic lipase (10). The model is supported by results obtained by other techniques, including limited proteolysis and mutagenesis (12–14). Thus, the overall three-dimensional structures of the lipases appears to be quite similar. In contrast to LPL, pancreatic lipase can exist as a catalytically active monomer (10). Thus, some important differences in folding or in conformational flexibility between these two proteins are expected. Because of instability and low solubility, the possibilities of using physical techniques for structural studies of LPL are limited. Little is known about the conformational differences between the active dimer and the catalytically inactive monomer. By continuous measurement of circular dichroism, under conditions in which the enzyme slowly lost its catalytic activity, a decrease in the content of secondary structure has been demonstrated (6). Conformational differences between active and inactive forms of LPL are also implicated by differences in their abilities to react with monoclonal antibodies (15).

Inactive LPL monomers can be demonstrated by sucrose density gradient centrifugation (16, 17). The affinity of the monomer to heparin is ~6000-fold weaker than that of the dimer (7), and the difference in heparin affinities can readily be used for separation of dimers and monomers by affinity chromatography on heparin columns (16–18). Functional studies have shown that, although the monomers are catalytically inactive, they retain several properties of the active dimer. Monomers are still able to interact with lipoproteins (19). In blood the monomers are mainly found together with LDL particles...
Bovine LPL was purified from milk (28). It was iodinated with 20 mM bis-Tris, pH 6.5, and immediately applied onto a gradient of NaCl in the bis-Tris buffer. Most of the active labeled LPL monomers are found, for example, in blood (22) and adipose tissue (23) and are also present in culture media from the expression of recombinant LPL in vitro (17, 24). It has been shown that the monomer/dimer ratio in adipose tissue is dependent on the nutritional state (23). In the fasted state more LPL is found in the inactive form (25), and therefore less of the plasma lipids are taken up for storage, whereas more is used in other tissues for energy production. Studies with actinomycin D indicate that some other protein with a short-lived mRNA regulates the dissociation of LPL into inactive monomers or prevents inactive LPL subunits from forming active dimers (25, 26). The details of this important control mechanism are still unknown, but the fact remains that monomeric LPL is present in most tissues and that monomeric LPL is the dominant form of LPL in blood. Recent studies indicate that the levels of this form in plasma correlate to disease (27). Many single-point mutations in the LPL gene result in the production of inactive LPL monomers (12, 18). It is therefore important to investigate the properties of the folded monomer and to understand its relation to the active dimeric form of LPL.

In the present investigation we have focused on mechanisms responsible for the spontaneous inactivation of LPL. We demonstrate that the subunits of dimeric LPL rapidly exchange partners. This may be a first step in the process that leads to inactivation of the enzyme. We have used compartmental modeling to study the inactivation mechanism, static light scattering to study aggregation, limited proteolysis to study differences in folding between inactive monomers and active dimmers, and bis-ANS to probe exposure of hydrophobic regions due to unfolding. The largest conformational changes on dissociation of the dimer were located to the middle of the LPL subunit and probably involve areas of subunit interactions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine LPL was purified from milk (28). It was iodinated by the lactoperoxidase method and separated from free NaI by chromatography on heparin-Sepharose as described (16). The amino coupling kit and CM5 sensor chips were from BIAcore (Uppsala, Sweden). Heparin was from Leo Pharma AB (Malmo¨, Sweden). Oregon Green 488 carboxylic acid, succinimidyl ester (OG), 5-carboxytetramethylrhodamine, succinimidyl ester (TMRA), and bis-ANS (4,4'-carboxylic acid, succinimidyl ester (OG), 5-carboxytetramethylrhodamine) were from Molecular Probes. LPL was characterized by endo-Glu-C was performed in 0.1M phosphate buffer, pH 7.8, with or without 1.0 M guanidinium chloride. The reactions were stopped by heating the samples at 90 °C for 15 min. The cleavage products were analyzed on Tricine-SDS-PAGE using 15.5% polyacrylamide gels.

**Labeling of LPL with OG and TMRA—**LPL (0.2–0.5 mg/ml) in 0.2 M NaHCO₃, 1 mM NaCl, pH 8.4, was incubated with OG or TMRA at 4 °C in the dark. After 1 h, the reaction was stopped by the addition of lysine to a final concentration of 1 mM. After 15 min, the mixture was diluted 5× with 20 mM bis-Tris, pH 6.5, and immediately applied onto a column of heparin-Sepharose. Labeled LPL was eluted by a linear gradient of NaCl in the bis-Tris buffer. Most of the active labeled LPL eluted with a peak at 1.1 M NaCl. The fractions with high LPL activity were pooled. The incorporation of OG and TMRA into LPL was characterized by the degree of labeling (DOL),

\[
DOL = \frac{A_{\text{max}}}{[\text{LPL}] \times \epsilon_{\text{max}}} \quad \text{(Eq. 1)}
\]

where \(A_{\text{max}}\) is absorbance of OG-LPL or TMRA-LPL at their absorbance maximum wavelength, \(\epsilon_{\text{max}}\) is extinction coefficient of OG or TMRA, and [LPL] is the concentration of the labeled LPL. The \(\epsilon_{\text{max}}\) values used were 70,000 for OG and 65,000 for TMRA. These values were from the website of Molecular Probes. The concentration of LPL was calculated from absorbance, \(A_{\text{max}}\) (29).

