Calcium Triggers Folding of Lipoprotein Lipase into Active Dimers*

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The active form of lipoprotein lipase (LPL) is a noncovalent homodimer of 55-kDa subunits. The dimer is unstable and tends to undergo irreversible dissociation into inactive monomers. We noted that a preparation of such monomers slowly regained traces of activity under assay conditions with substrate, heparin, and serum or in cell culture medium containing serum. We therefore studied the refolding pathway of LPL after full denaturation in 6 M guanidinium chloride or after dissociation into monomers in 1 M guanidinium chloride. In crude systems, we identified serum as the factor promoting reactivation. Further investigations demonstrated that \( \text{Ca}^{2+} \) was the crucial component in serum for reactivation of LPL and that refolding involved at least two steps. Studies of far-UV circular dichroism, fluorescence, and proteolytic cleavage patterns showed that LPL started to refold from the C-terminal domain, independent of calcium. The first step was rapid and resulted in formation of an inactive monomer with a completely folded C-terminal domain, whereas the N-terminal domain was in the molten globule state. The second step was promoted by \( \text{Ca}^{2+} \) and converted LPL monomers from the molten globule state to dimerization-competent and more tightly folded monomers that rapidly formed active LPL dimers. The second step was slow, and it appears that proline isomerization (rather than dimerization as such) is rate-limiting. Inactive monomers isolated from human tissue recovered activity under the influence of \( \text{Ca}^{2+} \). We speculate that \( \text{Ca}^{2+} \)-dependent control of LPL dimerization might be involved in the normal post-translational regulation of LPL activity.

Lipoprotein lipase (LPL) plays a central role in the metabolism of blood lipids (1, 2). Catalytically active LPL is a dimer with two identical glycosylated 55-kDa subunits connected to each other in a head-to-tail fashion by noncovalent interactions (3, 4). The enzyme is produced in parenchymal cells of adipose tissue and muscle, but is secreted for action on the vascular side of the endothelium, where it is bound to membrane-anchored heparan sulfate proteoglycans (1, 2). Dimerization of LPL occurs in the endoplasmic reticulum and is a prerequisite for activity (5–7). Recently, we showed that folding into the active form is dependent on endoplasmic reticulum-derived molecular chaperones (7). Previous studies have demonstrated that the two subunits of active LPL easily fall apart and that monomerization is accompanied by loss of catalytic activity (8). It has been suggested that the instability of the LPL dimer might be a built-in mechanism to limit the life span of LPL at the endothelium, where the enzyme is out of reach of normal control systems such as protein kinase-dependent phosphorylation and other intracellular regulatory systems (9, 10). Tissues normally contain a mixture of inactive monomers and active dimers of LPL (11). The dominating form in circulating blood is the inactive monomer, probably because this form has lower affinity for heparan sulfate proteoglycans and therefore detaches from the endothelial binding sites and appears in blood (12, 13). The activity of LPL in the major metabolic tissues (muscle and adipose tissue) is regulated according to the nutritional status (14, 15). Short-term regulation is mostly post-translational and involves changes in the dimer/monomer ratio (16, 17). The details of this regulation are not yet understood, but in adipose tissue, the balance between monomers and dimers appears to be controlled extracellularly (17).

The conversion of active dimers to inactive monomers was until now considered to be an irreversible process (8, 18). Based on the observation that the fractional rate for inactivation of LPL increases with decreasing concentrations of the lipase protein, it was concluded that reversible interactions occur between active monomeric and dimeric species of LPL and that dissociation into active monomers precedes the irreversible loss of activity, which occurs by unfolding of the monomers (8). Inactivation is accompanied by only a moderate change in secondary structure, indicating that the inactive monomer retains a folded shape (8). The decay to inactive monomers must be rapid because it has not been possible to isolate catalytically active monomers of LPL. Previous studies have shown that monomers of LPL can associate into higher oligomers (aggregates) both in vivo and in vitro (6, 8). For many proteins, removal of the denaturant leads to aggregation rather than correct refolding (19). In recent years, an understanding of protein folding and refolding has developed, and strategies for overcoming aggregation have been explored. In many cases, refolding can be achieved by manipulation with additives, including detergents (20, 21), osmolytes (22, 23), and artificial chaperones (24), and metal ions and salts (25–28).

The possibility that fully or partially denatured LPL refolds into the active dimeric form has not previously been systematically explored. We observed that LPL could regain traces of activity when incubated for long times (hours or days) under assay conditions with substrate, heparin, and serum as source of the activator apolipoprotein CII or in cell culture medium containing serum. In this investigation, we studied the refolding pathway of LPL after full denaturation in 6 M guanidinium chloride (GdmCl), after dissociation into inactive monomers in 1 M GdmCl, or by other means (temperature). We identified serum as the critical component in serum for reactivation of LPL and that refolding involved at least two steps. Studies of far-UV circular dichroism, fluorescence, and proteolytic cleavage patterns showed that LPL started to refold from the C-terminal domain, independent of calcium. The first step was rapid and resulted in formation of an inactive monomer with a completely folded C-terminal domain, whereas the N-terminal domain was in the molten globule state. The second step was promoted by \( \text{Ca}^{2+} \) and converted LPL monomers from the molten globule state to dimerization-competent and more tightly folded monomers that rapidly formed active LPL dimers. The second step was slow, and it appears that proline isomerization (rather than dimerization as such) is rate-limiting. Inactive monomers isolated from human tissue recovered activity under the influence of \( \text{Ca}^{2+} \). We speculate that \( \text{Ca}^{2+} \)-dependent control of LPL dimerization might be involved in the normal post-translational regulation of LPL activity.
factor promoting reactivation in crude systems. Further investigations demonstrated that calcium was the crucial component in serum necessary for the successful refolding of LPL from the molten globule, monomeric state to the more tightly folded, active, dimeric form. The rate-limiting step for complete refolding appears to be formation of a dimerization-competent monomer. Our results demonstrate that proline isomerization is a likely determinant of the slow kinetics for the calcium-induced reactivation of LPL.

