The Biochemistry of Hemolysin Toxin Activation: Characterization of HlyC, an Internal Protein Acyltransferase†

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ABSTRACT: Hemolysin toxin produced and secreted by pathogenic *Escherichia coli* is one of a family of cytolytic, structurally homologous protein toxins known as RTX (repeats in toxin) toxins. RTX toxins are products of a gene cluster, *CABD*. The *A* gene product, nontoxic hemolysin (proHlyA), is made toxic by posttranslational fatty acylation of two internal lysine residues. HlyC, the *C* gene product, is essential for acylation, and acyl-acyl carrier protein (ACP) is the acyl donor. HlyB and HlyD are involved in secretion of the toxin. ProHlyA and HlyC were separately subcloned, expressed, and purified, and acyl-ACPs with diverse radioactive acyl groups were synthesized. With these proteins, the conversion of proHlyA to HlyA by acyl transfer was assayed. Acyl-ACP was the obligate acyl donor. Acyl transfer was catalyzed by HlyC monomer, and an acyl-enzyme intermediate was shown. Reaction was inhibited by ACPSH but not by fatty acid or fatty-acyl CoA. K_m and V_{max} for HlyA were 0.94 μM and 7.5 pmol of acyl group transferred/min, respectively; K_m and V_{max} for myristoyl-ACP were 0.48 μM and 6.9 pmol/min. The kinetic parameters of different acyl-ACPs resembled a competitive inhibition as acyl group carbon chain length increased; K_m's increased while V_{max}'s remained unchanged. The different kinetic efficacies in the acyltransferase reaction of the ACPs with different acyl groups contrasted notably with the lytic powers of the corresponding acyl-toxins that they generated.

Pathogenic *Escherichia coli* secrete a toxic protein, hemolysin (HlyA),[1] which binds to and lyses mammalian cell membranes and, at lower concentrations, perturbs cell signal transduction and release of inflammatory mediators. It is one of a family of membrane-active toxins of similar mechanism. The family includes, among others, the leukotoxins of *Pasteurella* and *Actinobacillus* and the bifunctional adenylate cyclase hemolysin of *Bordetella pertussis* (*1*−*3*). These homologous toxins produced by Gram-negative bacteria are known as RTX (repeats in toxin) toxins. A repetitive glycine- and aspartate-rich nine amino acid sequence, the distinguishing structural feature for which the toxin family is named, formulates a Ca^{2+}-binding motif. The toxin arises from the expression of the *hlyCABD* gene cluster located on either the bacterial chromosome or the conjugative plasmids.

The *hlyA* gene product is nontoxic prohemolysin (proHlyA) which is converted intracellularly to mature toxin, HlyA, by the action of the *hlyC* gene product, HlyC (*4*). HlyC is required for transfer of a fatty-acyl group from acyl-ACP to proHlyA, converting it to HlyA which is secreted into the medium by the action of HlyB and HlyD proteins and TolC (*5*, *6*). Acylation is not essential for secretion (*4*), but acylation is the single factor that renders the protein toxic. The nature of in vitro HlyC-dependent modification was shown to be an internal acylation of the ϵ-amino groups of lysine residues 564 and 690 of proHlyA (*7*). HlyC apparently functions as an acyltransferase.

Internal fatty acylation of proteins is recognized as a significant mechanism for modification of protein behavior in signal transduction, and many toxins exert their effects through distortion of cellular signaling mechanisms. Numerous instances of protein internal fatty acylation, generally via thiol esterification of cysteine residues, have been reported. The acyltransferases catalyzing these reactions have been elusive, and, occasionally, the reactions have been proposed to proceed spontaneously. Recently, two protein-palmitoyl acyltransferases have been isolated (*8*, *9*). Although several instances of posttranslational protein modification by internal fatty acylation through amide linkage have been reported (*10* and references cited therein, *11*−*13*), the enzymes have not been isolated or characterized. Hitherto, there has been no study of the biochemistry of an enzyme catalyzing the formation of an internal amide by fatty acylation of a protein.

The role of HlyC as an enzyme needs clarification. Its amino acid sequence deduced from the DNA sequence of

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[1] Abbreviations: proHlyA, hemolysin A protoxin; HlyA, hemolysin A toxin; HlyC, acyl-ACP-proHlyA transacylase; ACP, acyl carrier protein; IPTG, isopropyl β-D-thiogalactopyranoside; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; bisTris, [bis(2-hydroxyethyl)amino]-tris(hydroxymethyl)methane; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.

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the *hlyC* gene does not resemble that of any known transacylase (*4*, *14*). There is no indication of the stoichiometry of its action which would define its role as a catalyst, and the latter point is confused by indications that enzyme, HlyC, and substrate, proHlyA, are produced together, presumably in similar amounts, in vivo from the same operon (*15*). Toxin formation has generally been assayed by hemolysis, an indirect, unstandardized assay of undefined stoichiometry. Definition of the function of HlyC and the reaction with which it is associated is necessary to dispel these ambiguities. To address these issues, HlyC and proHlyA were separately subcloned and expressed, and purified. Using purified, defined acyl-ACP (with radioactive acyl groups) and proHlyA, the acyltransferase activity of HlyC was studied, and the enzyme was characterized. Such an assay enabled the separation of two significant events; one, hemolysin toxin activation; and two, its lytic behavior, and the documentation of their different requirements. Also, insight has been gained into the biochemistry of a novel type of internal protein fatty acyltransferase that catalyzes the formation of an amide bond.

MATERIALS AND METHODS

Materials. ^{14}C-Labeled fatty acids and palmitoyl-CoA were from New England Nuclear except lauric acid which was from Amersham. American Radiolabeled Chemicals, Inc., supplied ^{3}H-labeled palmitoleic acid. Novagen was the source of pET plasmids, *E. coli* host strains HMS174, BLR(DE3), and BLR(DE3)pLysS, and alkaline phosphatase conjugated S-peptide. Agar for electrophoresis was from FMC. Spectrum Medical Industries, Inc., was the source of Spectra/Gel A4. Bacterial growth media were Luria broth and M9 minimal media. Human erythrocytes were collected and prepared from diatrizoate/Ficoll gradients (*16*).

