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A New Mutation Destroying Disulphide Bridging in the C-Terminal Domain of Lipoprotein Lipase

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Lipoprotein lipase (LPL) is one of two intravascular lipases involved in the lipolysis of the triglyceride core of circulating lipoproteins. The occurrence of patients with genetic deficiencies has provided insight into the structure and function relationships of this lipase. It is now known that LPL manifests a two domain structure with the N-terminal domain of greater structural and functional significance as it contains the active site and interfacial binding motifs. We report on a Cys418Tyr substitution in the C-terminal domain which disrupts the only disulphide bridge in the region and is associated with catalytic deficiency in post-heparin plasma. This result was unexpected as previous *in vitro* assessment of the functional significance of disulphide bridging had shown that while the 3, N-terminal disulphides were critical for enzyme function, loss of the only C-terminal disulphide minimally affected catalytic activity. We generated the Cys418Tyr mutant by site-directed mutagenesis and show that it manifests 48% of normal activity *in vitro*, while the companion variants, Cys438Ser and Cys418Ser-Cys438Ser, are less affected with activities at 76% and 78% of normal.

An absence of lipoprotein lipase (EC 3.1.1.34) (LPL) activity at the capillary endothelium results in Type I hyperlipoproteinemia, as characterised by chylomicronemia and very low levels of LDL and HDL cholesterol. Familial deficiencies generally manifest in childhood as episodes of eruptive xanthomata, abdominal pain and pancreatitis (1). Biochemically, patients have marked post-prandial chylomicronemia which persists into the fasted state, with triglyceride concentrations typically in excess of 15 mmol/l.

Chylomicronemic patients have been investigated in many laboratories, and over 50 mutations in the LPL gene have now been described and summarised in recent reviews (2,3). The majority of the amino acid substitutions described are localised to residues of the N-terminal domain and the resulting disruption of lipolytic activity attests to the functional and structural significance of peptide folding motifs of this domain (4,5,6). However, the bulk of data on the structure/function dynamics of LPL have been derived by extrapolation from the molecular model of the closely related pancreatic lipase (7,8) and from the study of laboratory generated variants which have been expressed in transfected COS cells. These studies have highlighted critical residues of the active site, interfacial binding region, active site loop, and heparin and lipid binding motifs. Recently the role of disulphide bridging in the functional stability of LPL was determined *in vitro* by generating variants with individually substituted cysteine pairs (9). LPL contains 4 disulphide bridges, 3 of which stabilise the folding of the N-terminal section with the fourth being in the smaller C-terminal domain. While disulphide bridging in the

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former was found to be critical for catalytic function, substitution of the C-terminal disulphide seemed to have minimal effect on lipolytic activity.

We now report on a patient who carries a C-terminal mutation which destroys the disulphide bridge in this region and show that this mutant is not active *in vivo.*

MATERIALS AND METHODS

The patient. The subject investigated is a 30 year old South African male of Dutch and Malay ancestry who was diagnosed as LPL deficient in childhood (10,11). The hypertriglyceridemia was discovered incidentally and only exceeded 15mmol/l after puberty when pancreatitis also occurred for the first time. Previous mutation analysis had revealed heterozygosity for the Ile194Thr substitution in exon 5 of the LPL gene (12). This mutation was shown, through transfection studies in COS cells, to yield a catalytically defective LPL species.

DNA analysis. Individual exons of the LPL gene were amplified and sequenced by standard methodologies using PCR primer sets and sequencing primers as described (13). Nucleotide and codon numbering was taken from the LPL cDNA sequence (GenBank accession number M15856).

RT-PCR of LPL mRNA was carried out on RNA isolated from cultured monocyte macrophages as described (14). Total RNA (1μg) was used for first strand cDNA synthesis with LPL cDNA being amplified in two overlapping segments using the primer set LPLcDNA6/7 for a 1047bp 5′ fragment with Sal 1 and Pst 1 terminal cloning sites and the primer set LPLcDNA1/8 for a 1026bp 3′ fragment with Bam H1 and Eco R1 terminal cloning sites.

