

# Identification of the Catalytic Triad of the Lipase/Acyltransferase from *Aeromonas hydrophila*

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*Aeromonas hydrophila* secretes a lipolytic enzyme that has several properties in common with the mammalian enzyme lecithin-cholesterol acyltransferase. We have recently shown that it is a member of a newly described group of proteins that contain five similar blocks of sequence arranged in the same order in their primary structures (C. Upton and J. T. Buckley, Trends Biochem. Sci. 233:178–179, 1995). Assuming that, like other lipases, these enzymes have a Ser-Asp-His catalytic triad, we used these blocks to predict which aspartic acid and histidine would be at the active site of the *Aeromonas* enzyme. Targeted residues were replaced with other amino acids by site-directed mutagenesis, and the effects on secretion and activity were assessed. Changing His-291 to asparagine completely abolished enzyme activity, although secretion by the bacteria was not affected. Only very small amounts of the D116N mutant appeared in the culture supernatant, likely because it is sensitive to periplasmic proteases it encounters en route. Assays of crude preparations containing this variant showed no detectable enzyme activity. We conclude that, together with Ser-16, which we have identified previously, Asp-116 and His-291 compose the catalytic triad of the enzyme.

*Vibrio* spp. secrete a 35-kDa lipase that has a distinctive reaction mechanism (18). The enzyme from *Aeromonas hydrophila* has been reasonably well characterized. Although it hydrolyzes both primary and secondary ester bonds in neutral lipids and phospholipids and, like other lipases, it is activated at an oil-water interface, it has the unusual ability to preferentially carry out acyl transfer from position 2 of glycerophospholipids to acyl acceptors, including cholesterol (3, 4, 6, 7), and it is often referred to as GCAT (glycerophospholipid-cholesterol acyltransferase). In this way it is similar to the important mammalian plasma enzyme lecithin-cholesterol acyltransferase. Both GCAT and lecithin-cholesterol acyltransferase appear to be members of the lipase “superfamily” (13, 16). However, the active-site serine (Ser-16) of GCAT is situated in the middle of the sequence G-X-S-X-S (14), whereas in most serine esterases, including many of the lipases, the nucleophilic serine is found in the sequence G-X-S-X-G. In the lipases for which the structures are available, this latter sequence forms a critically important loop between a  $\beta$ -strand and an  $\alpha$ -helix. Replacing the second serine with glycine in GCAT to normalize its sequence results in an inactive enzyme with an altered structure (22), indicating that the active site of this enzyme may not be the same as the active sites of lipases of known structure. The proximity of the active-site serine to the amino terminus of GCAT also distinguishes this enzyme from most lipases.

Recently we reported that a number of other proteins have the same G-X-S-X-S motif as *A. hydrophila* GCAT and that this motif is near the amino terminus in every case. We noted that these proteins contain four other blocks of sequence similar to sequences found in GCAT (Fig. 1). Some of these proteins are also known to be lipases, and this led us to suggest that they represent a new subfamily or group of enzymes (30). The heat-labile hemolysin of *Vibrio parahaemolyticus* is a mem-

ber of this group, as are a number of plant proteins of unidentified function (30).

Lipases typically contain a Ser-Asp-His catalytic triad, although Glu replaces Asp in the triad of the *Geotrichum candidum* lipase II (31). We have previously shown that Ser-16 is essential for the activity of GCAT (14). This residue is found in the first block in Fig. 1. We predicted that block III would contain the active-site aspartic acid (Asp-116) and that block V was the likely location for the active-site histidine (His-291). The prediction that GCAT should contain an essential histidine is not consistent with our earlier conclusion that none of the histidines in the enzyme are essential. We had arrived at this conclusion after observing that enzyme activity was not affected by replacing either His-175 or His-180 with asparagines by site-directed mutagenesis (14). These are the only two histidines in the 27-kDa fragment produced by trypsin which, we determined, retained some enzyme activity (15).

