

# Structure–function relationships of lipoprotein lipase: mutation analysis and mutagenesis of the loop region

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**Abstract** The molecular models of two microbial lipases and human pancreatic lipase (PL) have suggested the existence of common structural motifs including a buried active site shielded by an amphipathic surface loop. In an effort to explore the role of residues comprising the loop of lipoprotein lipase (LPL), we have used site-directed mutagenesis to generate three new LPL variants. In variant LPLM1 we deleted 18 amino acids leaving a loop of only 4 residues which resulted in an LPL protein inactive against triolein substrates. In contrast, two other LPL variants with only partial deletions, involving the apical section of the loop {LPLM2 (–8 amino acids) and LPLM3 (–2 amino acids)} manifested normal lipolytic activity. These findings indicate a critical requirement for the maintenance of charge and periodicity in the proximal and distal segments of the LPL loop in normal catalytic function. This is further highlighted by the detection of a mutation in the proximal section of the loop in a patient with LPL deficiency at position 225 which results in a substitution of threonine for isoleucine. ■ The intact catalytic activity of the partial deletion variants (LPLM2 and LPLM3) further suggests that the apical residues of the loop contribute minimally to the functional motifs of the active site. We support this postulate by showing that the conserved glycine in the apical turn section (G229) can be substituted by glutamine, lysine, proline, or threonine without significantly affecting catalytic activity.—Henderson, H. E., Y. Ma, M.-S. Liu, I. Clark-Lewis, D. L. Maeder, J. J. P. Kastelein, J. D. Brunzell, and M. R. Hayden. Structure–function relationships of lipoprotein lipase: mutation analysis and mutagenesis of the loop region. *J. Lipid Res.* 1993. 34: 1593–1602.

**Supplementary key words** gene defect • mutation • mutagenesis

Numerous lipases are active in the metabolism of dietary and endogenously synthesized triacylglycerols. Prominent amongst these in humans are the intravascular lipases, lipoprotein lipase (LPL) and hepatic lipase (HL), which function as pivotal enzymes in the metabolism of lipoprotein triacylglycerol and phospholipid. Both these

enzymes are close homologues of pancreatic lipase (PL) with which they form part of a dispersed gene family (1–3).

Lipoprotein lipase is the most comprehensively studied member of this gene family and structure–function studies at the protein level have revealed the presence of several functional domains. These include an active site, sites for the binding of polyanions such as heparin and heparan sulfate, an activator polypeptide (apoC-II), lipoproteins (interfacial binding site), and free fatty acids (4). Evidence in favor of specific residues involved in these functional domains has come from a variety of sources that include comparative analyses of the cDNA-derived amino acid sequences of LPL from human (5) and other mammalian species such as cow (6) and mouse (7), the characterization of naturally occurring mutations in the genes for LPL and HL (8–10), and the study of in vitro-generated variants of LPL and HL (11–14).

Furthermore, the high degree of amino acid homology among LPL, HL, and PL has allowed for the identification of functionally important residues by extrapolation from the molecular model of PL (15).

It is now known that the enzymes in this family each comprise two structural domains with the larger N-terminal sections containing the active sites. These sites manifest a common catalytic triad of serine, aspartic acid, and histidine. The N-terminal domain also carries some of the

Abbreviations: PL, pancreatic lipase; LPL, lipoprotein lipase; HL, hepatic lipase; PCR, polymerase chain reaction.

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residues of the polyanion binding sites and this same domain is presumed to interact with the activator polypeptide, apoC-II of LPL (14). In contrast, PL complexes with its activator, colipase, through residues on the C-terminal domain (16).

The amino acid sequences of numerous prokaryote and eukaryote lipases are now known (17, 18). Only one region of significant amino acid homology exists in all the lipases examined to date and comprises the active site serine and the immediate flanking residues (19). The molecular model of PL (15) shows that this conserved section is part of the multi-stranded beta sheet structural core of the catalytic domain and presumably is also part of the central domains of other lipases.

The three-dimensional structure of two fungal lipases, *R. miehei* and *G. candidum* has also recently been reported (20–22) and it is evident that several structural motifs are conserved in the lipolytic enzymes. One of these is a short amphipathic loop(s) that overlays the channel leading to the residues of the catalytic triad where it functions as a “lid.” The active sites are therefore buried in their respective molecules and are not accessible to lipid substrates; access must involve a repositioning of the flexible sections at the lipid–water interface. This is clearly demonstrated by the structural model of *R. miehei* where crystallization as a transition state complex reveals an exposed active site and a displaced loop that rests in an adjacent polar cavity (21, 23). Repositioning of the loop in the *R. miehei* lipase not only provides substrate access but also expands the hydrophobic surface around the active site. This expanded surface area presumably interacts with the non-polar core of the lipid micelle thereby stabilizing contact between enzyme and substrate. This interaction additionally creates a hydrophobic “seal” around the catalytic pocket that likely excludes bulk water, thereby allowing access of triglyceride to the active site through diffusion. This hypothesis of lipase action at the lipid–water interface predicts a crucial role for the loop section in masking the hydrophobicity of the active site and in stabilizing the interfacial binding region.