EXPERIMENTAL PROCEDURES

Materials—GdmCl, CaCl₂, trypsin, bovine cyclophilin A, and bovine serum albumin (BSA) were from Sigma. Heparin was from Leo Pharma AB (Malmö, Sweden). Heparin-Sepharose was prepared as described previously (29), and LPL was purified from bovine milk (30). The concentrations of LPL were determined from absorbance at 280 nm (A₂₈₀ nm = 1 corresponds to 0.60 mg/ml) or by the immunoadsorption described below. 4,4’-Dianilino-1,1’-binaphthyl-5,5’-disulfonic acid (dipotassium salt; bis-ANS) was from Molecular Probes. The concentration of bis-ANS was determined from the absorbance at 394 nm using A₃₉₄ nm = 16,000 cm⁻¹ M⁻¹ (31). Human and rat sera were collected against clotting of the blood. For some experiments, rat serum was heated for 30 min at 56 °C, followed by overnight incubation at 10 °C with 100 μg/ml phenylmethylsulfonyl fluoride and dialysis against 10 mM Tris-Cl (pH 7.4) containing 0.15 M NaCl and 2 mM EDTA. Lipoprotein-depleted serum and isolated lipoprotein fractions were prepared by sequential ultracentrifugation after additions of KBr to increase the denaturation state to the more tightly folded, active, dimeric form. The rate-limiting component in serum necessary for the successful refolding of LPL from the molten globule, monomeric state to the more tightly folded, active, dimeric form. The rate-limiting step for complete refolding appears to be formation of a dimerization-competent monomer. Our results demonstrate that proline isomerization is a likely determinant of the slow kinetics for the calcium-induced reactivation of LPL.

LPL Denaturation and Refolding—For gentle dissociation into folded but inactive monomers, native bovine LPL (0.8 mg/ml in 20 mM Bistris (pH 6.5) containing 1.2 M NaCl) was dialyzed overnight at 4 °C against 20 mM Tris-Cl (pH 7.4) containing 2 mM NaCl and 1 mM GdmCl (8). For full denaturation, native LPL (0.8 mg/ml) was dialyzed against 20 mM Tris-Cl (pH 7.4) containing 0.5 M NaCl and 6 M GdmCl or 8 M urea for 2 h. In other experiments, native LPL was mixed with 8 M GdmCl to a final concentration of 6 M GdmCl and incubated for the time specified in the figure legends. Refolding of fully denatured LPL was performed by dilution (50–100 fold) in 20 mM Tris-Cl (pH 7.4) containing 20 mM NaCl and 2.5 mM CaCl₂ or 20% (v/v) serum, followed by incubation at 25 °C for 5 h or for the times specified in the figure legends. For fluorescence measurements during refolding experiments, 10% glycerol was added to the buffer to stabilize LPL.

LPL Activity and Mass—LPL activity and protein mass were assayed as described previously (11). For activity measurements, 5 μl was incubated in a total volume of 200 μl of a mixture containing a phospholipid-stabilized emulsion of soybean triacylglycerols, with the same composition as Intralipid (10% Fresenius Kabi AG), into which tri-[9,10-³H]oleoylglycerol had been incorporated by the manufacturer. Heat-inactivated rat serum was present as a source of apolipoprotein CII (10 mg/ml) and heparin to stabilize the lipase (10 IU/ml). One milliunit of lipase activity corresponds to release of 1 nmol of fatty acid/min at 25 °C and pH 8.5. All samples were measured in triplicates, and the incubation time was adapted to give results in the linear range of the assay.

For measurement of LPL mass, affinity-purified chicken immunoglobulins (IgY) raised against bovine LPL was used for capture of the antigen on microtiter plates during overnight incubation. Bound LPL was detected with monoclonal antibody 5D2, raised against bovine LPL (a kind gift from Dr. John Brunzell, University of Washington, Seattle, WA), followed by peroxidase-labeled anti-mouse IgG. LPL purified from bovine milk was used as a standard. All samples were analyzed in three different dilutions.

Fluorescence Measurements—Fluorescence measurements were performed using a SPEX Fluoromax-2 fluorometer. The experiments were done at 25 °C in 20 mM Tris-Cl (pH 7.4) and 2 mM NaCl with or without 2.5 mM CaCl₂. For experiments involving refolding, the samples also contained 10% (v/v) glycerol to stabilize LPL. Stock solutions of bis-ANS (50 μM) were prepared in methanol. Bis-ANS was added in equimolar concentrations compared with the concentration of monomeric LPL immediately prior to the measurements. Fluorescence emission spectra for tryptophan residues were recorded at 300–400 nm with excitation at 295 nm. For bis-ANS, the emission spectra were recorded at 420–550 nm with excitation at 390 nm.

CD Measurements—CD measurements were carried out at 25 °C using a Jasco J-700 spectropolarimeter in the far-UV region (200–250 nm) and a cuvette with 1-mm path length. LPL was diluted in 20 mM Tris-Cl (pH 7.4) with or without 2.5 mM CaCl₂ to a concentration of 75 μg/ml. To reach an acceptable ratio of signal to noise, the buffer contained a reduced concentration of NaCl (0.3 M instead of 2 M) compared with that used in the other experiments. For all spectra, a reference sample containing the corresponding buffer was subtracted from the CD signal. The mean residue ellipticities were calculated according to the following equation: (θ) = 115(θ)obs/10LC, where θobs is the mean amino acid residue molecular weight of bovine LPL (33), L is the path length in centimeters, and C is the concentration of LPL in grams/ml (8).

Chromatography on Heparin-Sepharose—Separation was carried out as described previously (11). Briefly, 4 ml of refolded LPL (10 μg/ml) was diluted 10-fold in 20 mM Tris-Cl (pH 7.4) containing 10% glycerol before loading onto the column (1 ml of sedimented gel). After washing, the column was eluted by a linear gradient of NaCl in the same buffer (from 0.15 to 2.0 M), and 1-ml fractions were collected. The salt concentrations in the fractions were determined manually by conductometry using standard solutions of NaCl made up in the same buffer.