In this study we used the sequence blocks in the new subfamily to target residues for site-directed mutagenesis. We show that our previous conclusion was incorrect and that GCAT requires His-291 in block V for activity. This residue and Asp-116 in block III together with Ser-16 in block I are the likely members of the catalytic triad of GCAT.

## MATERIALS AND METHODS

**Bacterial strains and vectors.** *Escherichia coli* TG2 was grown at 37°C in Luria-Bertani medium and was routinely used for cloning. *Aeromonas salmonicida* CB3 was grown at 27°C (5) in Luria-Bertani medium supplemented with Davis minimal medium (19) and 0.2% (wt/vol) glucose. Where appropriate, the following antibiotics were added to the media: ampicillin (100  $\mu$ g/ml), kanamycin (40  $\mu$ g/ml), and rifampin (40  $\mu$ g/ml).

**Site-directed mutagenesis.** The changes that were introduced into GCAT are listed in Table 1. The necessary oligonucleotides were purchased from the University Core DNA Services, University of Calgary, Alberta. Site-directed mutagenesis was performed with the phagemid vector pTZ19U (Bio-Rad) and by a modification of the method of Kunkel (17). Confirmation of each single-nucleotide change was achieved by sequencing each entire mutant GCAT gene by the dideoxynucleotide chain-termination method (24) with Sequenase version 2.0 obtained from United States Biochemical Corporation. Each mutant gene was first inserted into the vector pMMB66EH (10) in *E. coli* TG2 and subsequently mobilized into *A. salmonicida* CB3 with *E. coli* MM297 containing the conjugative helper plasmid (8).

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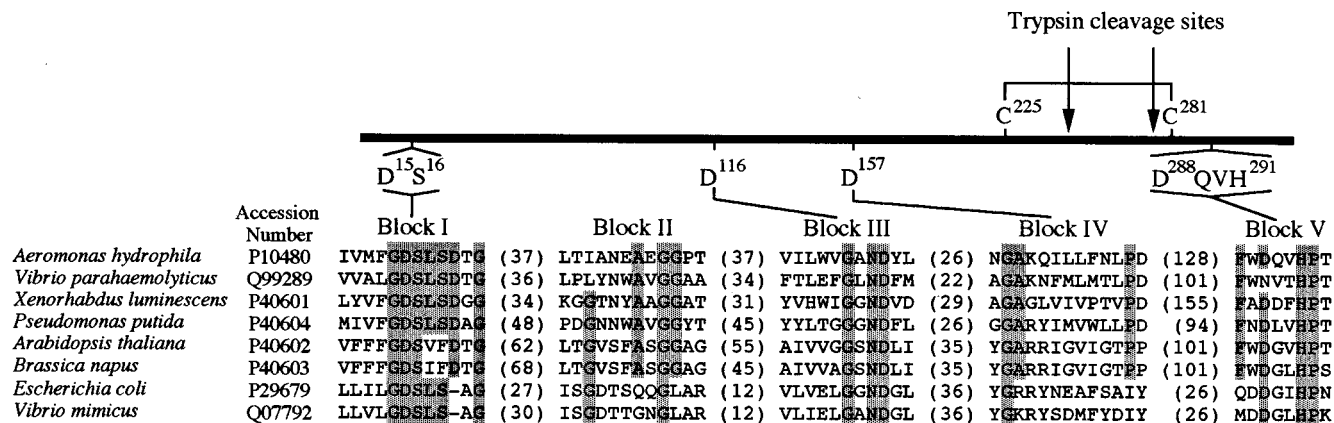


FIG. 1. Comparison of potentially important amino acids in GCAT with other members of the subfamily of proteins possessing the G-X-S-X-S/F motif. Amino acids that are conserved in at least six of the proteins are shown in shaded blocks. Numbers in parentheses indicate the number of amino acid residues between the conserved blocks.