Preparation of Acyl-ACPs. ACP was purified from *E. coli* strain BLR(DE3)pLysS containing a synthetic gene cloned into plasmid pMR19 (*17*). Following an overnight induction with 1 mM IPTG, cells from 1 L (∼7 g) were harvested, suspended in 30 mL of 100 mM Tris−glycine (pH 8), 25 mM EGTA, and frozen at −20 °C. Upon thawing, the mixture was sonified briefly and centrifuged at 12000*g* for 20 min. The supernatant solution was brought to 50% saturation with $(NH_4)_2SO_4$ at 4 °C, and the precipitate was discarded. The supernatant solution was diluted to ∼600 mL, brought to pH 6.1 with 10 M acetic acid, and applied to 50 mL of DE-52; the ACP was eluted as described (*18*). About 20 mg of ACP was obtained per liter of cells.

A variant of *E. coli* K-12, strain LCH68/pLCH6 (*19*), was used to synthesize the acyl-ACPs as described (*20*, *21*). Using ^{14}C-labeled fatty acids with a specific radioactivity of 20 Ci/mol, the following acyl-ACPs were made: lauroyl-, myristoyl-, palmitoyl-, stearoyl-, and oleoyl-. The specific radioactivity of ^{3}H-labeled palmitoleic acid was 200 Ci/mol. They were purified and evaluated as described (*18*) and stored in aliquots at −80 °C.

Construction of Recombinant DNA, Expression, and Purification of ProHlyA. The hemolysin genes were subcloned from plasmid pEK50 (*22*), a subclone of pHly 152 which had been isolated from the hemolytic *E. coli* strain PM152 (*23*).

ProHlyA expression involved cloning the 12 kb DNA fragment encoding HlyA, HlyB, and HlyD into the *Sal*I/*Bam*HI sites of pET-21b(+) (*24*). Following transformation into strain HB101 cells, the propagated recombinant DNA, designated pTXA1, was isolated and transformed into strains BLR(DE3) and BLR(DE3)pLysS cells for expression. Cells were grown in Luria broth.

Secreted proHlyA was obtained from the supernatant solution of a BLR(DE3)/pTXA1 culture. ProHlyA was precipitated at 65% $(NH_4)_2SO_4$ saturation. The precipitate was dissolved in 25 mM HEPES (pH 8), 5 mM EGTA, 2 mM DTT, 5 M urea and stored in aliquots at −20 °C. Urea-containing buffers were always freshly prepared, and proteins stored therein were monitored regularly for activity as a precaution for urea decomposition; 1.5 mg of protein was obtained/L.

ProHlyA was isolated from inclusion bodies in BLR(DE3)-pLysS/pTXA1 cells. Cells were harvested at $A_{600\ nm}$ 1.0 and suspended in 5 mL of 25 mM HEPES (pH 8), 5 mM EGTA, 2 mM DTT and stored at −20 °C. Upon thawing, cells were exposed briefly to ultrasound. Inclusion bodies were collected at 30000*g* for 25 min and washed with the following solutions: 0.5% sodium deoxycholate in 25 mM HEPES (pH 8), 5 mM EGTA, 2 mM DTT; twice with 0.1% sodium deoxycholate in the described HEPES buffer; and finally 3 times with HEPES buffer without deoxycholate. ProHlyA was solubilized in 25 mM HEPES (pH 8), 5 mM EGTA, 2 mM DTT, 5 M urea for 2 h. Following centrifugation, the clarified solution was divided into aliquots and stored at −20 °C. Each liter of culture yielded ∼8 mg of proHlyA.

Construction of Recombinant DNA, Expression, and Purification of HlyC. HlyC was expressed as an S-tag fusion protein using pET29b(+) and a PCR product from pEK50 encoding HlyC. Upper and lower primers, 5′-CTCGGTCATGAATATAAACAAACCATTAGAGAT-3′ and 3′-AAAATTAAAAAGTAATTGACCAATCGAGCTCGCC-5′, contained the restriction endonuclease sites *Bsp*HI and *Xho*I, respectively. The DNA sequence of the PCR product was determined to be that of *HlyC* (*25*) using the dideoxy method in a Promega TaqTrack system (*26*). The vector cut with *Nco*I and *Xho*I and the pared insert were ligated and transformed into HB101 cells for propagation of the plasmid designated pTXC1. Strain BLR(DE3)pLysS was used for expression. Cells were grown in minimal media at 37 °C, induced with 1 mM IPTG at $A_{600\ nm}$ 0.6, harvested 3 h later, and resuspended and stored as described above.

The fusion protein was isolated at 4 °C either from inclusion bodies or from the soluble portion of cells. Thawed cells were briefly exposed to ultrasound. Soluble fusion protein was obtained by centrifuging the cell lysate at 100000*g* for 1 h; it was stored at −20 °C. Alternatively, inclusion bodies were isolated from the cell lysate pellet (from 200 mL of culture) collected by centrifugation at 30000*g* for 25 min. Inclusion bodies were washed 3 times with 25 mL portions of 25 mM HEPES (pH 8), 5 mM EGTA, 2 mM DTT. The HlyC was solubilized in 7 mL of the same buffer which contained 5 M urea for 2 h. The fusion protein solution was clarified by centrifugation at 30000*g* for 30 min. The solution was processed one of two following ways:

First, to obtain highly purified fusion protein, an S-protein affinity medium was used. Fusion protein, 2 mg, was incubated overnight with 1 mL of Spectra/Gel A4 which contained S-protein affinity ligand in 25 mM HEPES (pH

8), 0.15 M NaCl, 0.01% Triton X-100, 2 M urea. The medium was washed with 50 mL of the same buffer followed by 30 mL of 10 mM bisTris (pH 6.2), 0.15 M NaCl, 2 M urea. Fusion protein, 0.9 mg, was released from the affinity column with 100 mM glycine (pH 3), 2 M urea and dialyzed against 25 mM MES (pH 6.1), 2 M urea followed by 25 mM HEPES (pH8), 5 mM EGTA, 1 mM DTT, 2 M urea. Finally, it was freed of urea by dialysis against 25 mM HEPES (pH 8), 5 mM EGTA, 2 mM DTT and clarified by centrifugation.

