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Studies on the Reaction Mechanism of a Microbial Lipase/ Acyltransferase Using Chemical Modification and Site-directed Mutagenesis*

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Aeromonas hydrophila **releases a protein which is a member of the lipase superfamily, similar in reaction mechanism to the important mammalian plasma enzyme lecithin-cholesterol acyltransferase. We have used chemical modification and site-directed mutagenesis of the protein to identify amino acids which may be involved in catalysis. The enzyme was unaffected by phenylmethylsulfonyl fluoride, but it was almost completely inhibited by another serine-reactive compound, diethyl *p*-nitrophenyl phosphate. A serine selectively modified by this reagent was identified by sequencing the amino-terminal region of the protein. It was located at position 16, in the short consensus sequence shared by the enzyme with other lipases. When this serine was changed to asparagine the product was an enzymatically inert protein which nevertheless retained the surface activity of the wild-type enzyme, suggesting its ability to bind to substrate was not changed. Diethylpyrocarbonate treatment drastically reduced the rate of acyl transfer by the native enzyme, but this did not appear to be due to modification of an essential histidine, since inhibition was not reversed by addition of hydroxylamine. We have shown that only two of the histidines in the enzyme can be involved in catalysis (Hilton, S., McCubbin, W. D., Kay, C. M., and Buckley, J. T. (1990) *Biochemistry*, 29, 9072–9078). Replacing both of these with asparagines had little or no effect on enzyme activity. These results indicate that, in apparent contrast to other lipases, histidine does not participate in the reaction catalyzed by the microbial enzyme. Since catalysis was not inhibited by sulfhydryl reagents, we conclude that a free cysteine is also not required for activity. This may distinguish the microbial enzyme from the mammalian acyltransferase.**

Most of the lipases found in nature appear to be members of an enzyme superfamily (1–6). They are all relatively nonspecific esterases, typically activated by binding to lipid-water interfaces. Some will also catalyze esterification reactions, and a few retain catalytic activity in non-aqueous systems. As a result, lipases have attracted considerable interest as biotechnological tools in recent years (7). The enzymes share a small consensus region which is thought to contain the active site. The sequence G-*X*-S-*X*-G is found within this region. This sequence is also found in other esterase families, including the family of serine proteases (8, 9). Recent x-ray crystallographic data obtained for pancreatic lipase and *Mucor miehei* triglyceride lipase suggests that, like serine proteases, the active sites of these enzymes contain a Ser-His-Asp triad (10, 11). This conclusion is generally supported by the results of a variety of chemical inactivation experiments. Hence most lipases are inhibited by diethylpyrocarbonate, inferring a role for histidine in enzyme activity. Although many lipases are resistant to some water-soluble serine-reactive reagents such as phenylmethylsulfonyl fluoride, it is generally believed that a serine residue participates in catalysis and perhaps in substrate binding (12). However, not all the data in the literature support the Ser-His-Asp model for lipase action. Chemical modification studies have suggested that a histidine residue is not required for the action of pancreatic lipase (13) or for hepatic lipase (14) and pancreatic lipase can still hydrolyze water soluble substrates after the serine of its proposed triad has been chemically modified (15).

The mammalian plasma enzyme lecithin-cholesterol acyltransferase and its microbial analogue glycerophospholipid-cholesterol acyltransferase (GCAT)[1] appear to be members of the lipase superfamily, as both contain sequences homologous to the lipase consensus sequence (16, 17). Their active sites must be in some way unusual however, since in the presence of an acyl acceptor such as cholesterol they preferentially catalyze acyl transfer rather than hydrolysis (18, 19). Recent evidence suggests that one or more cysteines participates in the transfer reaction catalyzed by lecithin-cholesterol acyltransferase (16, 20), but this does not appear to be the case for the microbial enzyme since its only two cysteines are in disulfide linkage (21).