**Fluorescence and Light-scattering Measurements—**These experiments were performed on a Fluoro-Max spectrofluorimeter. The conditions are detailed in the legends to Figs. 2, 5, and 6.

**Binding to Streptavidin-Agarose—**Equal volumes (0.1 ml) of radiolabeled LPL (0.05 mg/ml, 4000 cpm/µl) and biotinylated LPL (0.05–0.15 mg/ml) were incubated together for 1 min in 10 mM Tris, 0.5 mM NaCl, 1 mg/ml BSA, pH 7.4 at 4 °C. Of this incubation mixture, 0.05 ml was added to 2 ml of the same buffer containing 0.3 ml of streptavidin-agarose. The mixture was gently shaken for 5 min at 4 °C and centrifuged at 3000 \(\times g\) for 5 min. The radioactivity was determined in the top phase (containing unbound LPL) and in the lower phase (containing sedimented streptavidin-agarose and the bound LPL). Data are expressed as ratios of radioactivity in the sedimented agarose to the total radioactivity added to the system. Nonselective binding of radioactive LPL to streptavidin-agarose in the absence of biotinylated LPL was subtracted from the data.

**Binding Studies on BIAcore—**Details concerning conditions for these studies are described previously (7, 30). Experiments were performed on a BIAcore 2000 instrument. Biotinylated heparin or biotinylated LPL was bound to the matrix-coupled streptavidin. LDL and VLDL were then injected into these sensorchips in 10 mM Hepes, 0.15 mM NaCl, pH 7.4, at 25 °C.

**Activity Measurements—**Activity measurements with tributyrin as substrate were performed by continuous titration of butyric acid by a pH-stat (Methrome, Herisau, Switzerland) at 25 °C (13). Stock solutions of the substrate were prepared by sonication of tributyrin in a solution of gum Arabic/water, pH 7.4, at 25 °C. The analyses were performed on a home-made mass spectrometer at the National Institute of Chemical Physics and Biophysics (Tallinn, Estonia). The enzymatic activity was recorded at pH 8.5 in 0.15 mM NaCl containing 30 mM tributyrin and 0.2% gum Arabic. Titration was performed with 25 mM KOH. LPL activity on phospholipid-stabilized emulsions of long acyl chain triacylglycerols was measured as described in a previous study (23). Briefly, 5 µl of each sample was incubated in 200 µl of a mixture containing a phospholipid-stabilized emulsion of soy bean lecithin and cholesterol with the same composition as plasma (5:4; Fresenius-KABI, Uppsala, Sweden), into which tri-[9,10-3H]Holeoylglycerol had been incorporated on manufactory. Heat-inactivated rat serum was present as a source of apolipoprotein CII, BSA as fatty acid acceptor, and heparin to stabilize the lipase. One milliliter of lipase activity corresponded to the release of 1 nmol of fatty acid/min at 25 °C. All samples were assayed in triplicate.

**Mathematical Modeling of the Inactivation Process—**Mathematical modeling of the transfer of LPL between different states was done using SAAMII (simultaneous analysis and modeling), version 1.1 (SAAM Institute, Seattle, WA). This software allows compartmental modeling of complex systems using linear as well as nonlinear models. The error of the data was set to a fractional standard deviation of 0.1.

**Proteolytic Cleavage—**Limited cleavage of LPL by chymotrypsin and trypsin was carried out in 0.1 M Tris, 0.5 mM NaCl, pH 7.4, at 10 °C. In the case of monomeric LPL the cleavage reactions also contained 1.0 mM guanidinium chloride. The protease/LPL mass ratio was 5%, and the cleavage reactions were stopped by adding phenylmethylsulfonyl fluoride to a 10-fold molar excess over trypsin or chymotrypsin. Digestion by endo-Glu-C was performed in 0.1 M phosphate buffer, pH 7.8, with or without 1.0 M guanidinium chloride. The reactions were stopped by heating the samples at 90 °C for 15 min. The cleavage products were analyzed on Tricine-SDS-PAGE using 15.5% polyacrylamide gels.

