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Purification and Spectral Study of a Microbial Fatty Acyltransferase: Activation by Limited Proteolysis†

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ABSTRACT: A fatty acyltransferase with a reaction mechanism similar to that of mammalian lecithin: cholesterol acyltransferase has been purified from culture supernatants of a mutant *Aeromonas salmonicida* containing the cloned *Aeromonas hydrophila* structural gene. Typically, more than 35 mg of protein were isolated from 2 L of culture supernatant. The amino-terminal sequence, amino acid composition, and molecular weight of the purified protein corresponded to predictions based on the sequence of the gene, indicating that the signal sequence had been correctly removed during export but that no further processing had occurred. Analysis of the far-UV circular dichroic (CD) spectrum of the enzyme showed that it consists of 31% α-helix, 21% β-sheet, and 16% β-turn, with 12% of aperiodic form. Treatment of the purified protein with a variety of proteases resulted in nicking near the C-terminus. This led to an increase in enzyme activity against lipids in erythrocyte membranes and increased rate of hydrolysis of *p*-nitrophenyl butyrate. Activation was accompanied by a change in the CD spectrum and a change in its aggregation state. The trypsin cut site was located between the two cysteines in the enzyme. Evidence is presented that the cysteines are joined by a disulfide bond and therefore cannot participate in acyl transfer. This may distinguish the microbial enzyme from lecithin:cholesterol acyltransferase. This is the second extracellular *A. hydrophila* protein that we have shown can be activated by proteolysis after it is released.

Esterases that catalyze the hydrolysis of lipids are found everywhere in nature. Recently they have attracted considerable attention, not only because of their important and essential roles in the metabolism of plasma and cellular lipids but because of potential biotechnological applications in medicine, food science, and synthetic chemistry (Harwood, 1989). *Vibrio* species release an unusual lipolytic enzyme (MacIntyre et al., 1979), which shares several properties with plasma lecithin:cholesterol acyltransferase (LCAT). Like LCAT, the microbial enzyme catalyzes the transfer of the sn-2 fatty acid of lecithin to cholesterol (MacIntyre & Buckley, 1978). Both the equatorial hydroxyl and the trans-fused A:B ring are required for optimal transfer to occur (Buckley, 1982).

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When an acyl acceptor is not present, the two enzymes catalyze hydrolysis of lecithin, although they can be distinguished from most of the phospholipases because neither requires calcium. The microbial enzyme does not appear to distinguish between the sn-2 and sn-1 positions for hydrolysis, whereas LCAT shows some preference for the sn-2 position (Aron et al., 1973). Both enzymes will catalyze acyl transfer from a variety of other donors, including *p*-nitrophenyl esters (Buckley, 1983; Bonelli & Jonas, 1989).

Both LCAT and the microbial enzyme, which we have called glycerophospholipid:cholesterol acyltransferase (GCAT), contain a short amino acid sequence corresponding to the consensus sequence found in all of the lipases so far studied (Thornton et al., 1988; Jauhiainen & Dolphin, 1986). In spite of this, neither enzyme appears to be a true lipase. Thus, aside from the fact that they selectively carry out acyl transfer in the presence of an appropriate acceptor, they prefer phospholipid substrates, rather than triacylglycerols, and they do not seem to require the kind of interfacial activation that characterizes lipases such as the enzyme from pancreas (Verger & de Haas, 1976). The particular features of LCAT and GCAT that enable them to catalyze acyl transfer at interfaces are not understood. It is possible that hydrogen bonding between the hydroxyl of the acceptor and the ester linkage of the donor is important at the active site of GCAT (Buckley, 1983) and there is evidence that cysteine plays a role in LCAT-catalyzed acyl transfer (Jauhiainen & Dolphin, 1986; Jauhiainen et al., 1988). Whether or not this is also the case with GCAT is not known.

The microbial acyltransferase is smaller than LCAT and it is not glycosylated. In addition it has a much higher turnover number and it is far more stable (Buckley et al., 1982). For these reasons, and because it will catalyze ester formation even in aqueous systems, GCAT could be considered a candidate to replace some of the lipases currently used in biotechnological applications. Studies of its reaction mechanism should be much simpler than those using LCAT and should yield information that can be applied to the plasma enzyme. Purification of GCAT from *Aeromonas salmonicida* (Buckley et al., 1982) involves a rather complicated procedure, which requires detergents and yields only small amounts of protein. Recently we have cloned and sequenced the gene for GCAT from *Aeromonas hydrophila* (Thornton et al., 1988). Here we describe a much simpler purification of the enzyme from a mutant *A. salmonicida* containing the cloned *A. hydrophila* gene. The mutant was chosen because it produces greatly reduced amounts of its own extracellular enzymes, particularly proteases and chromosomally coded GCAT. We have used a similar system previously to purify the hole-forming toxin aerolysin of *A. hydrophila* (Buckley, 1990) and to isolate aerolysin modified by site-directed mutagenesis (Green & Buckley, 1990).