The loop region of LPL comprises 22 amino acids and is formed by disulfide bridging between Cys216 and Cys239. These residues are coded for by exons 5 and 6 which together with exon 4 code for some of the most conserved regions of the LPL sequence. It is not surprising, therefore, that approximately 90% of the missense mutations described in patients who manifest with LPL deficiency are found in these exons (8, 9). Many of these mutations occur between residues Ser172 and Gly210 in exon 5 which represents the longest conserved segment between LPL of different species. In contrast, no mutations have yet been described in the loop region which is poorly conserved between species.

In this manuscript we report on the first naturally occurring point mutation within the LPL loop region at

position 225, which significantly impairs catalytic activity towards long-chain triacylglycerol substrates. In contrast, we also show that the conserved glycine at position 229 is not essential for normal activity. We further show that deletion of 18 of the 22 amino acids from this region yields a defective enzyme while two smaller partial deletions are well tolerated. These data indicate variability in the structure–function constraints of different loop residues and also suggest that the residues deleted from the loops of the active mutants contribute little to the structural integrity of the interfacial binding region and the functional motifs of the active site.

## MATERIALS AND METHODS

### LPL mutagenesis

Site-directed mutagenesis of the full-length LPL cDNA cloned into the CDM8 expression vector was carried out as described (24, 25) where the two-primer method of Zoller and Smith (26) was used. Mutagenic primers for the loop deletions were chosen to contain 15 or 18 bases flanking the section of cDNA to be deleted. The amino acid residues substituted in generating the missense mutations and those deleted in creating the loop variants are shown in Fig. 1.

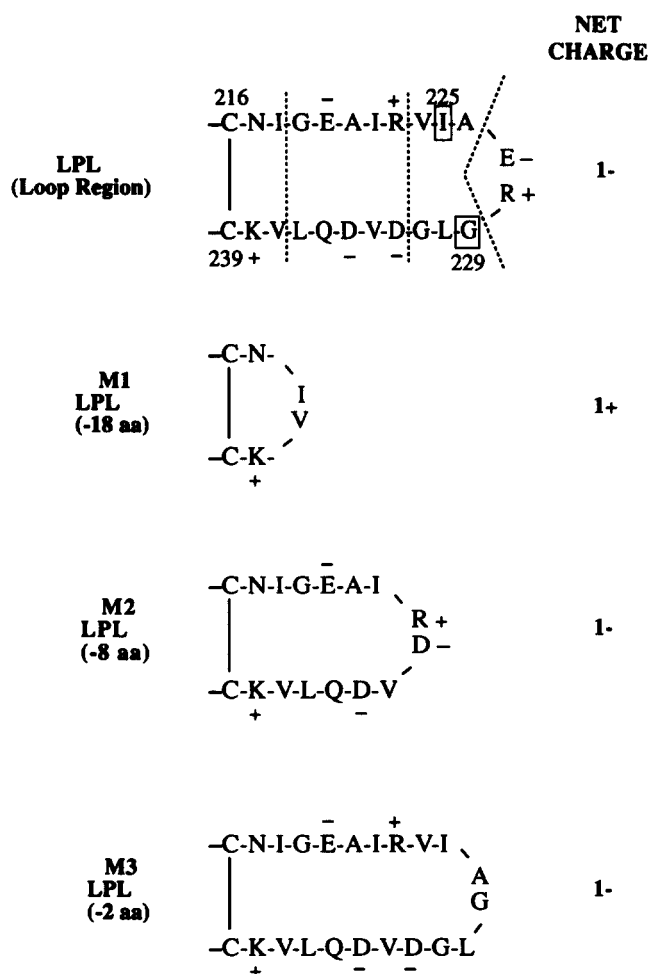
The I225→T substitution was created with primer (A) while the conserved glycine at position 229 (codon GGA) was substituted by lysine (AAA), proline (CCA), glutamine (CAA), and threonine (ACA) by using the degenerate oligomer (B). Loop mutant LPLM1 contained a deletion of 18 amino acids (–18 aa) being residues G219 to L236 and was generated using primer (C); mutant LPLM2 (–8 aa; residues V224 to G231) was generated by primer (D) and LPLM3 (–2 aa; residues E227–R228) using primer (E).

The nucleotide sequences of the 5 oligomers used were: (A). 5'-GCTATCCGCGTGACTGCAGAGAGAGGA-3' (27 bp); (B). 5'-TGCAGAGAGA(C/A)(C/A)ACTTGGAGAT-3' (22 bp); (C). 5'-CAGCCAGGATGTAACATT/GTGAAGTGCTCCCACGAG-3' (36 bp); (D). 5'-ATTGAGAAGCTATCCGC/GATGTGGACCAGCTAGTG-3' (35 bp); and (E). 5'-ATCCGCGTGATTGCA/GGACTTGGAGATGTG-3' (31 bp).