Sucrose Density Gradient Centrifugation—For determination of the aggregation state of LPL, triplicate samples of refolded LPL (10 μg/ml in 20 mM Tris-Cl (pH 7.4) containing 1 mg/ml BSA and 2 mM NaCl in a total volume of 200 μl were applied to linear sucrose density gradients (5–20% (w/v)) made up in 20 mM Tris-Cl (pH 7.4) containing 2.0 mM NaCl and 1 mg/ml BSA (total gradient volume of 3.6 ml). Centrifugation was carried out in a Beckman Coulter SW 60 rotor at 50,000 rpm for 16 h at 10 °C. After centrifugation, 238-μl fractions were collected by puncturing the bottom of the centrifuge tube using a needle attached by tubing to a pump. As a reference sample, dissociated monomeric LPL was prepared by treatment of native LPL with 1 M GdmCl in 20 mM Tris-Cl containing 0.5 mM ammonium sulfate and 0.2 M NaCl (8). This sample was diluted to a concentration of 2 μg/200 μl of the same buffer as used for the sucrose density gradients, and then 2 μg of native bovine LPL was added to produce a reference with both monomeric and dimeric forms of LPL. This sample (run in triplicate) was run in separate tubes from the samples of refolded LPL. Measurements of LPL activity and mass were made on the fractions as described above.

Preparation of a Heat-inactivated LPL Monomer, Followed by Refolding—Gentle thermal inactivation of LPL was accomplished by incubation at 25 °C until no measurable lipase activity was left (overnight). The sample (10 μg/ml LPL in 20 mM Tris-Cl (pH 7.4) containing 10% glycerol and 2 mM NaCl) was then divided into two groups, which were incubated with or without 2.5 mM CaCl₂ at 25 °C for 5 h.
ammonia buffer (pH 8.2) containing 1% Triton X-100, 0.1% SDS, and 2 Complete Mini proteinase inhibitor tablets (Roche Applied Science) per 100 ml. After centrifugation, 75 ml of the supernatant was used for chromatography on heparin-Sepharose (5 ml of gel) essentially as described above, but the gradient was run from 0.15 to 1.6M NaCl (total volume of 100 ml), and all buffers contained 1 mg/ml BSA. LPL activity and mass were assayed in each fraction as described above. The peak fraction from the inactive monomer (200 ng/ml in buffer containing 0.5 M NaCl) was used for refolding at 25 °C for 5 h. The concentration of NaCl was first adjusted to 2.0M by addition of solid NaCl, and the sample was divided into two groups, which were incubated with or without 2.5 mM CaCl2.

Trypsin Digestion—Refolded, previously fully denatured LPL (50 μg) in 20 mM Tris-Cl (pH 7.4) and 2 mM NaCl with a residual concentration of GdmCl amounting to 0.06 M was digested with 5 μg of trypsin (10% (w/w) LPL protein) at room temperature for 5 min. The reaction was stopped by addition of phenylmethylsulfonyl fluoride to a 10-fold molar excess over trypsin. The samples were then briefly dialyzed against 20 mM Tris-Cl (pH 7.4) and 2 mM NaCl to remove the remaining GdmCl. After addition of SDS to the samples to 0.05% (w/v), the dialysis buffer was changed to 0.05% SDS in water. After lyophilization, the samples were dissolved in sample buffer and heated to 95 °C before analyses by SDS-PAGE (15% acrylamide).

Double Jump Experiment—To study whether the refolding kinetics of fully denatured LPL depend on denaturation time, LPL (0.8 mg/ml in 10 mM Bistris (pH 6.5) and 1 mM NaCl) was denatured by addition of 8 M GdmCl to a final concentration of 6 M at 4 °C. After 1 min, 0.5 h, and 2 h, the denaturation was stopped by dilution of a sample in a solution of 20 mM Tris, 2.5 mM CaCl2, 0.1 mg/ml very low density lipoprotein (VLDL), and 2.0 M NaCl. Recovery of LPL activity was then followed for 6 h at 25 °C.

Effect on Refolding by a Peptidylprolyl Isomerase (Bovine Cyclophilin A)—Because cyclophilin A is sensitive to GdmCl, in these experiments, denaturation of LPL was carried out for 2 h at 4 °C in 8 M urea at pH 8.0 instead of in 6 M GdmCl. After this treatment, LPL was completely unfolded as evidenced by CD measurements. Refolding was initiated by dilution in 20 mM Tris-Cl (pH 7.4) containing 2 mM NaCl, 0.1 mg/ml VLDL, and 2.5 mM CaCl2 in the presence or absence of 1 μM bovine cyclophilin A (34). VLDL was included in these experiments to stabilize active LPL.

RESULTS

Fully Denatured LPL Can Refold into an Active State in the Presence of Serum—A previous study has shown that the catalytic activity of LPL can be stabilized by the presence of heparin, lipid substrates, and serum (30). We therefore investigated the effect of a combination of all these
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Identification of components in serum essential for reactivation of fully denatured LPL

LPL (500 µg/ml) was first denatured in 6 M GdmCl. Samples of fully denatured LPL were diluted 50-fold with 20 mM Tris-Cl (pH 7.4) and 2.0 M NaCl containing various supplements as indicated. After incubation for 5 h at 25 °C, the recovery of LPL activity was determined. Data are the means ± S.D. of at least five determinations. ND, not detectable.

| Additives                                      | Recovery of LPL activity |
|-----------------------------------------------|--------------------------|
| Human or rat serum (20% (v/v))                | 38 ± 4.5                 |
| Lipoprotein-depleted serum (20% (v/v), dialyzed against EDTA) | ND                       |
| LDL (0.4–1 mg/ml)                             | ND                       |
| VLDL (0.08–0.2 mg/ml)                         | ND                       |
| HDL (0.2–0.5 mg/ml)                           | ND                       |
| BSA (0.25–3 mg/ml)                            | ND                       |
| Glycerol (10%)                                | ND                       |
| Heat-inactivated rat serum (20% (v/v), 56 °C, dialyzed against EDTA) | ND           |
| Heat-inactivated human serum (20% (v/v), 56 or 100 °C, no dialysis) | 20 ± 4.3              |
| Human serum (20% (v/v), with 10–50 mM EDTA)   | ND                       |
| K⁺ (0.5–2 mM)                                 | ND                       |
| Mg²⁺ (0.5–5 mM)                               | ND                       |
| Zn²⁺ (0.01–1 mM)                              | ND                       |
| Ca²⁺ (0.1–2 mM)                               | ND                       |
| Ca²⁺ (2.5–5 mM)                               | 20 ± 3.1                 |
| Ca²⁺ (2.5 mM) + glycerol (10%) or BSA (0.1–1%)| 30 ± 3.1                 |
| Ca²⁺ (2.5 mM) + VLDL (0.08–0.2 mg/ml)         | 43 ± 4.3                 |
| Ca²⁺ (2.5 mM) + LDL (0.2–0.5 mg/ml)           | 43 ± 3.8                 |
| Ca²⁺ (2.5 mM) + HDL (0.4–1 mg/ml)             | 41 ± 3.3                 |