Materials and Methods

Strains, Plasmids, and Growth Conditions. The genotypes and sources of the strains and plasmids employed are listed in Table I. Bacteria were grown in LB (Maniatis et al., 1982) containing Davis buffer (Miller, 1972) and 0.2% (w/v) glucose. Kanamycin and rifampicin (40 μg/mL) and ampicillin (100 μg/mL) were added when required. Plasmid pJT2 was mobilized to CB3 with the helper plasmid pRK2013 by the same filter mating procedure employed earlier (Harayama et al., 1980).

Protein Purification. Overnight cultures of CB3 (pJT2) were diluted 1:100 into 400 mL of media and grown with shaking at 27 °C to an OD_{600} of 0.5. Production of GCAT was induced by the addition of isopropyl β-D-thiogalactoside to 1 mM and growth was continued for 24 h. All subsequent procedures were carried out at 4 °C. Cells were removed by centrifugation at 10000 rpm for 20 min and ammonium sulfate was added to the clear culture supernatant to 60% of saturation. The precipitate was recovered by centrifugation at 10000*g* for 20 min and suspended in 1/80th volume of 20 mM phosphate and 0.3 M NaCl, pH 6.0. After centrifugation for 15 min at 8000 rpm, the supernatant was discarded and the pellet was suspended in 1/200th of the original volume of the same buffer and centrifuged for 15 min at 8000 rpm. The resulting supernatant was applied to a 2.6 × 100 cm Sepharose S-300 (Pharmacia) column and proteins were eluted with the pH 6 buffer. Peak fractions were combined and protein was precipitated by the addition of ammonium sulfate to 85% of saturation. The precipitate was recovered by centrifugation and redissolved in 10 mL of 20 mM Tris HCl, pH 7.4. Residual ammonium sulfate was removed on a Sephadex G25 (Pharmacia) column and the desalted sample was applied to a 1.6 × 30 cm column of DEAE-Sepharose CL-6B (Pharmacia) equilibrated in the same Tris buffer. Proteins were eluted with a linear gradient of 250 mL of starting buffer and 250 mL of 20 mM Tris·HCl and 0.3 M NaCl, pH 7.4. All of the enzyme activity was recovered in a single peak at an NaCl concentration of approximately 0.15 M.

Table I: Bacterial Strains and Plasmids

strain	description	source
E. coli HB101	*recA*13, *hsdS*20, *ara*14, *proA*2, *lacY*1, *galK*2, *leuB*6, *rpsL*20, *xyl*5, *mt*/1, SupE44	E. E. Ishiguro
A. salmonicida Rif-1	ATCC 14174 Rif^r	this laboratory
CB3	Rif-1 cDNA::Tn*5*; Km^r	this laboratory
plasmids		
pRK 2013	Km^r, conjugative helper plasmid	E. W. Nester
pUW 964	pRK2013 kan::Tn*7xyz*::Tn5	S. Falkow
pJT2	pMMB66EHΩ(JT1, *Eco*RI–*Hin*dIII, 1.2 kb)Ap^r	this laboratory

Enzyme Assays. Three different assays were used to measure enzyme activity. One of these, using mixed micelles of phosphatidylcholine and cholesterol in Triton X-100, involved measurement of acyl transfer to radiolabeled cholesterol (Buckley, 1982). Another determined the production of glycerol phosphate produced by the transfer of fatty acids from the sn-2 position of phospholipids in erythrocyte ghost membranes, followed by hydrolysis of the sn-1 ester linkages (MacIntyre & Buckley, 1978). Hydrolysis of the monodisperse substrate *p*-nitrophenyl butyrate was also measured as described (Bonelli & Jonas, 1989).

CD Measurements. Measurements were made by using a Jasco 500C spectropolarimeter interfaced with a DP 500 N data processor in accordance with our standard methodology (McCubbin et al., 1987). The mean residue mass (110.505) used in calculating ellipticity was based on the sequence data. In addition to plotting data in terms of mean residue ellipticity versus wavelength, the secondary structures of GCAT and the trypsin-activated enzyme were determined by using the computer program CONTIN (Provencher & Glöckner, 1981), which analyzes CD spectra as the sum of the spectra of 16 proteins whose structures are known from X-ray crystallography. Program input was for mean residue ellipticities at 1-nm intervals from 240 to 190 nm (or the lowest attainable wavelength).

