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LIPOPROTEIN LIPASE: ROLE OF INTRAMOLECULAR DISULFIDE BONDS IN ENZYME CATALYSIS

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SUMMARY. Lipoprotein lipase (LPL) catalyzes the hydrolysis of the triacylglycerol component of triacylglycerol-rich lipoproteins. There are 4 cysteine pairs that are completely conserved among LPLs of all species known. We examined the functional importance of each of the cysteine pairs in enzyme catalysis by examining LPLs produced in Cos cells by transfection. Immunoreactive LPL was produced by vectors encoding the wildtype LPL and each of the 4 cysteine-pair mutant LPLs. Enzyme activity was detectable in the wildtype enzyme, but not in 3 of the 4 Cys→Ser mutant enzymes (C216S/C239S, C264S/C275S, and C278S/C283S). Interestingly, LPL activity was also present in the mutant (C418S/C438S), which affects the C-terminal cysteine pair, with a specific activity ~50% higher than that of wildtype. There is evidence that LPL contains two distinct domains consisting of the N-terminal three-quarters of the sequence connected by a flexible region to the C-terminal domain comprising the rest of the molecule. The conservation of catalytic function despite the disruption of the only disulfide bridge in the C-terminal domain of LPL indicates that the two domains can function independently of each other in enzyme catalysis.

Lipoprotein lipase (EC 3.1.1.34) (LPL) is an endothelial enzyme that plays a central role in lipoprotein metabolism. It hydrolyzes the triacylglycerol component of circulating chylomicrons and very low density lipoproteins (1,2). The enzyme belongs to a lipase superfamily that comprises primarily LPL, hepatic triglyceride lipase and pancreatic lipase (3). All three enzymes contain a serine protease-like catalytic triad consisting of the residues Ser-His-Asp. Among the three lipases, LPL is the most conserved during evolution (3). The three-dimensional structure of pancreatic lipase has been determined by x-ray crystallography (4). Although the x-ray structure of LPL is unknown, analysis of a large number of site-specific mutants expressed in vitro indicates that LPL probably has a three-dimensional structure very similar to that of pancreatic lipase (5-9).

One structural feature in LPL and hepatic lipase is the occurrence of four intramolecular disulfide bridges all of which are conserved among all species examined (3). Pancreatic lipase has the same conserved disulfides, but it also contains two additional disulfide bridges in the N-terminal region of the molecules which are absent in the two vascular lipases (LPL and hepatic lipase) (3). Intramolecular disulfide bridges are generally felt to be important for maintaining the properly folded conformation of proteins. In this communication, we examined the functional role

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of the four disulfide bridges in LPL with respect to its enzymatic activity by expressing site-specific human LPL mutants involving each of the cysteine pairs. We found that although all four disulfides were conserved in evolution, only three of them seemed to be required for the production of an active enzyme. Elimination of the C-terminal disulfide appears not to impair the catalytic function of LPL. This observation has interesting implications for the structure-function relationship of LPL.

MATERIALS AND METHODS

Human LPL cDNA expression vector. A 1786-bp human LPL cDNA was subcloned into M13mp19 and used as a template for site-specific mutagenesis. It contains 320 nucleotides in the 5' untranslated region, the entire coding region, and 38 nucleotides in the 3' untranslated region, and is bounded by an artificial EcoRI site in the 5' end and a natural EcoRI site in the 3' end (Figure 1).

Oligonucleotides (Table 1) synthesized on an Applied Biosystems Inc. 391 DNA synthesizer were 5'-phosphorylated and annealed with a single-stranded template. Mutagenesis was carried out as described by Kunkel (10). Mutant and wildtype LPL cDNAs were used to transfect E.coli CJ263 cells, and positive clones were identified by direct sequencing. Replicative form DNAs were isolated, digested with EcoRI, and inserted into the EcoRI site of p91023(B) (11). After transformation of E.coli DH5α cells, positive clones were isolated, and orientation of inserts was determined by restriction mapping.

