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Domain exchange: Characterization of a chimeric lipase of hepatic lipase and lipoprotein lipase

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ABSTRACT **Hepatic lipase and lipoprotein lipase hydrolyze fatty acids from triacylglycerols and are critical in the metabolism of circulating lipoproteins. The two lipases are similar in size and amino acid sequence but are distinguished by functional differences in substrate preference and cofactor requirement. Presumably, these distinctions result from structural differences in functional domains. To begin localization of these domains, a chimeric lipase was constructed composed of the N-terminal 329 residues of rat hepatic lipase linked to the C-terminal 136 residues of human lipoprotein lipase. The chimera hydrolyzed both monodisperse short-chain (esterase) and emulsified long-chain (lipase) triacylglycerol substrates with catalytic and kinetic properties closely resembling those of native hepatic lipase. However, monoclonal antibodies to lipoprotein lipase inhibited the lipase activity, but not the esterase function, of the chimera. Therefore, the chimeric molecule is a functional lipase and contains elements and characteristics from both parental enzymes. It is proposed that the N-terminal domain, containing the active center from hepatic lipase, governs the catalytic character of the chimera, and the C-terminal domain is essential for hydrolysis of long-chain substrates.**

Hepatic lipase (HL) and lipoprotein lipase are enzymes critical to processing of circulating lipoproteins. Together with pancreatic lipase, they form a highly homologous, but dispersed, gene family (1–3). Vitellogenin, a yolk protein, also shares substantial homology with these lipases but lacks enzymatic function (4, 5). The three-dimensional structure of pancreatic lipase, derived from x-ray diffraction patterns (6), shows two independent structural domains joined by a short spanning region. A similar three-dimensional structure is inferred for the other gene family members, HL and lipoprotein lipase, because of the high amino acid sequence homology (2), similarity in the location and number of disulfide bonds (7), and comparable function with regard to neutral lipid substrates. The putative pancreatic lipase catalytic center, formed by a triad of serine, histidine, and aspartate, is located in the globular N-terminal domain. The smaller C-terminal domain, although assigned no function, is superficially similar in both size and structure to fatty acid-binding protein (FABP) (8).

The effect of mutations on the function of HL (9, 10) and lipoprotein lipase (11) has been reported. N-linked glycosylation was required for lipoprotein lipase activity (11) but not for HL activity (10), and replacement of residues at or near the putative active site resulted in the production of catalytically incompetent proteins (9, 12). In addition, naturally occurring mutations, (deletions, duplications, truncations, and substitutions) have been characterized for both lipases (13). However, analyses of this kind often cannot fully define structure–function relationships. Such mutations commonly result in inactive enzyme, and, in the absence of crystal structure, the precise reason for inactivation remains open to conjecture. In many instances, global or indirect effects of these mutations are difficult to distinguish from altered chemical reactivity of critical residues or gross misfolding resulting in inactivation.

For these reasons, we have used an alternative strategy to investigate lipase structure–function relationships: intact domains were exchanged to assess functional roles played by large structural components. The strategy was based on reports describing the modular organization of proteins (14–16), with the intent of constructing a catalytically active chimeric lipase. Aside from important evolutionary considerations, the modular organization of proteins provides a means to investigate interchangeability of functional domains. We were motivated by the observation that members of the lipase gene family displayed different functional properties, yet amino acid homology was quite high. For example, lipoprotein lipase is a much less efficient phospholipase than HL, even though these enzymes have >50% amino acid identity. Basic differences in lipase characteristics provide an opportunity to evaluate the contribution of individual structural domains to enzyme function.