**Protein purification.** Wild-type and mutant GCAT enzymes were expressed in *A. salmonicida* CB3 and purified essentially as described previously (15), except that HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) replaced Tris as the buffer and fast-performance liquid chromatography with a Mono-Q Sepharose column (1.6 by 7.0 cm; Pharmacia) with a linear 0 to 1.0 M NaCl gradient replaced the DEAE-Sepharose step used before. The concentration of each purified protein was calculated with the previously determined extinction coefficient at 280 nm (15).

**Enzyme assays.** *E. coli* TG2 or HB101 cells that carried the wild-type or mutant GCAT genes were grown on Tween-peptone plates (28) containing 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG). The formation of opaque zones surrounding bacterial colonies was considered a qualitative measure of lipolytic activity. Esterase activity was measured by the method of Bonelli and Jonas (1) with 0.87 mM *p*-nitrophenyl butyrate as the substrate in 3% (vol/vol) acetonitrile–10 mM Tris (pH 7.4)–150 mM NaCl–0.01% (wt/vol) EDTA. Prior to the assay, purified GCAT was diluted to the desired concentration in 1% (wt/vol) sodium deoxycholate dissolved in phosphate-buffered saline (PBS) (23). Acyltransferase activity was measured with egg lecithin as the substrate and [<sup>14</sup>C]cholesterol as the acyl acceptor, as described before (3).

**Electrophoresis and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 12% acrylamide slab gels by the method of Neville (20). The proteins were subsequently stained with Coomassie brilliant blue or transferred to nitrocellulose and reacted with anti-GCAT antiserum. Staining of the immunoblots was achieved with a goat anti-rabbit alkaline phosphatase-conjugated second antibody (29). Alternatively, enhanced chemiluminescent detection (Amersham) was carried out with a horseradish peroxidase-conjugated second antibody.

**Pulse-chase labeling and immunoprecipitation.** Bacteria were grown to an optical density at 600 nm of 1.0 and induced for 15 min with 1 mM IPTG. The cells were washed twice with M9 medium (23) supplemented with 0.05  $\mu$ g of arginine per ml and 1 mM IPTG and then resuspended in the same medium containing 300  $\mu$ Ci of [<sup>35</sup>S]methionine (Amersham) per ml. Unlabeled methionine was added to a concentration of 500  $\mu$ g/ml after 1 min. Samples were taken at the times indicated in Fig. 3 and centrifuged. The cell pellets were each resuspended in 60  $\mu$ l of SDS buffer (1% SDS, 50 mM Tris [pH 8.0], 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) and boiled for 2 min. After adding 240  $\mu$ l of ice-cold Triton buffer (1.25% Triton X-100, 190 mM NaCl, 60 mM Tris [pH 7.4], 6 mM EDTA) and 20  $\mu$ l of 10% *Staphylococcus* cells (SAC; Calbiochem), the mixtures were incubated at 4°C for 2 h. The SAC were then removed by centrifuging for 5 min, 2  $\mu$ l of a polyclonal anti-GCAT antiserum was added to each supernatant, and the samples were incubated at 4°C overnight.

Fresh 10% SAC were washed twice with cold PBS and then incubated for 15 min at 4°C in the presence of 30 mg of bovine serum albumin (Sigma) per ml. Twenty microliters of the incubated SAC was added to each immunoprecipitation mixture, and the mixtures were incubated at 4°C for 1 h. The SAC in the immunoprecipitation mixtures were pelleted and washed twice in cold Triton buffer and once in cold 20 mM Tris (pH 7.4). The SAC were then resuspended in 30  $\mu$ l of sample buffer. The mixtures were boiled and centrifuged, and samples of the supernatants were separated by SDS-PAGE along with samples of the corresponding culture supernatants. Gels were fixed for 30 min in 7% acetic acid–30% methanol, washed for 30 min in Amplify (Amersham), and dried prior to autoradiography.