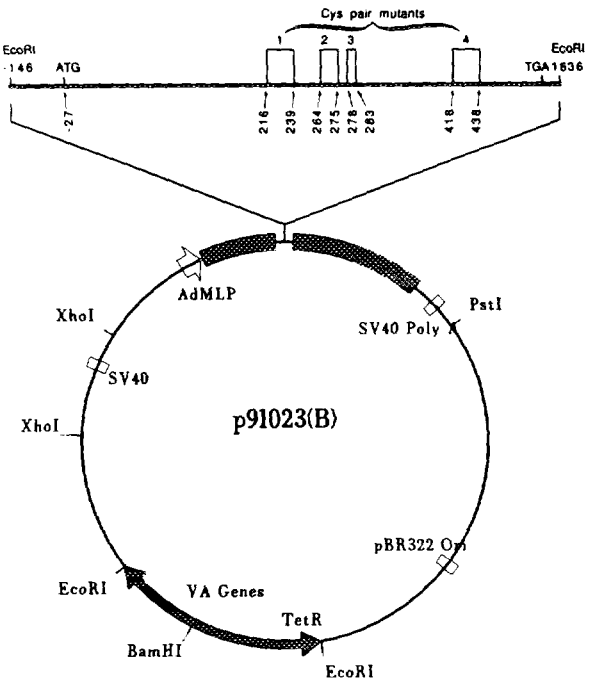

Figure 1. Map of human LPL expression vector. The vector is based on p91023(B) previously described by Wong et al. (11). The amino acid residue numbers of the 4 cysteine pairs (#1→4) are marked below the cDNA insert. The ATG codon is marked residue no. -27 because of a 27-residue signal peptide in human LPL.

Table 1. Oligonucleotides for site-specific mutagenesis

1.	$Cys^{216}\rightarrow Ser$	5' TTT CAG CCA GGA TCT AAC ATT GGA GAA 3'
	$Cys^{239}\rightarrow Ser$	5' CAG CTA GTG AAG TCC TCC CAC GAA CGC 3'
2.	$Cys^{264\&275}\rightarrow Ser$	5' GCC TAC AGG AGC AGT TCC AAG GAA GCC TTT GAG AAA GGG CTC AGC TTG AGT TGT 3'
3.	$Cys^{278\&283}\rightarrow Ser$	5' CTC TGC TTG AGT AGT AGA AAG AAC CGC AGC AAC AAT CTG GGC 3'
4.	$Cys^{418}\rightarrow Ser$	5' GTG ATC TTC AGT TCT AGG GAG 3'
	$Cys^{438}\rightarrow Ser$	5' TTT GTG AAA AGC CAT GAC AAG 3'

In vitro expression, LPL enzyme activity assay and quantitation of protein mass. COS M-6 cells (0.6-1 x 10^6 cells) were plated on T75 tissue culture flasks for 3 days and transfected with 20 μg of plasmid DNA per flask by the technique of Okayama et al. (12) using the DEAE dextran/dimethyl sulfoxide shock followed by a 3 h incubation with chloroquine. In all experiments, collecting media contained sodium heparin (40 μg/ml). The control flasks were subjected to the transfection protocol with p91023(B) only.

After 65 h transfection, aliquots of culture media were collected and cells were washed with phosphate-buffered saline, scraped into 2 ml of 25 mM NH_3/NH_4Cl (pH 8.1) containing 5 mM EDTA, and per ml: 40 μg heparin; 10 μg leupeptin; 1 μg pepstatin; 8 mg Triton x-100. Media and cell extracts were then flash-frozen in a dry ice/ethanol bath and stored at -70°C. Just before determination of LPL activity or protein mass, samples were thawed in ice water, made 0.2% in sodium deoxycholate, and rotated at room temperature for 5-10 min. Cell extracts and media were assayed for LPL enzyme activity as previously described (13). LPL activity is expressed in milliunits (1 milliunit = 1 nmole of fatty acid released/min). Rat serum (Sigma) was used as a source of apolipoprotein C-II in all the LPL assays. As a control, human LPL was purified from transfected CHO cells and assayed under the same conditions. LPL mass in media was determined by standard ELISA using human LPL as standard. A monoclonal antibody raised against purified bovine milk LPL (monoclonal antibody 40) (14) was used for the assay. LPL mass in cells was determined by sandwich ELISA (5). Monoclonal antibody 40 was used as first antibody coated on plates and polyclonal antibody generated from chicken against human LPL purified from transfected CHO cells was used as a second antibody to bind antigen (15).

