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The Disulfide Bond in the *Aeromonas hydrophila* Lipase/Acyltransferase Stabilizes the Structure but Is Not Required for Secretion or Activity

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***Vibrio* and *Aeromonas* spp. secrete an unusual 35-kDa lipase that shares several properties with mammalian lecithin–cholesterol acyltransferase. The *Aeromonas hydrophila* lipase contains two cysteine residues that form an intramolecular disulfide bridge. Here we show that changing either of the cysteines to serine does not reduce enzymatic activity, indicating that the disulfide bond is not required for correct folding. However, when either of the cysteines is replaced, the enzyme is more readily denatured by urea and more sensitive to degradation by trypsin than is the wild-type enzyme, evidence that the bridge has an important role in stabilizing the protein's structure. The two mutant proteins with serine-for-cysteine replacements were secreted by *Aeromonas salmonicida* containing the cloned genes, although the levels of both in the culture supernatants were lower than the level of the wild-type enzyme. When the general secretory pathway was blocked with carbonyl cyanide chlorophenylhydrazone, the cell-associated pools of the mutant enzymes appeared to be degraded, whereas the wild-type pool remained stable. We conclude that reduced extracellular levels of the mutant proteins are the result of their increased sensitivities to proteases encountered inside the cell during export.**

Aeromonas spp. secrete an unusual lipolytic enzyme called glycerophospholipid–cholesterol acyltransferase (GCAT) that is distinguished from most other lipases by its ability to also catalyze acyl transfer from phospholipids to acceptors such as cholesterol. In this and other ways, the enzyme resembles the important mammalian plasma enzyme lecithin–cholesterol acyltransferase (LCAT), although it is far more stable and therefore more amenable to in vitro studies (33). We have recently shown that GCAT is a member of a new group or subfamily of proteins that includes several other bacterial lipases as well as a large number of plant proteins (2, 38). These proteins differ from other lipases, including the mammalian enzymes and many bacterial lipases, in the way the regions containing the active-site residues are distributed and in the consensus sequence surrounding the nucleophilic serine. The significance of these differences to the structure and function of GCAT and the other proteins in the new group has not been determined.

Both *Aeromonas hydrophila* and *Aeromonas salmonicida* GCATs contain a single pair of cysteine residues that form a disulfide bond (15, 25) (Fig. 1). In the case of other lipases, such as LCAT (31) and lipoprotein lipase (23), a disulfide bridge is thought to be important to the structural integrity of the molecule and may be essential for secretion of the protein. Earlier reports that two of the free cysteines of LCAT are directly involved in catalysis have been largely discounted; however, there is some evidence that they are essential to activity (31). We have shown that trypsin will nick *A. hydrophila* GCAT between the cysteines, removing a small peptide and producing a 27-kDa protein connected to a 4.7-kDa C-terminal peptide by the disulfide bridge (14) (Fig. 1). This form of the protein is active against membranes, unlike the unnicked form, which we have shown has a lower surface activity (13). Since the 4.7-kDa peptide contains the active-site histidine of the enzyme (3), the disulfide bridge is required in the nicked enzyme. Whether it is required in the unnicked form has not been established.

Gram-negative bacteria can release proteins to the exterior in a variety of different ways. The most common route is called the general secretory pathway (GSP). It involves the products of the *sec* genes for inner membrane transit and another set of 12 or more gene products for secretion from the periplasmic space (30). Many proteins that follow this route are not secreted if their disulfide bonds are disrupted. These include a cellulase from *Erwinia chrysanthemi*, cholera toxin from *Vibrio cholerae*, and the heat-stable enterotoxin II from *Escherichia coli* (1, 27, 28). On the other hand, we have shown that even under reducing conditions, *A. hydrophila* and *A. salmonicida* secrete the channel-forming toxin aerolysin by the GSP (12). This appears to be because the protein can adopt a stable, secretion-competent structure in its reduced state, and it is the structure of the protein that is critical for secretion, not the presence or absence of cysteines or disulfide bonds per se (12). It would be very useful to know if this is also the case for other proteins.

In this communication we describe a study of the secretion, structure, and activity of GCAT mutants in which either of the cysteines has been replaced by serine. We show that the enzyme is secreted by the GSP and that, although neither the cysteines nor the disulfide bridge is required for secretion or activity, the bridge is essential to stabilize the protein's structure, making it much more resistant to proteases, including those it encounters on its way out of the cell.