Second, urea was removed by dilution from the inclusion body extract to give a preparation in which fusion protein was the majority of the protein. Using a polystaltic pump at a rate of ~5 mL/h, 117 mL of 25 mM HEPES (pH 8), 5 mM EGTA, 1 mM DTT was added overnight to 7 mL of 5 M urea extract of inclusion bodies. The resulting 0.3 M urea solution was clarified by centrifugation, and 7 mL of a freshly prepared 5 M urea extract of inclusion bodies was added at the same rate. The solution was brought to 0.3 M urea. The dilute renatured fusion protein served as a template for renaturation of the second 5 M urea aliquot (*27*). Fusion protein was precipitated overnight at 40% saturation $(NH_4)_2SO_4$, collected, and dissolved in the same buffer containing 0.3 M urea. Protein was measured to be 2 mg.

Assay of ProHlyA Acylation. The assay was done in microfuge tubes in a total volume of 100 μL in 100 mM HEPES (pH 8), 2 μM acyl-ACP, 12 μg of proHlyA (~2 μM), and HlyC (55 μg of soluble cell lysate, ~0.025 μM, or 10 μg of protein extracted from inclusion bodies, ~2 μM). Reaction was started by the addition of acyl-ACP. Following a 5 min incubation at 4 °C, reaction was stopped by placing the tubes in an 80 °C bath for 20 min followed by addition of 233 μL of saturated $(NH_4)_2SO_4$ in 500 mM Tris (pH 7). After 30 min at 4 °C, precipitated protein was collected by centrifugation. Acyl-ACP does not precipitate under these conditions. The pellet was washed and vortexed with 400 μL of 50% 2-propanol, and the precipitate was collected and dissolved in 1 mL of 0.2 M NaOH. The radioactivity of a 0.9 mL aliquot was measured in 5 mL of Ultima Gold Scintillation fluid. Assay blanks which contained heat-denatured HlyC measured about 30 cpm.

Chemical Cross-Linking of Proteins Involved in ProHlyA Acylation. The reaction was set up as described above for assay of proHlyA acylation, and any variations in substrates are given in the Figure 6 legend. The reaction mixes were exposed to 10 mM dimethyl suberimidate for 10 min at 25 °C. Reactions were halted by the addition of 1 M ammonium acetate to a final concentration of 100 mM, and proteins were precipitated at 4 °C with 10% trichloroacetic acid. After 1 h, precipitated protein was collected by centrifugation at 13600*g* for 5 min and washed with 500 μL of acetone, and the pellet was collected again by centrifugation. Protein was dissolved in 20 μL of 8 M urea and 20 μL of 2% SDS sample buffer and analyzed as described in the Figure 6 legend.

Assessment of HlyA Lytic Capability. HlyA as an agent of erythrocyte lysis was measured in 0.05% suspensions of erythrocytes as described (*28*). Assays of proHlyA activation were done as described above except reaction was stopped with 300 mM DTT which destroyed the thioester acyl donors. A dose−response curve was obtained for each separate acyl-HlyA formed from the corresponding acyl-ACP. To compare the lytic capabilities of the separate acyl-HlyA's, 20 μL aliquots of the reaction mixtures, each containing a differerent acyl-ACP, were assayed for erythrocyte lysis.

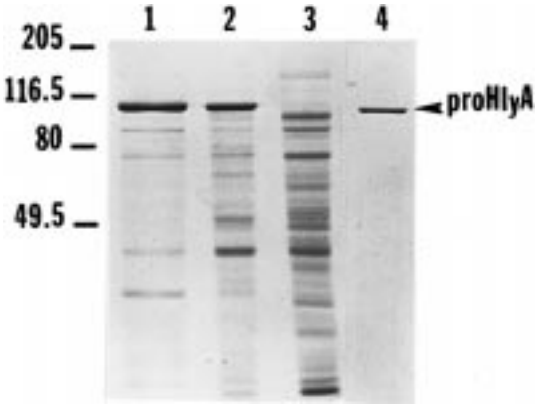

FIGURE 1: ProHlyA expression and purification. An SDS−10% PAGE showing distribution and purification of proHlyA. The arrow marks the position of the 107 kDa proHlyA. The gel was loaded as follows: lane 1, 10 μg of protein from the BLR(DE3)/pTXA1 cell growth supernatant solution; lane 2, 10 μg of the BLR(DE3)-pLysS/pTXA1 cell lysate; lane 3, 10 μg of lysate from cells that did not contain pTXA1; and lane 4, 2 μg of proHlyA purified as described in text. The migration of mass markers is indicated on the left in kDa.

Gel Electrophoresis and Western Blotting. SDS−PAGE followed the procedure of Laemmli (*29*). A Bio-Rad Semi-Dry Electrophoretic Transfer Cell was used according to the manufacturer's instructions for Western blots.

Protein Determination. Protein concentration was measured by the Bradford assay (*30*).

Determination of Kinetic Parameters. Reaction velocities were measured using the assay described above under conditions where rates were linear with time. Kinetic data were analyzed using a computer program previously described (*31*) which calculated kinetic parameters by several different methods and by the nonlinear regression analysis of Wilkinson (*32*); the different methods of computing kinetic constants agreed with *R* values greater than 0.96, or else the data were rejected. Reported kinetic constants were those computed from the Direct Linear analyses (*33*).

RESULTS

Expression and Purification of ProHlyA. Expression of proHlyA was independent of IPTG induction and different growth conditions. Figure 1 lane 2 shows proHlyA, M_r 107 000, present in the lysate of cells which contained pTXA1 while it was absent in cells which contained the vector, lane 3. As anticipated from the recombinant DNA which also encoded for HlyB and HlyD, proHlyA was also isolated from the culture medium (lane 1). Intracellular proHlyA was in inclusion bodies. Urea extraction of the isolated and washed inclusion bodies resulted in a preparation estimated by densitometry to contain >90% proHlyA (lane 4).