additives or human serum alone on the recovery of LPL activity after overnight incubation at 10 °C. For this, LPL fully denatured by treatment with 6 M GdmCl was diluted 50-fold in buffer with or without additives. We found that a few percent of the original LPL activity was recovered in buffer containing serum. The recovery was higher (~8%) when the buffer contained heparin in addition to serum (Fig. 1A). No activity was recovered in buffer with heparin and/or lipid substrate but no serum, indicating that serum was the most crucial component of the incubation medium. The presence of heparin increased the recovery of LPL activity when present together with serum (with or without substrate) (Fig. 1A). The recovery of activity was not enhanced by increasing the concentrations of heparin or serum, but we observed a strong dependence on the concentration of NaCl (Fig. 1B). Maximal recovery (~40%) was achieved in buffer containing 20% serum and 2.0 M NaCl (Fig. 1B). Heparin had a marginal effect at low concentrations of NaCl (<0.5 M), but was ineffective at higher concentrations (Fig. 1B). Reactivation did not occur in buffer with 2.0 M NaCl in the absence of serum, supporting the view that serum was crucial (data not shown). Further experiments demonstrated that reactivation was obtained also upon incubation at 25 or 37 °C (Fig. 1C). The time needed to reach optimal recovery of activity was shortened from 18 h at 10 °C to 2–5 h at the higher temperatures. The maximal yield of LPL activity was lower at 37 °C (only ~20%) than at 25 and 10 °C (at which it was 40%) (Fig. 1C). The recovery of LPL activity was dependent on the sufficient reduction of the residual concentration of GdmCl after dilution into the solution used for refolding. Maximal recovery was achieved when the GdmCl concentration was 0.1 M or less (Fig. 1D).

**Calcium Is the Crucial Component in Serum Responsible for Refolding of LPL into the Active State—**To determine the component(s) in serum necessary to support the reactivation of LPL, we first compared human and rat sera, which were found to be equally effective (TABLE ONE). Lipoprotein-depleted serum had no effect, and isolated lipoprotein fractions (VLDL, low density lipoprotein (LDL), and high density lipoprotein (HDL)) were also ineffective. Likewise, heat-inactivated rat serum (dialyzed against buffer containing EDTA after centrifugation) did not support reactivation of LPL, indicating that the necessary component might be heat-labile. This notion was overruled by the use of heated human serum recovered after centrifugation, but without dialysis. Dialysis against EDTA is a common procedure to recover lipoprotein-depleted serum as well as lipoprotein fractions after ultracentrifugation (35). When human serum was mixed with EDTA (or dialyzed against EDTA), it could no longer promote reactivation of denatured LPL (TABLE ONE). These results suggested that the crucial component in serum was chelated by EDTA. Among the metal ions tested, Ca²⁺ was the only one that could promote LPL activation (TABLE ONE). Compared with serum, Ca²⁺ (2.5–5 mM) gave a lower recovery of LPL activity (20% recovery compared with 38.5 ± 4.5% with serum). Elevated recovery of LPL activity by Ca²⁺ was observed if an additional agent, such as 10% glycerol or 0.1% BSA, was included in the refolding buffer, which presumably stabilized the active form. Similar effects were also seen upon addition of lipoproteins (VLDL, HDL, or LDL). A combination of lipoproteins and Ca²⁺ gave a similar recovery of LPL activity compared with serum (data not shown). The recovery was also similar whether or not LPL had been denatured in the presence of reducing agent (β-mercaptoethanol or dithiothreitol). In the following experiments, LPL was denatured in the absence of reducing agent, and 2.5 mM Ca²⁺ was used for refolding because the recovery with 1.25 mM Ca²⁺ was slightly lower and that with 0.5 mM Ca²⁺ was only 50% compared with the recovery obtained at 2 or 2.5 mM Ca²⁺.

**Fully Denatured LPL Rapidly Refolds into a Structured Molten Globule Conformational State Even in the Absence of Calcium Ions—**To investigate the mechanism for the calcium-induced reactivation of fully denatured LPL, changes in the secondary structure of LPL induced upon dilution from 6 M GdmCl were recorded by far-UV CD (Fig. 2A). LPL refolded within minutes after dilution, reaching a CD spectrum closely resembling that of LPL monomers formed by dissociation of active dimers in 1 M GdmCl. There was no additional measurable effect.

**TABLE ONE**

Identification of components in serum essential for reactivation of fully denatured LPL

| Additives                                      | Recovery of LPL activity |
|-----------------------------------------------|--------------------------|
| Human or rat serum (20% (v/v))                | 38 ± 4.5                 |
| Lipoprotein-depleted serum (20% (v/v), dialyzed against EDTA) | ND                       |
| LDL (0.4–1 mg/ml)                             | ND                       |
| VLDL (0.08–0.2 mg/ml)                         | ND                       |
| HDL (0.2–0.5 mg/ml)                           | ND                       |
| BSA (0.25–3 mg/ml)                            | ND                       |
| Glycerol (10%)                                | ND                       |
| Heat-inactivated rat serum (20% (v/v), 56 °C, dialyzed against EDTA) | ND           |
| Heat-inactivated human serum (20% (v/v), 56 or 100 °C, no dialysis) | 20 ± 4.3              |
| Human serum (20% (v/v), with 10–50 mM EDTA)   | ND                       |
| K⁺ (0.5–2 mM)                                 | ND                       |
| Mg²⁺ (0.5–5 mM)                               | ND                       |
| Zn²⁺ (0.01–1 mM)                              | ND                       |
| Ca²⁺ (0.1–2 mM)                               | ND                       |
| Ca²⁺ (2.5–5 mM)                               | 20 ± 3.1                 |
| Ca²⁺ (2.5 mM) + glycerol (10%) or BSA (0.1–1%)| 30 ± 3.1                 |
| Ca²⁺ (2.5 mM) + VLDL (0.08–0.2 mg/ml)         | 43 ± 4.3                 |
| Ca²⁺ (2.5 mM) + LDL (0.2–0.5 mg/ml)           | 43 ± 3.8                 |
| Ca²⁺ (2.5 mM) + HDL (0.4–1 mg/ml)             | 41 ± 3.3                 |
of the presence of calcium ions on the recovery of secondary structure, at least not on this short time scale (Fig. 2A). When the solutions were diluted another 10-fold from 1 to 0.1 M GdmCl, there was no further increase in secondary structure, indicating that the conformational state(s) were rather stable and well defined (data not shown).