**Mass Spectrometry and Sequence Analyses—**Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOP) mass spectrometry was used to analyze the products of the proteolytic cleavage reactions. The analyses were performed on a home-made mass spectrometer at the National Institute of Chemical Physics and Biophysics (Tallinn, Estonia). The matrix dihydrobenzoic acid was dissolved in 1 ml of a 1:1 mixture of 0.1% trifluoroacetic acid and acetonitrile. A 0.5-µl aliquot of the cleavage mixture was deposited on a stainless steel probe. The mass determination was accurate enough only for peptides smaller than 10 kDa. For larger peptides, N-terminal sequences were determined on an Applied Biosystems (Foster City, CA) 477A pulsed liquid-phase sequenator.

**RESULTS**

**Monomer Exchange Kinetics Studied by Fluorescence Resonance Energy Transfer—**To investigate whether the subunits of dimeric LPL are stably bound to each other or whether they...
were carried out under denaturating conditions (in 1 or 6M
and TMRA-LPL, respectively. Fig. 2
TMRA groups when the labeled variants of LPL were mixed,
not shown). These observations demonstrated that fluores-
cence increased between 540 and 570 nm on excitation at 480 nm (data
concentration-dependent with a tendency to saturation at higher
concentrations of TMRA-LPL. The effect was con-
sequential of the dye to LPL led to an increased degree of labeling at low
 maximal degrees of labeling were similar, 2.1 mol/mol for the OG-
and 2.2 mol/mol for the TMRA-LPL. In the following
experiments the labeling was between 1.9 and 2.1 mol/mol LPL
dimer.

When mixed together (Fig. 2A), TMRA-LPL significantly
quenched the fluorescence emission of OG-LPL, as measured
between 510 and 530 nm on excitation at 480 nm. A rapid
decline (half-life less than 5 s) in fluorescence of the OG-LPL
variant was observed after the addition of TMRA-LPL, but
at higher ratios (>4 mol/mol) the incorporation of label approached saturation (Fig. 1). The max-
imal degrees of labeling were similar, 2.1 mol/mol for the OG-
LPL and 2.2 mol/mol for the TMRA-LPL. In the following
fig. 2D demonstrates that flu-
cescence energy transfer did not occur when the experiments
were carried out under denaturing conditions (in 1 or 6 M
guanidinium chloride). The fluorescence resonance energy
transfer between the labeled proteins was not affected by the
presence of heparin (15 IU/ml) or by lipoproteins (LDL, 0.1 µg
protein/ml) in the incubation mixture (data not shown).
Subunit exchange between LPL dimers was also demon-
strated by binding experiments to streptavidin-agarose. In this
case 125I-labeled LPL was incubated with biotinylated LPL,
and then streptavidin-agarose beds were added. After centrifu-
gation, the radioactivity bound to the gel was determined.
Despite the high nonspecific binding of 125I-labeled LPL to the
matrix (20–30% of the total), there was a significant increase of
radioactivity after mixing of the radiolabeled enzyme with bi-
otinylated LPL (Table I). Because biotinylation or iodination has previously been shown not to affect the activity or the
structure of LPL (16), the increased binding of radi-
oactivity to the gel suggested formation of heterodimers of sub-
units from 125I-labeled LPL and biotinylated LPL, respectively.

Studies of Inactivation of LPL and Modeling of the Inactiva-
tion Process—Osborne et al. (6) reported that the relative inac-
tivation rate is faster at lower concentrations of LPL and pro-
posed that the inactivation is a two-step process including a
reversible dissociation of LPL dimers followed by an irreversi-
ble unfolding of the monomers. In the present study we revis-
itied the inactivation of LPL with the aim of analyzing the
inactivation process by compartmental modeling and also of
obtaining estimates for rate constants. The inactivation kinet-
ics was measured for a wide variation of LPL concentrations
under different conditions (Fig. 3). The effect of the concentra-
tion of the LPL protein was observed only under conditions in
which the enzyme was relatively stable, as in 0.3 M NaCl at
20 °C (Fig. 3A) or in 0.15 M NaCl with 15 IU of heparin/ml at
37 °C (Fig. 3B). In 0.15 M NaCl without heparin, where the
inactivation rate was fast, the effect of LPL concentration was
less pronounced (Fig. 3C). In this case, inactivation followed
simple monoexponential decay kinetics.

It was possible to fit each individual inactivation curve,
measured in 0.3 M NaCl, 20 mM Tris, 1 mg/ml BSA at 20 °C
(Fig. 3A), to the model proposed by Osborne et al. (6). However,
the calculated kinetic constants were systematically dependent
on the concentration of LPL. This indicated that additional
states of LPL were needed in the model to account for the data.
Evaluation of different kinetic models by global fitting led to
the model presented in Fig. 4, where an additional state (state
4) corresponding to reversible formation of inactive dimers/
 oligomers from the dimerization-competent monomers was
added. This model failed, however, to fit the inactivation curves
measured in the presence of heparin (data in Fig. 3B). A likely
explanation is that binding of different forms of LPL to heparin
increases the number of unknown parameters too much to
allow a valid mathematical solution.