## RESULTS

**Mutations of potentially important residues.** If all of the proteins in Fig. 1 have similar catalytic activities, it seems reasonable to suppose that three of the five sequence blocks they have in common will contain the active-site residues. We have already determined that Ser-16 is the active-site serine of GCAT. This residue is found in block I. Only block V contains a histidine in all of the proteins. In the case of GCAT, this is His-291, and this seemed the most likely candidate for a role in catalysis. None of the other four histidines of the *Aeromonas* enzyme appear in conserved regions of the sequence. What is more, we have already established that two of these four histidines, His-175 and His-180, are not required for activity (14) and a third, His-317, is the last residue in the molecule, an unlikely position for a catalytically essential amino acid. On the basis of this reasoning, His-291 was replaced with asparagine (Table 1).

Glutamate seemed an unlikely candidate for a catalytic triad residue in GCAT. Although block II of the *Aeromonas* enzyme contains two glutamates, there are none at corresponding positions in the other enzymes. Only blocks I and III of all of the proteins in Fig. 1 contain aspartates. For GCAT, these are Asp-15 and Asp-116, and these residues were each changed to asparagine. Asp-116 was also changed to glutamate and to alanine. Two other aspartates were also replaced with asparagines, Asp-157, which is found in block IV of all of the members of the group that have been shown to have lipolytic activity, and an aspartate corresponding to Asp-288, which is found in block V of all but one member of the group, the *V. parahaemolyticus* protein.

**Secretion of the mutant enzymes.** We have shown previously that proteins secreted by *Aeromonas* spp. must be correctly folded before they are released (12). There are at least two reasons for this. The native structure is required for transit

TABLE 1. Site-specific mutations in GCAT

Mutation	DNA codon change
D15N.....	GAC to AAC
D116A.....	GAC to GCC
D116E.....	GAC to GAG
D116N.....	GAC to AAC
D157N.....	GAT to AAT
D228N.....	GAT to AAT
H291N.....	CAC to AAC

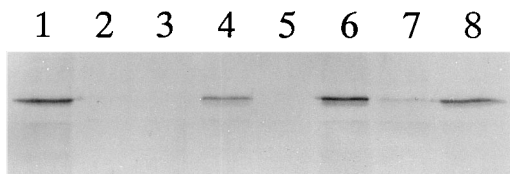


FIG. 2. Secretion of the GCAT variants by *A. salmonicida*. Cultures were grown to an optical density at 600 nm of 0.5 and were induced for 10 h with 1 mM IPTG. Fifteen microliters of each culture supernatant was separated by SDS-PAGE, and the gel was stained with Coomassie blue. Lane 1, wild type; lane 2, D15N; lane 3, D116A; lane 4, D116E; lane 5, D116N; lane 6, D157N; lane 7, D288N; lane 8, H291N.

through the general secretion pathway, and incorrectly folded proteins are rapidly degraded in the periplasm. Thus, the ability of *A. salmonicida* to secrete a cloned mutant protein is one measure of the effect of the mutation on the protein's structure. In Fig. 2, the levels of each of the mutant proteins in culture supernatants are compared with that of the wild type. Two of the changes, His-291 to Asn and Asp-157 to Asn, had no obvious effect on secretion. All of the other aspartate replacements profoundly reduced secretion of the mutant proteins. The D15N and D288N mutant enzymes could be detected on Coomassie blue-stained gels, although their levels were estimated to be less than 5% that of the wild type. The estimates were made by comparing Coomassie blue-stained gels containing the culture supernatants with a gel containing serial dilutions of a known amount of wild-type GCAT. The D116N variant could be detected only by Western blotting (immunoblotting; data not shown here). When the time dependence of its secretion was compared with that of the wild type by pulse-chasing and immunoprecipitating, it became clear that although the mutant protein appeared to be produced at the wild-type level, far less was released into the culture supernatant (Fig. 3). We have made similar observations with several variants of aerolysin, a toxin which also can normally be secreted by *A. salmonicida* (33, 34). No noticeable improvement in levels of secretion was observed by changing Asp-116 to alanine. However, when the Asp was changed to Glu, the D116E mutant GCAT was secreted reasonably well, although at a level that was still lower than that of the wild type (Fig. 2).