Expression, Assay, Purification, and Characterization of S-tag−HlyC Fusion Protein. The fusion protein was seen in SDS−PAGE of the total cell lysate as an intensely staining band at M_r 22 500 (Figure 2A, lane 3) localized mainly in the insoluble portion of the cell lysate (lane 1) while a small amount was present in the soluble portion (lane 2). A lysate of cells lacking the recombinant DNA (lane 4) contained none. A Western blot of the separated proteins (Figure 2B)

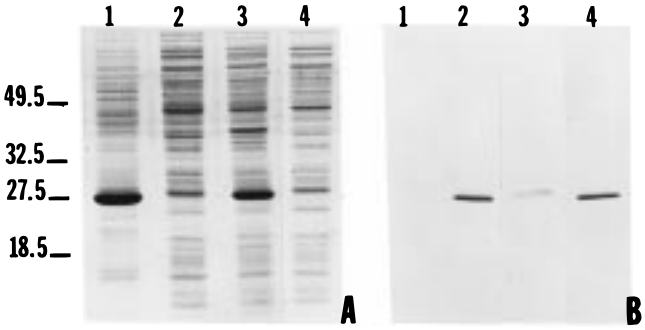

FIGURE 2: Expression of S-tag−HlyC. An SDS−15% PAGE (A) and its Western blot probed with S-protein−alkaline phosphatase conjugate (B) showing the cellular distribution of S-tag−HlyC fusion protein. Lanes in Panel A contained 10 μg of protein from the following sources: lane 1, insoluble fraction of cell lysate; lane 2, soluble fraction of the cell lysate; lane 3, cell lysate; lane 4, lysate of cells without pTXC1. The arrow shows the position of the fusion protein at 22.5 kDa. The gel in Panel B was loaded with the following proteins: lane 1, 3 μg of lysate of cells lacking pTXC1; lane 2, 1 μg of insoluble fraction of cell lysate; lane 3, 3 μg of the soluble fraction of the cell lysate; lane 4, 1 μg of the cell lysate. More protein was loaded into lanes 1 and 3 in order to visualize the fusion protein, if it were present. The migration of molecular mass markers is indicated on the left in kDa.

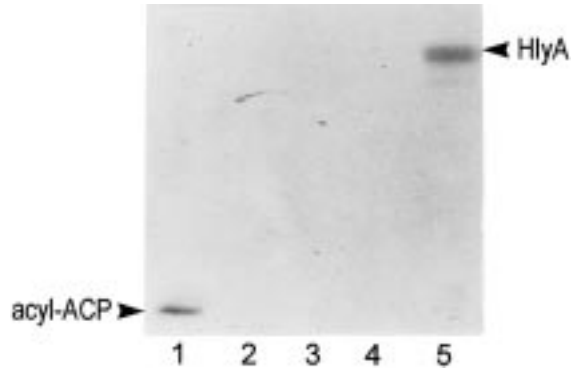

FIGURE 3: Dependence of acyl-ACP−proHlyA acyl transfer on HlyC. Fluorography of an SDS−12% PAGE of redissolved assay precipitates showing the transfer of radiolabeled fatty acid from acyl-ACP (M_r ~9000) to proHlyA forming radiolabeled HlyA (M_r 107 000). The method was that of Worsham and colleagues (*18*), and Kodak X-OMAT LS film was used. Although acyl-ACP does not precipitate under the conditions used to separate the reaction product HlyA, any entrapped thiol esters were removed by dissolving the assay precipitates in 100 μL of 25 mM HEPES (pH 8), 1 mM DTT, 5 mM EGTA, 5 M urea and treated with 100 μL of 2 M NH_2OH (pH 10) for 30 min at 37 °C. Following precipitation with cold trichloroacetic acid, samples were dissolved in SDS−PAGE sample buffer. [^{14}C]Palmitoyl-ACP, 0.4 μg, was applied to lane 1; other lanes contained material from assays. Lane 2 assay contained proHlyA, acyl-ACP, and lysate from cells which did not contain pTXC1. Lane 3 assay contained acyl-ACP and BLR(DE3)-pLysS/pTXC1 cell lysate. Lane 4 assay contained proHlyA and acyl-ACP. Lane 5 assay contained acyl-ACP, proHlyA, and BLR-(DE3)pLysS/pTXC1 cell lysate.

confirmed the presence of a small amount of the fusion protein in the soluble fraction which reacted with the S-peptide−conjugate probe (lane 3). No fusion protein appeared in lysates of cells which did not contain pTXC1 (lane 1) or which were not induced. The two dark bands (lanes 2 and 4) of the Western blot (Figure 2B) confirmed the identity of the intensely stained Coomassie Blue bands (Figure 2A, lanes 1 and 3) as fusion protein. Densitometric analysis indicated that >50% of the insoluble protein was the fusion protein. HlyC activity was assayed as described subsequently; sources of active enzyme included cell lysates, the 100000*g* supernatant solution of the cell lysate, and urea extracts of washed inclusion bodies.

ProHlyA acylation was measured using defined quantities of purified substrates (proHlyA and [^{14}C]palmitoyl-ACP) and isolated enzyme (HlyC) under reproducible reaction conditions. Radioactivity was transferred from acyl-ACP to proHlyA to form HlyA in the presence of HlyC. With proHlyA and palmitoyl-ACP concentrations each at 2 μM and HlyC about 25 nM in the assay, about 1500−3000 dpm were obtained routinely. HlyC source was 50 μg of soluble protein of the cell lysate, and its concentration was estimated from Western blot analyses and Novagen S-tag rapid assay kit. The specific activity of the cell extract soluble HlyC was ~200 nmol of fatty acyl group transferred min^{-1} (mg of protein)$^{-1}$ where HlyC was ~0.1% of the protein. In the absence of pTXC1 or its expression, there was no proHlyA−acyl-ACP acyltransferase activity.

Fluorography of SDS−PAGE of the precipitates from assays (Figure 3) showed the transfer of the radioactive fatty acyl group from palmitoyl-ACP (lane1) to a 107 kDa protein, HlyA (lane 5). There was no residual radioactively labeled palmitoyl-ACP following reaction termination and washing (lane 5). HlyA was labeled only when cell lysate containing HlyC was present; cell lysate without HlyC did not cause any transfer of radioactivity from acyl-ACP to proHlyA (lane 2). Reactions containing only HlyC and acyl-ACP or proHlyA and acyl-ACP (lanes 3 and 4, respectively) formed no HlyA. Densitometric analyses of fluorographs of HlyA formed in assays paralleled the assay of radioactivity incorporated into HlyA.

The fused S-tag did not influence acyltransferase activity. The acyl-ACP−proHlyA transacylase activities of two aliquots of soluble cell lysate were measured. One aliquot contained fusion protein while the other had been treated with thrombin to cleave the fusion protein to HlyC and S-tag. Fusion protein cleavage was monitored by S-peptide−conjugate probes of Western blots of SDS−PAGE. Exposure to thrombin overnight at 4 °C or for 2 h at 25 °C resulted in disappearance of S-peptide−probe reactivity upon SDS−PAGE Western blots (data not shown). Fusion protein and HlyC resulting from thrombin cleavage showed the same amount of acyltransferase activity.