Trypsin Activation for Structural Studies. In order to follow changes in secondary structure accompanying proteo-

Table II: Stages in the Purification of GCAT[a]

purification step	protein (mg)	total act.[b] (μmol/min)	sp act. [μmol/(min mg)]
culture supernatant	9600	1360	0.14
ammonium sulfate precipitate[c]	172	1350	7.8
Sepharose S-300	56	2620	46.7
DEAE-Sepharose	44	2350	68.7

[a] These results are typical of several purifications. [b] Activity was measured by using the *p*-nitrophenyl butyrate assay. [c] This is the second resuspended fraction described in the text.

lytic activation, purified GCAT (0.87 mg/mL) in 0.15 M NaCl and 20 mM Tris·HCl, pH 7.4, was treated with trypsin (protease–GCAT, 1:1000 wt:wt) at 25 °C in the sample cell of the spectrophotometer. Activated enzyme for sedimentation equilibrium, absorption, and CD spectroscopy was prepared in the same way, except that after 40 min, digestion was quenched by addition of a 2-fold excess of soybean trypsin inhibitor and the samples were dialyzed against incubation buffer for 48 h at 4 °C.

Computer-Assisted Analyses. Predictions of the secondary structure of the lipase were based upon a modification (Dufton & Hider, 1977) of the Chou and Fasman (1974) method. In addition, a composite surface profile was drawn showing the regions of the sequence most likely to lie on the surface of the protein. The program used to generate the data was obtained from the Alberta Peptide Institute (Department of Biochemistry, University of Alberta). It takes into account HPLC hydrophilicity parameters (Parker et al., 1986), accessibility (Janin, 1979), and flexibility (Karplus & Schulze, 1985).

Protein Concentration. Concentrations of GCAT before and after activation were determined from the absorbance at 279 nm, measured in a Perkin-Elmer λ5 spectrophotometer. Absorption coefficients were established by using a refraction method (Babul & Stellwagen, 1969) that correlates the absorbance of a dialyzed protein sample with its fringe count as determined from synthetic boundary experiments in the analytical ultracentrifuge. An average refraction increment of 4.1 fringes/(mg/mL) was used in the calculations. The extinction coefficient ($E_{1cm,279nm}$) of 1 mg/mL of native enzyme was 1.174, while that of GCAT after activation was 1.331.

Ultracentrifuge Experiments. A Beckman Model E analytical ultracentrifuge equipped with electronic speed control and an RITC temperature-control system was used for all experiments. The Rayleigh Interference optical system was used to measure fringe counts. The photoelectric scanning optical system was used for both the sedimentation velocity and sedimentation equilibrium experiments according to the existing methodology (Chervenka, 1970). Measurements of photographic plates for fringe counts were performed on a Nikon Model 6 microcomparator.

Other Methods. Protein was also determined by using a modification of the Lowry procedure (Markwell et al., 1978). Sodium dodecyl sulfate electrophoresis was carried out in 12% acrylamide slabs (Neville, 1971). Proteins were stained with Coomassie Blue. The amino acid composition of purified GCAT was measured by using an Applied Biosystems 420 A amino acid analyzer. Amino-terminal sequences were determined with an Applied Biosystems 470 A gas-phase sequenator.

Results

Purification of the Enzyme. The purification of GCAT from culture supernatants of CB3(pJT2) is summarized in Table II. Typically 35–45 mg of protein was obtained from 2 L of culture supernatant. Calculation of recoveries was impossible because the total activity increased during the gel filtration step, perhaps due to the removal of an inhibitor. The specific activity of the purified enzyme (Tables II and III) was comparable to the activity of the *A. salmonicida* GCAT we isolated earlier (Buckley et al., 1982) and several orders of magnitude higher than the activity of purified LCAT (Bonelli & Jonas, 1989). Upon electrophoresis, nearly all of the protein ran as a single band of 36.0 kDa. Sometimes several minor bands were seen, amounting to less than 1% of the total protein. They were not removed by repeating steps in the purification nor by hydroxylapatite chromatography. Unpublished observations and the results obtained below suggest that they may be produced by autoproteolysis.

Table III: Effect of Trypsin Treatment on the Activity of GCAT

substrate	enzyme act. [μmol of product/(min mg)][a] untreated	cut
p-nitrophenyl butyrate	49.5	113.2
PC–cholesterol mixed micelles	84.1	74.7
erythrocyte ghost lipids	22.5	87.3

[a] Results are the means of two separate determinations.