Mutant clones were detected by colony blot hybridization analysis using the mutagenesis oligomers as probes. All codon substitutions and deletions were confirmed by sequence analysis.

### Transfection of COS-1 cells

The mutant cDNAs were introduced into COS-1 cells by electroporation essentially as described (25). Cells were cultured for 48 h in DMEM/10% FCS containing heparin at 40 µg/ml and then for 12 h in fresh medium containing heparin. Medium was removed, snap frozen, and stored at –70°C.



**Fig. 1.** Amino acid sequence of the loop region of LPL. The isoleucine (I225), substituted by threonine in the proband, and the conserved glycine residue (G229), substituted by proline (P), threonine (T), glutamine (Q), and lysine (K), are boxed. Also shown are the sequences of the three loop deletion mutants with the deletion boundaries shown by dotted lines.

### Lipoprotein lipase activity and mass

LPL activity against triacylglycerol substrates was measured using a radiolabeled triolein-phosphatidylcholine emulsion as previously described (27). Results are given as nmol oleic acid/min per ml. The total medium volume per dish was 10 ml. LPL mass was measured by ELISA using the 5D2 monoclonal as the capture and conjugated antibody (28). Results are expressed as ng/ml.

### Subject

The proband was of Dutch ancestry and presented in early childhood with the classical features of LPL deficiency, including acute abdominal pain with pancreatitis, chylomicronemia with a markedly elevated plasma triglyceride level of 25 mmol/l, and complete deficiency of LPL activity in postheparin plasma but a normal incremental mass level of 331 ng/ml.

### DNA analysis

Genomic DNA from the proband was subjected to PCR amplification of the coding sequence of each of the 10 exons of the LPL gene (29). Each PCR-amplified exon was sequenced either directly or after cloning into a TA cloning vector (Invitrogen). Both strands were sequenced and DNA changes were confirmed by several rounds of repeat PCR and sequencing from different genomic DNA isolates. Nucleotide numbering is taken from that of the LPL cDNA sequence lodged in GenBank under accession number M15856.

### Analysis of secondary structure

Secondary structure of the loop region of LPL was analyzed using both the GCG programs and "Peptidestructure" (30) running on a VAX computer or the SAGA!! protein sequence analysis software running under MSDOS (DISoft; 25J Thems House, 144 Battersea Park Rd, London SW11 4NB). Hydrophobic moments and hydrophobicities were calculated using Eisenberg's consensus hydrophobicities (31) and a moving window of variable size.

### Computer modeling

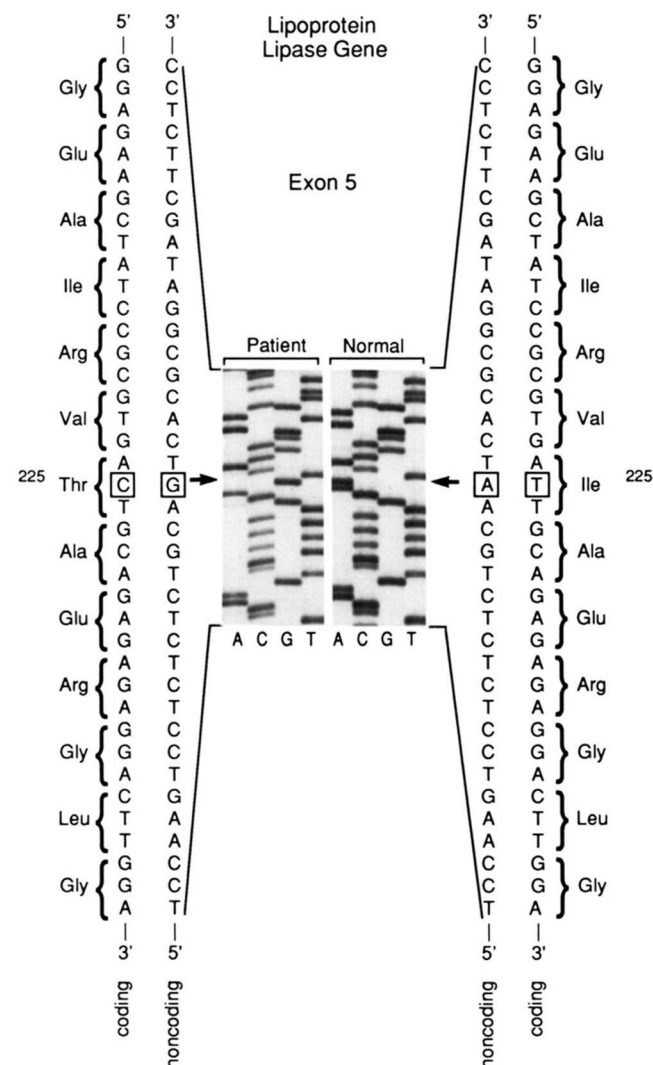
The crystallographic coordinates of human PL were kindly supplied by Dr. F. Winkler (Central Research Unit, F. Hoffman-La Roche, Basle). Molecular modeling was carried out using Biosym Insight II and Discover software on a silicon Graphics W-4D-35G workstation. To relax the high energy structures, the PL monomer and the mutant analogs were subjected to energy minimization and unrestrained molecular dynamics at 300 K for 10 picoseconds and the resulting structure was minimized. Non-bonded cutoffs of 10 Å were used in calculations. Deletions or amino acid replacements were made to the PL monomer to give the mutations that were homologous to LPL M1,2,3 and I225T. Comparative analysis of the primary sequence of PL with those of LPL and HL was carried out using the sequence alignment reported by Datta et al. (32).