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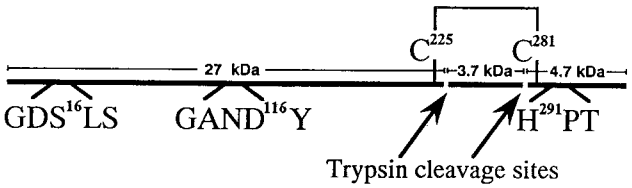

FIG. 1. Processing of GCAT by trypsin. The Ser-Asp-His catalytic triad residues and the two cysteines are numbered. The sequence encompassing amino acids 230 to 274 is removed by digestion with trypsin.

MATERIALS AND METHODS

Bacterial strains and vectors. The *Aeromonas* strains used in this study are described in Table 1. *E. coli* DH5αF′ was grown at 37°C in Luria-Bertani (LB) medium and used for cloning. *A. salmonicida* CB3 (5) was grown at 27°C in LB medium supplemented with Davis minimal medium and 0.2% glucose (24). *A. hydrophila* strains were grown at 27°C in LB medium. Where appropriate, ampicillin (100 μg/ml), kanamycin (40 μg/ml), and rifampin (40 μg/ml) were added to the media. Plasmid pJT2, which contains the wild-type GCAT gene, has been described previously (42).

Site-directed mutagenesis. The two cysteines in GCAT, Cys-225 and Cys-281, were separately replaced with serines by a modification of the method of Kunkel (21) as described earlier (3). The required oligonucleotides were purchased from the University Core DNA Services, University of Calgary, Calgary, Alberta, Canada. Each single-nucleotide change was confirmed by sequencing of the entire mutant GCAT gene (35). The mutant genes were first inserted into the broad-host-range vector pMMB66EH (11) by using *E. coli* DH5αF′ and then were mobilized into *A. salmonicida* CB3 by using *E. coli* MM297(pRK2013) as the helper strain (8). Under the conditions used in this study, this *A. salmonicida* strain produces no detectable chromosomal GCAT.

Protein purification and measurement of enzymatic activity. Wild-type and mutant GCAT enzymes were expressed in *A. salmonicida* CB3 and purified as described previously (3, 15). Where indicated, the cysteine mutants were also purified after culture supernatants were treated with 1 mM 4,4′-dithiopyridine (DTP). Acyltransferase activity was measured as described previously (4) by using egg lecithin as the substrate and [^{14}C]cholesterol as the acyl acceptor.

Stability studies. Wild-type GCAT was activated as described previously (15), by treatment with trypsin (0.5 μg/ml) for 50 min at room temperature followed by the addition of a 10-fold (wt/wt) excess of trypsin inhibitor. The stabilities of wild-type and trypsin-treated GCAT, as well as of the cysteine mutants, were assessed by plotting the change of the maximum fluorescence emission wavelength of tryptophans as a function of the urea concentration (39). Fluorescence measurements were performed with a PTI spectrofluorometer at 25°C. The excitation wavelength was 295 nm, and slit widths were 5.0 and 2.5 nm for excitation and emission, respectively. The buffer used for these studies contained 20 mM HEPES (pH 7.4), 150 mM NaCl, and the appropriate concentration of urea. The protein concentration was 0.3 to 0.4 mg/ml in all experiments. The samples were incubated for 4 h before each spectrum was taken. For each recorded spectrum, the Raman scatter contribution was removed by subtraction of the corresponding buffer blank. Trypsin and trypsin inhibitor present in the trypsin-treated GCAT samples did not significantly contribute to the fluorescence spectra.

Analysis of the reduction of GCAT by DTT and electrophoresis. Purified wild-type GCAT was incubated in the presence or absence of 5 mM dithiothreitol (DTT), with or without 0.5% sodium dodecyl sulfate (SDS), for 30 min at 37°C. A 10-fold excess of 0.3 M iodoacetamide in a buffer containing 50 mM NaH_2PO_4 (pH 7.0) and 1 mM EDTA was then added to each sample (36). Reduced GCAT was distinguished from the disulfide-bridged form by SDS-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions with 12% acrylamide slab gels (26).