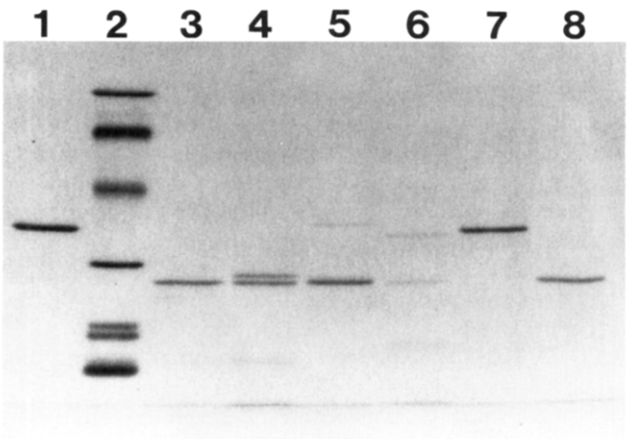

FIGURE 1: Effect of various proteases on the molecular weight of GCAT. The enzyme was exposed to 10 μg/mL of the specified proteases for 30 min at room temperature. Lane 1, untreated GCAT; lane 2, standards; lane 3, trypsin; lane 4, chymotrypsin; lane 5, thermolysin; lane 6, proteinase K; lane 7, *S. aureus* V8; lane 8, submaxillary ArgC.

Proteolytic Modification of the Purified Enzyme. The apparent molecular weight of the purified cloned enzyme is higher than the moelcular weight predicted from the derived amino acid sequence (31.3 kDa; Thornton et al., 1988) and considerably higher than the molecular weight of purified *A. salmonicida* GCAT (23.6 kDa; Buckley et al., 1982). However, when culture supernatants of AS440(pJT2) were used in the purification, the enzyme was reduced in size during a desalting step to an apparent molecular weight of 27 kDa (not shown here). Since, unlike CB3, AS440 releases several proteases, these results suggest that GCAT might normally be activated by limited proteolysis, perhaps in a manner similar to that observed for the extracellular protein aerolysin (Howard & Buckley, 1985). In order to examine protease sensitivity, GCAT was purified and treated with the enzymes specified in Figure 1. It may be seen that all except SV8 protease reduced the protein to approx. 27 kDa. Time-course studies with trypsin showed that one or more intermediate bands were produced early in the reaction (not shown here). Once formed, the 27-kDa product was quite resistant to further degradation by all the enzymes except proteinase K.

```
                                                                    1
Met Lys Lys Trp Phe Val Cys Leu Leu Gly Leu Val Ala Leu Thr Val Gln Ala Ala Asp Ser Arg
ATG AAA AAA TGG TTT GTG TGT TTA TTG GGA TTG GTC GCG CTG ACA GTT CAG GCA GCC GAC AGC CGT
                                                                              25
Pro Ala Phe Ser Arg Ile Val Met Phe Gly Asp Ser Leu Ser Asp Thr Gly Lys Met Tyr Ser Lys
CCC GCC TTC TCC CGG ATC GTG ATG TTT GGC GAC AGC CTC TCC GAT ACC GGC AAG ATG TAC AGC AAG

Met Arg Gly Tyr Leu Pro Ser Ser Pro Pro Tyr Tyr Glu Gly Arg Phe Ser Asn Gly Pro Val Trp
ATG CGC GGT TAC CTC CCC TCC AGC CCC CCC TAC TAT GAG GGC CGC TTC TCC AAC GGG CCC GTC TGG
      50
Leu Glu Gln Leu Thr Asn Glu Phe Pro Gly Leu Thr Ile Ala Asn Glu Ala Glu Gly Gly Pro Thr
CTG GAG CAG CTG ACC AAC GAG TTC CCG GGC CTG ACC ATA GCC AAC GAG GCG GAA GGC GGA CCG ACC
                  75
Ala Val Ala Tyr Asn Lys Ile Ser Trp Asn Pro Lys Tyr Gln Val Ile Asn Asn Leu Asp Tyr Glu
GCC GTG GCT TAC AAC AAG ATC TCC TGG AAT CCC AAG TAT CAG GTC ATC AAC AAC CTG GAC TAC GAG
                            100
Val Thr Gln Phe Leu Gln Lys Asp Ser Phe Lys Pro Asp Asp Leu Val Ile Leu Trp Val Gly Ala
GTC ACC CAG TTC CTG CAA AAA GAC AGC TTC AAG CCG GAC GAT CTG GTG ATC CTC TGG GTC GGC GCC
                                      125
Asn Asp Tyr Leu Ala Tyr Gly Trp Asn Thr Glu Gln Asp Ala Lys Arg Val Arg Asp Ala Ile Ser
AAC GAC TAT CTG GCC TAT GGC TGG AAC ACA GAG CAG GAT GCC AAG CGG GTG CGC GAC GCC ATC AGC
                                                150
Asp Ala Ala Asn Arg Met Val Leu Asn Gly Ala Lys Glu Ile Leu Leu Phe Asn Leu Pro Asp Leu
GAT GCG GCC AAC CGC ATG GTG CTG AAC GGC GCC AAG GAG ATA CTG CTG TTC AAC CTG CCG GAT CTG
                                                      175
Gly Gln Asn Pro Ser Ala Arg Ser Gln Lys Val Val Glu Ala Ala Ser His Val Ser Ala Tyr His
GGC CAG AAC CCC TCG GCC CGC AGC CAG AAG GTG GTC GAG GCG GCC AGC CAT GTC TCC GCC TAC CAC
                                                                  200
Asn Gln Leu Leu Leu Asn Leu Ala Arg Gln Leu Ala Pro Thr Gly Met Val Lys Leu Phe Glu Ile
AAC CAG CTG CTG CTG AAC CTG GCA CGC CAG CTG GCT CCC ACC GGC ATG GTG AAG CTG TTC GAG ATC

Asp Lys Gln Phe Ala Glu Met Leu Arg Asp Pro Gln Asn Phe Gly Leu Ser Asp Gln Arg Asn Ala
GAC AAG CAG TTT GCC GAG ATG CTG CGT GAT CCG CAG AAC TTC GGC CTG AGC GAC CAG AGG AAC GCC
225
Cys Tyr Gly Gly Ser Tyr Val Trp Lys Pro Phe Ala Ser Arg Ser Ala Ser Thr Asp Ser Gln Leu
TGC TAC GGT GGC AGC TAT GTA TGG AAG CCG TTT GCC TCC CGC AGC GCC AGC ACC GAC AGC CAG CTC
            250
Ser Ala Phe Asn Pro Gln Glu Arg Leu Ala Ile Ala Gly Asn Pro Leu Leu Ala Gln Ala Val Ala
TCC GCC TTC AAC CCG CAG GAG CGC CTC GCC ATC GCC GGC AAC CCG CTG CTG GCC CAG GCC GTC GCC
                        275
Ser Pro Met Ala Ala Arg Ser Ala Ser Thr Leu Asn Cys Glu Gly Lys Met Phe Trp Asp Gln Val
AGC CCC ATG GCT GCC CGC AGC GCC AGC ACC CTC AAC TGT GAG GGC AAG ATG TTC TGG GAT CAG GTC
                                  300
His Pro Thr Thr Val Val His Ala Ala Leu Ser Glu Pro Ala Ala Thr Phe Ile Glu Ser Gln Tyr
CAC CCC ACC ACT GTC GTG CAC GCC GCC CTG AGC GAG CCC GCC GCC ACC TTC ATC GAG AGC CAG TAC

Glu Phe Leu Ala His
GAG TTC CTC GCC CAC TGA TGA
```