## RESULTS

### DNA sequence analysis of the proband

LPL exonic sequence was found to be normal except for two single-base transitions that altered the primary sequence of the enzyme. These were a previously reported substitution of Arg243 by His (33) and a new T→C transition at nucleotide position 929, converting the ATT codon for isoleucine at position 225, to the ACT codon of threonine (Fig. 2). Sequence determination of cloned PCR products showed that these mutations resided on alternate alleles, confirming compound heterozygosity in this patient. The T→C transition of the I225→T substitu-





**Fig. 2.** Nucleotide sequence of a section of exon 5 from one LPL allele showing the T→C transition at position 929 resulting in the substitution of threonine for isoleucine at residue 225 in the loop region of LPL. Nucleotide and codon positions are taken from the published cDNA sequence (5) under GenBank accession # M15856.

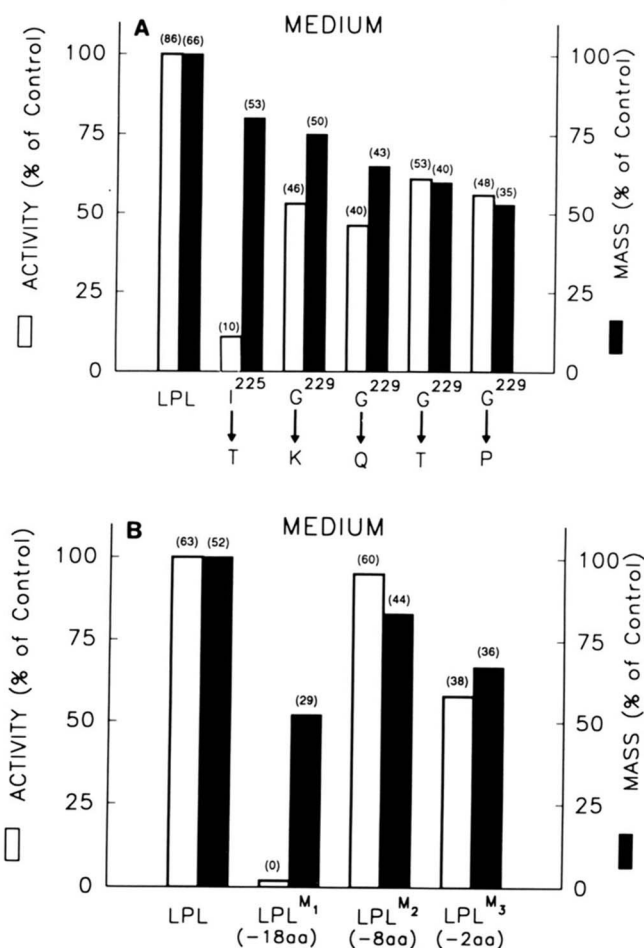
tion generates the CTGCAG palindrome of the restriction enzyme PstI and can readily be detected by amplification of exon sequence by PCR, digestion with PstI, and determination of the fragment pattern by agarose electrophoresis. This substitution occurs in exon 5 of the LPL gene and replaces one of the residues in the surface loop that covers the active site of the enzyme.

### Mutagenesis

**LPL activity and mass levels.** Medium from COS-1 cells transfected with the normal LPL cDNA revealed normal catalytic activity and mass while that from the cells transfected with the cDNA carrying the I225→T base transition revealed significantly impaired catalytic activity (close to 10% of normal). However, LPL immunoreactive

mass was present but only at 80% of the control value (53 vs. 66 ng/ml; **Fig. 3A**). In contrast, transfection of LPL cDNA with each of the four residue 229 variants revealed values for mass and activity which were approximately 50% of those obtained with the normal LPL cDNA transcript.