TABLE 1. Bacterial strains used in this study

Strain	Genotype or description	Source or reference
A. hydrophila Ah65	Wild type; Ap^r	7
A. hydrophila C5.84	Tn*5* Km^r Ap^r; leaky pleiotropic secretion mutant of strain Ah65	19
A. hydrophila L1.97	Tn*5* Km^r Ap^r; secretion-deficient mutant of Ah65	19
A. hydrophila S9	Ethylmethylsulfonate-mutagenized strain Ah65; pleiotropic secretion mutant	41
A. salmonicida CB3	Tn*5* Km^r Rif^r; protease-deficient mutant of wild-type strain AS440	5
Escherichia coli DH5αF′	ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96* F′[*proA*$^+$*B*$^+$ *lacI*^{q}ZΔM15]	22

TABLE 2. GCAT and aerolysin are secreted by the GSP in *A. hydrophila*

Strain	OD_{600} of cultures	Percent activity[a]			
		Aerolysin[b]		Lipase[c]	
		Cells	S/N[d]	Cells	S/N
Ah65	6.4	0	100	5	95
C5.84	5.6	64	36	58	42
L1.97	7.6	100	0	97	3
S9	8.2	100	0	100	0

[a] Activities are presented as percentage of total activity recovered from the cell and supernatant fractions.
[b] Aerolysin activity was determined by using its hemolytic titer (17).
[c] Lipase activity was measured by the acyltransferase assay (4).
[d] S/N, culture supernatant.

Pulse-chase labeling and immunoprecipitation. Bacteria were grown to an optical density at 600 nm (OD_{600}) of 1.0 and were induced for 15 min with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The cells were then washed twice in M9 medium (34) supplemented with arginine (0.05 μg/ml) and 1 mM IPTG and then were resuspended in the same medium containing [^{35}S]methionine (300 μCi/ml; Amersham). In some experiments 5 mM DTT was also present. After 1 min, unlabeled methionine was added to a concentration of 500 μg/ml. Where specified, 60 μM carbonyl cyanide chlorophenylhydrazone (CCCP) was added in order to block secretion. At the indicated times after the pulse, 300-μl samples were taken and culture supernatants were separated from whole cells by centrifugation for 45 s in a microcentrifuge. A 150-ml aliquot of the supernatant was carefully taken from the top of each sample, mixed with 50 ml of 4× sample buffer, and boiled. After the remaining supernatant was removed, whole cells were lysed open and immunoprecipitated as described elsewhere (3).

RESULTS

Secretion of GCAT by *A. hydrophila* mutants. In order to determine if GCAT is secreted by the GSP, the distribution of the enzyme in several *A. hydrophila* mutants was compared to the distribution of aerolysin, which we have previously established is secreted by this pathway. The results presented in Table 2 show that, as with aerolysin, GCAT was not released by two strains that are known to be defective in the GSP, S9 and L1.97, and the leaky pleiotropic secretory mutant C5.84 released comparably less GCAT and aerolysin than the wild-type strain. In every case the proteins remained associated with the cell.

Secretion and enzymatic activities of the cysteine mutants. In the past we have made several single-amino-acid changes to *A. hydrophila* GCAT, and we have expressed the altered protein in a mutant of *A. salmonicida* (CB3) that is deficient in a secreted protease, in order to obtain large quantities of the enzyme. Some of these changes had no measurable effect on secretion of the protein by CB3, whereas others resulted in no extracellular GCAT at all (3, 14, 33). The results in Fig. 2A show that, although the levels in the culture supernatants were somewhat lower than the level of wild-type GCAT, both the C225S and C281S GCAT mutants were secreted by CB3. Over 85% of the periplasmic marker β-lactamase remained within the cells at these times. Thus, we can conclude that the appearance of the mutant GCATs in the culture supernatants is not due to lysis of the cells and that the disulfide bridge is not absolutely required for secretion of the enzyme.

We were able to purify both mutant enzymes by our standard procedure. As their ability to catalyze acyl transfer was the same as that of the wild-type enzyme (Table 3), we can