To verify that HlyC was responsible for the acyltransferase activity, fusion protein was solubilized from washed inclusion bodies (Figure 4A, lanes 2 and 3) in buffered 5 M urea and affinity-purified. Application to S-peptide-liganded Sepharose 4A, followed by washing, elution at pH 3, and renaturation by dialysis to 25 mM Hepes (pH 8), 5 mM EDTA, 1 mM DTT, gave purified fusion protein seen as a single band at M_r 22 500 in Figure 4A, lane 4. The purified protein possessed acyl-ACP−proHlyA acyltransferase activity with a specific activity of 278 pmol of fatty acyl group transferred min^{-1} (mg of protein)$^{-1}$.

An S-peptide probe of an SDS−PAGE Western blot shows the purified fusion protein (Figure 4B, lane 5); following exposure to thrombin, the S-tag was removed by proteolytic cleavage (lane 4). A Coomassie-stained gel of the same material is shown in lanes 2 (after thrombin treatment) and 3 (before thrombin treatment). A barely visible band near the 32.5 kDa molecular mass marker is thrombin. The

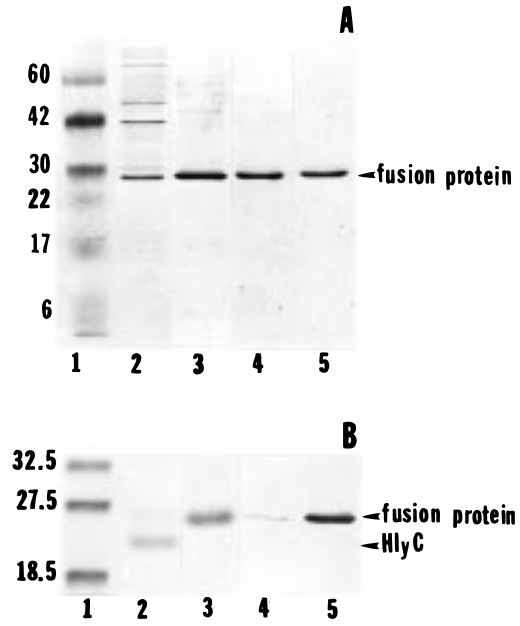

FIGURE 4: Purification of fusion protein and generation of HlyC. (A) SDS−15% PAGE illustrating the fusion protein purification described in the text. Proteins from the following sources were loaded in 5 μg aliquots: lane 1, mass standards in kDa; lane 2, unwashed inclusion bodies; lane 3, washed inclusion bodies; lane 4, pH 3 eluate from S-protein affinity chromatography; lane 5, TSK-250 HPLC (Figure 5) peak fraction of acyl-ACP−proHlyA acyltransferase activity. (B) SDS−15% PAGE and Western blot showing the thrombin cleavage of fusion protein. Proteins in lanes 1−3 were stained with Coomassie Blue, and proteins in lanes 4 and 5 were Western-blotted and probed with S-peptide−alkaline phosphatase conjugate. Lane 1, mass standards in kDa; lanes 2 and 3 each contained 5 μg of fusion protein, lane 2 after treatment with thrombin and lane 3 before thrombin treatment. Lanes 4 and 5 were each loaded with 1 μg of fusion protein after and before exposure to thrombin, respectively.

removal of the S-tag and its thrombin cleavage site changed the M_r of the protein band from 22 500 to ~19 000, the size of HlyC monomer deduced from its DNA sequence (18 458).

Although this preparation was highly purified and active, the relatively harsh conditions of purification and renaturation likely lowered the specific activity compared to that of soluble HlyC which had never been in inclusion bodies and not exposed to urea. More effective renaturation of HlyC from 5 M urea was obtained by removing the denaturant more quickly by dilution overnight while a second aliquot of washed inclusion bodies was stored at −20 °C. While the dilute, renatured HlyC was clarified, the stored aliquot of inclusion bodies was extracted with 5 M urea buffer and then centrifuged. The supernatant, 5 M urea solution was added to the clarified, dilute renatured HlyC which served as a template for its renaturation as described under Materials and Methods. The specific activity of the resulting enzyme solution, 679 pmol of acyl group transferred min^{-1} (mg of protein)$^{-1}$ where HlyC was estimated as >50% of the total protein, was more than twice that obtained when urea concentration was lowered by dialysis or chromatographic methods (not described). This preparation was used routinely for studies of the enzyme.

The size of the active form of HlyC was shown by size-exclusion HPLC to be M_r ~22 500 where fraction 29 showed the highest activty (Figure 5). Thus, HlyC was enzymatically active as a monomer. The largest $A_{280\ nm}$ peak in Figure 5,

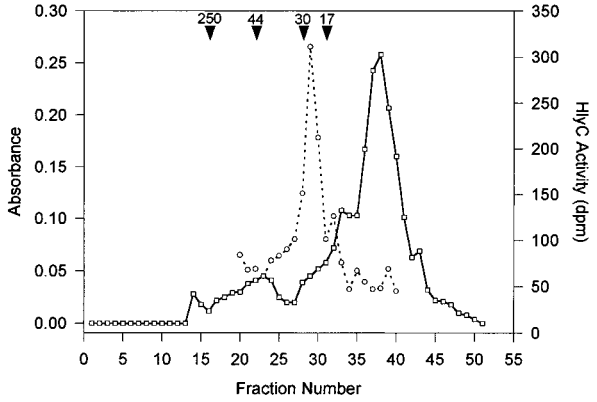

FIGURE 5: Size-exclusion HPLC showing M_r of acyl-ACP−proHlyA acyltransferase activity. Fusion protein, 0.3 mg in 25 mM HEPES (pH 8), 5 mM EGTA, 1 mM DTT, 0.3 M urea, extracted from inclusion bodies was applied to a TSK-250 column (Bio-Rad) on a Waters Model 510 HPLC column, and elution buffer was 25 mM HEPES (pH 7.2), 0.05 M NaCl. Fractions of 0.5 mL were collected and measured for $A_{280\ nm}$ (□) and acyl-ACP−proHlyA acyltransferase activity (○). Protein from the peak fraction of enzyme activity was analyzed by SDS−PAGE (Figure 4A, lane 5). Arrows denote elution peaks of calibration proteins of the indicated sizes in kDa.

fraction 38, was DTT in the sample applied. SDS−PAGE of the protein from the fraction which contained the highest acyltransferase activity (Figure 4A, lane 5) showed a single protein, the size of HlyC.