FIGURE 2: Deduced amino acid sequence of GCAT. This is a correction of our previously published sequence (Thornton et al., 1988). The signal sequence is highlighted in bold type. The two sequences detected by amino acid sequencing after trypsin treatment are underlined.

Identification of the Trypsin Cut Site. In an attempt to locate the site of trypsin action, native GCAT was treated with the protease and then subjected to amino-terminal sequencing. Two distinct sequences were obtained, one corresponding to the amino terminus that we earlier translated from the nucleotide sequence (Thornton et al., 1988). The other was not found in the derived sequence, but it was discovered by translating one of the other DNA reading frames, pointing to an error in the previously published sequence of the gene. The error was located by resequencing. The corrected sequence, presented in Figure 2, encodes a protein of 35.1 kDa, very close to the size of unprocessed purified GCAT observed by SDS–PAGE. In addition, the predicted amino acid composition is nearly identical with the measured composition (unpublished). Assuming that trypsin cut only at the site indicated in Figure 2, a molecular weight of 30.4 kDa would be predicted for the processed enzyme. This is somewhat larger than the 27-kDa protein we observe, suggesting that there is at least one other cut site nearer the amino terminus. Presumably any small peptides produced are lost during dialysis in preparation for sequencing.

Change in Molecular Weight on Reduction. When trypsinized GCAT was run in SDS–PAGE after reduction by boiling in sample buffer containing mercaptoethanol, it migrated as a protein of 27 kDa (Figure 3). A Coomassie Blue staining band could also be observed at the front of the gel. However, when the processed enzyme was dissolved in sample buffer without mercaptoethanol, a single band was obtained, corresponding in size to the sum of the 27-kDa protein and the 4.7-kDa peptide that would be produced from the trypsin cut site identified in Figure 2 to the C-terminus of the uncut protein (Figure 3). Since there are only two cysteines in the enzyme, one on either side of the cut site, this observation leads to the conclusion that normally they must be joined by a disulfide bond.