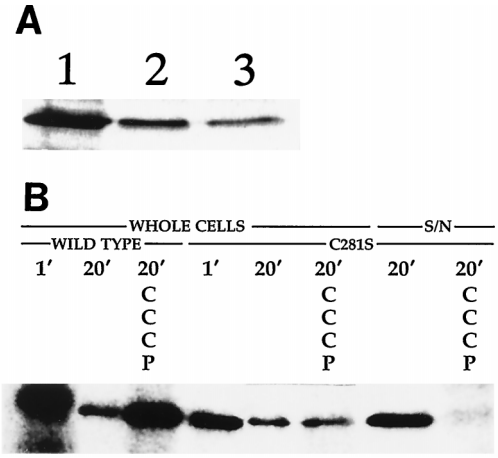

FIG. 2. Release of wild-type GCAT and the cysteine mutants into CB3 culture supernatants. (A) Cultures were grown to an OD_{600} of 0.5 and were induced for 16 h with 1 mM IPTG. Final OD_{600} values ranged between 5.3 and 6.0. Fifteen microliters of each culture supernatant in sample buffer was separated by SDS-PAGE. The separated proteins were stained with Coomassie blue. Lane 1, wild type; lane 2, C281S; lane 3, C225S. (B) Cultures were grown to an OD_{600} of 1.0 and induced for 15 min before the addition of [^{35}S]methionine (300 μCi/ml). Unlabeled methionine was added after 1 min, and then half of each culture was treated with 60 μM CCCP. Samples were taken at 1 and 20 min (1′ and 20′) after the start of the pulse and were prepared for SDS-PAGE as described in the text. Fifteen microliters of culture supernatants (S/N) and 7.5 μl of whole-cell samples were loaded onto the gels. The facts that proportionally more of the supernatant samples was applied and that there was no immunoprecipitation step in the preparation of the supernatants, thus reducing losses, account for the impression that there is more GCAT in the supernatant fractions. This panel was prepared with Photoshop, version 3.

conclude that the disulfide bridge is not required for enzymatic activity. Acyltransferase activity was considerably reduced when either the C225S or the C281S mutant was treated with DTP (Table 3), suggesting that the addition of the thiopyridyl group to the remaining cysteine in either mutant somehow altered the enzyme's structure or decreased substrate accessibility to the active site. Francone and Fielding (9) drew a similar conclusion in their study of the action of 5,5′-dithiobis(2-nitrobenzoic acid) on human LCAT. DTP had no effect on the wild-type enzyme (data not shown).

Effect of the Cys-to-Ser mutations on the stability of GCAT. The susceptibilities of the purified cysteine mutants to urea denaturation were compared to those of wild-type GCAT and the trypsin-activated wild-type enzyme. As shown in Fig. 3, both of the mutant enzymes were unfolded by urea concentrations 1.4 M lower than the concentration required to unfold wild-type GCAT. The fact that both mutations destabilize the protein to the same extent indicates that the effect is due to the removal of the disulfide bridge rather than to the serine replacements per se. Interestingly, treatment of wild-type enzyme with trypsin resulted in a similar degree of destabilization (Fig. 3). In untreated native GCAT, the 43 amino acids of the 4.7-kDa C-terminal peptide produced by trypsin treatment, which contain the active-site histidine, are linked to the rest of the protein by the sequence from 230 to 274 (which is removed by trypsin), as well as by the disulfide bridge (Fig. 1).

Proteolysis of the cysteine mutants. The 27-kDa polypeptide produced by nicking native GCAT with trypsin can be separated from the C-terminal 4.7-kDa peptide by SDS-PAGE under reducing conditions (15). Our previous study and the results in Fig. 4 show that this part of the nicked enzyme is resistant to further proteolysis by trypsin. A polypeptide of identical size was observed when the C225S and C281S mutants were treated with trypsin under the same conditions, but unlike the wild-type 27-kDa polypeptide, it was degraded by further exposure to the protease (Fig. 4).

Accessibility of the wild-type disulfide bond. We observed that although the wild-type and mutant enzymes migrated in the same way on SDS-PAGE under reducing conditions, wild-type GCAT migrated slightly differently in the absence of a reducing agent. Presumably, the disulfide bond prevents complete unfolding of the protein by SDS, thereby influencing its mobility. We took advantage of the difference in mobility of the reduced and unreduced forms of GCAT in order to determine the accessibility of the disulfide bridge in the native folded enzyme to DTT. The enzyme was exposed to the reducing agent for 30 min at 37°C, with or without SDS. After incubation, iodoacetamide was added to trap excess DTT, and the protein was examined by SDS-PAGE under nonreducing conditions. The results in Fig. 5a show that the enzyme's mobility was not affected by treatment with 5 mM DTT (that is, the disulfide bridge was not reduced) unless 0.5% SDS was present during the incubation. The effect of the detergent is presumably to unfold the protein, and the results therefore suggest that the disulfide bridge is normally buried within the wild-type enzyme.