Here we describe the construction and characteristics of a chimeric lipase composed of the rat HL catalytic domain (residues 1–329) directly linked to the human lipoprotein lipase C-terminal domain (residues 313–448). The junction site for the chimera was placed within a region analogous to that which joins the two domains of human pancreatic lipase (6) and corresponds in the cDNA to the splice site between exons 6 and 7 of both lipoprotein lipase (3) and HL (17). The chimera displayed catalytic properties of native HL with regard to both lipase substrates (emulsified long-chain triacylglycerols) and esterase substrates (monodisperse short-chain triacylglycerols). However, monoclonal antibody to lipoprotein lipase inhibited chimera lipase activity without affecting esterase function. These results indicated that the chimera was a functional lipase and possessed characteristics from both parental enzymes.

MATERIALS AND METHODS

Chimera Construction. A variation (18) of the polymerase chain reaction (PCR) was used to selectively amplify and link appropriate segments of the cDNAs for rat HL (2) and human lipoprotein lipase (19). The N-terminal primer (Xba5PrHL: 5′-TGACTCTAGATGGGAAATCACCTCCAAATC-3′) contained an *Xba* I restriction site and the first 21 coding nucleotides of the rat HL cDNA (2). The splicing primer (rHL329LPL313: 5′-CGAGCCCAGTCCCCCTTTAAAGTCTTCCATTACCAAGTAAAG-3′) consisted of 21 nucleotides encoding amino acids 323–329 of rat HL (2) followed by 21 nucleotides encoding amino acids 313–319 of human

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Abbreviations: HL, hepatic lipase; apo, apolipoprotein.
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lipoprotein lipase (19). The C-terminal primer (Bam3PLPL: 5′-ACGTGGATCCGAATTCACATGCCGTTCTTTG-3′) contained a *Bam*HI restriction site followed by reverse complement for nucleotides 1623–1643 in the 3′ untranslated region of human lipoprotein lipase cDNA (19). The chimeric and parental lipase cDNAs were cloned in the expression vector pSVL (Pharmacia), and the structure of the chimera was confirmed by complete nucleotide sequencing.

Expression. By use of Lipofectin (BRL) for transfection, the lipases were transiently expressed in COS-7 cells (20) according to the normal lipofection protocol (21) except that during the last 18–24 hr, heparin (Sigma) was added to the tissue culture medium at 20 units/ml. The culture medium was concentrated in Centricon-30 microconcentrators (Amicon) and stored at −80°C until assayed for enzymatic activity.

Genomic Sequencing. Sequencing of rat HL genomic sequences was accomplished by PCR amplification. Primers XbaExo6FWD (5′-CGATCTAGAGCCATAACCCAGACCATCAAAT-3′) and BamExo6REV (5′-AGCTGGATCCTTAAAGGGGGACTGGGCTCG-3′) were designed to amplify rat HL sequences between codons for Ala-249 and Lys-329, approximately corresponding to exon 6 of human HL (17). Amplification from Sprague–Dawley rat genomic DNA produced a single band of appropriate size upon electrophoresis in 1% agarose. DNA from the band was purified (22) and used in two sequencing strategies: (*i*) direct sequencing using an internal primer spanning codons for Leu-264 to Ser-272 in the sense direction and (*ii*) sequencing of plasmid DNA after cloning of the PCR product into the *Xba* I and *Bam*HI restriction sites of pSVL. Sequencing with primers flanking the plasmid cloning site showed that six independent clones carried identical sequences.

Lipases. Purified bovine lipoprotein lipase and rat HL were prepared as described (1, 23). Both preparations were homogeneous as judged by silver stain after SDS/PAGE.

Enzyme Assays. Lipase (24, 25) and esterase (26) assays were performed with radiolabeled triolein and tributyrin, respectively. Effects of antibodies on enzyme activity were assessed by incubating samples with antibodies for 1 hr at 4°C prior to determination of lipase or esterase activities. Rat serum (3%, vol/vol) is normally included in lipoprotein lipase activity assays as a source of the cofactor apolipoprotein (apo) C-II. The effect of apo C-II on lipase activities was measured by excluding serum in the preparation of the lipoprotein lipase assay substrate and including it in the HL substrate. Dependence of lipase activity on salt concentration was determined by addition of NaCl to 1 M.