Properties of the Dissociated LPL Monomer with Regard to
Activity and Aggregation—We observed that the time courses
for the inactivation of LPL were not the same when the remaining
LPL activity was assayed with different substrates. When
the inactivation was studied under conditions where LPL is
relatively stable, in solutions that contained 0.3–0.4 M NaCl at
20 °C as in Fig. 3A), the activity measured with tributyrin as
substrate was unchanged during the first 30 min (Fig. 5A).
After that a rapid decline was detected. In contrast, the activity
on the substrate with long acyl chain triacylglycerols (In-
tralipid) started to decrease already from the first minutes.
Fluorescence measurements performed on the enzyme during
inactivation under the same conditions showed a small but
reproducible decrease of tryptophan emission at 340 nm upon
excitation at 280 nm (Fig. 5B). The decrease occurred already
within the first 30 min, indicating conformational changes of
the protein that appeared to be parallel to the rapid decline of
activity measured against Intralipid. The model presented in

![Graph](image-url)
Fig. 4 can describe the inactivation data if we assume that dimerization-competent monomers and the higher oligomers are active, or become active, in the assay system with tributyrin but remain inactive when Intralipid is used as substrate. The explanation for this time-dependent difference was not investigated further.

A prominent property of the LPL monomer is that it tends to aggregate and precipitate (6). We have investigated the aggregation behavior under conditions used in the present study by simple static light scattering (Fig. 6). When LPL was incubated at physiological concentration of NaCl (0.15 M), aggregation was observed even during the first 5 min and at very low concentrations of LPL (5 nM). This was a direct demonstration of the fact that monomeric LPL has a strong propensity to aggregate (Fig. 6). The aggregation was much influenced by the concentration of NaCl. Increasing the salt concentration to 0.23 M NaCl led to a delay of about 20 min before prominent aggregation was seen. At 0.3 M NaCl or higher concentrations, essentially no aggregation was detected (an experiment at 0.4 M NaCl is shown in Fig. 6). Albumin and heparin were both able to prevent aggregation of LPL, even at 0.15 M NaCl, but probably by different mechanisms. Albumin directly inhibited formation of aggregates without any influence on the kinetics for the decline of activity (Ref. 6 and data not shown). These results indicate that under the conditions used for the studies of the inactivation process in Fig. 3A (0.3 M NaCl, 1 mg BSA/ml), resulting in the model presented in Fig. 4, aggregation was not a prominent event. This might be true also for the inactivation at 0.15 M NaCl in the presence of 1 mg BSA/ml, but light scattering was studied only for a low concentration of LPL, and we did not do any fluorescence energy transfer experiments.

**TABLE I**

| Added biotin-LPL (mg/ml) | Bound radioactivity (% of total) |
|-------------------------|---------------------------------|
| 0.06                    | 23 ± 5                          |
| 0.11                    | 33 ± 7                          |
| 0.15                    | 44 ± 6                          |

Each value is a mean of triplicate measurements. The nonspecific binding of 125I-labeled LPL (40,000 cpm/μl, 0.11 mg/ml), recorded in the absence of biotinylated LPL, was subtracted.

**Fig. 2. Quenching of OG-LPL fluorescence by TMRA-LPL.**

A, proposed model for subunit exchange. B, kinetics of quenching of OG-LPL fluorescence by TMRA-LPL. Broken line, stability of OG fluorescence under light (excitation 480 nm, emission 520 nm). The concentration of OG-LPL was 5 nM. Upper solid line, 5 nM TMRA-LPL was added to the OG-LPL solution. Lower solid line, 20 nM TMRA-LPL was added to the OG-LPL solution. C, fluorescence emission spectra of mixtures that contained 5 nM OG-LPL and different concentrations of TMRA-LPL. The excitation wavelength was 480 nm. The concentrations of TMRA-LPL were: 1–0 nM, 2–2 nM, 3–4 nM, 4–20 nM, 5–40 nM, and 6–53 nM. D, titration of OG-LPL by TMRA. $F_0$ is the fluorescence of OG-LPL at 520 nm on excitation at 480 nm in the absence of TMRA-LPL; $F$ is fluorescence at the same emission and excitation wavelengths but in the presence of various concentrations of TMRA-LPL. The experiments were performed in a solution containing 20 mM Tris, 0.3 M NaCl, and 1.0 mg/ml albumin at pH 8.0 and 10 °C (●) or, in addition, 1 M (○) or 6 M guanidinium chloride (▼).
with OG-LPL and TMRA-LPL to study exchange kinetics under this condition.