LPLcDNA6. 5′-TCAGTCGACTTGCTCAGCGCCAAAC-3′

LPLcDNA7. 5′-TTGCTGCAGCGGTTCTTTCTACAAC-3′

LPLcDNA8. 5′-CTAGGATCCATCTCTTGGGATACAGCC-3′

LPLcDNA1. 5′-TTCTTCACAGAATTCACATGCCG-3′

In-vitro mutagenesis and COS-1 cell transfections. For this study the entire coding sequence of LPL was cloned into the pcDNA3 vector (Invitrogen, San Diego, CA). Three LPL mutant cDNAs were generated by site-directed mutagenesis, one to replicate the Cys418Tyr substitution of our patient, another Cys438Ser, to substitute the companion cysteine and a double mutant Cys418Ser-Cys438Ser to remove both cysteines. While all three mutants effectively destroyed disulphide bridging the single and double serine mutants further minimised side chain distortions by exchanging hydroxyls for the thiol groupings. The Ile194Thr cDNA had been generated in a previous study and was used here as a positive control for catalytic deficiency in LPL. Mutant and normal cDNAs were introduced into COS-1 cells by electroporation as described (12).

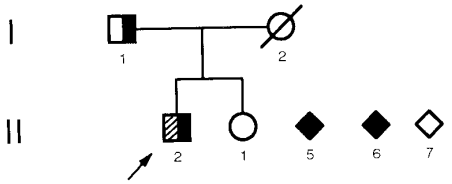
LPL activity and mass assays. LPL activity was determined in post-heparin plasma and COS cell culture medium using a radiolabelled ^{14}C-triolein/phosphatidyl choline emulsion as previously described (15). LPL activity in post-heparin plasma samples was taken as that fraction of the total lipolytic activity inhibited by the LPL monoclonal antibody 5D2 and was recorded as *nmol FFA/min/ml* plasma. For media, activities were calculated as *nmol FFA/min/dish* but were normalised against the activity of the COS cell transfection medium from the normal LPL construct which typically gave between 200-440 *nmol FFA/min/dish.* All samples were snap frozen in liquid N_2, stored at −70deg and thawed immediately before assay.

LPL mass in post-heparin plasma was calculated as dimer mass using the 5D2 monoclonal antibody (16) and recorded as *ng/ml* plasma. LPL mass in the COS cell transfection medium was calculated as total monomer mass using the 5F9 monoclonal antibody following guanidinium hydrochloride denaturation (17). Values are recorded as a percentage of the monomer mass in the COS cell transfection medium from the normal LPL construct.

Specific activities. The specific activities recorded for LPL in the COS cell media represent the ratio of normalised activity to that of the normalised total (monomer) mass. The specific activity of the LPL product of the normal construct is therefore, equal to 1.0.

RESULTS AND DISCUSSION

LPL activity in post-heparin plasma from our patient has been determined on several occasions and has consistently been found to be zero with near normal levels of heparin releasable mass (Fig. 1). Mutation analysis was therefore expected to uncover one or two catalytically defective LPL sequence variants. Determination of LPL cDNA sequence in this study, revealed a G→A transition at nucleotide position 1508. This resulted in a substitution at residue 418 of tyrosine for cysteine, which abolished the single conserved disulphide bridge in the C-terminal



Plasma Triglyceride	8.1	27	1.2	46	33	1.5
Plasma Cholesterol	8.8	12.4	5.4	9.3	5.2	5.6
LPL Activity	110	0	92	0	0	214
HL Activity	124	237	178	242	412	226
*LPL Mass	426	229	75	98	327	276

FIG. 1. Plasma lipid and lipase data for the patient, his family, two non-related LPL deficient subjects and a normal control. All samples were assayed in the laboratory of Dr. J. D. Brunzell, University of Washington, Seattle. *LPL mass represents the heparin releaseable mass. Our patient is shown as a compound heterozygote. Subject II5 and II6 are homozygous for the Gly188Glu and Ile194Thr mutations, respectively. Some of these data have been published previously (12). (□■) Ile194Thr allele; (▨■) Cys418Tyr allele.

domain (Fig. 2). Our patient is thus a compound heterozygote as he was previously found to be a carrier of the catalytically defective Ile194Thr substitution (12).

COS cell transfection experiments were carried out to determine the effect of the Cys418Tyr substitution, and the subsequent loss of disulphide bridging, on the catalytic function of LPL. This variant consistently registered a lower level of activity in the transfection media than the control LPL, with the average of 11 separate transfections being 48±7.4% of the control mean (Fig. 3). The Cys438Ser companion mutant and the double mutant, Cys418Ser-Cys438Ser were also found to retain catalytic activity but registered milder defects, giving respective activities of 76±10.4% and 78±23% of the control mean. (Fig. 3). While all three mutants lack disulphide bridging in the C-terminal domain, the lower activity of the Cys418Tyr variant