Antibodies. Polyclonal antibodies to lipoprotein lipase (23) and HL (25) were generated as described. These preparations are monospecific as determined by Western blot and immunoprecipitation analyses (25). Monoclonal antibody to bovine lipoprotein lipase was purchased from Oncogene Sciences (Mineola, NY). The monoclonal specificity was not known, although the antibody was reported to inhibit lipoprotein lipase activity. Antigen-binding (Fab) fragments derived from the intact monoclonal antibody were generated by papain cleavage and purified by protein A-Sepharose chromatography (Pierce). Silver staining of denaturing polyacrylamide gels of reduced Fab fragments detected a single band at 25 kDa.

RESULTS

PCR amplification in the presence of rat HL and human lipoprotein lipase cDNA and specific primers generated the chimeric molecule shown in Fig. 1. For convenience, this chimeric molecule is designated HL-chimera. Sequence analyses confirmed that the chimera contained the desired transition between rat HL and human lipoprotein lipase. However, several differences with the published rat HL cDNA sequence were noted in exon 6. The same differences were found upon resequencing the rat HL cDNA template clone. To determine whether these differences reflected true genomic sequence, exon 6 of HL was sequenced following PCR amplification from rat genomic DNA. All sequence differences detected in the chimera (Fig. 2) were also observed in genomic sequence and therefore reflect errors in the published rat HL cDNA sequence (2). The predicted changes in amino acid residues resulted in a more conserved sequence within the lipase gene family. For example, the corrected residue at position 286 in rat HL is also a serine in human HL. Similarly, the arginines now predicted at 311 and 313 are consistent with the arginine and lysine observed at the respective analogous positions in lipoprotein lipase.

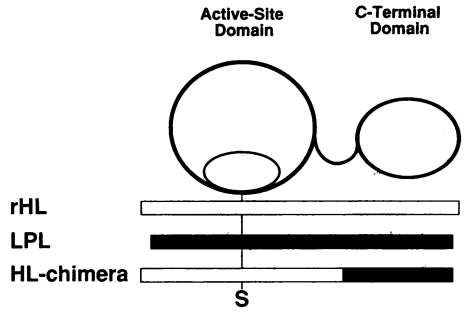

FIG. 1. Structure of the chimeric lipase. Rat HL (rHL; open bar) and human lipoprotein lipase (LPL; solid bar) are shown aligned at their putative catalytic serine residues (S). HL-chimera consists of the first 329 amino acids of rat HL (2) linked to the 136 C-terminal residues from human lipoprotein lipase (19). At the top is a lipase monomer model derived from the three-dimensional structure of pancreatic lipase, a closely related member of the lipase gene family. The N-terminal domain contains the putative catalytic serine residue, while the C-terminal domain consists of eight antiparallel β-strands present as two orthogonal sheets (6) similar in size and conformation to FABP (8).

HL-chimera hydrolyzed triolein substrate in a concentration-dependent manner (Fig. 3*A*). The rate of hydrolysis was linear over at least a 50-fold concentration range. Antibody inhibition studies demonstrated that HL-chimera contained both HL-specific and lipoprotein lipase-specific epitopes. Thus, monospecific antiserum to rat HL (25) readily and completely inhibited triolein hydrolytic activity present in media of HL- and HL-chimera-transfected cells (Fig. 3*B*). Inhibition of purified rat HL by this antiserum to rat HL was superimposable with that shown in Fig. 3*B* for HL and HL-chimera; purified bovine lipoprotein lipase activity was not affected by this antiserum (data not shown).