The LPL activity and mass levels in the medium of the COS-1 cells transfected with the loop deletion mutant cDNAs are given in **Fig. 3B**. Deletion of almost the entire 22 amino acid loop region (mutant LPLM1; -18 aa; **Fig. 1**) completely abolished catalytic activity towards the tri-olein substrate. In contrast, mutant LPLM2 containing a smaller 8-residue deletion, appeared to be unaffected as it showed 95% of control activity (60 vs. 63 nmol oleic acid/min per ml). Mutant LPLM3 however, showing only



**Fig. 3.** LPL activity and mass levels in the 12-h culture media from COS-1 cells transfected with the normal human LPL cDNA and the mutated cDNAs. **A:** cDNAs carrying single amino acid substitutions at positions 225 and 229 in the loop region of LPL. **B:** cDNAs carrying deletions for the loop mutants. Results are expressed as a percentage of the levels determined in the medium of cells transfected with the normal LPL cDNA. The actual values obtained are given in parentheses and are nmol oleic acid/min per ml for lipase activity and ng/ml for enzyme mass. I, Ile; G, Gly; K, Lys; T, Thr; Q, Gln; P, Pro.

a 2 amino acid deletion, was less active at 58% of control value (37 nmol oleic acid/min per ml).

Secondary structure analysis

*The LPL loop.* Predictions of secondary structure in the loop region of LPL vary remarkably depending on the specifics of the algorithm and implementation (Table 1).

In comparative secondary structure predictions we used the SAGA! and GCG program to analyze the loop structures of HL and PL. While the predictions for HL were essentially similar to those for LPL, the predicted structures for PL differed significantly. This must reflect the greater homology between LPL and HL (54–59%; GCG “Distance” program) than between PL and either of the other two lipases (20–30%). Further, neither of the three programs predicted the conformation of the PL loop as actually revealed in the molecular model of PL based on X-ray diffraction data (15).

DISCUSSION

In this manuscript we report on the first naturally occurring mutation in the surface loop covering the active site of LPL. Although this finding indicates a critical requirement for residues in the region, this does not apply to all amino acids as we also show that a closely situated conserved glycine can be substituted without dramatically affecting catalytic activity. Furthermore, we demonstrate that the loop motif is required for normal function, as deletion of most the motif destroys catalytic activity towards long-chain triacylglycerols. A completely intact loop is, however, not essential as partial deletions are relatively well tolerated. While this manuscript was being written two other reports on the activities of in vitro-generated loop mutants appeared in the scientific literature (35, 36). Both studies indicated that the loop region and secondary structure is critical for lipolysis. Here we have studied a different set of mutants and, in addition to confirming these findings, report on the importance of specific residues and sections of the loop.

The three-dimensional structures of unrelated lipid metabolizing enzymes such as lipases (15, 20–22), phospholipase A2 (39, 40), and cholesterol oxidase (41) have revealed the existence of several conserved structural motifs. All show a buried active site whose hydrophobic residues are shielded by a flexible surface loop. Access to the active site requires a conformational change through repositioning of the loop. While little is known about the mechanism of this repositioning, features such as hinge regions, amphipathic helices, and critical main domain interactions have been identified in the loop motifs of some enzymes (15, 21, 23). Given the proposed complexity of function, it is perhaps surprising that the mutation described in this manuscript is the first detected in the loop section of LPL, particularly as LPL-deficient patients have been extensively investigated worldwide and numerous mutations have been described with particular aggregation in exon 5 (Fig. 4).

Our finding that deletion mutant LPLM1, in which the length of the loop has been reduced to 4 residues, has no catalytic activity towards triolein indicates the critical requirement for the loop in lipolysis of long-chain triacylglycerols. Similar findings were reported by Dugi et al. (36) for their loop mutant in which the entire motif had been replaced by the 4 amino acid loop of protein kinase C. Both these findings are in keeping with the results of earlier studies on purified bovine LPL (42) where it was shown that scission of the loop by tryptic cleavage at the exposed Arg228 (now known to be centrally positioned in the loop) resulted in an enzyme incapable of hydrolyzing long-chain triacylglycerols but retained activity towards short-chain esters.

Removal of the entire loop region from the enzymes in the LPL/HL/PL family would not be expected to alter the alignment of the catalytic triad residues as they are not in contact with side chains of the loop residues. This was suggested by a study (36) of an inactive loop mutant, lacking almost the entire loop region, which was found to retain activity towards the short-chain ester tributyrin.