To detect changes in the global protein structure affecting the microenvironment of Trp residues, studies of the overall changes in intrinsic fluorescence were performed. Bovine LPL contains 8 tryptophan residues/subunit (33). Fig. 2B shows that LPL in 6 M GdmCl had an intrinsic fluorescence, with a maximal emission wavelength ($\lambda_{	ext{max}}$) at 354 nm, which is typical for an unfolded protein. Active dimeric LPL showed less intense fluorescence, with $\lambda_{	ext{max}}$ at 340 nm. This suggested that some Trp fluorescence was quenched in active dimeric LPL, probably due to folding or possibly shielding by interactions between the subunits. When LPL in 6 M GdmCl was diluted 50-fold to allow refolding, $\lambda_{	ext{max}}$ was significantly blue-shifted (from 354 to 338 nm). Monomeric LPL (dissociated from dimers in 1 M GdmCl) also demonstrated a $\lambda_{	ext{max}}$ at 338

FIGURE 3. Refolded LPL is monomeric and has low affinity for heparin. A, refolded LPL (10 µg/ml) after dilution to 0.1 M GdmCl was subjected to sucrose density gradient centrifugation as described under “Experimental Procedures” (3). A sample of bovine LPL containing both active dimers and inactive monomers (2 µg of each/200 µl) was used as a standard (●). The curves represent mean values from three centrifuge tubes run for each sample. LPL mass was assayed in the fractions. Fraction 1 represents the bottom of the gradient. B, LPL (10 µg/ml) in 4 ml of 20 mM Tris-Cl (pH 7.4) containing 0.15 M NaCl and 10% glycerol was subjected to chromatography on a 1-ml column of heparin-Sepharose (E). Fractions of 1 ml each were collected upon elution of the column by a gradient of NaCl (●). LPL mass was assayed in each fraction. Data are shown as the means of three determinations.
eluted from the heparin affinity column at heparin-Sepharose. We found that most of the refolded LPL protein was in two forms of LPL can therefore be separated by chromatography on a column with lower affinity for heparin compared with active dimers (11, 36), and the concentration to the bottom of tube, where it was difficult to recover (Fig. 2A). After cleavage, the sample was analyzed by SDS-PAGE (second lane). Corresponding samples with only trypsin (first lane) or only LPL (third lane) were run in parallel. The fourth lane contains molecular mass markers. The arrows indicate the band corresponding to trypsin and to the fragment originating from LPL as indicated.

To further investigate the properties of refolded LPL, we used the fluorescent probe bis-ANS, which is a commonly used detector of molten globule states in protein folding. This means that the protein has recovered most of its secondary structure and some of its tertiary structure, but that the conformation is in a much more dynamic state than in the fully folded, native protein (19). Fully denatured LPL did not bind bis-ANS, but when GdmCl was diluted to non-denaturing concentrations, a strong increase in bis-ANS fluorescence with a λmax at 478 nm was almost immediately observed for refolded LPL (Fig. 2C). The spectrum reached was clearly different from that of active dimeric LPL, which had a λmax for bis-ANS fluorescence emission at 485 nm, and that of dissociated monomers, which had a λmax at 478 nm. The fluorescence intensity of the dissociated monomer (in 1 M GdmCl) was increased compared with that of native dimeric LPL. These results indicated that the surface hydrophobicity increased upon dissociation of the LPL dimer into monomers and that the refolded monomer had somewhat different properties compared with the dissociated monomer.

LPL Refolded in the Absence of Calcium Is Monomeric—To investigate whether refolded LPL was monomeric or dimeric, we used sucrose density gradient centrifugation and compared the sedimentation behavior with that of a mixture of native dimeric LPL and dissociated inactive LPL monomers. About 80% of the refolded LPL protein sedimented in a peak corresponding to dissociated LPL monomers, whereas the rest of the protein was probably lost due to aggregation and sedimentation to the bottom of tube, where it was difficult to recover (Fig. 3A). Previous studies have shown that dissociated LPL monomers have lower affinity for heparin compared with active dimers (11, 36), and the two forms of LPL can therefore be separated by chromatography on heparin-Sepharose. We found that most of the refolded LPL protein eluted from the heparin affinity column at ~0.5 M NaCl (Fig. 3B). This was comparable with the elution behavior of monomeric LPL prepared by dissociation in 1 M GdmCl, whereas native dimers eluted at ~1.0 M NaCl (data not shown).

Refolding of LPL Starts from the C-terminal Domain—Like other members of the triacylglycerol lipase family, the LPL subunit probably forms two folding domains: a larger N-terminal domain (residues 1–310) and a smaller C-terminal domain (residues 311–448; numbers for human LPL). The observation from CD measurements demonstrating that most of the secondary structure was recovered after dilution of LPL denatured in 6 M GdmCl prompted us to investigate whether some regions or domains acquired native-like packing. For this, LPL refolded in the absence of Ca2+ was subjected to trypsin, and the initial products were analyzed by SDS-PAGE (Fig. 4). We identified two major protein bands, the upper of which corresponded to the added trypsin (confirmed by N-terminal amino acid sequencing). The lower 21-kDa band had the N-terminal sequence SQMPYK. This demonstrated that it originated from LPL due to cleavage at Arg306 (according to numbering for human LPL). Based on the apparent size of this trypsin-resistant fragment and considering the fact that there is an N-glycosylation site in the C-terminal folding domain, we concluded that this fragment corresponded to the whole C-terminal folding domain of LPL (Fig. 4).