In contrast, monoclonal antibody to bovine lipoprotein lipase (27) inhibited HL-chimera lipase activity, but not the lipase activity from medium of HL-transfected cells (Fig. 3*C*). This monoclonal antibody inhibited purified bovine lipoprotein lipase in a manner similar to that shown for

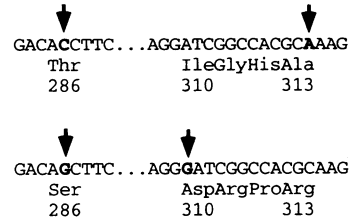

FIG. 2. Sequence corrections for rat HL. Nucleotide differences between the published rHL sequence (2) (*Upper*) and the sequence identified by PCR amplification of exon 6 of HL in rat genomic DNA (*Lower*) are marked by arrows. These nucleotide differences cause changes in the predicted identity of 5 amino acid residues.

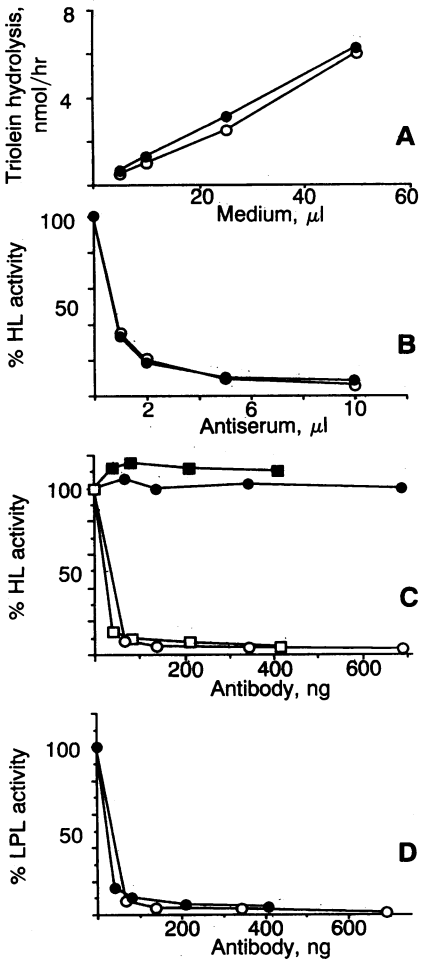

FIG. 3. HL and HL-chimera triolein hydrolysis and the effect of antibodies. (*A*) Triolein hydrolysis (24) as a function of medium from COS cells transfected with HL (●) or HL-chimera (○) cDNA. Medium from mock-transfected cells showed no lipase activity. (*B*) Effect of monospecific rabbit anti-rat HL antiserum on HL and HL-chimera lipase activity expressed in COS cell medium. A constant amount of HL and HL-chimera activity was incubated with various amounts of antiserum (100% lipase levels: 4.6 nmol/min per ml for HL; 4.1 nmol/min per ml for HL-chimera). After 60 min at 4°C, lipase activity was determined. (*C*) Effect of monoclonal antibody to bovine lipoprotein lipase (27) (●, ○) or monoclonal Fab fragments (■, □) on HL (●, ■) and HL-chimera (○, □) triolein hydrolase activity. Experimental conditions were the same as in *B* (100% lipase levels: 5.3 nmol/min per ml for HL; 3.1 nmol/min per ml for HL-chimera). (*D*) Effect of monoclonal antibody to bovine lipoprotein lipase (○) or monoclonal Fab fragments (●) on lipoprotein lipase (LPL) activity expressed in COS cells. The 100% lipase level was 5.5 nmol/min per ml.

HL-chimera (Fig. 3*D*). The epitope for the monoclonal antibody to lipoprotein lipase has not been previously described, but the inhibition of HL-chimera lipase activity shows that the epitope lies within the C-terminal 136 amino acids of lipoprotein lipase (residues 313–448). Significantly, antibody binding solely to the C-terminal domain of the chimera appears sufficient to abolish lipase activity. One explanation is that the monoclonal antibody binds to a functionally nonessential region but that its large size (≈150 kDa) inhibits catalysis by steric hindrance at a second, essential remote site. To examine this issue, the much smaller (≈50-kDa) Fab fragments were prepared from the inhibiting monoclonal antibody. Lipase inhibition by the Fab was essentially the same as for the parent antibody (Fig. 3*C*). Monospecific polyclonal anti-lipoprotein lipase serum also inactivated both bovine lipoprotein lipase (23) and HL-chimera lipase (data not shown).