**Limited Proteolysis of Monomeric LPL Compared with Dimeric LPL**—To obtain information about the location of possible conformational differences between the active dimer and the dissociated monomeric subunits, we used limited proteolysis. Susceptibility to treatment with trypsin, chymotrypsin, and endo-Glu-C was compared. For these studies we used LPL dissociated to monomers in 1 M guanidinium chloride, which was also present during treatment with the proteinases to prevent aggregation of monomers. We obtained similar results using monomeric LPL prepared by thermal dissociation of the dimer (data not shown). To localize the cleavage sites, SDS-PAGE in combination with sequence analyses was used. Additional information about the cleavage products was obtained by MALDI-TOF mass spectrometry. We focused on identification of the primary cleavage sites and also on identification of those parts of the monomer that remained intact after prolonged treatment with the proteinases, indicating that they were still tightly folded.

Previous studies had shown that chymotrypsin cleaves dimeric bovine LPL in the middle of the C-terminal domain, after Trp-392 (13). Prolonged incubation leads to cleavage also after Phe-313, located in the proposed link region between the two folding domains of the enzyme (21). In the present study we have shown that cleavage by chymotrypsin of the inactive LPL monomer occurs at several additional sites. An analysis of the cleavage products by SDS-PAGE is presented in Fig. 7A. Three main bands corresponding to ~23, 14, and 6.5 kDa were detected after Coomassie staining. Sequence analyses showed that the 23-kDa band corresponded to the part of LPL that starts with Ala-17 as the N-terminal residue. Thus, this peptide (CP1) covers the N-terminal part of the N-terminal folding domain. Two sequences of comparative length, starting with Thr-188 (designated CP3) and Leu-305 (designated CP2), were detected in the 14-kDa band. According to calculated molecular weights, CP2 probably spans the whole C-terminal folding domain and also includes residues 306–312, which form the link region between the N-terminal and the C-terminal folding domains (Fig. 7B). The fragment CP3 must correspond to the central part of the LPL molecule. The N-terminal sequence of the smallest peptide (CP4) started at Ser-393. The molecular mass of this peptide, determined by MALDI-TOF mass spectrometry, was 6714 Da. This value is in a good accordance with the calculated mass for the peptide containing residues 393–448.

In contrast to its being susceptible to trypsin and chymotrypsin (Fig. 7B), dimeric LPL was found to be totally resistant to treatment with endo-Glu-C, even after treatment for 24 h at a mass ratio of LPL/endo-Glu-C = 0.05. Treatment of monomeric LPL with endo-Glu-C at the same mass ratio led to the formation of three peptides (EP1, EP2 and EP3) within the first 30 min of treatment (Fig. 7A, right panel). The N-terminal sequences of peptides EP2 and EP3 both started from Tyr-166, whereas the N-terminal sequence of the peptide EP1 corresponded to the N-terminal sequence of intact LPL. The molecular mass determined by MALDI-TOF was 20,150 Da for EP1, 34,540 for EP2, and 25,710 Da for EP3. The total molecular...
mass for LPL was determined to 54,750 Da, which is close to the sum of the mass of EP1 and EP2. Prolonged incubation (up to 24 h) with endo-Glu-C led to complete cleavage of the peptides EP2 and EP3, whereas the amount of EP1 was only slightly decreased (data not shown). This indicated that the N-terminal part of the N-terminal domain forms a relatively tightly folded unit in the dissociated monomeric form of LPL.

In contrast to the results with chymotrypsin and endo-Glu-C, treatment of monomeric LPL with trypsin resulted in the rapid appearance of small peptides of less than 15 kDa (seen by SDS-PAGE, data not shown). This pattern was very different from the results with the LPL dimer, which was cleaved only at Arg-230 (31). Peptide analysis by mass spectrometry revealed a rapid cleavage by trypsin of the LPL monomer at residues Arg-2, Arg-10, and Arg-172.

Probing the Structure of Monomeric LPL with bis-ANS and Acrylamide—To investigate the possibility that hydrophobic areas are more exposed in the LPL monomer than in the dimer, we compared the binding of the fluorescent hydrophobic probe bis-ANS to both forms of LPL. In our previous studies we showed that bis-ANS is a potent inhibitor of LPL and that it interacts with the central part of the LPL dimer (32). Here we focused on binding of the dye to LPL in the presence of different concentrations of guanidinium chloride, with the aim of detecting possible conformational changes on dissociation. A marked increase in bis-ANS fluorescence was detected when the concentration of guanidinium chloride was increased to 1 M. At higher concentrations, the fluorescence started to gradually decrease, approaching zero at 2 M guanidinium chloride. Thus, the maximal fluorescence of bis-ANS was registered in the presence of 0.7–1 M guanidinium chloride (Fig. 8A). These are the lowest concentrations by which dimeric LPL can be completely dissociated into inactive monomers under the conditions used (6).

Conformational differences between monomeric and dimeric LPL were also probed by addition of acrylamide. When tryptophan residues are exposed on the surface of a protein, their fluorescence is quenchable by acrylamide (33). With LPL, acrylamide affected the fluorescence of dimers and monomers equally as much (Fig. 8B), indicating little or no change in the exposure of tryptophans after dissociation of the subunits.