**Purification of the mutant enzymes.** Using our standard purification procedure (15), we were able to isolate three of the variant GCAT proteins, those containing the changes D116E, D157N, and H291N. The other variants could not be

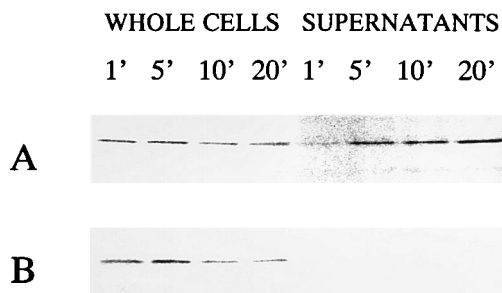


FIG. 3. Pulse-chase of D116N and wild-type GCAT expressed by *A. salmonicida*. Cultures were grown to an optical density at 600 nm of 1.0 and induced with 1 mM IPTG for 15 min before [ $^{35}$ S]methionine was added. Unlabeled methionine was added after 1 min, and samples were taken at the indicated times (in minutes) after the start of the pulse and prepared for SDS-PAGE as described in the text. (A) Wild type. (B) D116N variant.

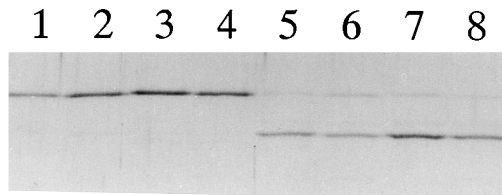


FIG. 4. Effects of trypsin treatment on purified GCAT variants. Purified protein samples were incubated with trypsin (1  $\mu$ g/ml) for 40 min at room temperature before SDS-PAGE was performed. Lanes 1 to 4, undigested samples; lanes 5 to 8, trypsin-digested samples; lanes 1 and 5, wild type; lanes 2 and 6, D116E; lanes 3 and 7, D157N; lanes 4 and 8, H291N.

purified, either because their concentrations in culture supernatants were too low or because they were more unstable than the wild type and were lost during the purification procedure.

**Proteolysis of mutant GCAT proteins.** We have shown that wild-type GCAT is nicked by trypsin to remove a peptide near the C terminus (Fig. 1), producing a protein containing a 27-kDa polypeptide joined to a 4.7-kDa fragment by a disulfide bridge. Only the 27-kDa fragment is visible after SDS-PAGE under reducing conditions. The nicked form of the enzyme is more surface active than the untreated protein, and it is resistant to further proteolysis (14, 15). Upon treatment with trypsin, the three mutant GCAT proteins that we were able to purify all showed a level of reduction in their molecular masses that was the same as that of wild-type GCAT (Fig. 4). This is another indication that the conformation of these proteins was not significantly different from that of the wild type.

In order to assess the consequences of trypsin treatment on the D15N and D288N variants, both of which we could not purify, we added the protease directly to culture supernatants. For both mutant enzymes, the fate of the 35-kDa band was compared with its fate in treated culture supernatant containing wild-type enzyme. The results in Fig. 5 show that the D288N GCAT was resistant to digestion by trypsin under conditions in which wild-type GCAT was nicked to produce the 27-kDa fragment. Even Western blotting failed to detect a 27-kDa fragment in the D288N supernatant under conditions in which a similar amount of the nicked wild-type GCAT was detectable (data not shown). The results in Fig. 5 also suggest that, in contrast to D288N GCAT and the wild-type enzyme, the D15N variant is completely degraded by trypsin. Thus, it would appear that the structures of the D15N and D288N enzymes are not the same as that of the wild type.