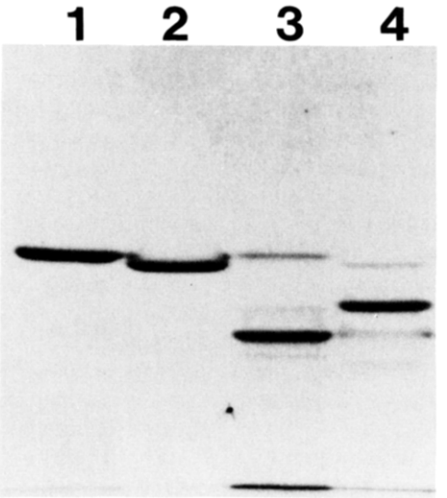

FIGURE 3: Mobility of native and trypsin-treated GCAT under reducing and nonreducing conditions. Samples were boiled 3 min in sample buffer before loading. Lanes 1 and 2, untreated GCAT; lines 3 and 4, trypsin-treated GCAT. Samples in lanes 2 and 4 were boiled in sample buffer without mercaptoethanol.

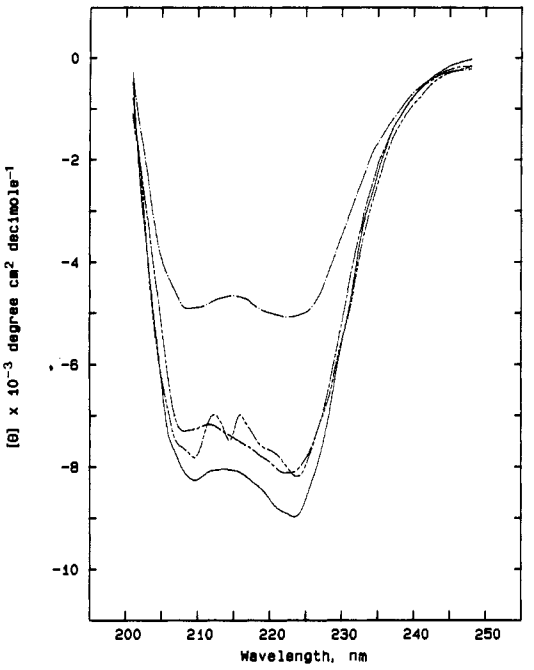

FIGURE 4: Far-UV CD spectra of native and trypsin-treated GCAT under reducing and nonreducing conditions. Spectra were determined in 0.1 M KCl and 50 mM Tris·HCl, pH 7.4, with and without 4 mM DTT. Protein concentrations used were 0.77 mg/mL (native GCAT) and 0.59 mg/mL (trypsin-treated GCAT). Cell path length was 0.0500 cm, temperature 25 °C. Solid line, native enzyme; (—-), native + DTT; (—--), trypsin-treated; (—), trypsin treated + DTT.

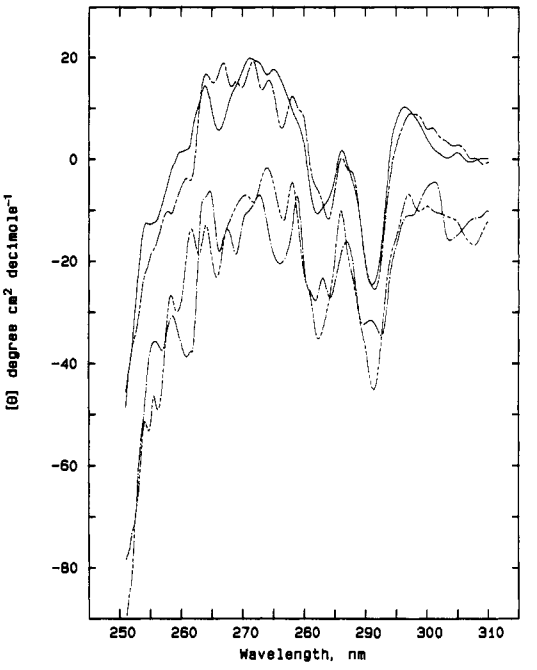

FIGURE 5: Near-UV spectra of native and activated GCAT. Solvent conditions and protein concentrations were as in Figure 4. Cell path length was 1 cm. The average of four scans was recorded. The legend is the same as in Figure 4.

Change in Aggregation State after Trypsin Treatment. Purified GCAT at a concentration of 0.427 mg/mL was found to have an S20,W of 2.76 S and a weight-average molecular weight of 32.3K by sedimentation equilibrium measurements. After treatment with trypsin, the S20,W increased to 3.93 S at 0.432 mg/mL and the weight-average molecular weight to 49.5K, indicating a change in the aggregation state of the enzyme (not shown here).

Activation of GCAT by Proteolysis. The effect of trypsin modification on the activity of the enzyme is shown in Table III. It may be seen that the change in activity observed depended on the assay used. There was more than a 4-fold increase in the production of glycerol phosphate from erythrocyte membrane phospholipids, which requires both acyl transfer and hydrolysis. The activity against *p*-nitrophenyl esters increased about 3-fold, whereas there was a slight decrease in production of cholesteryl ester in mixed Triton X-100 micelles.