Whereas HL activity requires no cofactor, lipoprotein lipase activity is strongly stimulated by apo C-II present in serum. HL activity is also distinguished from that of lipoprotein lipase by insensitivity to high ionic strength (e.g., 1 M NaCl). The triolein hydrolase activity of HL-chimera mimicked that of HL; i.e., it was neither stimulated by serum nor affected by NaCl concentration (Table 1). The lack of serum stimulation and the insensitivity to ionic strength are consistent with catalytic characteristics of HL, suggesting that these properties are due to structural elements in the HL catalytic domain (residues 1–329).

To more fully characterize the kinetic properties of HL-chimera, hydrolysis of emulsified long-chain (lipase) and monodisperse short-chain (esterase) triacylglycerol substrates was determined. Both HL and HL-chimera produced near-identical hyperbolic curves for triolein hydrolysis with increasing substrate concentration (Fig. 4*A*). Similarly, esterase activity (Fig. 4*B*), assessed with a tributyrin substrate (26), showed that esterase kinetic parameters of HL-chimera were essentially indistinguishable from those of native HL.

The monoclonal antibody to lipoprotein lipase bound to the C-terminal domain of the chimera and completely inhibited hydrolysis of emulsified triolein (Fig. 3*C*). Therefore, it was of interest to determine the effect of the antibody on HL-chimera esterase activity. Significantly, tributyrin esterase activity of HL-chimera was unaffected by the monoclonal antibody (Fig. 4*C*). Thus, the tributyrin substrate had complete access to the active site whether or not an antibody molecule was bound to the lipoprotein lipase portion of the chimera. In contrast, the triolein hydrolase activity of HL-chimera was completely inhibited by the monoclonal antibody (Fig. 3*C*), suggesting that the active site was not accessible to the emulsified substrate.

DISCUSSION

HL-chimera, composed of rat HL amino acid residues 1–329 and human lipoprotein lipase residues 313–448, was constructed and characterized. HL-chimera sequence analyses uncovered discrepancies when compared with the published sequence for rat HL (2). Sequence analyses of PCR-amplified rat HL exon 6 from genomic DNA confirmed the published sequence to be in error. The corrected sequence (Fig. 2) is located in the putative heparin-binding region of the enzyme and results in the substitution of two additional arginine residues into this region. A cluster of positively charged residues is consistent with this region functioning in ionic interaction with heparan sulfate proteoglycans (28, 29).

Catalytic properties of the chimera (Table 1) indicate that the salt-resistant lipase activity was attributable to the HL portion of the molecule (i.e., the HL catalytic domain). The inability of apo C-II in serum to stimulate HL-chimera lipase activity suggested that apo C-II may exert its stimulatory

Table 1. Lipase activity response to serum and NaCl

Enzyme	Serum (10 μg/ml) +	Serum (10 μg/ml) −	NaCl (1 M) +	NaCl (1 M) −
LPL	1.0	0.2	0.05	1.0
HL	0.47	1.0	1.0	1.0
HL-chimera	0.46	1.0	1.0	1.0

Medium from transfected cells was concentrated 10-fold and lipase activity was determined. For each lipase, values shown are normalized to the maximum activity observed when samples were assayed under optimum conditions. Maximum activities: lipoprotein lipase (LPL), 6.01 nmol/min per ml; HL, 3.54 nmol/min per ml; HL-chimera, 1.55 nmol/min per ml.