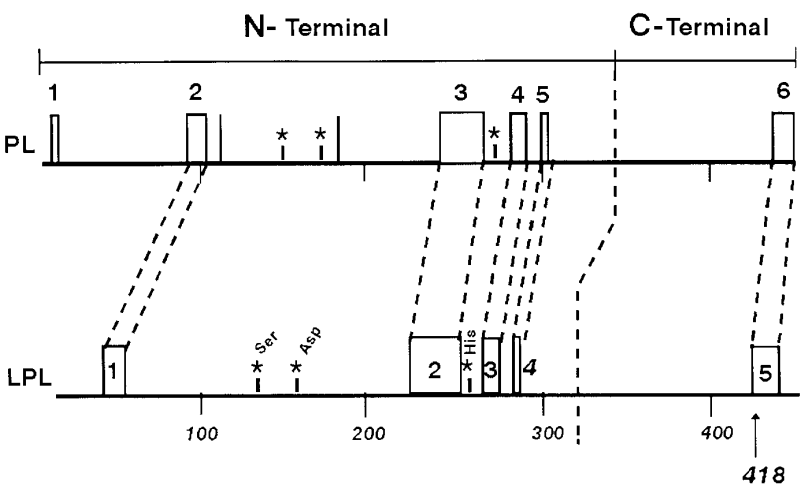


FIG. 2. A schematic comparison of the conserved disulphide bridges of PL and LPL. Cysteines are linked at their apices. Free cysteines are represented by vertical bars only. The catalytic triad residues are depicted as short bars topped by an asterisk. Disulphide bond positions are loosely to scale.

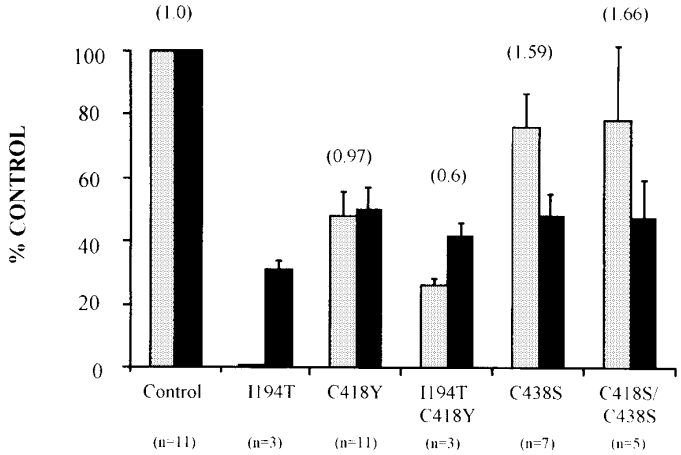


FIG. 3. LPL activity and mass levels in the COS cell transfection media. Results given as the mean plus standard deviation. The normalised specific activities are given in parentheses. Activities are depicted by the light panels and mass levels by the dark panels. I, Isoleucine; T, Threonine; C, Cysteine; Y, Tyrosine; S, Serine.

may be due to greater destabilisation of secondary or tertiary structure by the aromatic side chain and the lower hydrophobicity index of the tyrosine residue.

The total protein mass level of the Cys418Tyr mutant was also lower at 50±7% (n=11) than the mass level of the control LPL. This decrement closely parallelled the magnitude of the reduction in lipolytic activity giving a normal specific activity for the variant. In contrast, while the medium total monomer mass levels for the Cys438Ser and Cys418Ser-Cys438Ser mutants were also less than control LPL at 48±7.3% and 47±12.3% respectively, their mass reductions were proportionally greater than the corresponding activity losses, thereby generating specific activities significantly higher than 1.0 (1.78 and 1.69 respectively).

The normal specific activity of the Cys418Tyr mutant implies normal kinetics in enzyme secretion by COS cells and in the subsequent dissociation of the secreted active dimer into inactive monomer. Both the other mutants however, gave increased comparative specific activities, which could suggest that their dimer forms manifest heightened stability in the COS cell media. However, this appears unlikely as we have incubated aliquots of the harvesting media from each of these mutant enzymes (37deg for up to 2hrs) and have recorded reductions in activity (approx. 45%), equivalent to that of normal LPL (data not shown). Whatever the nature of this phenomenon, it is consistent, as an earlier study also reported a 50% increase in the specific activity of the identical 418/438 double cysteine mutant (9) using a different set of capture and tagged antibodies in their ELISA for mass determination.