The results of chemical modification experiments are often equivocal or ambiguous, unless they are supported by data obtained by genetic modification. We have cloned and sequenced the gene for GCAT, and we have developed a method for overexpression and purification of large amounts of the protein (17, 21). Here we describe the effects of various amino acid modifying reagents on enzyme activity as well as the consequences of changing selected amino acid residues by site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Vectors and Cell Growth—Most of the bacteria and vectors used have been previously described (21–23). *Escherichia coli* TG1 was supplied by the Amersham Corp.

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[1] The abbreviations used are: GCAT, glycerophospholipid-cholesterol acyltransferase; DEPC, diethylpyrocarbonate; PC, phosphatidylcholine.

Cell Growth—E. coli HB101 was grown at 37 °C in LB medium (24). TG1 was grown as recommended by the supplier. *Aeromonas salmonicida* Rif-1 was grown at 27 °C in LB medium buffered with Davis minimal media (25) and supplemented with 0.2% glucose. When appropriate, antibiotics were added to the following concentrations: ampicillin (100 mg/ml), rifampicin (40 mg/ml), kanamycin (40 mg/ml).

Site-directed Mutagenesis—Potentially important residues in the protein are identified in Fig. 1, and the changes which were made are listed in Table I. All mutagenesis reactions were performed using the methods of Eckstein *et al.* (26–28) with materials supplied by the Amersham Corp. Positive clones were screened initially by plaque hybridization or by dot blot hybridization. Final confirmation of the nucleotide changes in the clones was achieved by chain-terminating DNA sequencing using Sequenase Version 2.0 with materials and procedures supplied by the U. S. Biochemical Corp. Restriction analysis was also performed on positive replicative form DNA to ensure that the clones contained the same restriction sites as the wild-type DNA. Once a clone was confidently identified by these methods, the DNA was inserted into the vector pMMB66 (29) as we have described previously (22, 23).

Plasmid Manipulations and Bacterial Matings—Plasmid preparations and cloning procedures as well as bacterial matings between HB101 bearing mutagenized GCAT/pMMB66 constructs and *A. salmonicida* Rif-1 were carried out as before (22).

Protein Purification—All of the mutant proteins were purified according to our published procedure (21). Each migrated as a single band upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (30). The concentration of each purified protein was calculated from the extinction coefficient we have determined previously for the native enzyme (21).

Chemical Modifications—All reagents were freshly prepared and results were always compared to controls treated in parallel with the same buffers and solvents. The enzyme (1.4 mg/ml) was treated with serine-reactive phenylmethylsulfonyl fluoride or diethyl *p*-nitrophenyl phosphate at 5 mM final concentration in 20 mM Tris, 100 mM NaCl, 2 mM β-mercaptoethanol, pH 7.4, for 12 h at room temperature before assay. In order to study the effect of phenylboronic acid, the reagent was added directly to assay mixtures to a final concentration of 2 mM. Diethylpyrocarbonate inhibition was measured after treatment of the enzyme (0.5 mg/ml) for 20 min with 2.5 mM reagent in 100 mM NaH_2PO_4, pH 6.0, at room temperature. To test for reversal of diethylpyrocarbonate inhibition, the enzyme was further treated with 1 M neutral hydroxylamine for 1 h. Cysteine modification followed the procedure of Gargouri *et al.* (31). Enzyme (0.5 mg/ml), in 0.25 M Tris, pH 8.0, was incubated with 5 mM 5,5′-dithiobis-2-nitrobenzoic acid at room temperature for 18 h. Conversion of lysine residues by complete reductive methylation was performed essentially as outlined by Jentoft and Dearborn (32).

Enzyme Activity—Enzyme activity was measured in three ways. Acyl transfer from egg phosphatidylcholine to [4-^{14}C]cholesterol was determined as before (18). Hydrolysis of phosphatidylcholine was measured titrimetrically, essentially as described by van Oort *et al.* (33), using 3 mM diheptanoyl phosphatidylcholine in 10 mM $CaCl_2$, 5 mM Tris, pH 8.0. It was also estimated using a KSV surface barostat with didodecanoyl phosphatidylcholine monolayers at a surface pressure of 15 millineutons/m. The trough had a surface area of 495 cm^2. Enzyme was injected under the monolayer into the subphase (20 mM Tris, 150 mM NaCl, pH 7.4). Surface activities were also determined with the surface barostat using cylindrical 17-ml Teflon troughs and monolayers of sphingomyelin, which is not a substrate for the enzyme.