DISCUSSION

The results of the present study suggest that active LPL is a dynamic dimer in which the subunits rapidly exchange partners. This was shown by fluorescence energy transfer between differently labeled dimers, by streptavidin-agarose chromatography of a mix of biotinylated and 125I-labeled LPL, and by compartmental modeling of the inactivation kinetics. Osborne et al. (6) proposed a rapid equilibrium between dimers and monomers as a first step in the inactivation of LPL. Here we demonstrate that there is a rapid equilibrium between dimers and monomers even under conditions where LPL is relatively stable, for example at low temperature in the presence of high concentrations of salt (NaCl) or in the presence of heparin at physiological salt concentration (0.15 M NaCl). The rate of subunit exchange was too rapid to be determined by the techniques used in the present study, but the exchange was esti-
imated to occur in less than 5 s. Because the presence of heparin or lipoproteins did not markedly slow down the exchange rate, it is reasonable to assume that rapid dissociation/association of the LPL subunits occurs even under physiological conditions, at the vascular endothelium. Another implication is that the off-rates for binding of LPL to heparin and to lipoproteins must be even faster than the rates for subunit exchange. In studies of the interaction of LPL with heparin, using the biosensor technique, rate constants of 0.05–0.1 s⁻¹ were obtained previously (7). Thus, our data suggest that the effect of heparin on LPL stability is not due to keeping the subunits together in the dimer but rather to stabilizing the dissociated monomer. Stabilization was previously demonstrated also with short heparin fragments like octa- and decasaccharides (7).

Recently Lutz et al. (34) demonstrated that recombinant dimers, which have monomer subunits linked together in a head-to-tail fashion by a short peptide, were secreted from cells to a much lower degree than the normal noncovalent dimers. Thus, rapid dissociation/association might be important for the intracellular transport or processing of the LPL protein. In addition, the dissociation/association equilibrium may be important for the regulation of the activity of LPL at the vascular endothelium. The rapid dissociation of the dimers may expose LPL to factors that impair the reassociation of the monomers.
Thus, the unstable, noncovalent dimeric structure of LPL may be designed to allow termination of its activity on given signals and release of the inactive LPL protein to the blood.

Compartmental analyses revealed that the three-stage model proposed by Osborne et al. (6), active dimer → monomer → unfolded monomer, was not sufficient to describe the inactivation data. Assumption of an additional equilibrium between monomers and inactive dimers/oligomers was needed in our study. This was the simplest model that could describe the inactivation curves, but it remains speculative because the dimerization-competent monomers and the inactive, but reversible, dimers/oligomers could not be isolated. The rate of dimerization-competent monomers and the inactive, but reversible, dimers/oligomers was needed in our study. This was the simplest model that could describe the inactivation curves, but it remains speculative because the dimerization-competent monomers and the inactive, but reversible, dimers/oligomers could not be isolated. The rate of inactivation was dependent on the concentration of LPL also with heparin. This suggested that the same main stages in the inactivation process were operating as in the absence of heparin, but the system became too complex to be solved by mathematical modeling. Under conditions in which the enzyme was less stable (in 0.15 M NaCl) the concentration dependence almost disappeared, indicating that a monomolecular step became rate-limiting. This might be due to an increased dissociation rate for the active dimers or to a decreased association rate for dimerization-competent monomers. Thus, the values for the individual rate constants presented in Fig. 4 are highly dependent on the conditions under which the inactivation is studied. An interesting observation was that a small change in tryptophan fluorescence and the previously reported change in circular dichroism (6) occurred in parallel with the loss of activity against long acyl chain substrates, whereas the loss of activity against tributyrin was markedly delayed. This may indicate that intermediate form(s) could be active against a readily available substrate that does not require the presence of apolipoprotein CII, while still being completely inactive against substrates resembling the natural lipoproteins. An alternative conclusion is that association to active dimers was promoted during the assay with tributyrin.