The importance of the disulfide bridge to the secretion and stability of wild-type GCAT. We wanted to determine whether the reduced form of wild-type GCAT could be secreted normally. Since it was not possible to reduce the disulfide bond in native GCAT once it had formed without denaturing the protein, we attempted to block oxidation during folding within the cell by adding DTT to the medium while pulse-chasing the enzyme in CB3. We have shown that this is an effective way to prevent formation of the disulfide bonds in the periplasmic pool of proaerolysin (12). The rates of secretion of GCAT in the presence and absence of DTT in the medium are compared in Fig. 5b. The wild-type and mutant GCATs were all secreted by CB3. Interestingly, the amount of wild-type enzyme released in the presence of DTT was comparable to the amount of C281S secreted in the absence of the reducing agent, indicating that the effect of DTT is to lower the rate of secretion of the wild-type protein by preventing formation of the disulfide bond and not by causing some secondary effect on the cells. In support of this conclusion, we found that the presence of DTT did not further reduce the amount of the C281S mutant enzyme appearing in the culture supernatant (data not shown). These observations are comparable to those we have made while studying the secretion of aerolysin (12).

In order to determine if the disulfide bond in wild-type GCAT is normally formed in the cell before secretion and to get evidence that it is not formed in the presence of DTT, we repeated the pulse-chase experiments in media that also contained trypsin. The results are presented in Fig. 6. The GCAT

TABLE 3. Effects of amino acid changes and DTP treatment on acyltransferase activity

Mutation	DTP	Percent wild-type acyltransferase activity[a]
None	−	100
C225S	−	90
	+	16
C281S	−	110
	+	32

[a] Specific activities were determined by using the slopes of lines relating enzyme concentration to activity. Correlation coefficients exceeded 0.98 in all cases.

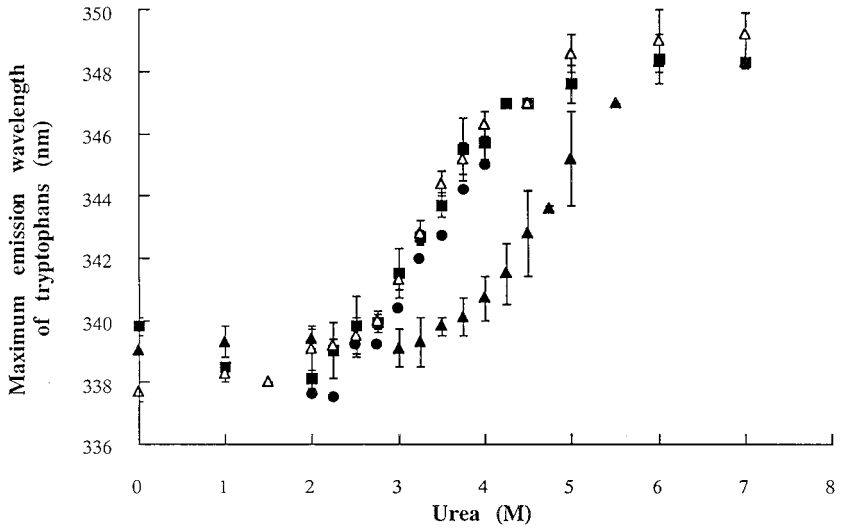

FIG. 3. Urea denaturation of wild-type GCAT (▲), trypsin-activated GCAT (■), and the purified GCAT cysteine mutants C281S (△) and C225S (●). The maximum emission wavelength of tryptophans was measured for each.

released into medium that did not contain DTT was correctly processed by the trypsin, generating the 27-kDa product. However, the enzyme released when DTT was present was completely degraded. Since the results given above (Fig. 5a) showed that this concentration of DTT could not reduce oxidized GCAT after the enzyme had folded unless SDS was present, the degradation observed in Fig. 6 likely means that the bridge had never formed. Thus, we can conclude that bridge formation is not required for GCAT secretion, as we did for aerolysin secretion previously (12). Since reduced GCAT is susceptible to complete degradation by trypsin, we can also conclude that in the two cysteine mutants, it is the absence of the bridge that makes them sensitive to the protease, rather than the Cys-to-Ser mutation that each contains.