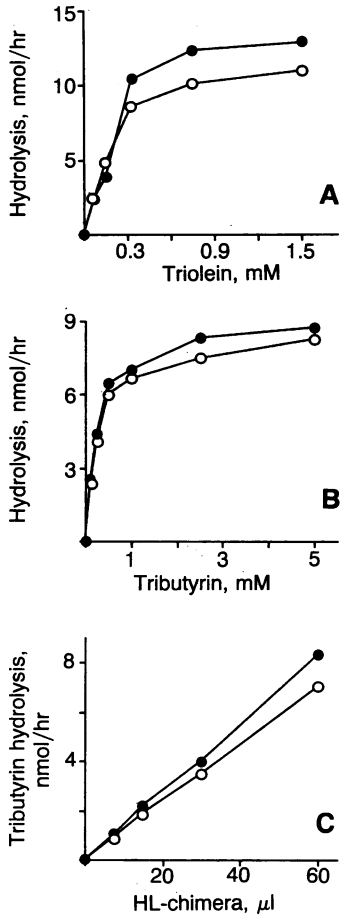

FIG. 4. Rate of hydrolysis of triolein and tributyrin substrates. (*A*) Rate of triolein hydrolysis as a function of triolein concentration. ●, Medium of COS cells transfected with rat HL; ○, medium of HL-chimera transfection. (*B*) Rate of tributyrin hydrolysis as a function of substrate concentration. Tributyrin substrate was used as a measure of esterase activity (26). Symbols are as in *A*. (*C*) Tributyrin esterase activity as a function of HL-chimera concentration in the presence (○) or absence (●) of 5 ng of monoclonal antibody to lipoprotein lipase.

effects via the lipoprotein lipase catalytic domain. This suggestion is supported by experiments on another chimeric lipase molecule, containing the lipoprotein lipase catalytic domain coupled to the HL C-terminal domain. In this case, apo C-II stimulated the chimeric lipase activity (unpublished work).

HL-chimera was the functional equivalent of native HL with respect to hydrolysis of emulsified (Fig. 4*A*) and monodisperse (Fig. 4*B*) substrates. These results indicated that the HL-chimera catalytic domain operates normally with little or no interference due to linkage with the lipoprotein lipase C-terminal domain. By the criteria of lipase and esterase activity, the lipoprotein lipase C-terminal domain shares a common functionality with the HL C-terminal domain, while retaining the epitope recognized by the lipoprotein lipase monoclonal antibody (Fig. 3*C*).

Previous studies have led to the conclusion that HL has separate sites for catalysis and lipid binding (26, 30). This conclusion was partly based on findings that protease-treated HL failed to hydrolyze triolein, whereas tributyrin activity was unaffected. In addition, a proteolytic fragment with esterase activity was partially separated from a lipid-binding fragment (30). Apparently, the HL fragment with esterase activity was unable to interact with emulsified substrates and therefore was inactive as a lipase. The inhibition of the HL-chimera lipase activity by monoclonal antibody (or Fab) to lipoprotein lipase demonstrated the importance of the C-terminal domain for hydrolysis of emulsified substrates (Fig. 3*C*). However, bound antibody did not block access to the catalytic site, since HL-chimera activity on a monodisperse substrate was not inhibited (Fig. 4*C*). A simple explanation for these findings is that the lipid substrate normally interacts at a site distant from the catalytic site, and the presence of the antibody either prevents binding to this region or prevents bound substrate from reaching the catalytic site. A smaller, nonaggregated substrate such as tributyrin may enter the catalytic site directly, unaffected by bound antibody (Fig. 4*C*). The precise mechanism of monoclonal antibody inhibition of chimera lipase activity awaits further experimentation. However, our results are consistent with HL having separate sites for catalysis and lipid binding and suggest that binding of Fab to the C-terminal domain resulted in the loss of lipase, but not esterase, function.