The propensity of the LPL monomer to unfold, aggregate, and precipitate is not easily compatible with available biophysical techniques for studies of protein structure and interactions. Therefore, we were mostly confined to indirect studies and to spectroscopy. Osborn et al. (6) had previously shown by measurements of circular dichroism that there is a minor conformational change in LPL accompanying dissociation/inactivation but that the inactive monomers are folded and conformationally stable for many hours. Limited proteolysis is a well-established tool to monitor conformational changes. Proteinases cleave peptide bonds of folded proteins more readily in regions that are flexible, unstructured, and exposed (35, 36). Cleavages may occur either in the intact protein at initial cleavage sites or subsequently in the fragments formed after the initial cleavage. Thus, the most valuable information about the conformation of a protein comes from analyses of the initial cleavage sites. These are usually exposed on the surface of the protein and are not located in α-helices or β-sheets (35). Based on modeling, Hubard et al. (37) concluded that the sites of limited proteolysis require an unfolded stretch composed at least 12 amino acid residues. In the dimeric LPL, such sites are found in the lid that covers the entrance to the active site (31) and in the middle of the C-terminal folding domain (13). The monomeric form of LPL was cleaved at several additional sites, most of which were located in the middle of the subunit, suggesting that conformational changes had mainly occurred there. An alternative possibility is that this region is part of the dimerization interface and is therefore shielded in the dimer but becomes exposed, and more accessible for proteinases, after dissociation. According to the model of LPL, residues Phe-187 and Tyr-304 are located in an α-helix and β-sheet, respectively, and thus should not be accessible to cleavage. Because chymotrypsin cleaved rapidly at both of these sites, we propose that conformational changes occur in these areas. Furthermore, residues Arg-172 and Glu-165 are located in an exposed loop according to the model, but cleavage after these residues occurred only after dissociation to monomers. This may suggest that also these residues are in, or close to, the region that is involved in the subunit interaction in the LPL dimer. The conditions used were selected to promote stability of the dimer during the experiment, favoring the dimeric state and the stability of the dissociated monomers. Because no cleavage occurred in native LPL by endo-Glu-C, a likely explanation is that additional conformational changes were needed in the exposed loops before they could be attacked by the proteinases. Such changes occur during the irreversible conversion of the monomer to the inactive state. In contrast to chymotrypsin and endo-Glu-C, trypsin was able to open up the monomer structure for secondary cleavages at many sites. Although dimeric LPL is knicked by trypsin only in the lid covering the active site, monomeric LPL was rapidly cleaved to small fragments.

Natural mutations in the LPL gene, leading to functional LPL deficiency (hyperlipoproteinemia type I), are clustered in the regions coded by exons 5 and 6 (1, 12, 14, 18, 38), which correspond to the central part of LPL. The natural mutants are either completely inactive or have severely reduced activity. Peterson et al. (38) demonstrated that the dimeric structure of some of the mutants is less stable than that of the wild-type form of LPL. They also selected monoclonal antibodies that specifically recognized the inactive, presumable monomeric form (15). This supports our conclusion that the central part of LPL may play an essential role in the dimerization of LPL and that conformational changes occur in this region on dissociation of the dimer. Other regions may also contribute to the stability of the dimer. Keiper et al. (39) demonstrated increased stability of human LPL by the introduction of two hydrophilic residues (Tyr-Lys) at position 415 and 416 replacing hydrophobic residues (Val-Ile) in the C-terminal domain.

Binding studies with the hydrophobic probe bis-ANS suggested exposure of more hydrophobic areas on the LPL monomer than on the dimer. The addition of acrylamide quenched the tryptophan fluorescence of both active dimers and inactive monomers to an equal extent, indicating that no major difference in folding around tryptophan residues had occurred. The eight tryptophans of LPL are concentrated into two regions. There is one cluster of tryptophans in the middle of the C-terminal domain composed of residues Trp-384, Trp-392, Trp-395, and Trp-396. Another tryptophan-rich region is located in the N-terminal part of the N-terminal folding domain. Our results indicated that those regions were essentially unperturbed and that the overall structure of LPL must remain largely compact and folded in the monomeric state. This is in good accordance with the results from the proteolytic cleavage, suggesting that the main conformational changes occur in the central part of the LPL subunit. The dissociation may expose additional hydrophobic areas, which may be prone to aggregation. Aggregation was, however, relatively slow at low temperature and at high salt concentration, indicating that the contribution of ionic interactions to formation of large aggregates was more important than hydrophobic interactions. According to the model of LPL the charged residues are unevenly distributed in positive and negative clusters, so that LPL resembles a dipole (10).

In summary, we have shown that LPL dimers are readily exchanging subunits, supporting the idea that the dimers are in equilibrium with dimerization-competent monomers. This form of LPL is still hypothetical, because it has not been pos-
sible to isolate. From our modeling we cannot deduce with certainty whether the intermediate monomer is active, inactive, or possibly active only against simple substrates like tributyrin. This state may associate reversibly to inactive dimers/oligomers, or alternatively, it may go through an irreversible conformational change to an inactive but still folded monomer of the type that exists in tissues and blood. This monomeric form appears to have a relatively stable, defined conformation. The main differences in conformation between the inactive monomer and the active dimer were found in the middle part of the subunit. We conclude that this part is probably engaged in subunit interaction in the active dimer.

Acknowledgments—We thank Dr. J. Subbi (National Institute of Chemical Physics and Biophysics, Tallinn, Estonia) for help with MALDI-TOF mass spectrometry, Dr. P.-I. Ohlsson (Department of Chemical Physics and Biophysics, Tallinn, Estonia) for the amino acid sequence analysis, and Solveig Nilsson for preparing the bovine LPL.