**Enzyme activities of GCAT aspartate mutants.** Three methods were used to compare the enzymatic activities of the GCAT variants (Table 2). The formation of white halos of insoluble calcium salts of free fatty acids on Tween-peptone plates (28) was used as a crude qualitative measure of expression of activity by *E. coli*. This method has two advantages: it is

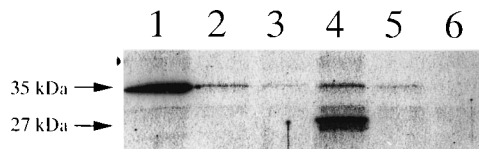


FIG. 5. Effects of trypsin treatment on GCAT in culture supernatants. Culture supernatants were incubated with trypsin (1  $\mu$ g/ml) for 40 min at room temperature. The supernatants were obtained from the experiment whose results are shown in Fig. 2. Twenty microliters of each trypsin-digested and undigested supernatant was separated by SDS-PAGE, and the gel was stained with Coomassie blue. Lanes 1 to 3, undigested samples; lanes 4 to 6, trypsin-digested samples; lanes 1 and 4, wild type; lanes 2 and 5, D288N; lanes 3 and 6, D15N.

TABLE 2. Effects of amino acid changes on enzyme activity

Mutation	Tween activity <sup>a</sup>	% Wild-type sp act <sup>b</sup> by:	
		Hydrolysis	Acyl transfer
Wild type	+	100	100
D15N	-	ND <sup>c</sup>	ND
D116A	-	ND	ND
D116E	-	60.9	4.1
D116N	-	ND	ND
D157N	+	142	96
D288N	-	0 <sup>d</sup>	10 <sup>d</sup>
H291N	-	0	0

<sup>a</sup> Formation of halos (+) or no formation of halos (-) surrounding *E. coli* colonies grown on Tween-peptone plates (28).

<sup>b</sup> Specific activities were determined with the slopes of lines relating enzyme concentration to activity. Correlation coefficients exceeded 0.98 in all experiments.

<sup>c</sup> ND, not detected.

<sup>d</sup> Specific activities were estimated by directly comparing the enzyme activity of the GCAT variant with that of an equivalent amount of wild-type GCAT in culture supernatants.

extremely sensitive and it can detect activity whether or not the enzyme is secreted. Assays using *p*-nitrophenyl butyrate hydrolysis or cholesterol ester formation were used for quantitative estimates of esterolytic and acyl transfer activity in fractions containing wild-type or mutant GCAT.

*E. coli* cells expressing the gene encoding the D116N variant showed no halo when grown on Tween-peptone plates, in contrast to cells producing comparable amounts of the wild-type GCAT (not shown here). In addition, culture supernatants from *A. salmonicida* expressing the D116N protein were completely devoid of acyltransferase activity in spite of the fact that the protein could be detected by Western blotting (see above). These culture supernatants were also incapable of *p*-nitrophenyl butyrate hydrolysis.

*E. coli* cells carrying the D116E variant also produced no halo on Tween-peptone plates (Table 2). In spite of this, the purified mutant protein was capable of hydrolyzing *p*-nitrophenyl butyrate at 60% the level of the wild type. Interestingly, there was a much greater difference in its level of acyltransferase activity, which was only 4% that of the wild type (Table 2).

The D157N protein could be distinguished from the wild-type GCAT protein only by virtue of its slightly increased level of hydrolytic activity with *p*-nitrophenyl butyrate as a substrate. Its level of acyltransferase activity was the same as that of the wild type, as was its level of lipolytic activity on Tween-peptone plates (Table 2).

Although we were unable to purify the D288N protein, we made a qualitative comparison of its level of activity with that of the wild type in culture supernatants by matching amounts used in the acyltransferase assay on the basis of band intensity on Coomassie blue-stained SDS-polyacrylamide gels. This led us to conclude that the D288N protein has approximately 10% of the acyltransferase activity of wild-type GCAT. Interestingly, the mutant enzyme appeared to be completely inactive against *p*-nitrophenyl butyrate (Table 2).

**Enzyme activity of the H291N mutant.** No hydrolytic or acyltransferase activity could be detected with the purified H291N protein. Moreover, *E. coli* cells expressing the H291N protein showed no lipolytic activity on Tween-peptone plates (Table 2).