Other Methods—The amino acid compositions of purified proteins were measured using an Applied Biosystems 420 A amino acid analyzer. Amino-terminal sequences were determined with an Applied Biosystems 470 A gas phase sequenator.

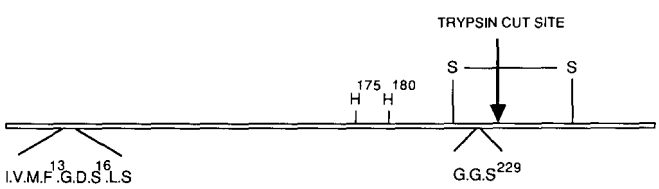

FIG. 1. **Potentially important regions in the primary sequence of the lipase.** The amino acids which were changed are numbered. The other three histidines in the protein are not shown as they are all on the carboxyl-terminal side of the trypsin cut site.

TABLE I

Site-specific mutations in GCAT

Amino acid	Original codon	Mutant codon	New amino acid
Phe^{13}	TTT	TCT	Ser
Ser^{16}	AGC	AAC	Asn
His^{175}	CAT	AAT	Asn
His^{180}	CAC	AAC	Asn
Ser^{229}	AGC	AAC	Asn

RESULTS

Chemical Modification of GCAT—The effects of various chemical treatments on native GCAT are summarized in Table II. Sulfhydryl-reactive 5 mM 5,5′-dithiobis-2-nitrobenzoic acid did not inhibit hydrolysis and had only a modest effect on acyl transfer. We have also found (not shown here) that iodoacetamide does not alter enzyme activity. These results are not surprising, as we have shown that the only two cysteines in the enzyme are in disulfide linkage (21). Like some other lipases (12, 34), GCAT was not inhibited by phenylmethylsulfonyl fluoride, perhaps because of the physical properties of the reagent. However, both acyl transfer and hydrolysis were reduced more than 75% by treatment with diethylpyrocarbonate and to a somewhat lesser extent by phenylboronic acid, both of which also interact with serine residues. Acyl transfer was also inhibited by DEPC, which normally indicates the participation of a histidine residue in the active site (35). However, the inhibition was not reversed by hydroxylamine, suggesting that an essential residue other than histidine had been changed (35, 36). Furthermore, hydrolysis of monodisperse phosphatidylcholine was unaffected by this reagent. Reaction of some enzymes with DEPC may result in the derivatization of lysine residues (36–38) and this could account for the inhibition of acyltransfer we observed. The fact that modification of lysine residues by reductive methylation of GCAT led to a 80% loss of acyl transfer activity and a 45% loss in hydrolytic activity (Table II) strengthens this conclusion.

Identification of a Serine Modified by Diethylpyrocarbonate—The results in Table II indicated that a serine residue is required for enzyme activity. Since the lipase consensus sequence in GCAT is located very close to the amino terminus of the protein (Fig. 1), we reasoned that we could determine

TABLE II

Effect of chemical inhibitors on PC-cholesterol acyltransfer and PC hydrolysis

Treatment	Percent control activity[a]	
	Hydrolysis	Acyl transfer
Phenylmethylsulfonyl fluoride	113.0 ± 3.8	103.0 ± 3.5
Phenylboronic acid	47.1 ± 3.3	39.1 ± 3.5
Diethyl *p*-nitrophenyl phosphate	17.6 ± 0.2	25.9 ± 1.4
5,5′-Dithiobis-2-nitrobenzoic acid	101.1 ± 14.1	88.9 ± 15.4
DEPC	96.9 ± 4.7	30.1 ± 4.7
DEPC + NH_2OH	100.5 ± 9.7	30.1 ± 2.8
Reductive methylation	55.2 ± 4.1	28.3 ± 2.0

[a] Results are expressed as percent of comparable control activity mean ± S.E. for three determinations. Control activity was in the range 184–238 μmol/min/mg in the hydrolysis assay and 40–56 μmol/min/mg in the acyl transfer assay.

if one of the serines in this sequence was modified selectively by diethylpyrocarbonate by simply sequencing the enzyme after treatment. When this was done, recovery of serine at position 16 was 80% lower than for untreated enzyme, corresponding to the inhibition of acyl transfer we observed in Table II. Recoveries of other serines on either side of this position in the segment sequenced were not affected (data not shown).