CD Spectra. The far-UV CD spectrum of native GCAT was measured in a very short path length cell (0.0103 cm, data not shown). Two minima of almost equal magnitude were found: $[\theta]_{208nm} = -9150°$ and $[\theta]_{225nm} = -9140°$. A maximum was observed at 193 nm with $[\theta]_{193nm} = 13\,520°$. Computer analysis of these data showed that unprocessed GCAT consists of 31% α-helix, 21% β-sheet, and 16% β-turns with 12% of aperiodic form. The spectrum is somewhat unusual because one of the minima is at 225 nm, rather than the normal location at 221–222 nm. CD data for the enzyme dissolved in buffer containing 50% helix-forming solvent trifluoroethanol showed 38% α-helix, 34% β-sheet, 10% β-turn, and 18% aperiodic form. This is a surprisingly small change and it suggests that there is little propensity for the native protein to form additional α-helical structure.

The change in far-UV ellipticity accompanying trypsin activation of the enzyme was monitored. There was a rapid loss of structure over the first 2 or 3 min, which was followed by a more gradual reduction in ellipticity.

The spectrum of native GCAT was remeasured over the wavelength region 250–200 nm in a 0.05-cm cell as shown in Figure 4. CONTIN analysis yielded 27% α-helix, 21% β-sheet, and 16% β-turn with 36% random coil. After exposure to trypsin, the calculated values changed to 24% α-helix, 26% β-sheet, and 20% β-turn with 30% aperiodic form. Both these spectra were obtained in the absence of DTT. The slight discrepancy between the two sets of values recorded for the native enzyme reflects the loss of information resulting from the inability of the instrument to measure down to 190 nm in the 0.05-cm cell.

The far-UV spectra for native and trypsin-activated GCAT measured in the presence of the reducing reagent DTT are also found in Figure 4. Disulfide reduction led to a diminution in ellipticity of both forms of the enzyme; however, the effect was much more dramatic with trypsin-treated protein.

The near-UV CD spectra for native and activated forms of GCAT are shown in Figure 5. The spectrum of the native enzyme is characterized by a positive peak near 297 nm, a sharp negative trough centered at 291 nm, a small positive peak at 286 nm, and another minimum around 282 nm. The remainder of the spectrum consists of a broad positive peak with fine structure imposed on it. The effect of DTT was to produce only minor changes in fine structure. The band positions are essentially unchanged in the spectrum of the activated form of the protein, although the whole spectrum is more negative and the effect of DTT is more pronounced.

The near-UV spectra of proteins in general results from the aromatic amino acids tyrosine, phenylalanine, and tryptophan being located near asymmetric optically active centers. Since native GCAT contains 13 tyrosines, 6 tryptophans, and 15 phenylalanines, some or all of which may contribute to the observed spectrum, it is not surprising that the spectrum is rather complicated. Obviously, no direct assignment of residues to CD bands is possible.

Predicted Secondary Structure–Comparison with Experimental Values. Use of the Chou–Fasman analysis on the amino acid sequence of native GCAT predicted 29.7% α-helix,

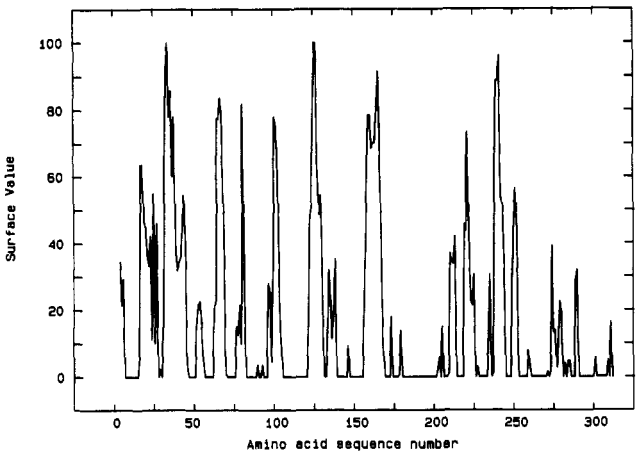

FIGURE 6: Composite surface plot of native GCAT. See the text under Computer-Assisted Analyses for details.

21.7% β-sheet, and 21.5% β-turns with 27.1% of aperiodic form.