The structure of human pancreatic lipase derived from x-ray crystallographic studies forms the basis of current models for HL and lipoprotein lipase. Even though there are structural variations between these enzymes, it is clear from their overall homology that they have basically similar structures. Recently crystallized fungal lipases show remarkably similar structural features, although they are unrelated genetically to the lipase gene family (31, 32). These fungal lipases also have a twisted β-pleated-sheet core with a serine–histidine–aspartate catalytic triad, an arrangement similar to that of serine proteases. Access to this triad is blocked by a "lid" domain. When substrate enters, the lid folds back (33) allowing substrate to enter the catalytic groove, simultaneously exposing a large surrounding hydrophobic surface that is presumably involved in lipase interfacial activation (34).

In spite of similar structural features, fungal and mammalian lipases have at least one major structural distinction: the fungal lipases lack a C-terminal domain and yet appear to carry out all lipase functions. This difference may be due to the subunit structure of the enzymes. The fungal lipases function as monomers (35, 36), whereas lipoprotein lipase and HL appear to be active as dimers; sedimentation equilibrium, gel filtration, and radiation inactivation studies of lipoprotein lipase clearly support the contention that it is a dimer in solution (37–39), whereas gel filtration indicates that HL is an active oligomer in solution (40). Similar studies have not been reported for pancreatic lipase; however, dimer crystals have been described, with two molecules per asymmetric unit (6). Inactivation of HL-chimera by the anti-lipoprotein lipase monoclonal antibodies (Fig. 3*C*) indicates that binding of antibody to the C-terminal domain disrupts lipase, but not esterase, catalysis. Perhaps binding of antibody causes dimer dissociation. If the dimer structure is necessary for enzyme binding to large insoluble substrates, antibody-induced dissociation could render the enzyme inactive as a lipase while leaving the monomer able to utilize monodisperse molecules as substrates. Conversely, the antibody may not cause dimer dissociation but instead interfere with binding of lipid substrates or the presentation of substrate to the active-site cleft. In either case, insights afforded by the structure of fungal lipases cannot be extended to the function of the C-terminal domain of the lipase gene family; the functional advantage and significance of such a structure remain unknown.

With the dimeric structure of the lipase gene family as a starting point and the crystal structure of pancreatic lipase as a guideline (6), two possible models for the enzymes emerge. The domains on each subunit can be arranged in a head-to-head (Fig. 5*A*) or a head-to-tail (Fig. 5*B*) fashion. In both cases, a twofold axis of symmetry exists. The head-to-head dimer would position the C-terminal domains together and

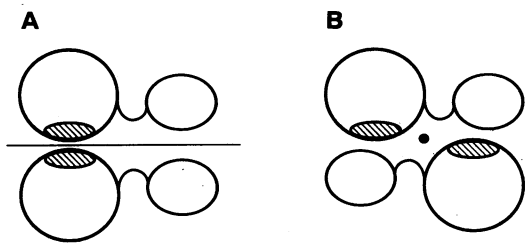

FIG. 5. Possible lipase dimer molecules. (*A*) Head-to-head dimer with axis of symmetry (line) in the plane of diagram. (*B*) Head-to-tail dimer with axis of symmetry (dot) perpendicular to the plane of diagram. The larger circle represents the N-terminal domain, containing the active-site region (hatched oval). The smaller circle is the C-terminal domain, where the monoclonal antibody to lipoprotein lipase binds.

maximize the distance to catalytic centers. In this case, the antibody might inhibit by interfering with a substrate anchor function of the paired C-terminal domains. However, this possibility appears unlikely since fungal lipases lack the C-terminal domain and yet are able to hydrolyze emulsified substrates. By contrast, head-to-tail dimers place the C-terminal domain on one subunit in close proximity to the active site on the other subunit. In this conformation (Fig. 5*B*), it is possible to envisage how the C-terminal domain could play a direct role in substrate binding or presentation. The observed Fab inhibition of lipase activity (Fig. 3*C*) could be a result of blocked lipid substrate binding or incorrect presentation of bound substrate to the catalytic site on the other subunit. In this situation, the C-terminal domain could function to determine enzyme substrate specificity. Chimeric lipases should play an important role in testing these alternative models of the lipase molecule and the function of the C-terminal domain.

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