Site-directed Mutagenesis of Ser^{16}—In order to further evaluate the importance of Ser^{16}, this residue was replaced with asparagine by site-directed mutagenesis. The modified enzyme, which behaved exactly like native GCAT during purification, was completely unable to catalyze either acyl transfer or hydrolysis (Table III).

Surface Activity of the Ser^{16} *Mutant Enzyme*—Either the serine at position 16 might participate directly in the catalytic reaction, or it might be an essential part of a separate lipid binding site. Separate binding and catalytic sites, each containing serine, have been proposed for other lipases (12). In order to determine if the binding ability of the enzyme was altered by changing this serine to asparagine, the surface activity of the mutant and wild-type GCAT were compared using monolayers of sphingomyelin. It may be seen in Fig. 2 that the two proteins are indistinguishable, leading to the conclusion that Ser^{16} is not important in binding to the lipid-water interface.

Site-directed Mutagenesis of His^{175} *and His*^{180}—We have shown previously that GCAT can be nicked by treatment with trypsin producing two polypeptides joined by a disulfide bridge, and that the larger 27-kDa polypeptide retains acyl transfer activity after it is reduced and freed from the 4-kDa peptide by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21). The 27-kDa protein contains only two histidines (Fig. 1). If a histidine is required for enzyme activity, it can only be one of these. But the results in Table III show that enzyme activity was not destroyed when either was changed to asparagine. Indeed, a mutant protein in which both histidines were substituted was also active. The reduction in the number of histidines was confirmed by amino acid analysis. The double His mutant and the His^{180} mutant were somewhat less active than the wild-type enzyme, perhaps a sign that His^{180} is important in maintaining enzyme conformation.

Site-directed Mutagenesis of Potential Binding Regions—Two possible substrate binding sites were identified, based on our limited knowledge and examination of the primary structure of the enzyme. The first is the hydrophobic string of amino acids which precedes Gly^{14} in the active site and the second is a sequence containing G-G-S^{229} which is found in many lipases and which has been claimed to be important for activity (Fig. 1; Ref. 12). Phe^{13} in the first sequence was changed to a Ser in order to modify the hydrophobicity of this region. However, *A. salmonicida* did not produce detectable amounts of the mutant protein, presumably because Phe^{13} is close enough to the amino terminus to be important in its secretion across either the inner or outer membrane, or because the conformation of the protein is altered in some way that causes it to be rapidly degraded. The enzyme changed at Ser^{229} was expressed, and it was purified in the usual way. Its activity was indistinguishable from the activity of the native enzyme (Table III).

Activity of Modified GCAT against Monolayers of Phosphatidylcholine—The activities of the mutant proteins were also compared to wild type using phosphatidylcholine monolayers as substrates. It may be seen from the results in Table IV that all of the mutant proteins behaved as native GCAT except for the Ser^{16} mutant which was completely inactive, just as it was in the other assays. Interestingly, GCAT treated with DEPC was considerably less active than untreated enzyme and the lag time (an indication of the rate of surface penetration) was increased. This may indicate that reaction with DEPC diminishes the ability of the enzyme to bind to substrate at a lipid-water interface.