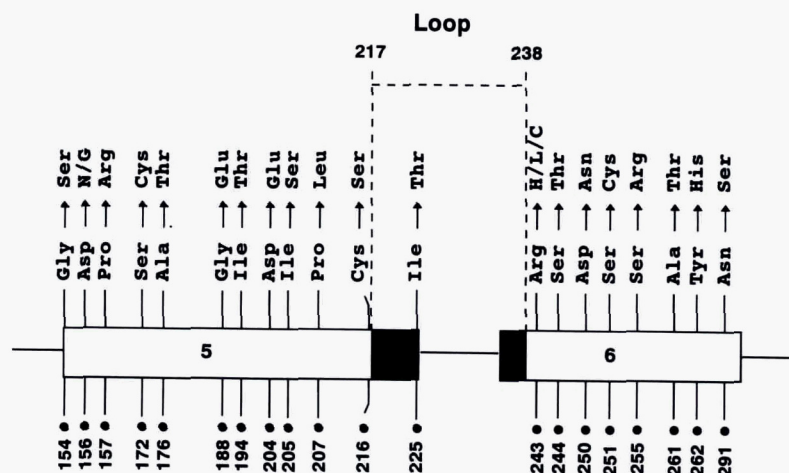
TABLE 1. Predictions of secondary structure in the loop region of lipoprotein lipase

Predictions	Algorithm + Program
216 225 239	
LPL loop CNIG EAI R V I A E R G L G D V D Q L V K C	
ttttt . . . . . ttttt BBB . . . . .	CF—SAGA!!
. . . . . HHHHHHHHH . . . h h h h h h .	CG—GCG peptide structure
tt . . . . . tttttt B B B B B .	GOR—GCG peptide structure
. . HHHB B BHHHHHHBBB B T B B B .	CF—not stated (35)
.HHHHHHHHHHHTTTTHHHHHHHH.	GN—“Helwheel” program (36)

H, strong alpha helix; t, turn; ., random coil; h, weak alpha helix; B, beta sheet. CF, Chou-Fasman (34); GOR, Garnier-Osguthorpe-Robson (37); GN, Garnier-Novotny (38).