Stability of the cell-associated GCAT pool. We have found that release of proaerolysin from the periplasm of *A. salmonicida* can be blocked by energy poisons such as CCCP (40). The results in Fig. 2B show that this is also the case with GCAT. Although most of the radiolabeled wild-type enzyme had left the cell within 20 min after the chase in the absence of CCCP (a time course similar to that which we have observed previously with aerolysin [17]), in the presence of CCCP the level inside the cell remained high after 20 min, much higher than the level in untreated cells at the same time. We can also conclude that, as with aerolysin (12), the wild-type enzyme that is prevented from leaving the cell is not subject to rapid degradation. This was not the case with C281S GCAT. It is clear from the results in Fig. 2B that although the protein did not appear in the supernatant when CCCP was present, it could not be recovered from the cells. In contrast to the wild-type protein, there was far less in the cells after 20 min than after 1 min; indeed, there was no more than in untreated cells after 20 min. Presumably, the mutant protein is more sensitive to degradation by periplasmic proteases than native GCAT. This may be because it is less tightly folded, consistent with the trypsin results described above, or because it is unable to interact as well as wild-type enzyme with some component of the GSP machinery which can provide protection from proteolysis. It should be stressed that we were monitoring GCAT without its signal peptide in these experiments. PreGCAT is visible only if CCCP is added at the same time as the radiolabeled methionine (not shown).

DISCUSSION

The results presented here provide strong evidence that the *Aeromonas* lipase GCAT is secreted via the GSP. The cell-associated lipase has the same apparent mass as the extracellular enzyme, an indication that the signal peptide has been removed and hence that the protein has crossed the inner membrane. As with aerolysin, transfer from this pool to the exterior can be blocked by CCCP (12) (Fig. 2B). Perhaps the most compelling evidence is that *A. hydrophila* mutants with defective *exe* genes are unable to secrete the lipase (Table 2).

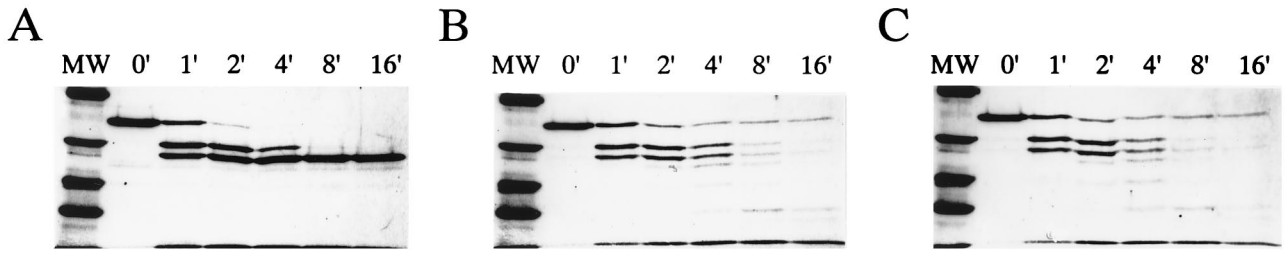

FIG. 4. Effect of trypsin treatment on wild-type GCAT (A) and the purified GCAT cysteine mutants C281S (B) and C225S (C). Purified protein samples were incubated with trypsin (1 μg/ml) for the indicated time intervals (e.g., 1 min [1′]) at room temperature before being boiled for 5 min in sample buffer. SDS-PAGE was then performed on the samples, and the separated proteins were stained with Coomassie blue. MW, molecular-mass standards (45, 31, 21.5, and 14.4 kDa).