The most interesting observation in this study is the demonstration that the Cys418Tyr mutant retains a substantial level of activity in the COS cell experiments but is not active in the patient, as shown by the classical Type I phenotype now manifested and the consistent, recent documentation, of zero activity in post-heparin plasma. Theoretically, our patient should have yielded activity levels between 12 and 25% of normal, given the 48% of normal activity recorded for the mutant *in vitro.* Low levels of this magnitude are well within the discriminatory capability of the lipolytic assay used in this study. LPL activity levels recorded in post-heparin plasma collected from Type I patients have thus far, shown good correlation with the activities recorded for the mutant enzymes when expressed in the COS cell transfection system. This has been true for patients with zero LPL activity and is proving consistent as increasing numbers of patients with partial deficiencies as low as 6% of normal, are being

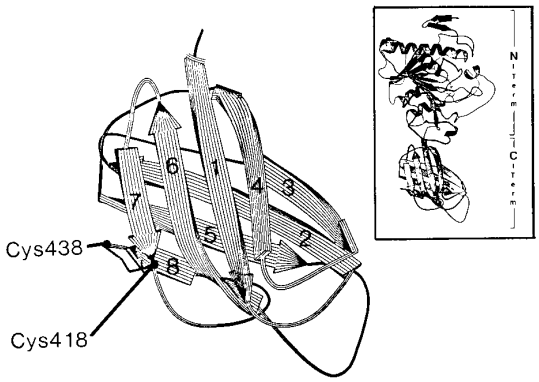


FIG. 4. An alpha-carbon ribbon diagram of the C-terminal domain of human pancreatic lipase as published (7). The location of cysteines 418 and 438 of LPL are indicated on the diagram where they have been superimposed on the homologous cysteines of PL which occur at positions 433 and 439, respectively. A ribbon diagram of the entire PL molecule is given in the inset and clearly shows the structural division into N- and C-terminal domains.

investigated at the molecular level (18,19,20,21). The discrepancy manifest in our study may be one of the first indications that not all mutant lipases behave similarly in the two systems. It is possible that the post translational processing and transport of this particular LPL variant is abnormal and has not been adequately tested by the monolayer COS cell cultures. The further possibility of a dominant negative effect in our patient, through pairing of the Cys418Tyr allele with the Ile194Thr mutation, is discounted by our transfection data. If this were the situation, co-transfection experiments with the Ile194Thr construct would, most likely, have abolished the activity of the secreted hetero-dimer. Our *in vitro* experiments however, gave a secreted product with a 40-50% reduction in lipolytic function, discounting a dominant negative effect (Fig. 3).

Our patient is a compound heterozygote for two missense mutations. Accordingly, we cannot determine whether the significant amount of heparin releasable LPL mass (229 ng/ml; Fig. 1) represents the product of one or both mutant alleles, particularly as the catalytically defective Ile194Thr mutant shows little impairment in transport to the capillary endothelium (12). This distinction will only be realised when the Cys418Tyr mutation appears in a patient in the homozygous form or is coupled with a frameshift or deletion mutation, leading to the absence of a gene product from the alternate allele.

Details of the disulphide bridging patterns of LPL (4) have been derived from the molecular model of the closely homologous pancreatic lipase (PL). The crystal structure of PL shows a total of 14 cysteines, 12 of which are in disulphide linkage with 5 pairs occurring in the N-terminal domain, and only one in the C-terminal region (Fig. 2). The primary sequence of LPL shows 4 fewer cysteines but all 10 thiols are disulphide bridged to give five pairs, analogous to those of PL (Fig. 2). Our data clearly shows that the loss of C-terminal disulphide bridging in LPL, does not completely destroy catalytic activity *in vitro* indicating that conformational change induced in the C-terminal peptide folding pattern does not exceed the structural tolerances which govern the functionality of this region. While speculation on the nature of this conformational change in LPL through reference to the molecular model of PL (Fig. 4), must be treated with caution, it is likely that the loss of disulphide bridging will lead to separation of the weakly hydrogen bonded *beta*-strands 8 & 5, and a destabilisation of secondary structure as far back as the end of *beta*-strand 7. Further destabilisation is unlikely as the anti-parallel strands 6 and 7 are extensively hydrogen bonded. While this is evidence for some

tolerance in the loss of secondary structure in the terminal 25-30 amino acids of LPL, a loss of primary sequence through premature termination is not compatible with catalytic activity *in vitro* if more than the terminal 10-12 residues are removed (22). In contrast to the C-terminal domain, the disruption of disulphide bridging for any of the three cysteine pairs of the N-terminal domain completely abolishes normal catalytic function (9). These Cysteine pairs occur within the central highly conserved region of this domain and must therefore be essential for the normal folding and function of the active site motifs.

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