Ca2+ Supports Additional Conformational Changes Necessary for Reactivation of LPL—To investigate whether the effect of calcium on the reactivation of LPL was due to interaction between Ca2+ and the partially refolded LPL monomers, we performed studies of the effects of Ca2+ on the Trp fluorescence and surface hydrophobicity of refolded LPL. There was an immediate effect on the bis-ANS fluorescence intensity upon addition of Ca2+ (Fig. 5A), indicating that rapid conformational changes occurred already within minutes after Ca2+ binding. With time (hours), further changes occurred, so the bis-ANS emission spectrum approached (but did not coincide with) that for native dimeric LPL. There was a slight red shift to λmax ~ 481 nm (Fig. 5A). A decreased intrinsic fluorescence was also observed upon addition of Ca2+ (Fig. 5A), occurring roughly in parallel with the change in extrinsic fluorescence (data not shown).
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To investigate whether proline isomerization contributed to the slow rate of dimerization, the process was slow, temperature-dependent, and also highly dependent on other conditions, indicating that an energy barrier had to be overcome. What is the rate-limiting step? Dimerization is a second-order reaction, and therefore, different concentrations of LPL should change the kinetics of reactivation if formation of dimers is rate-limiting. The influence of residual concentrations of GdmCl > 0.1 M on the refolding reaction (see Fig. 1D) limited the possible concentration range to a 10-fold difference of LPL (0.8–8.0 μg/ml). The refolding rates were the same within this range (Fig. 7A), suggesting that dimerization was not such as not rate-limiting.

Proline cis/trans-isomerization is known to be a rate-limiting step in the folding of many proteins (37). Bovine LPL has 20 proline residues: 16 are in the N-terminal domain, and 4 are in the C-terminal domain (38). To investigate whether proline isomerization contributed to the slow not shown). These results demonstrated that hydrophobic areas exposed on the surface of refolded, inactive, monomeric LPL (in the molten globule state) were changed by binding of Ca2+. This might reflect the time-dependent conformational changes corresponding to the activation/dimerization of LPL.

To further study the role of Ca2+ in reactivation, the Ca2+-chelating agent EDTA was added during refolding. In the experiment in Fig. 5B, LPL was refolded at 25 °C for 3 h with 2.5 mM Ca2+ in the presence of 0.1 mg/ml VLDL (for stabilization) before EDTA was added. Upon further incubation for an additional 2 h, almost twice as much activity was recovered. When EDTA was added after reactivation for 3 h, no further increase in LPL activity was observed. To exclude the possibility that EDTA might destabilize native LPL, we incubated native LPL with different concentration of EDTA (5–25 mM). No loss of activity was observed during the following 2 h at 25 °C, demonstrating that, under the conditions used, LPL was fully stable, independent of the presence or absence of EDTA (Fig. 5B). When native LPL was incubated in buffer containing 2 mM NaCl and 25 mM EDTA, but in the absence of VLDL, LPL activity was markedly decreased with time (Fig. 5C). The inactivation rate was the same with or without EDTA (data not shown), but it was reduced in the presence of 2.5 mM Ca2+ (Fig. 5C). The reason for the lack of continued reactivation of refolded LPL after addition of EDTA was probably that EDTA chelated Ca2+ and thereby prevented the necessary conformational changes allowing reactivation. Thus, Ca2+ appears to have a catalytic effect on reactivation, but does not seem to remain bound to active dimeric LPL.

Ca2+ Induces Dimerization of Partially Refolded LPL—To investigate whether the conformational changes induced by Ca2+ led to dimerization, sucrose density gradient centrifugation was performed after refolding of fully denatured LPL for 5 h at 25 °C in the presence of Ca2+. The peak of activity of reactivated LPL sedimented to the same position in the gradient as that of native dimeric LPL when run in the same experiment for reference (Fig. 6A). This demonstrated that LPL that had regained activity in the presence of Ca2+ was in fact dimeric. Analyses of LPL mass in the fractions demonstrated that more than half of the refolded LPL mass had not been converted to dimers, but sedimented as dissociated LPL monomers. Some LPL was found in the bottom of the tube, probably due to aggregation (Fig. 6B). In contrast, no dimeric LPL was produced from refolded LPL incubated for 5 h without Ca2+, and in this case, more of the protein was lost as aggregates (Fig. 6B). Chromatography on heparin-Sepharose demonstrated that the recovered LPL activity eluted in the same position in the salt gradient as did the native dimeric LPL activity (data not shown).

Proline Isomerization Is Rate-limiting for the Ca2+-dependent Reactivation of LPL—Reactivation from the partially folded, but inactive, monomeric state appeared to involve both additional folding and dimerization. The process was slow, temperature-dependent, and also highly dependent on other conditions, indicating that an energy barrier had to be overcome. What is the rate-limiting step? Dimerization is a second-order reaction, and therefore, different concentrations of LPL should change the kinetics of reactivation if formation of dimers is rate-limiting. The influence of residual concentrations of GdmCl > 0.1 M on the refolding reaction (see Fig. 1D) limited the possible concentration range to a 10-fold difference of LPL (0.8–8.0 μg/ml). The refolding rates were the same within this range (Fig. 7A), suggesting that dimerization was not such as not rate-limiting.

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reactivation kinetics, two strategies were used. First, LPL was initially unfolded in 6 M GdmCl for different times (1 min, 0.5 h, and 2 h) before refolding was initiated by dilution. Most of the proline residues remain in their native configuration shortly after unfolding, but isomerization leads to new equilibria of \textit{cis}/\textit{trans}-configurations with increasing unfolding times. Therefore, different reactivation rates can be expected depending on the length of the unfolding time. The results in Fig. 7 show that a 2-h reactivation time was needed for maximal recovery of the activity of LPL that had been denatured in 6 M GdmCl for 1 min. After denaturation for 2 h, a 2.5-fold longer reactivation time (5 h) was needed. Second, we investigated whether addition of a proline isomerase (bovine cyclophilin A) could accelerate the reactivation of LPL denatured for a long time (2 h). As shown in Fig. 7C, cyclophilin A significantly increased the reactivation rate of LPL in the presence of Ca$^{2+}$, but

**FIGURE 6. Reactivated LPL is dimeric.** Fully denatured LPL (10 μg/ml) was reactivated by incubation at 25 °C in 20 mM Tris-Cl (pH 7.4) containing 10% glycerol and 2 mM NaCl with (●) or without (○) 2.5 mM Ca$^{2+}$ for 5 h and was then subjected to sucrose gradient centrifugation. The sedimentation was compared with that of a standard containing both native bovine LPL and dissociated monomers (2 μg of each/200 μl) (■) run in another experiment. The results are the means from triplicate tubes for the two refolded samples. LPL activity (A) and mass (B) were measured in each fraction. Fraction 1 was collected from the bottom of the tube.