TABLE III

Effect of amino acid modifications on PC-cholesterol acyl transfer and PC hydrolysis

Amino acid modified	Specific activity[a]	
	Hydrolysis	Acyl transfer
None (wild type)	228 ± 5.3	46.9 ± 3.9
Ser^{16}	0	0
His^{175}	216 ± 13.2	53.3 ± 0.3
His^{180}	192 ± 7.5	42.4 ± 1.4
His^{175} + His^{180}	181 ± 15.1	41.7 ± 1.7
Ser^{229}	234 ± 8.0	40.0 ± 3.7

[a] Specific activities are all means of three determinations of mean ± S.E. Units are μmol/min/mg.

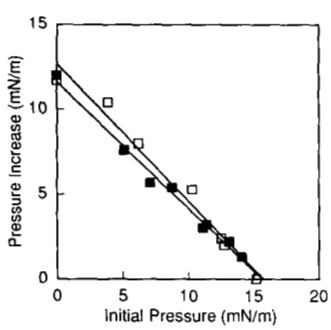

FIG. 2. **Surface activity of native enzyme and the Ser^{16} mutant.** The proteins (40 μg) were injected under monolayers of sphingomyelin at the initial surface pressures indicated and the change in surface pressure was measured. ■, native protein; □, Ser^{16} mutant.

DISCUSSION

Perhaps the most remarkable conclusion which can be drawn from the present study is that histidine does not participate in the reaction catalyzed by the microbial lipase. Reports that histidine is essential for lipolytic activity of other enzymes have been based on results obtained by chemical modification, but the pitfalls of relying entirely on this approach are illustrated by our findings. Thus, although the acyl transfer reaction catalyzed by GCAT was inhibited by DEPC, the enzyme remained active when both of the histidines which could be involved in the reaction were replaced. Because DEPC inhibition was not reversed by hydroxylamine, it seems probable that the inhibition of acyl transfer that we recorded was due to the modification of one or more lysine residues or the amino terminus of the protein. The residue

TABLE IV

Activity of modified lipases against PC monolayers

Modified lipase	Percent control activity[a]	Percent control lag time[a]
Ser^{16}	0	N.D.
His^{175}	117	91
His^{180}	96	113
His^{175} + His^{180}	96	111
Ser^{229}	125	79
DEPC-treated native enzyme	49	233

[a] Results are the means of duplicate determinations which did not differ by more than 5%. N.D., not determined.

which is changed is more likely to be involved in binding at the lipid-water interface than in catalysis, as hydrolysis of short chain phosphatidylcholine was not affected by DEPC treatment, yet hydrolysis of phosphatidylcholine monolayers was inhibited. In addition, the lag time (the time taken by the enzyme to reach maximum velocity) was increased, a sign that the ability of the enzyme to bind to or penetrate the interface may have been diminished by the reagent. The positively charged amino-terminal amino group is known to be essential for binding of phospholipase A_2 to its substrate (39), and a lysine near the amino terminus of gastric lipase is required for the activity of this enzyme (40). The fact that GCAT activity was inhibited by reductive methylation is further evidence that a primary amino group rather than histidine has an important role in GCAT action. The results of reductive methylation were somewhat different from those using DEPC, since phosphatidylcholine hydrolysis was also affected, although less so than was acyl transfer. Perhaps there is only one DEPC-reactive lysine, which plays a role in the assay measuring acyl transfer but not in the PC hydrolysis assay, and one or more others, also affected by reductive methylation, which have some important general function in enzyme structure.

From our results it seems clear that Ser^{16} is either at the active site of the microbial enzyme, or that it is necessary for maintenance of the protein's conformation. A similar conclusion has been reached by Davis *et al.* (41) for Ser^{147} in the consensus sequence of hepatic lipase, the only other lipase for which catalysis has been studied by site-directed mutagenesis. Although further experiments to identify other important functional groups in GCAT will be necessary, a role for histidine or cysteine as a general base in ester hydrolysis or acyl transfer appears to have been ruled out by our results. Thus this enzyme seems to have a reaction mechanism quite different from those proposed for other lipases and for mammalian lecithin-cholesterol acyltransferase. Whether GCAT is unique, which appears unlikely as the active serine is within the lipase consensus sequence, or whether future experiments will change our thinking about the mechanisms of the other enzymes, remains to be seen.

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