Composite Surface Plot. This analysis is an attempt to predict which of the residues lie on the surface of the folded molecule. The approach has been used to predict antigenic sites on proteins. We found it of interest to look at the disposition of tryptophan residues, which in most proteins are few (six for GCAT) and important as the major source of intrinsic protein fluorescence. The results in Figure 6 indicate that tryptophan residues 48, 111, 232, and 287 are buried, while residue 122 is probably only partially exposed. Only residue 79 appears to be on the surface. We found that the fluorescence emission maximum of the enzyme occurs at 336 nm, a figure that is consistent with the predominantly hindered nature of five of the six trp residues (data not shown). It is also of interest to note that, with the exception of β-turn number 7 (residues 113–116) in the Chou–Fasman analysis, all of the turns are predicted to occur on the surface. The lipase consensus sequence (residues 9–18) is located beneath the surface, where it has been found in the enzyme from *Mucor miehei* as well as in pancreatic lipase (Winkler et al., 1990; Brady et al., 1990).

Discussion

The molecular weight, amino acid composition, and amino-terminal sequence of the purified *A. hydrophila* enzyme all are consistent with predictions based on translation of the structural gene. This is evidence that the GCAT signal sequence is correctly removed during export by the *A. salmonicida* mutant CB3 but that no further processing occurs. *A. hydrophila* GCAT is larger than the enzyme isolated earlier from a wild-type strain of *A. salmonicida* (Buckley et al., 1982). But this strain exports several proteases and at least one of them can process the cloned hydrophila enzyme. It seems likely therefore that the *A. salmonicida* acyltransferase had been processed in a similar way during purification.

The purified *A. hydrophila* enzyme was sometimes contaminated with traces of the processed, suggesting that there may be some proteolysis during purification. Pancreatic lipase has been shown to have weak proteolytic activity (Kaimal & Saroja, 1989) and this may be a property of lipases in general, as they appear to contain the same Asp-His-Ser catalytic triad as the serine proteases (Winkler et al., 1990; Brady et al., 1990). Thus the appearance of lower molecular weight proteins during GCAT purification may be due to autoproteolysis. Interestingly, the 4.7-kDa carboxy-terminal peptide produced by protease treatment and reduction is not necessary for enzyme activity as the 27-kDa band retains its acyltransferase activity when recovered from SDS–PAGE gels (unpublished observations). We made a similar observation with the *A. salmonicida* protein obtained in the same way (Buckley et al., 1982).

It has been suggested that cysteine may be involved in the acyl-transfer reaction carried out by LCAT, although a cysteine residue does not appear to be required for the hydrolytic mechanism (Jauhiainen & Dolphin, 1986; Jauhiainen et al., 1988). There are only two cysteines in *A. hydrophila* GCAT, and since our results show that normally they must be in disulfide linkage, it is most unlikely that either could play a role in enzyme activity. It would seem, therefore, that the reaction mechanisms of the mammalian and microbial enzymes are different.

There have been a number of reports of changes in the kinetics of lipolytic enzymes after limited proteolysis. The activity of the *Staphylococcus hyicus* lipase against lipid substrates is increased by treatment with trypsin (van Oort et al., 1989). But, although lipoprotein lipase (Bengtsson & Olivecrona, 1981), porcine pancreatic lipase (De Caro et al., 1986), and gastric lipase (Bernbäck & Bläckberg, 1989) retain their ability to hydrolyze water-soluble substrates after trypsin cleavage, hydrolysis of triglyceride emulsions is lost or much reduced. The effect of proteolysis on the kinetics of the microbial acyltransferase is less clear cut and may depend on the physical state of the substrate.

As with GCAT, trypsin treatment of lipoprotein lipase generates two fragments that are held together by a disulfide bridge. However, in contrast to the microbial enzyme, there is only a slight change in conformation as judged by circular dichroism and the enzyme is dimeric before and after proteolysis (Bengtsson-Olivecrona et al., 1986). There are several observations that support the spectroscopic evidence that the acyltransferase undergoes a major change in structure as a result of proteolysis. Our estimates of the molecular weight of GCAT by ultracentrifugation indicate that the enzyme is monomeric before trypsin treatment but that it may dimerize after proteolysis. In addition we have found that native GCAT cannot penetrate lipid monolayers at surface pressures above approximately 20 mN/m, whereas after it is nicked it will degrade monolayers at pressures above 40 mN/m (unpublished observations). Since the surface pressures of natural membranes are thought to be approximately 30 mN/m (Demel et al., 1975; Blume, 1979), unprocessed enzyme may be inactive against the bacteria's own membranes. If this is the case then extracellular processing, as with aerolysin, protects the bacterial cell from damage during export.

This is the second extracellular *A. hydrophila* protein that we have shown can be modified by limited proteolysis after release. The hole-forming toxin aerolysin is released as an inactive precursor that is converted to active toxin by proteolytic removal of about 20 amino acids from the C-terminus (Howard & Buckley, 1985; Garland & Buckley, 1988). This presumably protects the bacteria from self-inflicted damage during export.

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