**FIGURE 7. Studies of possible rate-limiting steps for reactivation of LPL.** A, effect of LPL concentration on the reactivation rate of refolded LPL. The concentrations of LPL were 0.8 μg/ml (●), 4.0 μg/ml (■), and 8.0 μg/ml (▲). Reactivation was carried with 2.5 mM Ca$^{2+}$ in the presence of 0.1 mg/ml VLDL at 25 °C for the indicated times, followed by assay of LPL activity. The residual concentration of GdmCl was <0.1 M in all samples. Data are shown as the means ± S.D. (n = 3). B and C, effect of proline isomerization on reactivation of LPL. Bovine LPL (0.8 mg/ml) was denatured by addition of 8 M GdmCl in 20 mM Tris (pH 7.4) to a final concentration of 6 M and incubated for 1 min (○), 0.5 h (●), and 2 h (▲) at 4 °C. Refolding was initiated by rapid dilution in the refolding buffer containing 2.5 mM Ca$^{2+}$ at 25 °C. The residual concentration of GdmCl was 0.1 M. Aliquots were taken at different times and assayed for LPL activity (B). To investigate whether cyclophilin A could catalyze the reactivation of LPL that had been denatured in 6 M GdmCl, LPL was denatured for 2 h as described for B, but 6 M GdmCl was replaced with 8 M urea to avoid the inactivation of cyclophilin A. Reactivation of LPL by Ca$^{2+}$ was performed in the absence (○) or presence (▲) of 1 μM cyclophilin A (cyc, cycA). In addition, the reactivation was also checked in the presence of 1 μM cyclophilin A, but without Ca$^{2+}$ in the refolding buffer (●-●). LPL activity is expressed as a percent of the activity in the original nondenatured LPL protein. Data are shown as the means ± S.D. (n = 3).
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Three sources of monomeric LPL were used: 1) dissociated LPL in 1 M GdmCl prepared as described under “Experimental Procedures” and diluted to 10 μg/ml in 20 mM Tris-Cl (pH 7.4) containing 0.1 mg/ml VLDL and 2 mM NaCl, 2) a fraction of the monomer peak from human placenta (200 mg/ml) prepared as described under “Experimental Procedures” and supplemented with additional NaCl to a final concentration of 2 mM, and 3) heat-inactivated LPL (10 μg/ml) in 20 mM Tris-Cl (pH 7.4) containing 10% glycerol and 2 mM NaCl prepared as described under “Experimental Procedures.” All three samples were incubated with and without 2.5 mM Ca2+ prepared as described under “Experimental Procedures.” All three samples were incubated at 25 °C for 5 h. The recovery of activity was calculated from the LPL mass, as determined by enzyme-linked immunosorbent assay, assuming a maximal specific activity of 300 milliunits/μg LPL. Data are shown as the means ± S.D. of triplicate determinations.

Reactivation still did not occur without Ca2+. These results suggest that proline isomerization (rather than dimerization) is the rate-limiting step for the Ca2+-dependent reactivation of LPL from partially refolded monomers.

Ca2+ Triggers Reactivation of LPL Dissociated in 1 M GdmCl, Inactive Monomeric LPL Found in Human Tissues, and Thermally Inactivated LPL—It was important to investigate whether the effect of Ca2+ on LPL was general or whether it was confined to the partially refolded form of LPL obtained after full denaturation in GdmCl. Therefore, we studied the reactivation of monomers prepared by dissociation of dimeric LPL in 1 M GdmCl; monomers isolated from human tissues (placenta), and monomers created by thermal inactivation of LPL (25 °C, overnight). After dilution of LPL from 1 to 0.1 M GdmCl, a maximum of ~30% of the LPL activity was recovered upon incubation in buffer with Ca2+ (Fig. 8). In the absence of Ca2+, there was no reactivation. With inactive LPL isolated from human placenta, >10% of the expected activity was recovered upon incubation in the presence of Ca2+, whereas no activity was detected in the absence of Ca2+. In the case of LPL inactivated by temperature, <10% of the protein was reactivated. At higher concentrations of LPL and at higher temperatures, visible aggregates were formed. It was not possible to reactivate these aggregates simply by dilution and incubation with Ca2+, but solubilization of the protein in 6 M GdmCl and then dilution was necessary (data not shown). These results indicated that Ca2+ could trigger refolding of inactive monomeric LPL irrespective of how the monomer was formed.

DISCUSSION

In this study, we have demonstrated, for the first time, that inactive LPL can form active LPL dimers with the assistance of calcium ions. This was true for bovine LPL, fully or partially unfolded in GdmCl or by heat treatment, and for inactive LPL from human tissue. Apart from calcium, elevated concentrations of NaCl and the presence of stabilizers (such as serum, lipoprotein(s), heparin, glyceral, and BSA) were needed to obtain the maximal yield of active LPL. However, none of these components alone or in combinations were able to support reactivation of LPL if Ca2+ was not present. This suggests that these other factors either stabilize the correctly folded monomer or prevent aggregation of partially folded LPL molecules. It is well known that successful refolding of denatured proteins is strongly hampered by their propensity for misfolding and aggregation (39). We did not explore whether the stabilizing effect of VLDL was due to direct interaction with LPL or possibly to generation of small amounts of lipolytic products (such as free fatty acids and glycerol) during the long times required for refolding. Detergents (such as Triton X-100, SDS, and deoxycholate) and long chain fatty acids have previously been shown to increase the solubility and stability of LPL (40).

Fig. 9 summarizes schematically the molecular transitions that we have found in this and a previous study (18). Our data indicate that the refolding of fully denatured LPL into active dimers proceeds via two main steps: 1) a rapid formation of inactive monomers in the molten globule state and 2) a slow reactivation of the inactive monomers involving additional folding and dimerization. Only the second step is dependent on calcium. It was not possible to isolate the correctly folded and therefore dimerization-competent, monomeric, intermediate form. We could therefore not determine whether Ca2+ only promotes the necessary folding or whether Ca2+ is also directly involved in the dimerization process (Fig. 9, step 3). Furthermore, we could not deduce whether reactivation of dimers dissociated in 1 M GdmCl or dissociated by heating follows the same pathway as reactivation of fully denatured LPL (Fig. 9, step 4). Reactivation was independent of whether the LPL protein had been denatured in the presence of reducing agent to break intramolecular disulfide bonds. This was in accord with a previous study on the effects of molecular chaperons on the recovery of LPL activity from transfected cells (7). Although calreticulin increased the activity substantially, only marginal effects of coexpression with protein-disulfide isomerase were found, indicating that formation of the proper disulfide bonds was not rate-limiting for folding of LPL.