Characterization of HlyC-Dependent ProHlyA Activation. The reaction rate was linear with respect to HlyC concentration, and the amount of product formed was linear with time. Michaelis−Menten kinetics were observed. With myristoyl-ACP held constant at 1 μM, proHlyA had a K_m^{app} of 0.94 ± 0.06 μM and a V_{max}^{app} of 7.5 ± 0.16, and myristoyl-ACP had a K_m^{app} of 0.48 ± 0.1 μM and a V_{max}^{app} of 6.9 ± 0.49 with proHlyA held constant at 2 μM (Table 1). Diverse acyl-ACPs served as substrates, but when [^{14}C]palmitoyl-CoA was substituted for radiolabeled acyl-ACP, there was no incorporation of radioactivity into proHlyA. Furthermore, if unlabeled palmitoyl-CoA or free fatty acid was added to a reaction containing radioactively labeled acyl-ACP, there was no effect on the rate of HlyA activation. If, however, ACPSH which contained no acyl group was added to the reaction in addition to acyl-ACP, reaction was inhibited (unpublished data, M. S. Trent). The presence of urea, up to 3 M, had no effect on the assay. Storage of HlyC in >0.5 M urea, however, was not tolerated. A variety of cations and anions were examined, and the assay was insensitive to ionic strength and ions, with a notable exception. Ca^{2+} was inhibitory. Deleterious effects of Ca^{2+} on proHlyA activation were seen at assay ion concentrations above 50 μM, and concentrations >200 μM practically halted toxin formation.

The acyltransferase activity was relatively stable. It remained constant indefinitely at 4 or −20 °C. Several freeze−thaw transitions were tolerated with no diminution of activity. It was destroyed quickly by higher temperatures or by storage in buffers containing >0.5 M urea.

Chemical cross-linking of proHlyA, [^{14}C]acyl-ACP, and HlyC in different combinations followed by SDS−PAGE and fluorography resulted consistently in visualization of discrete protein−protein complexes (Figure 6). Dimethyl suberimidate treatment of solutions which contained all three

Table 1: Kinetic Parameters of Different Acyl-ACP's in the Transacylase Reaction[a]

acyl-ACP	$K_m$app	% of myristoyl-ACP K_m^{app}	V_{max}^{app}	% of myristoyl -ACP V_{max}^{app}	V^{app}/K_m^{app}
lauroyl-	0.46 ± 0.05	96	5.66 ± 0.18	83	12.31
myristoyl-	0.48 ± 0.1	100	6.86 ± 0.49	100	14.28
palmitoyl-	0.96 ± 0.08	200	8.52 ± 0.18	124	8.87
palmitoleoyl-	0.91 ± 0.22	190	2.24 ± 0.18	33	2.46
stearoyl-	8.50 ± 0.53	1771	7.02 ± 0.03	102	0.83
oleoyl-	6.36 ± 0.64	1325	5.94 ± 0.33	87	0.93

[a] K_m^{app} is μM, and V_{max}^{app} is pmol incorporated/min. The assay procedure and computation of kinetic parameters were given under Materials and Methods. Kinetic parameters are apparent values for the particular conditions given including the concentration of the fixed substrate, proHlyA, which was 2 μM.

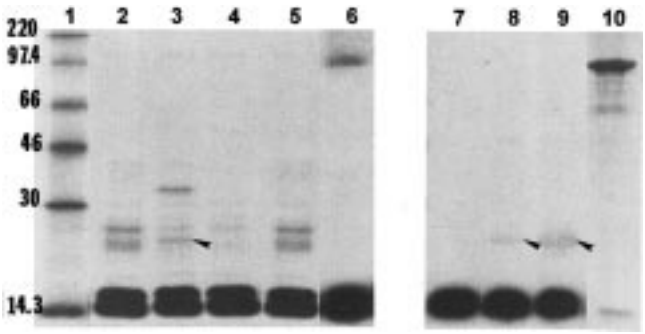

FIGURE 6: Fluorographic demonstration of acyltransferase reaction proteins which cross-linked in the presence of dimethyl suberimidate and the formation of an acyl-enzyme intermediate (arrow) with and without dimethyl suberimidate treatment. Lane 1 contained ^{14}C-labeled mass standards in kDa. Lanes 2 through 6 contained combinations of acyltransferase reaction proteins exposed to dimethyl suberimidate, subjected to SDS−12% PAGE, and fluorographed as described (Worsham et al., 1993). Kodak BioMax film was used. Each assay treated with dimethyl suberimidate contained 2 μM [^{14}C]myristoyl-ACP plus the following proteins: lane 2, none; lane 3, 2.5 μM HlyC; lane 4, 2.5 μM HlyC that had been exposed to 5 M urea overnight and in the reaction; lane 5, 2.3 μM proHlyA; lane 6, 2.5 μM HlyC + 2.3 μM proHlyA. Lanes 7 through 10 were assay mixes which were incubated for 10 min at 4 °C and were not exposed to dimethyl suberimidate, but they were prepared for electrophoresis in the same way as the chemically cross-linked proteins. Each contained 2 μM [^{14}C]myristoyl-ACP plus the following proteins: lane 7, none; and lane 8, 10 μM HlyC. Lanes 9 and 10 arose from a single 200 μL acyltransferase assay which contained the indicated acyl-ACP + 20 μM HlyC; it was divided into two 100 μL aliquots after incubation. To one aliquot was added nothing (lane 9) while 10 μM proHlyA was added to the other (lane 10); after an additional 10 min incubation at 4 °C, both were processed as described.