RESULTS AND DISCUSSION

The human LPL cDNA was cloned into the EcoRI site of the expression vector p91023(B) which had been used to produce high level expression of a number of proteins (11). It gives good level expression of human LPL. In this series of experiments, ~90% of the LPL activity was secreted into the medium and ~10% was extracted from the cells. The secreted LPL activity and that extracted from the transfected COS cells were stimulated about 4-5 fold by the addition of apolipoprotein C-II (provided in the form of rat serum, which by itself had no LPL activity), a known activator of LPL (16). Activity increased from 220.56 mU/dish to 894.72 mU/dish in an experiment using the wildtype expression vector in a pilot experiment.

Site-specific mutant human LPL constructs were used for expression in parallel experiments. The four pairs of Cys residues were mutated in turn to Ser pairs. As shown in Fig. 2, transfection using three of the four mutant LPL constructs (nos. 1→3) failed to produce any detectable LPL activity either in the medium or intracellularly. Interestingly, the last mutant construct (no. 4), C418S/C438S, produced an enzyme with readily detectable LPL activity at about 85% of the wildtype LPL, both intracellularly and in the culture medium (Fig. 2).

To determine whether the absence of LPL activity following transfection of the first three mutant LPL constructs was caused by a complete failure of production of the mutant LPL proteins, or by the production of inactive mutant enzymes, we measured the mass of immunoreactive LPL

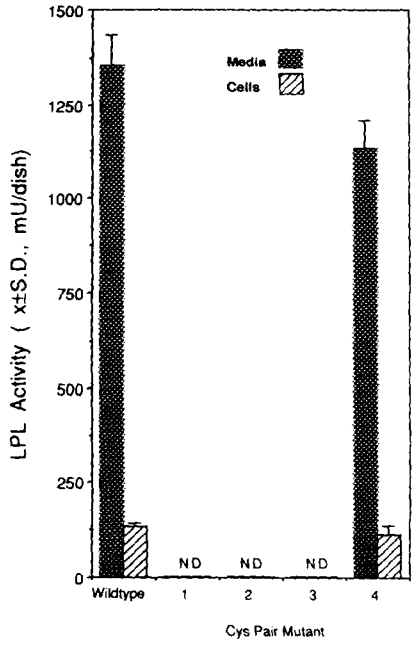

Figure 2. LPL enzyme activity in transfected Cos cells. Cys pair mutants are: 1, C216S/C239S; 2, C264S/C275S; 3, C278S/C283S; and 4, C418S/C438S.

(Table 2) using an ELISA assay as described in Materials and Methods. The assay was linear and could detect 0.5 ng of protein. We found that these three mutant constructs directed the production of diminished amounts of LPL immunoreactive protein which was, however, readily detectable by our assay. Therefore, mutant human LPLs with replacements of each of the N-terminal three Cys pairs by Ser pairs were enzymatically inactive.

We next quantified the amount of immunoreactive LPL produced by the LPL mutant construct (no. 4) containing a pair of Ser residues replacing the C-terminal Cys pair. We found

Table 2. Enzyme activity, immunoreactive mass, and specific activity of wildtype and mutant hLPL produced in COS M-6 cells

	LPL Activity (milliunits/dish)		LPL Mass (μg/dish)		Specific Activity (milliunits/μg)	
	media	cells	media	cells	media	cells
Wildtype	1355.7 ± 78.3	133.2 ± 9.2	7.6	0.81	183.9 ± 33.5	163.6 ± 11.0
Cys→Ser Mutants						
1. 216 & 239	0	0	1.6	0.22	0	0
2. 264 & 275	0	0	1.7	0.16	0	0
3. 278 & 283	0	0	2.2	0.41	0	0
4. 418 & 438	1132.1 ± 76.6	114.7 ± 22.2	3.8	0.47	287.8 ± 37.0	241.9 ± 39.0

These results were calculated on one set of experiments with triplicate transfections. Values are mean ± S.D.

that this mutant LPL was produced at ~50% of the wildtype enzyme both intracellularly and in the medium. Since its total activity was ~85% of wildtype, the specific activity of this mutant LPL was actually ~50% higher than wildtype both intracellularly and following secretion in to the medium (Table 2).