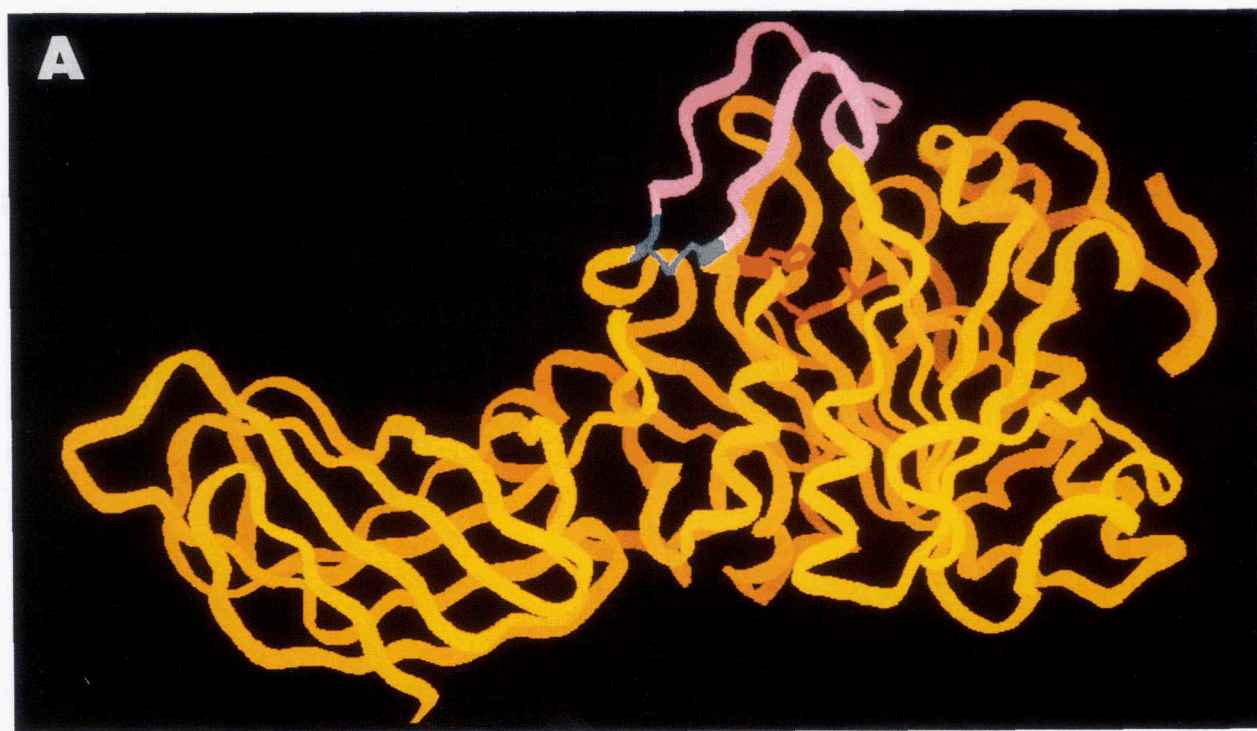


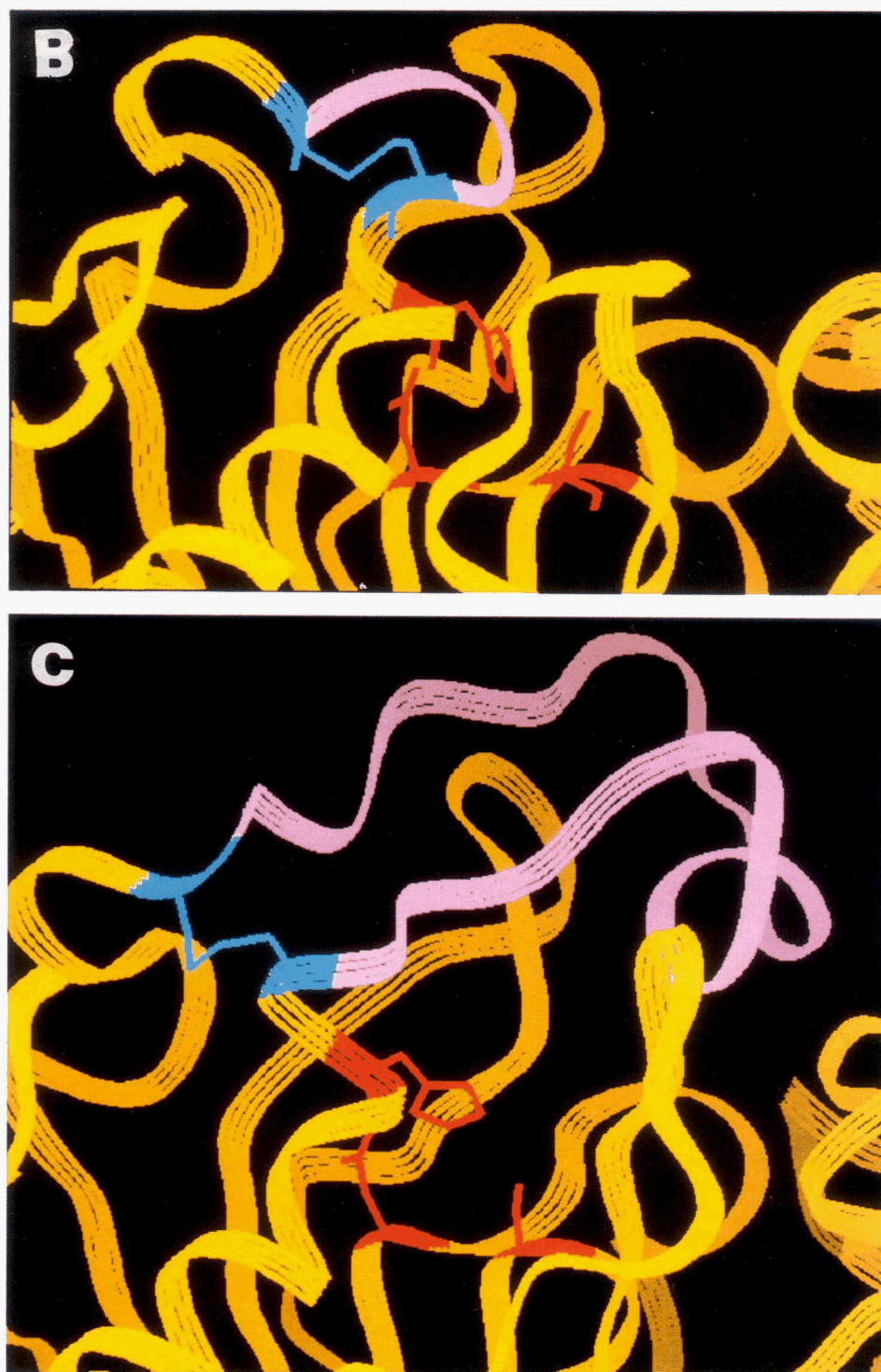
**Fig. 4.** Exons 5 and 6 of the LPL gene showing the regions coding for the amino acid residues of the loop motif. This motif comprises residues 217 to 238 and is depicted in black. Also shown are the locations of the described missense mutations in the LPL gene. References to these mutations and others in the LPL gene are summarized in 8 and 9.



The molecular basis for the loss of catalytic activity in the LPLM1 mutants is not clear. This may involve an essential requirement for the loop in the binding of substrate at the active site as suggested by Dugi et al. (36). Alternatively, it may involve a primary requirement for the loop in the conformational change of other segments of the molecule whose side chains are in intimate contact with the loop and which also hinder substrate access to the active site. This occurs in PL where the loop residues are in contact with the side chains of residues of two nonconserved loops (residues 75–79 and 212–216) (15) and likely

also applies to the active site of LPL. Support in favor of the loop being involved in maintenance of the conformation of other regions comes from our computer modelling studies of PL where removal of all but 4 residues of the loop induced a significant shift in the spatial orientation of an adjacent loop comprising residues 208 to 216 of PL (Fig. 5, A+B). It is possible that a similar alteration has occurred in the orientation of the adjacent loops in our inactive mutant LPLM1 and that access to the catalytic site is permanently interrupted. It is equally feasible, however, that these loop-deficient mutants are inactive, as the loop





**Fig. 5.** Molecular modeling of PL reveals the N and C terminal domains (yellow) and the loop (purple) linked by disulphide bonds (blue) shielding access to the catalytic site (red) (A). Deletions of PL homologous to the deletion of 18 amino acids in LPL induce a significant shift in orientation of the adjacent loops, significantly altering the channel to the active site (B). In contrast, deletion of 2 residues from PL does not appear to significantly alter the conformation of the channel to the catalytic triad (C).