proteins, the complete reaction mixture, resulted in the detection of HlyA only, at M_r 107 000, with no evidence of heterotrimer or heterodimers at higher molecular weights (lane 6). Exposure of a solution of [^{14}C]acyl-ACP and proHlyA to dimethyl suberimidate (lane 5) did not result in any complexes except those generated from [^{14}C]acyl-ACP alone (lane 2). Similar treatment of a solution of [^{14}C]acyl-ACP and HlyC resulted in a complex at M_r ~34 500, a heterodimer (lane 3). The M_r ~34 500 band was not seen when [^{14}C]acyl-ACP alone was subjected to chemical cross-linking. In addition, a faint, unique band was seen at M_r ~23 000 (arrow), the size expected for an acyl-HlyC monomer. If HlyC was denatured by exposure to 5 M urea prior to chemical cross-linking, no heterocomplex or acyl-HlyC was formed (lane 4). When a solution of HlyC and [^{14}C]acyl-ACP was prepared with no exposure to dimethyl suberimidate and subjected to SDS−PAGE and fluorography, a band at M_r ~23 000 was evident (lanes 8 and 9); the presence of acyl-HlyC−S-tag was confirmed by reaction of the Western-blotted band with S-protein-conjugated alkaline phosphatase (data not shown). Furthermore, as seen by comparing lanes 8 and 9, the amount of acyl-enzyme increased when the concentration of HlyC in the solution was increased. A solution containing HlyC and [^{14}C]acyl-ACP was prepared and divided into two parts. Nothing was added to one portion while proHlyA was added to the other. Both solutions were processed like the other protein solutions in Figure 6. The portion which had no added proHlyA contained acyl intermediate (lane 9). The acyl intermediate disappeared from the other portion upon addition of proHlyA, and HlyA formation ensued (lane 10).

Different Acyl-ACPs as Substrates Compared with Erythrocyte Lysis by the Different Acyl-Toxins. The kinetic parameters of acyl-ACPs containing different acyl groups were determined (Table 1). Proportionality constants, V_{max}/K_m (directly proportional to k_{cat}/K_m), were used to compare catalytic efficiencies, and myristoyl-ACP was the most effective substrate. Its kinetic parameters were used as a reference for comparing those of the other substrates, shown as percentages of myristoyl-ACP values in Table 1. Except lauroyl-ACP, the percentages of myristoyl-ACP K_m fluctuated among the various acyl-donors while there was not much variation among the V_{max} percentages, except for palmitoleoyl-ACP. Without the latter acyl-ACP, a double-reciprocal plot of the different acyl-ACPs showed unchanging *y* axis intercepts and increasing slopes, as carbon chain length increased (data not shown). This pattern describes a plot of competitive inhibition. The two shortest chain length saturated acyl-ACPs were the most effective substrates largely because of their low K_m values, close to 0.5 μM (Table 1). Increasing chain lengths of the saturated fatty acyl-ACPs resulted in increasing K_m values, 0.96 μM for palmitoyl-ACP and 8.5 μM for stearoyl-ACP. The proportionality constants decreased strikingly with increasing chain length. There was not much difference between oleoyl- and stearoyl-ACPs, but palmitoleoyl-ACP was a much less efficient substrate than palmitoyl-ACP. The K_m values, however, for each of the two unsaturated fatty acids were lower than that of the corresponding saturated homologue, so shortening of carbon atom chain length perhaps contributed to lowering K_m's.

The erythrocyte lytic abilities of the different HlyA's formed by the different acyl-ACP donors did not correspond to the amount of acyl group transferred. Thus, the extent of lysis depended upon the nature of HlyA's fatty acyl group rather than the amount of toxin (Table 2). Acyltransferase assays each contained an identical amount of a different acyl-

Table 2: Comparison of Substrate Efficacies of Acyl-ACP's with Lytic Abilities of the Corresponding Acyl-HlyA's

acyl-ACP	amount of acyl group transfer (V/K_m)[a]	amount of erythrocyte lysis (A_{543})[b]	lysis per unit of acyl group transferred[c]
lauroyl-	12.31	0.739 ± 0.004	0.60
myristoyl-	14.28	0.438 ± 0.038	0.31
palmitoyl-	8.87	0.614 ± 0.012	0.69
palmitoleoyl-	2.46	0.451 ± 0.005	1.83
stearoyl-	0.83	0.785 ± 0.069	9.46
oleoyl-	0.93	0.813 ± 0.112	8.74

[a] The proportionality constant is a measure of the rate of transfer of the respective acyl groups to proHlyA. [b] Lysis of 2% solutions of erythrocytes caused by the addition of equal sized aliquots of acyl-transferase assays which had been assembled with equal concentrations of the respective acyl-ACP's. Lysis was measured, as described in the text, by the A_{543} of 1:10 dilutions of the respective erythrocyte lysis assay supernatant solutions. [c] The amount of lysis per unit of acyl group transferred was calculated by dividing the A_{543} (corrected for the 1:10 dilution) of the respective solutions (column 3) by the corresponding proportionality constant for the acyltransferase activity of that particular acyl-ACP (column 2).

ACP donor, and, following reaction, each had formed different amounts of HlyA as predicted by Table 1. If toxicity was independent of the nature of the acyl group on the toxin, hemolysis (Table 2, column 3) should parallel toxin formation (column 2), which it did not, and the lysis per unit of acyl group transferred should be similar for all the different acyl toxins, which it was not. Although stearoyl-ACP and oleoyl-ACP were the least effective acyl group donors, the lesser amounts of toxins produced from them were most lytic as shown in column 4 of Table 2. The most effective acyl-ACP substrate, myristoyl-ACP, produced product, myristoyl-HlyA, that caused roughly half of the lysis that stearoyl-HlyA caused, but myristoyl-ACP-supported toxin activation produced >17 times more acyl-toxin than did that employing stearoyl-ACP. An assay which contained palmitoyl-CoA instead of acyl-ACP did not activate toxin; nor did assays which contained only two of the three essential components.

DISCUSSION

All the proteins involved in rendering proHlyA toxic were highly purified except the proHlyA which was >90% pure; both intracellular proHlyA and secreted proHlyA served as substrates in the reaction. It is unlikely that an unrecognized HlyC-dependent acyltransferase activity could be present as a contaminant in each of these disparate proHlyA preparations. The role of HlyC as the acyltransferase was confirmed (*34*, *6*) by showing that the purified fusion protein was the single factor in bringing about toxin formation in the presence of acyl-ACP and proHlyA. Furthermore, although HlyC and proHlyA may be produced in similar amounts in vivo, the catalysis of the internal protein fatty acylation was a typical enzyme−substrate relationship. Catalysis ensued with less than stoichiometric amounts of HlyC relative to proHlyA and fatty acyl groups transferred. The specific activity of the enzyme isolated from the soluble portion of the cell lysate was approximately 300 times greater than the best renaturation achieved from inclusion bodies, and it had a roughly estimated turnover number of 4450 mol/min, a respectable enzymatic activity. The cell-soluble extract HlyC would likely be more representative of the *in vivo* enzyme catalyzing toxin activation than that from inclusion bodies. HlyC extracted from inclusion bodies was, however, much purer. Aggregation in inclusion bodies has been shown to be protein-specific, and, thus, relatively pure proteins are extracted from the inclusion body state (*35*).