## DISCUSSION

The results of this study strongly indicate that His-291 and Asp-116 are members of the catalytic triad of the *A. hydrophila* lipase, together with Ser-16, on which we reported previously (14). When His-291 was replaced with asparagine, the resulting protein was completely inactive, in spite of the fact that it was secreted at the same level as that of the wild type and it was correctly processed by trypsin, both signs that the three-dimensional structure of the protein had not been affected by the mutation. It is the only histidine in the enzyme that is found in the same block in all members of the new family (Fig. 1). As we discussed in the introduction, in a previous communication we concluded that a histidine was not involved in GCAT catalysis. This was based on the observation that the 27-kDa fragment produced by trypsin retained activity when it was separated from the 4.7-kDa fragment (which contains His-291) by SDS-PAGE under reducing conditions. We have no reasonable explanation for this result, which is obviously inconsistent with the present findings and with the generally held view that all of the serine esterases have a histidine in their active sites. We can suggest only that somehow the refolding of the 27-kDa peptide after reduction and SDS treatment gave rise to a structure that had residual lipase activity.

We replaced four aspartates, one at a time, but deciding which aspartate is essential for the catalytic activity of the enzyme was more difficult than for histidine. This is because at least three of the changes, D15N, D116N, and D288N, not only affected the activity of the enzyme but reduced secretion so that we could not get enough protein to permit purification. All of these mutants appeared to be physically different from the wild type, accounting for the reduced secretion rates and for activity changes. Only the D157N change seemed to have no effect on the enzyme's secretion or activity.

Although an aspartic acid corresponding to Asp-15 is found in block I of all of the proteins in Table 1, this residue is unlikely to be part of the catalytic triad. In all lipases for which the triad is known, the nucleophilic serine precedes the aspartate in the sequence. What is more, in all lipases of known structure, the Asp, Ser, and His of the triad are located in separate loops (2, 9, 11, 21, 25, 26, 32). We have found that other changes in the same region of GCAT (F13S and S18G) also result in decreased secretion (22), and this may suggest that the general secretory pathway system is particularly sensitive to changes in the amino terminus.

There are several reasons to believe Asp-288 is also not involved in catalysis. It is very close to His-291, whereas the members of the catalytic triad are found in separate loops, as noted above. In addition, there is no aspartate at all in block V of the *V. parahaemolyticus* protein in Fig. 1, yet this protein has been shown to be a lipase (27). Thus, it was not surprising to find that, in contrast to the D116N variant of GCAT, the variant D288N retained an estimated 10% of the level of wild-type acyltransferase activity.

It seems reasonable to conclude that Asp-116 is essential for catalysis. It is the only aspartate conserved in the same block of all members of the family, and it is correctly positioned between the catalytic serine and histidine. The D116N mutant was completely inactive under all conditions we tested. Even when we overexpressed the mutant in *E. coli* behind a T7 promoter, under conditions in which wild-type GCAT similarly expressed was easily measured, we were unable to detect any activity (not shown here). When Asp-116 was replaced with glutamate, the resulting variant was secreted, although at reduced levels, and it was 60% as active as the wild type in the *p*-nitrophenyl butyrate assay. Interestingly, D116E GCAT had

no detectable activity against Tween 80 and far less acyltransferase activity than the wild type, suggesting that its substrate specificity is different from that of the wild type. Comparable observations have been made with the lipase II from *G. candidum* (31). When the active-site glutamate residue of this enzyme was changed to aspartate, the mutant enzyme exhibited 44% of the level of wild-type activity against triolein but only 13% of the level of wild-type activity against tributyrin (31).

In conclusion, *Aeromonas hydrophila* likely has the same catalytic triad as other lipases and the three amino acids appear in the same order in the GCAT sequence as in the sequences of other lipolytic enzymes. What distinguishes GCAT and the other members of the lipase subfamily with related sequence blocks from other lipases remains to be determined. In any case, our ability to use these blocks to identify the triad of GCAT is strong circumstantial evidence that the Asp, Ser, and His in the corresponding blocks of the other proteins will be catalytic residues. This knowledge should help in determining the function and in studying the properties of these proteins.

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