REFERENCES

1. Merkel, M., Eckel, R. H., and Goldberg, I. J. (2002) J. Lipid Res. 43, 1997–2006
2. Mead, J. R., Irvine, S. A., and Ramji, D. P. (2002) J. Mol. Med. 80, 753–769
3. Preis-Landl, K., Zimmermann, R., Hammerle, G., and Zechner, R. (2002) Curr. Opin. Lipidol. 13, 471–481
4. Stein, Y., and Stein, O. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 185–194
5. Santamarina-Fojo, S., and Dugi, K. A. (1994) Curr. Opin. Lipidol. 5, 117–125
15. Peterson, J., Fujimoto, W. Y., and Brunzell, J. D. (1992) J. Lipid Res. 33, 1165–1170
16. Olivecrona, G., and Lookene, A. (1997) Methods Enzymol. 286, 102–116
17. Zhang, L., Wu, G., Tate, C. G., Lookene, A., and Olivecrona, G. (2003) J. Biol. Chem. 278, 29344–29351
18. Hata, A., Rödiger, D. N., Sutherland, S. D., Emi, M., Kwong, L. K., Shubha, J., Lubbers, A., Guy-Grand, B., Basdevant, A., Iervasi, P.-H., Wilson, D. E., and Lalouel, J.-M. (1992) J. Biol. Chem. 267, 20132–20139
19. Pentikainen, M. O., Ornin, K., and Kovanen, P. T. (2000) J. Biol. Chem. 275, 5694–5701
20. Vielle, E., Joven, J., Fernández, M., Villar, S., Brunzell, J. D., Olivecrona, T., and Bengtsson-Olivecrona, G. (1993) J. Lipid Res. 34, 1555–1564
21. Nykänen, A., Bengtsson-Olivecrona, G., Lookene, A., Moestrup, S. K., Petersen, C. M., Weber, W., Beisiegel, U., and Gliemann, J. (1993) J. Biol. Chem. 268, 15046–15055
22. Olivecrona, G., Hultin, M., Savonen, R., Skottova, N., Lookene, A., Tugrul, Y., and Olivecrona, T. (1995) in Atherosclerosis X (Woodford, F. P., Davignon, J., and Sniderman, A. D., eds) pp. 250–253, Elsevier, Amsterdam
23. Bergö, M., Olivecrona, G., and Olivecrona, T. (1996) Biochem. J. 313, 893–898
24. Krapp, A., Zhang, H. F., Ginzinger, D., Liu, M. S., Lindberg, A., Olivecrona, G., Hayden, M. R., and Beisiegel, U. (1995) J. Lipid Res. 36, 2362–2373
25. Bergö, M., Wu, G., Ruge, T., and Olivecrona, T. (2002) J. Biol. Chem. 277, 11927–11932
26. Wu, G., Olivecrona, G., and Olivecrona, T. (2003) J. Biol. Chem. 278, 11925–11930
27. Hanyu, O., Mida, T., Obayashi, K., Ikarashi, T., Suda, S., Kaneko, S., Hirayama, S., Suzuki, K., Nakamura, Y., Yamatani, K., and Aizawa, Y. (2004) Atherosclerosis 174, 385–390
28. Bengtsson-Olivecrona, G., and Olivecrona, T. (1991) Methods Enzymol. 197, 345–356
29. Olivecrona, T., Bengtsson, G., and Osborne, J. C., Jr. (1992) Eur. J. Biochem. 214, 629–633
30. Lookene, A., Savonen, R., and Olivecrona, G. (1997) Biochemistry 36, 5267–5275
31. Bengtsson-Olivecrona, G., Olivecrona, T., and Jörnvall, H. (1986) Eur. J. Biochem. 161, 281–288
32. Lookene, A., Zhang, L., Tougu, V., and Olivecrona, G. (2003) J. Biol. Chem. 278, 37183–37194
33. Efthimiopoulos, M. E., and Ghiron, C. A. (1981) Anal. Biochem. 114, 199–227
34. Lutz, E. P., Kako, Y., Yagyu, H., Heeren, J., Marks, S., Wright, T., Melford, K., Ben Zeev, O., Radner, H., Merkel, M., Bensadoun, A., Wong, H., and Goldberg, I. J. (2004) J. Biol. Chem. 279, 238–244
35. Hubbard, S. J. (1998) Biochim. Biophys. Acta 1382, 191–206
36. Fontana, A., deLaureto, P. P., DeFilippis, V., Scaramella, E., and Zambonin, M. (1997) Fold. Des. 2, R17-R26
37. Hubbard, S. J., Beynon, R. J., and Thornton, J. M. (1998) Protein Eng. 11, 349–359
38. Petersen, J. A., Ayoubi, A. F., Ma, Y. H., Henderson, H., Reina, M., Deeb, S. S., Santamarina-Fojo, S., Hayden, M. R., and Brunzell, A. D. (2002) J. Lipid Res. 43, 398–406
39. Keiper, T., Schneider, J. G., and Dugi, K. A. (2001) J. Lipid Res. 42, 1180–1186