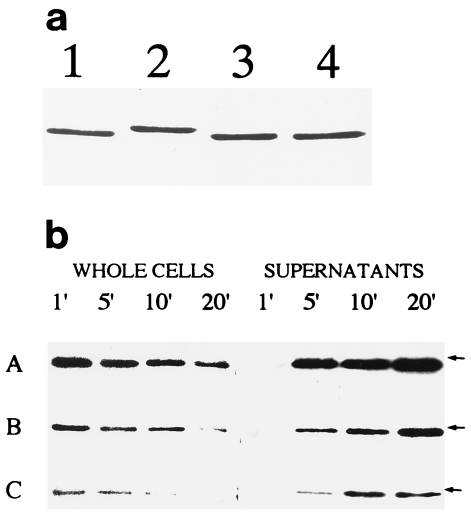

FIG. 5. Inaccessibility of the disulfide bond in wild-type GCAT and effect of DTT on the secretion of GCAT. (a) Purified GCAT was incubated for 30 min at 37°C without additions (lane 4) or in the presence of the following reagents: 5 mM DTT (lane 1), 5 mM DTT and 0.5% SDS (lane 2), and 0.5% SDS (lane 3). The samples were quenched with 0.3 M iodoacetamide before SDS-PAGE was carried out under nonreducing conditions. The gel was stained with Coomassie blue. (b) Pulse-chase conditions were the same as for Fig. 2B. See the legend to Fig. 2B for details of sample preparation. Rows: A, untreated cells expressing wild-type GCAT; B, cells expressing wild-type GCAT that were incubated with 5 mM DTT; C, untreated cells expressing C281S. Arrows indicate where oxidized GCAT (containing the disulfide bridge) would migrate.

There have been several reports that secretion of other gram-negative lipases requires the participation of periplasmic chaperones or colipases that are needed for correct folding of the enzymes (6, 10, 18, 20). It is clear from the present studies, as well as from our earlier work (42), that *A. salmonicida* is capable of secreting large amounts of GCAT expressed from a plasmid that contains only the enzyme's structural gene. Thus if a chaperone is required, it must be a constitutive component of the bacteria's periplasm, present in large enough quantities to accommodate the large amounts of GCAT expressed from the plasmid. Alternatively, expression of the lipase may trigger a response similar to the σ^E-dependent stress response in *E. coli* (32).

By replacing each of the two cysteines in GCAT with serines, we have produced two mutant proteins that lack the wild-type disulfide bridge. Both of them had near-UV circular dichroism spectra much like the spectrum of native GCAT (data not shown), and both had wild-type enzymatic activity (Table 3), indicating that the loss of the bridge caused no major structural alterations. It seems that the primary role of the bridge is to stabilize the three-dimensional structure of the protein. In its absence, GCAT becomes much more sensitive to urea denaturation (Fig. 3) and to degradation by proteases (Fig. 4). Without the disulfide bridge to anchor it, the 4.7-kDa peptide may tend to move away from the rest of the protein, exposing other sites to proteases. This would account for the observation that at early times, the same 27-kDa product is produced by trypsin digestion of the wild-type and mutant proteins. At later times, without the protection of the smaller peptide, the mutant 27-kDa polypeptides are degraded (Fig. 4).

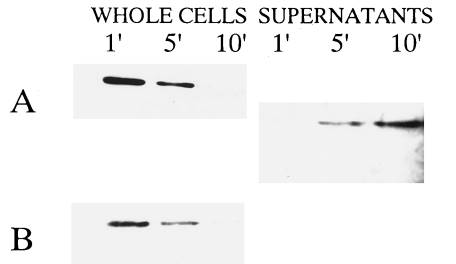

FIG. 6. Effect of DTT on the secretion and trypsin processing of wild-type GCAT expressed by CB3. Pulse-chase conditions were the same as in Fig. 2B, except that trypsin (1 μg/ml) was present in the chase medium. Samples were taken at the indicated times (1′, 1 min) after the start of the pulse and were prepared for SDS-PAGE as described in the text. Volumes loaded onto the gel were the same as for Fig. 2B. (A) Untreated cells expressing wild-type GCAT; (B) cells expressing wild-type GCAT that were incubated with 5 mM DTT.

The fact that both mutant proteins were secreted by *A. salmonicida* is also reason to believe that they are structurally similar to the wild-type protein. There is accumulating evidence that secreted proteins must fold into their native conformations before they can be released from the cell via the GSP (1, 12, 16, 28, 29, 37). Although the disulfide bridge does not seem to be absolutely required for secretion, removing it clearly reduced the amount of protein that appeared outside the cells (Fig. 2A). By taking advantage of the susceptibility of the two cysteine mutants to proteolysis and of the ability of CCCP to block release from the cell, we were able to show directly for the first time that the apparent decrease in secretion was likely due to increased protein susceptibility to proteases encountered in transit. Thus, although the cell-associated pool of wild-type GCAT remained quite constant in the presence of CCCP, little of the C281S mutant remained after 20 min, a sign that it had been degraded. We have found that a number of other GCAT mutants (14), as well as aerolysin mutants and products of aerolysin fusion with alkaline phosphatase (41), do not reach the cell's exterior. Our unpublished data indicate that this is not necessarily because a secretion signal has been altered. In some cases, as with the cysteine mutants described here, it is clear that they are unable to withstand the proteases they encounter along the GSP.

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