Studies by far-UV CD showed that most of the secondary structure of LPL was rapidly recovered when the protein was diluted from 6 to 1 M GdmCl with or without Ca2+. The recovery of tertiary structure did not fully reach the same state compared with monomers produced by dissociation of active dimers in 1 M GdmCl. The difference was not due to the presence of the remaining GdmCl because the intrinsic fluorescence of dissociated monomeric LPL did not change upon further dilution of GdmCl. In the absence of NaCl, the LPL protein precipitated when the concentration of GdmCl was decreased further, illustrating that the partially folded monomer was more prone to aggregate than the native protein. We found a similar behavior in a previous study in which we followed inactivation of LPL upon formation of dissociated monomers (18). The propensity to aggregate is probably due to exposure of a larger hydrophobic surface area, as was demonstrated previously for the dissociated monomer (18) and here for the refolded monomer.

Upon dilution of completely unfolded LPL, the λmax for intrinsic tryptophan fluorescence was immediately decreased and blue-shifted, indicating that, as expected, refolding restricted the exposure of Trp residues to water. The increase in binding of bis-ANS after dilution demonstrated that surface-exposed hydrophobic areas were restored upon refolding. Compared with the active dimeric form, the intrinsic fluorescence was less quenched in the refolded LPL monomer, and the
surface hydrophobicity was higher. This indicated that dimerization involved hydrophobic areas and that it required (or induced) a more compact folding. The refolded, monomeric, intermediate form is most likely in a molten globule conformational state, representing a free energy minimum. The kinetic barrier to reach the active dimeric state appears to be too high to occur at a reasonable rate without the presence of Ca$^{2+}$. Thus, the refolded monomer is dimerization-incompetent, like the dissociated monomer (18), explaining why inactivation of LPL has been previously considered to be irreversible.

In the presence of Ca$^{2+}$, active dimers were slowly formed from the partially refolded LPL monomers as demonstrated by sedimentation analyses and heparin-Sepharose chromatography. Conformational changes occurred as a result of Ca$^{2+}$ binding and could be observed by changes in intrinsic and extrinsic fluorescence. When EDTA was added during the refolding/dimerization process, the reactivation was immediately stopped. EDTA is often used as a component of the protease inhibitor mixture added to stabilize LPL in tissue extracts and in buffer solutions (8). Because previous and present experiences show that the presence of EDTA or EGTA is compatible with LPL activity, it must be concluded that chelatable Ca$^{2+}$ is not present in native LPL, but that Ca$^{2+}$ is transiently bound to the monomeric intermediate forms during the assisted refolding.

The study of extrinsic fluorescence showed that the surface hydrophobicity of refolded LPL decreased in a time-dependent manner during reactivation in the presence of Ca$^{2+}$ and that there was a slight red shift of $\lambda_{\text{max}}$ toward that seen for native LPL. These changes illustrate increased packing of the tertiary structure of LPL. In agreement with this, the recovery of activity was clearly both time- and temperature-dependent, indicating that the reactivation process required molecular motions. The relative reactivation rate was independent of the concentration of LPL, but was faster after short-term denaturation compared with long-term denaturation. This suggested that the population of monomers was heterogeneous upon initiation of refolding from fully denatured LPL. Only the subpopulation with proline residues in the native isomeric forms had a sufficiently low energy barrier to pass for dimerization/reactivation. In agreement with the conclusion that proline isomerization contributed to the slow kinetics of reactivation, accelerated recovery of LPL activity was seen upon addition of a proline isomerase (cyclophilin A). There was, however, no reactivation with cyclophilin A in the absence of Ca$^{2+}$, implying that reactivation of LPL required not only proline isomerization, but also other conformational changes in LPL. The main part of this rearrangement most likely occurred in the N-terminal domain. Based on the experiments with limited proteolysis, the C-terminal domain seemed to fold without assistance of Ca$^{2+}$, implying that reactivation of LPL required not only proline isomerization, but also other conformational changes in LPL. The main part of this rearrangement most likely occurred in the N-terminal domain. Based on the experiments with limited proteolysis, the C-terminal domain seemed to fold without assistance of Ca$^{2+}$, implying that reactivation of LPL required not only proline isomerization, but also other conformational changes in LPL. The main part of this rearrangement most likely occurred in the N-terminal domain. Based on the experiments with limited proteolysis, the C-terminal domain seemed to fold without assistance of Ca$^{2+}$, implying that reactivation of LPL required not only proline isomerization, but also other conformational changes in LPL. The main part of this rearrangement most likely occurred in the N-terminal domain. Based on the experiments with limited proteolysis, the C-terminal domain seemed to fold without assistance of Ca$^{2+}$, implying that reactivation of LPL required not only proline isomerization, but also other conformational changes in LPL. The main part of this rearrangement most likely occurred in the N-terminal domain.
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(6, 7, 41). We found that the Ca2+-assisted refolding of LPL appears to involve two steps, which may be similar to the two-step binding/release mechanism of the chaperone system in the endoplasmic reticulum. Interestingly, calreticulin is involved in Ca2+ homeostasis (42). Thus, the initial folding of LPL in the endoplasmic reticulum might be assisted by both chaperons and Ca2+.

Our data support the view that the active dimeric form of LPL is in a meta-stable, high energy state that easily falls down to the more stable monomeric state(s) in one way or the other (8, 18). Stein and co-workers (43) reported previously that LPL in medium from cultured heart cells lost activity more rapidly if the medium contained dialyzed rather than undialyzed serum and that recovery of LPL activity from the cells was restored if the dialysate was added together with the dialyzed serum. The authors concluded that a low molecular weight, positively charged monomeric state(s) in one way or the other (8, 18). Stein and co-workers (8, 18). Stein and co-workers (8, 18). Stein and co-workers (8, 18).

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