All four Cys pairs in LPL examined in this study are completely conserved in five vertebrate species including human, bovine, mouse, guinea pig and chicken (3). In a previous study on the analysis of LPL structure, we constructed a conservation index (CI) to display conserved features spanning the entire LPL sequence across the five vertebrate species, a CI of 1.0 signifying perfect conservation (i.e., complete identity) of a rolling average of a window of 9 amino acid residues (3). We found that all eight Cys residues occur at or near individual peaks of CI. This observation strongly suggests that the four pairs of Cys residues are probably extremely important for proper LPL folding and function. The complete loss of activity of LPL mutants (nos. 1→3) involving the three N-terminal Cys pairs is consistent with this interpretation. Moreover, these Cys residues occur within the central very highly conserved region of the lipase superfamily. The first Cys pair (C216/C239) flanks a surface loop shielding the catalytic center (7). This surface loop, which occurs in all lipases, must be repositioned before the substrate can gain access to the catalytic residues (4). Replacement of the Cys pair flanking this loop probably affects the ability of this loop to assume the correct position for enzyme catalysis, and explains the total loss of catalytic activity in this LPL mutant (no. 1).

We believe that the most interesting observation of this study is the fact that removal of the C-terminal disulfide bridge does not have any apparent deleterious effect on the catalytic activity of the LPL. In fact, the mutant LPL without this disulfide bridge is some 50% more active than the wildtype enzyme. The last two Cys residues comprising this disulfide bond are also located at the peaks of CI, indicating that they are in regions of relative conservation during evolution. We note, however, that the height of the CI peak for the last Cys (C438) is relatively low at around 0.6, whereas all the other Cys residues except Cys 264 were located within CI values of 0.8 or higher. This somewhat lower conservation of sequences flanking the last Cys may reflect the fact the elimination of this last disulfide bond does not appear to be detrimental to LPL enzyme activity.

The crystal structure of pancreatic lipase indicates that the protein can be separated into two distinct domains, an N-terminal domain comprising the N-terminal three quarters of the sequence connected to a separate domain comprising the C-terminal quarter of the molecule by a flexible connecting peptide (4). An analogous domain arrangement in LPL is strongly supported by data generated from site-specific mutant LPLs (5 - 9) and LPL/hepatic lipase chimeric molecules where an LPL N-terminal domain is ligated to a hepatic lipase C-terminal domain and vice versa (17). The N-terminal domain contains the catalytic center of the molecule whereas the C-terminal domain may function as a modulator of substrate specificity and heparin-binding. LPL, hepatic lipase and pancreatic lipase show considerable secondary structural and functional similarity (but little primary sequence homology) to a number of fungal lipases (18, 19), both classes of lipase showing a conserved β-εSer-α motif (20, 21) that must have been the product of convergent evolution. It is noteworthy that the fungal lipases share the α/β-hydrolase fold structure of the N-terminal domain of LPL/hepatic lipase/pancreatic lipase superfamily, characterized by a catalytic core formed by β-

strands connected by α-helices. It is also noteworthy that the fungal lipases do not have a second domain corresponding to the C-terminal domain of LPL (and hepatic lipase as well as pancreatic lipase). Our experiments indicate that, indeed, disruption of the structure of the C-terminal domain of LPL by removal of its only intramolecular disulfide bond does not impair catalytic activity. This observation supports the conclusion that the N- and C-terminal domains of LPL can function independently of each other in enzyme catalysis.

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