**Fig. 6.** Amino acid sequence alignment of the loop regions of human LPL, PL, and HL. The conserved glycine is boxed while asterisks mark positions of conserved hydrophobicity. The alignment is taken from Datta et al. (32) while residue numbering is taken from the cDNA-derived sequences for LPL (5), PL (43), and HL (32).

216                   \*                   225                   229                   \*                   239  
 L P L C N I G E A I R V I A E R G L G D V D Q L V K C  
 238                   262  
 P L C K K N I L S Q I V D I D G I W E G T R D F A A C  
 231                   254  
 H L C H F L E L Y R H I A Q H G F N A I T Q T I K C

region is primarily involved in the binding of lipid substrates, and that this property is essential for access to the active site (36).


The secondary structures of the loop motifs in LPL and HL are unknown at present. Although it is possible that these structures will be similar to that of the homologous PL, which has an extended conformation comprising a short apical helix with flanking sections of random coil (Fig. 5), several factors suggest a different secondary structure. First, sequence homology between the lipases over the loop regions is low as these sections show only a single amino acid identity with each other while 4 residues only are homologous when hydrophobicity indexes are considered (Fig. 6). Secondly, *in vitro* mutagenesis experiments involving the loop motif of LPL (35, 36) have shown that the entire loop of this enzyme can be replaced by that of HL without affecting catalytic activity. Interchange with that of the PL loop, however, produces an inactive chimeric protein. These features suggest divergent secondary structures for the loop regions of LPL and PL.

To examine this further we compared the computer-derived predictions of secondary structure for the loop sections in all three enzymes (data not shown). We were unable, however, to draw any firm conclusions as the predictions were found to vary for each lipase depending on the computational approach used and also none of the programs used actually predicted the conformation of the loop observed in the molecular model of PL derived from X-ray diffraction data. These predictions, however, were compatible with a similar loop geometry for LPL and HL which is distinctly different from that of PL.

Prior mutagenesis studies in the loop region of LP (35, 36) have demonstrated a critical requirement for maintenance of periodicity and charge particularly in the proximal and distal segments of the LPL loop. Data from our patient support these findings as the I225T substitution occurs in the proximal section of the loop motif and lies within a triplet of hydrophobic residues (Table 1). The introduction of the hydrophilic hydroxyl group of threonine into this triplet presumably abolishes a critical hydrophobic interaction of the loop residues with either the lipid substrate or the main domain of the molecule.

To determine whether the entire loop is required for

normal function, we generated two mutants, LPLM2 and LPLM3, with partial deletions in this region. The introduced deletions spanned 8 and 2 residues, respectively, and involved the apical section of the loop and were designed to maintain the symmetry and net charge of the loop (Fig. 1). In contrast to the catalytically defective activity of LPL M1, both LPL M2 and LPL M3 were found to hydrolyze triolein, indicating that the N and C terminal sections of the loop are required for normal function. Other loop deletion mutants have also previously been generated (35) but were found to be inactive. Although these deletions involved similar numbers of residues, those deleted were not sequential and were selected to maximally disrupt the secondary structure of the loop. These findings again point to the critical requirement for preferred structure in the loop of LPL.

The near-normal functional functioning of mutants LPLM2 and LPLM3 indicate that the residues at the tip of the LPL loop do not form an indispensable part of the catalytic channel or active site motifs which are generated at the lipid-water interface. To further explore this interpretation we generated a series of mutants carrying different substitutions at position 229 which lies within the apical region of the loop segments. Glycine is found at this position and was selected for substitution as it is the only amino acid conserved in the loop regions of the three lipases (Fig. 6). Conservation was presumed to indicate a requirement for conformational freedom particularly as the loop must undergo a conformational change during activation at the lipid-water interface. Residues of differing side chain chemistries were introduced and comprised proline, threonine, lysine, and glutamine. None of these substitutions yielded a significantly catalytically defective enzyme (Fig. 3A), indicating a high degree of tolerance for sequence variation in the apical segment of the LPL loop. This finding further supports the possibility of redundancy in the apical section of the loop as revealed by the normal functioning of the partial deletion mutants. In contrast, the finding of the first naturally occurring mutation in the proximal section of the loop, together with the complete loss of catalytic activity following deletion of 18 amino acids from the loop, supports the critical role for this region of the loop in maintaining normal catalytic function of LPL. 



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