The acyltransferase assay was highly reproducible, quantifiable, and, as shown by fluorography and densitometric analysis, directly measured product formation. The erythrocyte lysis assay widely used to assess HlyA, and indirectly HlyC activity, was shown not to be a good measure of HlyA formation *in vitro* unless appropriately qualified, because of the dependence of HlyA's lytic ability upon the nature of the HlyA fatty acyl group rather than the quantity of HlyA (discussed below). The acyltransferase assay also enabled measurement of characteristics which might independently affect cell membranes and subsequently lysis such as ions, temperature, etc. on transacylation alone. This is important for the study of the enzyme and the protein substrates of the enzyme. For example, Ca^{2+} has been shown to be required for binding the toxin to target cells (*36*, *37*). In contrast, Ca^{2+} alone among numerous ions investigated inhibited toxin acylation as measured by the direct assay. Ca^{2+} inhibition of transacylation likely arose from its effect on proHlyA conformation (*38*).

The effect of different fatty acyl groups acylating the toxin on the lytic abilities of the respective fatty acyl-toxins has not been previously addressed. Different acyl-ACPs varied in their efficacies as substrates for the transacylase while the acyl-toxins exhibited yet a dissimilar ranking of power as lytic agents shown in Table 2, column 4. Neither the lytic response nor the acyl donor capability of the respective acyl groups was accounted for by increases in hydrophobicity and membrane affinity associated with the addition of carbon groups to a fatty acyl chain (*39*). Substrate efficacies declined with increasing chain length; there was no such trend in the lytic behaviors of HlyA's bearing different acyl groups. An abrupt increase in lytic capability was, however, seen in stearoyl-HlyA relative to palmitoyl-HlyA where the lysis per unit of acyl group transferred rose 14-fold. The introduction of double bonds into an acyl chain decreases its hydrophobicity and its affinity for membranes (*39*); no structural generalization was apparent, however, regarding acyl group unsaturation and behavior as an acyl-donor. The toxins acylated with 16 carbon chain length saturated and unsaturated fatty acids were present in different quantities but produced amounts of lysis which were close. In contrast, the toxins acylated with 18 carbon chain length fatty acids produced similar amounts of toxin and lysis regardless of saturation. Perhaps distinct acyl groups favor separate characteristics such as toxin aggregatability and different efficiencies of membrane insertion which contribute to the overall lytic efficacy of a particular toxin. Additionally, the acylation of a protein may have a significant but as yet undefined effect on its higher order structure. Furthermore, this effect, conceivably, may vary depending on the nature of acyl-HlyA's fatty acyl group. While some fatty acyl groups clearly generate a more lytic toxin, others may somehow enhance inactivation of the toxin, a relatively unstable protein. Although acyl transfer is directly measured in the HlyC assay described herein, whether one or both of the proHlyA acylation sites was acylated by each of the different acyl-ACP donors is not known. Welch and co-

workers have shown that acylation at only one or the other of the two potential sites has differential effects on hemolytic activity (*40*, *41*). The different lytic efficacies of the HlyA's bearing different acyl groups could stem, in part, from unequal acylation of the two potential acylation sites. Since the extent of lysis depends on carbon chain length and unsaturation of the acyl group, lysis without experimental qualification is not a valid measure of proHlyA activation. Furthermore, the identity and number of fatty acyl groups on HlyA produced in vivo are not known.

Acylation of the toxin was more sensitive to fatty acyl group structure than was lysis, but fatty acids or fatty acyl-CoA's had no effect on the acyltransferase reaction velocity in contrast to ACPSH which inhibited. Thus, fatty acyl groups per se were not recognized by HlyC while ACPSH was recognized. Large variations in the K_m values of the various acyl-ACPs pointed to crucial structural differences among the various acyl-ACPs. These differences could be related to different acyl chain lengths fitting an HlyC acyl group binding site for acyl-ACP. This hypothetical HlyC acyl group binding site likely operated secondary to protein−protein recognition in view of the fact that fatty acids alone were not inhibitory. Alternatively, slight but significant conformational variations among acyl-ACP's cannot be ruled out. NMR studies of ACPSH and octanoyl-ACP suggest that there are not large structural differences among the acyl-ACPs which served as acyltransferase substrates (*42*). Some degree of conformational variation among the acyl-ACPs studied is, however, established by their different migrations on conformationally sensitive nondenaturing PAGE in the presence of urea (*43*). The fatty acyl chain strengthens the native structure in proportion to the carbon chain length (*43*, *44*). A dynamic structure illustrated as a model involving two conformers provides the best fit of NMR analysis of ACP structure (*45*). With such a structure, different fatty acyl groups may exert distinct transitions among conformers of the acyl-protein.

Recognition between acyl-ACP and HlyC was underscored by the demonstration of their binary complex through chemical cross-linking and by the appearance of an acyl-HlyC intermediate. The failure of Issartel and co-workers (*5*) to detect the acyl-enzyme intermediate probably stemmed from the fact that they did not use purified, defined acyl-ACPs to study the enzyme, since the ratio of enzyme to acyl-ACP affects the amount of acyl-enzyme detected (Figure 6, lanes 8 and 9). That the acyl-enzyme intermediate is a step on the main pathway in the reaction sequence was shown by its disappearance upon proHlyA addition with the concomitant appearance of HlyA. It was not a nonreactive, dead-end product; it was a viable intermediate whose detection depended upon the lack of proHlyA, the acyl acceptor. The formation of heterodimer, acyl-ACP−HlyC, shown by chemical cross-linking corroborated the HPLC demonstration that monomer was the active form of HlyC rather than dimer as reported (*6*). The heterodimer and the formation of an acyl-enzyme intermediate support a substituted enzyme mechanism for the acyltransferase reaction, and this is currently being investigated. The transfer of the acyl group from the acyl-enzyme to proHlyA was more elusive, and there was no evidence of any heteromolecular complexes with proHlyA/HlyA. The lack of much variability among V_{max}^{app} values while K_m^{app}'s varied extensively suggests that formation of the acyl-enzyme intermediate varies among acyl-ACP reactants and that acyl transfer from the acyl-enzyme to proHlyA proceeds at similar rates among acyl-enzyme species.

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